A Novel Caspr Mutation Causes the Shambling Mouse Phenotype by Disrupting Axoglial Interactions of Myelinated Nerves

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Abstract

The neurological mouse mutation shambling (shm) exhibits ataxia and hindlimb paresis. Positional cloning of shm showed that it encodes contactin-associated protein (Caspr), which is required for formation of the paranodal junction in myelinated nerves. The shm mutation is a TT insertion in the Caspr gene that results in a frame shift and a premature stop codon at the COOH-terminus. The truncated Caspr protein that is generated lacks the transmembrane and cytoplasmic domains. Here, we found that the nodal/paranodal axoplasm of shm mice lack paranodal junctions and contain large mitochondria and abnormal accumulations of cytoplasmic organelles that indicate altered axonal transport. Immunohistochemical analysis of mutant mice showed reduced expression of Caspr, contactin, and neurofascin 155, which are thought to form a protein complex in the paranodal region; protein 4.1B, however, was normally distributed. The mutant mice had aberrant localization of voltage-gated ion channels on the axolemma of nodal/paranodal regions. Electrophysiological analysis demonstrated that the velocity of saltatory conduction was reduced in sciatic nerves and that the visual response was attenuated in the primary visual cortex. These abnormalities likely contribute to the neurological phenotype of the mutant mice.

Key Words: Contactin-associated protein, Myelin, Optic nerves, Paranodes, Sciatic nerves.

INTRODUCTION

Saltatory conduction of nerve impulses in myelinated nerves depends on highly specialized axonal domains termed the node of Ranvier, the paranode, the juxtaparanode, and the internode. The paranodes flanking the node of Ranvier are one of the major attachment sites between the axon and myelinating glial cells, that is, oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS) (1–5). A septate-like adhesive junction is formed at the paranode between the axolemma and myelin loops. The paranode acts as an electrical and a biochemical barrier between nodal and internodal membrane compartments. In addition, the paranodal junction is thought to play a role in molecular communication between the neuron and the glial cell. Thus, multiple roles of the paranodes are crucial for maintaining the normal formation of myelinated nerves.

Disruption of axoglial interaction at the paranodes results in severe pathological conditions in a variety of mouse mutants that are deficient in paranodal and myelin molecules (6–11). All of these so-called paranodal mutants lack a normal paranodal junction and display ataxia, motor deficits, and dramatically reduced nerve conduction velocities. Altered paranodal structures have also been reported in human demyelinating diseases such as Charcot-Marie-Tooth disease (CMT), an inherited degenerative neuropathy of the PNS (12, 13), and multiple sclerosis (14, 15).

Mutant phenotypes in mice often closely resemble human disease phenotypes; therefore, these mouse mutations can provide valuable models to understand how the diseases develop and to test ways to prevent or treat the corresponding diseases (16–18). Shambling (gene symbol, shm) was originally discovered in the Jackson Laboratory in 1960 and has been propagated in our laboratory for 20 years. Mutant mice exhibit ataxia and motor deficits resulting in hindlimb paresis. Here, we identify the mutated gene and show that it encodes contactin-associated protein, Caspr (also termed paranodin or NCPI), a major component of the paranode (19–21). This is the first report of a mutation of the Caspr gene.

MATERIALS AND METHODS

Animals

Heterozygous shambling mice (SHM-shm/+) are maintained at the Research Institute of Environmental Medicine in Nagoya University under approval of the ethics committee.
for animal experiments of the Institute and in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. Animals are housed in a room at 22°C ± 1°C with a light-dark cycle of 12 hours each. Food and water are given ad libitum. MSM mice, an inbred strain originating from Japanese wild mice (Mus musculus molossinus), were also used in the genetic analysis. The MSM mice were supplied by the National Institute of Genetics (Mishima, Japan).

Genetic Mapping

Genomic DNA was prepared from mouse tail tissue by standard phenol-chloroform extraction and ethanol precipitation methods. Fifty nanograms of DNA were subjected to polymerase chain reaction. The primers used for this analysis amplify DNA fragments of different sizes from SHM-shm/+ and MSM mice. Primer sequences and allele sizes are available from the Mouse Microsatellite Data Base of Japan (www.shigen.nig.ac.jp/mouse/mmdbj/microsatelliteMapAction.do? chromosome = 11).

The shm locus has been mapped to chromosome 11 and has been found to be located 1.5 ± 0.7 cM from the Re locus (22). To reduce this region, classical genetic mapping was used. In brief, heterozygous SHM-shm/+ mice were crossed with C57BL/6J (B6) inbred mice to generate heterozygous B6-shm/+ congenic mice. The genotypes of F2 offspring (total number of mice, 202) were analyzed using microsatellite markers adjacent to the Re locus. Because of the low rate of recombination in the original cross, a second cross between SHM-shm/+ mice and MSM+/+ mice was also performed (total number of mice, 389).

Reverse Transcription–Polymerase Chain Reaction and Sequencing

Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using the SuperScript III Preamplification System for First Strand cDNA Synthesis (Invitrogen Corp, Carlsbad, CA). Primers to amplify DNA fragments of different sizes from SHM-shm/+ and MSM mice. Primer sequences and allele sizes are available from the Mouse Microsatellite Data Base of Japan (www.shigen.nig.ac.jp/mouse/mmdbj/microsatelliteMapAction.do? chromosome = 11).

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RNA Extraction and Northern Blot Analysis

Total RNAs were extracted from brains of mutant mice and their normal littermates on postnatal day (P) 22 to 28 (n = 3 for each) by the acid-guanidine-phenol-chloroform method (23). Fifteen-microgram aliquots of total RNA were used in the identification of Caspr mRNA. The procedures for RNA denaturation, electrophoresis, and hybridization were as previously described (24). Caspr cDNA was labeled with [α-32P]dCTP (specific activity, 111Tbq/mmol) (PerkinElmer Life and Analytical Sciences, Boston, MA) using a Random Primer DNA labelling Kit Ver. 2 (Takara Bio Inc, Otsu, Japan) and used as a probe. After hybridization, the radioactivity of the band representing Caspr mRNA was measured using a Fuji Bioimage Analyzer (BAS 2000; Fuji Photo Film, Tokyo, Japan).

The hybridized membrane was reprobed with cDNA for 18S ribosomal RNA as a control.

Western Blot Analysis

Total proteins were extracted from the cerebellum, spinal cord, and sciatic nerves of normal and mutant mice on P22 to P28 (n = 3 for each). Aliquots of 80 μg protein were separated on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA) with Trans-blot SD (Bio-Rad, Hercules, CA). After washing with Tween–Tris-buffered saline, the membranes were incubated with anti-Neurexin/Caspr (BD Biosciences, San Jose, CA) in Can Get Signal® solution I (Toyobo Co Ltd, Osaka, Japan) overnight at 4°C, followed by incubation with anti-mouse horseradish peroxidase–conjugated IgG (Jackson ImmunoResearch, West Grove, PA) in Can Get Signal® solution II for 1 hour. Specific reactivity was visualized with Supersignal® West Pico enhanced chemiluminescence substrate (Pierce, Rockford, IL) and an ATTO cool saver system (Atto, Osaka, Japan).

Electron Microscopy

Normal and mutant mice at P46 to 4 months old (n = 3 for each) were anesthetized with Nembutal and transcardially perfused with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer. The spinal cord, optic nerves, and sciatic nerves were dissected, and the tissues were cut into small pieces. The tissue pieces were postfixed with 1% OsO4 in phosphate buffer for 1 hour. After dehydration through graded alcohols, the tissues were embedded in epoxy resin. Semithin sections were cut and stained with toluidine blue for light microscopy. Ultrathin sections were examined with an electron microscope (JEM-1210; JOEL Ltd, Akishima, Japan).

Quantification of Nodal Gaps and Paranodal Loop Gaps

Ultrathin longitudinal sections of the sciatic nerve were examined in 2 sets of mutant and wild-type pairs of mice. Electron micrographs of the node of Ranvier were taken at magnification 5,000× for measurement of the length of the node and at 20,000× to quantify gaps between the axon and glial membrane; the images were scanned and digitized in Photoshop. The gap between the axonal surface and the center of each paranodal loop was measured as a straight line from the outer leaflet of the axon membrane to the outer leaflet of the opposing glial cell membrane. Data from mutant mice and normal littermates were compared statistically using Student t-test.

Preparation of Teased Sciatic Nerves and Sections From Optic Nerves and Spinal Cord

Sciatic nerves were removed from mutant and littermate normal mice from P14 to 2 months of age (n = 6 for each, mostly P22–P24). The nerve tissues were treated with collagenase (3.6 mg/mL collagenase Type 1; Wako Inc, Osaka, Japan) for 0.5 to 2 hours and then teased apart with fine needles. The teased sciatic nerve fibers were mounted on glass slides and fixed with 4% paraformaldehyde in PBS for

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15 to 60 minutes. The nerves were stored in PBS at 4°C until use. For staining with an anti-neurofascin (neurofascin 155 [NF155]) antibody, the nerves were fixed for an additional 15 minutes in Zamboni fixative.

For tissue sections, normal and shm mice aged 1 to 2 months (n = 3 for each) were fixed by cardiac perfusion with 4% paraformaldehyde in PBS, and the lumbar spinal cords were removed. The spinal cords were cryoprotected with 30% sucrose and frozen in liquid nitrogen. The tissues were sectioned at 10 to 16 μm on a cryostat.

**Immunofluorescence Microscopy**

After several washes in PBS, the nerves or tissue sections were permeabilized with 0.3% Triton X-100 in PBS for 30 minutes and blocked in 3% goat serum/5% bovine serum albumin in PBS at room temperature for 1 hour. They were incubated at 4°C overnight with primary antibodies in PBS containing 1% bovine serum albumin. The following antibodies were used: affinity-purified mouse monoclonal and rabbit polyclonal antibodies to Caspr (1:200 and 1:2000) (25, 26); mouse anti-contactin (BD Biosciences); rabbit anti-NF155 (a gift of H. Baba, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan); rabbit anti-protein 4.1B (Protein Express, Cincinnati, OH); mouse anti-sodium channel (Sigma, St Louis, MO); and rabbit anti-potassium channel (Kv1.1) (Chemicon, Temecula, CA). Nerves were washed for 1 hour at room temperature in PBS before application of Alexa488- or Alexa568-labeled antibodies (Jackson Immunoresearch). Nerves stained with a fluorescent secondary antibody were washed in PBS, mounted in VECTASHIELD (Vector Lab, Inc, Burlingame, CA), and analyzed with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss MicroImaging, Jena, Germany).

**Electrophysiology**

Normal and mutant mice aged 2 to 3 months (n = 4 for each) were anesthetized with pentobarbital sodium (50 mg/kg, intraperitoneally). The sciatic nerve along with its continuing tibial nerve was removed, put on an array of Ag-AgCl electrodes, and covered with warmed mineral oil. The distal part of the nerve was stimulated, and the compound action potential (CAP) was recorded at the proximal part. The following stimulus parameters were used: pulse width 10 milliseconds and current intensity at 10% more than the intensity that elicited maximal CAP amplitude. The conduction velocity was determined by the conduction delay between the stimulus artifact to the peak of CAP and the distance (12 mm) between the stimulation cathode and the recording electrode. The temperature of the mineral oil in the recording chamber was measured (range, 28°C–33°C), and the conduction velocity was corrected to that at 37°C (27). The CAPs were amplified, digitized, and analyzed with PowerLab and Scope 3.7 (AD Instruments, Spechbach, Germany).

Visual evoked potential (VEP) recording was carried out in normal and mutant mice aged 3 to 4 months (n = 4 for each) that had been anesthetized with a mixture of fentanyl (0.1 mg/kg) and droperidol (5 mg/kg). The mice were secured onto a stereotaxic instrument, and a small portion of the skull overlying the visual cortex was carefully drilled and removed leaving the dura intact. The VEPs were recorded from the binocular region of the visual cortex (2.7 mm lateral and 3.5 mm posterior to the bregma). The tip of a tungsten microelectrode (FHC Inc, Bowdoinham, ME; 0.5 MΩ) was placed about 500 μm below the cortical surface. The position of the tip was adjusted to achieve the maximal VEP amplitude. Visual stimulation consisted of full-field sine wave gratings of 100% contrast (mean luminance, 25 cd/m²) at 0.05 cycle per degree was reversed at 1 Hz. Stimuli were generated by a VSG2/5 card (Cambridge Research System, Kent, UK) and presented on a computer monitor. The display was positioned 28.5 cm in front of the mouse and centered on the midline, thereby occupying 60 degrees × 100 degrees of the visual field. Mean luminance was determined by a photodiode placed in front of the computer screen and was 50 cd/m². Electrical signals were amplified and filtered (high-pass filter, 0.01 Hz; low-pass filter, 1 kHz). The VEP amplitude was determined by the average for 150 stimuli presented to the eye contralateral to the recorded hemisphere.

**RESULTS**

**Neurological Phenotype of Shambling Mice**

Shambling mutant (shm/shm) mice were easily distinguished from their normal littersmates by their smaller size and wobbly gait caused by hindlimb weakness. The abnormal locomotion was first noted around P14 to P15 by slow motor activity and lack of coordination in hindlimb movement. After 3 weeks, the body sizes of the mutant mice barely increased. An ataxic gait and trembling body were also noticeable by 3 weeks of age. The mutant mice adopted an abnormal posture in which their hindlimbs extended laterally, which worsened with age so that their hindlimbs were held stiffly up and off the cage floor (Fig. 1A). The motor deficit was also progressive, and occasionally the mice became immobile as they aged. Many mutant mice died around weaning and special care measures such that reducing the number of pups in the litter and giving them ground food were required to aid their survival beyond weaning.

**Identification of the Gene Responsible for the shm Phenotype**

To identify the gene mutation responsible for the shm phenotype, we performed positional cloning using microsatellite DNA markers. Analysis of the F₂ offspring from an initial cross of SHM-shm/+ and B6 mice indicated that the shm gene was localized within a 4.5-megabase (Mb) interval between markers D11Mit58 and D11Mit59. To further narrow this region, we used a second cross between SHM-shm/+ and MSM/+ mice. Analysis of 389 F₂ mice from this cross-localized shm to an interval of 0.24 Mb between D11Oda21 and D11Oda27; we developed these as new microsatellite DNA markers (Fig. 1B).

From the mouse genome databases, 17 genes were identified in this 0.24-Mb region (Fig. 1C). We selected 14 of these, amplified their cDNAs in brain tissues from mutant and normal mice, and sequenced the resulting cDNAs. We found a TT insertion in exon 22 of the contactin-associated protein (Caspr) gene (Fig. 1D). Caspr maps at 100.8 Mb on...
chromosome 11. The gene contains 24 exons and a 4,158-bp open reading frame encoding 1,385 amino acids. The TT insertion caused a frame shift resulting in an altered sequence of 45 amino acid residues at the C terminus. In addition, the insertion generated a stop codon at amino acid residue 1287 of the protein so that 98 amino acid residues were deleted from the C terminus of the mutated Caspr protein. The deleted region contained the transmembrane and cytoplasmic

FIGURE 1. Identification of the Caspr gene in shambling mice. (A) Phenotypes of shambling mice. Left: Normal (+/+) and mutant (shm/shm) littermates at P21. Right: 4-month-old mutant mouse. The arrow indicates a stiff and extended hindlimb (left) and the characteristic hindlimb paresis (right). (B) Haplotype recombination frequencies in F2 offspring (SHM+/shm × MSM+/+) localize the mutated gene to the region between microsatellite DNA markers D11Oda21 and D11Oda27. (C) Schematic diagram of the mutant locus. Upper: Physical map of the polymorphic markers between D11Oda21 and D11Oda27. Lower: Schematic diagram of the genes present in the region between the 2 DNA markers. (D) The mutation in the Caspr gene of shm mice. A TT insertion (double arrows) has occurred in exon 22. (E) Partial alignment of the deduced amino acid sequences of the wild-type (wt) Caspr protein and the truncated (shm) protein.
Expression of Caspr mRNA and Protein in the shm Mice

Next, we examined whether expression of Caspr mRNA and protein was affected in the brains of shm mutant mice. Northern blot analysis detected a 5.5-Kb band for Caspr mRNA in brains of both normal and shm mice, and their expression levels were similar (Fig. 2A). Expression of the Caspr protein was examined by Western blot using antibodies against the extracellular domain of Caspr. The Caspr protein was detected as a single band of the expected molecular mass of 190 kd in wild-type mice (+/+), and a smaller band with the expression level reduced in the mutant (shm/shm) mice. Western blot analysis in which Caspr protein expression was compared between wild-type (+/+) and mutant (shm/shm) mice. The mutant protein is expected to lack the transmembrane and intracellular domains.

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Expression of the Caspr protein was examined by Western blot using antibodies against the extracellular domain of Caspr. The Caspr protein was detected as a single band of the expected molecular mass of 190 kd in the normal cerebellum, but in the shm cerebellum, a smaller and weaker band was present (Fig. 2B). The same results were obtained for the spinal cord and sciatic nerves (data not shown). These data indicate that a truncated Caspr protein was produced as expected, and that its expression level was decreased in the shm CNS and PNS.

Morphological Analysis

Light microscopy of sciatic nerves and the white matter of the spinal cord revealed that the numbers of myelinated axons, thickness of the myelin sheath, and diameter of the axons were similar in normal and mutant mice (Figs. 3A–D). We did not attempt to quantify these characteristics.

By electron microscopy, sciatic nerves, node, and paranodes appeared to form normally in shm mice, but the axonal cytoplasm occasionally contained abnormally large mitochondria and showed accumulation of mitochondria and other organelles at the nodal and paranodal regions (Figs. 3E–H). Furthermore, the lengths of the node were longer in shm mice than in normal mice (1,625.2 ± 163.5 nm, n = 6 vs 1,091.6 ± 55.2 nm, n = 4; p < 0.05), indicating that the nodes were widened in the mutant mice.

At the highest magnification used, myelin loops were arrayed sequentially and in close apposition to the axonal membrane in normal paranodes in shm mice (Figs. 5A, B). The periodic transverse dense bands were present between the loops and the axonal membrane, termed the paranodal junctions (Figs. 4A, C). In contrast, paranodal junctions were absent from the paranodes of shm sciatic nerves, although paranodal loops faced the axolemma and compact myelin formation appeared normal (Fig. 4B). Measurement of the space (gap) between paranodal loops and the axonal plasma membrane indicated that the gaps in mutant mice were significantly wider than those in normal mice (13.12 ± 1.11 nm, n = 18 vs 5.97 ± 0.37, n = 9; p < 0.001).

In the CNS paranodes of shm mice, in addition to the absence of the paranodal junctions (Fig. 4D), the loops faced away from the axonal membrane and were frequently everted (Fig. 4E). Occasionally, the loops were layered over the everted loops (Fig. 4E). Electron-dense material was occasionally present between myelin loops and axolemma at the paranodes (Fig. 4D). Together, these data indicate that the axoglial junctions are disrupted at the paranodes of shm mutant mice.

The localization and distribution of proteins at the paranodal regions including the node and juxtaparanode were investigated next. We first determined whether the mutant Caspr protein was located to the paranode of shm mice by performing an immunohistochemical analysis of sciatic nerves using different anti-Caspr antibodies. The antibody that recognizes the cytoplasmic domain of Caspr (R6061) did not stain the paranodal regions of shm sciatic nerves, whereas staining was present at the paranode when an antibody directed to the extracellular domain of Caspr (M275) was used (Fig. 5A). The intensity of Caspr staining varied among the paranodes in shm sciatic nerves, however. In addition to the normal profile expected of Caspr staining (Figs. 5A, B), some paranodal regions showed faint and diffuse staining with the Caspr antibody (Fig. 5B). Caspr staining was not often encountered at the paranodal region of shm sciatic nerves (Fig. 5C). These observations are consistent with the Western blot analysis in which Caspr protein expression was decreased in the mutant mice.

To assess how often Caspr staining was present at the paranodal regions of shm myelinated nerves, we performed double staining with anti-Caspr and anti-Kv1.1 antibodies. The anti-Kv1.1 antibody stained K’ channels at the juxtaparanode in normal myelinated nerves and at the paranode in shm myelinated nerves. We quantified the frequencies of the parasagittal loops.
paranodal staining profiles, including nodes and juxtaparanodes, and classified them as having or lacking Caspr staining; 24.19% ± 9.19% (mean ± SEM, n = 3, P22 mice) of the paranodal regions showed Caspr staining (Figs. 5C, D). Thus, shm Caspr was present in the paranodal region of approximately one quarter of the sciatic nerves, although there was variation in the staining pattern (Figs. 5Ba, Bb). The remainder of the paranodes lacked Caspr, presumably as a result of generation of the mutant Caspr protein. Because the shm Caspr lacks transmembrane and cytoplasmic domains (Fig. 2C), it could not form a stable association at the plasma membrane (28). An alternative explanation is that the mutant Caspr is transported less efficiently to the axon from the soma. We found that the extracellular domain of the shm Caspr protein is intact. Normally, the extracellular domain of Caspr interacts with contactin, and this interaction is essential for the axonal transport of Caspr (29, 30).

To investigate this issue, we examined spinal cord neurons to determine whether Caspr was retained in the soma. In normal mice, Caspr staining was observed at the paranode within the white matter of the lumbar spinal cord. By contrast, staining was weaker in the white matter in mutant mice compared with normal mice. The mutant mice occasionally showed staining that was confined to the neuronal soma in the ventral horn (Fig. 5E). These findings suggest that the mutated Caspr was synthesized in the soma of motor neurons but was only partially transported to the axon.

To determine whether the truncated Caspr protein of the shm mutant affects the organization of paranodal components, we examined expression and localization of paranodal proteins by immunohistochemistry in shm sciatic nerves. Contactin is a glycosyl phosphatidylinositol-anchored IgSF cell adhesion molecule (31). Caspr and contactin form an adhesion complex that is essential for the formation of the paranodal junction (7, 32). In normal sciatic nerves, contactin immunostaining was restricted to the paranode (Fig. 6A). In shm sciatic nerves, however, contactin displayed a range of staining patterns, but the normal paranodal profile was rarely detected (Fig. 6A). Therefore, we counted the number of paranodal profiles with and without contactin staining by double labeling with anti-Kv1.1 antibodies and found that 52.84% ± 10.11% (mean ± SEM, n = 3, P22–24 mice) of paranodal regions showed contactin staining (Fig. 6B). As for Caspr, contactin immunoreactivity in

FIGURE 3. Histology of myelinated nerves in normal and shuffling mice. (A–D) Cross sections of sciatic nerves from normal (A) and shm mutant mice (B) and of the ventral regions of the spinal cords of normal (C) and shm mutant mice (D). Insets show higher magnification images. There are no obvious differences in axon or compact myelin formation between normal and mutant mice. Plastic-embedded sections stained with toluidine blue. (E–H) Electron micrographs of sciatic nerves from normal (E) and shm mutant mice (F–H). The axoplasm of normal myelinated nerves are enriched in tubular and filament organelles (E). In the mutant mice, there are abnormal organelles (i.e. aggregated lysosomes) (F) arrows) and swollen mitochondria (Mit) (G) in the nodes. Mitochondria are also abundant in the paranodal region (H). (A–D) P50; (E) 4 months; (F–H) P50.
lumbar spinal cord neurons of *shm* mice was also retained in the somata (data not shown).

Neurofascin 155 is a cell adhesion molecule expressed on the glial surface on the opposing paranodal loop (11, 33). It is believed that a complex of Caspr and contactin binds to NF155 at paranodes (33, 34). Neurofascin 155 staining was detected at the paranodal region; however, the immunoreactivity was extremely low compared with that of Caspr and contactin (Fig. 6C). Nevertheless, where NF155 staining was associated with Caspr staining (i.e. where Caspr was concentrated in the paranodal regions), NF155 was also present; and where Caspr was minimal, NF155 was also less evident.

The cytoplasmic domain of the normal Caspr protein interacts with protein 4.1B, providing a potential link with the actin cytoskeleton. This interaction is necessary to anchor a Caspr-contactin complex to the axonal membrane (28). Immunostaining for protein 4.1B revealed that it was expressed and distributed normally at the paranodes in *shm* sciatic nerves. Double staining for protein 4.1B and Caspr showed that protein 4.1B staining was apparently normal even when Caspr staining was barely detectable in the paranodal region (Fig. 6D).

We next examined whether altered distribution of paranodal molecules affected localization of the voltage-gated channels. The Na⁺ channels at the node and the K⁺ channels at the juxtaparanode are functionally crucial for generating action potentials and for propagating saltatory conduction. In the double staining for Na⁺ channels and K⁺ channels (Kv1.1) in normal axons, both were separated at each domain by a paranodal junction (Fig. 7A). In *shm* mutant mice, Na⁺ channels and K⁺ channels were closely located (Fig. 7B). The Na⁺ channels were restricted to the node, whereas the K⁺ channels were adjacent to the nodes (Fig. 7C). Thus, the K⁺ channels were mislocalized at the paranode in *shm* sciatic nerves.

**Electrophysiological Analysis of Sciatic Nerves and Optic Nerves**

We investigated whether disruption of the paranodal junction and the altered distribution of ion channels affected conduction of nerve impulses in *shm* mutant mice. Nerve conduction velocity was assessed by measuring CAPs in sciatic nerves isolated from normal and mutant mice. The electrophysiological properties of the sciatic nerves in the genotypes are summarized in the Table, and the differences in CAP waveforms are illustrated in Figure 8A. The CAPs of mutant mice exhibited markedly reduced peak-to-peak amplitude and delayed onset compared with those of normal mice. The mean conduction velocity in mutant mice was significantly reduced to 45.1% of that of normal mice (p < 0.0001; Table). Similarly, the reduction in CAP peak-to-peak amplitudes

![FIGURE 5. Expression and localization of the Caspr protein. (A-D) Sciatic nerves. (E) Spinal cords. (A) Caspr staining is not detected in *shm* mutant mice using an antibody against the cytoplasmic domains (R6061), but an antibody against the extracellular domain (M275) stains the paranodes of mutant mice. (B) The distribution pattern of Caspr at the paranodal region in *shm* mice. Arrows indicate the node. An arrowhead indicates Schmidt-Lanterman incisures. (C) Double labeling of Caspr (green) with Kv1.1 (red) for identification of paranodal profiles. Arrows indicate the node. (D) Percentage (%) of Caspr staining at paranodal regions after staining using anti-Kv1.1 antibody. Paranodal profiles were analyzed from 3 normal and 3 *shm* mice (100 each). (E) No cells in the ventral horn of a normal mouse are stained (left); large motor neurons express Caspr in an *shm* mutant mouse (arrows in right panel). (A) P24 to 30; (B) P14 to P24; (C) P22 to P24; (E) P30.](image-url)
was statistically significant (Table). The minimum voltage threshold required to elicit a CAP was also significantly higher in mutant mice than normal mice (Table). Overall, therefore, these data indicate that saltatory conduction in sciatic nerves in shm mice is impaired because of the disruption of the paranodal junctions and altered distribution of ion channels.

We also recorded VEPs from the primary visual cortex of shm and normal mice. The VEPs elicited in the mutant mice in response to contralateral eye stimulation were far smaller than those of normal mice (Figs. 8B, C). The mean amplitude of VEPs was significantly smaller (p < 0.01) in mutant mice than control mice (Fig. 8D). Likewise, the latency of VEPs was significantly longer (p < 0.04) in mutant mice than normal mice (Fig. 8E). Thus, the data indicate that the pathway to the primary visual cortex was severely affected in shm mutant mice.

**DISCUSSION**

**Caspr Mutation in shm Mice**

The shm mouse was identified in the fifth generation of mice after spermatogonial X-irradiation (35). Although it was discovered more than 4 decades ago, however, the responsible gene had not been identified nor had its pathogenesis been investigated in depth before this study. To date, no Caspr gene mutations have been reported in rodents or humans. We found that a TT insertion in exon 22 resulted in a stop codon in exon 23 of the Caspr gene of shm mice (Fig. 1D). The shm mutation apparently escaped from

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<th>TABLE. Electrophysiological Analysis of Sciatic Nerves</th>
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Measurements are expressed as mean ± SEM.

*p < 0.0001, **p < 0.001, by Student t-test.

CV, conduction velocity; PkAmp, peak-to-peak amplitude.
nonsense-mediated mRNA decay as the premature termination codon is located less than 55 bp from an exon-exon interaction in the second exon (36, 37). A truncated Caspr protein lacking the transmembrane and cytoplasmic domains was generated, and expression of the abnormal protein was low in shm mice (Fig. 2B). The shm mice therefore provide a unique model for investigating the role(s) of the extracellular domain and the cytoplasmic domain of the Caspr protein in the formation and function of paranodal junctions. Thus, we investigated whether a normal paranodal complex was organized and a normal paranodal junction structure was formed in myelinated nerves of shm mice.

Expression of the Truncated Caspr Protein

The extracellular domain of the Caspr protein binds laterally to contactin; this association is required for the intracellular transport of Caspr from the endoplasmic reticulum and for targeting the protein to the cell surface at the paranode (29, 30). In addition, the cytoplasmic domain of the Caspr protein is necessary for retention of the Caspr-contactin complex at the paranodal junction (28). Because the extracellular domain of the mutated Caspr protein is intact, we anticipated that a Caspr-contactin complex would be inserted into the plasma membrane at the paranode. It was also likely that the truncated Caspr protein would not be capable of being immobilized on the paranodal axolemma and would be internalized in the axon at the paranodal region. Our analysis of shm mice revealed a variety of distribution patterns for the mutated Caspr protein: a certain amount of the protein was retained in the soma of motor neurons in the ventral horn of the spinal cord (Fig. 5E), whereas it was also clearly located in some paranodes and adjacent to the paranodes (Figs. 5B, C; 6C, D). Our observations indicate that the axonal transport of Caspr is somewhat aberrant in shm mutant mice. Because there was a faint diffuse staining in the paranodal region (Fig. 5B), some of the truncated Caspr protein might also be internalized within the axon. We suspect that the mutated Caspr protein cannot form a normal paranodal complex in myelinated nerves. Indeed, we observed that paranodal junctions were absent from the sciatic nerves of shm mice (Fig. 4B).

In summary, the mutated Caspr protein in shm mice was expressed and distributed at the paranode, although the amounts of protein were significantly reduced. In addition, the aberrant expression and distribution of the truncated Caspr influenced paranodal organization because the expression and distribution of other paranodal molecules were also reduced in this region.

Disruption of Paranodal Junctions in shm Myelinated Nerves

The most prominent defects of paranodes in shm mice were disruption to the paranodal junctions; in particular, the absence of transverse bands and widened gaps between the axonal membrane and paranodal loops. The disruption to paranodal junctions differed between the CNS and the PNS. In the CNS, electron-dense materials were occasionally present, indicating residual material from the paranodal junctions, and myelin loops were more disorganized, with the loops facing away from the axon (everted loops) (Fig. 4).
These findings are consistent with those from Caspr KO mice (6). Absence of transverse bands at the paranodal junctions have also been reported in the PNS of contactin KO (7), NF155 KO (11), and CGT KO (8) mice.

The disruption to the paranodal junctions in shm mutant mice caused the aberrant distribution of voltage-gated ion channels, that is, the Na+ and K+ channels. The Na+ channels were more diffuse at the node: we found that the nodal region was significantly wider and the stained Na+ channels at the node were longer. Preliminary results from a normal P21 mouse gave a mean length for Na+ channels of 1.35 ± 0.04 μm (n = 74) and from a mutant P21 mouse a mean of 1.80 ± 0.05 μm (n = 114). The K+ channels, which are normally distributed in the juxtaparanode, were mislocated to the paranodal region next to the Na+ channels at the node in mutant mice (Fig. 7). The paranodal junctions provide a barrier for segregation of Na+ channels and K+ channels, which is crucial for producing action potentials for conduction velocity in myelinated nerves (1, 2, 5). The disruption of this barrier may be responsible for the aberrant nerve conduction in shm mutant mice. Mislocalization of K+ channels was also found in Caspr KO (6) and contactin KO (7) mice.

Aberrant Nerve Impulse Conduction in shm Mice

Rapid impulse propagation via saltatory conduction in myelinated nerves is critically dependent on the insulating function of myelin. In the shm sciatic nerve, the conduction velocity and the peak-to-peak amplitudes of compound action potentials were markedly reduced possibly caused by disrupted paranodal junctions. In shm myelinated nerves, there were no distinct differences in either myelin formation in the axons or the ratio of fiber diameter to myelin thickness (Figs. 3A–D). Reduction of conduction velocity has also been reported in Caspr KO (6) and contactin KO (7) mice. Aberrant nerve impulse conduction in peripheral nerves likely contributes to the hindlimb weakness commonly displayed by these mice.

We also found that the visual pathway was affected in shm mutant mice. The smaller amplitude of VEPs was probably caused by disruption of the axoglial interaction at the paranode (Fig. 4D). Furthermore, the disruption of axoglial interaction at the paranodes may also affect the conduction velocity in optic nerves because the latency of VEPs was prolonged in mutant mice. This is the first report of a defect of nerve impulse conduction in the CNS in mice with a deficiency for paranodal molecules. The VEPs are widely used to assess the function of the visual pathways in both health and disease (39, 40). In shm mutant mice, the amplitude of VEPs was very small compared with those in normal mice, indicating that visual function was severely affected. In hypomyelinated shiverer mutant mice, a significant increase in latency has also been observed, but there is no consistent difference in the amplitudes of the main peak of the VEPs (41). The small amplitude of VEPs in mutant mice may be ascribed to the conduction failure of nerve fibers mediating visual signals from the retina to primary visual cortex in addition to the prolonged conduction velocity. In summary, a failure to segregate Na+ and K+ channels at the nodes and juxtaparanodes caused by disruption of paranodal axoglial junction and altered expression of paranodal proteins is accompanied by aberrant nerve conduction in the PNS and the CNS. These abnormalities likely contribute to the neurological phenotype of shm mutant mice.

An Animal Model for Axodegenerative and Possibly Demyelinating Disease

The nodal/paranodal axoplasm of shm mice had abnormally large mitochondria and accumulation of cytoplasmic organelles (Figs. 3F–H), suggesting that axonal transport is affected in the nerves. Altered neuron-glial interaction at the paranodes has been reported in human demyelinating diseases such as CMT and multiple sclerosis. The CMT phenotype can be produced by more than 30 different mutations, but to date, there is no evidence that Caspr can cause CMT (42). In CMT, however, the paranodal junction is absent and the structure of the node of Ranvier is altered (13), implying that loss of the interaction between glial cells and neurons results in demyelination and axonal degeneration (12). Multiple sclerosis is characterized by the presence of multiple chronically demyelinated lesions and axonal loss, and immunolocalization of Caspr and nodal/juxtaparanodal proteins is diffuse along the axons and not concentrated in these regions (14, 15), suggesting that aberrant location of Caspr may be an early indicator of impending myelin loss in multiple sclerosis. Taken together, we propose that a neurological mutant mouse, shambling, which is a newly identified mutation of Caspr, could be a useful animal model for human axodegenerative and possibly demyelinating diseases.

REFERENCES


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