Calnexin Deficiency Leads to Dysmyelination*§

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Calnexin is a molecular chaperone and a component of the quality control of the secretary pathway. We have generated calnexin gene-deficient mice (cnox−/−) and showed that calnexin deficiency leads to myelopathy. Calnexin-deficient mice were viable with no discernible effects on other systems, including immune function, and instead they demonstrated dysmyelination as documented by reduced conductive velocity of nerve fibers and electron microscopy analysis of sciatic nerve and spinal cord. Myelin of the peripheral and central nervous systems of cnox−/− mice was disorganized and decompacted. There were no abnormalities in neuronal growth, no loss of neuronal fibers, and no change in fictive locomotor pattern in the absence of calnexin. This work reveals a previously unrecognized and important function of calnexin in myelination and provides new insights into the mechanisms responsible for myelin diseases.

The endoplasmic reticulum (ER)5 is the first compartment in the secretary pathway responsible for protein synthesis, posttranslational modification, and correct folding. The resident molecular chaperones ensure that only correctly folded proteins leave the ER. Calnexin is a type I ER membrane protein, a major component in assuring the quality control of the secretary pathway, and together with calreticulin and the oxi-

doreductase ERp57, it promotes the correct folding of newly synthesized glycoproteins (2). Calnexin and calreticulin bind monoglucosylated carbohydrate on newly synthesized glycoproteins, whereas ERp57 catalyzes rearrangements of disulfide bonds within the calnexin/calreticulin substrate proteins (2). Despite its ubiquitous expression, the absence of calnexin has a different effect in different organisms. Calnexin deficiency is lethal in Schizosaccharomyces pombe but not in Saccharomyces cerevisiae (3), Dictyostelium (4, 5), or Caenorhabditis elegans (6, 7). The loss of calnexin affects phagocytosis in Dictyostelium (4, 5) and promotes necrotic cell death in C. elegans (7). It has been reported that deletion of the calnexin gene in a mouse results in early postnatal death (1), and thus the molecular consequences of calnexin deficiency could not be studied.

Here we show that calnexin deficiency in the mouse did not result in early postnatal death (1). These animals developed myelopathy with no discernible effects on other systems, including immune function. The phenotype was linked to slow nerve conduction velocities in the absence of calnexin with evidence of peripheral axon dysmyelination. The dysmyelinating phenotype described here underscores the emerging importance of calnexin and ER-associated pathways as contributors to these severe neurological disorders.

EXPERIMENTAL PROCEDURES

Generation of Calnexin-deficient Mice—Gene trapping with the trap vector pGT1TMpfs was used to generate the calnexin gene disrupted embryonic stem cells, designated KST286. The cell line KST286 was from the Gene Trap Resource (BayGenomics, University of San Francisco, San Francisco, California). The KST286ES cell line was generated from the 129P2 (formerly 129/Ola) embryonic stem cell line, the E14Tg2A.4 subclone. Parental cell lines (CGR8 and E14Tg2A) were established from delayed blastocysts. Embryonic stem cells were microinjected into 3.5-day-old C57BL/6J blastocysts to generate chimeric mice (8). Chimeric males were analyzed for germ line transmission by mating with C57BL/6J females, and the progeny were identified by PCR analysis, β-galactosidase staining, and Western blot analysis. All of the animal experimental procedures were approved by the Animal Welfare Program at the Research Ethics Office of the University of Alberta and con-

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The abbreviations used are: ER, endoplasmic reticulum; MHC, major histocompatibility complex; RT, reverse transcription; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; TCR, T cell receptor.

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**Genotype Analysis of Calnexin-deficient Mice**—Genomic DNA was isolated from mouse tails by lysis with a buffer containing 10 mm Tris, pH 8.0, 150 mm NaCl, 10 mm EDTA, 0.5% SDS, and proteinase K digestion followed by phenol-chloroform extraction. An inverse PCR technique was used to identify the gene trap insertion site in the calnexin gene. Briefly, genomic DNA was first digested with BfaI restriction enzyme that cleaves at frequent intervals and digests the gene trap vector near the 3’-terminal end. The resulting DNA fragments were ligated under conditions that favor intramolecular circularization of single fragments. The nucleotide sequence located at the 3’-terminal end of the gene trap vector was then selectively amplified using inverse DNA primers (INVF1, 5’-TCAAGGCCAGTTACATGATCC-3’; and INVR1, 5’-AGGCCA-TACCACACCGAGCG-3’) derived from the nucleotide sequence of the gene trap vector. The resulting PCR product was amplified a second time using nested DNA primers (F2, 5’-TCAAGGCCAGTTACATGATCC-3’; and R2, 5’-CGGCTGACACCGAGATGC-3’), purified, and sequenced. The PCR product obtained corresponded to the gene trap vector sequence. To verify the integration site allow detection of the wild-type allele integration vector sequence with primer R3 was used to detect the gene trap allele insertion (see Fig. 1). DNA primers that flank the integration site allow detection of the wild-type allele (Primers F1 (exon 7), 5’-GGCCAGATCGAGATCTGAGACACC-3’; and R3 (intron 7–8), 5’-CACACAGGGGTATGGGGCTGTTTCAG-3’), whereas DNA primer F2 within the insertion vector sequence with primer R3 was used to detect the gene trap allele insertion (see Fig. 1).

To determine that no alternative splicing around the interruption cassette took place, RNA was isolated from wild-type, heterozygote, and homozygote calnexin-deficient mice (see Fig. 1A). DNA primers that flank the site of insertion allow detection of the wild-type allele (Primers F1 (exon 7), 5’-GGCCAGATCGAGATCTGAGACACC-3’; and R3 (intron 7–8), 5’-CACACAGGGGTATGGGGCTGTTTCAG-3’), whereas DNA primer F2 within the insertion vector sequence with primer R3 was used to detect the gene trap allele insertion (see Fig. 1A).

**Western Blot Analysis**—Two distinct polyclonal rabbit anti-calnexin antibodies were used: SPA-860 (Stressgen Biotechnologies) raised against a synthetic peptide corresponding to the C terminus of calnexin (amino acid residues 575–593) and SPA-865 (Stressgen Biotechnologies) raised against a synthetic peptide near the N terminus. Antibodies were used at 1:1000 and 1:500 dilutions, respectively. Prepartation of cell extracts, Western blot analysis, and immunostaining of wild-type and calnexin-deficient cells were carried out as described previously (21). Twenty μg of cell and brain tissue extracts and 200 ng of purified recombinant protein (C-tail and N+P domain) was loaded for analysis of calnexin protein expression. The membranes were stripped with a buffer containing 1% SDS, 100 mm β-mercaptoethanol, and 50 mm Tris-HCl, pH 6.8. Anti-glycerolaldehyde-3-phosphate dehydrogenase antibodies (1:500; Abcam) were used to normalize for protein loading.

**Electrophysiology Measurements**—Newborn, 1-day-old, and 2-day-old mice were used for the electrophysiological experiments (10). The spinal cord was pinned ventral side up in a recording chamber and perfused with oxygenated Ringer’s solution containing 111 mm NaCl, 3.08 mm KCl, 11 mm glucose, 25 mm NaHCO3, 1.18 mm KH2PO4, 1.25 mm MgSO4, and 2.52 mm CaCl2 at room temperature. Electroneurogram recordings were made by placing bipolar suction electrodes on a combination of the second and fifth lumbar ventral roots (LI2-L2IL5) (10). The second lumbar ventral roots consist of primarily flexor motor axons, and the L5 ventral roots consist of primarily extensor motor axons; therefore fictive locomotion involves alternation between IL2 and IL5 as well as alternation between IL2 and rIL2. Electroneurogram signals were amplified, bandpass-filtered (100 Hz-1 kHz), digitized, and collected using Axospect software (Axon Instruments). Rhythmic fictive locomotor activity was induced by the addition of 5 μm 5-hydroxytryptamine and 10 μm N-methyl-d-aspartic acid to the Ringer’s solution (10).

**Multifiber Motor and Sensory Conduction**—Multifiber motor and sensory conduction studies were carried out in mice briefly anesthetized with isoflurane, using protocols previously reported (11). In brief, sciatic-tibial motor fibers were supramaximally stimulated at the sciatic notch and knee, and a compound muscle action potential was recorded (base line-peak amplitude) from the motor end plate of tibial innervated dorsal interosseous foot muscles. Motor conduction velocity was calculated for the notch to knee segment. For sensory conduction, digital hind paw nerves were supramaximally stimulated, and the sciatic-tibial sensory nerve action potential (base line-peak
amplitude) was recorded from the knee after averaging (5–10 times). Stimulation and recording were carried out using E2 subdermal platinum electrodes (Grass/Astrobotm). All of the recordings were carried out with near nerve temperatures maintained at 37.0 ± 0.5 °C.

**Histological and Electron Microscopy Analyses**—The mice were decapitated, and the brains were rapidly removed and flash frozen in 2-methylbutane on solid carbon dioxide (12). Serial coronal sections of 20 μm were obtained, and the sections were thaw mounted on charged slides. To visualize neuronal cell bodies and astrocytes, immunoassays were performed using antibodies recognizing the neuron-specific marker mouse anti-neuronal nuclei (1:1,000; NeuN, Chemicon) and glial fibrillary acidic protein (1:750; Dako), respectively. The fresh frozen sections were brought to room temperature, postfixed in buffered formalin, and taken through graded ethanol washes. The sections were incubated in a humidifying chamber with 1% hydrogen peroxide to quench endogenous peroxidase enzyme activity and were subsequently blocked with universal blocking serum (Dako) containing 0.2% Triton X-100. The sections were washed with phosphate-buffered saline (PBS) and incubated with the primary antibodies for 1 h at room temperature followed by the secondary antibody (rabbit anti-mouse, 1:200; Dako) for 30 min. Then the sections were washed with PBS and incubated for 30 min with an avidin-biotin complex (1:100; Vector Laboratories) and washed three times, and immunoreactivity was visualized with 3,3′-diaminobenzidine tetrahydrochloride. The sections were then rinsed, dehydrated in a series of ethanol washes, and mounted with Permount.

To assess myelinated axons, Weil's stain for myelin was used. Fresh frozen sections were postfixed in buffered formalin, rinsed with water, and dehydrated. The sections were then incubated for 45 min at 55 °C in Weil's staining solution containing 10% hematoxylin and 4% ferric ammonium sulfate solution preheated to 55 °C. The sections were then washed with tap water and differentiated macroscopically with 4% ferric ammonium sulfate and microscopically with Weigert's differentiator (potassium ferricyanide with borax).

For electron microscopy analysis, the mice were euthanized by decapitation, and their brains and spinal cord tissues were removed. The following regions of the brain were dissected: rostral spinal cord, medulla, cerebellum, diencephalon, fornix, striatum, internal capsule, corpus callosum, and motor cortex. Primary fixation was carried out at 4 °C for 4 h in a freshly prepared solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in 100 mM cacodylate, pH 7.2 (13). Spinal cord and sciatic nerve samples were obtained following fixation by perfusion or by euthanasia by cervical displacement and dissection, followed by fixation by immersion. Identical results were obtained for samples fixed by tissue perfusion or by fixation by immersion of the tissue in fixative. In both cases, the fixative used was 2.5% glutaraldehyde and 0.1 M sodium cacodylate, pH 7.0. The samples were processed for electron microscopy and examined with a Hitachi Transmission Electron Microscope H-7000.

For morphological analysis of a comprehensive range of tissues, the organs were obtained following euthanasia by cervical displacement and dissection. The samples were fixed in Zinc-Formal Fixx (Fisher) overnight, then processed, and embedded in paraffin blocks, after which 5-μm sections were cut and placed onto Histobond slides (Fisher). The sections were rehydrated to water and stained with Harris hematoxylin and alcoholic eosin Y (Electron Microscopy Sciences) as per standard histology protocols, followed by mounting with Entellan medium (Electron Microscopy Sciences).

**Flow Cytometry**—The thymus, lymph nodes, and spleen were harvested from the mice following euthanasia. Single-cell suspensions were generated, and 2 × 10^6 cells were aliquoted into the wells of a 96-well plate for antibody staining. All of the antibody incubations were carried out for 30 min on ice in a fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% fetal calf serum and 0.02% sodium azide), and the cells were washed twice with FACS buffer following antibody incubations. The cell events were collected with a BD FACS Canto II flow cytometer and analyzed with FlowJo software (Treestar).

**Retinal Analysis**—For retinal analysis of calnexin-deficient mice, the following antibodies were employed: the 1D4 monoclonal anti-rhodopsin antibodies raised against a synthetic peptide located at the C terminus of bovine rhodopsin (amino acid residues TETSQVAPA) (from R. Molday, University of British Columbia, Vancouver, Canada) (14), polyclonal rabbit anti-M-opsin antibodies (a gift from C. Craft, University of Southern California, Los Angeles, CA) (15), and polyclonal rabbit anti-melanopsin antibodies raised against a synthetic peptide located at the N terminus of mouse melanopsin (amino acid residues QTLSSLVRPGPSDM) (a gift from I. Provencio, University of Virginia, Charlottesville, VA) (16). Whole retinas from both wild-type and calnexin-deficient mice were sonicated in Laemmli buffer, and 2.5 μg of protein were loaded per lane for detection of rhodopsin using the 1D4 antibody. For detection of calnexin, M-opsin, and melanopsin, 7.5 μg of protein was loaded per lane. The proteins were separated by electrophoresis in SDS-PAGE (12% acrylamide) and electroblotted onto nitrocellulose membranes. The immunoreactive proteins were visualized using horseradish peroxide-conjugated goat anti-mouse or anti-rabbit IgG (Invitrogen) followed by ECL detection (Amersham Biosciences).

For histological analysis of eye tissue, calnexin-deficient and wild-type mice were subjected to intracardiac perfusion with a modified Karnovsky’s fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 100 mM phosphate buffer. Eye cups were fixed and processed as previously described (17). 500-nm tissue sections were stained with a 0.25% azure II, 0.25% methylene blue stain containing 0.25% sodium borate in water.

**Miscellaneous Procedures**—Axon cultures (Camperon cultures) were set up and maintained as previously described (18). Protein concentration was estimated using a Bio-Rad DC protein assay (19). To determine the number of neurons, wild-type and cnx^-/- mice were anesthetized and fixed by sequential intracardiac perfusion of PBS and 4% paraformaldehyde in PBS. Brain and whole spinal cord were dissected out, postfixed in the same fixative overnight, processed for paraffin embedding, and cut in 10-μm-thick coronal sections. After paraffin removal in xylol and graded alcohols, the sections were counterstained with cresyl violet. Spinal cord motoneurons exhibiting clear
nucleus/nucleolus in layer IX were counted every 10 sections. Similarly, the number of brain stem facial motoneurons was counted every three sections. Ca\(^{2+}\) measurements were carried out using 1 mM Fura 2-acetoxyethyl ester as described previously (20). Ca\(^{2+}\) measurements were analyzed in response to 1 \(\mu\)M thapsigargin and 600 nM bradykinin using a Photon International Technology fluorometer at excitation of 340 nm and emission of 380 nm. For electrocardiogram analysis, ECG Leads I, II, III, AVR, and AVL of nonseeded transgenic and control mice were simultaneously recorded using E for M ECG amplifiers (PPG Biomedical Systems Inc., Pleasantville, NY) (21).

RESULTS

Calnexin-deficient Mouse—Fig. 1A summarizes the gene targeting strategy used to generate the calnexin gene knock-out mice. The calnexin gene was disrupted by random gene trapping using a cassette containing the \(\beta\)-galactosidase-neomycin genes. Using specific primers F1, F2, and R3 (Fig. 1A), we determined the site of insertion to be preceding the first nucleotide of intron 7–8 (Fig. 1A). DNA sequence analysis confirmed that the interruption cassette was inserted directly following exon 7. Primers F1 and R3 correspond to the calnexin gene, whereas primer F2 corresponds to a sequence within the \(\beta\)-galactosidase gene in the insertion cassette. We used these primers for PCR-driven amplification of genomic DNA to genotypen the mice. As expected, analysis of DNA isolated from wild-type mice showed only a 316-bp DNA product with F1/R3 primers (Fig. 1B) and no DNA product when F2/R3 primers were used (Fig. 1B). Analysis of genomic DNA from \(\text{cnx}^{-/-}\) mice showed amplification of a 941-bp DNA product with the use of F2/R3 primers and no DNA product with primers F1/R3, indicating that both alleles of the calnexin gene were interrupted by the insertion cassette (Fig. 1B). In contrast, PCR analysis of genomic DNA from heterozygote mice with F1/R3 and F2/R3 primers produced both 316- and 941-bp DNA fragments corresponding to the presence of both wild-type and calnexin gene interrupted alleles, respectively (Fig. 1B). To determine that there was no mRNA alternative splicing around the interruption cassette, RT-PCR analysis was carried out of RNA isolated from wild-type, heterozygote, and calnexin-deficient brain tissue using a specific set of primers (Fig. 1C). Fig. 1D shows that mRNA encoded by exons 1–7, prior to the interruption cassette, was transcribed with no detectable alternative splicing near the interruption cassette. No RT-PCR product was detected with primers covering exons 8–15 of the calnexin gene (Fig. 1, C and D). Similarly, no RT-PCR product was seen when primers 7F (exon 7) and primers 8R and 12R (exon 8 and 12, respectively) were used (Fig. 1, C and D). As expected, RT-PCR analysis of \(\text{cnx}^{-/-}\) and \(\text{cnx}^{-/-}\) RNA, but not wild-type RNA, with primers covering exon 7 and insertion cassette, resulted in a specific DNA product (Fig. 1D). Western blot analysis revealed that there was no detectable expression of calnexin protein when both alleles of the gene were interrupted (Fig. 1, E and F). Identical results were obtained with a calnexin antibody specific for the N-terminal ER luminal portion (Fig. 1E) or an antibody that recognizes the C-terminal cytoplasmic domain (Fig. 1F). We concluded that the expression of calnexin protein was fully inactivated.

Absence of Calnexin Results in Impaired Nerve Conduction Velocity—The heterozygote mice had a normal phenotype, being viable and fertile. Intercrossing of heterozygote females and males was carried out to generate homozygote calnexin-deficient mice. In stark contrast to the results of Denzel et al. (1), we did not observe early postnatal death in mice with the complete loss of calnexin. Instead, newborn calnexin-deficient mice were indistinguishable from wild-type and heterozygote littermates with respect to their size, weight, and external appearance. However, a size difference between wild-type and calnexin-deficient (\(\text{cnx}^{-/-}\)) mice became apparent as early as 7 days after birth, and a marked size discrepancy was evident 14–16 days following birth, resulting in \(\text{cnx}^{-/-}\) mice that are 30–50% smaller than their wild-type littermates. Calnexin-deficient mice showed neurological abnormalities manifested by a gait disturbance with instability, splaying of the hind limbs, ataxia, tremors, lower limb motor defects, and a rolling walk (Fig. 2A and supplemental video).

To evaluate neuronal status, we carried out morphological analysis of the brain tissue, counted motoneurons of the spinal cord, and examined neuronal growth and function in the absence of calnexin. We did not observe any significant changes in the gross morphology of the brain in calnexin-deficient mice (Fig. 2B). Examination of the motoneuron distribution in the spinal cord indicated that although the spinal cord was shorter in the absence of calnexin (consistent with their smaller size), the motoneuron distribution was comparable with that of wild type (Fig. 2C). Furthermore, a careful count of motoneurons in wild-type and calnexin-deficient mice revealed no difference in the number of motoneurons (Fig. 2C). Similarly, amplitudes of the compound muscle action potentials, indices of motor axon innervations, were not altered in \(\text{cnx}^{-/-}\) mice. Neuronal growth in \(\text{cnx}^{-/-}\) neurons was investigated by culturing sympathetic neurons in compartmentalized cultures (18). We observed no difference in neuronal growth in the absence of calnexin (Fig. 2D).

To assess neuron status in calnexin-deficient mice, we examined pharmacologically induced lower limb walking movements in the isolated spinal cord. To evoke fictive locomotion (characterized by the oscillatory bursting of motor neurons in a step cycle period of 2–4 s), we applied 5 \(\mu\)M 5-hydroxytryptamine (serotonin) and 10 \(\mu\)M \(N\)-methyl-D-aspartic acid (Fig. 3A) to isolated spinal cords (10) taken from wild-type and \(\text{cnx}^{-/-}\) neonatal mice. Electroneurograms were recorded from the second and fifth lumbar ventral root on the left side (i.e. IL2, IL5) and the second lumbar ventral root on the left and right side (i.e. IL2, rL2). Appropriate alternation between bursts was noted in wild-type and calnexin-deficient preparations (Fig. 3A), indicating that the fictive locomotor pattern was undisturbed in the \(\text{cnx}^{-/-}\) mouse.

Next, we tested for electrophysiological parameters of motor and sensory neurons in calnexin-deficient and wild-type mice. Fig. 3B shows that motor nerve conduction velocities were significantly slowed in the absence of calnexin. There was a significant difference between wild-type and calnexin-deficient motor conduction velocities at values of 43.1 ± 2.5 and 31.0 ± 3.2 m/s, respectively (\(n = 4; p = 0.01\)) (Fig. 3B). Wild-type and calnexin-deficient mouse compound muscle action potential
FIGURE 1. Generation of a calnexin-deficient mouse. A, random gene trapping was used to generate the calnexin-deficient mice. An interruption cassette (pGT1Tmplfs cassette, in red) containing β-galactosidase and neomycin genes was inserted into the calnexin gene. The numbers indicate the locations of calnexin gene exons (in blue). Forward (F1 and F2) and reverse (R3) primers are indicated with arrows. B, PCR analysis of genomic DNA isolated from wild-type (wt), heterozygote (cnx+/H11001/H11002), and homozygote (cnx−/H11002/H11002) calnexin-deficient mice. Forward (F1 and F2) and reverse (R3) primers were used as indicated for A. A DNA product of 941 bp amplified with primers F2 and R3 identifies successful cassette insertion and interruption of the calnexin gene (cnx−/H11002/H11002), whereas a DNA product of 316 bp amplified with primers F1 and R3 indicates the presence of the wild-type allele (wt). The presence of both 941- and 316-bp DNA products identifies heterozygotes. C, schematic representation of calnexin mRNA. The location of the insertion cassette (in red) is shown. The location of specific primers used for RT-PCR analysis in D is indicated in the figure. D, RT-PCR was carried out using wild-type (wt), heterozygote (cnx+/H11001/H11002), and calnexin-deficient (cnx−/H11002/H11002) RNA isolated from brain tissue. Pairs of specific DNA primers used for the analysis are indicated. E and F, Western blot analysis of wild-type, heterozygote, and calnexin-deficient tissues and wild-type and calnexin-deficient fibroblasts with anti-calnexin antibodies. The location of molecular weight markers is indicated to the left of the gel. In E, anti-N terminus (N+P domain) calnexin antibodies were used. In F, the blot was probed with anti-C terminus calnexin antibodies. N+P, N+P domain of calnexin; C-tail, cytoplasmic C-terminal domain of calnexin. The asterisk and ns designate the nonspecific reactive protein band. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
amplitudes were preserved at 9.2 and 10.0 mV, respectively. Sensory nerve conduction velocities were also reduced in the cnx/H11002 mice (Fig. 3C). The sensory nerve conduction velocity in wild-type mice was 46.1 m/s with a significant decrease in sensory nerve conduction velocity (38.2 m/s) in the absence of calnexin (Fig. 3C). The amplitude of the sensory nerve action...
Calnexin-deficient Mouse

FIGURE 3. Functional analysis of calnexin in neuronal tissue. A, the fictive locomotor pattern is undisturbed in the cnx 
−/− mouse. Electroneurograms were recorded from the second and fifth lumbar ventral root on the left side (i.e. IL2, IL5) and the second lumbar ventral root on the left and right side (i.e. IL2, rl2) of the spinal cord in the wild-type (wt, left traces) and cnx −/− (right traces) mice. Fictive locomotion was evoked with 5 mM serotonin (5-hydroxytryptamine) and 10 mM N-methyl-d-aspartic acid. The spinal cords used were obtained from newborn, 1-day-old, and 2-day-old mice (n = 4). Note the appropriate alternation between bursts in both cases. B, motor nerve conduction velocity was significantly slower in the absence of calnexin. C, sensory nerve conduction analysis of wild-type (wt) and calnexin-deficient (cnx −/−) mice. Motor nerve conduction velocity was significantly lower in the absence of calnexin. Six sex-matched sets of wild-type and calnexin-deficient mice of 4–5 months of age were examined for motor and sensory nerve conduction. The asterisks indicate significant differences (p = 0.01).

potentials in the wild-type mice was also comparable between the groups: 14.1 μV in controls compared with 10.9 μV in the calnexin-deficient animals. The sensory nerve action potential amplitude reflects the number of excitable myelinated axons that can be recruited by stimulation. Taken together, these results indicate that neuronal growth and neuron number were not altered in the absence of calnexin, but there was a significant decrease in the nerve conduction velocity in cnx −/− mice.

Dysmyelination in the Calnexin-deficient Mouse—Myelin surrounds axons and allows for rapid nerve conduction that is essential to nervous system function. Loss of myelin leads to reduced nerve conduction velocity, and therefore we tested whether myelination was affected in the absence of calnexin. First, we carried out electron microscopic analysis of spinal cord and sciatic nerve in calnexin-deficient mice to examine, at a higher resolution, whether myelin formation was impaired in the absence of calnexin. Calnexin-deficient spinal cords had a thinner, wavy, and decompacted myelin in the absence of calnexin (Fig. 4, A–D), indicating that the absence of calnexin affects myelination of the spinal cord. A different kind of myelination defect was apparent in the sciatic nerve. Electron microscopic analysis of calnexin-deficient sciatic nerve revealed, in addition to wavy and decompacted myelin, a hypermyelination that appeared to invade the neuronal areas (Fig. 4, E–H). The findings resembled “G fibers” or tomaculae that are described in human hereditary neuropathy with sensitivity to pressure palsy or focally folded myelin described in Charcot-Marie-Tooth disease 4B. To test whether calnexin deficiency resulted in a reduced amount of myelin in nervous tissue (hypomyelination), the g ratio was calculated for calnexin-deficient and wild-type spinal cord and sciatic nerve. The g ratio is defined as the ratio of the axonal diameter divided by the diameter of the axon plus the thickness of myelin sheath. Calculation of the g ratio revealed that calnexin deficiency resulted in modest hypomyelination in the spinal cord and did not affect myelin sheath thickness in the sciatic nerve (cnx −/−/−) spinal cord (g ratio was 0.78 ± 0.05 (n = 40) compared with wild-type spinal cord at 0.71 ± 0.05 (n = 40); cnx −/−/− sciatic nerve g ratio was 0.73 ± 0.09 (n = 10) compared with wild-type sciatic nerve at 0.71 ± 0.07 (n = 10); n represents the number of neurons measured from representative electron micrographs). We concluded that in the absence of calnexin there was no significant reduction in myelin but defective formation and compaction of myelin sheaths. These findings indicate significant changes in the peripheral and central nervous systems of calnexin-deficient animals and help to explain the neuronal phenotype and decreased nerve conduction velocity in cnx −/−/− mice.

Histological analysis of the brain tissue from wild-type and cnx −/−/− animals was carried out to evaluate the consequences of calnexin deficiency on various regions of the central nervous system. In all of the brain regions examined, neuronal cell bodies, visualized using anti-neuronal specific nuclear protein (NeuN) antibodies, appeared normal and healthy in both the wild-type (Fig. 5A) and calnexin-deficient (Fig. 5B) mice. However, large white matter tracts were variably affected. In calnexin-deficient animals the rostral corpus callosum was thinner and, particularly at the medial rise, displayed areas of patchy and irregular myelination or dysmyelination (Fig. 5, C and D). However, the axons appeared to be spared. In the absence of calnexin, the internal capsule (Fig. 5F) displayed less branching and was narrower than that observed in the wild type (Fig. 5E), suggesting fewer myelinated fibers traveling between the periphery and cerebral cortex. Patchy areas of myelination were...
evident in the cerebral peduncle of the calnexin-deficient mouse (Fig. 5, G and H). The cerebellar peduncles of the cnx<sup>−/−</sup> mouse were also characterized by a patchy, loose myelination pattern (Fig. 5, I and J). At low resolution, white matter tracts of the spinal cord did not show obvious dysmyelination, and the cell bodies of the horns appeared normal and healthy. An increased number of glial fibrillary acid protein-positive astrocytic fibers was observed in the absence of calnexin (Fig. 5L) compared with the wild type (Fig. 5K). The perpendicular organization of the glial fibers in the calnexin knock-out mice was similar to that observed early in central nervous system development, suggesting that in the absence of calnexin, spinal cord development was altered.

Calnexin Deficiency Specifically Affects Myelination—Considering that calnexin is a ubiquitously expressed ER-associated protein, both the specificity of the neurological phenotype and the effect on myelination in cnx<sup>−/−</sup> mice were surprising. We expected that calnexin deficiency may also affect other tissues, and this may have been masked by the predominant neurological phenotype described above. However, we found no gross histological abnormalities in heart, lung, pancreas, spleen, femur, skeletal muscle, colon, liver, kidney, or stomach in the absence of calnexin (Fig. 6).

Given the role that calnexin plays in the early events of MHC Class I protein folding (22) and the fact that calnexin is able to associate with cell surface CD3 complexes (23) and regulate T
cell receptor (TCR) assembly (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes. Unexpectedly, calnexin deficiency had no impact on the assembly of TCRαβ (24), we anticipated that the elimination of calnexin would lead to aberration of these processes.
These findings further support our conclusions that calnexin plays a critical and specific role during myelination. The molecular chaperone function of calnexin is essential for proper formation of the myelin sheaths, for which there are no compensatory mechanisms provided by other ER chaperones, including homologous calreticulin. Yet, evidently, there must be compensatory redundancy in the other tissues of calnexin-deficient mice, including in the immune system.

Mutations in the *Drosophila* homolog of the calnexin gene (calnexin 99A) lead to severe defects in rhodopsin expression (28), suggesting that calnexin deficiency may also affect visual pigments. However, the complete lack of calnexin did not affect expression of rhodopsin, M-opsin, or melanopsin in mouse retinas. Consistent with Western blot analysis, the photoreceptor outer segments did not display significant defects in the cnx−/− mice (supplemental Fig. S2). However, there was an increased number of nuclei in the outer and inner nuclear layers, and the nuclei were disorganized. There was also vacuolization in the retinal pigment epithelial layer, indicating that calnexin was required for proper function of the retinal pigment epithelial layer and the retina.

The systematic and comprehensive analysis described here reveals that calnexin plays an important role in the pathogenesis of peripheral neuropathies. The specificity of calnexin deficiency toward myelin proteins and myelination is intriguing and supports early observations of transient association of myelin glycoprotein PMP22 with calnexin (29). PMP22 accounts only for a small fraction of peripheral nervous system myelin, whereas P0 is a major peripheral nervous system myelin glycoprotein (30). Both myelin proteins are involved in the compaction and maintenance of myelin (30). In the absence of calnexin, it is possible that PMP22 folding and function are modified, leading to dysmyelination. Our findings have identified a previously unknown role for calnexin in myelination and myelin diseases and as a novel contributor to the diversity of neurological disorders.

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