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Genome-Wide Screen Identifies Drug-Induced Regulation of the Gene Giant Axonal Neuropathy (*Gan*) in a Mouse Model of Antiretroviral-Induced Painful Peripheral Neuropathy

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Abstract

Painful peripheral neuropathy is a debilitating complication of the treatment of HIV with nucleoside reverse transcriptase inhibitors (NRTIs). Patients are living longer with these drugs; however many develop excruciating, unremitting, and often treatment-limiting neuropathy that is resistant to conventional pain management therapies. Improving patient comfort and quality of life is paramount and depends on a clearer understanding of this devastating side effect. The mechanisms underlying the development of NRTI-induced neuropathy, however, remain unclear. Using a mouse model of NRTI-induced neuropathy, the authors conducted an unbiased whole-genome microarray screen to identify molecular targets in the spinal dorsal horn, which is the location where integration of ascending sensory transmission and descending modulatory effects occur. Analysis of the microarray data identified a change in the gene giant axonal neuropathy 1 (*Gan1*). Mutation of this gene has been linked to the development of giant axonal neuropathy (GAN), a rare autosomal recessive condition characterized by a progressive sensorimotor neuropathy. *Gan1* has not been previously linked to nerve pathologies in other populations. In this study, downregulation of the *Gan1* gene and the gene protein product, gigaxonin, was validated via quantitative polymerase chain reaction ([qPCR] gene expression) and Western blot analyses (protein level). Our report is the first to suggest that *Gan1* might be a novel molecular target in the development of NRTI-induced peripheral neuropathy with implications for new therapeutic approaches to preventing or reducing a significant side effect of HIV treatment.

Keywords

microarray; painful peripheral neuropathy; chronic pain; gigaxonin; giant axonal neuropathy; HIV/AIDS; HAART; NRTI

Nearly 40 million children and adults worldwide are living with HIV/AIDS (UNAIDS, 2007). In the United States, it is estimated that over 1 million persons are currently infected with HIV and an average of 50,000–60,000 new cases are diagnosed each year (Centers for Disease Control [CDC], 2008; Hall et al., 2008). The highly active antiretroviral therapy (HAART) used to treat HIV is often initiated early in the infection before patients develop symptoms of AIDS and has been credited with extending the lives of HIV patients. HAART

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involves the combined use of multiple anti-HIV drugs, usually including a protease inhibitor and one or more of the nucleoside reverse transcriptase inhibitors ([NRTIs] Simpson, 2002; White, 2001). Each of these drugs has a different mechanism of action and they work synergistically to interrupt the life cycle of the HIV.

HAART is extremely effective in reducing viral load and maintaining and/or improving immune function. However, HAART is also associated with significant side effects including diarrhea, pancreatitis, liver failure, and peripheral neuropathic pain. The symptom burden of these side effects leads one-in-four HIV-infected patients to limit or stop treatment to improve their comfort and quality of life (d'Arminio Monforte et al., 2000). Thus, a clear understanding of the mechanisms underlying the development and persistence of the HAART side effects is of utmost importance.

Painful peripheral neuropathy is one of the most prevalent side effects of HIV treatment and is a complication of HIV infection, itself, occurring in approximately 35% of patients (Moore, Wong, Keruly, & McArthur, 2000; Schifitto et al., 2002; So, Holtzman, Abrams, & Olney, 1988). The drug-induced painful peripheral neuropathy resulting from HAART is most closely associated with the dideoxynucleoside (d-drug) family of NRTIs, including stavudine, lamivudine, and zalcitabine (Dalakas, 2001; Moore et al., 2000; White, 2001). The mechanism of action for the d-drugs is to block the function of reverse transcriptase, an enzyme that is necessary for the HIV virus to build new DNA from RNA. However, the d-drugs also strongly inhibit g-polymerase, resulting in a dose- and time-dependent decrease in levels of intracellular mitochondrial DNA, especially in the liver, skeletal muscle, and peripheral nerves (Walker, 2003; for review see Dalakas, 2001 and Simpson, 2002). The mitochondrial toxicity occurring in the long sensory nerves of the lower extremities destabilizes peripheral nerve health, leading to the dieback of distal small sensory fibers and epidermal denervation and possibly contributing to the development of neuropathic pain (McCarthy et al., 1995; Polydefkis et al., 2002; for review see Dorsey & Morton, 2006).

Patients with NRTI-induced painful peripheral neuropathy present with increasingly debilitating and intractable pain that begins bilaterally in the feet. The symptoms include painful responses to normally innocuous stimuli such as the touch of socks and bed sheets (allodynia), exaggerated painful symptoms to noxious stimuli (hyperalgesia) and significant spontaneous pain (Brinley, Pardo, & Verma, 2001; Cornblath & McArthur, 1988; Wulff, Wang, & Simpson, 2000). Further, these patients frequently alter their gait to avoid the pain that results from placing pressure on the soles of the feet, limiting their mobility and increasing their risk for falls (Verma, 2001). While painful symptoms associated with peripheral neuropathies of all etiologies are difficult to treat, NRTI-induced neuropathic pain is particularly difficult to manage, as few drugs used to manage neuropathic pain, alone or in combination, are effective in relieving HIV patients' pain (Simpson, 2002). Although withdrawal from HAART can significantly improve neuropathic symptoms within 16 months, in some cases withdrawal can also exacerbate painful symptoms and not all patients will recover from this disorder after the drugs are stopped (Berger et al., 1993). Moreover, cessation of therapy is not usually a viable option because drug treatment is necessary to maintain virologic control and a functional immune system (Moyle & Sadler, 1998; Simpson, 2002; Verma, 2001). Thus, there is a great need for new interventions and discovery-based research to identify novel therapeutic opportunities that can improve pain management and quality of life for patients with NRTI-associated peripheral neuropathic pain.

Several research groups have examined mechanisms underlying antiretroviral-induced neuropathic pain in rodent models. These studies have linked persistent allodynia to microgliosis (Wallace et al., 2007), sensory C-fiber axonal "dieback" (Wallace et al., 2007),

altered intracellular calcium buffering secondary to drug-induced mitochondrial damage in dorsal root ganglion neurons (Joseph, Chen, Khasar, & Levine, 2004), enhanced neurotransmitter release and increased chemokine signaling in the spinal dorsal horn (Bhangoo et al., 2007; Joseph et al., 2004). These findings are based on tissue sampling and analysis that occurred relatively late in the course of symptoms, suggesting that the drug effects linger or representing evolved nerve pathology. They do little, however, to illuminate the mechanisms underlying the development of allodynia.

To explore the developmental mechanisms of this disorder in more detail, we generated a mouse model of NRTI-induced painful peripheral neuropathy by giving a single weight-based intravenous injection of stavudine that produced a robust tactile allodynia within 24 hr of drug treatment. Using this mouse model, we examined molecular changes that occurred in the dorsal horn of the spinal cord, which is the principal region where integration of ascending pain transmission and descending pain modulatory effects occur. We chose to exploit the power of an unbiased, whole-genome approach to examine these changes. In contrast to a more traditional candidate approach where prior research and theory drives hypothesis testing of known molecules of interest, use of whole-genome microarray technology allows exploration of gene changes at specific time points following drug treatment and increases the likelihood of discovering novel molecular targets involved in either known or novel pain pathways.

Material and Method

Animals

We conducted all experiments using adult male C57BL/6J mice (20–30 g; Jackson Laboratories, Bar Harbor, ME). All mice were on a 12:12 hr light:dark cycle with food and water available ad libitum. We followed the International Association for the Study of Pain (IASP) guidelines for investigations of pain in animals (Zimmerman, 1983). The Institutional Animal Care and Use Committee of the University of Maryland School of Medicine approved these experiments. In accordance with these guidelines, we used the minimum numbers of animals to meet the rigor necessary for this series of experiments (see Table 1).

Animal Model Generation

Age-matched mice received a single 50 mg/kg intravenous (IV) dose of 2', 3'-dideohydro-3'-deoxythymidine (Sigma Aldrich, St. Louis, MO; brand name: Zerit; generic name: stavudine) into the tail vein. Control mice received a weight-based dose-equivalent volume of physiological saline vehicle via tail vein. We selected stavudine as the agent as this is a widely prescribed NRTI in clinical use and is associated with significant neuropathic pain. Although patients are generally administered stavudine orally, previous studies have shown that both oral and intravenous administration routes produce similar nocifensive behavioral profiles in rodents (Joseph et al., 2004). Thus, we chose to use the intravenous route to minimize the handling stress to the animals associated with daily oral gavage.

For the injection, the mice were placed in a Broome Style Rodent Restrainer (Plas Labs, Lansing, MI) with the tail extending from the end. The tail was vasodilated by immersion in a warm water bath (40–42°C) for 15–30 s prior to injection. A 100- μ l Hamilton syringe with a 1/2 inch 30g needle was used for the injection. The lateral tail vein was located and the tail was immobilized between the thumb and forefinger. The needle was inserted, bevel up, at a 10° angle in the rostral direction. We injected the solution slowly while watching closely for the vein to blanch and to ensure that there was no detectable swelling of the tail near the injection site. Following needle removal, we applied pressure to the injection site for 15–30

s to stop bleeding and avoid hematoma formation. Total weight-based injectate volumes for drug- and vehicle-treated animals ranged from 40 to 60 μ l.

Nocifensive Behavioral Testing

The nocifensive behavior of paw withdrawal from a mechanical stimulus was used to assess the development of tactile allodynia. A series of von Frey filaments (Touch Test Sensory Evaluator Kit, myNeurolab.com, St. Louis, MO), with bending forces that ranged from 0.04g to 1.40g, was used to deliver the tactile stimuli. Naïve mice were tested before drug administration to determine their tactile threshold, defined as the fiber with the smallest bending force that elicited three aversive responses (paw withdrawal) out of five trials. Tactile allodynia was determined to be present if the response threshold shifted to the left, such that a previously nonnoxious fiber with a bending force less than the naïve threshold fiber elicited three aversive responses out of five trials. Two groups of mice (drug group $n = 6$ and vehicle group $n = 6$) were tested pre-drug (naïve) and then 1, 7, 14, 21, and 28 days after drug administration to observe changes in their behavioral responses over time.

For behavioral testing, the mice were placed in individual Plexiglas cubicles (8.5 cm in length $\times$ 4 cm in height $\times$ 4 cm in width) on an elevated wire mesh platform and allowed to acclimate for approximately 1 hr, during the course of which exploratory and grooming activity ended. Each von Frey filament was applied to the hind paw until the filament just bent and was held in place for 5 s or until the mouse withdrew its paw. Each filament was tested five times on each hind paw. A positive (aversive) response to the stimulus was defined as a brisk withdrawal, with or without shaking or licking, of the hind paw either during or immediately upon removal of the filament application. The tactile stimuli were applied to the plantar surface of the hind paw, starting with the 0.4g filament. If the 0.4g filament elicited three positive responses out of five trials, then the mouse was tested moving downward through the filament series toward 0.04g until the filament with the smallest bending force to elicit three positive responses was identified and recorded as the threshold fiber. If the 0.4g filament did not elicit three positive responses, then the mouse was tested moving upward through the series toward 1.4g until the filament with the smallest bending force to elicit three positive responses was identified and recorded as the threshold fiber. Mice that are naïve to experimental treatment typically show a positive response to the 0.6g–1.4g fibers, while mice that are treated with stavudine typically respond positively to the filaments in the 0.04g–0.4g range. Because stavudine is a systemic drug, the thresholds of both hind paws were averaged for each mouse. In all nocifensive tests, the observer was blind to condition. Data are presented as the mean gram bending force $\pm$ standard error of measurement (*SEM*).

RNA Extraction

Once nocifensive responses were established in drug-treated animals, two early time points were identified as having potential to illuminate molecular targets involved in the development of allodynia. Day 1 was selected because of the robust nocifensive behavioral response present 24 hr following drug administration. Day 3 was selected as a likely early point to capture molecules that are involved in the development of neuronal plasticity in the spinal dorsal horn to sustain the robust tactile allodynia. Separate cohorts of animals were used for each time point for microarray and quantitative polymerase chain reaction (qPCR; see Table 1). The mice were administered weight-based doses of stavudine or physiological saline (vehicle) on Day 0 for RNA extraction on Days 1 and 3 after drug treatment for both the microarray analysis and qPCR assay.

For the tissue harvest, the mice were euthanized by cervical dislocation followed by rapid removal of the spinal cords, which were immediately flash frozen on dry ice. The lumbar

region of each spinal cord was isolated and dorsal horn dissected from ventral horn. A standard TRIzol extraction protocol (Invitrogen, Carlsbad, CA) was used to extract RNA from the dorsal horn tissue samples, with each sample representing a single experimental animal. The RNA was further purified using the RNeasy Mini Kit (Qiagen, Valencia, CA). The quality and purity of the samples was analyzed by spectrophotometry and using the Experion RNA StdSens analysis kit (Bio-Rad, Hercules, CA). Samples with a 260/280 ratio of approximately 2.0 and two sharp peaks that corresponded to the 18S and 28S RNA on the RNA gel were considered of sufficient purity to be used in the microarray analysis and qPCR assays.

RNA Labeling and Microarray Processing

All microarrays were processed by one person in the same laboratory, following a standardized lab protocol to minimize nonbiological technical bias. Purified total RNA (3 µg) was reverse transcribed into cDNA using a 3'-Amplification One-Cycle cDNA Synthesis Kit (Affymetrix, Affymetrix, Inc., Santa Clara, CA, P/N 900431). The synthesized double-stranded cDNA was then purified using a GeneChip sample cleanup module (Affymetrix, P/N 900371) and used as a template in the subsequent synthesis of the biotin-labeled cRNA. A GeneChip IVT Labeling Kit (Affymetrix, P/N 900449) was used to synthesize the biotin-labeled cRNA and quantified per manufacturer guidelines. To monitor the labeling process independently from the quality of the starting RNA samples, a set of poly-A controls (Affymetrix, P/N 900433) were amplified and labeled together with all samples.

Gene-expression analysis was performed with the Affymetrix GeneChip Mouse Genome 430 2.0 array (Affymetrix, P/N 900496), which contain 45,000 probe sets and reports the gene-expression level of transcripts and variants from over 34,000 mouse genes. A total of 15 µg of synthesized cRNA from each sample was hybridized on the gene chip in a hybridization oven at 45°C for 16 hr at 60 rpm. All probe arrays were scanned with an Affymetrix GeneChip Scanner 3000 with digital image data processed using the Affymetrix GeneChip Operating Software 1.4 (GCOS, Affymetrix).

Microarray Data Analysis

The microarray data were provided as a flat file (.dtf). The .dtf file was extracted using the GeneChip Operating Software, and raw data files (.cel) for each hybridization were generated. Raw .cel files were then subjected to background correction and intrachip normalized using JMP Genomics v3.2 software (SAS, Cary, NC). Briefly, background correction is the process of correcting probe intensities on any one array using probe information only on that array. Normalization is the process of removing nonbiologic variability between arrays from the analysis. The software breaks the array into regions and within each grid determines a signal-to-noise value for that grid. For each probe, the background/noise adjustment takes the weighted average of the grid background/noise values, with weights dependent on distance from the probe location to the center of each grid. The robust multichip average (RMA) method (Irizarry et al., 2003) fits a robust model to the log transformed raw data using a quantile approach with a median polish algorithm of probe and chip effects to predict chip expression with a robust multichip average. Differences in gene expression were analyzed using repeated measures analysis of variance. To correct for multiple testing, we applied the false discovery rate (Reiner, Yekutieli, & Benjamini, 2003) with an a priori alpha of .05. Thus, only genes that were significantly different at $p < .05$ after correction were considered for further analysis.

qPCR Verification of Identified Target Gene

The identification of significant changes in expression of the giant axonal neuropathy 1 (*Gan1*) gene through microarray was validated using a quantitative real-time qPCR technique. This approach uses the PCR to detect, amplify, and quantify the absolute number of copies of a specific DNA sequence in a sample relative to a normalized DNA sample. Two cohorts of 8 mice (drug group $n = 4$ and vehicle group $n = 4$ per cohort, see Table 1) were administered weight-based doses of stavudine or physiologic saline vehicle on Day 0 for RNA extraction on Days 1 and 3 after drug treatment. Harvested RNA was reverse transcribed using the Superscript II reverse transcriptase and random primers (Invitrogen). We performed 40 cycles of qPCR using the Lightcycler 480 SYBR Green I Master Mix (Roche Applied Science, Indianapolis, IN). Because fluorescence dye is used to monitor the double-stranded DNA, plotting fluorescence as a function of temperature as the thermal cycler heats through the dissociation temperature of the DNA produces a DNA melting curve. The position and shape of this DNA melting curve can be used to differentiate amplified DNA sequences separated by less than 2°C in melting temperature. The relative abundance of each transcript was computed using Roche Lightcycler 480 Relative Quant software (Roche Applied Science). The following *Gan1*-specific amplification primers, which span at least two intron/exon boundaries, were selected: forward: 5'-ATG CCC ACT GAA AGA GAG GTT-3'; reverse: 5'-TGG CAG GGA TGC ATA GGT TCT GAT-3' (Integrated DNA Technologies, Coralville, IA). We used the β -actin gene as the reference gene with these specific primers: forward: 5'-TGT GGT GCC AGA TCT TCT CCA TGT-3'; reverse: 5'-TGT GGT GCC AGA TCT TCT CCA TCT-3' (Integrated DNA Technologies).

Western Blot Analysis of Gene Protein Product

To determine whether changes to the *Gan1* gene result in changes to the expressed level of its protein product, gigaxonin, 9 mice received stavudine or physiologic saline via tail vein on Day 0 for tissue harvesting on Days 1 and 3 after drug treatment (see Table 1 for cohort breakdown).

Euthanization and spinal cord removal and preparation followed the same procedure as described above. The spinal dorsal horn tissue was mechanically homogenized in lysis buffer with protease and phosphatase inhibitors (Tris-buffered saline [pH = 8.0] plus 10% glycerol, 0.1% Triton X-100, protease and phosphatase pellets [Roche Applied Science]). SDS (sodium dodecyl sulfate), solubilized tissue extracts were incubated at 100°C for 5 min, fractionated on 4–12% NuPAGE bis-tris gels (Invitrogen) and transferred to a nitrocellulose membrane. After blocking in nonfat dried milk, membranes were incubated overnight at 4°C with a primary antibody to gigaxonin (anti-gigaxonin 1:500, Santa Cruz Biotechnology, Santa Cruz, CA) followed by incubation with a peroxidase-conjugated secondary antibody (anti-rabbit IgG 1:4000, Amersham Pharmacia Biotech, Piscataway, NJ) and visualized by chemiluminescence (Amersham Pharmacia Biotech). Blots were quantified by scanning autoradiographs into ImageJ (NIH, Bethesda, MD, version 1.62) imaging software to determine the optical density of each band. After processing, to standardize samples for protein loading quantities, the blots were stripped and reprobed with a primary antibody to actin (anti-actin 1:500, Sigma Aldrich) followed by incubation with a peroxidase-conjugated secondary antibody (anti-rabbit IgG 1:4000, Amersham Pharmacia Biotech) and visualized by chemiluminescence (Amersham Pharmacia Biotech). The blots were quantified again by scanning autoradiographs into ImageJ (NIH) imaging software to determine the optical density of each band.

Statistical Analysis

The behavioral data, expressed as mean gram force $\pm$ SEM, were analyzed using one-way analysis of variance (ANOVA). The microarray data (reported as percentage change) were analyzed using repeated measures ANOVA with false discovery rate correction to control multiple testing error. Post hoc testing was done using Tukey's honestly significant difference (HSD). The qPCR and Western blot data (reported as percentage change) were analyzed using repeated measures ANOVA with post hoc testing by Tukey's HSD.

Results

Stavudine Produces Robust Mechanical Allodynia

Mice treated with stavudine developed a robust tactile allodynia by 24 hr after drug administration, with a significant decrease in withdrawal threshold compared to the naïve threshold ($F_{(1)} = 130.55$; $p = .000$), which persisted for at least 28 days. Mice treated with saline demonstrated no change from their naïve threshold (see Figure 1). The significant decrease in the threshold of the stavudine-treated mice within 1 day of receiving the drug suggests that changes within the nervous system that lead to the development of severe tactile allodynia occur rapidly after the drug insult.

Gan1 Is Downregulated in Stavudine-treated Mice

Analysis of the microarray data identified several genes with expression levels that changed following stavudine treatment. One of the genes that we identified as changing in response to stavudine treatment is *Gan1*, which was significantly downregulated after stavudine administration compared with vehicle-treated control mice ($F_{(2)} = 31.62$, $p = .0005$, using false discovery rate (FDR) correction for multiple testing), and post hoc testing revealed the significant difference between vehicle- and stavudine-treated mice to be at 3 days after drug administration ($p = .005$; Figure 2A). To confirm this microarray result, we harvested RNA from the spinal dorsal horn tissue of two new groups of mice and examined *Gan1* expression via qPCR. Results from this analysis were in agreement with our microarray results, with *Gan1* significantly downregulated ($F_{(2)} = 11.023$, $p = .010$) at both 1 ($p = .011$) and 3 ($p = .025$) days after stavudine treatment (Figure 2B).

Gigaxonin Protein Is Downregulated in Stavudine-Treated Mice

After verifying that the *Gan1* gene was downregulated via qPCR, we asked whether the protein product, gigaxonin, was also downregulated after stavudine administration. This is an important question, because mRNA transcript quantity does not always translate into changes in absolute amount of protein expression. The autoradiographic images of a representative immunoblot are presented in Figure 3A. In this blot, each lane represents the protein from a single mouse. The ratio of gigaxonin protein to actin was calculated and the results were quantified. As shown in Figure 3B, gigaxonin protein is significantly downregulated compared with the vehicle control ($F_{(2)} = 14.55$, $p = .005$). Post hoc testing indicated a significant difference between the vehicle and Day 3 stavudine groups ($p = .005$) and between the Days 1 and 3 stavudine groups ($p = .020$).

Discussion

The goal of this study was to use microarray technology to elucidate novel molecules in the spinal dorsal horn that are involved in promoting the development of chronic painful peripheral neuropathy following antiretroviral therapy to treat patients with HIV/AIDS. We reasoned that this unbiased, whole genome approach would be more likely to yield novel results than a more traditional, candidate-based approach. In addition, rather than limiting our search to a subset of genes using a more focused microarray, we chose to exploit the

power of the whole-genome array, the Affymetrix Mouse 430 2.0 gene chips, so that we could evaluate the entire mouse transcriptome.

From the results of the microarray analysis, we identified several genes that are either increased or decreased after stavudine treatment. We are currently exploring the roles that these genes play in the development and persistence of neuropathic pain. One, *Gan1*, which we found to be decreased in the spinal dorsal horn of stavudine-treated mice at both 1 and 3 days after drug administration compared with control mice, is known to have physiological relevance for its role in the development of GAN, a rare genetic disease. The locus for the human homolog, *GAN*, occupies a 590-kb interval on chromosome 16q24.1. Seventeen exons produce the gene product of 4,677 base pairs that encodes for the 1,794 amino acid protein gigaxonin. The disorder is associated with more than 20 distinct mutant alleles evenly distributed across the 11 exons (Asbury, Gale, Cox, Baringer, & Berg, 1972; Bomont et al., 2000). GAN was first described in 1972 by Berg, Rosenberg, and Asbury as an autosomal recessive disease that causes severe motor and sensory fiber neuropathy in the peripheral and central nervous system. Peripheral symptoms of the disease, which include progressive sensory fiber loss and distal weakness causing gait disturbance, are typically evident within the first 4 years of life, although rare cases occur as late as 10 years of age (Bomont et al., 2000). Most GAN patients also have distinctly curly hair. Central nervous system involvement can manifest in mental retardation, seizure disorders, and deficits in upper motor neuron function (for review, see Ouvrier, 1989). Pathological hallmarks of the disease include sensory and motor fiber loss, axonal swelling and neurodegeneration (Asbury et al., 1972).

The protein gigaxonin is expressed ubiquitously. Functionally, it is most closely related to the BTB Broad-Complex, Tramtrack, and Bric-a-Brac/kelch superfamily of proteins because it contains distinctive kelch repeats that are critical for protein–protein interactions (Timmerman, De Jonghe, & Van Broeckhoven, 2000). Gigaxonin interacts with microtubule-associating protein 1B to maintain the integrity of the cytoskeleton and to promote neuronal stability (Ding et al., 2006). In a transgenic mouse model of *Gan1*, it has been demonstrated that gigaxonin disruption, for example due to a quantitative decrease in the amount of protein expressed, leads to impairments in the ubiquitin-proteasome system. When this system functions improperly due to gigaxonin loss, the microtubule-associated protein 8 (MAP8) cannot be degraded, leading to alterations in axonal transport that can result in neuronal death (Ding et al., 2006).

In our mouse model of stavudine-induced painful peripheral neuropathy, we demonstrate a significant decrease in both the *Gan1* gene and the protein product gigaxonin at 1 and 3 days after stavudine administration. The decrease in expression of gigaxonin is associated with the development of nocifensive behavior in the mouse, suggesting the development of allodynia. The nocifensive behaviors we report in the mouse are qualitatively similar to the type of chronic pain that patients with HIV/AIDS taking antiretroviral NRTI drugs experience. This, the first report of the potential role for gigaxonin in the development of antiretroviral-induced painful peripheral neuropathy, suggests that stavudine causes downregulation of the gene *Gan1*, leading to a significant decrease in the amount of gigaxonin expressed in the spinal dorsal horn. Although the mechanisms underlying a role for gigaxonin in producing stavudine-induced allodynia in the mouse model remain unclear, future in vitro and in vivo studies will focus on elucidating the role for this protein. Our results have potential implications for the management and treatment of HIV/AIDS patients experiencing painful peripheral neuropathy as well as for patients with GAN. The potential development of new treatment modalities for managing NRTI-induced painful peripheral neuropathy will have a direct impact on the nursing care of this patient population and will

create a need for nursing research into the efficacy of these new treatments for improving the patients' health status and quality of life.

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Figure 1. Stavudine produces robust tactile allodynia by Day 1 after drug administration that persists for at least 28 days. Mice were behaviorally tested for their naïve withdrawal threshold and then received a single dose of stavudine (50 mg/kg/IV) or a dose-equivalent volume of saline. At 1 day and then weekly thereafter, mice were tested for the presence of tactile allodynia. *Significantly different from naïve; $p < .001$ by analysis of variance.

Figure 2.

Stavudine produces a significant downregulation of the gene giant axonal neuropathy 1 (*Gan1*) in the spinal dorsal horn 1 and 3 days after drug administration. A, Microarray analysis of RNA extracted from the spinal dorsal horn of stavudine-treated mice revealed that *Gan1* was significantly downregulated at 3 days (white bar; $p = .0005$) after drug administration, compared with saline-treated mice (black bar; saline set to 100%). B, Quantitative polymerase chain reaction (qPCR) validation of the microarray results confirmed that *Gan1* was significantly downregulated in the spinal dorsal horn of stavudine-treated mice at 1 day (gray bar; $p = .011$) and 3 days (white bar; $p = .025$) after drug administration, compared with saline-treated mice (black bar; saline set to 100%).

*significantly different from saline-treated mice.

Figure 3.

Gigaxonin protein is significantly decreased in the spinal dorsal horn of stavudine-treated mice. A, A representative autoradiograph of an immunoblot probed with an antibody specific for gigaxonin (top panel) followed by probing with an antibody to actin (bottom panel). In this blot, each lane represents the protein from an individual mouse. B, Quantification of the mean protein amount for each experimental group in the Western blot demonstrates that gigaxonin protein is significantly decreased in the spinal dorsal horn of stavudine-treated mice 3 days after drug administration compared to both vehicle-treated mice ($**p = .005$) and stavudine-treated mice 1 day after drug ($*p = .020$).

NOTE: qPCR = quantitative polymerase chain reaction

Table 1

Study Variables and Number of Animals Used for Each Experiment

Study Arm	Total # of Mice	Day 1 Time Point	Day 3 Time Point
Nocifensive testing	12	Drug group $n = 6$, tested from naïve through 28 days; vehicle group $n = 6$, tested from naïve through 28 days	
Microarray analysis	12	Drug group $n = 3$; vehicle group $n = 3$	Drug group $n = 3$; vehicle group $n = 3$
qPCR analysis	16	Drug group $n = 4$; vehicle group $n = 4$	Drug group $n = 4$; vehicle group $n = 4$
Protein analysis	9	Drug group $n = 3$; vehicle group $n = 3$	Drug group $n = 3$

NOTE: qPCR = quantitative polymerase chain reaction.