Gene targeting of GAN in mouse causes a toxic accumulation of microtubule-associated protein 8 and impaired retrograde axonal transport

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Mutations in gigaxonin were identified in giant axonal neuropathy (GAN), an autosomal recessive disorder. To understand how disruption of gigaxonin’s function leads to neurodegeneration, we ablated the gene expression in mice using traditional gene targeting approach. Progressive neurological phenotypes and pathological lesions that developed in the GAN null mice recapitulate characteristic human GAN features. The disruption of gigaxonin results in an impaired ubiquitin–proteasome system leading to a substantial accumulation of a novel microtubule-associated protein, MAP8, in the null mutants. Accumulated MAP8 alters the microtubule network, traps dynein motor protein in insoluble structures and leads to neuronal death in cultured wild-type neurons, which replicates the process occurring in GAN null mutants. Defective axonal transport is evidenced by the in vitro assays and is supported by vesicular accumulation in the GAN null neurons. We propose that the axonal transport impairment may be a deleterious consequence of accumulated, toxic MAP8 protein.

INTRODUCTION

Giant axonal neuropathy (GAN), first described in 1972 (1), is a severe, autosomal recessive motor and sensory neuropathy affecting both the peripheral nervous system and the central nervous system (CNS). Onset of symptoms usually occurs before the age of 4 years, with rare cases occurring up to the age of 10. The typical clinical manifestations of GAN for the first 7 years of the life are clumsiness, followed by progressive and predominant distal weakness and sensory loss with a gait disturbance. CNS involvement includes electroencephalographic abnormalities, mental retardation, seizures and defective upper motor neuron function (2,3). Curly or kinky hair is not a constant finding but appears in most of the patients (4,5). Death nearly always occurs by the third decade and is common by late adolescence (3). Pathologically, GAN is characterized by giant axonal swellings, fiber loss and neurodegeneration (6,7). At the ultrastructural level, GAN is characterized by generalized cytoskeletal abnormalities. In addition to densely packed neurofilaments (NFs) with displacement or segregation of other axonal organelles, reduced microtubule density and accumulation of intermediate filaments (IFs) outside of the nervous system are also noticed in biopsies from GAN patients (4–6,8–12). The recessive inheritance implies that GAN is a loss of function disorder.

The disease locus of GAN was assigned to a single locus on chromosome 16q24.1 (13–15) and the GAN gene encoding a protein, called gigaxonin, was identified (16). To date, 24 distinct mutations have been identified in 20 GAN patient families (16–18). The mutations were evenly distributed throughout the coding sequence of 11 exons. They included one insertion mutation at seventh amino acid, which leads to...
a premature termination, two deletions in the BTB domain, seven nonsense mutations in the kelch repeat domain and 14 missense mutations scattered over the entire coding region. The gene discovery represents an important step towards understanding the pathogenesis of the disorder. However, the mechanism by which disruption of the gene leads to devastating neurodegeneration was not fully elucidated.

Gigaxonin belongs distantly to BTB–kelch-repeat superfamily (16,19). Much has been learned in recent years about the functions of BTB-containing proteins that involve in many aspects of cellular processes, such as actin cytoskeleton interaction (20), cell morphology and growth (21), cytoplasmic sequestration of transcription factors (22) and possibly viral pathogenesis (23). Recent works suggest that the BTB–kelch proteins are involved in ubiquitin–proteasome system (UPS) and may define a new class of substrate-specific adaptors of Cul3-based E3 ligase complexes (24–26). It has been showed that BTB protein Keap1 targets the transcription factor Nrf2 for ubiquitination (27–29). The devastating clinical features occurring in GAN patients indicate an essential role of gigaxonin in axonal structure and neuronal function. Determining the molecular pathways that involve gigaxonin may contribute significantly to understanding the pathogenesis of GAN as well as other neurodegenerative disorders.

RESULTS

The GAN null mouse is a model of human GAN

The recessive inheritance of GAN and the disruptive mutations identified in human GAN patients imply that the disorder is due to a loss of gigaxonin function. To elucidate molecular mechanisms underlying the disorder, we ablated the GAN gene using traditional gene targeting. The targeting vector was constructed as depicted in Figure 1A and a 5 kb fragment containing exons 3, 4 and major portion of 5 was replaced by the 1.8 kb Neo gene cassette. An EraRV restriction digestion site is deleted in the targeted allele. (B) Genotyping of the targeted mice. The genomic DNAs were extracted from mouse tails. WT3, Neo1 and mGigDiag32 primers were used to amplify a PCR product of 328 bp for mutant (mut) and 436 bp for wild-type (wt). −/−, homozygous null; +/−, heterozygous; +/+ wild-type. (C and D) Brains and spinal cords of 30-day-old wt and KO mice were used to prepare protein lysates and mRNAs. (C) RT–PCR analysis was performed using primers to N-terminal gigaxonin and actin to confirm the null samples. (D) The protein samples were immunoblotted with antibodies to gigaxonin and actin.
These data confirmed that the gene targeting in mouse had successfully resulted in ablation of gigaxonin expression. A prominent feature of most GAN null mutants is a progressive deterioration in motor function with onset varying from 6 to 10 months. A substantial reduction of spontaneous movement is the initial sign of neurological abnormalities. The subclinical motor deficits were evidenced by established criteria utilizing specialized testing apparatus. Footprint tests recording locomotion displayed comprised motor functions and gait disturbance of the null mice at the early phenotypic stage of age 6–10 months (Fig. 2). Shortly afterward, most of the null mutants could be distinguished from their littermate controls by bizarre limb positioning and overall weakness (Fig. 3B, C and E) occasionally with signs of spasticity and seizure. The involvement of weakness started from hind limbs (Fig. 3C) and progressed to the forelimbs. By the age of 12 months and beyond, some of the null mice exhibited muscle wasting and progressive weight loss (Fig. 3B and E). Later during progression, the effort to walk for the phenotypic null mice becomes more severely compromised. Surprisingly, only very few GAN null mutants died prior to the age of 12 months. In addition to neurological problems, many human GAN patients display strikingly kinky or curly hairs. Likewise, hair phenotypes could be also found in some of the GAN null mice; some had no whiskers (Fig. 3E) and others displayed abnormal or fragile hairs (Fig. 3F). The heterozygous deficient mice bear a striking resemblance to the clinical manifestations of human GAN patients, although with milder course and significantly slower progression.

Pathological lesions in the null mice include axon loss involving both myelinated and unmyelinated nerve fibers, with a variable severity ranging from ∼18–27% in the peripheral nerves at the age of 9–12 months. On average, the axons are enlarged, but the featured giant axons found in human GAN patients could not be seen in the GAN null mice (Fig. 4G–I). Ultrastructural studies on phenotypical null mice revealed enlarged axons distended with NFs (Fig. 4E). The densely packed NFs displaced or segregated other axonal organelles (Fig. 4C), a feature characteristic of human GAN pathology. Pronounced axonal swelling and degeneration with thin myelin sheath is observed in both peripheral nervous system (Fig. 4B and F) and CNS (Fig. 4C). At later stages, axons in the phenotypic null animals are marked by vesicular accumulation (Fig. 4F), suggesting the impairment of axonal transport.

We conclude that the phenotypes and the pathological features of GAN null mice validate this null mouse as a model of human GAN.

Gigaxonin interacts directly with MAP8
To elucidate the molecular mechanisms underlying the disorder, we set out to understand the physiological functions of gigaxonin by identifying its binding partners using yeast two-hybrid screen. Using full-length gigaxonin as bait, a novel protein was also identified in addition to MAP1B-LC (30,31). Functional characterization of this protein revealed two microtubule-binding domains with distinctive affinities, one at each chain (32). We named it as microtubule associated protein 8 (MAP8). The C-terminal microtubule-binding domain of MAP8 was also identified by another group in transfected cells and reported as MAP1S (33). Post-natally, the protein expression was primarily found in the nervous system, with barely detectable levels in most tissues outside the nervous system (32). The specific interaction between gigaxonin and MAP8 was further tested in mammalian cells. When co-expressed in COS7 cells with MAP8, the gigaxonin protein no longer displayed a diffuse distribution (30), but co-localized with MAP8 along microtubules (Fig. 5C–E). The presence of the two proteins in co-immunoprecipitations also confirmed their association (Fig. 5B, lane 3). To precisely define the domains responsible for the protein interaction, we mapped their binding sites. Gigaxonin was dissected into the C-terminal kelch-repeat and N-terminal BTB domains. The yeast two-hybrid results indicated that the kelch repeat domain contains the direct binding site for MAP8 (Fig. 5A). Moreover, in co-transfected COS7 cells, the C-terminal
kelch repeat domain co-aligned with MAP8 (data not shown), supporting the notion that kelch-repeat motif is important for the interaction. Next, we examined which domain of MAP8 is the target for gigaxonin’s association. Furthermore, the co-localization of kelch-repeat domain with the C-terminus of MAP8 (MAP8-CT) on the actin filaments (Fig. 5F–H) was consistent with the results of the yeast two-hybrid screen. Thus, through its kelch-repeat domain, gigaxonin interacts directly with the C-terminus of MAP8. These results also suggest that the binding of MAP8 to gigaxonin does not affect its cytoskeletal associations.

To assess whether GAN-associated mutations disrupt the interaction of gigaxonin with MAP8, we analyzed three mutations for a possible loss of the specific association of gigaxonin with MAP8: the truncating mutation (18insA) and two nonsense mutations (R242X and R293X). As assessed by immunoblot analysis, all three mutations caused unstable protein expression, either undetectable for the 18insA mutant or significantly reduced in the cases of R242X (data not shown) and R293X mutants (Fig. 5B, lane 2). As revealed by co-immunoprecipitations, the R293X mutant failed to be pull-downed by the Flag-tagged MAP8-LC (Fig. 5B, lane 4), in contrast to its wild-type counterpart (Fig. 5B, lane 3). Furthermore, of the few detectable cells co-transfected with MAP8-CT and R242X (data not shown) or R293X (Fig. 5I–K), the mutant proteins were diffusely accumulated in the cytoplasm and had completely lost their binding to the C-terminus of MAP8/MAP1S (Fig. 5I–K), demonstrating that an effective interaction of the two proteins requires a functional kelch-repeat domain.

Gigaxonin negatively regulates the protein level of MAP8

Gigaxonin is reported to bind directly with ubiquitin-activating enzyme E1 (UBE1) through its BTB domain and plays an important role in controlling MAP1B’s protein degradation (31). We next determined whether disruption of the gigaxonin–MAP8 interaction also leads to protein accumulation of MAP8. Total lysates of mouse brain, spinal cord and sciatic nerves of wt and the null were analyzed by immunoblotting with antibodies to MAP8-HC and actin (Fig. 6A) or to dynein intermediate chain (DIC) and MAP8-LC (Fig. 6B). As judged by three independent assays, the absence of gigaxonin led to substantially increased levels of MAP8 protein in comparison with unchanged DIC or actin controls in the null animals (Fig. 6A and B). Densometric scans of western blots, normalized with levels of DIC and actin, indicate a nearly 1.5–3-fold increase of MAP8 level in the null samples at the age of 10–15 months (Supplementary Material, Fig. S1). To determine whether this increase was due to upregulated gene transcription, we analyzed MAP8 transcript in wt and the null animals. In contrast to the increase in protein, RT–PCR and real-time PCR revealed no change in
transcription of MAP8 in comparison to actin in the null samples (Fig. 6C and D). Thus, the increased level of MAP8 was due to protein accumulation.

The accumulation of MAP8 protein in the gigaxonin null mice prompted us to investigate whether gigaxonin plays an important role in controlling the degradation of MAP8. PC12 cells, which express endogenous gigaxonin and MAP8, were selected for assays. As the initial assessment, the protein stability of MAP8 was evaluated by utilizing cycloheximide (CHX) to suppress protein translation. As determined by immunoblot assays using anti-MAP8-LC (Fig. 7A) or anti-MAP8-HC (data not shown), the endogenous MAP8 is quite stable; the majority of the protein was still present 8 h after CHX treatment (Fig. 7A, lanes 1–7). Moreover, the protein level remains constant when both proteasome inhibitor (PI) and CHX are present (Fig. 7A, lanes 8–12), suggesting that a functional UPS is required during MAP8 degradation. Following the protein stability assays, the effect of gigaxonin on the protein levels of endogenous MAP8 was examined in Flag-gigaxonin or GFP-transfected PC12 cells. A transfection efficiency of ~15–20% was achieved for both Flag-gigaxonin and GFP constructs. In contrast to GFP (Fig. 7B, lanes 3 and 4), overexpression of gigaxonin was accompanied by a consistent reduction of MAP8-LC protein level 20–50 h post-transfection in PC12 cells (Fig. 7B, lanes 1 and 2). The moderate reductions detected at 24 h could be accounted by the fact that the reduction only occurred in 15–20% of the gigaxonin-overexpressing cells. Importantly, this gigaxonin-mediated reduction could be prevented by addition of PI to the gigaxonin overexpressing cells (Fig. 7B, lanes 5 and 6). These findings demonstrate that gigaxonin promotes destabilization of MAP8 protein and, in view of the fact that PI reverses MAP8 turnover, suggest the involvement of UPS in its degradation.

If the gigaxonin-controlled protein degradation of MAP8 is mediated by the UPS, the protein should become covalently conjugated with poly-ubiquitin when the degradation is inhibited. We tested this hypothesis on both exogenous (Fig. 7C, lanes 1 and 2) and endogenous (Fig. 7C, lanes 3 and 4) MAP8 proteins. To facilitate the assays of ubiquitination, an

Figure 4. Pathological lesions were found in the null animals. (A–F) Ultrastructural analysis (EM) on wt (A and D) and the null axons (B, C, E and F). Cross-section (A–C) and longitudinal (D–F) section of wt (A and D) and KO (B, C, E and F) samples revealed swollen axons (B), densely accumulated NFs (E), thin myelin sheath (indicated by arrows in B, C and F), completely disorganized cytoarchitecture and heavily accumulated membranous structures (C, from brain and F, from sciatic nerve) in the null animals. (G–I) Semi-thin sections of sciatic nerves from wt (G) and GAN null mice (H and I) at the age of 28 weeks show averagely enlarged axons with signs of degeneration in the null samples. Bar represents 500 nm for (A–D), 750 nm for (E and F) and 12 μm for (G–I).
excess of ubiquitin was supplied. Indeed, co-transfection of HA-tagged ubiquitin and Flag-MAP8-LC led to formation of an apparently typical ubiquitin ladder in the presence of PI, equivalent to the conjugation of poly-ubiquitin tags on the Flag-MAP8-LC (Fig. 7C, lane 2). Similarly, endogenous MAP8-LC was also poly-ubiquitinated when an excess of ubiquitin and PI were supplied, displaying the high molecular shifts of MAP8 concentrated by immunoprecipitations (Fig. 7C, lane 4). When a similar analysis was applied on cultured cortical neurons isolated from wt and the null animals, endogenous MAP8 in the GAN null cells failed to be poly-ubiquitinated in the absence of gigaxonin (Fig. 7D, lane 2). Significantly, when HA-gigaxonin was re-introduced into the GAN null neurons by transient transfections (Fig. 7D, lane 3), the characteristic high molecular shifts of MAP8 poly-ubiquitination, co-immunoprecipitated by anti-Flag-ubiquitin and detected with anti-MAP8-LC, was not only restored but also enhanced (Fig. 7D, lane 3), compared with that in wt neurons (Fig. 7D, lane 1). In contrast, the gigaxonin mutant-associated GAN, HA-R293X (Fig. 7D, lane 4), exercised no recovering effect (Fig. 7D, lane 4), providing additional evidence that MAP8 ubiquitination requires functional gigaxonin. We conclude that, in contrast to its mutant counterpart, gigaxonin has essential role in linking poly-ubiquitin tags to MAP8. Thus, mutations in gigaxonin could cause failure in UPS function leading to substrate overstabilization which may have toxic consequence on affected cells.

Figure 5. The kelch-repeat domain of gigaxonin binds directly to the C-terminus of MAP8. (A) pGBK7-BTB domain or pGBK7-kelch was mated to pGADT7-MAP8-C, showing no binding with BTB, but a positive reaction for the kelch-repeat domain of gigaxonin in yeast. (B) Co-immunoprecipitations were conducted on total lysates of COS7 cells co-transfected either with full-length gigaxonin (HA-Gig-F, lane 3) or mutant gigaxonin (HA-R293X, lane 4) and MAP8 light chain (Fl-M8-LC). The cells were co-IPed with anti-Flag beads and probed with anti-HA antibody. The arrows indicate the wt gigaxonin band in lane 3 and the absence of the mutant band in lane 4. The total lysates of co-transfected cells were loaded as controls (lanes 1 and 2). Asterisk indicates IgG chains. (C–E) Co-transfection of HA-gigaxonin (HA-Gig-F) and Flag-MAP8 (Fl-M8-F) in COS7 cells showed co-localization of the two proteins. (F–H) The kelch-repeat domain of gigaxonin (HA-Gig-C) and the GFP tagged C-terminus of MAP8 (GFP-M8-CT) displayed identical staining patterns in the co-transfected COS7 cells. (I–K) R293X mutant gigaxonin (HA-R293X) cannot bind the cytoskeletal associated C-terminus of MAP8 in COS7 cells. Bar represents 12 μm.

Accumulation of MAP8 is toxic to neurons in vitro

Gigaxonin apparently functions to control protein degradations of its binding partners including MAP1B (31), TBCB (34) and MAP8. To address whether MAP8 accumulation also contributes significantly to the pathogenesis of GAN, we tested the effect in cultured neurons. The cortical neurons isolated from wt or GAN null mice were cultured in parallel. During the initial 4–6 days in vitro (DIV), abundant neurites were seen in all cultures and formed a dense neurite network indistinguishable between wt and null neurons (data not shown). However, after ~10–12 days in culture, neurites of the GAN null neurons became progressively shorter, sparser and beaded (Fig. 8A and B); in contrast, wt neurites continuously grew denser and longer in the following weeks (Fig. 8C). By 17 days, there was a marked reduction (up to 90%) in cell density in cultured null neurons (Supplementary Material, Fig. S2) with apparent protein accumulation of MAP8 (Fig. 8A and B).

Next, we overexpressed MAP8 protein in wt neurons at DIV 2–3 using transient transfections to elucidate whether MAP8 accumulation alone is sufficient to cause neurodegeneration. A transfection efficiency of ~5–10% was achieved for both the GFP control and MAP8. Within the first 24–48 h post-transfection, no significant differences across all cultured neurons were observed. However, filamentous bundles (insets in Fig. 8E) were seen in a subset of MAP8 overexpressing neurons after 48–72 h post-transfection; ranging from 15–20% of transfected neurons in five independent experiments. By days 4–6 post-transfection, ~60–70% of the MAP8 overexpressing neurons began to show beaded and shortened neurites with aggregates in cell bodies (Fig. 8F–I). As judged by DAPI staining, all MAP8 transfected neurons displayed extensive nuclear disintegration or condensation 4–6 days after transfection. Shortly thereafter, an overwhelming majority of dying neurons completely lost any sign of normal morphology, a phenotype recapitulating
degeneration and cell death occurring in the cultured GAN null neurons. In contrast, GFP-expressing and untransfected wt neurons were indistinguishable over the entire test period, displaying long axonal processes with significant branching (Fig. 8D). These findings demonstrate that overexpression/accumulation of MAP8 contributed importantly to the neurodegeneration and cell death occurring in GAN null neurons. The formations of filamentous bundles and aggregates were observed to precede devastating neuronal death.

To further evaluate the pathogenic contribution of accumulated MAP8, scrambled or specific MAP8 interfering RNAs (siRNA) co-expressing GFP were introduced into gigaxonin null neurons at DIV 3–5 to prevent MAP8 accumulation. As revealed by immunofluorescence microscopy, no significant changes could be observed within the first 24 h. However, 3 days after transfection, an apparent reduction of MAP8 protein level could be seen in the MAP8 siRNA expressing null neurons. By days 7–10 post-transfection, the MAP8 siRNA expressing neurons display substantially improved survival rate (Supplementary Material, Fig. S3) in comparison with untransfected null neurons (Fig. 8L and M). In contrast, the null neurons transfected with scramble siRNA begin to undergo degeneration with MAP8 protein accumulating and aggregating in the cells (Fig. 8J and K). Fourteen days after transfection, survived MAP8 siRNA expressing neurons still displayed numerous elaborate neurite structures (Fig. 8N), whereas, the scrambled siRNA expressing neurons were morphologically undetectable. Thus, we conclude that the accumulation of MAP8 contributes directly to the pathogenesis of neurodegeneration in GAN. Consistently, although much improved, MAP8 siRNA expressing neurons could not be maintained for as long as wt neurons, strongly suggesting that accumulated MAP8 protein is not the only pathogenic factor; other toxic factors including accumulated MAP1B (31) are involved in neurodegeneration and cell death occurring in GAN.

**MAP8 overexpression alters the microtubule network and traps motor protein in aggregates**

How the toxicities mediated by MAP8 accumulation required further investigation. One possibility is that aberrant MAP8 accumulation alters microtubule networks or dynamics that may interfere with the movement of motor proteins leading to impaired axonal transport. We tested this possibility in cells by overexpressing MAP8 over a prolonged period. In COS7 cells, overexpression of MAP8 caused alterations of the microtubule network exhibiting a wavy and bundled pattern (Fig. 9A and B) or forming aggregates (Fig. 9C and D). Most interestingly, the accumulation of MAP8, but not the control protein (Supplementary Material, Fig. S4), trapped dynein motor protein into insoluble aggregates, as revealed by immunostaining using anti-DIC (Fig. 9E and F). This finding was further substantiated by immunofluorescent analysis on cultured GAN null neurons, displaying a similar pattern of aggregates positive for DIC and MAP8 antibodies (Fig. 9I and J). We propose that MAP8 protein accumulation causes an aberrant microtubule network that interferes with motor protein function leading to disrupted axonal transport; this explanation could account for the apparent vesicular accumulation in the null neurons (Fig. 4F).

**Retrograde axonal transport is impaired in the gigaxonin null neurons**

We wish to explore the posited link between abnormal distribution and accumulations of vesicles in GAN null axons and impaired axonal transport. The presence of aggregated DIC and Golgi dispersion prompted us to assess retrograde transport in neurons of dorsal root ganglia (DRG) isolated from post-natal day 0 animals of wt and GAN null, and cultured in a microfluidic chamber (35). At DIV 6–15, the DRG neurons were incubated with BtNGF-QD605, a streptavidin-Quantum dot 605 conjugated to biotinylated NGF (36), to label a subfraction of vesicular structures arising from endocytosis. Retrograde transport was subsequently monitored using time-lapse confocal microscopy. The movements of BtNGF-QD605-labeled vesicles within axons were analyzed for both their frequency and the persistence. Indeed, in comparison with that in wt neurons at DIV 6–8, retrograde movement of BtNGF-QD605-associated vesicles was reduced and discontinued in ~30% of the null samples (Fig. 10A) (Supplementary Material, Movie S1; Fig. S5). At DIV 13 and thereafter, the traced vesicles in wt neurons exhibit processive or sustained motion towards cell bodies (Fig. 10C) (Supplementary Material, Movie S3; Fig. S5). In contrast, in ~81% of GAN null neurons, the frequency of the labeled vesicle movements were nearly diminished in neurites (Fig. 10B and S2 movie), revealing severely impaired retrograde axonal transport.

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**Figure 6. Ablation of gigaxonin causes the protein accumulation of MAP8.**

(A and B) About 25 µg of total lysates of brain (Br), spinal cord (SP) and sciatic nerve (SC) from wild-type (WT, lane 1s) or gigaxonin knockout (KO, lane 2s) mice were analyzed using antibodies to MAP8-HC (M8-HC) and actin in (A) or to DIC and MAP8-LC (M8-LC) in (B). The substantial accumulation of MAP8 in the null sample. (C and D) RT–PCRs (C) and real-time PCRs (D) were conducted on mRNAs of WT or KO using multiple primer pairs to MAP8-LC and actin. In contrast to the protein levels, MAP8 mRNA levels are unchanged.

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**Figure 8.** Ablation of gigaxonin causes the protein accumulation and aggregation of MAP8 in cultured gigaxonin null neurons. In contrast to the protein levels, MAP8 accumulation of MAP8 in the null sample. (A and B) About 25 µg of total lysates of brain (Br), spinal cord (SP) and sciatic nerve (SC) from wild-type (WT, lane 1s) or gigaxonin knockout (KO, lane 2s) mice were analyzed using antibodies to MAP8-HC (M8-HC) and actin in (A) or to DIC and MAP8-LC (M8-LC) in (B). The substantial accumulation of MAP8 in the null sample. (C and D) RT–PCRs (C) and real-time PCRs (D) were conducted on mRNAs of WT or KO using multiple primer pairs to MAP8-LC and actin. In contrast to the protein levels, MAP8 mRNA levels are unchanged.
Taken together, our data demonstrate that gene targeting of \textit{GAN} results in toxic accumulation of proteins including MAP8 leading to aberrant cytoskeletal network and defective vesicle transport in the null animals. Impaired retrograde axonal transport preceded the observable signs of degeneration may directly and importantly contribute neurodegeneration and cell death in human GAN.

\textbf{DISCUSSION}

In this report, we describe gigaxonin as a distinct cytoskeleton regulatory protein, important for maintaining a physiological protein level of MAP8 through ubiquitin-mediated degradation. Loss of gigaxonin’s function leads to a substantial increase in MAP8 protein level that has detrimental effects
on neurons, leading to progressive neuronal degeneration. The accumulations of vesicles, mitochondria and other membranous structures in GAN null neurons correlate with a marked impairment of retrograde axonal transport. Our study points to impaired retrograde axonal transport as a contributing factor on pathogenesis of GAN.

GAN is a severe neurodegenerative disorder; this class of diseases is among the most puzzling and devastating illnesses in medicine. The causes of neuronal degeneration and death are still largely unknown. Because of the scarcity of disease tissues and the inability to conduct rigorously controlled investigations with human subjects, the pathogenesis of GAN was difficult to investigate prior to the development of animal models. On the basis of its recessive inheritance and the types of mutations in gigaxonin, it was proposed that the neurodegeneration occurring in GAN patients is the consequence of a loss of gigaxonin function(s). Using conventional gene targeting, we generated GAN null mice which developed progressive motor weakness as well as abnormalities in non-neuronal tissues. Although bearing a striking resemblance to the clinical manifestations of human GAN, the phenotypic features displayed by GAN null mice were less severe. The onset of observed motor deficit in the null mice was much later (3–7 years old in human patients versus 6–10 months in mouse) and the phenotypes progressed more slowly and moderately. Also GAN null mice appear to have a relatively normal life span because most of them survived over the age of 20 months. Cytopathologically, the prominent features found in GAN null mice are similar to those in human GAN patients, including NF accumulation, an abnormal microtubule network, mitochondrial swelling and thinned myelin sheaths. However, the giant axons in peripheral nerves that are characteristic of human GAN could have been hardly seen in GAN null mice. The later disease onset, slower progression, less severe axonal pathology and longer life span in the mouse model are unexplained, but could result from a number of factors including reduced motor stress for peripheral nerves in the rodent, which walks on four relatively short limbs. A portion of the null animals display no observable or very subtle neurological phenotypes suggesting that some genetic modifiers may exist. Nevertheless, our studies provide compelling evidence that the GAN null mice generated

Figure 8. Overexpression of MAP8 provokes neurites degeneration and cell death. (A–C) Cortical neurons from KO (A–B) and WT (C) day 16 embryos were cultured for 15 days. The neurons were stained by anti-MAP8-LC after culture. (D–I) wt cortical neurons were cultured for 48–72 h prior to transfections of GFP (D) or Flag-MAP8 (E–I). The transfected neurons were processed for immunofluorescence microscopy using mouse anti-Flag (E–I) or GFP (D). The Flag-M8 transfected neurons show bundle formation (insets in E), neurite fragmentation (F), intracellular aggregates or practically no neurites (G–I). (J–N) Cortical neurons (DIV3-5) transfected with scrambled siRNA (J–K) or MAP8 siRNA (L–N) were stained with rabbit anti-MAP8-LC and mouse anti-GFP at DIV 14 (J–M) or at DIV 19 (N). The bar in (A) represents 50 μm for (A–C and J–N), 60 μm for (D) and 10 μm for (E–I).
herein are an animal model of human GAN. This model can now be used to observe disease progression, to conduct a comprehensive examination throughout the disease course and to analyze the pertinent biological pathway(s) and the pathology of the disorder well beyond what is available in patients. Of more general interest, this genetic model of neurological diseases in mice may help to elucidate the pathogenesis of other more prevalent neurological disorders associated with cytoskeletal aberration and UPS deficits.

In addition to MAP1B-LC, gigaxonin can directly bind to MAP8, a new member of microtubule-associated protein family (32,33). It is noteworthy that an efficient binding of gigaxonin to MAP8 requires functional kelch-repeat domain. At least six out of 24 distinct mutations identified in human GAN patients (16–18) result in a complete loss of the kelch repeats, which may consequently lead to disruption of the gigaxonin–MAP8 interaction. Our results on the three tested mutations, 18insA, R242X and R293X, provided direct evidence for a disruption. The interaction between gigaxonin and MAP8 is apparently required for the degradation of MAP8 protein. The overexpression of gigaxonin in transfected cells significantly increased UPS-mediated MAP1B degradation, suggesting an involvement of gigaxonin’s function in the UPS pathway (31,34). Over the years, the ubiquitin system of intracellular protein degradation has been implicated in the control of many fundamental cellular processes. Defects in this system have been directly linked to the development of human diseases, including cancer and neurodegenerative diseases (37–39). Our results provide additional evidence linking a defect in UPS-mediated protein degradation with a human neurodegenerative disorder.

MAP8 joins a growing family of classical microtubule-associated proteins. It has a generalized pattern of tissue distributions and contains direct binding sites to microtubules and actin microfilaments. Emerging evidence indicates that MAP expression levels are under tight control in response to morphological and functional requirements of neuron (40,41). Regardless of the details of how gigaxonin overexpression accelerates protein degradation in transfected cells, the significant protein accumulation of MAP8 in GAN null animals verifies that gigaxonin is a critical player in controlling the protein levels of MAP8 in addition to MAP1B and TBCB (31,34). Significant protein reduction of MAP8 through RNAi decreased but did not entirely eliminate death of GAN null neurons in culture. This suggests that accumulation of MAP8 is sufficient to cause neurodegeneration, but does not serve as the only factor. Thus, gigaxonin may be involved in degradation of proteins and/or in other important pathways. The mechanisms underlying cell death mediated by MAP8 accumulation must be elucidated. Several possibilities have been proposed. One possibility is that MAP8 accumulation may interfere with the movement of motor proteins by directly competing for or blocking adjacent binding sites on the microtubule surface. Several studies suggest this possibility, as MAP2 can inhibit the motility of dynein or kinesin in vitro (42,43). Another possibility is that aberrant elevation of MAP levels affects transport processes indirectly by altering microtubule dynamics. Our data suggest that accumulated MAP8 protein alters microtubule network and traps the dynein motor protein into aggregates. The market accumulations of membrane structures in the GAN null neurons and the results of our transport assays point to impaired retrograde axonal transport as a potential factor causing neurodegeneration. Our study speaks to the pathogenesis of human GAN. It is now possible to envision that gigaxonin disruption, either directly or indirectly, represents a central pathological step in several neurodegenerative diseases that feature cytoskeletal abnormalities and aberrant dysfunction of axonal transport. Whether the featured demyelination of axons in GAN is a primary defect directly involving gigaxonin function or is a pathogenic consequence requires to be elucidated. Whether the densely packed NFs are a compensatory response
to the altered microtubule network or whether gigaxonin primarily involves in IF network through a yet unknown factor awaits further investigations.

MATERIALS AND METHODS

Generation of antibodies

Gigaxonin antibodies were generated and purified as described previously (30). A purified GST fusion protein encompassing N-terminal portion of the light chain or a synthetic peptide located within the heavy chain was used for raising anti-M8-C or anti-M8-N antibodies in rabbits, respectively. The antisera were affinity purified and tested against purified recombinant protein or total cell lysates from Flag-M8-HC or Flag-M8-LC transfected cells prior to use.

Generation of the GAN knockout mice

A 16 kb mouse genomic DNA fragment was cloned from the mouse 129Sv/Ev lambda genomic library. The targeting vector was constructed as depicted in Figure 1A. We made our 3'-arm relatively short to make PCR screening and mouse genotyping easier, whereas the long 5'-arm was to enhance homologous recombination. A 1.5 kb PCR DNA fragment amplified with primer pair of GANYA1 and GANYA2 was used as the 3'-short arm. Primer GANYA1 is located 1.65 kb downstream of exon 5, whereas primer GANYA2 is located 150 bp downstream of exon 5. The short arm was inserted into the 5' of the Neo gene cassette using the MluI site. The long arm was a 7.3 kb SpeI genomic fragment. In this strategy, exons 3–5 containing ~5 kb genomic region were replaced by the 1.8 kb Neo gene cassette. For the screening strategy, as depicted in Figure 1A, the primer pair of Neo1 and mGigDiag31 was designed for assuring 3' arm homologous integration and the pair of Neo 2 and mGig-Diag52 for confirming 5' arm integration. Of a total of ~300 ES clones screened with sequential PCRs for both arms, three targeted ES clones were scored positive for desired homologous recombination, which were confirmed by Southern blot analysis. Sufficient numbers of high-contribution chimerics were generated from these three independent ES clones which successfully contributed to the germline transmission. Heterozygous offspring derived from all three independent ES clones were mated to produce homozygous mutants, and their offspring were genotyped to determine whether they carry the gigaxonin null mutants (Fig. 1B). To confirm unequivocally that the expected targeting event had taken place, we analyzed gigaxonin’s expression at both mRNA and protein levels, confirming that the gene targeting successfully resulted in ablation of gigaxonin’s expression in mouse (Fig. 1C and D).

Electron microscopy

WT and gigaxonin null animals were sacrificed by intravenous perfusion with 2% paraformaldehyde and 0.05% glutaraldehyde. The dissected brain and sciatic nerves samples were post-embedded as described previously (30). The sections were analyzed under a Philips CM10 microscope.

Cortical neuron culture

Cortical neuron cultures were prepared from mouse embryos at days 15–18 (E15–E18). The procedures were similar to those described in Current Protocol of Neurobiology. Briefly, the mouse embryo brains were removed and enzymatically dissociated with 0.25% trypsin (Sigma) in dissociation buffer (15 mM HEPES, 1% sodium bicarbonate in 1 x Hanks buffer). The dissociated cells were then plated on 15 mm diameter cover slips coated with poly-L-lysine (50 μg/ml) at a density of 100–160 cells/mm². The cortical neurons were then grown in neurobasal medium containing B27, 0.5 mM L-glutamine and 1% Penicillin–Streptomycin supplements suggested in Gibco protocol (Gibco). The cultures could be maintained up to 4 weeks.
For transfections, the cortical neurons were seeded at a density of 1–1.5 × 10^3 cells/well in a 12-well plate. The expression vectors of Flag-MAP8 or pEGFP-C2 were transfected at culture days 3–5 using OptiMEM® and LIPOFECTAMINE 2000™ according to manufacturer’s instructions (Invitrogen, Rockville, MD, USA). The cultures were terminated for immunofluorescent microscopy analyses at various time points for assays. For siRNA experiments, the pZ-OFF EGFP vector was the generous gift from Dr Craig Garner’s laboratory. The following oligos for the siRNA constructs (Genscript, USA) were cloned into the pZ-OFF EGFP vector using BglII and HII sites. MAP8 siRNA: CTGCAACTGTAGCTGCCAAGA; scrambled siRNA: CGGCTTGACCACATCATGAGCTA.

**Real-time PCR**

Real-time PCR was performed using Mx3000P QPCR system (Stratagene) and SYBR Green Master Mix (Stratagene 600548). The MAP8 primer sequences were as follows. Forward: 5’-CAGCCGCCAGTGGGCTG-3’ and reverse: 5’-GGTAAGCCAGGC CAGTTATAC-3’. A total of 100 ng of mouse cDNA were mixed according to manufacturer’s instructions. After initial incubation at 95°C for 10 min, the samples were amplified for 40 cycles at 95°C for 30s, 58°C for 1 min and 72°C for 30s. Data were analyzed using the comparative quantification method by the 2^(-ΔΔCt) equation, ΔΔCt = ΔCt (gigaxonin-null) − ΔCt (wild-type), where ΔCt is the threshold cycle (Ct) value of the housekeeping gene (GADPH) subtracted from the Ct value of the target gene (MAP8).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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