Ankyrin<sub>G</sub> Is Required for Clustering of Voltage-gated Na Channels at Axon Initial Segments and for Normal Action Potential Firing

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Abstract. Voltage-gated sodium channels (NaCh) are colocalized with isoforms of the membrane-skeletal protein ankyrin<sub>G</sub> at axon initial segments, nodes of Ranvier, and postsynaptic folds of the mammalian neuromuscular junction. The role of ankyrin<sub>G</sub> in directing NaCh localization to axon initial segments was evaluated by region-specific knockout of ankyrin<sub>G</sub> in the mouse cerebellum. Mutant mice exhibited a progressive ataxia beginning around postnatal day P16 and subsequent loss of Purkinje neurons. In mutant mouse cerebella, NaCh were absent from axon initial segments of granule cell neurons, and Purkinje cells showed deficiencies in their ability to initiate action potentials and support rapid, repetitive firing. Neurofascin, a member of the L1CAM family of ankyrin-binding cell adhesion molecules, also exhibited impaired localization to initial segments of Purkinje cell neurons. These results demonstrate that ankyrin<sub>G</sub> is essential for clustering NaCh and neurofascin at axon initial segments and is required for physiological levels of sodium channel activity.

Key words: ankyrin<sub>G</sub> • sodium channel • neurofascin • clustering • action potential

Clustering of voltage-gated sodium channels (NaCh) at axon initial segments, nodes of Ranvier, and postsynaptic folds of the neuromuscular junction is vital for generating sufficient local current to overwhelm membrane capacitance and resistance, and to initiate and propagate a self-regenerating action potential. Understanding the mechanisms for formation and maintenance of these NaCh-enriched domains represents a challenge with important implications for the physiology of excitable membranes as well as general issues of cell polarity and membrane structure.

Initial clues regarding possible molecular neighbors of the NaCh that could determine membrane localization came from observations that the NaCh copurified with the membrane skeletal protein ankyrin and associated with ankyrin in in vitro assays (Srinivasan et al., 1988). Ankyrin subsequently was localized at axon initial segments and nodes of Ranvier (Kordeli et al., 1990; Kordeli and Bennett, 1991), sites where the existence of a high density of NaCh has been well documented (Catterall, 1981; Ellisman and Levinson, 1982; Wollner and Catterall, 1986). Ankyrin also was localized to the NaCh-enriched regions of the neuromuscular junction (Flucher and Daniels, 1989). The isoforms of ankyrin localized at nodes of Ranvier, and axon initial segments have been identified as 480- and 270-kD alternatively spliced variants of ankyrin<sub>G</sub> (Kordeli et al., 1995). Ankyrin<sub>G</sub> is present in the postsynaptic folds of the neuromuscular junction (Kordeli et al., 1998; Wood and Slater, 1998) and is associated with NaCh at early stages in morphogenesis of the node of Ranvier (Lambert et al., 1997). Ankyrin<sub>G</sub> also is associated with NaCh clusters in the dystrophic mouse in regions lacking myelination (Deerinck et al., 1997), as well as clusters of NaCh induced in cultured retinal ganglion neurons (Kaplan et al., 1997). Together, these results provide circumstantial evidence for a role of ankyrin<sub>G</sub> in the clustering of NaCh to specific membrane regions.
The ankyrins are a family of spectrin-binding proteins associated with the cytoplasmic surface of the plasma membrane in many cell types. Ankyrins associate via their membrane-binding domains with several ion channels in addition to the voltage-gated Na channel, as well as calcium release channels and the L1CAM family of cell adhesion molecules (Bennett et al., 1997). The nodal/initial segment ankyrinG isoforms are distinguished by a serine/threonine-rich domain glycosylated with O-GlucNAc residues (Zhang and Bennett, 1996) and also contain an extended tail domain (Chan et al., 1993; Kordeli et al., 1995). The membrane-binding domain of ankyrins contains four subdomains, each composed of six ankyrin repeats (Michaela and Bennett, 1993). Biochemical studies (Michaely and Bennett, 1995a,b) suggest that ankyrin is multivalent and could form lateral homo- and/or hetero-complexes between integral membrane proteins.

Isolomers of neurofascin and NrCAM, members of the L1CAM family of ankyrin-binding cell adhesion molecules (Hortsch, 1996), are colocalized with ankyrinG at the nodes of Ranvier in peripheral nerve and axon initial segments of Purkinje cell neurons (Davis et al., 1993, 1996). Clustering of neurofascin and NrCAM precedes redistribution of ankyrinG, 480/270 kD and the NaCh during development of the node of Ranvier, which has prompted the hypothesis that these cell adhesion molecules may define the initial site for subsequent assembly of ankyrinG and the NaCh (Lambert et al., 1997).

This study evaluates the cellular and physiological consequences of the knockout of a cerebellar isoform of ankyrinG in mice. In this mutant mouse, we studied the targeting of neurofascin and NaCh and the firing properties of Purkinje cells in cerebellar slices. Immunofluorescence evidence suggests that NaCh are absent from axon initial segments of granule cells of mutant mice. In addition, Purkinje cells demonstrate severe deficits in action potential firing in response to somatic current injection, suggesting a reduced density or impaired localization of NaCh in mice lacking cerebellar ankyrinG. Neurofascin also exhibited impaired targeting to initial segments of Purkinje cell neurons. Together, these results provide direct evidence for an essential physiological role of ankyrinG in the normal function of NaCh as well as targeting of NaCh and a companion cell adhesion molecule to axon initial segments.

Materials and Methods

Targeted Disruption of Exon1b Locus

A 14-kb genomic DNA covering exon1b and upstream promoter sequence was isolated from a mouse 129S6/EV genomic library (Stratagene, La Jolla, CA) and subcloned into pBS vector (Stratagene). A Neo gene flanked with SpeI and Apal sites was inserted into the Smal site. The resulting DNA was ligated with the tk gene and subcloned into pGEM11Z (Promega Corp., Madison, WI), linearized with NotI, and electroporated into an R1 ES cell line. Among 200 clones selected by G418 (200 µg/ml) and ganciclovir (2 µM), six positive clones were identified by Southern blot. They were expanded and injected into blastocysts. Male chimeras were bred to C57BL/6J to generate F1 offsprings. Heterozygous F1 mice were bred to generate the homozygous mutant mice. The exon1b-null (mutant) mice were confirmed by standard Northern, Southern, and Western blots. To reduce the variability of the mutant mouse phenotypes caused by different genetic backgrounds, the mutant mice and their littermates were kept at 50% C57Bl/6 and 50% C57B129 genetic background. All of the six founders from six independent ES cells gave rise to mutant mice with a similar phenotype.

Northern, Southern, and Western Blots

Northern blots were performed as described (Kordeli et al., 1995). The multiple tissue blot was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). The blot shown in Fig. 2 was run according to the glyoxal/DMSO method outlined in Sambrook et al. (1989). The exon1b, exon1e, and spectrin-binding domain (common) probes were generated by PCR using the following primers: exon1e, sense, 5'-GAATTCGGGCGCTGCAGC3'; antisense, 5'-GAATTCTTTCCTCCCCCTCTT3'; exon1b, sense, 5'-TGGAGATGGCCCTGCTG3'; antisense, 5'-GACGTCGTCGTTTGT3'; common region probe, sense, 5'-GTGAACCTGGTCTCAGGC3'; antisense, 5'-GCATATCACGCTCTGTA3'. The blots were hybridized with the exon1b probe first and then stripped and rehybridized with exon1e probe. The blot was stripped again and hybridized with a probe to common to all the isoforms. The results were obtained by 1-d exposure. After each strip, the blot was exposed for at least 2 d to confirm the complete removal of the previous signal. Tail DNA was iso-

In Situ Hybridization

Whole brains from control or mutant mice were frozen on a piece of luminal foil on powdered dry ice and then processed for cryosectioning. The sections (15 µm) were collected on poly-l-lysine–coated slides and air-dried. After fixation in ice-cold 4% paraformaldehyde, the sections were stored in 95% ETOH at 4°C. The sections were prehybridized in “mini-list” solution (50% formamide, 4× SSC, 10% dextran sulfate) and then hybridized overnight with 100 µl of minilist solution containing 50 pg of 32P-labeled probes per section at 42°C. The probes were synthetic 45-mer oligomers: exon1e, 5'-CCT TGG GTA CCT CTA ATC AGG CAT AGG GCA GCA TAG CCC TCG CAG3'; exon1b, 5'-CCT TCC TGT AGA TCA GGG GAG TAT AGA CGA GAC AGA AGA TCA CCT3'. They were freshly labeled before use with a tailing kit (Boehringer Mannheim Corp., Indianapolis, IN). The sections were then washed with 1× SSC at 60°C, dried, and exposed to Kodak XAR-5 film (Rochester, NY).

Immunocytochemistry

The immunofluorescence labeling was performed according to published procedures (Lambert et al., 1997). Polyclonal anti–sodium channel, neurofascin, and NrCAM antibodies were described elsewhere (Davis et al., 1996; Lambert et al., 1997). A polyclonal anti-type II sodium channel from Upstate Biotechnology (Lake Placid, NY) was used with similar results (data not shown). In addition, the following sodium channel antibodies were used: a polyclonal antibody against looip of sodium channel and a polyclonal antibody against both the common epitope (TTEQKYYNA-MKKLGSKSKK) of type I and II NaCh generated in this laboratory, and two type I NaCh–specific antibodies, two type II NaCh–specific antibodies, and two type II anti–ankyrin, antibody is described elsewhere (Zhang and Bennett, 1996). Monoclonal anti–alkaline phosphatase antibody was purchased from Boehringer Mannheim Corp. Monoclonal antineurofascin antibody NN18 was from Sigma Chemical Co. (St. Louis, MO).

Electrophysiology

Parasagittal slices of cerebellar vermis were prepared from mice using standard techniques (Edwards, 1995). Mice (postnatal day P2) were anesthetized by inhalation of methoxyflurane and decapitated. The brain was immediately removed and immersed in ice-cold oxygenated artificial cerebrospinal fluid (O2-ACSF) containing (in mM) 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 1.25 NaHPO4, 26 NaHCO3, 25 glucose, pH 7.4. Using a vibratome, 300-µm parasagittal slices were cut and incubated for 1 h before experimentation in O2-ACSF at an elevated temperature (31–32°C), after which the bath was allowed to cool to room temperature (~22–23°C).

Whole-cell patch clamp recordings were performed using minor modifications of standard methods (Kornner et al., 1990). A cerebellar slice
was submerged in a recording chamber and held in place under a grid of nylon threads glued to a U-shaped platinum frame. The slice was perfused with room temperature O₂-ACSF (with 10 μM bicuculline added) at 1–2 ml/min, and Purkinje cells were visually identified with a 40× ceramic-coated water immersion objective. Recordings in the current clamp mode were made using a patch pipette (1.6–2.6 MΩ) filled through the tip with (in mM) 140 KCl, 8 NaCl, 10 Hepes, 0.5 EGTA and backfilled with the addition of 4 MgATP, 0.3 TrisGTP. Using a Dagan 3900 amplifier equipped with a 3911A Whole Cell Expander, data were filtered at 5 kHz and acquired (3.3–50 kHz) using pCLAMP software (Axon Instruments, Inc., Foster City, CA).

**Results**

**Cerebellum-specific Knockout of AnkyrinG in Mouse Brain**

AnkyrinG cDNAs isolated from human brain (Kordeli et al., 1995) and mouse kidney (Peters et al., 1995) have distinct NH₂-terminal amino acid sequences immediately preceding the ankyrin repeats (Fig. 1A). A cDNA isolated from rat brain has an NH₂-terminal sequence nearly identical to that of the isoform isolated from mouse kidney (Fig. 1A). These considerations combined with genomic sequence data suggested the possibility of two classes of ankyrinG transcripts, each with distinct promoter elements (data not shown). Northern blot analysis confirmed that one exon (exon1b) is expressed only in mouse brain, while the other exon (exon1e) is expressed in brain as well as heart, skeletal muscle, and kidney (Fig. 1B, left and middle). AnkyrinG transcripts from lung and some isoforms in heart and kidney did not hybridize with exon1b or exon1e, suggesting the existence of a third set of transcripts and possibly an additional promoter (Fig. 1B, right).

In situ hybridization using probes specific to exon1b and exon1e revealed selective expression of the two exons in adult mouse brain (Fig. 1C). Exon1e-containing ankyrinG transcripts are expressed throughout the frontal brain with elevated levels in hippocampus, caudate putamen, and frontal cortex. Exon1b, in contrast, is most strongly expressed in cerebellum, but it is also detectable in the brain stem and thalamus at low levels. Developmental patterns of expression of these transcripts have not yet been examined.

The selective expression of exon1b in the cerebellum suggested a strategy for targeted gene disruption resulting in preferential loss of cerebellar ankyrinG, while maintaining ankyrinG expression in other tissues as well as other regions of the nervous system. We produced mutant mice with a neomycin gene incorporated in reverse orientation into the brain-specific exon1b by homologous recombination (Fig. 2A). The success of the targeted disruption was confirmed by Southern blot (Fig. 2B). The specific knockout of exon1b-containing ankyrinG transcripts in homozygous mutant mice was confirmed by Northern blot with probes specific for exon1b and exon1e (Fig. 2C). No exon1b-containing transcripts were detected in the mutant mice, whereas the level of expression of exon1e-containing transcripts was unaffacted (Fig. 2C, middle). In situ hybridization performed on mutant mouse brain sections re-
revealed no compensation of the exon1e-containing ankyrinG transcripts in cerebellum, while exon1b-containing ankyrinG transcripts were undetectable (data not shown). Immunoblots using an antibody to the tail domain of ankyrinG (anti-270/480 ankyrinG; Zhang and Bennett, 1996), which recognizes proteins derived from both classes of transcripts, revealed almost complete absence of ankyrinG protein in cerebellum of the mutant mouse (Fig. 2 D). Similar data were obtained using an antibody to the spectrin-binding domain of ankyrinG (data not shown). The mutant mouse forebrain exhibited only a moderate reduction of ankyrinG, suggesting that the alternative, exon1e-containing isoforms are normally expressed in forebrain.

The region-specific knockout of ankyrinG was further confirmed by immunofluorescence labeling (Fig. 3). AnkyrinG was almost completely missing in mutant mouse cerebellum (Fig. 3 A). Some ankyrinG immunoreactivity in the white matter of mutant mouse cerebellum was still detectable, suggesting that these fibers contain exon1e-ankyrinG. The expression of ankyrinG in hippocampal neurons of mutant mice was unaffected compared with the wild-type control mouse, indicating that these neurons also use exon1e-ankyrinG (Fig. 3, C and D).

**Mutant Mice Develop Progressive Ataxia**

Mice with the exon1b-ankyrinG \(-/-\) genotype (hereafter referred to as mutant mice) were born in nearly Mendelian ratios (55/211) and had no obvious abnormality until P16–17. Mutant mice then developed characteristic cerebellar defects with symptoms of abnormal gait and tremor and reduced locomotion. When the mutant mice were prodded to walk, their hindlimbs were uncoordinated, and they fell frequently (Fig. 4). This phenotype had a variable time course of onset and severity but could generally easily be observed in mutant mice as young as P16. The ataxia became more severe with increasing age. The mutant mice are fertile but do not breed well. Some premature death of mutant mice occurred between 4 and 6 mo and in some cases was preceded by uncontrollable jumping and convulsions. Mice with the exon1b-ankyrinG \(+/-\) genotype (heterozygotes) were phenotypically normal and could not be distinguished from wild-type mice \((+/-)\) for exon1b-ankyrinG.

**Loss of Targeting of NaCh to Axon Initial Segments of Neurons in Mutant Mouse Cerebellum**

At least three types of NaCh are expressed by neurons of mouse cerebellum (Westenbroek et al., 1989, 1992; De Miera et al., 1997). The type II NaCh is the major NaCh expressed by granule cells (De Miera et al., 1997), while NaCh6/Scn8a/CerIII (Burgess et al., 1995; Schaller et al., 1995; De Miera et al., 1997) is expressed in Purkinje cells and is likely one of the contributors to the generation of Purkinje cell action potentials (Raman et al., 1997). Type I NaCh are expressed in Purkinje neurons (Westenbroek et al., 1989; De Miera et al., 1997) but are not concentrated at axon initial segments (data not shown). Our NaCh antibodies were raised against peptides shared by type I and II NaCh (Lambert et al., 1997) and reacted with NaCh at nodes of Ranvier of peripheral nerve (Lambert et al., 1997) and axon initial segments of granule cells on tissue sections (Fig. 5 D). In addition, the molecular layer, which is composed of unmyelinated axons of granule cell neurons, was also labeled with this antibody, as previously reported (Westenbroek et al., 1989, 1992).
AnkyrinG is confined to initial segments of axons of granule cells (Fig. 3B) as well as Purkinje neurons (see below), as previously reported (Kordeli et al., 1995). AnkyrinG at axon initial segments is clearly resolved in cultured granule cells (data not shown). This distribution pattern is also true in cerebellum sections (Fig. 5B), where sodium channels colocalized with ankyrinG at the initial segments of granule cells (Fig. 5F, see the enlarged view in the inset).

Immunofluorescence indicates that NaCh were not concentrated at initial segments of granule cells from mutant mice (Fig. 5C), while NaCh were highly concentrated at granule cell initial segments and colocalized with ankyrinG in wild-type mice (Fig. 5D). Mutant mouse cerebella retain strong labeling of NaCh in the molecular layer, where no ankyrinG is present, even in normal mice. Immunoblot data indicated the levels of NaCh protein detected by this antibody were the same in the mutant and control mouse cerebella of animals less than 40 d (data not shown). Although ankyrinG is absent from the molecular layer, ankyrinG is highly expressed in this region (Kunimoto et al., 1991), and this or some other protein such as syntrophin (Gee et al., 1998) could interact with NaCh in the unmyelinated granule cell axons. NaCh could not be consistently visualized in Purkinje cell initial segments with our antibody or with eight other antibodies (see Materials and Methods). These negative results suggest either that the NaCh did not react strongly with antibody because of problems with fixation or some other experimental parameter, or that axon initial segments of these neurons have a specialized NaCh isoform that is not recognized by available antibodies. However, the electrophysiological data presented below strongly support the conclusion that the density of NaCh in Purkinje neuron initial segments is dramatically reduced in the mutant mice.

Abnormal Physiology of Purkinje Cells of Mutant Mice

We studied the ability of Purkinje cells to fire action potentials upon injection of current at the soma in whole-cell patch-clamp recordings from cerebellar slices. Experiments were done blind to genotype, although the motor phenotype of each mouse was obvious. After analysis, the genotype of each animal was determined by Southern blot from tail-snip specimens. Wild-type (+/+ ) and heterozygote (+/−) mice could not be distinguished by cage behavior nor electrophysiological properties and were thus combined for data analysis as normal mice. Purkinje cells in cerebellar slices from mutant mice (−/−) were, however, impaired in their ability to fire action potentials. Three features were notable. Purkinje cells from mutant mice had a higher threshold to initiate action potentials, did not always fire an action potential in response to a short, 1-ms current injection, and showed a slower rate of maximal firing of multiple action potentials in response to long current injections. In Purkinje cells from normal mice, a single, “all-or-nothing” action potential (Fig 6A, left) could always be elicited from a 1-ms pulse (10/10 cells from 7 animals). In contrast, most cells from mutant mice showed only passive membrane responses and were unable to ini-
because of the knockout of a cerebellar isoform of ankyrinG. This is consistent with the immunofluorescence evidence for reduced localization of NaCh in cerebellar granule cell initial segments (Fig. 5) and neurofascin in Purkinje cell initial segments (Fig. 7, below).

**Reduced Concentration of Neurofascin at Axon Initial Segments of Purkinje Neurons**

Neurofascin and NrCAM colocalized with ankyrinG at initial segments of Purkinje neuron axons and the nodes of Ranvier of peripheral nerve (Davis et al., 1996; Lambert et al., 1997). The L1CAM family member(s) targeted to granule cell neuron initial segments has not been defined. In control mouse cerebellum, ankyrinG and neurofascin are colocalized at the initial segments of Purkinje cell axons (Fig. 7 F), with low levels of neurofascin also localized along the cell body plasma membrane, as previously reported (Davis et al., 1996). In the mutant mouse cerebellum, however, neurofascin was uniformly distributed at the plasma membrane of Purkinje neuron cell body and axon initial segment (Fig. 7 E). Some elevation of neurofascin was found in the molecular layer of ankyrinG-deficient mice. The same observations were made with an antibody to NrCAM (data not shown).

**Neuronal Degeneration in the Mutant Mouse Brain**

The cerebella of adult mutant mice (>5 mo old) is substantially smaller than those of control littermates, but the frontal brains were similar in size (data not shown). The molecular layer of the adult mutant mouse cerebellum was reduced in thickness, and the number of Purkinje cells was reduced by 60% based on staining with neurofilament antibody, hematoxylin and eosin, or toluidine blue (data not shown, n = 6). An antibody to calbindin, a Purkinje cell marker, revealed large gaps in the molecular layer of mutant mice where cell bodies and dendritic arbors of Purkinje cells were absent (Fig. 8 A). Purkinje cells of mutant mice are likely to provide ineffective or inappropriate signaling to their target neurons because of an abnormal input from granule cells and from their own deficiencies in action potential initiation and firing rates. A potential consequence of abnormal signaling to the deep nuclei is that Purkinje cells would not receive sufficient neurotrophic factors by retrograde transport. These considerations may explain the progressive loss of Purkinje neurons in ankyrinG mutant mice. Interestingly, progressive, late-onset Purkinje cell loss was also found in jolting mice with a mutation in the Scn8a NaCh expressed in Purkinje cells (Boakes et al., 1984; Dick et al., 1986; Kohrman et al., 1996).

**Discussion**

This study demonstrates that ankyrinG is essential for normal targeting of NaCh to axon initial segments of granule cells and for normal firing properties in Purkinje cells. Targeted disruption of ankyrinG gene expression in the cerebellum results in progressive ataxia, abolishes targeting of voltage-gated NaCh to axon initial segments of granule cells, impairs action potential firing in Purkinje neurons, and results in progressive Purkinje neuron degeneration. Neurofascin and NrCAM, members of the L1CAM family...
of ankyrin-binding cell adhesion molecules, also exhibited reduced concentration at Purkinje cell axon initial segments in mutant mice. Although the molecular type of NaCh expressed by the Purkinje neurons could not be identified by immunocytochemistry, the physiological data (Fig. 6) strongly support the idea that NaCh localization to the initial segment is impaired and/or NaCh density is reduced because of the cerebellar knockout of ankyrinG. This is consistent with the immunocytochemical evidence for reduced targeting of NaCh in cerebellar granule cells (Fig. 5) and neurofascin in Purkinje cells (Fig 7).

The demonstration that ankyrinG is essential for clustering of sodium channels and neurofascin at the initial segments extends previous observations that ankyrinG coclusters with neurofascin and NaCh during morphogenesis of nodes of Ranvier (Davis et al., 1996; Lambert et al., 1997). The ankyrin membrane binding domain has distinct binding sites for the NaCh, located on subdomains 3 and 4 (Srinivasan et al., 1992), and for neurofascin, located on subdomains 2 and 3 as well as 3 and 4 (Michaely and Bennett, 1995b). Therefore, ankyrinG could form heterocomplexes between NaCh and neurofascin in vivo by di-

Figure 5. Loss of sodium channel clustering at initial segments of cerebellar granule cell axons of mutant mice. Cerebellar brain sections from mutant mouse (A, C, and E) and the wild-type control littermate (B, D, and F) were double-stained with antibodies to ankyrinG (A and B, green in E and F) and sodium channel (C and D, red in E and F). One typical example of colocalization of ankyrinG and NaCh at the initial segments of granule cells in wild-type mouse cerebellum (B, D, and F, arrows) was magnified and shown in the inset. The initial segment was marked with an arrowhead. Bars, 20 μm.
Figure 6. Deficits in action potential initiation and firing in mutant mice. Whole-cell current clamp recordings of Purkinje cells in cerebellar slices from (A) a normal mouse or (B) a mutant mouse with cerebellar knockout of ankyrinG. Cells were hyperpolarized to −80 mV and the membrane potential responses to 1-ms (left) or 50-ms (right) current injections at the soma were recorded. Dashed lines indicate −80 and 0 mV, as marked. (C) Comparison of the maximal action potential firing rate for 50–100-ms current injections delivered to Purkinje cells from wild-type (+/+; n = 3), heterozygous (+/−; n = 7), and mutant (−/−; n = 7) mice. A series of depolarizing current injections from 0.1–1.9 nA in 0.1-nA increments was delivered until the maximal firing rate was reached. (D) Comparison of the minimum current injection required to elicit the first action potential in Purkinje cells from normal (n = 10) and mutant (−/−; n = 7) mice for stimuli of 10, 50, or 100 ms duration. The normal group includes both wild-type and heterozygous mice since the physiological and behavioral phenotypes of the two groups were not different. Data (means ± SEM) were analyzed by a two-tailed t test. *P < 0.001.

The role of ankyrinG in assembly of NaCh at nodes of Ranvier and the neuromuscular junction is not addressed in this study because of the selective disruption of ankyrinG in the cerebellum and the lack of antibodies recognizing NaCh of Purkinje neurons. However, similarities between initial segments and other sites of NaCh concentration support the prediction that ankyrinG is also required for restriction of NaCh at these domains. Nodes of Ranvier, the neuromuscular junction, and axon initial segments each have specialized features as well. Thus NaCh/ankyrinG assemblies are likely to include additional proteins that perform specific functions adapted to each cell domain. Syntrophins, for example, associate with NaCh and are concentrated at the neuromuscular junction (Gee et al., 1998; Shultz et al., 1998). The β subunits of NaCh also are candidates to mediate important domain-specific interactions (Isom et al., 1995; Isom and Catterall, 1996).

The role of ankyrinG in molecular events leading to assembly of the specialized plasma membrane domain at axon initial segments remains an important issue for future investigation. One conclusion from this study is that signals for ankyrin-based targeting of NaCh to axon initial segments are likely to involve additional interacting proteins that have yet to be identified. Neurofascin and NrCAM have been proposed to directly assembly, first of ankyrin at nodes of Ranvier and other sites, followed by the localization of NaCh (Lambert et al., 1997). However, the finding that neurofascin is not distributed normally in the absence of ankyrinG (Fig. 7) suggests that ankyrinG is required for the concentration of neurofascin as well as the NaCh at least at axon initial segments. It is of interest that restriction of 270-kD ankyrinG to axon initial segments of cultured dorsal root ganglion neurons requires multiple domains in addition to the membrane-binding domain (Zhang and Bennett, 1998). These findings imply the existence of unidentified ankyrin-binding protein(s) upstream in the pathway to formation of the initial segment specialized domain.

The finding that ankyrinG is required for the normal physiological function of NaCh is an example of a general principle of the critical importance of spatial organization of ion channels in cells of metazoan animals and the requirement for cytoplasmic proteins for proper cellular targeting. Other instances demonstrated in animal models include the role of rapsyn in the organization of acetylcholine receptors at the neuromuscular junction of mice (Gautam et al., 1995), and of the PDZ protein, Discs-Large (Dlg), in the organization of potassium channels and Fasciculin II in neuromuscular junctions of Dro-
AnkyrinG and Clustering of Voltage-gated Sodium Channels

Apparantly, evolution of mechanisms for spatial organization has proceeded through convergent pathways for different types of channels, with the selective advantage of rapid and precise response to stimuli. Ion channels with polarized localization in cells that associate with ankyrin include the Na/K ATPase (Nelson and Veshnock, 1987) and Na/Ca exchanger (Li et al., 1993). The Na/K ATPase requires ankyrinG119 for delivery to the plasma membrane in cultured cells (Devarajan et al., 1997) and may also require ankyrinG for targeting to basolateral domains of epithelial cells in tissues. Voltage-gated NaCh are members of a super-family that also includes voltage-gated channels for K⁺, and Ca²⁺ (Armstrong and Hille, 1998). It will be of interest to determine if ankyrin-binding and ankyrin-dependent cellular targeting are features shared by other members of the voltage-gated channel family. Deciphering the molecular code for cellular targeting of ion channels and most likely other signaling molecules is an issue connecting the fields of cell biology and physiology and is equivalent in functional significance to understanding primary and tertiary structures of these proteins.

**Figure 7.** Redistribution of neurofascin in Purkinje cells of mutant mice. Sections of cerebellum from P40 mutant mouse (−/−; A, C, and E) and the wild-type control littermate (WT; B, D, and F) were stained with antibodies to ankyrinG (C and D, green in E and F) and neurofascin (A and B, red in E and F). Without ankyrinG, neurofascin was distributed uniformly at the plasma membrane of Purkinje cells (A, arrows). In normal mice, neurofascin was highly concentrated at the initial segments of Purkinje cells (B, arrowheads). Bar, 20 μm.

**Figure 8.** Neurodegeneration of Purkinje cells in mutant mice. Sections of cerebellum from a 5-mo-old mutant mouse (A) and the wild-type control littermate (B) were stained with antibodies to calbindin. The results show a dramatic reduction of Purkinje cells in the adult mutant mice. Bars, 50 μm.
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