Genetica **122:** 37–45, 2004.

Allelic mutations of the sodium channel SCN8A reveal multiple cellular and physiological functions

Miriam H. Meisler, Nicholas W. Plummer, Daniel L. Burgess, David A. Buchner & Leslie K. Sprunger

Department of Human Genetics, University of Michigan, Ann Arbor, MI 48109-0618, USA (Phone: +1-734-763-5546; Fax: +1-734-763-9691; E-mail: meislerm@umich.edu); Current addresses: N.W. Plummer, NIEHS, Research Triangle Park NC; D.L. Burgess, Department of Neurology, Baylor College of Medicine, Houston TX; D.A. Buchner, Life Sciences Institute, University of Michigan, Ann Arbor MI; L.K. Sprunger, College of Veterinary Medicine, Washington State University, Pullman, WA

Key words: allelic series, hypomorphs, med, missense mutations

Abstract

Allelic mutations of Scn8a in the mouse have revealed the range of neurological disorders that can result from alternations of one neuronal sodium channel. Null mutations produce the most severe phenotype, with motor neuron failure leading to paralysis and juvenile lethality. Two less severe mutations cause ataxia, tremor, muscle weakness, and dystonia. The electrophysiological effects have been studied at the cellular level by recording from neurons from the mutant mice. The data demonstrate that Scn8a is required for the complex spiking of cerebellar Purkinje cells and for persistent sodium current in several classes of neurons, including some with pacemaker roles. The mouse mutations of Scn8a have also provided insight into the mode of inheritance of channelopathies, and led to the identification of a modifier gene that affects transcript splicing. These mutations demonstrate the value of mouse models to elucidate the pathophysiology of human disease.

Overview

The spontaneous mouse neurological mutant 'motor endplate disease' (*med*) arose in 1958 at the University of Edinburgh (Duchen, Searle & Strich, 1967). This autosomal recessive mutation resulted in muscle atrophy, paralysis and death. Two allelic mutations were later identified at the Jackson Laboratory, med^{J} and *jolting* (Sidman, Cowen & Eicher, 1979). Elegant morpholgical, physiological and electrophysiological studies in the laboratories of Duchen and Harris in the 1970s and 1980s led to the prediction that the mutation affected an ion channel gene involved in release of neurotransmitter from the nerve terminals of motor neurons. In 1992, an insertional mutation with a similar phenotype was discovered by Brett Spear at the University of Kentucky. We demonstrated allelism of the new mutation by noncomplementation with med^{J}, and demonstrated that the disrupted gene was a novel neuronal sodium channel (Burgess et al., 1995; Kohrman et al., 1995). Fortunately, the first three alleles had been preserved, and Dave Kohrman went on to identify the molecular lesions in all three alleles (Kohrman, Harris & Meisler, 1996a; Kohrman et al., 1996b). The *Scn8a* allele series provides the first demonstration of the *in vivo* physiological and clinical consequences of mutations in a neuronal sodium channel gene. In addition to the visible neurological disorders, electrophysiological studies of neurons isolated from these mice have revealed some of the unique cellular functions of the Scn8a channel. The phenotypes of the mouse mutants provide a prediction of the types of human patients that may carry mutations in voltage gated sodium channels. The first such mutation was found in our laboratory earlier this year (Trudeau et al., 2000). The *Scn8a*

allele series demonstrates the heterogeneity of disorders resulting from different mutations in a single gene, and illustrate the value of multiple alleles for elucidating cellular and physiological function.

The voltage-gated sodium channel α subunit gene family

The voltage-gated sodium channels are responsible for the rising phase of the action potential in neurons and muscle cells. The mammalian genome contains 10 closely related sodium channel genes with highly conserved protein sequence but divergent tissue-specific expression. These genes are located in four paralogous chromosome segments that also contain the HOX gene clusters (Plummer & Meisler, 1999). Four of the sodium channel genes are expressed at a high level in the central nervous system (CNS): *SCN1A*, *SCN2A*, *SCN3A*, and *SCN8A*. Most neurons in the CNS appear to express all of these channels, albeit in different proportions. Although their *in vitro* channel biochemical activities are very similar, null alleles of Scn1a, Scn2a and Scn8a in the mouse are all lethal, demonstrating that they are not functionally redundant *in vivo* (Burgess et al., 1995; Planells-Cases et al., 2000; W. Catterall, pers. commun.). Analysis of rescue by chimeric channels may provide insight into the unique functions associated with each family member. Physiological studies of these mice will be essential for understanding the role of each channel in the intact nervous system.

Characteristics of sodium channel SCN8A

SCN8A is one of the most abundant voltage-gated sodium channels in the brain (Schaller et al., 1995), and is the major channel at the nodes of Ranvier in mature myelinated axons (Caldwell et al., 2000; Krzemien et al., 2000; Schaller & Caldwell, 2000; Tzoumaka et al., 2000). Additional sites of subcellular localization in the CNS include dendrites, presynaptic and postsynaptic membranes, and nonmyelinated axons. Because it is found at synapses, modulation of SCN8A activity by genetic variation or physiological modification may be expected to influence synaptic strength, learning and memory.

An insertional allele of the *Scn8a* locus, *Scn8a*^{tg}

The insertional mutation of *Scn8a* arose during a long-term study of expression of the alpha fetoprotein promoter in transgenic mice (Spear, 1994; Ramesh, Ellis & Spear, 1995). Homozygous transgenic mice of one line, A4, exhibited a neurological syndrome that began with abnormal gait at 2 weeks of age and led to paralysis and death by 3 weeks of age. The new mutation was genetically mapped approximately 10 cM distal to the position of the med locus on the consensus map of mouse chromosome 15 (Kohrman et al., 1995). Because of the similarity in the phenotypes of A4 and *med*, we tested for alleleism by crossing A4 transgenic heterozygotes with heterozygous *med*^{J} mice from the frozen embryo bank at The Jackson Laboratory. The expected frequency of 25% of compound heterozygote offspring were recovered in the F2, and these animals displayed the typical *med* phenotype, demonstrating that the new mutation was allelic with *med*^{J}.

Cloning of the Scn8a gene

To identify the mutated gene, we generated a cosmid library of genomic DNA from *Scn8a*^{tg} homozygotes and screened it by hybridization with the transgene; the methods were described (Meisler et al., 1997) and are still appropriate for the isolation of new insertional mutants. Junction clones containing both transgene and adjacent DNA from the insertion site were analyzed and found to contain exons from a novel sodium channel (Burgess et al., 1995). A deletion of ≤20 kb in the middle of the *Scn8a* gene removed several exons and inactivated the gene.

The human ortholog of the new gene, *SCN8A*, was isolated and mapped to a conserved linkage group on chromosome 12q13 (Burgess et al., 1995). The structure and sequence of the human gene have been determined (Plummer et al., 1998). The orthologous rat cDNA was independently isolated in 1995 (Schaller et al., 1995), and the human cDNA was subsequently isolated from dorsal root ganglion neurons (Dietrich et al., 1998). This channel has been variously designated NaCh6, CerIII and PN4; the new recommended nomenclature is $Na_v1.6$.

The original *med* mutation is a null allele of *Scn8a*

The original *med* line and the *jolting* allele were maintained by Dr John Harris at the Neuromuscular Disease Unit in Newcastle, UK, for more than 20 years, and he kindly provided breeding pairs. Analysis by RT-PCR and genomic sequencing demonstrated that the original *med* mutation was caused by insertion of a transposable L1 element into exon 2 (Figure 1). Aberrant splicing of the mutant transcript disrupts the open reading frame and truncates the protein close to the N-terminus (Kohrman, Harris & Meisler, 1996a). Thus both the original allele and the transgene insertion allele are nulls, and exhibit identical phenotypes (Table 1).

Failure of motor nerve function in Scn8a null mice results in juvenile lethality

Homozygous $Scn8a^{med}$ and $Scn8a^{medtg}$ mice develop severe muscle atrophy, weakness, and progressive paralysis that begins in the hind limbs. Between 17 and 23 days of age, the sciatic nerve exhibits reduced conduction velocity, prolonged refractory period, and widening of the non-myelinated gaps at the nodes of Ranvier (Agnaut-Petit et al., 1982; Rieger et al., 1984; Bournaud et al., 1987; Füchtbauer, 1987; Kearney, 2002). These changes are accompanied by failure of transmission at the neuromuscular junction, with somewhat faster progression in biceps than in triceps (Duchen & Stefani, 1971). Terminal sprouting of the motor nerves, muscle fiber atrophy, and appearance of central nuclei indicative of fiber regeneration (Duchen, Searle & Strich, 1967; Duchen & Stefani, 1971) demonstrate that the motor neurons do not properly innervate the muscle in homozygous null mice, which do not survive beyond 1 month of age.

The severe phenotype and hind limb paralysis of the null mutants can be understood in the light of recent evidence that Scn8a is the major channel at the nodes of Ranvier (Caldwell et al., 2000). The total sodium current of motor neurons increases three fold during the first week of life in normal mice, and most of this increase appears to be due to Scn8a, since there was no increase in Scn8a null mice (Garcia et al., 1998). Scn8a is also the major nodal channel in nerves innervating the diaphragm muscle, and paralysis of the diaphragm may be the cause of death. Because of the early lethality, the null mutant cannot be used to study the effects of Scn8a deficiency in adult brain. Heterozygotes for the null mutations are phenotypically normal, demonstrating that 50% of normal levels of Scn8a is adequate.

A hypomorphic allele of *Scn8a* results in severe muscle weakness and dystonic postures

The med^{J} mutation arose 30 years ago at the Jackson Laboratory in a linkage stock carrying the closely linked visible marker *Caracul* (*Ca*). A 4 bp deletion in the splice donor site of intron 3 results in abnormal splicing and skipping of exons 2 and 3 (Kohrman, Harris & Meisler,

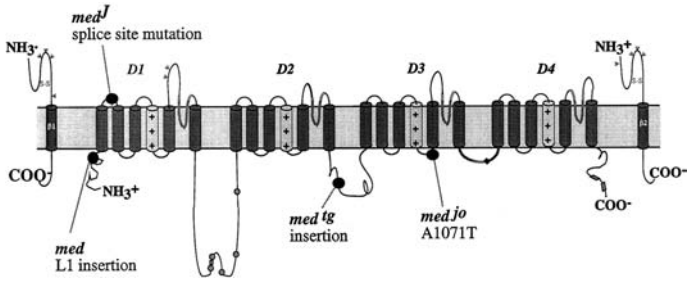

Figure 1. Positions of four allelic mutations of the mouse sodium channel Scn8a. The voltage-gated sodium channels contain four homologous domains (D1 to D4) each with six transmembrane segments (S1 to S6). The domains are separated by cytoplasmic loops and a short inactivation linker. The sequences of the transmembrane domains are more conserved than the cytoplasmic loops. The positively charged S4 segments act as voltage sensors and move outward from the membrane in response to depolarization. The associated transmembrane $\beta 1$ and $\beta 2$ subunits are also shown.

Table 1. Four allelic mutations in the mouse SCN8A gene

Mutant name	Allele	Effect on protein	Molecular mutation	Neurological phenotype	Physiological phenotype	Cellular phenotype
med	*Scn8a*^{med}	Null	Spontaneous Line1 insertion	Paralysis	Muscle atrophy, slowed nerve conduction, neurotransmitter release at NMJ	Sprouting of motor neurons, muscle atrophy
med^{J}	*Scn8a*^{medJ}	Hypomorph(12% level of transcript)	4 bp splice site deletion	Weakness, dystonia		P-cells, DCN, motor neurons
jolting	*Scn8a*^{jo}	Amino acid substitution	Nucleotide substitution	Tremor ataxia		P-cells abnormal, motor neurons normal
TgNA4Bs	*Scn8a*^{tg}	Null	Nontargetted transgene insertion	Paralysis	Same as above	P-cells motor neurons cortical pyramidal cells?

P-cell – cerebellar Purkinje neuron; DCN – dorsal cochlear nucleus; NMJ – neuromuscular junction.

1996a). The resulting protein is truncated in Domain 1 (Figure 1). However, *Scn8a*^{medJ} is not a true null allele because approximately 12% of the transcripts are correctly spliced (Kearney et al., 2002). This low level of expression prevents paralysis, but homozygotes have severe muscle weakness and cannot support their own weight. They also exhibit frequent episodes of sustained abnormal postures of the limbs and body (Sprunger et al., 1999). These dystonic postures persist for periods from 5 to 10 s in young animals, and for several minutes in older animals. An example is shown in Figure 2, left panel. These postures resemble human dystonia, although it is not known whether the pathogenesis is of central or peripheral origin. Imbalance in the strength of flexor and extensor muscles could be responsible, but abnormal currents in higher centers of motor control may also contribute the dystonic phenotype. A 'floxed' allele of *Scn8a* has been developed, in order to resolve these alternatives using tissue specific inactivation of the channel (S. Levin & M. Meisler, genesis 2004). Inactivation of *Scn8a* in cerebellar Purkinje cells reproduces a subset of the null phenotype (manuscript in preparation).

A missense mutation of *Scn8a* results in tremor and ataxia

The *jolting* mutation arose at the Jackson Laboratory in 1965 (Sidman, Cowen & Eicher, 1979). A single nucleotide change results in an alanine to threonine substitution in an evolutionarily conserved cytoplasmic linker domain (Figure 1) (Kohrman et al., 1996b). This mutation causes a 14 mV positive shift in the voltage dependence of activation of the channel (Kohrman et al., 1996b; Smith & Goldin, 1999). *Scn8a*^{jo} homozygotes have chronic ataxia with an unsteady, wide-based gait, as well as a rhythmical tremor of head and neck that is induced by attempted movement (Dick, Boakes & Harris, 1985). Neuromuscular transmission is normal, and the mice are fertile and have a normal lifespan. Cerebellar Purkinje cells show a lack of spontaneous activity and there is progressive loss of Purkinje cells after 6 months of age (Dick, Boakes & Harris, 1985; Harris, Boakes & Court, 1992; see below). The characteristics of the four *Scn8a* mutants are summarized in Table 1.

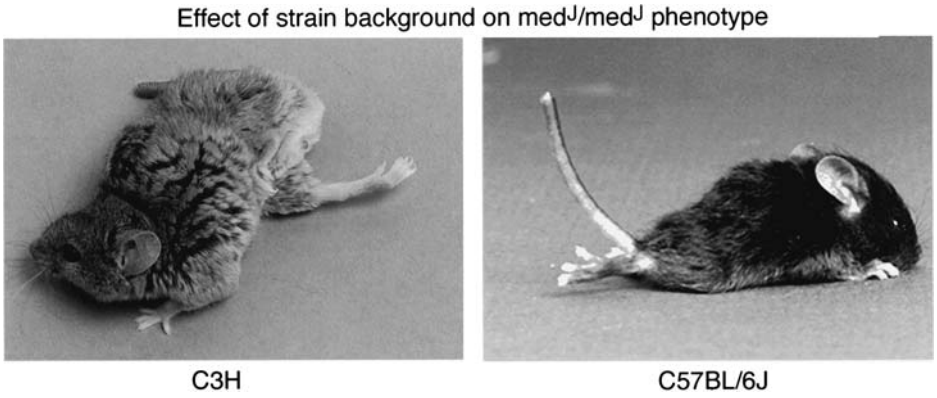

Figure 2. A modifier gene alters the clinical consequences of low levels of Scn8a in homozygous *med*^{J} mice. The 8 month old homozygote on strain C3H exhibits muscle weakness and dystonic postures. The 3 weeks old animal on strain C57BL/6J is paralyzed. The difference is caused by the Scnm1 modifier locus on chromosome 3.

Scn8a is essential for complex spiking in cerebellar Purkinje cells

The most striking cellular effect of the *Scn8a* mutations described to date is the effect on cerebellar Purkinje cells. These cells are distinguished by their spontaneous slow firing pattern and their ability to generate a series of action potentials after stimulation. This complex spiking activity is thought to be involved in integration of signals for motor control. Recordings of currents from Purkinje cells demonstrate loss of complex spiking in both *Scn8a*^{tg} and *Scn8a*^{jo} mice (Figure 3, right panel). The similar effect of the null and the missense mutation indicates that the Purkinje cell is particularly dependent on Scn8a. This functional requirement is consistent with the high level of Scn8a expression that was detected in Purkinje cells (Schaller et al., 1995; Krzemien et al., 2000). *Scn8a* mutations also affect two other characteristic

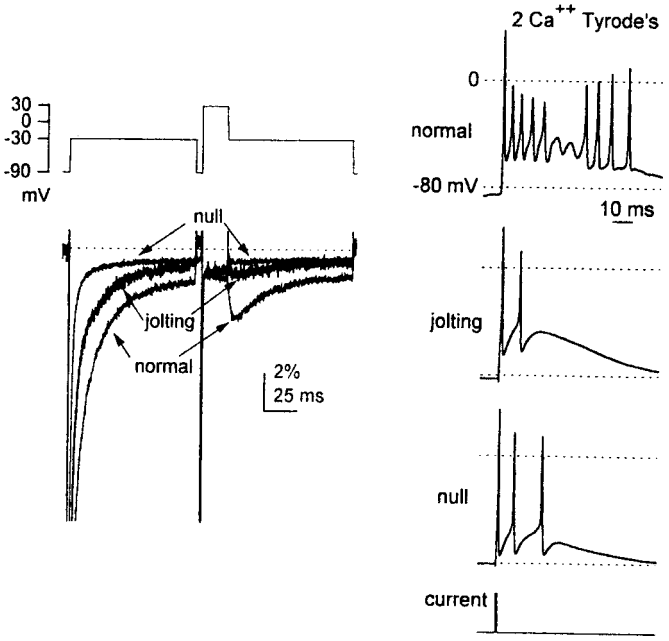

Figure 3. Two characteristic currents of cerebellar Purkinje cells are reduced in mice with mutation in Scn8a. Left, resurgent current and persistent currents; right, complex spiking. Reprinted from Raman et al. (1997) with permission of the publisher.

sodium currents of Purkinje cells, the persistent current and the resurgent current (Figure 3, left panel) (Raman et al., 1997). It is interesting that the small change in voltage dependence of *Scn8a*^{jo} has a profound effect on currents in Purkinje cells but does not impair the generation of action potentials in motor neurons.

Cartwheel cells of the dorsal cochlear nucleus are ontologically related to cerebellar Purkinje cells and exibit similar firing patterns. Currents recorded from the dorsal cochlear nucleus in brain slices from *Scn8a*^{medJ} and *Scn8a*^{jo} mice demonstrated extensive loss of these cells as well (Chen et al., 1999). Persistent current is a feature of many pacemaker neurons and pyramidal cortical cells, and the *med* mutants alter these as well (Raman et al., 1997; Maurice et al., 2001).

Threshold quantitative requirement for SCN8A: how much is enough?

For most enzyme deficiencies, a few percent of normal activity is sufficient to prevent disease, but for structural proteins like collagen, haploinsufficiency is common. We generated mice with various levels of Scn8a by combining different alleles (Table 2). The results suggest that the critical threshold for survival is between 6 and 12% of normal levels, and the threshold for normal movement is between 12 and 50% of the normal concentration.

Dominant or recessive inheritance of sodium channel mutations

The mode of inheritance of ion channel mutations is determined by the nature of the channel defect and the function of the mixed channel population in heterozygous cells. Sodium channel α subunits function as monomers. Many human mutations in ion channels result in incomplete inactivation of the channel and are inherited as dominant disorders (Bulman, 1997). In contrast, the *Scn8a* alleles in the mouse are recessive. This can be understood in terms of the functional effects of each allele. *Scn8a*^{jo} encoded channels require an extra 14 mV of depolarization before the channel is activated. In heterozygotes, the wildtype channels activate at normal voltage and can initiate the action potential. Thus both types of inheritance may occur, depending on the functional characteristics of the mutant protein.

Table 2. Quantitative threshold for SCN8A function (+, wildtype; −, null)

Genotype	Amount	Phenotype
+/+	100%	Normal
+/−	50%	Normal
med^{J} /med^{J}	12%	Dystonia, muscle weakness
med^{J}/−	6%	Lethal
−/−	0	Lethal

Digenic inheritance: an unlinked modifier locus of the hypomorphic *Scn8a*^{medJ} allele

The influence of secondary genetic factors on the severity of human monogenic disorders is becoming increasingly evident (Dipple & McCabe, 2000). Inbred strains of mice carry different alleles at many loci. Interacting genes that modify a mutant phenotype may be identified by crossing a mutant onto different strains. The *Scn8a*^{medJ} mutation has been tested on five strain backgrounds. Muscle weakness and dystonic postures were observed on four strains: C3H, DBA, A/J and 129/SvJ (Figure 2, left panel). However, on strain C57BL/6J, *Scn8a*^{medJ} homozygotes mimic null homozygotes and do not survive (Figure 2, right panel). A modifier gene on chromosome 3, designated sodium channel modifier 1 (*Scnm1*), co-segregates with lethality in crosses between C57BL/6J and each of the other strains (Sprunger et al., 1999, Buchner et al., 2003a). Thus C57BL/6J appears to carry a unique, 'susceptible' allele of the modifier *Scnm1*. The orthologous human chromosome band 1q21 is a gene-dense region containing several genes with neuronal functions. We used positional cloning to identify this novel gene and its mechanism of interaction with Scn8a (Buchner, Trudeau & Meisler, 2003b). The modifier gene encodes an U1C zinc finger protein with nuclear localization that acts on the *med*^{J} transcript to increase the proportion of correctly spliced transcripts. The isolation of *SCNM1* is one of the first demonstrations of the mechanism of interaction of a modifier gene with a primary disease gene.

Insights from multiple alleles of *Scn8a*

If we had been limited to analysis of a null allele of *Scn8a*, we would not have guessed that mutations in this gene could result in tremor, ataxia, or dystonia, all serious human medical concerns. We would also have been unable to study the role of the channel in the adult nervous system. Within the next few years we anticipate a significant increase in the number of human neurological disorders associated with sodium channel mutations. We are likely to discover a heterogeneous group of amino acid substitutions causing mild alterations in channel properties, like the SCN1A mutations in GEFS plus (Escayg et al., 2000). Because of the critical role of sodium channels in neuronal signaling, structural variants may also be susceptibility factors in polygenic disease, including the common psychiatric disorders, through genetic interaction with subtle variants in other proteins. We have identified rare coding variants of SCN1A, SCN2A and SCN3A in 5% of patients with familial autism (Weiss et al., 2003). Different classes of neurons may vary in their susceptibility to specific mutations, as we have seen for the *jolting* mutation in Purkinje versus motor neurons.

The phenotypes of *Scn8a* mutant mice have directed our attention to specific human patients for mutation testing. We anticipate that at some point in the future all admissions to neurological or psychiatric services will be routinely screened for channelopathies, using a sequencing chip that can detect variation in all 10 sodium channel genes. A wealth of information on structure/function relationships will then become available. Introduction of human disease mutations into the orthologous mouse gene as transgenes or targeted mutation will be useful for verifying causality and for analysis of pathogenic mechanisms (Figure 4). Recordings from neurons isolated from mutant mice provide data about mutant channels that goes beyond the information that can be obtained from expression in *Xenopus* oocytes or transfected mammalian cells. Crosses with mice carrying mutations in other genes can reveal genetic interactions and modifiers influencing clinical phenotypes. Analysis of multiple alleles in the mouse can provide a depth of understanding of *in vivo* gene function that is otherwise unavailable.

Acknowledgements

Our work on the alleles of *Scn8a* has been supported by research grants from the National Institute of General Medicine (GM24872), the National Institute of Neurological Disease and Stroke (NS34509), the March of Dimes and the Wilson Medical Research Foundation.

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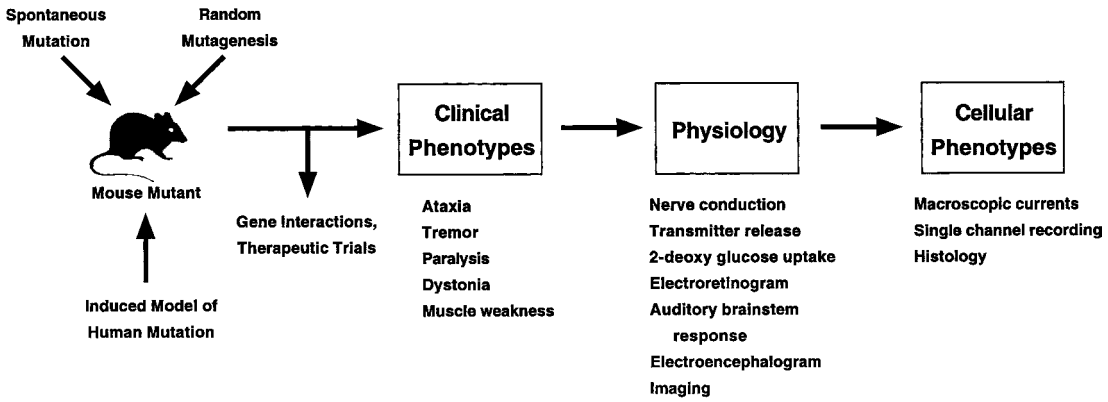

Figure 4. Experimental analysis of neurological dysfunction in mutant mice. The neurological mutations can arise spontaneously, or be introduced to the mouse germ line by chemical mutagenesis *in vivo* or in ES cells, or by homologus recombination in ES cells. For the case of ion channel mutations, electrophysiological studies on neurons isolated from mutant mice will reflect the *in vivo* functional alteration of the mutant channel more accurately than assays in *Xenopus* oocytes or transfected mammalian cells.

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Note added in proof: Three new ENU-induced alleles of Scn8a that cause recessive disease have recently been described (Buchner et al., Mammalian Genome 15: 344–351, 2004). A floxed allele for conditional inactivation of Scn8a has been generated (Levin and Meisler, Genesis, 2004, in press).