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Autonomic Nervous System Involvement in the Giant Axonal Neuropathy (GAN) KO Mouse: Implications for Human Disease

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Abstract

PURPOSE—Giant axonal neuropathy (GAN) is an inherited severe sensorimotor neuropathy. The aim of this research was to investigate the neuropathologic features and clinical autonomic nervous system (ANS) phenotype in two GAN knockout (KO) mouse models. Little is known about ANS involvement in GAN in humans, but autonomic signs and symptoms are commonly reported in early childhood.

METHODS—Routine histology and immunohistochemistry was performed on GAN KO mouse specimens taken at various ages. Enteric dysfunction was assessed by quantifying the frequency, weight, and water content of defecation in GAN KO mice.

RESULTS—Histological examination of the enteric, parasympathetic and sympathetic ANS of GAN KO mice revealed pronounced and widespread neuronal perikaryal intermediate filament inclusions. These neuronal inclusions served as an easily identifiable, early marker of GAN in young GAN KO mice. Functional studies identified an age-dependent alteration in fecal weight and defecation frequency in GAN KO mice.

CONCLUSIONS—For the first time in the GAN KO mouse model, we described the early, pronounced and widespread neuropathologic features involving the ANS. In addition, we provided evidence for a clinical autonomic phenotype in GAN KO mice, reflected in abnormal gastrointestinal function. These findings in GAN KO mice suggest that consideration should be

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

given to ANS involvement in human GAN, especially when considering treatments and patient care.

Keywords

Giant axonal neuropathy; Intermediate filaments; Gigaxonin; Autonomic nervous system; Neurodegenerative disease

INTRODUCTION

Giant axonal neuropathy (GAN, OMIM# 256850) is a rare, hereditary, severe motor and sensory axonal neuropathy with central nervous system (CNS) involvement [1]. Onset of symptoms is typically at 3–4 years of age and commonly heralded by clumsiness of gait. During the second decade of life, patients typically become wheelchair-dependent and progressively lose coordination and strength in their upper extremities. Death nearly always occurs by the second or third decade. The pathologic signature of GAN is giant axonal swellings, which are focally distributed and filled with densely packed accumulations of structurally normal neurofilaments (NFs).

From a clinical standpoint, GAN is characterized by a distally predominant peripheral neuropathy manifested by hypotonia, muscle atrophy, tendon contractures, and areflexia [1]. CNS features include prominent cerebellar dysfunction, reflected in nystagmus, dysarthria, dysmetria and ataxia. Detailed neuropathologic analyses of GAN have been rendered only in a limited number of postmortem studies.

GAN is caused by autosomal recessive loss-of-function mutations in the *GAN* gene that encodes the protein gigaxonin [1]. Gigaxonin plays a pivotal role in cytoskeletal organization and degradation of intermediate filaments (IFs). Loss of gigaxonin leads to dysregulation and accumulation of IFs such as NFs (NF-H, NF-M, NF-L), peripherin, alpha-internexin, desmin, keratin and vimentin [2, 3]. Three mouse models have been developed for GAN by knocking out part of the endogenous *GAN* gene [4, 5, 6]. All three models mirror the IF dysregulation and peripheral nerve pathology seen in human GAN but exhibit a much milder phenotype.

During the course of a comprehensive pathologic characterization of the GAN KO mouse, we encountered the very early involvement of the autonomic nervous system (ANS). Here, described for the first time, we document the pronounced and widespread involvement of the enteric, parasympathetic and sympathetic ANS in the GAN KO mouse. Implications of this autonomic neuropathy for human GAN are discussed.

MATERIALS AND METHODS

Animals

GAN KO breeders with a deletion of GAN exons 3-5 (GAN/Y) were obtained from Y. Yang, and GAN KO breeders with a deletion of GAN exon 1 (GAN/J) were obtained from J.P. Julien; both lines were maintained at the University of North Carolina at Chapel Hill (UNC–CH). GAN KO mice were generated by breeding a homozygous KO male with a

heterozygous female. Consistent with the previous reports [4,6], heterozygous GAN mice are phenotypically normal and were used as controls. Mixed sex and age-matched littermates from both GAN KO models were used in these studies. Mice in the 4- and 12-month-old cohorts for pathological studies were used as controls in other studies in the lab and had received vehicle injections 2-4 weeks prior to harvest. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23] and approved by the UNC–CH Institutional Animal Care and Use Committee. All mice were kept on a 12 h light-dark cycle with access to food and water *ad libitum*.

One-hour stool collection

To assess gastrointestinal function, fecal pellet output and water content were assessed as previously described [7] in male GAN KO and heterozygote littermates at 3.5 (GAN/Y), 12 (GAN/Y) and 22 (GAN/J) months of age. Each mouse was individually placed in a novel clean cage for a 60-min collection period. Immediately after expulsion, fecal pellets were collected and placed into pre-weighed 1.5 mL microcentrifuge tubes that were sealed to avoid evaporation. Tubes were weighed to obtain the wet weight of the stool and the stool was then dried overnight at 65°C and reweighed to obtain the dry weight. The fecal water content was calculated by taking the difference of the wet and dry weight expressed over the wet stool weight. All mice were tested at the same time each day (2-4 P.M.).

Tissue preparation

Mice were anesthetized at 4, 12 or 24 months of age with an overdose of avertin (0.04 mL/g of a 1.25% solution). For the 4-month-old cohort, tissues were immediately removed and drop-fixed in 10% neutralized buffered formalin (NBF). For the 12- and 24-month-old cohorts, mice were first perfused with phosphate-buffered saline containing 1 μg/mL heparin and then perfused with either 4% paraformaldehyde or 10 % NBF. Tissues were then removed and immediately fixed in 10% NBF at room-temperature for at least one week. Following fixation, whole spines were de-calcified overnight in Formical-4 and divided into cervical, thoracic and lumbar regions. Tissues were paraffin embedded and sectioned at 5 μm thickness for histological analysis.

Histological analyses

Hematoxylin and eosin (H&E) staining was performed on tissue sections, and light microscopic evaluation for pathological abnormalities was performed with the examiner blind to genotype. Standard immunohistochemical procedures were used to immunostain serial sections of tissues with antibodies for IF proteins and to counterstain with hematoxylin. Analyses of IF aggregates in paravertebral sympathetic-chain ganglia were performed using rabbit anti-peripherin (Abcam: ab4666; 1:2500), rabbit anti-vimentin (Abcam, Ab92547; 1:250), rabbit anti-alpha-internexin (Abcam, Ab40758; 1:250) and mouse anti-NF-heavy chain, hypophosphorylated (Covance, SMI-32R; 1:5000). Rabbit anti-peripherin (Abcam: Ab4666; 1:1000) was used to stain IF aggregates in the enteric nervous system. Images were captured at 60x and 100x with oil immersion using the Olympus BX61 microscope (Olympus America), the QImaging Retiga 4000R camera (Surrey) and Volocity 6.2.1 software (Perkin Elmer, Inc.). Figures were created using Adobe Photoshop CS4.

Statistical Analyses

Data are presented as the mean ± standard error of the mean. Data from the 1 h stool collection study were analyzed by two-way ANOVA with genotype and age as independent variables followed by post hoc analysis with Bonferroni's multiple comparison test using GraphPad Pris m version 6.04 software (GraphPad Software). $P < 0.05$ was considered to be statistically significant.

RESULTS

Enteric nervous system

In 4-month-old GAN KO mice, light microscopic examination of H&E-stained sections revealed eosinophilic inclusions within neuronal cell bodies in the myenteric (Auerbach's) plexi of the esophagus, stomach and the small and large intestines and the submucosal (Meissner's) plexi of the s mall and large intestines (Fig. 1a). Histologically identical neuronal perikaryal inclusions were found in the myenteric and submucosal plexi of 12- and 24-month-old GAN KO mice (Fig. 1b). The neuronal inclusions in the enteric ANS showed strong positivity for peripherin (Fig. 1c). Similar pathological features were present in both GAN/J and GAN/Y mice. Age-matched control mice had no neuronal inclusions in enteric ganglia.

Parasympathetic nervous system

In 4-month-old GAN KO mice, light microscopic examination of H&E-stained sections of systemic organs revealed numerous neuronal perikaryal inclusions within parasympathetic ganglia of the salivary glands (Fig. 1d), larynx, trachea, heart, bladder, kidney and gastrointestinal tract. In 24-month-old GAN KO mice, light microscopic examination of H&E-stained sections of systemic organs showed abundant neuronal perikaryal inclusions within parasympathetic ganglia of salivary glands, trachea, heart, bladder and gastrointestinal tract. The neuronal inclusions in the 4- and 24-month-old KO mice were histologically indistinguishable. Similar pathological features were present in both GAN/J and GAN/Y mice. Control mice had no neuronal inclusions in the parasympathetic nervous system.

Sympathetic nervous system

In 24-month-old GAN KO mice, light microscopic examination of H&E-stained sections of cervical, thoracic and lumbar paravertebral sympathetic ganglia and prevertebral sympathetic ganglia (celiac, superior/inferior mesenteric ganglia) revealed abundant, round to oval, brightly eosinophilic neuronal perikaryal inclusions (Fig 2a). Similar pathological features were present in GAN/J and GAN/Y mice. Control mice had no neuronal inclusions in sympathetic ganglia. Immunohistochemistry (IHC) for IF proteins showed diffuse, strong peripherin positivity within the neuronal inclusions and focal positivity within neuronal processes (Fig 2b). Controls showed only focal positivity within neuronal processes. IHC for vimentin and alpha internexin revealed immunoreactivity within the neuronal perikaryal inclusions and diffuse background staining of neuronal perikaryal cytoplasm and processes. Controls showed diffuse background staining of neuronal perikaryal cytoplasm and

processes. IHC for SMI-32 showed variable immunoreactivity of neuronal perikaryal cytoplasmic inclusions, ranging from negative to strongly positive, and focal immunoreactivity within neuronal perikaryal cytoplasm. Controls showed only focal immunoreactivity within neuronal perikaryal cytoplasm.

Central nervous system

In the 4-month-old GAN KO mice, light microscopic examination of H&E stained sections showed only occasional neuronal perikaryal inclusions in the cerebral cortex (Supplementary material Fig 1). There were no neuronal inclusions or axonal swellings in the spinal trigeminal nucleus of the medulla. Sections of cervical, thoracic and lumbosacral spinal cord showed no neuronal inclusions or axonal swellings. Preganglionic autonomic neurons within the intermediolateral (IML) columns of thoracic cord had no perikaryal inclusions. Age-matched control mice had no neuronal inclusions in the CNS.

In the 24-month-old GAN KO mice, light microscopic examination of H&E-stained sections revealed numerous neuronal perikaryal inclusions throughout the cerebral cortex. There were scattered neuronal inclusions and numerous axonal swellings in the spinal trigeminal nucleus of the medulla. Sections of cervical, thoracic and lumbosacral spinal cord showed numerous axonal swellings within the dorsal horns and the dorsal columns. Preganglionic autonomic neurons within the IML columns of thoracic cord had no perikaryal inclusion (Fig. 2c). Age-matched control mice had neither inclusions nor axonal swellings.

Gastrointestinal and urinary systems

Macroscopic examination of freshly dissected GAN KO and control mice at 24 months of age showed that GAN KO mice commonly had smaller intestinal tracts, including caecum, containing smaller fecal pellets in the large intestines when compared to intestinal tracts and fecal pellets in control mice (Supplementary Material Fig. 2a). At necropsy, the urinary bladders of GAN KO mice were frequently “full” when compared to control mice whose bladders were frequently “empty” (Supplementary Material Fig. 2b). After bladders were removed and manually emptied, GAN KO bladders were still noticeably enlarged compared to control mice (Supplementary Material Fig. 2c). Similar gross pathological features were present in both GAN/J and GAN/Y mice.

Based on macroscopic and microscopic abnormalities, gastrointestinal function was examined in 3.5-, 12-, and 22-month-old male GAN KO mice and controls by measuring stool expulsion, weight and water content over a 1 h period. Analysis of the total fecal pellets expelled showed that age significantly increased propulsive motility [$F(2, 41) = 3.44$, $p < 0.05$]. Additionally, GAN KO mice had increased fecal pellet expulsion compared to controls [$F(1, 41) = 11.77$, $p < 0.01$] with no difference in 3.5-month-old mice whereas significant differences were found in 12- ($p < 0.05$) and 22-month-old mice ($p < 0.05$) (Fig. 3a). While the total dry weight of all fecal pellets over the 1 h period was similar between controls and GAN KO mice at all ages (data not shown), the average dry weight of the individual expelled fecal pellets were significantly less in GAN KO mice as compared to controls [$F(1, 41) = 7.13$, $p < 0.05$] and with increasing age [$F(2, 41) = 13.78$, $p < 0.0001$]

(Fig. 3b). The stool water content was unchanged between GAN KO and control littermates and for all age groups examined (Fig. 3c).

DISCUSSION

Although the behavioral phenotype of the GAN KO mouse model is mild and stands in contrast to the pronounced clinical findings in human GAN, the pathological phenotype of the GAN KO mouse model is strong and shares similar morphological features with the human disease. Most notably, the signature ultrastructural feature of human GAN, giant axonal swellings packed with accumulations of IFs [1], is very prominent in the GAN KO mouse. The GAN KO mouse also shows the involvement of both peripheral nerves and the CNS. Here, described for the first time in the GAN KO mouse, we document the presence of neuronal perikaryal inclusions in neurons of the enteric ANS and in postganglionic neurons of the sympathetic and parasympathetic ANS. These neuronal inclusions were much more frequent in neurons of the ANS than in neurons of the CNS in the 4-month-old KO mice and served as an easily identifiable early marker of GAN in the young KO mice. GAN/Y KO mice were initially reported to have a severe phenotype with the onset of motor function deficits as early as 6 months of age [4], but others have been unable to detect a motor phenotype in GAN KO mice up to 14-months-of-age [5, 6]. Here we show for the first time that abnormalities in gastrointestinal function can be detected in two different lines of GAN KO mice and at an earlier age than the onset of a motor phenotype. These findings suggest that the involvement of the ANS comes early in the overall disease progression of GAN.

ANS involvement in human GAN has been left largely uninvestigated. There is one published case report of a young child with GAN in whom ultrastructural examination of a rectal biopsy revealed abnormal neurons with perikaryal IFs “arranged into interwoven bundles” in the myenteric plexus [8]. Gastrointestinal symptoms, including lactose intolerance, constipation, obstipation, and reflux and regurgitation, are prominent and present in early childhood in GAN patients [1]. Additional signs and symptoms pointing to disturbances in autonomic regulation, such as reduction or loss of sweating ability, blood pressure instability with orthostatic hypotension, and heat intolerance are reported by GAN patient advocacy groups and professional organizations (http://www.hannahshopefund.org). These signs and symptoms of autonomic dysfunction appear early, supporting our findings in the GAN KO mice that ANS involvement is an early feature of GAN.

Preliminary assessment of gastrointestinal function in GAN KO mice showed increased stool expulsion as compared to age-matched controls. ANS dysfunction reflected in increased stool frequency compared to control mice has been reported in several mouse models of Parkinson's disease [9-11]. GAN KO mice had smaller fecal pellets as compared to controls, whereas the fecal water content was unchanged, suggesting that food intake and/or motility may be affected in GAN KO mice while water absorption in the gut is normal. Further tests examining food consumption, gastric emptying and gut motility are warranted to characterize the full spectrum of enteric dysfunction in GAN KO mice.

The identification of early, widespread ANS involvement in GAN KO mice should be considered as treatment strategies are developed for human GAN. Studies in GAN KO mice

have demonstrated that gigaxonin gene transfer can reverse the intracellular IF aggregates present in GAN [12], leading to gene transfer being the first proposed therapy for GAN. Validation of therapeutic efficacy and viral vector delivery systems with GAN KO models [4, 6] has provided the springboard for the development of the intrathecal (IT) scAAV9/JeT-GAN viral vector employed in the Phase I gene therapy clinical trial for the treatment of children with GAN, recently announced by NIH/NINDS (https://clinicaltrials.gov/ct2/show/NCT02362438). This gene transfer approach was not specifically designed to address the IF accumulations in the ANS. If this ANS pathology is present and clinically significant in humans, a future comprehensive treatment for GAN that includes the ANS may require an altered gene transfer approach or a combinatorial treatment strategy.

In conclusion, our systematic survey of neuropathologic alterations in the GAN KO mouse revealed neuronal intracytoplasmic abnormalities that were pronounced and pervasive throughout the ANS. An autonomic phenotype in GAN KO mice was demonstrated by abnormalities of gastrointestinal function. Such observations extend the pathologic characterization of the GAN KO mouse model and may provide an explanation for symptoms suggestive of ANS dysfunction in GAN patients. Further investigation of ANS dysfunction in human GAN may be warranted to (1) augment standardized, clinically meaningful measurements for the rate of unmitigated disease progression in natural history studies [13], (2) promote continued development of alternative outcome measures for therapeutic effect in clinical trials, and (3) expand quality of life (QoL) metrics for GAN patients and their families [14].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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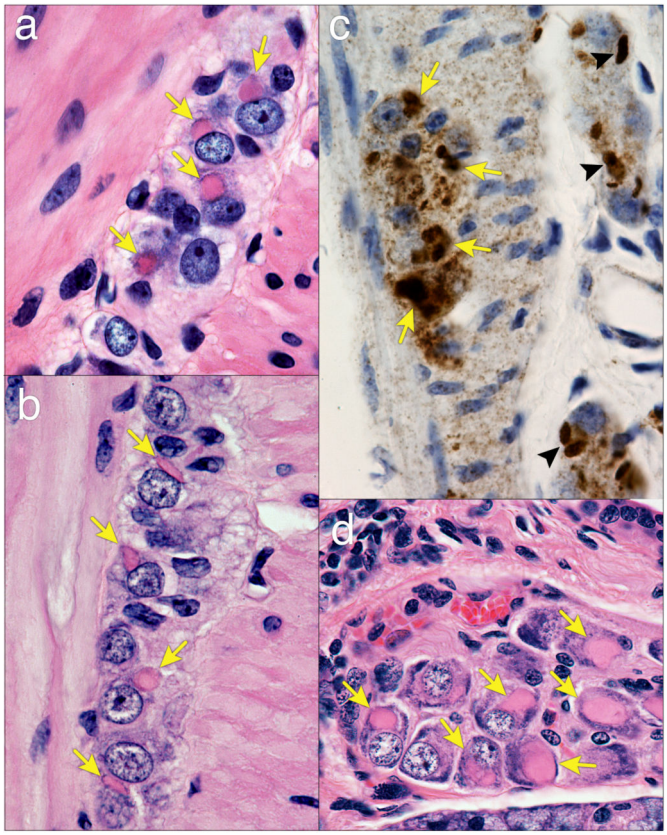

Fig. 1.

Neuronal inclusions in the enteric and parasympathetic ANS of GAN KO mice. **a** GAN KO (4-month-old) enteric ganglia myenteric plexus of large intestine reveals eosinophilic neuronal perikaryal inclusions (arrows) (H&E; original magnification x100). **b** GAN KO (24-month-old) enteric ganglia myenteric plexus of large intestine shows eosinophilic inclusions within neuronal cell bodies (arrows) (H&E; original magnification x100). **c** GAN KO (12-month-old) enteric ganglia neuronal intracytoplasmic inclusions within the submucosal plexus (arrowheads) and myenteric plexus (arrows) of the small intestine (duodenum) show strong peripherin IF protein immunoreactivity (arrows) (peripherin IHC; original magnification x100). **d** GAN KO (4-month-old) parasympathetic ganglia in salivary gland reveals abundant eosinophilic neuronal perikaryal inclusions (arrows) (H&E; original magnification x60)

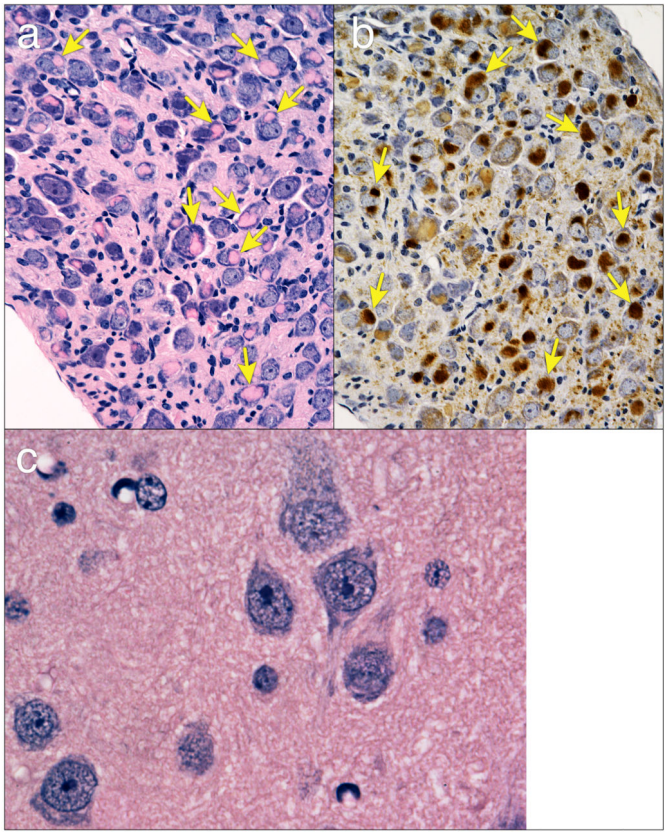

Fig.2.

Sympathetic neurons in the 24-month-old GAN KO mice. **a.** GAN KO postganglionic neurons within thoracic sympathetic paravertebral ganglia show abundant, eosinophilic neuronal perikaryal inclusions (arrows) (H&E; original magnification x40). **b.** GAN KO neuronal cytoplasmic inclusions within postganglionic neurons of thoracic sympathetic paravertebral ganglia show strong peripherin IF protein immunoreactivity (arrows) (Peripherin IHC; original magnification x40). **c.** GAN KO preganglionic sympathetic neurons within the intermediolateral (IML) columns of thoracic spinal cord show no inclusions (H&E; original magnification x100)

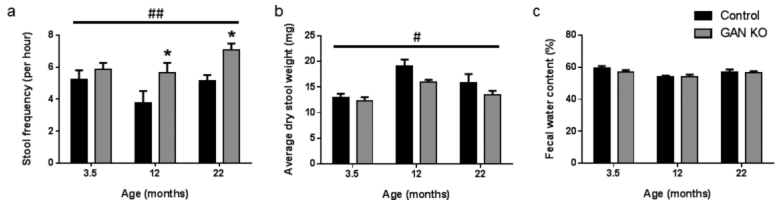

Fig.3.

Gastrointestinal function in GAN KO mice. Animals were placed individually in new cages and fecal pellets were collected over a 1-hour period. **a.** GAN KO mice have increased stool frequency compared with age-matched control mice at 12 and 22 months of age. **b.** Overall, GAN KO mice have a decreased average fecal pellet weight compared to control mice. **c.** The amount of fecal water is unchanged between GAN KO and control mice. Data were analyzed via two-way ANOVA (genotype × age) with Bonferroni's post-hoc analysis. #$p < 0.05$, ##$p < 0.01$ (main effect of genotype indicated) and *$p < 0.05$ (post-hoc analysis). Results represent the average measurements for two trials ± SEM for 7-9 animals per cohort