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▶ **To cite this version:**

Sandrine Cestèle, Massimo Mantegazza, William Catterall. Sodium channelopathies of skeletal muscle and brain. Physiological Reviews, 2021, 101 (4), pp.1633-1689. 10.1152/physrev.00025.2020 . hal-03411361

HAL Id: hal-03411361
https://hal.science/hal-03411361

Submitted on 19 Aug 2022

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SODIUM CHANNELOPATHIES OF SKELETAL MUSCLE AND BRAIN

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Running Title: Sodium Channelopathies

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Abstract

Voltage-gated sodium channels initiate action potentials in nerve, skeletal muscle, and other electrically excitable cells. Mutations in them cause a wide range of diseases. These channelopathy mutations affect every aspect of sodium channel function, including voltage sensing, voltage-dependent activation, ion conductance, fast and slow inactivation, and both biosynthesis and assembly. Mutations that cause different forms of periodic paralysis in skeletal muscle were discovered first and have provided a template for understanding structure, function, and pathophysiology at the molecular level. More recent work has revealed multiple sodium channelopathies in the brain. Here we review the well-characterized genetics and pathophysiology of the periodic paralyses of skeletal muscle, and then use this information as a foundation for advancing our understanding of mutations in the structurally homologous α subunits of brain sodium channels that cause epilepsy, migraine, autism, and related co-morbidities. We include studies based on molecular and structural biology, cell biology and physiology, pharmacology, and mouse genetics. Our review reveals unexpected connections among these different types of sodium channelopathies.

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I. INTRODUCTION TO SODIUM CHANNELOPATHIES

Inherited sodium channelopathies are genetic diseases caused by mutations in sodium channels (4, 357), which are the primary focus of this review. In some diseases, alterations in sodium channel expression or function can occur through other means than genetic mutations. These acquired sodium channelopathies are of great interest, but they are not our focus here. The inherited periodic paralyses of skeletal muscle were the first recognized channelopathies (264, 357). Their genes were mapped to the locus of the skeletal muscle sodium channel gene *SCN4A* that encodes the sodium channel $Na_V1.4$, and both genetic and physiological studies confirmed a causal relationship between mutations in that gene and specific phenotypes of the periodic paralyses (264, 357). After these first studies, it has been shown that mutations in the *SCN1A* gene encoding the brain sodium channel $Na_V1.1$ cause genetic epilepsy (218), mutations in the *SCN8A* gene encoding the $Na_V1.6$ channel cause paralysis in mice (218, 221), and mutations in the *SCN9A* gene encoding $Na_V1.7$ cause pain syndromes (91). Similarly, a large number of mutations in the *SCN5A* gene, encoding the cardiac sodium channel $Na_V1.5$, cause inherited arrhythmias (170). In this article, we will review the periodic paralyses as the foundation for studies of channelopathies, and we will consider the ever-increasing number of mutations in brain sodium channels that cause genetic forms of epilepsy, autism, and cognitive dysfunction. Recent review articles have covered other aspects of this large field, including inherited pain syndromes (21), inherited cardiac arrhythmias (1, 31, 231), and channelopathies caused by mutations in the non-pore-forming auxiliary β subunits of sodium channels (36, 100).

A. The Sodium Channel Protein Complex

Voltage-gated sodium channels initiate and conduct action potentials in nerve and muscle cells (144). Sodium channel proteins isolated from nerve and muscle are complexes of a large, pore-forming α subunit of 250 kDa with one or two β subunits of 30-40 kDa (FIGURE 1A (52)). The α subunits are composed of 24 transmembrane segments organized in four homologous domains containing six transmembrane segments in each (FIGURE 1B (52)). The short intracellular linker between domains III and IV serves as the inactivation gate (FIGURE 1B, C). The three-dimensional structure of the core functional unit of the sodium channel was first revealed by X-ray crystallographic studies of the homotetrameric ancestral bacterial sodium channel Na_VAb (FIGURE 1 D-F (259)). As expected from structures of voltage-gated potassium channels, the pore is formed by the S5 and S6 segments in the center of a square array of four subunits, and the voltage sensor is a bundle of four transmembrane alpha-helices (S1-S4), connected to the

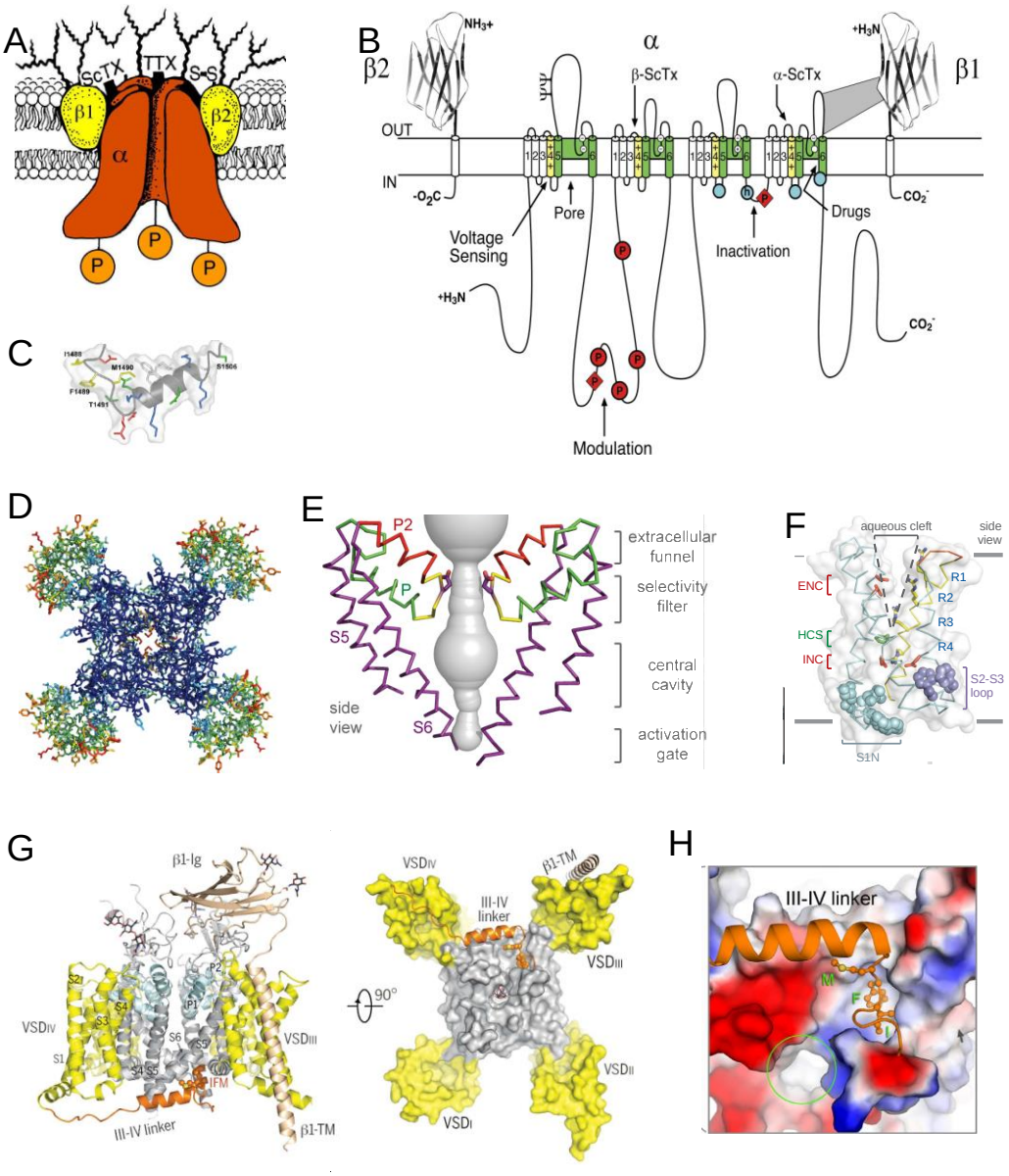


FIGURE 1. Structure of voltage-gated sodium channels revealed step-by-step over >30 years. A. Cartoon model of purified brain sodium channels with α and β subunits ca. 1986. ScTx, scorpion toxin; TTX, tetrodotoxin; P, protein phosphorylation. Adapted from (54). **B.** Transmembrane folding diagram of the sodium channel α subunit with key functional domains indicated ca. 2000. Blue circle with h, inactivation particle with IFM motif; Empty blue circles, inactivation gate receptor. Adapted from (52). **C.** Structure of the inactivation gate peptide of the brain $Na_V1.2$ channel determined by NMR. Adapted from (52). **D.** Top view of the structure of Na_VAb determined by X-ray crystallography ca. 2011. Blue, pore module; green, voltage sensors. Adapted from (259). **E.** Structure of the pore of Na_VAb. Adapted from (259). **F.** Structure of the voltage sensor of Na_VAb. Adapted from (259). **G.** Structure of Nav1.4 with β1 subunit ca. 2018. The Domain III-IV Linker (brown) serves as the fast inactivation gate. Adapted from (250). **H.** Close-up of the IFM motif of the inactivation gate (brown) interacting with its receptor. Adapted from (250).

pore by the S4-S5 linker (56, 259). The structures of eukaryotic nerve and skeletal muscle sodium channels have been determined by cryogenic electron microscopy (cryo-EM), including human sodium channels from nerve and skeletal muscle (FIGURE 1G, H (250, 304, 379)). The structure of the functional core of these channels is virtually identical to Na_VAb (259), which was used as a search template to solve the initial structure (379). These detailed analyses of eukaryotic sodium channels reveal the structure of the complex of α and β subunits, the conformations of the selectivity filter and fast inactivation gate, and the structures of portions of the large intracellular and extracellular domains that are not present in the bacterial sodium channels.

Numerous proteins can interact with the core sodium channel complex formed by α and β subunits, leading to heterogeneous multi-molecular complexes that can be specific of different cell types or different cell sub-compartments (1, 80, 92, 338). These interactions play a role in trafficking and localization of the core channel complex, as well as in modulation of its functional properties, often by means of post-translational modifications. They can also be implicated in rescuing folding/trafficking defective mutants, as we have outlined below in Section III-C. Interestingly, recent studies of cardiac $Na_V1.5$ channels indicate that there can also be interactions of α subunits: dimers of α subunits can be isolated from cardiac tissue, and activities consistent with the existence of functional dimers are observed upon expression in noncardiac cells (71). The two channel proteins in these dimers are functionally coupled, and mutations in one channel protein can influence the biophysical and functional properties of its partner (71, 72). Biochemical studies revealed that $Na_V1.1$ and $Na_V1.2$ channels can also form dimers (71). These surprising findings could have implications for recessive $Na_V1.1$ and $Na_V1.2$ sodium channelopathies, in which wild-type and mutant sodium channels are co-expressed in neurons and other cell types *in vivo*. Although some of the complexity of functional phenotypes observed in sodium channelopathies may arise from changes in interaction with associated proteins and functional dimerization of α subunits, we will not review them further here because they have been less studied in comparison with the direct effect of mutations of the core sodium channel complex, and some defective interactions implicated in channelopathies have been recently reviewed (80, 338).

B. Structural Basis for Sodium Channel Function

Detailed structure-function studies using mutagenesis, electrophysiology, and molecular modeling have given a two-dimensional map of the functional parts of sodium channels (FIGURE 1B (52, 203)). Channelopathy mutations affect all aspects of sodium channel function. The

structural and molecular mechanisms for sodium channel function are introduced in the paragraphs below, and the impact of different classes of channelopathy mutations are considered in the subsequent sections.

1. Voltage-dependent activation

Voltage-dependent activation of sodium channels is initiated by voltage-driven outward movement of the positive gating charges, usually arginine residues, in the S4 transmembrane segments of the voltage sensors (29, 52, 147, 314). The voltage sensor is a four-helix bundle with a substantial aqueous cleft that faces the extracellular milieu (FIGURE 1F). The gating charges in the S4 segment are usually arginine residues spaced at three-residue intervals, which span the membrane (R1-R4; FIGURE 1F). Upon depolarization, the S4 segment moves outward, exchanging ion pair partners and transporting the arginine gating charges through the hydrophobic constriction site (HCS, FIGURE 1F), which serves to seal the voltage sensor and prevent transmembrane movement of water and ions. Changes in membrane potential drive the S4 segment inward and outward in response to hyperpolarization and depolarization, moving the gating charges through the HCS and across the complete transmembrane electric field. This '*sliding-helix*' mechanism of gating charge movement (54) has been confirmed for sodium channels by extensive mutagenesis, disulfide-locking, molecular modeling, and most recently determination of the high-resolution structure of Na_VAb by cryo-EM (56, 84-86, 204, 371, 381). Similar experiments on potassium channels led to a consensus model for this voltage-sensing mechanism (355). This outward movement of the S4 segment initiates a conformational change in the voltage sensor, which is transmitted to the pore by a twisting motion of the S4-S5 linker (259, 371, 381). The intracellular ends of the S6 segments cross and interact closely to form the closed activation gate, which opens in an iris-like motion in response to the voltage-dependent conformational changes in the voltage sensor (6, 56, 185, 259, 371). Structures of the sodium channel in closed and open states reveal a substantial movement of the amino acid side chains at the intracellular ends of the S6 segments, from a closed conformation with an orifice of less than 1 Å to an open conformation with an orifice of to 8.5 Å (6) to 10.5 Å (185). Determination of the structure of the resting state of a voltage-gated sodium channel fits closely with the expectations of this gating model, and provides a complete picture of voltage sensing, electromechanical coupling, and pore opening at the atomic level (371). Currents generated by the activation of sodium channels are studied with voltage clamp experiments, in which the transmembrane potential is controlled and the current elicited at different potentials is recorded (FIGURE 2A).

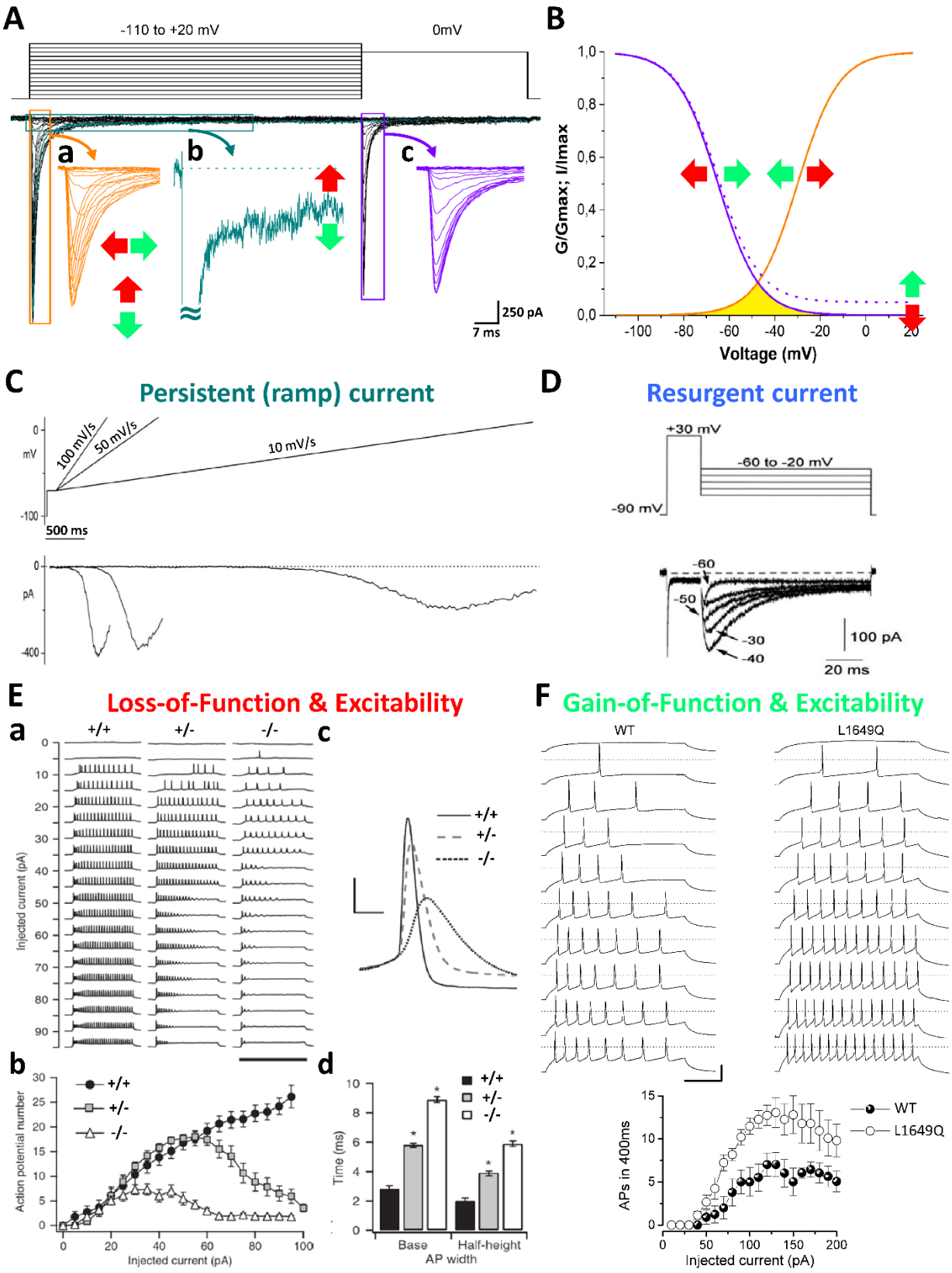

A
-110 to +20 mV
0mV
a
b
c
250 pA
7 ms
B
G/Gmax; I/Imax
Voltage (mV)
C
Persistent (ramp) current
100 mV/s
50 mV/s
10 mV/s
500 ms
D
Resurgent current
+30 mV
-60 to -20 mV
-90 mV
-60
-50
-30
-40
100 pA
20 ms
E
Loss-of-Function & Excitability
+/+
+/-
-/-
Injected current (pA)
Action potential number
Time (ms)
Base
Half-height
AP width
F
Gain-of-Function & Excitability
WT
L1649Q
APs in 400ms
Injected current (pA)

FIGURE 2. Functional properties of sodium channels. Functional properties and their modifications induced by mutations involved in channelopathies are studied performing voltage clamp experiments that follow the general methods introduced by Hodgkin and Huxley in their seminal work (145). In modern experiments aimed at identifying functional effects of mutations, sodium channels are often expressed in transfected cell lines that do not have endogenous channels of interest, and currents are evoked controlling the membrane potential in voltage clamp mode by means of the whole-cell configuration of the patch-clamp technique. **A.** Representative recordings of families of $Na_V1.1$ sodium currents with the two-pulse voltage protocol shown above. Inward sodium currents are a negative quantity by convention and therefore plotted downward. The first pulse is a step depolarization at different potentials (up to +20 mV) activating inward sodium currents (inset a), which then undergo fast inactivation during the 100-ms depolarization. These recordings determine the maximal current amplitude and the kinetics of fast inactivation from the open state within a few milliseconds. Current amplitude can be increased by gain of function mutations (depicted by the green vertical arrow), or decreased by loss-of-function mutations (red vertical arrow). Sodium currents can be prolonged by gain-of-function mutations (green horizontal arrow), or shortened by loss-of-function mutations (red horizontal arrow). The small fraction of current with slower inactivation is called persistent sodium current (inset b), in which a single trace is shown. Gain-of-function mutations can increase its amplitude, whereas loss-of-function mutations can decrease it. The second pulse at 0 mV evokes sodium currents (inset c) whose amplitude decreases according to the amount of fast inactivation induced by the first pulse. Scale bars refer to the black traces. Similar voltage protocols with longer depolarizations (up to tens of seconds) can be used to study slow inactivation. **B.** The activation curve (orange) is obtained by plotting the normalized peak conductance of the currents in inset a as a function of the stimulus potential. The fast inactivation curve (violet) is obtained plotting the normalized peak values of the currents displayed in inset c as a function of the potential of the first pulse. Gain- and loss-of-function mutations can induce shifts of the curves (horizontal arrows). The current elicited at membrane potentials in which activation and inactivation curves overlap (yellow area) is called window current. When there is persistent current, the inactivation curve shows a baseline at positive voltages (solid line 0% persistent current; dashed line 5% persistent current). **C.** The persistent current can be elicited also with slow depolarizing voltage ramps that inactivate the fast transient current; of note, relatively fast voltage ramps can elicit a mixed persistent and fast current, whose amplitude depends on the kinetics of fast inactivation, and very slow voltage ramps can induce inactivation of persistent current. Modified from (337). **D.** The resurgent sodium current is generated in some cell types during repolarizations from positive voltages to moderately negative potentials, and gain of function mutations can increase it, whereas loss-of-function mutations can decrease it. Modified from (269). **E.** Loss-of-function mutations of sodium channels decrease neuronal excitability. Inset a, Loss of action potential firing in GABAergic neurons from *Scn1a* knock-out mice. Action potential discharges, recorded in current clamp mode with the whole-cell configuration of the patch clamp technique, elicited injecting depolarizing currents of increasing amplitude in control (+/+), heterozygote $Scn1a^{+/-}$ (+/-) and homozygote $Scn1a^{-/-}$ (-/-) GABAergic neurons. Inset b, input-output relationships of the number of action potentials versus the injected current show a large reduction of excitability in $Scn1a^{-/-}$ neurons and less severe reduction in $Scn1a^{+/-}$ neurons. The rheobase (ie., the minimum depolarizing current that elicits an action potential) is not modified in this model, but loss of function of some sodium channels can increase it. Inset c representative single action potentials, recorded for each genotype of GABAergic neurons and elicited by the same injected current amplitude (35 pA), show reduced amplitude with $Na_V1.1$ loss of function, as well as increased width and half-width, which are defined as the width at the base and at the half-maximum amplitude of the action potential. Inset d. Modified from (382). **F.** Action potential discharges recorded in cultured GABAergic neurons transfected with wild type $Na_V1.1$ or the familial hemiplegic migraine gain-of-function L1649Q mutant show increased excitability when the mutant

is expressed, as quantified in the input-output relationships (bottom panel). Modified from (61). Sodium channel gain of function can also decrease rheobase and modify features of action potentials (e.g. amplitude, slope, width); not shown.

A surprising finding from structure-function studies of both sodium and potassium channels was the ability of the voltage sensor to serve as a proton-conducting (320) or cation-conducting (315, 342) pathway after mutation of the arginine gating charges. Neutralization of the gating charges of voltage-gated sodium and potassium channels causes a voltage-dependent leak current to flow through the mutant voltage sensors continuously. These gating pore currents (or omega currents) are voltage-dependent because the leak occurs only when the mutant residue replaces the arginine gating charge in the HCS that seals the voltage sensor against transmembrane ion movement (315, 342). In sodium channels, mutation of the R1 and R2 gating charges causes gating pore current in the resting state, whereas mutation of R2 and R3 gives gating pore current in the activated and inactivated states (315). These characteristics are consistent with the sliding-helix model of voltage sensor function, which predicts that the gating charges slide through the HCS in sequence during activation and deactivation of the voltage sensor (315). Mutant gating pores can be highly selective for protons when histidine is substituted for arginine (320), but they are not highly selective among monovalent cations when other amino acid substitutions are present (314, 315, 342). However, mutant gating pore often have a high conductance to guanidine (314, 342), likely because it fits well in the space left by removal of the guanidine-containing arginine side chain.

2. Sodium conductance and selectivity

Sodium conductance is mediated by the pore domain formed by the S5 and S6 segments and the P loop between them. As for potassium channels, sodium selectivity is mediated by the P loops in the four pore domains of sodium channels, which interact with Na^+ as it approaches and enters the ion selectivity filter (62, 234, 259, 349). However, in sharp contrast to potassium channels, the outward-facing edge of the ion selectivity filter is composed of a square array of four glutamate residues in bacterial Na_V channels (259) or a square array of four different amino acid residues, Asp-Glu-Lys-Ala, in vertebrate Na_V channels (250). This high field-strength site partially dehydrates the approaching Na^+ ion and allows only Na^+ to pass efficiently (62). A dunking motion of the glutamate residues accompanies the partial dehydration of Na^+ and its inward movement through the selectivity filter (62).

3. Fast inactivation

Within 1-2 msec after opening, the fast inactivation gate formed by the intracellular linker connecting domains III and IV folds into the pore and inactivates it (FIGURE 1B, C (52, 203). In voltage clamp experiments, some properties of fast inactivation can be studied analyzing the current decay during step depolarizations and the decrease of the amplitude of the current elicited by the second depolarizing pulse in a two-pulse protocol (FIGURE 2A, B). Fast inactivation does not have its own intrinsic voltage sensor. Structure-function studies indicate that outward movement of the gating charges in the S4 segment of the voltage sensor in domain IV plays a key role in coupling activation to fast inactivation (47, 63, 277, 303). A series of key amino acid residues in this linker, Ile-Phe-Met, serves as the classically defined 'inactivation particle', which folds into the inner mouth of the pore and blocks sodium conductance (171, 367). The structure of the fast inactivation gate contains an alpha-helical motif preceded by two turns that present the key interacting residues in the Ile-Phe-Met motif to the intracellular mouth of the pore, where they are bound and block ion permeation (FIGURE 1C, H (250, 278)). The ‘receptor’ that binds the inactivation gate to the intracellular end of the pore is formed by amino acid residues in the S4-S5 linkers in domains III and IV and the intracellular end of the S6 segment in domain IV (215-217, 250, 304, 310, 333).

The intracellular C-terminal domain can modulate the properties of fast inactivation (208), possibly interacting with the fast inactivation gate (232). Consistently, this domain is implicated in modulations that modify inactivation properties, including G protein βγ subunits that increase the persistent sodium current (209). The persistent current is a slowly inactivating fraction of the sodium current, which is generated because fast inactivation is not complete, even at very depolarized potentials (78) (FIGURE 2A, C). Its kinetics of inactivation shows multiple time constants and there is a residual component even after depolarizations of tens of seconds (74). Although it is often a small fraction of the peak transient sodium current, it can have important effects on cellular excitability. Distinct from the persistent current, the window current is a non-inactivating current generated in the sharp window of membrane potentials where the curves of voltage dependence of fast inactivation and activation overlap, and it is important for boosting subthreshold depolarizations. Another subthreshold sodium current is the resurgent current, an unusual transient sodium current elicited by repolarizations that follow strong depolarizations and can contribute to spontaneous and high-frequency firing (188, 269) (FIGURE 2D). It has been proposed that this current is produced by a putative intracellular blocking factor that would bind to open sodium channels and prevent fast inactivation.

4. Slow inactivation

During long single depolarizations or long trains of depolarizations, sodium channels enter a slow inactivated state, from which recovery requires prolonged repolarization (359). Slow inactivation has a different structural basis than fast inactivation. Slow inactivation of bacterial sodium channels is caused by an asymmetric collapse of the pore involving amino acid residues in the ion selectivity filter and the full length of the pore-lining S6 segments (56, 258, 387). This mechanism is conserved in vertebrate sodium channels, in which analogous amino acid residues in the selectivity filter and the pore-lining S6 segments undergo conformational changes in the slow inactivation process (8, 119, 236, 359). The close structural similarity in the core transmembrane regions of bacterial and mammalian sodium channels (<2 Å root mean square deviation in backbone structure) supports close similarity in the underlying mechanisms of slow inactivation.

These structure-function models of sodium channels derived from a combination of mutagenesis, electrophysiological analysis, and structural studies have provided valuable templates for understanding the deleterious effects of mutations that cause sodium channelopathies. Impairments of voltage-dependent activation, generation of gating pore current through the voltage sensor, altered coupling of activation to pore opening, and defects in ion conductance, fast inactivation, and slow inactivation have all been associated with channelopathies of skeletal muscle and brain sodium channels. These defects can have a direct effect on action potential generation and their features (FIGURE 2E, F).

II. $Na_V1.4$ AND PERIODIC PARALYSIS

The periodic paralyses are rare genetic diseases with a dominant pattern of inheritance within families (114, 157, 357). They cause episodic flaccid paralysis, sometimes accompanied by episodic stiffening of skeletal muscles, which are associated with a wide range of physiological and environmental factors, including exercise, temperature, and changes in serum potassium levels that result from exercise and food intake (43, 157, 357). As outlined below, these diseases are generally caused by heterozygous mutations in the *SCN4A* gene encoding the skeletal muscle sodium channel $Na_V1.4$ (157, 357) (FIGURE 3A, B).

A. Hyperkalemic Periodic Paralysis

In HyperPP, affected individuals have episodes of flaccid paralysis associated with high levels of serum potassium (157, 357). Electrophysiological studies of skeletal muscle fibers from patients

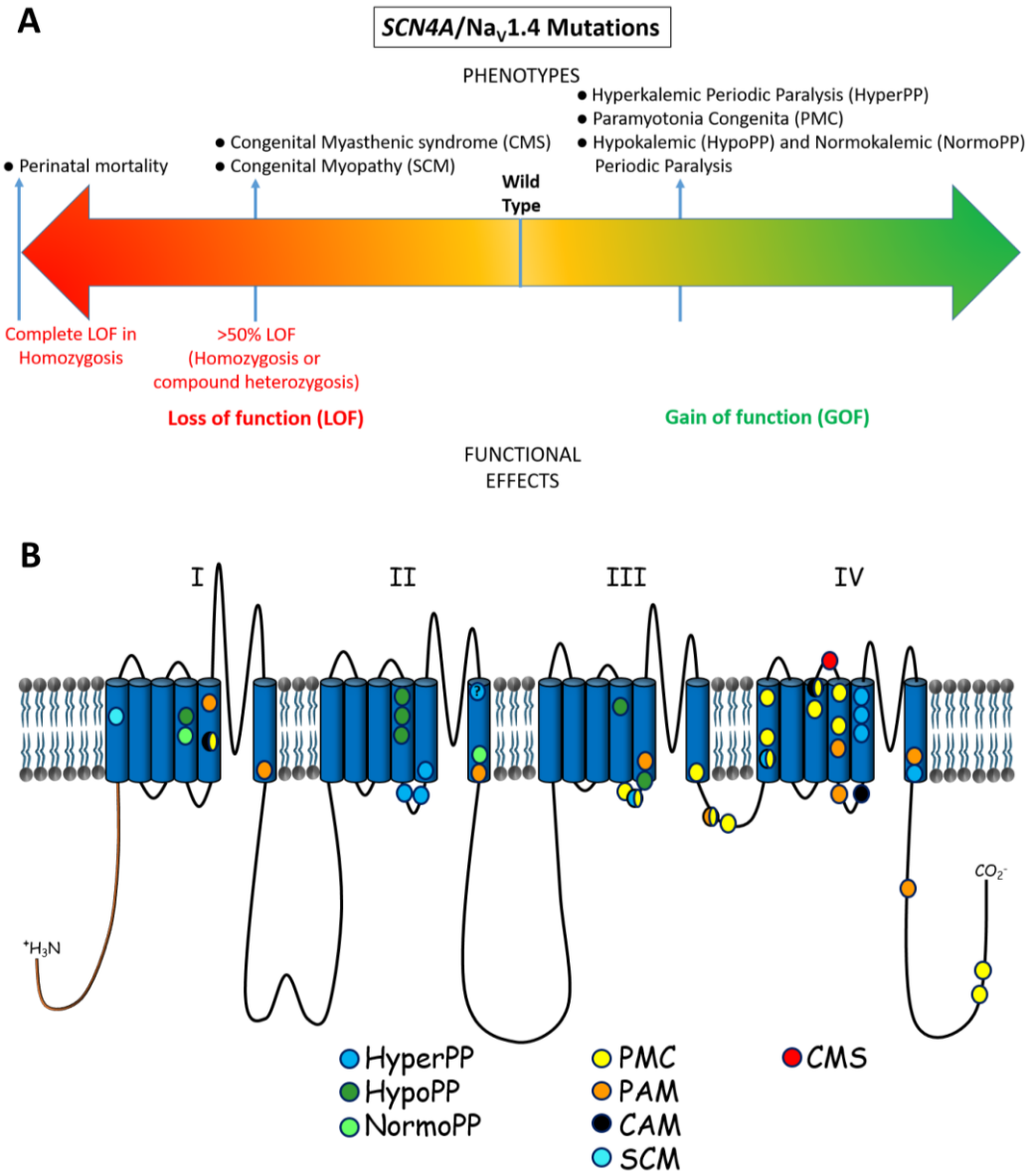


FIGURE 3. Spectrum of mutations and phenotypes for *SCN4A*/Na$_V$1.4. A. Phenotypic spectrum. Most *SCN4A* mutations cause Na$_V$1.4 gain of function with different mechanisms, including induction of gating pore current, leading to specific clinical entities. A minority of mutations cause loss of function, inducing clinical symptoms when the loss is >50% (homozygosis or compound heterozygosis). The few patients with complete loss of function mutations in homozygosis showed perinatal lethality. **B.** Molecular map of *SCN4A* mutations color-coded as indicated: Hyperkalemic Periodic Paralysis (HyperPP), Hypokalemic Periodic Paralysis (HypoPP), Normokalemic Periodic Paralysis (NormoPP), Paramyotonia Congenita (PMC), Potassium-aggravated myotonia (PAM), Cold-Aggravated Myotonia (CAM), Congenital Myopathy (SCM), and Congenital Myasthenic Syndrome (CMS).

revealed impaired inactivation of the sodium current and increased persistent sodium current that caused depolarization, block of action potential generation, and flaccid paralysis (182). These studies implicated the skeletal muscle sodium channel as either a direct or indirect target of HyperPP mutations. Genetic mapping identified the *SCN4A* gene encoding the skeletal muscle $Na_V1.4$ channel as a prime target of these mutations (115, 268). In landmark studies, Ptacek et al (266) and Rojas et al (279) cloned and characterized mutations that cause Hyperkalemic Periodic Paralysis (HyperPP) in the *SCN4A* gene encoding the skeletal muscle sodium channel $Na_V1.4$, the first discovery of the genetic basis for an ion channelopathy. Sequencing of several mutations in this gene from individuals with HyperPP and their close relatives definitively identified *SCN4A* as the site of mutations that cause this disease (266). Mutations that cause HyperPP are concentrated in the S4-S5 linkers in domains II and III and in the S6 segments near the intracellular end of the pore in domain IV (FIGURE 4A, B, *blue circles* (307)). These segments are crucial for coupling of voltage sensor movement to pore opening and for forming the receptor for the fast inactivation gate as it folds in and blocks the pore (215-217, 250, 304, 310, 333). Structure-function studies reveal that disruption of the protein interactions that keep the fast inactivation gate closed impairs fast inactivation, allows re-opening of sodium channels, and causes persistent sodium currents (171, 367). Therefore, as expected from these structure-function studies, many HyperPP mutations impair fast inactivation (eg., (44, 45)). HyperPP mutations also impair slow inactivation (18, 138, 359). Mutations in distinct locations cause a variable mixture of impairment of fast and slow inactivation (18). These dual functional deficits may work together by first producing prolonged depolarization following action potentials due to impaired fast inactivation, which leads to persistent depolarization in the absence of effective slow inactivation.

B. Paramyotonia Congenita and Potassium Aggravated Myotonia

Paramyotonia Congenita (PMC) causes episodic paralysis of skeletal muscle, which initially presents as stiffness and rigidity triggered or aggravated by low temperature and is often followed by flaccid paralysis and muscle weakness (130, 183, 275, 284). PMC mutations mapped to the same gene as HyperPP (268), and cDNA cloning and sequencing of mutations from affected and unaffected members of the same families demonstrated that PMC is caused by mutations in the *SCN4A* gene encoding $Na_V1.4$ like HyperPP (265, 267). PMC mutations are clustered in the inactivation gate and nearby transmembrane segments and in the S4-S5 linker in domain III (FIGURE 4A, B (307)).

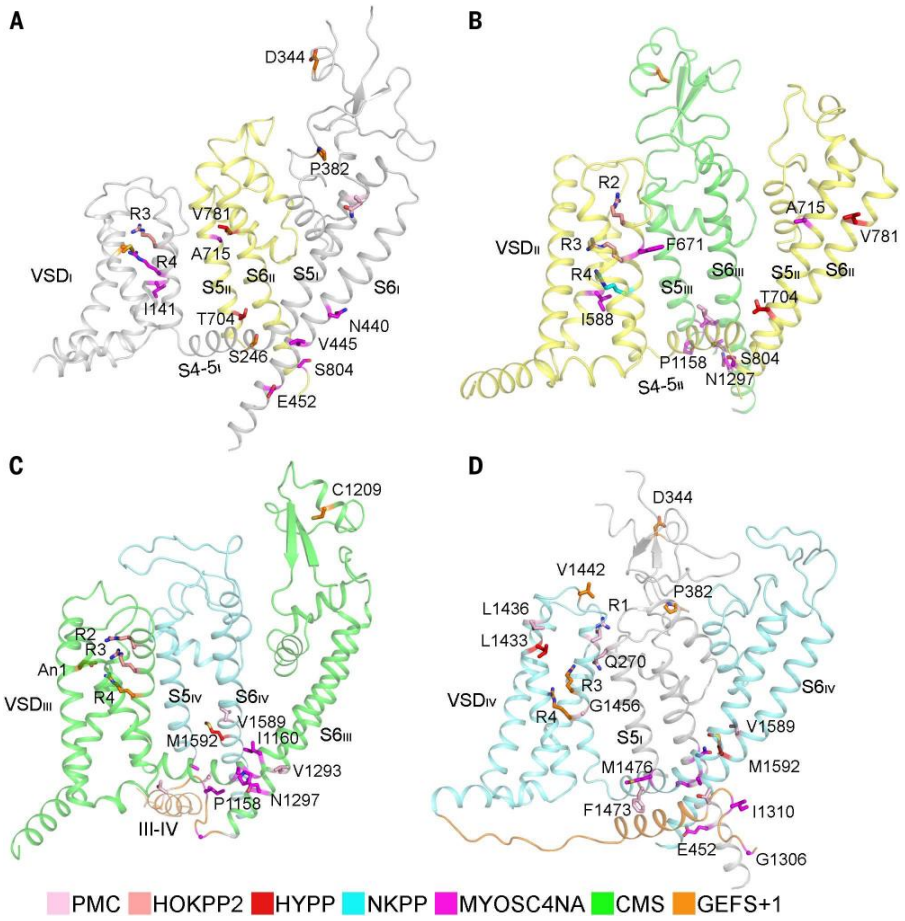


FIGURE 4. Structural features of $Na_V1.4$ mutations. A-D. Structural location of periodic paralysis mutations. PMC, paramyotonia congenita; HOKPP2, hypokalemic periodic paralysis; HYPP, Hyperkalemic Periodic Paralysis; NKPP, Normokalemic periodic paralysis; MYOSCN4A, myotonia caused by SCN4A mutations; CMS, congenital myasthenic syndrome; GEFS+1, Generalized epilepsy with febrile seizures plus-1. Adapted from (250).

They are also clustered in the S4 segment in domain IV, which is thought to trigger fast inactivation (19, 265). Physiological studies of muscle fibers dissected from patients exhibited an unusual persistent depolarization of myofibers, which was blocked by tetrodotoxin and therefore required the activity of the mutant sodium channels (187). Single channel recordings revealed many late openings following depolarization of muscle fibers from PMC patients, but not in fibers from control individuals (187). These results pointed to gain-of-function effects of PMC mutations on sodium channels. Electrophysiological analysis of the functional changes in the mutant $Na_V1.4$ channel expressed from cDNA in cultured nonmuscle cells revealed impaired inactivation of the sodium currents, caused by a combination of slowed and less steeply voltage-dependent fast inactivation and accelerated recovery from fast inactivation (34, 35, 113, 228, 380). These

functional changes would cause persistent sodium current and re-opening of single sodium channels in cells expressing these mutants. These gain-of-function effects lead to generation of inappropriate trains of action potentials and to uncoordinated twitching or myotonia. Although these mutations impair inactivation and increase persistent sodium current, they do not cause an increase in resting sodium levels in muscle fibers; however, cold challenge during exercise does cause an increase in intracellular sodium levels (365). Increased intracellular sodium during the bursts of action potentials that drive forceful muscle contractions would reduce the driving force for sodium current, depolarize muscle fibers, and cause failure of action potential generation. These cellular events may lead to the flaccid paralysis that follows episodes of myotonia and muscle stiffening in PMC.

Potassium-aggravated myotonia (PAM) is a milder disease than PMC. Stiffness and myotonia are initiated during exercise, and they may persist for hours, but they are not followed by flaccid paralysis (307). Muscle stiffness is aggravated by elevated serum potassium, but is unaffected by cold (307). Mutations that cause this disease are scattered along the intracellular surface of the sodium channel, especially in domain IV, and they include amino acid residues identified as important components of the fast inactivation gate and its receptor region (FIGURE 4A, B (307)). The functional effects are milder than those that cause PMC, but there is one mutation in the fast inactivation gate that causes both diseases, depending on the family and the genetic backgrounds of the patients (227).

C. Hypokalemic and Normokalemic Periodic Paralysis

Hypokalemic Periodic Paralysis (HypoPP) causes episodic flaccid paralysis associated with low potassium levels in serum and high potassium levels in skeletal muscle (157, 283, 357). Muscle fibers are depolarized and have a high internal sodium concentration, which leads to loss of excitability (157, 283, 357). Mutations in the *CACNAL1* gene encoding the skeletal muscle calcium channel $Ca_V1.1$ are the most frequent cause of HypoPP (158). In contrast to the broad range of mutations that cause HyperPP and PMC, mutations that cause HypoPP always change the first or second arginine gating charge in an S4 segment to a neutral amino acid residue (213, 307, 308). Introduction of a HypoPP mutation into mice reproduced the potassium-sensitive paralysis and weakness characteristic of HypoPP, further confirming the causative role of these gating charge mutations in the disease (376). Surprisingly, analogous mutations in the first or second gating charges (R1 and R2) in the S4 segment of the $Na_V1.4$ skeletal muscle sodium channel also cause this disease in humans and mice (FIGURE 4A, B (39, 323, 375, 376)), further

highlighting the pathogenic role of these gating charge mutations in HypoPP. The rare related disease, Normokalemic Periodic Paralysis (NormoPP), causes similar flaccid paralysis at normal levels of serum potassium (358). Remarkably, it is caused by mutations of the gating charge arginine in the third (R3) position in S4 segment of $Na_V1.4$ (358). This characteristic genetic feature that is shared by HypoPP and NormoPP mutations suggests a unique common mechanism of action of these mutations that are exclusively in gating charges. However, even though HypoPP mutations have a variety of effects on fast and slow inactivation of $Na_V1.4$ channels, none of these effects are potent enough to be the primary cause of the symptoms of HypoPP or NormoPP (159, 175, 323).

The enigma of the mechanism of HypoPP and NormoPP was resolved by discovery of gating pore currents that are induced by HypoPP and NormoPP mutations (312-314, 325, 326). As described above under Structure and Function of Sodium Channels, mutation of gating charge arginine residues to neutral amino acid residues can cause gating pore current through the mutant voltage sensor. Based on these earlier findings, it was natural to hypothesize that HypoPP mutations cause pathogenic gating pore current. As expected from this hypothesis, pathogenic gating pore currents could be detected for HypoPP mutations in $Na_V1.4$ (313, 325). The inward currents conducted by the pathogenic gating pores is in the range of 1% of the peak sodium current, and they are nonselective among monovalent cations (313, 314, 325, 326). In contrast to the nonselectivity among monovalent cations, mutants having His substituted for Arg often conduct protons selectively (325, 326). HypoPP mutations in $Na_V1.4$ channels are located in the R1 and R2 gating charges, and they cause gating pore current in the resting state (313). Even though the gating pore currents are small, they are constant and calculations indicate a large effect on sodium entry into muscle fibers, consistent with the increased intracellular sodium levels and depolarized membrane potentials in HypoPP muscle (313, 325).

In contrast to HypoPP, mutations that cause NormoPP specifically neutralize the R3 gating charge (358). They cause gating pore current in the activated and inactivated states, because the R3 gating charge is located in or near the HCS in the activated state of the voltage sensor (312). The voltage dependence of pathogenic gating pore current is complex: it is activated at strongly depolarized potentials but deactivated only at much more negative potentials resulting in a hysteresis loop (312). The low conductance of the mutant gating pore would not be pathogenic if the gating pore were only open briefly during an action potential. However, voltage clamp analysis showed that the mutant gating pore is open in both activated and slow-inactivated $Na_V1.4$

channels (312). Because slow inactivation accumulates during the long trains of action potentials that induce forceful muscle contraction, the mutant gating pore is open for sufficiently long intervals to cause periodic paralysis and degeneration of muscle fibers.

HypoPP and NormoPP mutations have been introduced into the bacterial sodium channel Na_VAb in order to study the structural basis for gating pore current (154). The central pore currents and gating pore currents generated by these mutants were analogous to those observed in pathogenic mutations in $Na_V1.4$ channels (FIGURE 5A, B (154)). The central pore currents were typical for sodium channels (Figure 5A, B, Left). However, the mutants exhibited marked gating pore currents (FIGURE 5 A, B, center and right). The HypoPP mutant R2S conducted gating pore leak currents at negative membrane potentials in the resting state (FIGURE 5A, center and right, blue), whereas the R3G mutant conducted gating pore leak currents at positive potentials in the activated state (FIGURE 5B, center and right, red). As expected from physiological studies, these mutations cause a voltage-dependent transmembrane conductance pathway through the voltage sensor itself that is clearly observed by X-ray crystallography (FIGURE 5C). The diameter of the pathogenic gating pore is 2-3 Å, consistent with conductance of dehydrated sodium ions at the low rate characteristic of gating pore current. The complete water-filled pathway through which the sodium ion moves is highlighted in magenta in FIGURE 4C, right, as revealed by analysis with the program MOLE (154).

Although gating pore current is nonselective, its primary pathogenic effects are caused by inward leak of Na^+. Quantitative estimates indicate that entry of Na^+ through the open gating pore would increase the resting leak of Na^+ more than two-fold and possibly as much as ten-fold (313, 314, 325, 326). This increase in inward leak of Na^+ causes elevation of intracellular Na^+ from ~15 mM in controls to a range of 19 mM to 25 mM in patients with different mutations (160). Muscle fibers are also depolarized from -87 mV in controls to a range of -74 mV to -77 mV in patients (160). These deficits in patients were significantly worsened by local cooling of muscle fibers, which has no significant effect on controls. The alterations in ion gradients and membrane potential also exacerbate a bi-stable state of excitability, in which the membrane potential jumps from hyperpolarized to depolarized levels in muscle fibers from patients much more than in controls (160). Similar effects were observed in a mouse model of $Na_V1.4$ HypoPP, including gating pore current, persistent depolarization, impaired action potential generation, and episodic weakness without myotonia (375). Although mutations in HypoPP are heterozygous, the gating pore current of the defective sodium channel causes dominant-negative physiological impairment of action

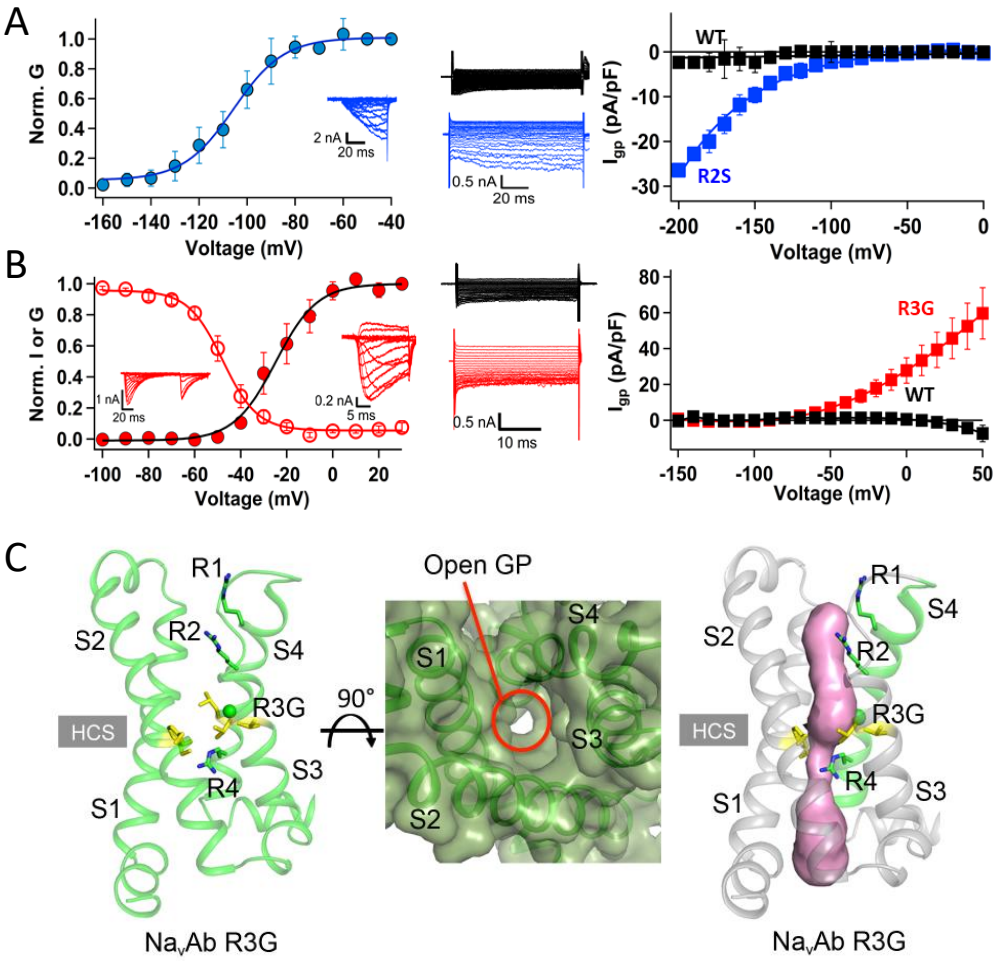


FIGURE 5. Pathogenic gating pore current and structure. A. Left, Central pore Na^+ currents (inset) and G/V curve for Na_vAb/R2S recorded during 200-ms depolarizations from -200 mV to the indicated potentials. Center. Leak Na^+ currents for WT (black) and Na_vAb/R2S (blue). Note the larger negative leak currents in Na_vAb/R2S due to gating pore current. Right. I/V curves for nonlinear leak currents for Na_vAb/R2S (blue) or Na_vAb/WT (black) elicited by depolarization from -100 mV to the indicated potentials. **B.** Central pore Na^+ currents (inset) and G/V curve for Na_vAb/R3G from a holding potential of -160 mV (filled circles). Voltage dependence of steady-state inactivation (open circles) for Na_vAb/R3G. Center. Leak Na^+ currents for Na_VAb/R3G (red) or Na_VAb/WT (black) for voltage steps from 0 mV to the indicated potentials. Right. I/V curves for nonlinear leak currents for Na_vAb/R3G (red) or Na_VAb/WT (black, n=11). Note the larger positive leak currents in Na_vAb/R3G due to gating pore current. **C**. Structure of a pathogenic gating pore in a Normokalemic Periodic Paralysis (NormoPP) mutation. HCS, hydrophobic constriction site; GP, gating pore; R1-R4, gating charges; S1-S4, transmembrane segments; magenta shading, water accessible space determined by MOLE2. Adapted from (154).

potential firing by the wild-type sodium channels through reduction of the sodium gradient and inactivation by chronic depolarization. Low extracellular potassium levels contribute to episodes

of flaccid paralysis in two parallel ways. First, low potassium reduces the effectiveness of sodium efflux mediated by the Na,K-ATPase, which exchanges extracellular potassium for intracellular sodium. This effect exacerbates the increase in intracellular sodium and membrane depolarization. Second, the reduced extracellular potassium increases the shift to the depolarized state by changing the ratio of the bi-stable hyperpolarized and depolarized states of membrane potential (160).

D. Congenital Myasthenic Syndrome and Congenital Myopathy

Myasthenias are characterized by development of weakness and fatigue during continuous use of skeletal muscles (102, 112). Most of the myasthenia syndromes are caused by failure of synaptic transmission, similar to myasthenia gravis (102, 112). However, rare cases of failure of action potential firing downstream of synaptic transmission have been discovered (102, 112). The Congenital Myasthenic Syndrome (CMS) is a recessively inherited disease most often caused by homozygous mutations that neutralize a gating charge Arg in the S4 segment in domain IV of $Na_V1.4$ (3, 131). Similar symptoms have been observed in patients with heterozygous mutations in the S4-S5 linker in domain I and the S4 segment and S3-S4 linker in domain IV (101, 347). These mutations cause a large negative shift in the voltage dependence of fast inactivation, slower recovery from inactivation, and in some cases, dramatically accelerated slow inactivation (3, 131). Altogether, these mutations greatly reduce the sodium current in transfected cells and impair action potential firing in skeletal muscle fibers (43). Their effects are most pronounced during sustained trains of action potentials, which trigger forceful muscle contraction. Early in an action potential train, generation of action potentials is nearly normal; however, as the train continues, increasing numbers of the mutant and wild-type sodium channels enter the fast- and slow-inactivated states, and eventually action potential generation fails causing fatigue (43).

Congenital myopathy is characterized by neonatal or early-onset weakness (273). It is caused by mutations in many genes. Fewer than twenty families worldwide have been identified with congenital myopathy caused by recessive *SCN4A* mutations (383). The mutations that have been analyzed have loss-of-function phenotypes, including complete or nearly complete loss of functional expression and large positive shifts in the voltage dependence of channel activation (383). The degree of impairment of muscle function correlates with the extent of loss of function of the mutant $Na_V1.4$ channels. The inheritance of full loss of function mutations on both *SCN4A* alleles causes a particularly severe phenotype resulting in early lethality.

E. Genotype-Phenotype Correlations in Periodic Paralysis

The periodic paralyses have characteristic differences in their effects on action potential sodium currents and on sodium leak, which determine the pathology of these muscle diseases (43). Gain of function mutations cause HyperPP and PMC by producing $Na_V1.4$ channels that are hyperactive, conduct excess persistent sodium current and/or re-open frequently after inactivation. In HyperPP, these functional effects at the cellular level cause repetitive action potential firing leading to sustained depolarization, inactivation of both wild-type and mutant $Na_V1.4$ channels, and episodes of weakness or flaccid paralysis (139). In PMC, changes in the kinetics of fast inactivation slow its onset and accelerate its recovery, thereby producing mutant sodium channels that are ready to re-activate immediately upon repolarization from the preceding action potential. These changes lead to inappropriate repetitive firing of action potentials that cause stiffness, the clinical hallmark of myotonia (43).

The pathogenesis of HypoPP and NormoPP is remarkably different from HyperPP and PMC (43, 312, 313, 325, 375). Mutations in the R1, R2, and R3 gating charges cause pathogenic gating pore currents, which leak protons and/or sodium into the cell, reduce the sodium gradient, depolarize the cell, and inactivate both wild-type and mutant $Na_V1.4$ channels due to depolarization. The constant inward gating pore current destabilizes the bi-stable resting membrane potential in skeletal muscle and causes oscillating changes that impair the electrophysiological stability of the muscle fiber (43). Gating pore current is a gain-of-function effect at the molecular level, but it causes loss of contractile function at the cellular level by these diverse mechanisms. Adult patients with HypoPP and NormoPP experience pathologic changes in their skeletal muscle fibers with advancing age, and a similar vacuolar pathology with disruption of transverse tubules and triad junctions is observed in mice bearing a HypoPP mutation in $Ca_V1.1$ (376), but the long-term outcomes of sodium channel HypoPP mutations in skeletal muscle of mutant mice have not yet been studied in detail. In humans, it is likely that the prolonged elevation of intracellular sodium and prolonged depolarization in HypoPP are responsible for local edema and changes of intracellular calcium signaling that cause degenerative cellular effects over time. Swelling and degeneration of muscle fibers increases progressively during the disease in response to altered ionic homeostasis. Repair mechanisms are not sufficient to counteract these cellular pathologies, leading to progressive failure of muscle function in addition to periodic paralysis.

III. BRAIN SODIUM CHANNELS: $Na_V1.1$ AND EPILEPSY

Epilepsy is caused by excess synchronized action potential generation in the brain, which in turn depends directly on sodium channels (128). Sodium channels are the molecular targets for major antiepileptic drugs, including phenytoin, carbamazepine, and lamotrigine (206). Therefore, it is not surprising that sodium channels are important molecular targets for mutations that cause epilepsy. Four sodium channel genes are expressed in the brain: $Na_V1.3$, which is expressed primarily in embryonic and early postnatal brain, plus $Na_V1.1$, $Na_V1.2$, and $Na_V1.6$, which are expressed increasingly in development and remain highly expressed in the mature brain (15, 126, 174, 344, 348). These three adult sodium channel types are all broadly expressed in the brain, but they differ in expression in different types of neurons and in localization in specific subcellular compartments of neurons. Early studies revealed differential expression of sodium channel types in hippocampal neurons, with $Na_V1.1$ primarily in cell bodies and $Na_V1.2$ primarily in dendrites and unmyelinated axons (368). $Na_V1.1$ is the dominant sodium channel in interneurons (382), whereas $Na_V1.6$ and $Na_V1.2$ are the dominant sodium channel types in excitatory neurons and their myelinated axons (33, 348, 350). It is likely that the differential expression and subcellular localization of these sodium channel subtypes prevents effective functional compensation when one of these channel types is mutated, even though their functional properties are similar.

All sodium channel types that are highly expressed in the brain are targets for epilepsy mutations, which cause epileptic syndromes with a wide range of severity and co-morbidities (38, 128, 219). The *SCN1A* gene that encodes the $Na_V1.1$ channel is the most frequently mutated gene in sodium channel epilepsies, and these mutations cause a broad spectrum of epilepsy syndromes from mild febrile seizures to intractable, drug-resistant developmental and epileptic encephalopathies (55, 202, 299) (FIGURE 6).

A. Dravet Syndrome

Dravet Syndrome is a developmental and epileptic encephalopathy that was first described clinically by Dr. Charlotte Dravet in Marseille (France) in the late 1970's in an exceptional clinical investigation for its time (97, 123). Children with this disease develop normally for six to nine months. The first symptom of the disease is usually febrile seizures, which evolve over a few months to frequent spontaneous seizures that become drug-resistant. Affected children lose developmental milestones that had previously been achieved, and they have severe cognitive impairment, autistic-like behaviors, ataxia, and disruption of circadian rhythms and sleep quality. Before the pathophysiology of the disease was well-understood, more than 30% of children died prematurely from Sudden Unexpected Death in Epilepsy (SUDEP) or from accidents related to

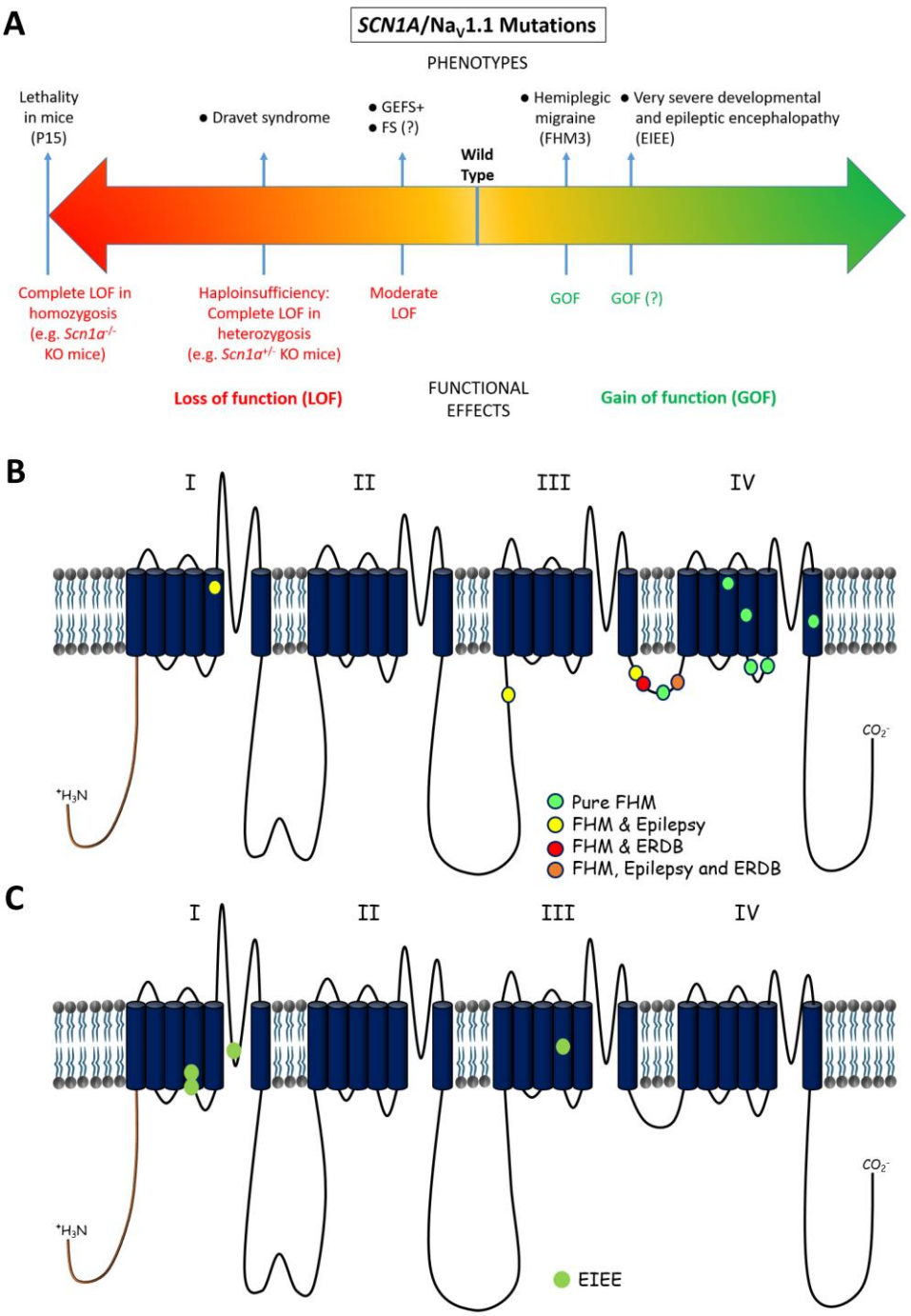


FIGURE 6. Spectrum of mutations and phenotypes for *SCN1A*/Na$_V$1.1. A. Phenotypic spectrum of *SCN1A* mutations. Most *SCN1A* mutations cause epileptic phenotypes, including the severe developmental and epileptic encephalopathy Dravet Syndrome and the milder form genetic epilepsy with febrile seizures plus (GEFS+), which however shows large phenotypic variability and can include severe cases. *SCN1A* variants may be also involved in febrile seizure phenotypes (FS) that can include development of temporal lobe epilepsy with hippocampal sclerosis. These forms are caused by loss of function of Na$_V$1.1 in heterozygosis, with often complete loss of function for Dravet syndrome, which is modeled by *Scn1a*$^{+/-}$ knock out mice. Gain of function of Na$_V$1.1 has been identified for hemiplegic migraine (FHM3) mutations and

proposed for mutations that cause an extremely severe Early Infantile Epileptic Encephalopathy (EIEE), although functional studies for this form have been performed for a single mutation. **B.** Molecular map of the $Na_V1.1$ sodium channel with the location of hemiplegic migraine mutations color-coded as indicated (ERDB: elicited repetitive daily blindness). **C.** Molecular map of the $Na_V1.1$ sodium channel with the location of *SCN1A* EIEE mutations. Mutations causing other phenotype are not shown because they are too many for a graphical representation.

their seizures and impaired cognition. Even with modern care, more than 15% of affected individuals die prematurely (77), and most of those who survive to their teenage years have IQ's in the range of 50 and require lifelong care (97, 123).

Dravet Syndrome is caused by genetically dominant loss-of-function mutations in $Na_V1.1$, which lead to deletions, truncations, nonsense codons, and single amino acid substitutions located throughout the coding exons of the gene (69, 70, 118, 328). Mutations that cause the truncation of $Na_V1.1$ lead to pure haploinsufficiency (14). More than 80% of patients diagnosed with Dravet Syndrome have a mutation in $Na_V1.1$ (77). Moreover, because only the 6 kb exons of the gene that code for the $Na_V1.1$ protein are normally sequenced, it is likely that many of the other patients diagnosed with Dravet Syndrome have mutations in the remaining 94% of the 100-kb *SCN1A* gene that cause loss of expression of the $Na_V1.1$ protein and result in Dravet Syndrome.

1. Loss of action potential firing in inhibitory interneurons in mouse models of Dravet Syndrome

It was a paradox that loss-of-functions mutation in a sodium channel would cause epilepsy. This paradox was resolved through studies of mouse models of Dravet Syndrome, which have spontaneous generalized tonic-clonic seizures that are easily observed by video recording or by electroencephalography (FIGURE 7A (201, 243, 382)). Electrophysiological analysis of these mice revealed a selective loss of sodium currents and action potential firing in GABAergic interneurons in the hippocampus (55, 201, 243, 382). Subsequent studies have shown a similar selective loss of electrical excitability in the GABAergic Purkinje neurons in the cerebellum, in the GABAergic neurons of the reticular nucleus of the thalamus, and in both parvalbumin-expressing and somatostatin-expressing interneurons in Layer V of the cerebral cortex (FIGURE 7B (83, 162, 164, 331)). In each case, GABAergic inhibitory neurons were unable to sustain long trains of action potentials, which is required for effective control of the firing of excitatory neurons in neural circuits.

Studies of local synaptic circuits are also consistent with this phenotype. Recordings of spontaneous, action potential-driven excitatory postsynaptic currents and inhibitory postsynaptic

currents in the CA1 neurons in hippocampal slices showed that the frequency of inhibitory postsynaptic currents was decreased (133, 140).

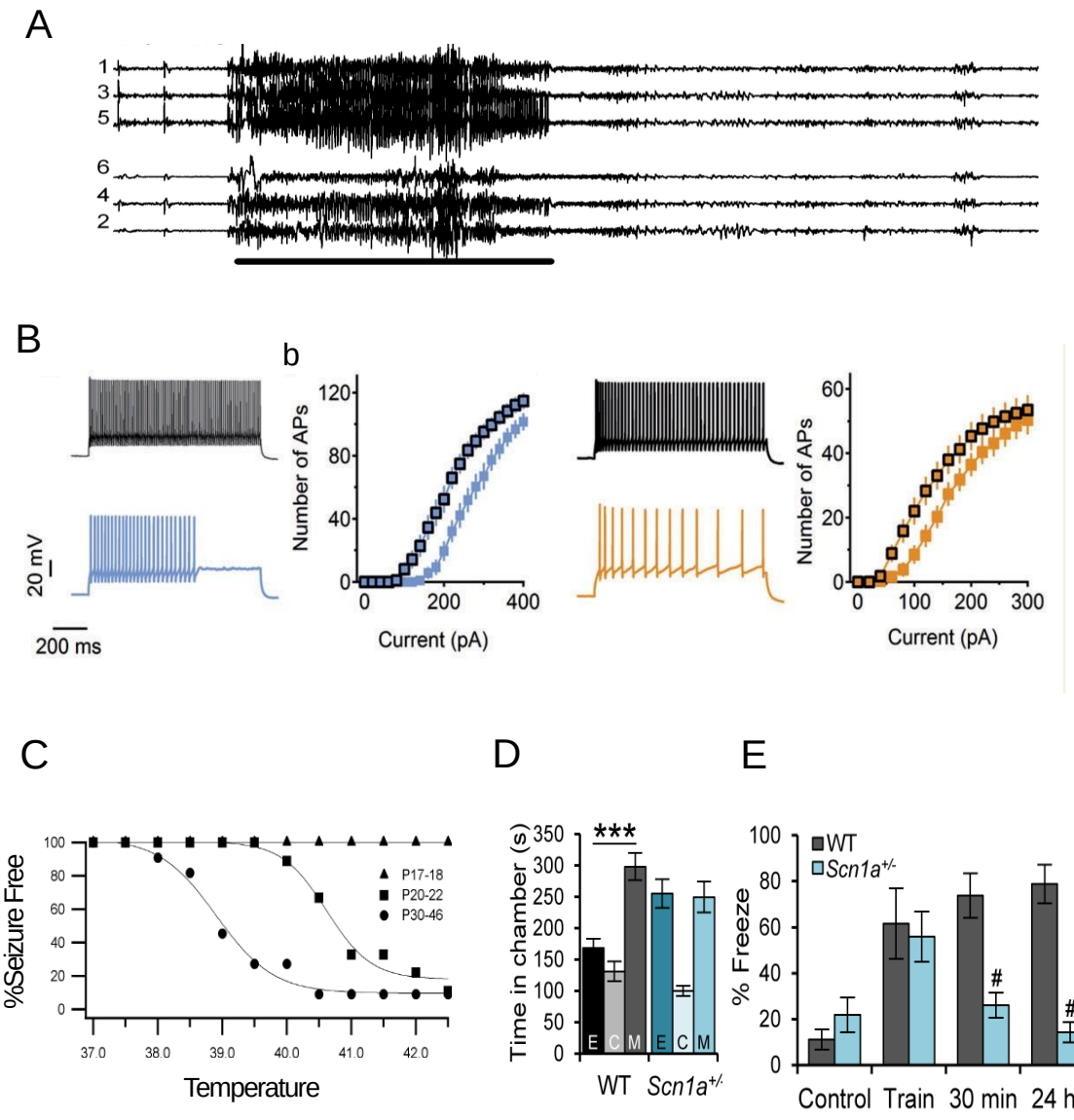


FIGURE 7. Cellular and systems phenotypes of Dravet Syndrome in mice. A. Electroencephalogram of a generalized tonic-clonic seizure in a Dravet Syndrome mouse. **B.** Loss of firing in interneurons. APs, action potentials; pA, picoamperes of stimulating current. *Left*, parvalbumin-expressing interneurons in Layer V of the cerebral cortex. Black, wild-type; blue, DS. *Right*, somatostatin-expressing interneurons in Layer V of the cerebral cortex. Black, wild-type; gold, DS. Adapted from (331). **C.** Thermal induction of seizures. **D.** Autistic-like social interaction behavior. **E.** Context-dependent fear conditioning. Adapted from (133).

In response to that decrease, the frequency of excitatory postsynaptic currents was increased, and the hippocampal network became hyperexcitable (133, 140, 194). In recordings of

hippocampal circuit function *in vivo*, the frequency of occurrence of sharp waves and sharp-wave ripples was reduced, and the frequency of individual ripples within a single sharp-wave ripple was also significantly reduced (64). These findings are consistent with impairment of action potential firing by parvalbumin-sensitive inhibitory basket cells in the hippocampus, whose firing provides critical timing information for sharp-wave ripples (42). Thus, action potential firing in interneurons is impaired, the ratio of excitatory to inhibitory synaptic activity in neural circuits is consistently increased, and the timing of circuit function is altered in the hippocampus in the early stages of Dravet Syndrome in mice. Moreover, the hippocampus is directly implicated in the generation of seizures (194), and selective heterozygous deletion of $Na_V1.1$ in the hippocampus is sufficient for causing thermal seizures and cognitive deficit characteristic of Dravet Syndrome (321).

In the cerebral cortex, disynaptic inhibition between neighboring pyramidal neurons in Layer V, which is mediated by somatostatin-positive frequency-accommodating interneurons (306), was strikingly impaired in DS mice (331). In brain slices of cerebral cortex *ex vivo*, the excitability of parvalbumin-positive and somatostatin-positive interneurons was reduced in DS mice, and optogenetic silencing of those neurons led to circuit hyperexcitability (83). However, studies of network function *in vivo* did not reveal consistent changes in the cerebral cortex, suggesting that compensatory mechanisms may be engaged in the in vivo experimental setting (83). Of note, dysfunctions might be more visible when the circuit is challenged and involved in high frequency activity, as observed in the hippocampus (194).

2. Febrile seizures in a mouse model of Dravet Syndrome

Dravet Syndrome usually begins with febrile seizures during a fever, a hot day, or a hot bath (97, 245). This characteristic of Dravet Syndrome is mimicked in genetically modified mice by thermally induced seizures (240). Slow increase in the core body temperature of DS mice into the fever range of 38-41^{o}C generates myoclonic seizures followed by generalized tonic-clonic seizures (FIGURE 7C). As in children with Dravet Syndrome, susceptibility to thermally induced seizures is age-dependent, with onset at a similar developmental time near the age of weaning, followed by more severe seizures and premature death (FIGURE 7C (65, 97, 240)).

3. Genetic evidence for a primary role of interneurons in Dravet Syndrome

Genetic evidence for a primary role of inhibitory interneurons in Dravet Syndrome has come from specific gene deletion with the Cre-Lox method (66, 242). Deletion in forebrain interneurons using the Dlx promoter/enhancer, which is specifically expressed in developing GABAergic interneurons

that arise in the medial ganglionic eminence and migrate to cerebral cortex, hippocampus, thalamus, and other forebrain structures (263), recapitulates the thermally induced seizures, spontaneous seizures, and premature death of mice observed with global deletion of $Na_V1.1$ channels (66). In contrast, specific deletion of $Na_V1.1$ in excitatory neurons ameliorates the phenotypes of Dravet Syndrome (242). These results clearly demonstrate that the primary cause of the core symptoms of Dravet Syndrome is failure of normal action potential firing by GABAergic interneurons. Consistently, single-cell transcriptomic data from humans and mice have confirmed that *SCN1A* is predominantly expressed in inhibitory neurons (98), and transcranial magnetic stimulation paradigms applied to Dravet Syndrome patients have disclosed reduced intracortical inhibition *in vivo* (322).

4. Co-morbidities in a mouse model of Dravet Syndrome

The major co-morbidities of Dravet Syndrome are also caused by failure of firing of GABAergic interneurons (51). (i) Ataxia is correlated with failure of action potential firing of the GABAergic Purkinje neurons of the cerebellum, which are crucial for coordination of movement (164). (ii) Cognitive deficit in context-dependent fear conditioning (FIGURE 7D) and spatial learning, and autistic-like behaviors in social interaction tests (FIGURE 7E) are all observed in mice in which $Na_V1.1$ channels have been specifically deleted in forebrain inhibitory interneurons (133, 335). (iii) Impaired sleep quality is also observed in mice with specific deletion of $Na_V1.1$ channels in forebrain interneurons, and it is correlated with failure of firing of the GABAergic interneurons of the reticular nucleus of the thalamus, which set sleep rhythms by driving sleep spindles (162). Failure of firing of these neurons has also been observed in different epileptic *Scn1a* models (140); however, one study puzzlingly reported hyperexcitability of these neurons in brain slices from *Scn1a*$^{R1407X/+}$ mice (276). (iv) The circadian rhythm defect is also correlated with failure of firing of the GABAergic neurons of the suprachiasmatic nucleus of the hypothalamus, and it is rescued by enhancement of GABAergic neurotransmission with the benzodiazepine clonazepam, implicating failure of firing of interneurons as the primary cause of this defect (133). Thus, Dravet Syndrome is an interneuronpathy in which an increase in excitation:inhibition balance in neural circuits throughout the brain causes the multifaceted co-morbid disease phenotypes.

The Cre-Lox method has also been used to probe the functional roles of different classes of interneurons in the multifaceted phenotypes of Dravet Syndrome (Table 1 (281)). Nearly all of the interneurons in the cerebral cortex can be divided into three classes identified by their expression of parvalbumin, somatostatin, or the 5-HT3a receptor (285, 343). Heterozygous deletion of $Na_V1.1$

channels in parvalbumin-expressing interneurons causes pro-epileptic effects and autistic-like behavior (281). Deletion in somatostatin-expressing interneurons causes pro-epileptic effects and hyperactivity (281). Deletion in these two classes of interneurons together gives synergistic effects on epilepsy, premature death, and cognitive deficit (281). In contrast, deletion in 5-HT3a receptor-expressing interneurons has much milder effects (Table 1 (369)). It has been suggested that 5-HT3a receptor-expressing interneurons do not express $Na_V1.1$ (378). However, single cell RNA sequencing investigations suggest expression of $Na_V1.1$ in these neurons (257, 334), and recent studies have shown reduced excitability of a subset of 5-HT3a receptor-expressing interneurons (irregular spiking VIP positive interneurons) in layer II-III of the primary somatosensory and visual cortex (125, 370). Altogether, these studies indicate that altered excitation:inhibition balance in brain circuits, induced mainly by failure of action potential firing by parvalbumin-expressing interneurons, somatostatin-expressing interneurons, or both, induces both the core symptoms and the co-morbidities of Dravet Syndrome.

Interneuron	Epilepsy	SUDEP	Hyperactivity	Autistic Behavior	Cognitive Deficit
PV	++	+/-	-	+	-
SST	+	-	+	-	-
PV + SST	+++	++	+	+	+
5-HT3R	-	-	-	+/-	-
All: Dlx-Cre	++++	++++	+	+	++

Table 1. Functional Impacts of Deletion of $Na_V1.1$ Channels in Specific Classes of Interneurons.

5. SUDEP in a mouse model of Dravet Syndrome

SUDEP is the most serious result of Dravet Syndrome, and parents and caregivers live in fear of this devastating outcome (123, 235, 309). In C57BL/6J mice, Dravet Syndrome mutations cause a wave of high incidence of SUDEP on P21-P28 (163, 243, 382). Specific deletion of $Na_V1.1$ channels in forebrain interneurons is sufficient to cause the primary wave of SUDEP during P21 to P28, indicating that epilepsy itself causes premature death at this time rather than other parallel effects of deletion of $Na_V1.1$ channels in heart or other peripheral tissues (163). Consistent with this conclusion, specific heterozygous deletion in the heart does not cause SUDEP (163). SUDEP

in DS mice is correlated with dramatic bradycardia (163), respiratory disturbances (172), and sudden cardiac arrest following generalized tonic-clonic seizures (163). Sudden death is prevented by peripheral administration of N-methylscopolamine, a peripherally restricted inhibitor of muscarinic acetylcholine receptors (163). These results point to overactive parasympathetic cholinergic outflow from the CNS during and following generalized tonic-clonic seizures, resulting in hyperactivation of cardiac muscarinic acetylcholine receptors, as the primary mechanism underlying SUDEP during P21-P28 in this model of Dravet Syndrome (163). Abnormal parasympathetic regulation may disturb both respiration and cardiac function; however, prevention of sudden death by treatment with the non-CNS-penetrant muscarinic antagonist N-methylscopolamine administered in the peripheral circulation supports the conclusion that hyperactivation of muscarinic acetylcholine receptors in the heart, resulting in severe bradycardia and ventricular arrhythmia, is the proximate cause of death in the Dravet Syndrome model in C57BL/6J mice during P21-P28 (163).

6. Genetic background effects in Dravet Syndrome

Genetic background effects are important in Dravet Syndrome. Complete loss-of-function truncation mutations cause differing degrees of disease severity in Dravet Syndrome patients with different genetic backgrounds (97), and dramatic genetic background effects are observed for identical mutations in different strains of mice (282, 382). In the first study of Dravet Syndrome in mice, the C57BL/6J strain was found to be much more susceptible to the disease mutation compared to the 129SvJ strain (382). For that reason, most subsequent studies have been carried out on mice bred for more than ten generations into C57BL/6J. In this genetic background, Dravet Syndrome mice recapitulate all of the complex symptoms and co-morbidities of the human disease, as reviewed above. Physiological studies of Dravet Syndrome mice in the C57BL/6 genetic background and the 129SvJ genetic background revealed less severely impaired action potential firing and less severely impaired sodium channel-dependent boosting of excitatory postsynaptic currents recorded in GABAergic inhibitory neurons in 129SvJ mice (282). These milder effects of the DS mutation on action potential firing in 129SvJ mice may contribute to the milder phenotype of DS in these mice. Gene mapping has identified a subunit of $GABA_A$ receptors as a contributing molecular factor to this difference in disease severity, again pointing to defects in inhibitory neurotransmission as fundamental in Dravet Syndrome (137).

Dravet Syndrome has also been studied in *Scn1a*$^{+/-}$ mice of mixed genetic backgrounds generated by crossing C57BL/6J with 129S6.SvEvTac (135, 226). The resulting 50:50 F1

generation mice have a milder epileptic phenotype than pure-bred C57BL/6J, and they require temperatures in the range of 42.5ºC for thermal induction of seizures (135). This temperature range is characteristic of heat stroke, so the seizures induced at that temperature may not be epileptic in origin. These mice have not been analyzed for co-morbidities of Dravet Syndrome to date. Thus, at this stage, they are a less complete model for studies of Dravet Syndrome than pure-bred C57BL/6J.

7. Compensatory effects in Dravet Syndrome

In addition to *SCN1A*, three other sodium channel genes are broadly expressed in the brain (344), but they do not effectively compensate for loss of $Na_V1.1$ channels in Dravet Syndrome. Early studies revealed increased expression of $Na_V1.3$ channels in the hippocampus (382) and increased activity of $Na_V1.6$ channels in the Purkinje cells of the cerebellum (164), but neither of these compensatory changes is sufficient to prevent the symptoms of Dravet Syndrome (164, 382). In mice with mixed 50:50 C57BL/6J:129S6.SvEvTac genetic background, up-regulation of sodium current was observed in dissociated hippocampal pyramidal neurons from P21 mice (226). Increased sodium channel activity at P21 may be important for generation of the first spontaneous seizures, which occur at this age in Dravet Syndrome mice. In parvalbumin-expressing layer II-III interneurons in brain slices from the primary somatosensory cortex of these mixed-breed mice, action potential firing was diminished at P18-21, but returned to normal at P35-56 (110). These results open the possibility that normalization of action potential firing in these neurons takes place with increasing age, which would correspond to late childhood and early teenage years in humans when seizures in Dravet Syndrome become less severe and more effectively treated with antiepileptic drugs (123). However, the reduction of sodium current and the impairment of action potential firing in cortical neurons is less prominent than in hippocampal neurons in C57BL/6 mice (83, 194, 331, 382). Therefore, it will be important to determine if the excitability of these highly sensitive hippocampal neurons, as well as of cortical interneurons of other layers and cortical regions, also returns toward wild-type in older mice or remains low and continues to create hyperexcitability in neuronal circuits in the adult.

8. Antiepileptic therapies tested in animal models of Dravet Syndrome

Mouse models of Dravet Syndrome have been used to test both pharmacological treatments and gene therapy-like approaches. The first studies tested the effect of drugs and treatments already used in the clinic on seizures induced with hyperthermia or the convulsant flurothyl. The ketogenic

diet (a high fat/low carbohydrates and protein diet used as an alternative treatment for refractory epilepsy) was effective in reducing the sensitivity to flurothyl-induced seizures (99). Stiripentol (an antiepileptic approved specifically for Dravet Syndrome) or combinatorial therapy with stiripentol plus clobazam (a positive allosteric modulator of GABA-A receptors) were effective in reducing convulsant-induced seizures (46). Moreover, clonazepam (a positive allosteric modulator of GABA-A receptors) and tiagabine (a presynaptic GABA reuptake inhibitor) were effective individually in increasing the threshold for thermal induction of seizures and gave synergistic beneficial effects when given together (239). Overall, these results established the Dravet Syndrome mouse model as a potentially valuable asset in testing new drugs and drug combinations in Dravet Syndrome.

More recent studies have tested drugs that are not yet used extensively in the clinic. The synthetic neuroactive steroid SGE-516 is a potent positive allosteric modulator of both synaptic and extrasynaptic GABA-A receptors. It increased threshold for hyperthermia-induced seizures, decreased frequency of spontaneous convulsive seizures, and prolonged survivals (136), consistent with other GABA-A receptor enhancing treatments. GS967, a non-conventional sodium channel blocker that preferentially inhibits persistent sodium current, was unexpectedly effective in reducing frequency of spontaneous seizures and mortality (2). The effect was probably related to a reduction of the excitability of excitatory neurons, which was enhanced in chronic administration because it reduced expression of $Na_V1.6$ channels. A recent study reported reduction of seizures with chronic administrations of the peptidic toxin Hm1a, which can act as a specific $Na_V1.1$ enhancer at nanomolar concentrations (274). However, delivery and dosage in clinical settings are challenging problems for this approach, in particular considering that at slightly higher concentrations Hm1a can hit numerous other targets (106).

Cannabidiol (CBD), a non-psychotropic cannabinoid extracted from *Cannabis sativa*, is increasingly used to treat refractory epilepsy, and a small clinical trial showed a beneficial effect on Dravet Syndrome patients (89). Clinical trials in Dravet Syndrome are challenging because of the small number of patients, the wide age range of patients, and the differences in background standard-of-care medications that they receive concomitantly. Nevertheless, consistent with these clinical trial results, CBD can reduce the duration and severity of hyperthermia-induced seizures and the frequency of spontaneous convulsive seizures of *Scn1a*$^{+/-}$ mice under carefully controlled laboratory conditions (166), further supporting its therapeutic efficacy. When tested in brain slices *in vitro*, CBD increased inhibitory neurotransmission and decreased excitatory

neurotransmission in the granule cell neurons of the dentate gyrus of the hippocampus, as measured by the frequency of spontaneous inhibitory and excitatory postsynaptic currents. These effects were caused by direct increase of the frequency of action potentials in GABAergic neurons and a resulting decrease in dentate granule cell neurons. This latter effect may be due in part to the reduction of persistent current generated by $Na_V1.6$ channels, which can be inhibited by CBD in transfected nonneuronal cells expressing $Na_V1.6$ (124, 256). CBD binds in the pore of an ancestral sodium channel, providing a molecular mechanism for this inhibitory effect (289). Notably, CBD's actions on mouse dentate granule cells from *Scn1a*$^{+/-}$ mice did not depend on activation of CB1 cannabinoid receptor, but were potentially mediated by antagonism of the lipid-activated G protein-coupled receptor GPR55 (166).

Surprisingly, *Scn1Lab*$^{-/-}$ mutant zebrafish have provided a model of Dravet Syndrome that can be efficiently used in drug screens (9, 95). These screens have identified serotoninergic drugs that in some cases were effective when tested in Dravet Syndrome patients, and more detailed studies identified 5-HT2B receptors as the potential drug target (127, 316). Clinical trials are in progress to test the efficacy of previously developed drugs that target serotonin receptors for prevention of seizures in Dravet Syndrome (*https://www.epygenix.com/pipelines/*). Although the motor dysfunction induced in zebrafish by *Scn1a* mutations is not similar to seizures in mammalian brain and the concentration of drug candidates used in this system are much higher than typical for human pharmacology, the successes to date indicate that phenotypic screens in this novel experimental system have promise for discovery of unexpected classes of drug candidates for treatment of intractable epilepsy.

Gene therapy approaches have been recently developed for upregulating the wild-type *Scn1a* allele in *Scn1a*$^{+/-}$ mice. One study identified a novel antisense non-coding RNA (SCN1ANAT) implicated in a physiological mechanism that downregulates the expression of *Scn1a* (148). Antisense oligonucleotides (AntagoNATs) targeting and degrading SCN1ANAT were able to specifically upregulate *Scn1a* expression in vitro and in vivo following intrathecal administration in the brain of a Dravet Syndrome mouse model and a wild-type non-human primate (148). Four weekly injections of AntagoNATs decreased frequency of spontaneous convulsive seizures and increased the threshold for hyperthermic seizures. Moreover, the treatment partially rescued the excitability of parvalbumin-positive hippocampal GABAergic neurons. A more recent study used antisense oligonucleotides targeted to *Scn8a* for reducing expression of $Na_V1.6$, which is the primary sodium channel driving firing of excitatory neurons. A single treatment of Dravet

Syndrome mice with *Scn8a* antisense oligonucleotides at P2 reduced spontaneous convulsive seizures and delayed mortality onset from 3 weeks to beyond 5 months of age (186), a surprisingly long-lasting beneficial effect that is consistent with the therapeutic benefits obtained by heterozygous gene deletion of $Na_V1.6$ in another study (211). An additional approach used the CRISPR-ON system with a catalytically dead Cas9 (dCas9) to upregulate *Scn1a* expression (73). A specific single guide RNA (sgRNA) that increases *Scn1a* gene expression levels in cell lines and primary neurons with high specificity was identified. Treatment with this CRISPR-ON strategy was able to increase $Na_V1.1$ protein levels and rescue excitability of GABAergic neurons from *Scn1a*$^{+/-}$ mice in primary culture. Delivery of the *Scn1a*-dCas9 activation system to *Scn1a*$^{+/-}$ neonates (before disease onset) using adeno-associated viruses enhanced excitability of parvalbumin-positive interneurons in brain slices and increased threshold for hyperthermic seizures (73). Most recently, Han et al (2020) used a Targeted Augmentation of Nuclear Gene Output (TANGO) approach to increase the expression of functional $Na_V1.1$ channels in Dravet Syndrome mice (134). They observed a substantial decrease in spontaneous seizures and SUDEP when the specific antisense oligonucleotides were injected in newborn mice.

These methods hold great promise for treatment of Dravet Syndrome in children who can be identified by gene sequencing early in life before their symptoms arise, although genotype-phenotype relationships are not always straightforward. Altogether, these genetic approaches can have a high level of specificity for $Na_V1.1$ channels and reverse the effects of loss-of-function of this channel, but the timing of treatment, method of delivery, and half-life of therapeutic agents are still challenging problems that need to be solved.

B. Genetic Epilepsy with Febrile Seizures Plus

The first pathogenic $Na_V1.1$ mutations were identified in Genetic Epilepsy with Febrile Seizure Plus (GEFS+) (105), which is characterized by a large phenotypic spectrum. Disease onset is between 3 months and 6 years with febrile/hyperthermic seizures that continue to occur into adulthood. Spontaneous afebrile seizures develop later, and the most severe phenotypes in the GEFS+ spectrum are similar to Dravet Syndrome (388). GEFS+ is genetically heterogeneous. Mutations are often identified in large families with autosomal dominant segregation and incomplete penetrance. Mutations of $Na_V1.1$ have been identified in about 20% of GEFS+ families, and they are all missense mutations.

GEFS+ $Na_V1.1$ mutations were also the first to be functionally investigated in vitro (199). For this study, the cDNA of the long human splice variant was used, and the observed effect on $Na_V1.1$ current was reduction of inactivation leading to increased persistent sodium current. This gain-of-function effect would be consistent with enhanced neuronal excitability, but that was opposite to the predicted effect of truncating DS mutations that had been previously identified (70). However, the same group reported loss-of-function for other GEFS+ mutations, in some causing complete loss-of-function, as for a DS missense mutation that was studied in parallel (198). Although for a few other GEFS+ mutations a net gain-of-function effect was reported un early work, studies performed thereafter have consistently shown that the general functional effect identified in transfected cells of both DS and GEFS+ $Na_V1.1$ mutations is loss of function, (201). The initial variability of functional effects was probably generated by the smaller functional effect of GEFS+ mutations compared to Dravet syndrome mutations, and by the experimental conditions used for the functional studies; in particular, the cellular background and the type of cDNA (including the splice variant) can have an important modulatory effect. Notably, the R1648H $Na_V1.1$ GEFS+ mutation, which has been reported as a gain-of-function in an investigation that used heterologous expression systems (199), has been expressed in transgenic mice and its functional effects have been studied in dissociated neocortical neurons (332). This study showed that in a neuronal cell background it induces loss-of-function, but with modifications that are neuron subtype-specific: slower recovery from inactivation and increased use-dependent inactivation in bipolar GABAergic interneurons, but negative shift of voltage dependence of inactivation in pyramidal neurons.

Clinical phenotypes observed in patients carrying the R1648H mutation show large variability, ranging from mild GEFS+ to severity approaching that of Dravet Syndrome (87, 105). A gene-targeted knock-in mouse model of the R1648H mutation was subsequently generated (*Scn1a*$^{R1648H/+}$) to correlate neuronal dysfunctions with phenotypic features (210). In the first studies, it was reported that the phenotype of this model is characterized by hyperthermic and spontaneous seizures (210) and sleep dysfunctions (253), which were milder in comparison with mice carrying truncating mutations that model Dravet Syndrome.

Experiments performed in brain slices obtained from *Scn1a*$^{R1648H/+}$ mice (140) have shown hypoexcitability of GABAergic interneurons in cerebral cortex, hippocampus, and thalamus, as quantified by the reduced number of action potentials in input-output relationships, without detectable modifications in excitatory neurons.

Reduced post-hyperpolarization firing in GABAergic neurons of the reticular nucleus of the thalamus was also observed (140), as reported in global knock-out *Scn1a*$^{+/-}$ mice (162). Hypoexcitability of GABAergic neurons caused a reduction in action potential-induced postsynaptic GABAergic currents and tonic GABAergic current, as well as abnormal thalamo-cortical and hippocampal spontaneous network activities, which showed pathologic high frequency oscillations that were not observed in control. Thus, the basic pathological mechanisms observed in *Scn1a*$^{R1648H/+}$ mice are similar to those observed in models of Dravet Syndrome truncating mutations, supporting the conclusion that loss-of-function is responsible for the range of GEFS+ phenotypes of this mutation. However, the observed phenotype is milder, recapitulating the mildest phenotypes in the GEFS+ spectrum, rather than the more severe phenotype approaching that of Dravet Syndrome observed in some R1648H patients. The spectrum of phenotypes observed in these patients is consistent with the hypothesis that loss of function mutations in *SCN1A* cause a range of phenotypes, of which Dravet Syndrome is the most severe (55).

Interestingly, a recent study has shown that synergistic interactions between the genetic mutation and seizures can cause pathologic remodeling in *Scn1a*$^{R1648H/+}$ mice that leads to a severe Dravet Syndrome-like phenotype (291) (FIGURE 8). To exclude the contribution of spontaneous seizures, this study used *Scn1a*$^{R1648H/+}$ mice in a 50%-50% 129:C57Bl/6 F1 genetic background, which are asymptomatic and do not show spontaneous seizures, but have hyperthermia-triggered seizures. Short seizures (less than 1 min) were induced experimentally with hyperthermia or with the convulsant flurothyl (1 seizure/day for 10 days, starting at P21). Hyperthermia triggered seizures only in *Scn1a*$^{R1648H/+}$ mice, generating a chronic severe epileptic phenotype and cognitive/behavioral defects of hyperactivity, reduced social interaction, and deficits in learning and memory in adulthood, without major modifications in cytoarchitecture or neuronal death (291). Increased excitability was observed in hippocampal dentate granule cells but not CA1 pyramidal neurons, consistent with pathological remodeling that is not directly related to the effect of the mutation on GABAergic neurons. Flurothyl triggered similar seizures in both *Scn1a*$^{R1648H/+}$ mice and wild-type littermates, but led to chronic epilepsy, behavioral/cognitive dysfunctions and hyperexcitability of hippocampal granule cells in *Scn1a*$^{R1648H/+}$ mice, which showed a phenotype similar to that observed after induction of hyperthermic seizures. Thus, these results show that *Scn1a* genetic mutations/variants, even if mild *per se*, can make the brain sensitive to the deleterious effect of seizures, even short ones, and increase the risk of second hits to develop severe phenotypes. Strikingly, the interaction between the *Scn1a* mutation and seizures is necessary for generating pathological remodeling leading to a severe phenotype. This is relevant

for both GEFS+ and Dravet Syndrome, suggesting that seizures should be controlled to avoid phenotypic worsening, and also for other common epilepsies in which *SCN1A* genetic variants have been identified as risk factors (76, 104, 150, 169).

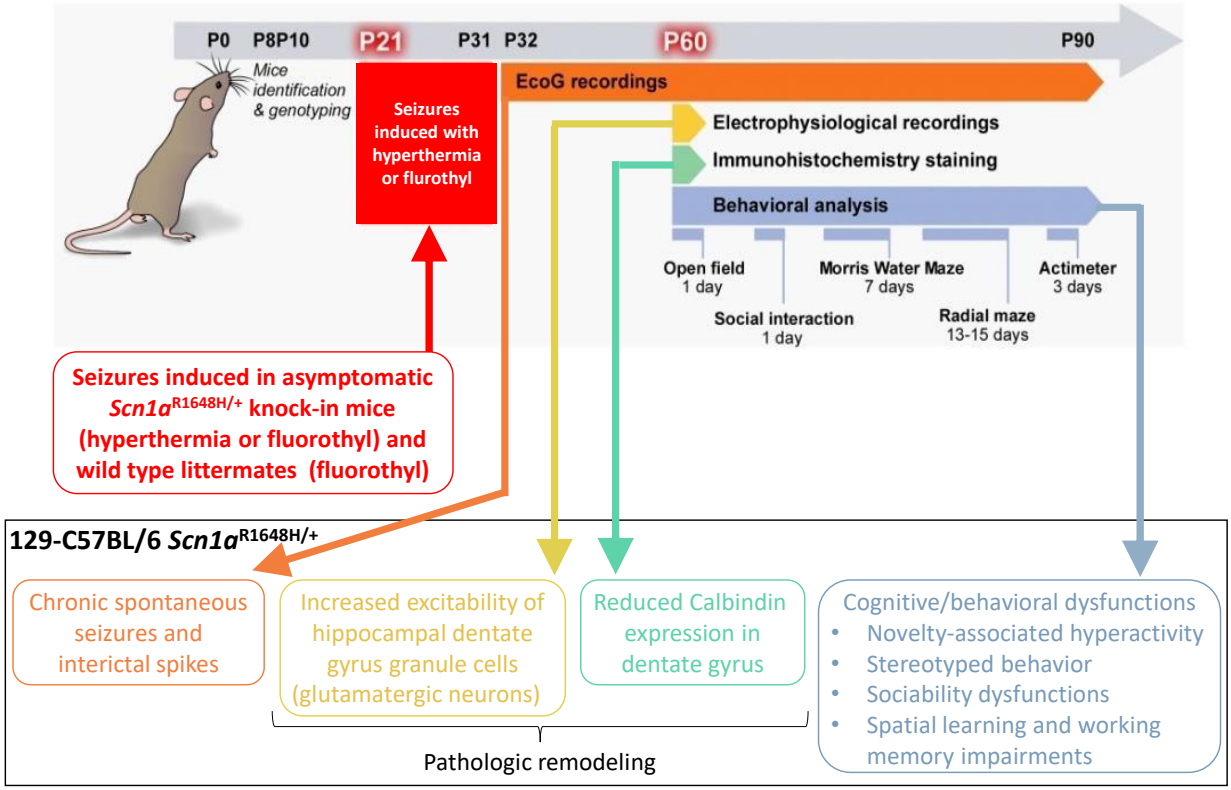


FIGURE 8. Synergistic interactions between *SCN1A* mutations and seizures leading to pathologic remodeling and development of severe phenotypes. Top. Protocol timeline. Bottom. Outcomes of the experiments that showed the *Scn1a*$^{R1648H/+}$ knock-in mice in the 129-C57BL/6 genetic background have an asymptomatic phenotype. The induction of short repeated seizures had no effect on WT mice, but it transformed the asymptomatic phenotype of *Scn1a*$^{R1648H/+}$ mice into a severe Dravet syndrome-like phenotype, including frequent spontaneous seizures and cognitive/behavioral deficits. In these mice, there were no major modifications in cytoarchitecture or neuronal death, but increased excitability of hippocampal dentate gyrus granule cells and increased expression of calbindin, consistent with a pathological remodeling of neuronal functions. Thus, an *SCN1A* mutation is a prerequisite for a long-term deleterious effect of seizures on the brain, indicating a clear interaction between seizures and the mutation (two-hit) for the development of a severe phenotype generated by pathological remodeling. Adapted from (291).

These results, emphasizing the importance of seizures in the severity of *SCN1A* phenotypes, contrast with data obtained down-regulating $Na_V1.1$ expression in the hippocampus of wild-type rats by means of RNA interference, which showed cognitive deficits without seizures (20, 290). However, the phenotypic effects obtained with RNA interference experiments could be enhanced

by the large (>70%) reduction of $Na_V1.1$ expression, which is larger than the 50% reduction expected from haploinsufficiency (14).

C. Rescuable Folding Defective Epileptogenic $Na_V1.1$ Missense Mutants

The severity spectrum of $Na_V1.1$-related epilepsies could be a continuum and depend on the amount of loss-of-function of the mutant: a mild impairment of $Na_V1.1$ function would cause mild phenotypes, whereas a more complete loss-of-function would cause severe phenotypes (55). Interestingly, some $Na_V1.1$ missense mutations cause loss of function because of folding/trafficking defects that lead to channel degradation in intracellular compartments (338). These mutants can often be rescued by incubation of the cells at low temperature or by interacting proteins that probably stabilize the correct folding conformation in the endoplasmic reticulum (ER) (13, 286, 287, 330, 340) (FIGURE 9A). Similar interactions may rescue the mutants *in vivo*, regulate the extent of loss-of-function, and modulate the phenotype in this way. Possibly, the complete loss-of-function observed for some GEFS+ mutants in functional studies *in vitro* may be caused by lack of rescue in the experimental conditions used and, conversely, lack of rescue *in vivo* could induce complete loss-of-function of Dravet Syndrome mutants that show only mild loss-of-function in some *in vitro* expression systems. Notably, folding-defective $Na_V1.1$ missense mutants can also be partially rescued by interactions with small drugs (pharmacological chaperones), which bind to them in the ER and probably stabilize the correct folding conformation. Similar to interacting proteins, they can bind to rescued channels targeted to the plasma membrane and modify their properties (FIGURE 9A). Interestingly, a peptide toxin selective for sodium channel, whose binding site is in the S1-S2 and S3-S4 loops of domain II (58), has been engineered to function as ER-resident pharmacological chaperone (13) (FIGURE 9A, B). In fact, the cDNA of the β toxin from the venom of the scorpion *Centruroides sculpturatus Ewing* (CsEI) was synthetized optimizing the sequence for expression in human cells, and adding a Calreticulin ER targeting sequence and a KDEL ER retention motif. Its expression in cells transfected with a plasmid containing the synthetic DNA was able to rescue some folding-defective mutants, without being released in the extracellular medium and not modifying functional channels in the plasma membrane. These results showed that interaction in the ER can be sufficient for rescuing folding-defective mutants, circumventing potential unwanted side effects of classical pharmacological chaperones, and these rescuing approaches may be used for developing therapeutic strategies (338). Importantly, epileptogenic $Na_V1.1$ mutants do not show gain-of-function changes in functional properties upon rescue (13, 286, 287, 330, 340). Folding-defective mutants have also been identified in familial hemiplegic migraine (61, 90) (see below). These mutants show gain of

function even when partially rescued, because of the modifications of their intrinsic gating properties.

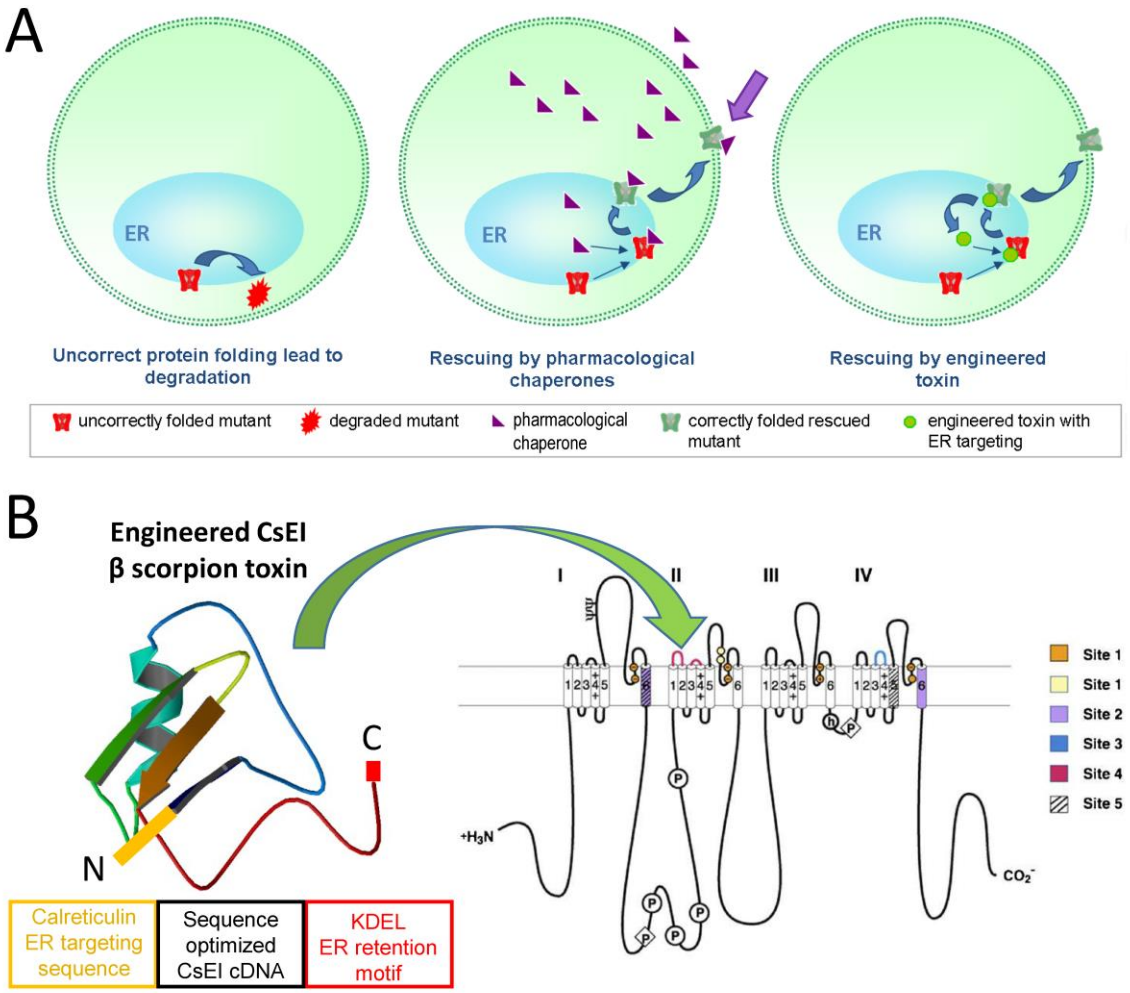


FIGURE 9. Rescue of expression and function of *SCN1A*/Na$_V$1.1 mutants. A. Mutations of Na$_V$1.1 and protein folding. Left. Folding-defective mutants are recognized as incorrectly folded proteins by the quality control system of the endoplasmic reticulum (ER) and degraded. Center. Folding defects can be rescued by molecular interactions with interacting proteins or pharmacological chaperones, which probably act by stabilizing a correct folding conformation and preventing degradation. They also interact with rescued proteins by modifying their functional properties (purple arrow), which can be a major drawback for therapeutic applications of pharmacological chaperones. Rescue of epileptogenic Na$_V$1.1 folding-defective mutants attenuates the loss of function but, different than for FHM mutations, never induces gain of function. Right. Engineered peptide toxins targeted to the endoplasmic reticulum can rescue Na$_V$1.1 folding-defective mutants, but unlike pharmacological chaperones, do not interact with rescued proteins at the plasma membrane**. B.** Left. The cDNA of CsEI β scorpion toxin, sequence-optimized for expression in human cells, was engineered to target the peptide to the endoplasmic reticulum by insertion of an N-terminal calreticulin ER targeting sequence (orange) and a C-terminal KDEL ER retention motif (red); cells transfected with a plasmid containing the engineered cDNA expressed the engineered ER-resident toxin that rescued some Na$_V$1.1 mutants and was not released extracellularly, as tested with immunoblots of the extracellular medium and with

functional evaluations (13). Right, interaction of the toxin with sodium channels at Neurotoxin Receptor Site 4 in domain II; toxin binding sites are colour coded as illustrated. Adapted from (13) and (58).

D. Febrile Seizures

Febrile Seizures (FS) affect 2-4% of children and are defined as seizure events associated with fever, with onset after the age of 6 months and spontaneous remission by the age of 6 years, but without pathologic or traumatic cause (255). They are classified as a distinct pathological entity rather than epilepsy. Approximately, 3–8% of children with FS develop epilepsy later in life, in particular idiopathic generalized epilepsy or temporal lobe epilepsy with hippocampal sclerosis (TLE-HS), although the causal link between FS and the subsequent development of these epilepsies is still debated (255). In contrast, children in GEFS+ families present with febrile/hyperthermic seizures early in life that can continue to occur in adulthood, but they do not develop temporal lobe epilepsy with hippocampal sclerosis (388). Interestingly, these features are shared by Dravet Syndrome patients, who do not develop hippocampal sclerosis, even if they have frequent febrile status epilepticus (50, 129), and by animal models carrying *Scn1a* epileptogenic mutations, in which there is no evident neuronal death (243, 291, 382).

A genetic predisposition has been proposed for FS, and using large, multi-generation families with presumed autosomal dominant inheritance, several chromosomal loci for FS have been identified (12). The FEB3 locus contains one of the few genes for which a causal role has been proposed: *SCN1A.* Consistently, the *SCN1A* M145T missense mutation was identified in all 14 affected members of a large family showing pure FS that always ceased by the age of 6 years, and 3 of them subsequently developed TLE-HS (75, 207). Functional studies of sodium currents in transfected tsA-201 cells have shown that M145T causes a partial loss of function, inducing 60% reduction of current density and a 10-mV positive shift of the voltage dependence of activation, consistent with hypoexcitability (207). Although it has been proposed that this family could be included in the GEFS+ spectrum (233), it fits better with the clinical features of typical FS (75), including the subsequent development of TLE-HS, which is not observed in GEFS+. Moreover, a genome-wide association study has subsequently identified significant association between the *SCN1A* gene and TLE-HS that develops following a history of FS (169). However, it is not clear why *SCN1A* mutations that induce quantitatively similar loss of function of $Na_V1.1$ can cause either GEFS+ or typical FS with possible development of TLE-HS.

E. $Na_V1.1$ Mutations in an Extremely Severe Early Infantile Epileptic Encephalopathy

Recently, heterozygous *de novo SCN1A* mutations have been implicated in an additional epileptic phenotype: developmental and early infantile epileptic encephalopathy (EIEE) that is even more severe than typical *SCN1A* Dravet Syndrome, with seizure onset between 6 and 12 weeks, profound cognitive and motor impairments, and hyperkinetic movement disorder. Sequencing of *SCN1A* in the nine patients that were initially identified showed that eight of them had a recurrent missense mutation, T226M, and one child had the missense mutation P1345S (288). One more case, which was retrospectively thought to have a phenotype consistent with *SCN1A* EIEE, carried the V422L variant (246). Moreover, a subsequent study identified the S228P *SCN1A* mutation in a patient showing overall features that are consistent with *SCN1A* EIEE, but disease onset occurred in the first hours of life (317), which is different than the other *SCN1A* EIEE patients described. This feature is puzzling because *SCN1A* is thought to be expressed primarily later in postnatal development. Pathogenicity of S228P was supported by the exclusion of additional genetic variants by performing whole exome sequencing and analyses of deletions/duplication and copy number variants targeting all protein-coding exons, exon-intron boundaries, and selected non-coding clinically-relevant variants (317), although pathogenic contribution by other non-coding regions or by other yet undetectable epigenetic factors cannot be excluded.

Functional studies were performed for the T226M mutation expressed in transfected Chinese hamster ovary (CHO) cells using a semi-synthetic cDNA of the $Na_V1.1$ long splicing variant, modified for reducing spontaneous mutagenesis in bacteria (22). T226M showed mixed effects on functional properties that could lead to either gain of function, because of a hyperpolarizing shift of the activation curve, or loss of function, because of a hyperpolarizing shift of the inactivation curve and faster time course of inactivation. To shed some light on the overall functional effect of T226M, which could not be directly predicted from these mixed dysfunctions, the dynamic action potential clamp method was used (24). Simulations of action potential firing were generated with a “hybrid cell” based on a computational model in which the sodium conductance was obtained in real time from the whole-cell recording of a CHO cell expressing either wild-type or T226M channels. Model hybrid cells with the T226M conductance displayed a negative shift in rheobase and depolarization block with cessation of action potential firing upon injection of depolarizing current stimuli that instead generated repetitive action potential firing in control cells. Similar results were obtained with traditional fully computational model neurons. The interpretation of these results was that the overall effect of T226M on $Na_V1.1$ is consistent with gain of function at the molecular level, which causes pathological facilitation of action potential

firing and depolarization block in GABAergic interneurons, leading to a dominant negative effect at the cellular and *in vivo* levels. This form of depolarization block was hypothesized to produce a larger decrease of inhibition in neuronal networks than the haploinsufficiency that is typical of Dravet Syndrome.

The results of these functional and cell modeling studies are controversial, because gain of function is a common effect observed for migraine $Na_V1.1$ mutations that do not cause epilepsy(205) (see below). Moreover, the effects of T226M on $Na_V1.1$ are very similar to those observed for other epileptogenic mutations, such as W1204R (13, 318), which causes mild epilepsy. This complicates interpretation of the data obtained with T226M. To resolve this discrepancy, functional investigation of the mutation T226M should be completed with studies in real neurons, and results should be supported by functional studies of other $Na_V1.1$ EIEE mutations.

IV. $Na_V1.1$ AND MIGRAINE

Migraine is a highly prevalent, debilitating, episodic disorder characterized by a severe and long-lasting headache that can coincide with nausea and neurological signs (111, 261). In about 30% of the patients, headaches are preceded by transient symptoms, including visual effects, called aura. The pathophysiology of migraine is complex, and some understanding has emerged from the studies of a rare monogenic form of migraine with aura, familial hemiplegic migraine (FHM), in which the attacks are associated with hemiparesis (i.e. transient, one-sided motor weakness). It is noteworthy that the features of migraine attacks in FHM, except for the motor symptoms, are identical to common migraine with aura, and most FHM patients also have attacks of migraine without motor symptoms, suggesting similar pathophysiological mechanisms for FHM and common migraine. (111, 261). Mutations in 3 genes have been identified as the causes of FHM: *CACNA1A,* encoding the α_{1a} subunit of the $Ca_v2.1$ neuronal calcium channel (FHM1) (247); *ATP1A2,* encoding the $\alpha 2$ subunit of the astrocytic sodium/potassium pump (FHM2) (81), and *SCN1A,* encoding the $Na_v1.1$ sodium channel (FHM3) (93). As reviewed above *SCN1A* is the most frequently mutated gene in epilepsy, with hundreds of mutations identified in a range of epilepsy syndromes with different phenotypes (202). Thus far, 11 mutations in the *SCN1A* gene have been shown to cause FHM3, including sporadic *de novo* mutations in non-familial cases (205). They are all missense mutations, mainly clustered in domain IV of the $Na_V1.1$ channel (FIGURE 6B).

The different types of FHM can show distinct clinical phenotypes. FHM1 and 2 are often characterized by complex phenotypes, comprising permanent cerebellar signs/ataxia and intellectual disability, as well as severe hemiplegic attacks lasting days, which are sometimes followed by loss of consciousness, coma, and in some cases even death (261). Interestingly, thus far there are no FHM3 patients with these features (205). Moreover, epilepsy has been reported as a co-morbidity in FHM. However, in contrast to *CACNA1A* and *ATP1A2* mutations, in the few FHM patients carrying *SCN1A* mutations who also present with seizures, hemiplegic migraine attacks are always independent from seizures, and in general the two phenotypes do not temporally overlap (205). A few patients show a further FHM3-specific co-morbidity: elicited repetitive daily blindness (ERDB), which is independent from hemiplegic migraine attacks and has been reported in one of the four patients carrying the F1499L mutation in one family, and in four of the five patients carrying the Q1489H mutation in a different family (351). Notably, in two additional independent FHM3 families carrying the F1499L mutation, the phenotype was pure hemiplegic migraine without ERDB (300).

Functional studies of seven mutations causing pure hemiplegic migraine or both hemiplegic migraine and epilepsy have been undertaken and have provided major insights into pathophysiological mechanisms as described below. Thus far, there are no functional studies of mutations that cause hemiplegic migraine and ERDB.

A. Folding Defective FHM3 Mutants Leading to Gain of Function upon Rescue

The initial functional studies of FHM3 mutations engineered in human $Na_V1.1$ cDNAs generated controversial results, with gain of function identified for some mutations and loss of function for others (60, 161). In particular, the mutation L1649Q, which causes pure hemiplegic migraine in a family with seven carriers, was engineered into the long $Na_V1.1$ spice-variant cDNA and did not generate quantifiable sodium current in a transfected human embryonic kidney cell line, suggesting a complete loss of function (161). In the same study, using cell-surface biotinylation and immunoblot analysis, it was shown that WT and L1649Q channels had similar levels of total protein expression, but L1649Q exhibited a 10-fold lower level of cell surface expression compared to WT (161), reflecting impaired trafficking to the plasma membrane. Puzzlingly, this complete loss of function is similar to the effects of most mutations causing Dravet Syndrome. A subsequent study (61) investigated L1649Q using the short $Na_v1.1$ human splice variant, which is the predominant variant expressed in the brain (197, 297). Results under control conditions

were similar to those of (161); however, this study showed that the L1649Q mutant is folding-defective, as it can be rescued by incubation of the cells at 30°C, a typical feature of folding-defective mutants (27), or by co-expression with interacting proteins (61). Strikingly, L1649Q was partially functional when expressed in transfected cortical GABAergic neurons in primary culture at physiological temperature. Evidently, the neuronal cell background was sufficient to induce a partial rescue, probably because of interactions with endogenous proteins. Analysis of the gating properties of L1649Q channels showed both gain- and loss-of-function modifications that were similar in a human cell line and in neurons. Investigation of the overall effect of the mutant by means of voltage stimuli mimicking action potential discharges showed that frequency-dependent inactivation was reduced, consistent with better capacity to sustain high-frequency firing than WT. In fact, current clamp recordings in transfected neurons showed that expression of L1649Q-$Na_V1.1$ induces hyperexcitability that is greater than with expression of WT-$Na_V1.1$. A computational model in which the effects of this mutation were implemented showed that a limited partial rescue (30% in the model) is sufficient for transforming the apparently non-functional L1649Q mutant into a gain-of-function mutant (61).

Interestingly, loss-of-function mutations that lead to overall gain of function upon rescue are recurrent in FHM3. The mutation L1670W, causing pure hemiplegic migraine in two unrelated families, was studied in a human cell line and in cortical neurons in culture using the short $Na_V1.1$ human splice variant (90). The results showed that L1670W is characterized by nearly complete loss of function in transfected non-neuronal cells maintained at physiological temperature, similar to L1649Q. Incubation of the transfected cells at 30°C or transfection into cortical neurons was able to partially rescue the function of the mutant. Therefore, L1670W, similar to L1649Q, is a folding-defective mutant. Analysis of the functional effects of rescued L1670W revealed numerous modifications, in most cases consistent with destabilization of the inactivated states. In fact, the study of the overall effect of the mutation using voltage stimuli mimicking action potential discharges indicate that L1670W induces a gain of function both in a human cell line and in cortical neurons (90). The mutation L1670W was studied by another group with a synthetic $Na_V1.1$ clone, expressed for functional analysis in a human cell line (28). Similar to (22) (see above), the synthetic clone was generated to reduce spontaneous mutagenesis in bacteria expressing plasmids that contain human $Na_V1.1$ cDNA. In this case, it was formed with a $Na_V1.5$ cDNA backbone in which codons were modified to obtain the $Na_V1.1$ amino acid sequence. With this strategy, the $Na_V1.1$ protein should be produced, but RNA processing could be different compared to that of the native $Na_V1.1$ gene sequence. The functional effects observed were consistent with destabilization of inactivation and were similar to those observed after rescue in (90). However,

in the experimental conditions of (28), L1670W was evidently already partially rescued in the cells used for functional studies, because current density was reduced less than in (90).

Overall, these mutants show nearly complete loss of function in some conditions, but they are transformed into gain of function upon rescue of folding defects. This feature is specific to FHM mutants, because epileptogenic $Na_V1.1$ mutants can be partially rescued, but do not become gain-of-function (see above).

B. Functional Studies of Other Mutations Causing FHM

Two other *SCN1A* mutations causing pure FHM3 have been functionally studied. The mutation L1624P was identified in a three-generation family containing five patients with FHM attacks, showing high individual clinical variability with respect to attack duration, severity, and frequency, but without comorbidities. Functional studies of L1624P revealed numerous dysfunctions in general consistent with a severe destabilization of fast inactivation, leading to gain of function and neuronal hyperexcitability (109). A further mutation, F1174S, was engineered into the synthetic $Na_V1.1$ clone outlined above, and the data obtained from expression and electrophysiological analysis in a human cell line showed results that are also consistent with destabilization of fast inactivation leading to gain of function (28).

Other functional studies have investigated mutations identified in families with FHM and epilepsy. The mutation Q1489K was the first FHM3 mutation reported and was identified in 22 carriers from three families (93). One carrier had benign focal epiliesy of childhood that remitted before the onset of FHM, and a single febrile seizure was reported in two carriers before the onset of FHM (93). The functional study of this mutation was initially performed using the cardiac $Na_V1.5$ isoform to bypass the difficulties encountered to manipulate and express the human $Na_V1.1$ clone. In this study, the only altered gating property was the recovery from fast inactivation, which was accelerated by the mutation (93). Two further studies analysed the effect of this mutation by introducing it into the human $Na_V1.1$ cDNA. One study introduced the mutation in the short human $Na_V1.1$ splice variant, and analyzed its effects in a human cell line and in neurons in primary culture (60). The other study used the long splice variant expressed in a human cell line (161). Both studies found that Q1489K exhibited numerous contrasting functional defects in voltage-clamp recordings, interpreted as preponderant loss of function in (161) and preponderant gain of function in (60). Current-clamp recordings reported in (60) showed that neurons transfected with Q1489K generated more action potentials than neurons transfected with WT $Na_V1.1$, consistent with gain of function and hyperexcitability.

The FHM mutation L263V was identified in the only reported family in which hemiplegic migraine co-segregated with epilepsy in one branch of the family (49), but another branch showed pure hemiplegic migraine. Moreover, the same group identified a second family carrying the same mutation, in which the phenotype was pure hemiplegic migraine, consistent with polygenic inheritance in the first family (11). Functional analysis of L263V using the long $Na_V1.1$ splice variant indicated that the mutation induces gain of function because of destabilization of both fast and slow inactivation (161). Recently, L263V has been engineered in the first FHM3 gene-targeted mouse model (153), which shows a phenotype that is consistent with migraine, but does not show seizures (see below).

The heterozygous T1174S missense mutation was found in a three-generation family in which three affected individuals in a branch had febrile seizures and/or focal epilepsy, whereas two additional relatives in another branch had only typical FHM3. To investigate the pathophysiological mechanisms leading to these different phenotypes in the same family, both functional studies and simulations with a computational model were performed (59). The study showed that T1174S induces divergent functional effects that can be consistent with both gain and loss of function: positive shift of the activation curve (loss of function), deceleration of the recovery from fast inactivation (loss of function) and increase of persistent sodium current (gain of function). Notably, the increase in persistent current was a labile property for this mutant in long-lasting whole-cell recordings, indicating that it is most likely caused by sensitization of the mutant channel to neuromodulation by intracellular signaling pathways. Both functional and modeling data suggest that the overall functional effect of the mutation can be regulated. The increase of the persistent current may be dependent of the action of modulators that switch the effect of the mutation form loss of function (epilepsy) to gain of function (FHM). Interestingly, this mutation has been identified in another family with migraine and motor impairments (122), but it has also been identified in patients with different epileptic phenotypes, although with incomplete penetrance (177). Thus, T1174S could be a variant that needs modifiers or co-factors to be pathogenic, which could also switch its effect from mainly pro-epileptic to mainly pro-migraine.

C. Overall Pathophysiological Mechanisms in FHM3

The functional studies outlined above have shown that FHM3 mutations induce overall gain of function of $Na_V1.1$ and neuronal hyperexcitability. This has allowed us to infer a possible pathophysiological mechanism. In fact, as illustrated in previous sections, $Na_V1.1$ is expressed mainly in GABAergic inhibitory neurons, in which it has a key role in the generation of action potentials in the axon initial segment (140, 191, 243, 382). The hyperexcitability of GABAergic

neurons could cause increased extracellular K^+, neuronal depolarization, and initiation of cortical spreading depression (CSD), a wave of transient network hyperexcitability leading to a long-lasting depolarization block of neuronal firing that is the electrophysiological correlate of migraine aura and a proposed pathological mechanism of migraine headache (60, 205, 260) (FIGURE 10). In fact, we have shown that hyperexcitability of GABAergic neurons induced with the selective $Na_V1.1$ enhancer Hm1a or with optogenetic stimulation triggers CSD specifically in the neocortex, because of extracellular accumulation of K^+ initially generated by GABAergic neurons' spiking, which eventually engage also glutamatergic neurons (68). Consistently, we have shown in a recent computational model that the increase of extracellular K^+ generated by the spiking of a hyperexcitable GABAergic neuron can be sufficient to induce depolarizing block, the cellular correlate of CSD, of a connected pyramidal excitatory neuron (88).

It is worthwhile to emphasize that pathophysiological mechanisms of epilepsy and migraine can share some similarities, because hyperexcitability of cortical neuronal networks can be involved in both these diseases. In particular, as reported above, it has been proposed that some FHM3 mutations, such as L263V, can cause both hemiplegic migraine and epilepsy in the same family, although there is no evidence of association between migraine attacks and seizures (205). The first FHM3 mouse model, a knock-in that expresses the L263V *Scn1a* mutation, has been recently reported (153). Interestingly, these mice show spontaneous CSD events as well as facilitation of CSD induction with electrical stimulations, recapitulating what has been shown in patients having migraine with aura. No seizure-related or other abnormal behaviors were observed, and electrographic recordings did not reveal epileptic activity during a total recording time of 2228 h in 24 animals. Thus, the model replicates a migraine phenotype without epileptic co-morbidity, and the pathophysiological mechanism is consistent with facilitation of CSD initiation. Notably, this is different in comparison with other FHM models, for example knock-in mice carrying the S218L FHM1 mutation of $Ca_V2.1$ calcium channels, which exhibit facilitation of CSD induction and spontaneous generalized tonic-clonic seizures (352). A feature of L263V *Scn1a* mice that is not consistent with the human FHM3 phenotype is the high mortality rate, with only about 10% of heterozygous mutant mice surviving to the age of P60. Different from epileptic *Scn1a* models, maintaining the mutation in either the genetic background of pure C57BL/6J or mixed 50:50 C57BL/6J-129/SvJ had no significant impact on survival of L263V *Scn1a* mice.

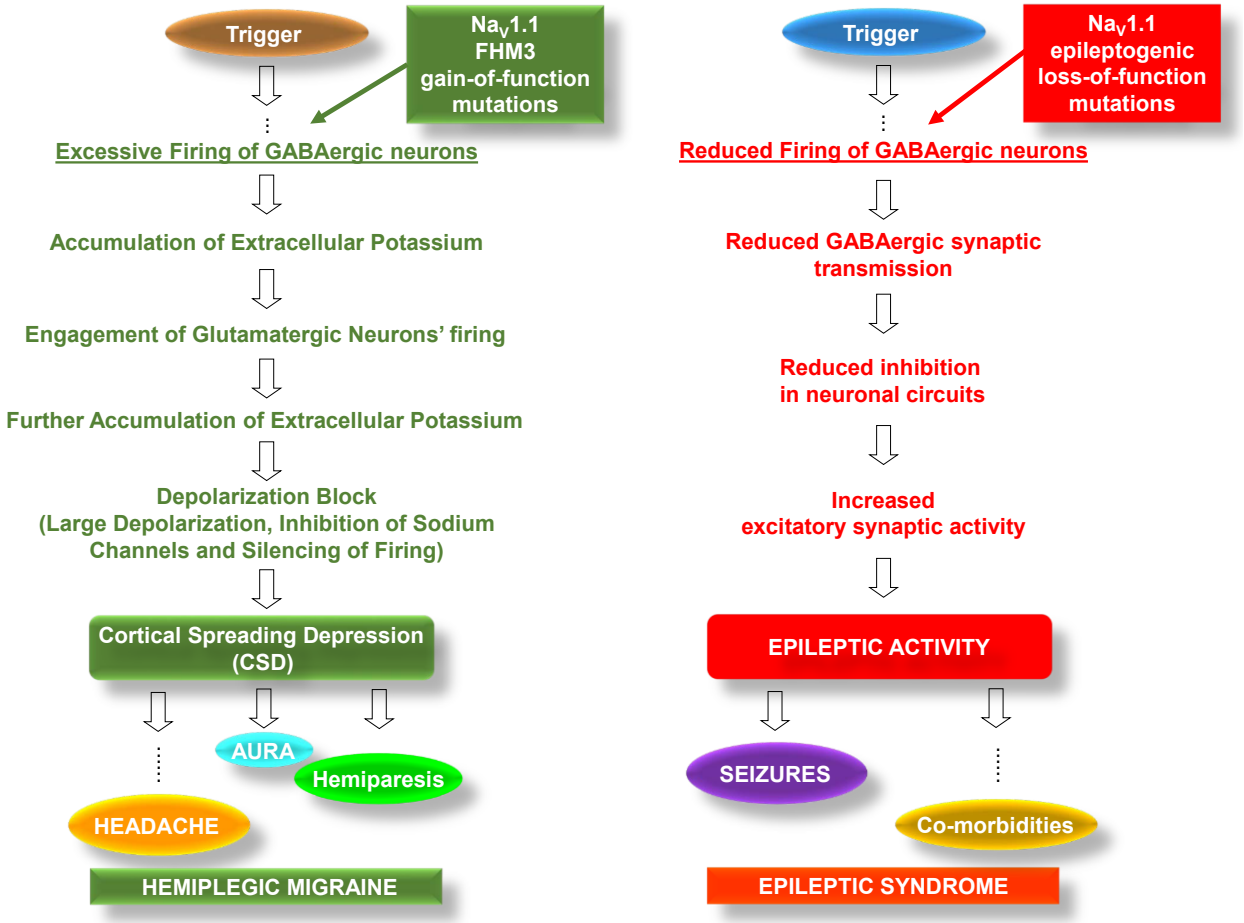


FIGURE 10. Comparison of proposed mechanisms for generation of migraine attacks by gain-of-function $Na_V1.1$ FHM3 mutations and seizure generation by $Na_V1.1$ epileptogenic mutations. Left. In migraine the genetic background is important for determining an intrinsic threshold for migraine attacks which is modulated by internal and external factors (trigger stimuli). Emotional stress and minor head trauma are among the most common triggers of hemiplegic migraine attacks (111, 261). Some triggers are thought to induce excessive neuronal firing and consequently lead to extracellular K^+ accumulation, which can eventually lead to long-lasting neuronal depolarization and silencing of firing caused by inactivation of sodium channels (depolarization block). These events produce cortical spreading depolarization (CSD), a wave of transient network hyperexcitability leading to a long-lasting depolarization block of neuronal firing. CSD directly causes aura, and it could also induce headache by activating trigeminal nociceptors) and hemiparesis (205, 260). FHM3 $Na_V1.1$ gain-of-function mutations can lower the triggering threshold of migraine attacks by increasing excitability of GABAergic neurons, which can induce spike-dependent accumulation of extracellular potassium that engages the spiking of glutamatergic neurons, eventually leading to depolarizing block and CSD initiation. Extracellular accumulation of the excitatory neurotransmitter glutamate plays a minor role in this proposed GABAergic neuron-dependent mechanism (68). Right. Similar to migraine, it is hypothesized that epileptogenic mutations lower the threshold for seizure generation, although seizure triggers are less clearly identified than migraine triggers (205). Epileptogenic $Na_V1.1$(loss-of-function mutations lower seizure threshold by reducing firing of GABAergic neurons, which leads to reduced GABAergic synaptic transmission and reduced inhibition in neuronal circuits, which can lead to generation of epileptic activity. Co-morbidities could be generated by both seizures and the direct effect of the mutation (51, 55, 202).

V. $Na_V1.1$ AND AUTISM SPECTRUM DISORDER

Autism spectrum disorders (ASD) are a group of neurodevelopmental disorders that are defined by impairments in social interactions along with restricted and repetitive behaviors. High-throughput sequencing has implicated numerous genes in autism spectrum (237, 238, 293, 294), including *SCN1A* discussed here and *SCN2A* discussed below. These studies included sequencing of 'trios'; that is, a set of DNAs from two parents, an unaffected sibling, and an affected individual. This approach identified *de novo* mutations in $Na_V1.1$ in the affected individuals that are not present in parents or siblings and therefore are causative for autism. Consistent with these genetic results, behavioral studies of Dravet Syndrome patients with *SCN1A* loss-of-function mutations (26, 176, 189) revealed autistic features in a large fraction of affected individuals. Moreover, autistic-like behaviors, including social interaction deficit and repetitive behaviors, were observed in Dravet Syndrome *Scn1a*$^{+/-}$ mice analyzed under carefully controlled laboratory conditions (133, 335). Because Dravet Syndrome is caused by loss-of-function mutations in $Na_V1.1$ channels, which cause specific loss of excitability in GABAergic interneurons, it is likely that these autistic behaviors result from impaired firing of interneurons. Direct evidence for this conclusion came from studies of mice with $Na_V1.1$ channels specifically deleted in forebrain GABAergic inhibitory neurons (133), which exhibited both impaired social interactions and repetitive behaviors that could be rescued by treatment with low-dose benzodiazepines that enhance the postsynaptic response to GABA.

VI. $Na_v1.2$ AND EPILEPSY

A. Functions of $Na_V1.2$ Channels in Neurons

Early studies showed that $Na_V1.1$ and $Na_V1.2$ channels are differentially localized in neurons in the hippocampus—$Na_V1.1$ in the cell bodies of excitatory and inhibitory neurons vs. $Na_V1.2$ channels in unmyelinated axons and dendrites of excitatory neurons (344, 368). The $Na_v1.2$ sodium channel is widely expressed in the central nervous system, particularly in cortical and hippocampal glutamatergic pyramidal cells (149, 193). In myelinated axons, Na^+ channels are clustered at high density at the nodes of Ranvier to allow saltatory conduction. $Na_V1.2$ are the main channels in nodes of Ranvier during early postnatal development and are largely replaced by $Na_v1.6$ channels during development of maturing nodes of Ranvier (32, 167). Moreover, a high density of Na^+ channels is present at the axonal initial segment (AIS) which is, for this reason, the primary site for generation of action potentials in neurons (33, 53, 374). Similar to nodes of Ranvier, in the first days of rodents' postnatal development, $Na_v1.2$ is the main sodium channel

of the AIS; after the 10th day of postnatal life, it is partially replaced by $Na_v1.6$, which becomes the main sodium channel in the distal part of the AIS, whereas $Na_v1.2$ is segregated in the proximal part (33, 193, 271). This separation can be functionally important, as it has been proposed that $Na_v1.6$ channels in the distal AIS promote action potential initiation, whereas $Na_v1.2$ channels in the proximal AIS promote backpropagation to the soma (149). In adult rodents, $Na_V1.2$ is still present in thin processes, presumably distal unmyelinated portions of pre-terminal axons (244).

In these cellular contexts, $Na_V1.2$ channels are in position to drive action potential generation, back-propagation, and possibly contribute to epilepsy. It was surprising to discover $Na_V1.2$ channels in high density in dendrites of hippocampal neurons in early immunocytochemical studies (368). Later work revealed back-propagating action potentials moving from cell bodies into many types of dendrites (e.g., (327)). Moreover, recent work with heterozygous $Na_V1.2$ knockout mice has shown that these channels are crucial for back-propagating action potentials in dendrites of prefrontal cortex pyramidal neurons (319). Back-propagating action potentials are thought to distribute and integrate action potential generation and calcium signaling in the large dendrites of cortical pyramidal neurons (179). In this cellular context, recent work has shown that $Na_V1.2$ channels are crucial in a spectrum of diseases, including epilepsy, autism, and other neuropsychiatric disorders (FIGURE 10A).

The possible involvement of $Na_V1.2$ mutations in epilepsy was initially proposed in 2001, with the identification of the variant R187W in a family in which the proband developed complex partial seizures after an initial diagnosis of FS plus (FS+) (329). However, the small pedigree did not show clear co-segregation with the disease phenotype and there was bi-lineal inheritance of epilepsy in the family, so it is possible that there are other genes implicated in the phenotype of this patient (152).

B. $Na_v1.2$ Channels in Benign Familial Neonatal/Infantile Seizures

The first epileptic syndrome clearly associated with mutations of *SCN2A* was Benign Familial Neonatal/Infantile Seizures **(**BFNIS) (25, 143). This autosomal dominant disorder is caused by missense mutations with high penetrance and is characterized by afebrile seizures with onset between 3 and 6 months of age and spontaneous remission within the first year of life, without subsequent neurological deficits. BFNIS is a relatively mild syndrome within the spectrum of

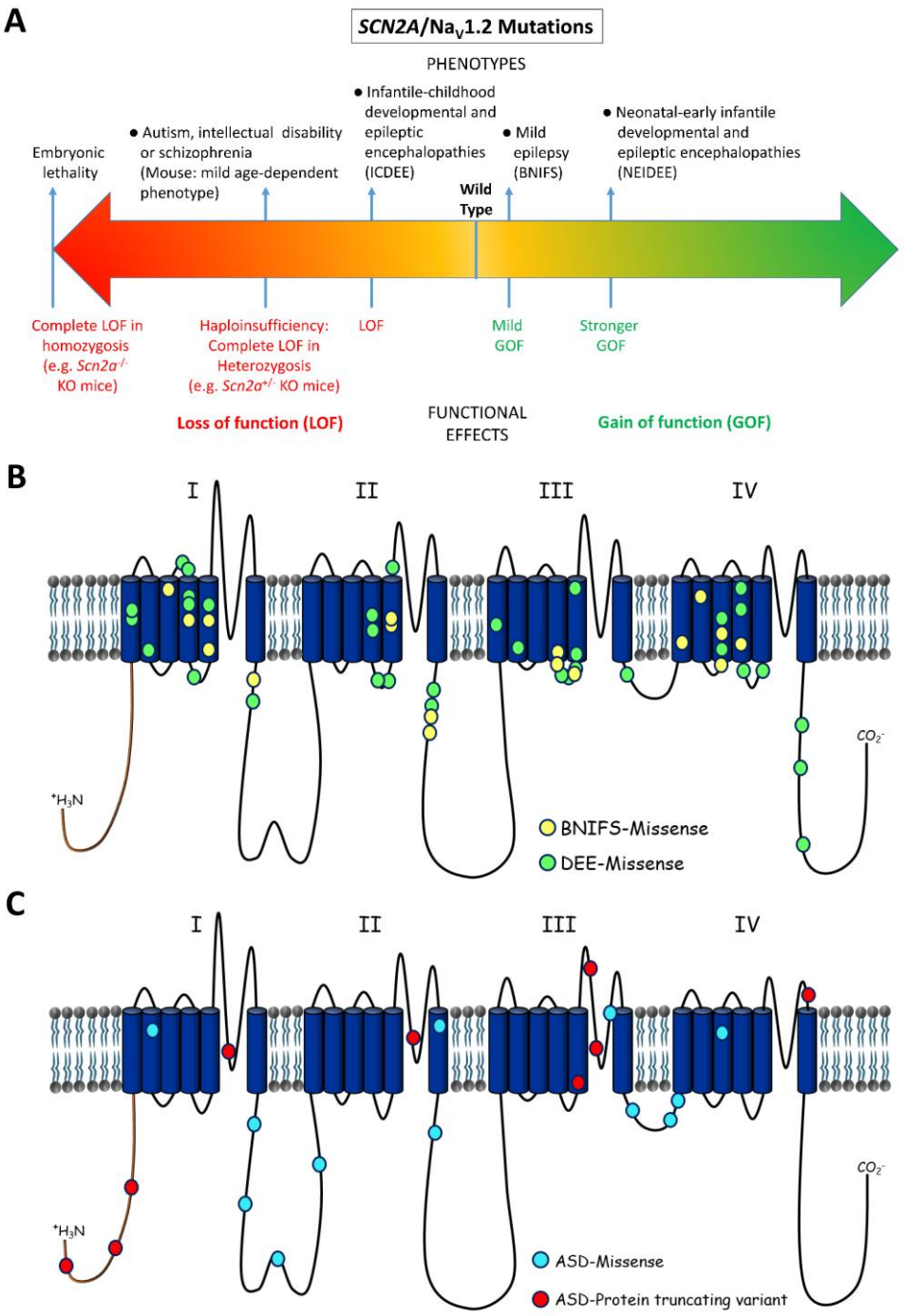


FIGURE 11. Spectrum of mutations and phenotypes for *SCN2A*/Na$_V$1.2. A. *SCN2A*/Na$_V$1.2 mutations inducing mild gain of function cause benign neonatal-infantile familial seizures (BNIFS) with onset between 3 and 6 months of age. Neonatal-early infantile developmental and epileptic encephalopathies (NEIDEE) with onset before 3 months are in general caused by mutations that induce larger gain of function. Mutations inducing loss of function cause infantile-childhood developmental and epileptic encephalopathies (ICDEE) with onset after 3 months. Complete loss of function in heterozygosis (haploinsufficiency) can lead to behavioral/cognitive phenotypes without epilepsy: autism spectrum disorder (ASD), intellectual disability, or schizophrenia. Haploinsufficiency in *Scn1a*$^{+/-}$ mice causes a relatively mild and age-dependent phenotype

including short absence-like seizures, autistic/schizophrenic traits and memory dysfunctions. Homozygote *Scn1a*$^{-/-}$ knock-out mice show embryonic mortality. **B.** Molecular map of the $Na_V1.2$ sodium channel with the location of 19 missense *SCN2A* mutations in benign neonatal-infantile familial seizures (BNIFS) (yellow) and 49 missense *SCN2A* mutations in developmental and epileptic encephalopathy (DEE: both ICDEE and NEIDEE) (green). **C.** Molecular map of the $Na_V1.2$ sodium channel with the location of 12 missense (red) and 9 protein-truncating (blue) *SCN2A* variants identified in autism spectrum disorder (ASD) cases.

$Na_V1.2$ channelopathies (FIGURE 11A, B). Following the initial studies, several other missense mutations that map broadly across the channel structure have been identified in *SCN2A* that are responsible for BFNIS, further confirming BFNIS as a $Na_V1.2$ channelopathy (FIGURE 11B)(142, 324, 386).

The first functional analysis of $Na_V1.2$ BFNIS mutations was performed by Scalmani et al. in 2006 (296). Four BFNIS mutations L1330F, L1563V, R223Q and R1319Q, were studied using the rat $Na_v1.2$ isoform and transfected pyramidal and bipolar-shaped (GABAergic) neocortical neurons in primary culture. Classical voltage clamp experiments showed gain of function effects for L1330F and L1563V, with mild modifications of gating properties, whereas mixed gain and loss of function effects were observed for R223Q and R1319Q. Action potential-clamp experiments showed that R223Q and R1319Q induce an increase of both subthreshold and action sodium currents. Thus, the overall functional effect was consistent with neuronal hyperexcitability for all four mutations studied.

A subsequent study challenged these results, investigating three of those mutations (R1319Q, L1330F and L1563V) using the human adult $Na_V1.2$ isoform expressed in a human cell line (225). This study reported a reduction in current density and cell surface expression (quantified using biotinylation), together with mild and contrasting alterations of gating properties. Overall, the functional outcome was consistent with mild loss of function, opposite to the first study of these mutations.

However, further studies confirmed that BFNIS mutations cause gain of function of $Na_V1.2$. These investigations have often been performed comparing effects in “neonatal” and “adult” human $Na_V1.2$ splice variants (168, 295), because BFNIS onset is in the first months of life. Gain of function has been observed for the L1563V BFNIS mutation (377), studied using both neonatal and adult splice variants expressed in a human cell line. This study showed that the adult WT variant can sustain increased excitability compared to the neonatal WT variant, because of mild

modifications of gating properties. Notably, L1563V did not induce significant functional effects in the adult isoform, but it caused the neonatal isoform to behave like the adult one, consistent with gain of function and increased neuronal excitability. However, a subsequent study from the same group of the L1563V mutant expressed in Chinese hamster ovary (CHO) cells (23) reported effects in the adult variant that were similar to those previously observed with the neonatal isoform (377). In this study, simulations obtained with the dynamic action potential clamp technique (see above) predicted that L1563V would induce mild neuronal hyperexcitability also when inserted into the adult variant. Moreover, a further recent study confirmed a gain of function for L1563V engineered in the adult variant expressed in a human cell line (16). Thus, L1563V can induce mild gain of function in both neonatal and adult splice variants.

Another study compared the effect of two BFNIS mutations, M252V and V261M, on human neonatal and adult $Na_V1.2$ variants transiently expressed in a human cell line (193), showing that both mutations induce gain of function by altering different gating properties. Functional effects were observed in both neonatal and adult variants for V261M, but only in the neonatal variant for M252V. Further studies investigated the BNFIS mutations Y1589C (180), V280E, and K908E (181) using the human adult $Na_V1.2$ variant transiently expressed in a human cell line, showing an overall gain of function for all mutations due to modifications of several gating properties.

Therefore, the common functional effect of BFNIS mutations is a mild gain of function consistent with hyperexcitability of excitatory neurons. Functional studies have shown that the major factor for the transient generation of seizures limited to the first months of life is probably the age-dependent differential expression and localization of $Na_V1.2$ channels in neuronal subcompartments, rather than specific effects of the mutations on the $Na_V1.2$ neonatal splice variant.

C. $Na_v1.2$ Channels in Developmental and Epileptic Encephalopathies

Since the initial identification of *SCN2A* mutations in BFNIS (143), the phenotypic spectrum of epilepsies caused by *SCN2A* mutations has expanded considerably, in particular including severe developmental and epileptic encephalopathies (DEE) (292, 372, 373) (FIGURE 11A). Similar to BFNIS, these mutations map broadly across the $Na_V1.2$ channel structure (FIGURE 11B)

The first $Na_V1.2$ mutation reported in a severe epilepsy was the de novo heterozygous truncation R102X, identified in a patient showing pharmaco-resistant seizures with onset at 1 year and 7

months of age, severe intellectual and psychomotor retardation, and autistic traits (16, 165). R102X causes a large truncation, sparing just a cytoplasmic N-terminal fragment of the $Na_V1.2$ protein and inducing complete loss of function, as confirmed by more recent work of the same group that compared features of knock-in mice expressing the R102X mutation to *Scn1a*$^{+/-}$ knock-out mice (244).

After the identification of R102X, other *SCN2A* mutations have been identified that cause infantile and childhood DEE (ICDEE), representing about 40% of the total *SCN2A* DEE cases (372, 373) (FIGURE 11A). Phenotypes of *SCN2A* ICDEE patients can be correlated to the age of onset. Patients with onset between 3 months and 1 year of age in general show a West Syndrome phenotype (infantile spasms as seizure type, EEG with hypsarrhythmia, and developmental regression), with possible evolution into a Lennox-Gastaut phenotype (multiple seizure types, EEG with diffuse spike-and-wave and paroxysmal fast activity, and intellectual disability). *SCN2A* ICDEE patients with onset at more than 1 year of age often show variable seizure phenotypes that cannot be classified as an established epileptic syndrome, including tonic-clonic, myoclonic, and absence seizures, with developmental/cognitive delay and autistic traits that can appear before seizure onset.

Notably, about 60% of *SCN2A* DEE have onset in the first 3 months of life, mostly in the neonatal period (372, 373). These neonatal-early infantile DEE (NEIDEE) patients show intellectual disability in all the cases. About 50% of them have unclassifiable epileptic phenotypes with variable seizure types, whereas the others have phenotypes that fit the features of two epileptic syndromes: Ohtahara syndrome (neonatal-onset spasms or tonic seizures, and EEG with burst suppression pattern, sometimes evolving into West syndrome and/or Lennox-Gastaut phenotypes) or epilepsy with early infantile migrating focal seizures (EIMFS: multiple types of focal seizures that migrate from one hemisphere to the other). *SCN2A* DEE mutations generally arise *de novo*, and about 80% are missense (FIGURE 11B). However, a few mutations are recurrent in different patients: for example, A263V, R853Q and L1342P, which cause ICDEE; and E999K and R1882Q, which cause NEIDEE (17, 373).

The first functional study of a *SCN2A* NEIDEE mutation (I1473M) appeared controversial because it showed a net gain of function (241), which is opposite to the effect of the original R102X ICDEE mutation (165). However, more recent studies have delineated a clearer correlation between gain-of-function effects and clinical phenotypes (141). Functional studies of the A263V mutation,

identified in a patient with neonatal-onset seizures and childhood-onset episodic ataxia, provided additional evidence that *SCN2A* mutations involved in neonatal/early infantile seizures induce gain of function (192). In fact, A263V expressed in a human cell line induced a prominent gain of function in both neonatal and adult $Na_V1.2$ variants because of several modifications of gating properties that are consistent with destabilized inactivation. A subsequent report confirmed these results, identifying *SCN2A* mutations in other patients with neonatal-onset seizures and ataxia: the same mutation reported before, A263V, in one patient; G1522A in a second patient; and the combination of G1522A and R1882G in the third patient. All of these mutations induced gain of function with increased persistent current, negative shifts of the voltage dependence of activation or positive shifts of inactivation (302). The clinical spectrum of these patients, and of others subsequently identified, is consistent with a milder NEIDEE characterized by childhood ataxia (301). Moreover, a report including data from 201 patients shed further light on genotype-phenotype relationships of DEE *SCN2A* mutations and suggested therapeutic implications (373). This work showed that truncating mutations, which are likely to cause loss of function, are found only in ICDEE, in general when onset is after 2 years of life. Consistent with this, antiepileptic sodium channel blockers are often effective in NEIDEE, but not in ICDEE, in which they often induce phenotype worsening. These findings are consistent with $Na_V1.2$ gain of function in NEIDEE and loss of function in ICDEE, a dichotomy that was supported in this study by the functional analysis of four mutations. In fact, two NEIDEE mutations showed gain of function because of large increase of persistent current (V423L) or accelerated recovery from fast inactivation (F1597L), whereas two ICFEE mutations showed loss of function because of a large negative shift of the voltage dependence of inactivation and faster current decay (P1622S), or a positive shift of the voltage dependence of activation (G899S).

Two further studies (23, 212) confirmed these results comparing two recurrent mutations and showing gain of function for R1882Q, implicated in NEIDEE, and loss of function for R853Q, implicated in ICDEE. R1882Q impaired fast inactivation and increased persistent sodium current (212). In contrast, R853Q reduced peak sodium current, enhanced fast inactivation, and reduced persistent sodium current. In Xenopus oocytes, the mutation R853Q, which is located in the S4 segment of domain II, reduced functional expression and induced a substantial inward gating-pore current at membrane potentials negative to –30 mV. This is an unusual functional effect, but it cannot be generalized to other ICDEE mutations, and its influence on the overall effect of the R853Q mutation is not clear. The second study used dynamic action potential clamp methods (23), predicting a dramatic increase in firing for R1882Q and a marked reduction in firing for

R853Q. Altogether, these results strongly support the conclusion that loss-of-function mutations primarily cause ICDEE, whereas gain-of-function mutations primarily cause NEIDEE.

Separation of mutations that induce the same type of functional effect (gain vs. loss of function) but cause phenotypes of different severity can have substantial clinical relevance, in particular for genetic counseling and for orienting therapies. Toward that goal, an “electrophysiological index” was developed for correlating functional effects and clinical severity based on parameters obtained from classic voltage-clamp experiments and was used to evaluate three novel and 21 previously characterized gain-of-function $Na_V1.2$ mutations (181). This electrophysiological index correlated well on average with clinical severity, but there were overlaps between some single mutations that did not allow consistent predictions of phenotypic severity based only on their functional properties analyzed by voltage clamp recording *in vitro*. These potentially important tools should be further developed and refined in future studies.

A recent study extended the comparison of the functional effects observed in the neonatal and adult splicing variant to five NEIDEE mutations (339). This comparison had been already attempted for BNIFS mutations with controversial results, and for the A263V NEIDEE mutation without observing differential effects (192) (see above). The five mutations (T236S, E999K, S1336Y, T1623N, and R1882Q) were identified in patients that had seizure onset in the first days of life. All mutations, expressed in a human cell line, exhibited gain-of-function modifications consistent with enhanced neuronal excitability. Only three of the five mutations (T236S, E999K, and S1336Y) exhibited greater dysfunctions in the neonatal variant compared with those observed in the adult one. Therefore, these studies showed that a larger effect in the neonatal variant is not a general property of mutations involved in NEIDEE with neonatal onset, similar to BNIFS mutations.

Overall, the consensus based on these extensive functional studies is that ICDEE mutations cause loss of function and that NEIDEE mutations cause gain of function, which is in general more pronounced than for BNIFS mutations.

VII. $Na_V1.2$: AUTISM SPECTRUM DISORDER, INTELLECTUAL DISABILITY AND SCHIZOPHRENIA

Interestingly, *SCN2A* mutations have also been identified in patients with neurodevelopmental dysfunctions, whose phenotypes include autism spectrum disorder (ASD) and/or intellectual

disability (ID), as well as other neuropsychiatric phenotypes such as schizophrenia (292, 372, 373) (FIGURE 11A). These mutations map broadly across the structure of $Na_V1.2$ (FIGURE 11C). Notably, *SCN2A* mutations have been reported as among the most frequent *de novo* mutations identified in ASD by genome sequencing of trios (237, 238, 292-294), even though their frequency is probably underestimated because many more epileptic patients are screened for *SCN2A* mutations compared to ASD/ID patients (292). Although seizures have been observed in some of these patients after the onset of neurodevelopmental dysfunction, epilepsy is not a major feature of their phenotype.

A. $Na_v1.2$ Channels and Autism Spectrum Disorder

Functional analysis of 10 *SCN2A* mutations identified in ASD patients without seizure was performed using the adult splicing variant of $Na_V1.2$ expressed in a human cell line (17) . Two nonsense mutants (C959X and G1013X) and one frameshift mutant (S686fs), which generate truncated $Na_V1.2$ proteins, as well as 4 missense mutants (R379H, R937H, R937C and C1386R) caused complete loss of $Na_V1.2$ activity. Three additional missense mutants (D12N, D82G and T1420M) induced partial loss of function because of reduction of current amplitude and/or modifications of gating properties. Altogether, the data demonstrated that the most common effect of ASD-related missense and frame-shift mutations was loss of channel function. The missense mutants often caused complete block of ion conduction, although three of these mutants were conductive and showed only partial loss of function. A computational model predicted that all of the mutations studied would induce a deficit of neuronal excitability. Considering the expression pattern and distribution of $Na_V1.2$ channels during development, the authors suggested that these defects in neuronal excitability would result in a persistent change in neuronal circuit function, which together with neurodevelopment changes could result in induction of permanent ASD behaviors. Mouse models have been used to test this hypothesis (see below), although those available thus far are knock-outs that do not carry a mutation identified in patients.

B. $Na_v1.2$ Channels and Intellectual Disability

De novo heterozygous mutations of *SCN2A,* identified by exome sequencing studies in patients with sporadic ID without seizures, were functionally evaluated in a recent study, using the human adult $Na_V1.2$ variant transiently expressed in a human cell line. The three ID mutations were the frameshift L611Vfs*35 (190, 272), the stop codon W1716X (373), which both induce a large truncation, and the missense R937C (272). Their effects were compared with those of the NEIDEE mutation E1803G (254), the ICDEE mutation L1342P (132), and the BNIFS mutation

L1563V. ID mutations showed complete loss of function because they did not produce measurable currents, similarly to most of the ASD mutants investigated in (373) (see above). The NEIDEE mutant E1803G had a shallower slope of the voltage dependence of inactivation, which induced a partial positive shift and an increase of the window current, both gains of function consistent with the effect of other NEIDEE mutants. The ICDEE mutation L1342P induced contrasting modifications of gating properties, which can lead to an overall loss of function, similar to most of the ICDEE mutations studied. A mild gain of function was confirmed for the BNIFS L1563V mutation. These findings confirm the results obtained in other functional studies of $Na_V1.2$ mutations involved in epilepsy and support the hypothesis that complete loss-of-function usually leads to ID or ASD without epilepsy; however, it is not clear yet why some complete loss-of-function mutations cause ICDEE and not ID or ASD without epilepsy (372, 373).

C. $Na_V1.2$ Channels and Schizophrenia.

Schizophrenia is a neurodevelopmental disorder whose pathophysiology is largely unknown, characterized by incoherent or illogical thoughts, abnormal behavior, and altered perception, with abnormalities of brain function. The first report that showed possible involvement of *SCN2A* variants in schizophrenia was a genome-wide association study (GWAS) of 860 schizophrenic patients and 510 controls that revealed a significant association of general cognitive ability with polymorphisms in *SCN2A* and thereby implicated the $Na_V1.2$ channel in the pathophysiology of cognitive impairments in schizophrenia (94). The same study showed reduced expression of *SCN2A* mRNA in post-mortem prefrontal cortex tissue samples from schizophrenic patients compared to controls. Subsequent gene-sequencing studies have identified rare *SCN2A* variants in schizophrenic patients, including the missense mutation R850P discovered by exome sequencing of 623 schizophrenia trios (117), the missense mutation V1282F discovered in two cases, and the truncating mutation E169X discovered by sequencing *SCN2A* in 980 schizophrenic patients (48). The two missense mutations have uncertain functional effects, whereas the large truncation is predicted to cause complete loss of function. Functional studies are warranted in order to clarify better the genotype-phenotype relationship of these mutations, and to gain insight into the specific pathophysiological mechanism underlying this neurodevelopmental disorder compared to ASD and ID.

VIII. MOUSE GENETIC MODELS OF $Na_V1.2$ CHANNELOPATHIES

Although studies using heterologous expression systems are essential for screening the functional effects of numerous mutations and have given insights into the alterations of $Na_V1.2$

channel function caused by *SCN2A* mutations, gene-targeted mouse models carrying human variants are important tools for investigating the mechanisms of *SCN2A* mutations in different subtypes of neurons, disclosing their effects on neuronal networks, and linking them to behavioral dysfunctions. For instance, as outlined above, the neuropathogenic mechanisms of mutations of $Na_V1.1$ have emerged from studies of a gene-targeted mouse model, showing that $Na_V1.1$ is the major sodium channel in inhibitory neurons, and thus providing an explanation for the network disinhibition in patients with loss of function $Na_V1.1$ mutations in Dravet Syndrome and other diseases (51, 382). The mouse models of *SCN2A* mutations studied extensively thus far either delete *Scn2a* or express a truncated non-functional form of $Na_V1.2$.

The first $Na_V1.2$ mouse model was generated more than 20 years ago, before the identification of the first human pathogenic *SCN2A* mutation, in order to obtain insights into the physiological functions of $Na_V1.2$ (262). It is a gene knock-out that completely blocks $Na_V1.2$ expression by deletion of the first *Scn2a* exon. These global homozygous knockout mice (*Scn2a*$^{-/-}$) showed perinatal death and therefore suggested a crucial role of $Na_V1.2$ for the nervous system, despite possible compensatory effects of other sodium channels. In contrast, heterozygous *Scn2a*$^{+/-}$ mice did not show an overt phenotype, even though hippocampal pyramidal neurons dissociated from P5-P9 *Scn2a*$^{+/-}$ mice had 50% reduction of sodium current. Later follow-up studies did not detect convulsive seizures in *Scn2a*$^{+/-}$ mice (224). However, several recent papers based on this original mouse line and other lines more recently developed do indeed show epileptic features. A recent study (244) compared the *Scn2a*$^{+/-}$ global knock-out mouse developed in (262) with two other *Scn2a* mouse lines: a knock-in mouse model carrying the nonsense R102X *SCN2A* mutation that causes ICDEE (165) (see above), and conditional mouse lines with a *Scn2a* deletion in specific brain areas and cell types. The results showed that adult mice with both *Scn2a*$^{+/-}$ global knock-out and *Scn2a*$^{+/R102X}$ genotypes exhibit a mild epileptic phenotype of short absence-like seizures (FIGURE 12A), which are associated with spike-and-wave discharges (SWDs) in EEG recordings and are sensitive to the anti-absence seizure drug ethosuximide. This phenotype was more prominent in heterozygous conditional mice in which *Scn2a* was deleted specifically in dorsal-telencephalic excitatory neurons, whereas seizures were not detected in mice with selective *Scn2a* deletion in inhibitory neurons. A follow-up study showed that heterozygous *Scn2a* deletion in corticostriatal, but not corticothalamic neurons, was sufficient for the generation of SWDs, suggesting that impaired cortico-striatal excitatory transmission is a possible mechanism for absence-like seizures in this model (229).

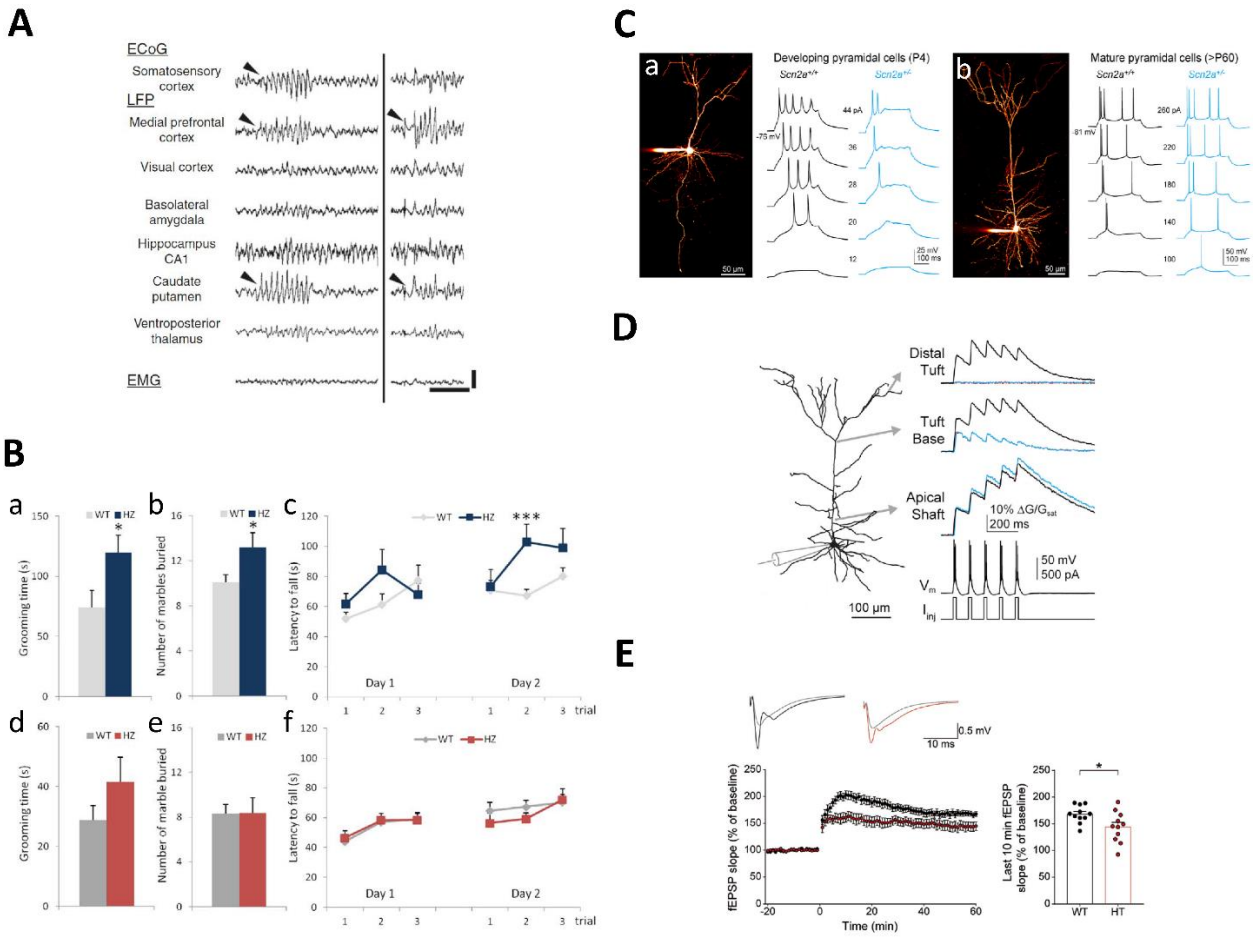


FIGURE 12. Phenotypes of heterozygous *Scn2a* knockout (*Scn2a*$^{+/-}$) mice. A. Absence-like seizures with spike and wave discharges (SWDs) associated with electromyogram (EMG) suppression are observed in 10–27 weeks old *Scn2a*$^{+/-}$ mice, as shown by electrocorticogram and multisite local field potential (ECoG-LFP) recordings, which reveal the predominant appearance of LFP epileptiform discharges in medial prefrontal cortex (mPFC) and caudate putamen (CPu). Black arrowheads indicate the onset of SWD. Scale bars: vertical, 500 µV; horizontal, 1s. Modified from (244). **B.** Stereotyped behaviors consistent with autistic-like traits are present in young but not adult *Scn2a*$^{+/-}$ (HZ) mice. Inset a. Time spent in self-grooming. Inset b. Number of marbles buried. Inset c. Persistence in repetitive rotarod trials, which evaluates repetitive motor behaviors. These stereotyped behaviors are not observed in adult (P60-95) mice. Modified from (184). **C.** Impaired somatic excitability. Inset a. Severe impairment in developing cortical pyramidal neurons from P4-7 *Scn2a*$^{+/-}$ mice. Morphology of a developing pyramidal cell (left) and action potential discharges (right). Inset b. Moderate impairment in mature neurons from adult *Scn2a*$^{+/-}$ mice (>P60). Morphology of a mature, thick-tufted pyramidal cell (left) and action potential discharges (right). Modified from (319). **D.** Back-propagating dendritic excitability is impaired in mature cortical layer 5 pyramidal neurons of *Scn2a*$^{+/-}$ mice. Calcium transients evoked by trains of action potential duplets were recorded at various locations throughout the apical dendrite. in WT neurons, duplets reliably evoke calcium transients throughout the apical dendrite, whereas calcium transients in *Scn2a*$^{+/-}$ neurons rapidly diminish in amplitude with increasing distance from the soma, becoming virtually absent in the most distal dendritic branches. Modified from (319). **E**. Decreased LTP induced by theta-burst stimulation (10 trains of 4 pulses at 100 Hz) at hippocampal Schaffer collaterals-CA1 synapses of three-month-old floxed *Scn2a*$^{+/-}$ mice (red) compared to WT littermates. Modified from (305).

Other recent studies of *Scn2a* mouse lines focused on behavioral and cognitive alterations, as well as cellular and local network defects. Behavioral and cognitive alterations identified in adult (>P60) *Scn2a*$^{+/-}$ global knock-out mice are quite mild. One study showed novelty-induced exploratory hyperactivity, decreased anxiety, and quite mild dysfunctions of social behavior (336), which may be interpreted as autistic- and/or schizophrenic-like features, whereas another study showed only trends towards dysfunctions in social behavior (319). Similarly, social behavior was not modified in a novel global heterozygous conditional floxed *Scn2a* knock-out mouse line (305), in which social approach, social communication, and repetitive behaviors were investigated. Tests of cognitive features showed enhanced fear memory in two studies (305, 336), one of which also showed mildly impaired spatial learning and memory in the Morris water-maze (305), and a third study showed only trends towards dysfunctions in a few other learning tasks (319). Spatial learning dysfunctions were studied in detail in another study (223). Slowed hippocampus-related spatial learning and abnormalities in hippocampal replay were observed, including decreased cell assembly reactivation strengths and truncated hippocampal replay sequences, which may be involved in consolidation of spatial memories. In contrast, no changes to single place cells or cell assemblies were observed during encoding of spatial information. Two studies analyzed autistic features in newborn and juvenile (up to P21) mice, including ultrasonic vocalizations, but these investigations did not show abnormalities (305, 336).

In contrast with the very mild behavioral dysfunctions observed in adult mice and the absence of them in newborn/juvenile ones, a recent study (184) identified more evident autistic features in young (P22-P45) global *Scn2a*$^{+/-}$ mice. Social communication quantified with ultrasonic vocalizations was strongly reduced, impairment in social interactions was very mild, and several motor stereotypies were observed (FIGURE 12B). These young *Scn2a*$^{+/-}$ mice also showed decreased memory performance in the novel object recognition test and a trend in the Y maze test. Moreover, they were less anxious and resigned than WT littermates. Consistent with the other studies, adult *Scn2a*$^{+/-}$ mice (>P60) showed a much milder phenotype, because only social communication was significantly reduced. Thus, behavioral-cognitive phenotypes are more prominent in young global *Scn2a*$^{+/-}$ mice than in adults.

Two studies that performed behavioral tests also investigated cellular/local network defects in the pre-frontal cortex (319), which may be particularly important for autistic and schizophrenic features, and in the CA1 area of the hippocampus (305), which may be particularly important for memory dysfunction. Investigations in prefrontal cortex brain slices of global *Scn2a*$^{+/-}$ mice (319)

showed, as expected, reduced excitability of cortical Layer 5 pyramidal neurons across developmental periods, including reduced action potential threshold and maximal firing frequency in neonatal neurons, and reduced action potential slope after P20 (FIGURE 12C). In contrast, excitability of cortical parvalbumin- and somatostatin-positive GABAergic neurons, as well as excitability of pyramidal neurons in the CA1 area of the hippocampus, were not altered in neurons from P34-P40 animals. Surprisingly, back-propagating action potentials in mature neurons were more attenuated in distal dendrites than in the soma, because $Na_V1.2$ haploinsufficiency severely impaired dendritic excitability (FIGURE 12D). Moreover, in juvenile (P27) mice, but not in neonates, both the frequency of miniature excitatory postsynaptic currents (mEPSCs) and the AMPA:NMDA ratio of stimulated synaptic currents were reduced, and dendritic spine morphology was altered, consistent with a pathologic remodeling of synaptic properties. In contrast, no modifications of miniature inhibitory postsynaptic currents (IPSCs) were observed.

To determine if these defects are caused by neurodevelopmental dysfunctions induced in the neonatal period or directly by $Na_V1.2$ haploinsufficiency, a conditional floxed *Scn2a* knock-out line was crossed with a (CaMKIIa)-Cre line, which expresses Cre recombinase in neocortical pyramidal cells only after P10 (319). These mice, which are haploinsufficient for $Na_V1.2$ after P10, show the same dysfunctions observed in global *Scn2a*$^{+/-}$ mice, ruling out the involvement of early neurodevelopmental dysfunctions in causing phenotypes later in life. Moreover, injections of low-titer Cre-expressing adeno-associated virus at P28 were used to obtain sparse $Na_V1.2$ haploinsufficient cortical neurons. These neurons showed reduced action potential slope, AMPA:NMDA ratio, and long-term potentiation (LTP), as well as small alterations of spine morphology. Thus, network effects were not necessary for generating the dysfunctions observed in these experiments.

Recordings in the CA1 area of the hippocampus of brain slices obtained from 3-week-old floxed global *Scn2a*$^{+/-}$ mice (305) showed some differences in comparison with the data obtained in the prefrontal cortex (319). In fact, AMPA:NMDA ratio and basal evoked synaptic transmission at Schaffer collateral-CA1 synapses were not modified, and there was a reduction of spontaneous excitatory postsynaptic currents (sEPSCs, which depend on network excitability), but not of miniature excitatory postsynaptic currents (mEPSCs, which depend on synaptic properties). This is consistent with lack of modification of functional properties or number of synapses. Nevertheless, LTP induced by high-frequency stimulation was reduced (FIGURE 12E), although LTD was not modified. Excitability of CA1 pyramidal neurons showed quite mild, if any, impairment, and inhibitory synaptic transmission was not modified.

Overall, these studies show that *Scn2a*$^{+/-}$ mice have a relatively mild phenotype, including short absence-like seizures, spatial memory deficits but enhanced fear memory, plus autistic and possibly schizophrenic traits. Some features are age-dependent, in particular autistic traits are more prominent in young mice and tend to remit in adults. Moreover, recordings in brain slices of these models have shown that $Na_V1.2$, besides its established axonal role, has important dendritic functions in pyramidal neurons of the prefrontal cortex, where its haploinsufficiency impairs synaptic plasticity and synaptic strength, even when $Na_V1.2$ expression is reduced in single neurons late in postnatal development. Notably, $Na_V1.2$ haploinsufficiency may lead to differential synaptic remodeling in the hippocampus compared to the prefrontal cortex. It would be interesting to investigate whether developmental reversal of cellular/network dysfunctions may be related to the strong attenuation of autistic-like behaviors in adult mice.

In summary, it is remarkable that studies to date reveal mutations of *SCN2A*/$Na_v1.2$ that cause a wide range of neurodevelopmental disorders, including epilepsy of varying severity with neonatal, infantile, or childhood onset, ASD, ID, and possibly schizophrenia. The first line of functional studies gives some explanations about genotype/phenotype relationships: gain-of-function mutations are related to mild benign neonatal/infantile epilepsy and to neonatal/early infantile epileptic encephalopathy, whereas loss-of-function mutations are linked to infantile/childhood epileptic encephalopathy or neurodevelopmental disorders like autism and intellectual disability without seizures. However, it is more difficult to establish genotype/phenotype relationships within categories of these gain-of-function or loss-of-function mutants, because functional effects of mutations causing different phenotypes often overlap. Different from *Scn1a* models, the available *Scn2a* mouse models show much milder phenotypes than patients, who have more prominent autistic traits, cognitive deficits, and epilepsy. In particular, haploinsufficient knock-out models show only mild absence seizures, whereas most patients have more severe epilepsy and there are only few patients that show absence seizures alone. More studies are now required to shed light on detailed pathophysiological mechanisms specific for each disorder within the *SCN2A* spectrum. The development of further *Scn2a* mouse models carrying different types of human mutations may help to unravel in more detail the pathophysiological mechanisms of patients.

Thus far, the only mouse model of a specific human pathogenic mutation is the knock-in of A263V (298), a mutation that induces neonatal onset seizures and late onset episodic ataxia in patients and causes $Na_V1.2$ gain of function because of gating modifications in cellular expression systems

(192). However, these mice have been studied in much less detail than the knock-out models presented above.

IX. $Na_V1.6$: EPILEPSY AND MOVEMENT DISORDERS

As outlined above, $Na_V1.6$ is the main sodium channel in AIS and nodes of Ranvier of myelinated axons of excitatory neurons and is now implicated in epilepsy and movement disorders (FIGURE 13A, B). The first pathogenic mutations of $Na_V1.6$ were identified in the "motor endplate disease" (now termed *Scn8a*^{med}; (220)) spontaneous mutant mouse line, which is characterized by severe motor dysfunction, including dystonia, ataxia, tremor, and progressive paralysis of the hind limbs, and by juvenile lethality. This mouse line carries a recessive *Scn8a* mutation, which truncates the $Na_V1.6$ channel protein leading to complete loss of function (41, 173, 220). The closely related mutant mouse line *Scn8a*^{medJ} has a nearly complete loss of function and similar phenotype (173, 220). A milder phenotype in the *jolting* mouse (now termed *Scn8a*^{jo}) (220) is caused by a missense mutation in the S4-S5 linker in domain III, which positively shifts the voltage dependence of activation and thereby impairs channel function (174). In-frame deletion of three residues in the S6 segment in domain IV in the *Scn8a*^{9J} mouse line (156) leads to partial loss of function of $Na_V1.6$ and less severe phenotypes. Different from the phenotypes observed in homozygous mice, anxiety-like behavioral dysfunction was initially identified as the main phenotype of heterozygous mice carrying these mutations (220). These mouse lines do not show spontaneous convulsive seizures, but spike-wave discharges (SWD) during periods of behavioral arrest, the hallmark of absence epilepsy, are observed in heterozygous *Scn8a*^{med} and *Scn8a*^{jo} mice, as well as in a line carrying a chemically induced *Scn8a* missense mutation (252).

Recordings from neurons obtained from mouse lines carrying *Scn8a* loss of function mutations have shown that firing is reduced in cerebellar Purkinje cells (269, 270) and granule neurons (248), consistent with motor dysfunction phenotypes, but also in numerous other types of neurons, including hippocampal CA1 pyramidal neurons (280), subthalamic neurons (96), globus pallidus neurons (222), trigeminal mesencephalic neurons (103), retinal ganglion cells (353), and DRG sensory neurons (67). A reduction of transient, persistent, and resurgent sodium current has been observed in several of these neurons (79, 96, 103, 214, 248, 269, 280).

A recent investigation, which used conditional cre-lox lines that allow specific $Na_V1.6$ knock-out in different neuron subtypes, proposed that SWD are generated by reduced tonic firing of GABAergic neurons of the thalamic reticular nucleus, leading to an impairment of desynchronizing recurrent synaptic inhibition within the reticular nucleus and thus to hyperexcitation of thalamo-

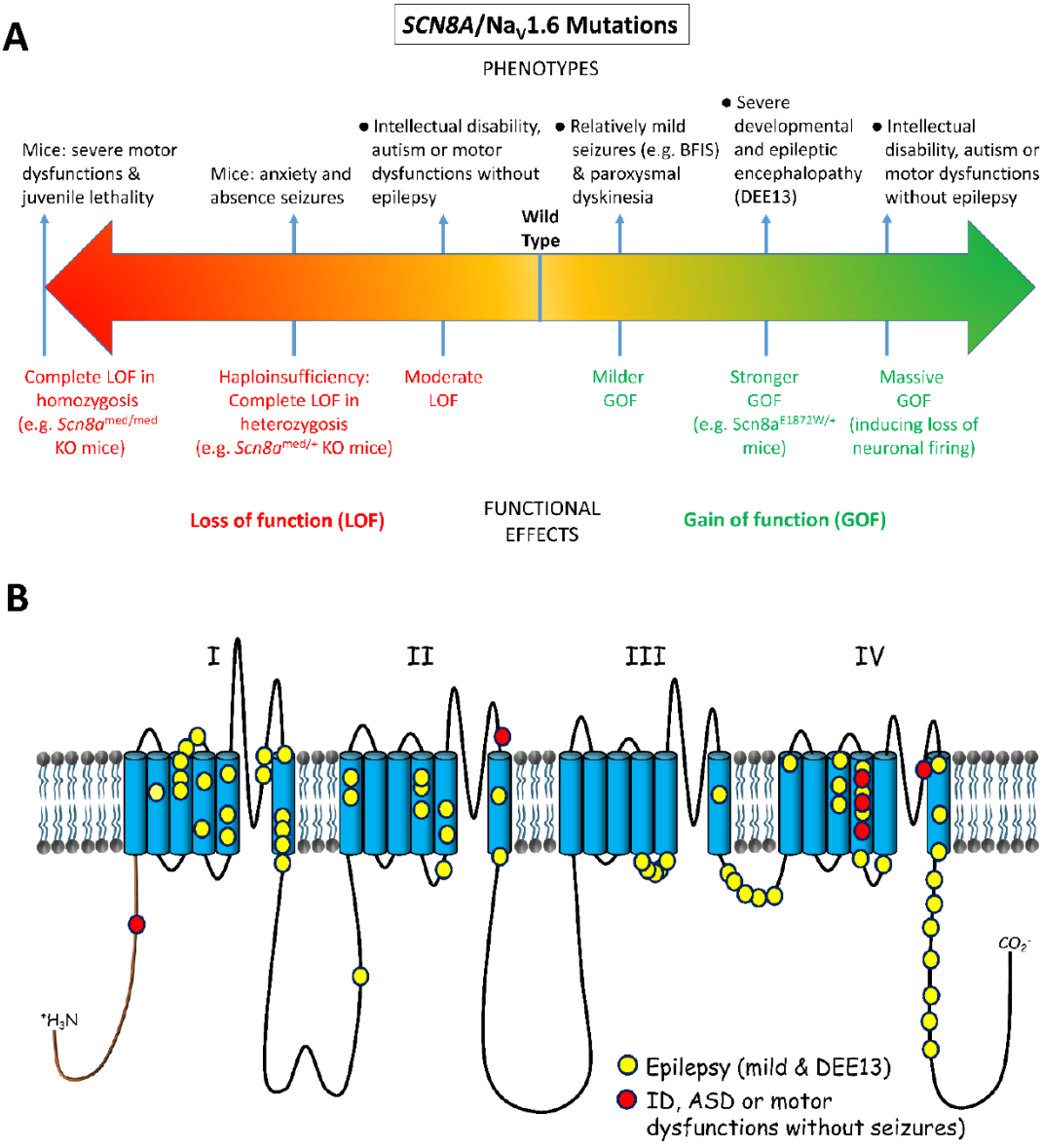


FIGURE 13. Spectrum of mutations and phenotypes for *SCN8A*/Na$_V$1.6. A. Phenotypic spectrum. A. Gain of function *SCN8A*/Na$_V$1.6 mutations can cause relatively mild epilepsy (e.g. BFIS), paroxysmal dyskinesia when the functional effect is moderate, or severe developmental and epileptic encephalopathy (DEE13) when the functional effect is stronger. Loss of function mutations can cause intellectual disability, autism or motor dysfunctions without epilepsy, which can be caused also by mutations that cause a massive gain of function inducing loss of neuronal firing by depolarizing block. Knock-in mice carrying gain of function mutations show hyperexcitability of excitatory neurons and phenotype consistent with DEE13. Knock-out mice reproducing complete loss of function in heterozygosis (haploinsufficiency) show anxiety and absence seizures. Mice with complete loss of function in homozygosis show severe motor dysfunctions and juvenile lethality. **B.** Molecular map of the Na$_V$1.6 sodium channel with the location of *SCN8A* mutations color-coded as indicated.

cortical circuits (200). Notably, in this study, specific decrease of $Na_V1.6$ expression in cortical excitatory neurons was antiepileptic, reducing convulsive seizures induced with flurothyl. Reduced epileptic activity was also observed previously in double heterozygous mice carrying a loss of function mutation in *Scn1a* that causes the Dravet Syndrome phenotype plus a loss of function mutation for $Na_V1.6$ that reduces action potential generation in excitatory neurons (211). These observations suggest that reduced expression of *Scn8a* protects against convulsive seizures by decreasing excitability of excitatory glutamatergic neurons.

The first human *SCN8A* mutation, introducing a premature stop codon that truncates $Na_V1.6$ causing complete loss of function, was identified in a heterozygous proband with cognitive impairment and cerebellar ataxia without seizures (345). However, this study was limited by lack of segregation in the small pedigree and incomplete information about family members. The second human mutation was identified by whole genome sequencing in a proband presenting with an infantile epileptic encephalopathy (early onset seizures, intellectual disability and ataxia), who died of sudden unexplained death in epilepsy (SUDEP) (356). The functional study of the missense mutation (N1768D) was carried out in neuroblastoma ND7/23 cells because $Na_V1.6$ does not express well in other cell lines normally used for functional studies of ion channels. The results showed a large increase in persistent current and incomplete sodium channel inactivation, consistent with a gain of function of $Na_V1.6$, leading to increased action potential firing in transfected neurons (356).

At present, a few hundred patients with *SCN8A* mutations have been reported with a wide phenotypic spectrum. The vast majority of the published *SCN8A* patients suffer from severe developmental and epileptic encephalopathy (DEE13), characterized by early onset epilepsy with multiple seizure types, infrequent febrile seizures, EEG abnormalities mainly in the temporo-occipital regions, severe intellectual disability, and movement disorders (121). Other patients show milder phenotypes, including benign familial infantile seizures (BFIS) with paroxysmal dyskinesia (120), and epilepsies with intermediate phenotypes (155). Furthermore, some patients carrying *SCN8A* mutations and showing intellectual disability, autism, or movement disorders without epilepsy have been reported (195, 361). Their phenotype is similar to that induced by the first human *SCN8A* mutation identified (345).

Although the first *SCN8A* disease mutation identified was truncating (345), thus leading to complete loss of function, the ones subsequently identified cause missense substitutions of

evolutionarily conserved amino acid residues that alter channel function. In contrast to pathologies caused by mutations in other sodium channels, more than 20% of patients have recurrent mutations, in particular at residues R1617 or R1872 (360). Functional studies of sixteen mutations have been carried out by expression *in vitro* in transfected ND7/23 cells and, in some studies, in transfected neurons in primary culture to investigate effects on excitability (82, 108, 195, 251, 356, 360, 361). Functional effects of mutations that cause DEE13 or milder epilepsy induce gain of function and include negative shifts of voltage dependence of activation, positive shifts of voltage dependence of inactivation, slowed channel inactivation, or increased persistent or resurgent current. These functional changes are all consistent with neuronal hyperexcitability, which has been observed in the studies that evaluated the effect on action potential discharges of transfected cultured neurons (108, 195, 356). Other mutations causing intellectual disability, autism, or movement disorders without epilepsy induce loss of function, which can be complete (195, 345, 361). Interestingly, a recent study has shown that functional analysis in transfected neurons is crucial for correctly disclosing the overall effect of some mutations and obtaining clear genotype-phenotype relationships (195). In fact, the mutation A1622D, identified in a patient with severe developmental delay and intellectual disability without epilepsy, caused a profound destabilization of the inactivated state leading to massive slowing of inactivation, positive shift of voltage dependence of inactivation, and increased persistent current, all effects causing gain of function at the channel level. However, expression in neurons showed that the large effects of this mutation lead to reduced excitability because of induction of depolarizing block of action potential firing. Moreover, the mutation E1483K causing mild epilepsy induced small and statistically insignificant modifications of the functional properties of the channel in ND7/23 cells, but increased firing in transfected neurons. Thus, it is important to include studies performed in neurons in future functional evaluations, as has been already done for $Na_V1.1$ (61, 90).

Two human *SCN8A* mutations causing DEE13 have been studied by engineering them in knock-in mouse models: the first mutation identified, N1768D, which was engineered in a standard knock-in (362), and the recurrent mutation R1872W, which was engineered in a conditional floxed knock-in (40). Both mice recapitulate in heterozygosis the basic features of *SCN8A* encephalopathy with spontaneous seizures, SUDEP, and mild impairment of motor coordination. Heterozygous N1768D mice show gain of function of sodium currents (increased persistent and resurgent current; positive shift of inactivation) and hyperexcitability in pyramidal neurons of the CA1 area of the hippocampus, layer II stellate neurons of the medial entorhinal cortex, and subiculum neurons of the hippocampus (196, 249, 366). In contrast, no changes were observed

in GABAergic bipolar neurons from the CA1 area or pyramidal neurons from the cortex and the CA3 area of the hippocampus (196, 249, 366). Conditional R1872W mice confirmed that excitatory neurons are the target of the functional effect, because restriction of R1872W expression to excitatory neurons induced seizure and SUDEP phenotypes that were similar to those of global expression, whereas restriction of R1872W expression to inhibitory neurons did not induce an overt phenotype (40). Notably, expression of R1872W in adulthood was sufficient to generate seizures and SUDEP, suggesting that successful therapies would probably require lifelong treatment (40). Cardiac features have been studied in heterozygous N1768D mice, showing that they have hyperexcitability of cardiac myocytes and increased parasympathetic tone leading to cardiac arrhythmia, which might be implicated in SUDEP (116). Thus, knock-in mice have confirmed that gain-of-function mutations of *SCN8A* are sufficient to induce hyperexcitability of some subtypes of excitatory neurons, generating severe seizures and a lethal phenotype, similar to the clinical features of DEE13. In contrast, spontaneous mouse models carrying loss of function $Na_V1.6$ mutations (220) show a phenotype that is similar to that of patients with intellectual disability and/or movement disorders without epilepsy. Future genetic studies of human pathologies characterized by motor dysfunctions may reveal that $Na_V1.6$ is more widely implicated in these pathologies.

Patients with *SCN8A* mutations are in general considered drug resistant. Knock-in *Scn8a* mice and transfected cells have been also used to identify potential new drug candidates for treatment of epilepsies caused by *SCN8A* mutations, showing that both new and old sodium channel blockers can be effective in reducing the functional effects of gain of function mutations (5, 7, 10, 40, 256, 366). Interestingly, it has been shown that DEE13 patients, which carry gain-of function mutations, can respond to high-dose treatment with the classical antiepileptic sodium channel blocker phenytoin (30), although another study cautioned regarding the long-lasting adverse effects of high-dose phenytoin treatment (37). Specific $Na_V1.6$ blockers are under development (e.g. https://www.xenon-pharma.com/product-pipeline/xen901-for-epilepsy/), and they might be more effective for long-lasting treatments than the classical drugs, which are not isoform-specific. Recently, antisense oligonucleotides that reduce *Scn8a* expression by 25 to 50% have been shown to delay seizure onset and lethality in both *Scn8a*$^{R1872W/+}$ and *Scn1a*$^{+/-}$ (Dravet Syndrome model) mice (186). Such genetic approaches offer a high level of specificity for inhibition of $Na_V1.6$ expression, if the challenges of drug delivery and drug half-life can be addressed.

X. $Na_V1.3$: EPILEPSY AND IMPAIRED NEURODEVELOPMENT

$Na_V1.3$, encoded by the *SCN3A* gene, is a still mysterious member of the sodium channel family, because its functions have not been completely determined, and, until recently, it had a marginal role in channelopathies. $Na_V1.3$ is broadly expressed in the brain at high levels during embryonic development, but postnatal expression is very low in both rodents and humans (15, 126, 311). In the past, the implication of $Na_V1.3$ in pathologies was limited to postnatal upregulation of its expression, observed in pain-related pathologic remodelling (364) or in homeostatic responses of epileptic rodent models, for example in *Scn1a* models (382). More recently, mutations of *SCN3A* have been identified in patients with different neurological phenotypes (FIGURE 14A, B).

The first one, the missense mutation K345Q, was identified in one patient in a study that screened a cohort of 18 children with pharmacoresistant childhood focal epilepsy. Functional analysis of this mutation, obtained by inserting the $Na_V1.3$ mutation into the cDNA of the $Na_V1.5$ cardiac channel, showed an increase in persistent current, consistent with gain of function (146). Functional analysis of this mutation performed subsequently using the human $Na_V1.3$ cDNA confirmed that it increases persistent sodium current in a transfected human cell line and induces hyperexcitability in transfected neurons (107). A genetic screen of pediatric patients with neonatal-childhood focal epilepsy identified four other *SCN3A* missense variants causing relatively mild phenotypes (354). All the variants identified in this study showed increased sodium current elicited with slow depolarizing ramps, although only one variant induced an increase of persistent sodium current elicited with step depolarizations, consistent with a preponderant effect on kinetics of fast inactivation for the three others. Thus, the results of the functional studies are consistent with neuronal hyperexcitability as the pathological mechanism of these $Na_V1.3$ mutations. Other variants have been identified in individuals with mild epileptic phenotypes, but with uncertain pathogenicity and without functional analysis (346, 363).

More recently, three de novo *SCN3A* heterozygous missense mutations (I875T in two cases, P1333L, and V1769A) have been identified in four unrelated early infantile developmental and epileptic encephalopathy (DEE62) patients, characterized by multifocal seizures, severe intellectual disability and, for the two cases with the I875T mutation, polymicrogyria (multiple small gyri creating excessive folding of the cerebral cortex) (384). The functional effect of the three mutations was studied in a human cell line transfected with a human $Na_V1.3$ cDNA, showing various mechanisms of gain of function consistent with neuronal hyperexcitability, including robust increases of persistent sodium current, negative shifts of voltage dependence of activation and positive shifts of voltage dependence of inactivation (384). After the first report, the recurrent

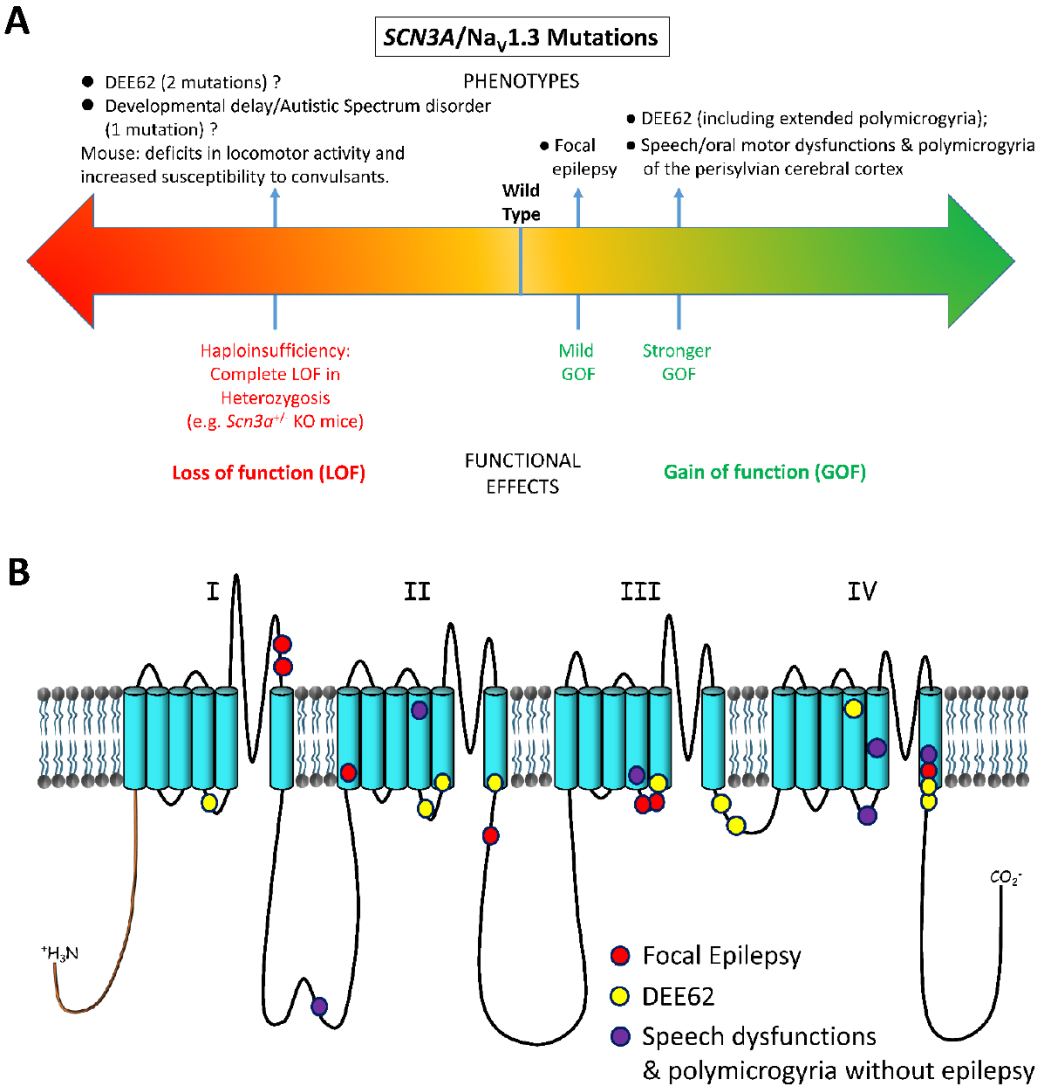


FIGURE 14. Spectrum of mutations and phenotypes in *SCN3A*/Na$_V$1.3. A. Phenotypic spectrum of Na$_V$1.3 mutations. Most *SCN3A*/Na$_V$1.3 pathogenic mutations cause gain of function, with milder ones identified in patients with focal epilepsy and stronger ones in patients with early infantile epileptic encephalopathy often including extended polymicrogyria (DEE62) or speech/oral motor dysfunctions and polymicrogyria limited to the perisylvian cerebral cortex without epilepsy. Loss-of-function mutations have been identified in two patients with DEE62 and one patient with developmental delay/ASD, but It is not clear how they can give rise to phenotypes that are similar to those of the gain-of-function mutations, which are the large majority. Haploinsufficiency in *Scn3a*$^{+/-}$ knock-out mice causes deficits in locomotor activity and increased susceptibility to convulsants, but not an overt epileptic phenotype or behavioral dysfunctions. **B.** Molecular map of the Na$_V$1.3 sodium channel with the location of *SCN3A* mutations color-coded as indicated.

I875T mutation was identified in two other unrelated patients with DEE62 and polymicrogyria (230), and two further *de novo* mutations were identified (without performing functional analysis) in two patients from another cohort (151), the M1765I mutation in one patient with DEE62 and severe polymicrogyria, and the L984del mutation in one patient showing intellectual disability with autistic features and pharmacoresponsive epilepsy, but no polymicrogyria.

Interestingly, five *SCN3A* heterozygous missense mutations have been recently identified in six unrelated families presenting with speech and oral motor dysfunctions associated with polymicrogyria of the perisylvian cerebral cortex, but that did not typically exhibit epilepsy (311). Disrupted cerebral cortical folding and neuronal migration were recapitulated in ferrets expressing the mutant $Na_V1.3$, extending previous work on mice and rats do not have cortical gyri. These studies in ferrets disclose a possible unexpected role of *SCN3A* in progenitor cells and migrating neurons involved in prenatal development of human cortical language areas. Interestingly, one of the mutations identified in this study (I875T) was also identified in a DEE62 patient with polymicrogyria (384). Functional analysis in a transfected human cell line using a human $Na_V1.3$ cDNA showed that these mutations induce gain of function, including increased persistent current and positive shift of voltage dependence of inactivation, similar to the effects reported in (384).

A further recent study (385), performed on a cohort of 22 patients, confirmed that *SCN3A*-related clinical phenotypes show a wide spectrum, including mild epilepsy with intellectual dysfunction, early infantile developmental and epileptic encephalopathy often associated with polymicrogyria, and speech and oral motor dysfunctions associated with polymicrogyria without epilepsy; ictal and non-ictal autonomic dysfunction or microcephaly can be additional clinical features. Relatively mild neonatal-childhood focal epilepsy, caused by the first *SCN3A* identified mutations (146, 354), would be the mildest phenotype in the spectrum. Onset is scattered between the neonatal and the early childhood period for all the phenotypes.

The functional studies in transfected human cell lines have shown that most of the *SCN3A* mutations identified in patients with severe phenotypes exhibit prominent gain of function, inducing in particular large increases of persistent sodium current, whereas variants identified in patients with milder phenotypes exhibit less pronounced gain of function (107, 311, 354, 384, 385). A puzzling finding is that loss-of-function caused by markedly reduced current density has been reported for three *SCN3A* variants: L247P in a child with focal epilepsy, developmental delay, microcephaly and autonomic nervous system dysfunctions (178), Y1669C in a child with

mild intellectual disability and autism spectrum disorder with no history of seizures or epilepsy (385) and K1506Nfs*18 in a DEE62 infant with profound developmental delay and without polymicrogyria (385). Heterozygous adult *Scn3a* knock-out mice were investigated as model of *SCN3A* loss-of-function mutations, but they do not show features of developmental and epileptic encephalopathy. In fact, they have increased susceptibility to induced seizures and deficits in locomotor activity, but no spontaneous seizures or abnormalities in other behavioral features (178). Interestingly, lack of plasma membrane targeting was reported for the L247P mutant, consistent with folding/trafficking defects (178). It would be interesting to test if *SCN3A* folding/trafficking mutants can be functionally rescued by expression in a neuronal cell background leading to gain-of-function, like some $Na_V1.1$ mutations (61, 90) (see above).

Most *SCN3A*-positive patients are drug resistant and, of note, patients that carry gain-of-function mutations are also reported as resistant to antiepileptic treatment with sodium channel blockers. Perhaps, earlier initiation of therapy or use of higher doses might be more efficacious, although we do not know yet whether epilepsy is directly caused by the $Na_V1.3$ dysfunction, by the brain malformations, or both.

Notably, patients carrying chromosomal deletions encompassing *SCN3A*, which should induce loss of function of $Na_V1.3$, show microcephaly, autistic features and language delay, but no seizures (57), and patients carrying chromosomal duplications encompassing *SCN3A*, which may induce gain of function of $Na_V1.3$ because of increased expression (but without modifications of gating properties), show neonatal seizures with normal intellectual development (341). Although disruption or duplication of neighboring genes are implicated in these chromosomal defects and may also contribute to these complex phenotypes, these findings are not consistent with some of the results presented above. These complex and variable phenotypes of *SCN3A* mutations may reflect an important role of *SCN3A* in neurodevelopment, which causes variable phenotypic outcomes through individual differences in neurodevelopmental processes and genetic background of affected individuals.

XI. LOOKING AHEAD

In addition to presentation of their physiology and pathophysiology, our review of sodium channelopathies of skeletal muscle and brain leads to several overarching themes with significance for the future.

- *Small effects matter.* Both skeletal muscle and brain sodium channels are involved in numerous human genetic diseases, in which their function can be either increased or decreased. Lessons from classic studies of skeletal muscle channelopathies taught us that small effects, such as impairment of sodium channel inactivation by only a few percent, are sufficient to be pathogenic. Mutations of brain sodium channels often follow this pattern, with relatively small effects on the voltage dependence and kinetics of sodium channel function leading to epilepsy, autism, and other neuropsychiatric disorders.
- *Functional analysis in detail is crucial*. Functional analysis in transfected cells is an important first step for performing screens of numerous mutants and may also be exploited for the development of diagnostic tests that could be included in clinical evaluations. However, specific experimental conditions for each gene should be better identified and standardized, in order to obtain robust functional evaluations. It will be important to use the correct cDNA clones, to express mutants in relevant subtypes of neurons, to study all the functional properties of sodium channels (including slow inactivation), and to perform experiments for investigating overall effects, including prediction of the effect on neuronal excitability (action potential clamp, dynamic clamp, computational modeling). These predictions and expression in neurons are particularly important for functional analysis of mutations causing loss of function that can be rescued or transformed into gain of function in neuronal cell backgrounds, as well as for mutations causing large gain-of-function effects, which may be equivalent at the cellular level to those that cause large loss of function, because they can induce depolarizing block of excitability.
- *Homeostasis often cannot compensate.* There are nine genes that encode voltage-gated sodium channels, and four of them are widely expressed in the brain. Nevertheless, even when homeostatic responses have been identified, in most of the cases they are not sufficient to rescue the effect of pathogenic mutations, especially those causing severe loss of function. Moreover, pathologic remodeling can often amplify the pathological effects of mutations. These complex responses to the initial direct functional effect of a mutation, which can be modulated both qualitatively and quantitatively by individual genetic background, are implicated in the substantial phenotypic variability that is observed in sodium channelopathies.
- *The genetic background of human nerve and muscle can determine the overall effects of mutations*. The use of human neurons, in particular neurons obtained with induced

pluripotent stem cell (iPSC) technologies, will complement phenotypic studies in the future. Thus far, only a few papers have been published on iPSC models of brain sodium channelopathies and there are none for skeletal muscle sodium channelopathies. Most of these studies have investigated $Na_V1.1$ mutations, and some technical problems related to variability and incomplete cellular maturation have limited their effectiveness, as recently reviewed (202). These technical problems may be solved in the future, but iPSC technologies are too time-consuming and expensive to be exploited for routine drug screening and clinical tests at this stage.

- *Animal models are essential.* Analysis of pathogenesis of sodium channel mutations *in vitro* is insufficient, although it can provide information on the initial direct functional effect of a mutation. Mutations should be introduced into animal models for testing therapeutic approaches *in vivo* and for identifying detailed mechanisms at the systems level, including homeostatic responses and pathologic remodeling. However, careful comparisons and critical evaluations of human and animal phenotypes are necessary to integrate phenotypic features and age-related specificities of diseases in different species, whose time course of development and complexity of brain and muscle function are so different.
- *Structure is coming.* Though we have not focused on this point here, the tidal wave of sodium channel structures that have emerged in recent years provide exceptional opportunities for defining common molecular and structural mechanisms of pathogenesis and using high-resolution structural data in structure-based drug design for seemingly intractable diseases.

XII. ACKNOWLEDGEMENTS

Research in the authors' laboratories presented here was supported by research grants from the Laboratory of Excellence "Ion Channel Science and Therapeutics" (LabEx ICST, ANR-11-LABX-0015-01, France), the ComputaBrain project (UCA-Jedi, ANR-15-IDEX-01, France), the European Commission (FP7 project 602531-DESIRE), the Foundation Famiglie Dravet Onlus (Italy), the Foundation Jérôme-Lejeune (France), the Interdisciplinary Institute for Modeling in Neuroscience and Cognition (NeuroMod) of the Université Côte d'Azur (France) (M.M. and S.C.) and the National Institutes of Health, U.S.A. (W.A.C.: R01 NS25704, R01 NS15751, and R35 NS111573). MM and SC are members of the "Fédération Hospitalo-Universitaire" InovPain (FHU-InovPain, France). W.A.C. sincerely thanks the Institut de Pharmacologie Moléculaire et Cellulaire, Université Cote d'Azur (Valbonne-Sophia Antipolis, France) and especially Directeur Professeur Jean-Louis Nahon, Directeur Adjoint Professeur Florian Lesage, and Directeur Emeritus Professeur Michel Lazdunski for their hospitality during his appointment as a Visiting Scientist in Autumn, 2018, when the concept, outline, and initial drafts for this review article were developed. The visit was sponsored in part by the Académie 4-Université Cote d'Azur (UCA-Jedi, ANR-15-IDEX-01). We thank all the members of our laboratories that have contributed to obtain the results presented in this review.

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