A Cellular Mechanism of Neuronal Loss in the Dorsal Root Ganglia of *Dystonia musculorum* (*dt*) Mice

Kuang-Wen Tseng, Kuo-Shyan Lu, and Chung-Liang Chien*

Department of Anatomy and Cell Biology, College of Medicine,
National Taiwan University, Taipei, Taiwan
Abstract

*Dystonia musculorum* (dt) is a mutant mouse with hereditary sensory neuropathy, and a defective bullous pemphigoid antigen 1 (BPAG1) gene is responsible for this mutation. In present study, we examined the distribution of neuronal intermediate filament proteins in the central and peripheral processes of the dorsal root ganglia (DRG) in adult dt mice by different approaches. We found that not only BPAG1 but also α-internexin was absent in the DRG neurons in adult dt mice. To study the relationship between the absence of α-internexin and the progressive neuronal loss in the DRG of dt mice, we further cultured DRG neurons from embryonic dt mutants. Immunocytochemical assay of cultured DRG neurons from dt embryos revealed that α-internexin was aggregated in the proximal region of axons and juxtanuclear region of the cytoplasm, yet the other intermediate filament proteins were widely distributed in all processes. The active caspase-3 activity was observed in the dt neuron with massive accumulation of α-internexin. From our observations, we suggest that (1) the interaction between BPAG1 and α-internexin may be one of the key factors involved in neuronal degeneration, and (2) abnormal accumulation of α-internexin may impair the axonal transport and subsequently turns on the cascade of neuronal apoptosis in dt mice.
Introduction

*Dystonia musculorum (dt)* is a naturally occurring hereditary neuropathy in mice that mainly affects the sensory arm of the nervous system (1). The phenotype can be recognized at between 1 and 2 weeks of age when homozygous dt mice have difficulty in moving due to progressive loss of coordination (2). Despite the dystonic movements and the rapidity of degeneration in juvenile dt animals, no consistent pathology has been detected either in motor neurons or in skeletal muscle except in the late stages of the degenerative process, and this pathology is especially prominent in the dorsal root ganglia (DRG) (1). The diameter of the nerve process and the neuron size of the DRG are smaller in dt mice than those in the wild type and these observations are hallmarks of their degeneration (2-5). Neuron loss in the DRG, a severely reduced number of nerve fibers, and giant axonal swellings filled with organelles and intermediate filaments (IFs) were further demonstrated in the dt mutant (6).

Ablation of the BPAG1 gene results in the dt mouse exhibiting rapid sensory nerve degeneration, dystonic movements, and severe ataxia (7), and the gene responsible for the dt phenotype was termed bullous pemphigoid antigen 1 (BPAG1) (8,9). BPAG1n is a neural isoform of BPAG1 and found in a variety of sensory and autonomic neuronal structures during development (10). Previous studies showed that BPAG1 is one of the cytolinker proteins that has an N-terminal actin-binding domain and a C-terminal microtubule-binding domain (7,9,11). It has also been demonstrated that the C-terminal tail domain of BPAG1n interacts with neuronal IFs, such as \(\alpha\)-internexin, peripherin and neurofilaments (NFs) *in vivo* (7,12,13).

In the mammalian nervous system, five IF proteins are specifically expressed in differentiated neurons: \(\alpha\)-internexin, peripherin, and the neurofilament triplet proteins (NF-L, NF-M, and NF-H) (14-16). NFs have long been considered to be the
major types of intermediate filaments (IF) expressed in mature neurons (17,18), whereas α-internexin and peripherin have been shown to be highly expressed in developing neurons (19-25). α-internexin and peripherin are also found in embryonic DRG neurons \textit{in vivo} (26,27), although the expression of α-internexin declines postnatally (28,29). The earlier expression of α-internexin may stabilize neurons and their processes and provide scaffolding for the co-assembly of the other IFs during development (26,30,31).

So far, it is not well characterized whether α-internexin and peripherin are co-expressed in DRG neurons of homozygous (dt/dt) mice. Therefore, the primary aim of the present study is to investigate the expression patterns and distribution of α-internexin and peripherin in degenerating axons of the central and peripheral processes of DRG in dt/dt mice \textit{in vivo}. To study the axonal degeneration pattern of the central as well as the peripheral processes of DRG neurons from dt mutant mice were also examined at ultrastructural level. Moreover, we collected and genotyped embryos from the heterozygous (dt/+) breeding and subsequently cultured DRG neurons from each genotyped embryo. The pattern of neuronal IFs in embryonic DRG neurons and cell death events of DRG neurons from dt homozygous embryos were extensively examined by immunocytochemistry, TUNEL assay, and conventional electron microscopy.
Materials and methods

Mice

B6C3Fe-ala-Dst<sup>dt/dt</sup> mice, carrying a natural mutation in the BPAG1 gene were used in the present study. Experimental dt/dt, dt/+, and wild type mice were collected from the littermates of heterozygous breeding pairs obtained from Jackson Laboratories (Bar Harbor, Maine, USA). Embryonic day 15.5 (E15.5) embryos, postnatal day 1 (P1) and 8 weeks mice were collected for following studies. The genotype of each littermates was assayed by RT-PCR according to their expression patterns of BPAG1n from the RNA preparation of spinal cords (10). Care and treatment of animals were in accordance with standard laboratory animal protocols. A total of 40 adult mice (dt/dt, dt/+, and wild type) were collected from 6 littermates of heterozygous breeding pairs for the following studies.

RT-PCR assays

Mice were scarificed by cervical dislocation after anesthesia with choral hydrate (i.p., 400 mg/kg of body weight). Expression patterns of BPAG1n, α-internexin, peripherin and β-actin were assayed by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA from the DRG and spinal cord was prepared using Trizol reagent and converted to cDNA using a reverse primer and reverse transcriptase (Invitrogen, Carlsbad, CA, USA). To amplify the cDNA, we used Taq DNA polymerase and PCR, consisting of 40 cycles at 94°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min. Specific PCR primer sequences were prepared as follow:

BPAG1n primers (5’-GAC GAG AAG TCG GTG ATA ACC TAT G-3’ and 3’-CTG TTT GAG TAG GA CGG GCT T-5’; producing a 511 bp fragment); α-internexin primers (5’-ATC TGG AGA AGA AGG TGG AGT CG-3’ and 3’-CAA GCG GTT GGA TTT GCT CGT; producing a 256 bp fragment); peripherin primers (5’-ATG
AGC CAT CAT CAC TCG TCG GGC C-3’ and 3’-TCC TGG ATC GCC GCG AGT
TCG TCT; producing a 248 bp fragment). β-actin was applied as a positive control
and its primers were 5’-AAC CAT GAG GGA AAT CGY GCA C-3’ and 3’-AGT
CAA GGG AAT CGG CAG AAT G-5’ (producing a 419 bp fragment).

In situ hybridization

Mice were anesthetized and perfused with phosphate-buffered 4%
paraformaldehyde. DRG were isolated from dt/dt and wild type mice. Tissues were
frozen in isopentane supercooled with liquid nitrogen, embedded in OCT (Miles, Inc.,
Elkhart, IN, USA), cut into 10 µm cryostat sections on silanized slides (DACO,
Tokyo, Japan), and allowed to dry for 30 min. Sections were kept at -80°C for further
studies or were post-fixed in 4% paraformaldehyde for in situ hybridization. After
washing in PBS, sections were acetylated with acetic anhydride and placed in
prehybridization solution. This solution was replaced with hybridization solution
containing a digoxigenin (DIG)-labeled cRNA probe. cRNA probes, prepared from
selected BPAG1n, α-internexin, and peripherin cDNA ,were incubated with the
sections in a humidified chamber at 60°C overnight. Stringency washes were
performed at 60°C in 0.2X SSC (sodium chloride/sodium citrate) for 2 hr after
hybridization. Finally, sections were blocked with 10% normal goat serum for 1 hr,
incubated with alkaline phosphatase-conjugated anti-DIG antibody (1:500; Roche,
Mannheim, Germany) at 4°C overnight, and color detected using an NBT/BCIP
solution (Sigma, St. Louis, MO, USA).

Antibodies

Monoclonal peripherin (MAB1527) and α-internexin (MAB5224) antibodies,
and polyclonal peripherin (AB1530), α-internexin (AB5354), and PGP 9.5 (AB1761)
antibodies were obtained from Chemicon (Temecula, CA, USA). Polyclonal active
caspase-3 antibody was obtained from BD Biosciences (San Jose, CA, USA). The
polyclonal NF-M antibody was a gift from Dr. Ronald Liem (Columbia University,
New York, NY. USA).

**Double immunohistochemistry**

Whole E15.5 embryos and P1 pups were collected for cryostat sections. DRG
and nerves dissected from the adult were post-fixed in 4% paraformaldehyde at 4°C
overnight and then rinsed in PBS. Cryostat sections were blocked at 37°C for 30 min
with 10% goat serum and 0.3% Triton in PBS and incubated with primary antibodies
at room temperature for 1.5 hr. Immune complexes were revealed with
FITC-conjugated rabbit anti-mouse IgG (1:100) and Rhodamine-conjugated goat
anti-rabbit IgG (1:200) antibodies (Sigma).

Samples were mounted with CrystalMount (Biomeda, Foster City, CA, USA)
and examined under a Leica TCS SP2 confocal spectral microscope (Leica
Microsystems, Heidelberg, Germany).

**Electron microscopy**

Mice were deeply anesthetized by choral hydrate (i.p., 400 mg/kg of body
weight) and perfused with a fixative containing 4% paraformaldehyde and 1%
glutaraldehyde in 0.1 M cacodylate buffer. DRG were dissected out and immersed in
the same fixative at 4°C overnight. After post-fixation in 1% osmium tetroxide (OsO₄),
tissue samples were dehydrated in a graded series of ethanol, and then embedded in
epoxy resin. Ultrathin sections (70 nm thickness) were collected on copper grids,
doubly stained with uranyl acetate and lead citrate, and examined in a Hitachi 7100
electron microscope (Hitachi, Tokyo, Japan).
**Immunohistochemistry for cutaneous tissues**

After perfusion, cutaneous tissues from footpads were collected and 30 µm cryostat sections were cut. The floating sections were rinsed in PBS, incubated in 3% hydrogen peroxide for 30 min to eliminate endogenous peroxidase activity, and finally blocked for 1 hr in PBS containing 5% goat serum and 0.5% Triton X-100 (Sigma). Sections were incubated with the primary antibody against PGP9.5 at 4°C overnight. Labeling was accomplished with a Vector ABC kit (Vector Labs, Burlington, Canada) and 3, 3’-diaminobenzadine (DAB) reaction (Sigma).

We quantified epidermal innervation according to the modified protocol in a coded fashion (32). PGP 9.5-immnopositive nerve fibers in the epidermis of each footpad were counted at a magnification of 400X with a light microscope. Each individual nerve with its branches inside the epidermis was counted as one. For nerves with branches in the dermis, each individual epidermis nerve was counted separately. Epidermis nerve density was derived and expressed as the number of fibers per millimeter of the epidermis length along the upper margin of the stratum corneum in each footpad.

**Hot-plate test**

The hot plate test was applied to measure response latencies of mice (modified from the protocol suggested by Woolfe and MacDonald (33)). The temperature of the hot plate was maintained at 48°C. A mouse was placed onto the hot plate and the lapsed time between placement and licking of the paws was recorded as the index of response latency. Each animal was tested at least 3 times. All data were collected and analyzed by Student’s t test and p<0.001 was taken as the significant level.
Cell culture for embryonic DRG

For each experiment, 30-35 ganglia from each mouse embryo at embryonic 15.5 day (E 15.5) were dissected, collected, and maintained in Leibovitz L-15 medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS and 100 μg/ml penicillin/streptomycin as previously described (34). To genotype each embryo from the heterozygous breeding, the spinal cord of the embryo was collected for RT-PCR analysis.

DRG collected from each embryo were treated with 0.25% trypsin for 20 min at 37°C. Cells were dissociated physically by pipetting, then plated in 12-well tissue culture dishes (Corning, NY, USA) and allowed to attach to the poly-D-lysine (Sigma) substrate. Culture medium was composed of Neurolbasal medium (Gibco) supplemented with 20% FBS, 2% glucose, 2.5 mM L-glutamine, 2% B-27, and 50 ng/ml NGF (R&D Systems, Minneapolis, MN, USA). Cultured cells were collected at 3 or 5 day in vitro (DIV) for further analysis.

Immunocytochemistry for cultured DRG neurons

For immunocytochemistry, DRG neurons from wild type and dt/dt mice were cultured on cover glasses in a cell culture dish. At 3 DIV and 5 DIV, cells were washed in PBS, fixed in methanol at -20°C for 10 min, equilibrated in PBS, blocked for 30 min with 5% normal goat serum, incubated with the primary antibodies overnight, and washed extensively with PBS. Cells were then incubated for 1 hr with the secondary antibodies and fluorescent Hoechst 33342 (Sigma), and again washed with PBS. Subsequently, cells were mounted and observed under a Leica TCS SP2 confocal spectral microscope.
Terminal deoxynucleotidyl Transferase (TdT)-Mediated dUTP-Biotin Nick-End Labelling (TUNEL) assay

The assay was performed by In Situ Cell Death Detection Kit following the protocol suggested by the manufacturer (Roche, Indianapolis, IN, USA). Cultured DRG cells were grown on poly-D-lysine coated coverslips for 5 days, fixed with 4% paraformaldehyde for 10 min and washed with PBS before blocking with 0.3% H2O2 in methanol. After the second washing in PBS, samples were incubated in the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and the deoxynucleotide mixture at 37°C for 60 min followed by the immunostaining of anti-fluorescein antibody conjugated with horse-radish peroxidase at 37°C for 30 min. Finally, samples were rinsed in PBS and placed in 0.05% DAB solution (Sigma) for color development. All cells were examined and analyzed in a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany) with DIC observation.

Electron Microscopy for cultured DRG

For conventional electron microscopy, cultured DRG cells were fixed with 4% glutaraldehyde in PBS for 2 hr at 4 °C, then rinsed 3 times in PBS prior to post-fixation with 1% OsO4, dehydrated in a graded series of ethanol and embedded in an Epon-araldite mixture. Ultrathin sections were obtained and stained with uranyl acetate and lead citrate before examining with a Hitchi-7100 electron microscope (Hitachi, Tokyo, Japan).
Results

Analysis of BPAG1n, α-internexin, and peripherin mRNAs of DRG from wild type, dt/+ and dt/dt mice

We first demonstrated the expression patterns of BPAG1n, α-internexin, and peripherin mRNAs from wild type, dt/+, and dt/dt mice by RT-PCR (Fig. 1A).

BPAG1n mRNA could be detected in DRG of wild type and dt/+, but not dt/dt mice. BPAG1n mRNA expression was much less in dt/+ than that in wild type mice. A pair of dt/+ mice were set up for mating and dt/dt mice could be found in the breeding littermates. Surprisingly, α-internexin mRNA could be detected in the spinal cord and brain, but was completely absent in DRG of dt homozygous mice. Peripherin mRNA could be detected in the DRG, spinal cord and brain of all genotypes (Fig. 1A).

BPAG1n, α-internexin and peripherin mRNAs appeared to be abundant in the DRG of the adult wild type mice, yet BPAG1n and α-internexin mRNAs were absent in dt/dt mice. The lack of expression of α-internexin mRNA in the DRG, but not in the spinal cord and brain, of BPAG1 deficient dt/dt mice on RT-PCR assay encouraged us to do the following in situ hybridization and immunohistochemistry studies.

Absence of BPAG1n and α-internexin mRNAs in DRG neurons of dt/dt mice

The absence of BPAG1n and α-internexin in the DRG of dt/dt mice was further confirmed by in situ hybridization. Expression patterns of BPAG1n and α-internexin as well as peripherin mRNA in DRG neurons of wild type and dt/dt mice were investigated. Using antisense cRNA probes of BPAG1n, α-internexin, and peripherin, we detected the expression of BPAG1n, α-internexin, and peripherin mRNAs in wild type DRG neurons (Fig. 1B1-B3). In DRG neurons of dt/dt mice, we could not detect
any signal of BPAG1n and α-internexin (Fig. 1B4 and B5), but the signal of peripherin was present (Fig. 1B6). Our data revealed that α-internexin mRNA was expressed in DRG neurons of wild type mice (Fig. 1B2), but was turned off in the DRG of dt/dt mice (Fig. 1B5).

**Lack of α-internexin intermediate filaments in central process nerves of dt/dt mice**

We used antibodies against α-internexin, peripherin, and NF-M to analyze the protein levels in the central and peripheral processes of wild type (Fig. 2A-H) and dt/dt mice (Fig. 2I-P). α-Internexin and NF-M could be found in the central processes (Fig. 2C and D), and peripherin and NF-M were visible in the peripheral processes (Fig. 2E and F) of wild type mice. In dt/dt mice, as we predicted, α-internexin was completely absent in the central process nerves of dt/dt mice (Fig. 2K). However, we still observed NF-M in the central processes (Fig. 2L), and peripherin and NF-M in the peripheral processes (Fig. 2M and N) of dt/dt mice. In the central processes, α-internexin and NF-M were visible in wild type mice (Fig. 2C and D), but only NF-M was visible in dt/dt mice (Fig. 2L and P). These results clearly demonstrate that α-internexin was absent in DRG neurons of dt/dt mice, but the expression of peripherin and NF-M was not affected.

**Loss of sensory nerve endings and autonomic nerves in the skin of dt/dt mice**

In an attempt to define the relationship between decreased BPAG1 in the DRG and degenerating sensory nerve endings in the dt/dt mutant, the general neuronal marker of PGP 9.5 was used for cutaneous tissue staining. In wild type mice, abundant epidermal nerves and autonomic nerves that innervate sweat glands were found in the skin of footpads (Fig. 3A and B). However, in the skin of dt/+- and dt/dt
mice, only residual epidermal and autonomic nerves were visible (Fig. 3C, D, E and F). There were associated changes in the epidermis nerves in \( dt/+ \) and \( dt/dt \) mice. In wild type mice, the epidermis nerves had a dense, linear and continuous pattern of immunoreactivity to PGP 9.5 (Fig. 3A and B). In \( dt/+ \) mice, the PGP 9.5-immunoreactivity in the epidermis nerves became faint and had a beaded appearance as seen in Figure 3C and D, and the reduction in the nerves was most significant in \( dt/dt \) mice (Fig. 3E and F).

After statistical analysis of the cutaneous innervation (Fig. 3G), the epidermal nerve density was 52.8 ± 4.2 fibers/mm in wild type mice. The reduction in the epidermal nerve density was statistically significant in \( dt/+ \) mice (41.2 ± 3.1 fibers/mm, \( P < 0.001 \)) and in \( dt/dt \) mice (15.8 ± 2.4 fibers/mm, \( P < 0.001 \)) as compared to that in the wild type.

**Increased paw-withdrawal latency in \( dt/+ \) mice**

The phenotype of \( dt/dt \) mice could be easily judged by their uncoordinated locomotion. However, it is not easy to distinguish the heterozygous mutant from the wild type mice by their phenotype. To distinguish the heterozygous mutant from the wild type mice, a modified hot plate test was applied. Using the hot plate test, a nociceptive deficiency was observed in \( dt/+ \) mice but not in the wild type (Fig. 3H and I). Any change in nociception in \( dt/+ \) mice could affect the results of the inhibitory avoidance test. We observed that the paw-withdrawal latency in \( dt/+ \) mice was significantly longer than in wild type mice (Fig. 3J). The accuracy of this phenotyping procedure was confirmed by further histopathological examination. Indeed, the result was confirmed by examining the progressive loss of cutaneous innervation in \( dt/+ \) mice. To avoid the burn injury, we didn’t apply the hot plate test on the \( dt/dt \) mutant in this study.
**IFs disorientated in the surviving axons of the central processes of DRG from \(dt/dt\)**

Because of \(\alpha\)-internexin deficiency in the DRG of \(dt/dt\) mice, further pathological examinations were applied to illustrate the axonal morphology from \(dt/dt\) together with wild type mice. Large myelinated nerve fibers were observed in the peripheral and central processes from the wild type DRG at a light microscope level (Fig. 4A and B). A greater number of degenerated fibers and fewer myelinated axons in the peripheral and central processes of the \(dt/dt\) mutants were also noted (Fig. 4C and D). From the electron microscopy, we found that IFs and microtubules were oriented parallel to the long axis of axons from the wild type mice (Fig. 4E and F), and IFs were disoriented in the surviving axons of both peripheral and central processes from \(dt/dt\) mutants (Fig. 4G, H, I and J). The disorganization of IFs was more severe in the central processes than that in the peripheral processes (Fig. 4J and I). From these observations, it could be implied that \(\alpha\)-internexin deficiency accelerates the central process degeneration in DRG of \(dt/dt\) mice.

**Expression patterns of neuronal IFs and the immunoreactivity of active caspase-3 in DRG neurons**

Confocal microscopy revealed that \(\alpha\)-internexin and peripherin could be detected in all growing processes of DRG neurons from wild type embryos at 3 DIV (Fig. 5A). However, in the DRG neurons from \(dt/dt\) embryos, \(\alpha\)-internexin was only aggregated in the juxtanuclear region of the cytoplasm, yet the peripherin was widely distributed in all processes (Fig. 5B). At 5 DIV, DRG neurons from wild type embryos displayed numerous longer neurites, and \(\alpha\)-internexin and peripherin were colocalized in all processes (Fig. 5C). Nevertheless, abnormal accumulations of IFs
were found not only in the soma but also in the proximal region of cell processes from the cultured DRG neurons of dt/dt mutant (Fig. 5D). Massive accumulations of α-internexin were significantly increased in cell bodies and processes of DRG neurons from dt/dt mutant at 5 DIV by a statistic analysis (Table 1). Ultrastructural patterns of cultured DRG neurons from dt/dt embryos were further examined. Aggregations of IFs were found in the perikaryon of DRG neurons at 3 DIV (Fig. 5E). In addition, a high density of IFs and some entrapped organelles could also be observed in swelling axons of DRG neurons in the same preparation (Fig. 5F).

To illustrate the relationship between α-internexin aggregation and cell death, the immunostaining of active caspase-3 was applied. At 3 DIV, no immunopositive cell with active caspase-3 was found in the cultured DRG neurons from wild type and dt/dt embryos (Fig. 6A and B). At 5 DIV, very few immunopositive cells with active caspase could be detected in the DRG neurons from wild type embryos (Fig. 6C), whereas a number of neurons were labeled with active caspase-3 and their nuclei were in an eccentric position exhibiting α-internexin accumulation (Fig. 6D). With a statistical analysis, the percentage of active caspase-3 immuno-positive neurons was significantly increased in the cultured neurons with α-internexin accumulation from dt/dt embryos compared with that from wild type (Table 2). These results implied that the cell death were associated with abnormal accumulations of α-internexin aggregations. It also suggested that the deficient in the interaction between BPAG1 and α-internexin may enhance the process of cell death by accumulation of α-internexin in the cytoplasma and processes of cultured neurons from dt/dt embryos.

To confirm the cytotoxic effect of α-internexin aggregation in DRG neurons in vivo, the caspase-3 activity was also examined at E15.5 and P1 stages by immunohistochemistry. No immunopositive neuron with active caspase-3 was found in the DRG from wild type and dt/dt embryos at E15.5 (Fig. 6E and F). At P1 stage,
active caspase-3 still could not be detected in the DRG from wild type (Fig. 6G). However, it could be detected in some DRG neurons of $dt/dt$ where $\alpha$-internexin aggregations were also found in their soma (Fig. 6H).

**TUNEL reactions and ultrastructural patterns of the cell death on the fifth day from $dt$ mutant**

Besides the immunocytochemical reaction of active caspase-3 could be detected in the DRG neurons of $dt/dt$ embryos, cell death was further investigated with TUNEL assays. At 5 DIV, almost no TUNEL staining could be observed in the cultured DRG cells from wild type embryos (Fig. 7A). However, the positive TUNEL staining with dark-brown reaction products could be identified in the eccentric nuclei of some cultured DRG cells from $dt/dt$ embryos (Fig. 7B). Accordingly, ultrastructural patterns of cell death in cultured DRG neurons from the wild type and $dt/dt$ embryos were examined at 5 DIV. A typical round nucleus was found in the cultured DRG neuron of wild type embryos (Fig. 7C). Nevertheless, nucleus with characteristic condensed chromatin and mitochondria with abnormal swelling patterns were found in some cultured DRG neurons from $dt/dt$ embryos only (Fig. 7D). From these observations, we conclude that the process of cell death in cultured DRG cells from $dt/dt$ embryos is through the caspase-dependent apoptotic pathway.
Discussion

Different expression patterns of IFs in central and peripheral processes of DRG neurons

Both α-internexin and peripherin mRNAs have been found in embryonic DRG neurons (28,35). However, in the adult, α-internexin is expressed primarily in the CNS (19,26,29) while peripherin is predominantly expressed in neurons of the PNS (22,36,37). Western blotting revealed that α-internexin protein was virtually absent from homogenates of adult sciatic nerve (29), suggesting that α-internexin gene expression in the adult PNS may be very low (28). However, a recent study showed that α-internexin could be found in the sciatic nerve of wild-type and dt/dt mice at age of 3 weeks (13). Nevertheless, our data showed that α-internexin mRNA is still highly expressed in adult DRG neurons of wild type (Fig. 1B2), and its protein transported to the central processes instead of the peripheral processes (Fig. 2C). To explain discrepancies among these studies, it is possible that α-internexin in sciatic nerve may originate from motor neurons or it is a residual protein in the distal axons of sensory neurons at postnatal stage. Besides, we can not rule out the possibility that discrepancies among these studies may be due to the specificity of antibodies. Interestingly, in our study of embryonic DRG culture, α-internexin and peripherin could be detected in all growing processes of DRG neurons from wild type embryos (Figure 5A and 5C). This may also imply that there is no sorting signal for α-internexin or peripherin in the neurite outgrowth of sensory neurons in culture system. From this observation, we suggest that the distributions of α-internexin and peripherin in the axonal processes of the DRG may be induced by some cues from their peripheral or central targets.

Absence of α-internexin in DRG neurons from dt/dt mice
α-internexin may play a unique role in axonal development and provide a scaffold to facilitate subsequent NF deposition within the axonal cytoskeleton (28,38). However, inhibition of α-internexin expression did not prevent neuritogenesis, but this inhibition did prevent continued outgrowth of existing neurites (39). It is surprising that α-internexin was absent from DRG neurons of dt/dt mice in this study, as this had not been observed in dt pathology. Although α-internexin was absent in the central processes of the DRG in dt/dt mice as compared to that in the wild type, the expression of neurofilaments proteins such as NF-M could still be detected. In the study of knockout mice, although α-internexin was absent, these animals developed normally to adulthood without any obvious behavioral or anatomical abnormalities and produced normal offspring. Moreover, the absence of α-internexin did not interfere with neurite extension of cultured DRG neurons (40). However, α-internexin is upregulated in injured neurons, and it is suggested that in the absence of this protein, regeneration may be perturbed (41). Interestingly, our quantitative analysis revealed fewer myelinated axons in the central than that in the peripheral processes (data not shown). In considering all the data, we suggest that the loss of α-internexin expression in the sensory components of the central processes of BPAG1 deficient mice may consequently cause these nerves to degenerate more rapidly than peripheral nerves.

Degeneration of nerve terminals in BPAG1 deficient mice

Previous studies on morphometry indicated that there are significant reductions in myelinated and unmyelinated nerves in the dorsal root and sciatic nerves of dt/dt mice, corresponding to 63 % of nerve fibers of wild type mice (42). The fact that dt/dt mutants were impaired relative to ataxic controls (dt/+ and wild type) in motor coordination (43) is well-known, but reports on the cutaneous innervation of dt/dt mice are limited. The present study further characterized the sensory nerve terminals
in the skin of \( dt/dt \) mice and showed that the reduction in cutaneous innervation is robust, that is, the skin of hind paws of \( dt/dt \) mice is completely denervated in early postnatal period. We also quantified epidermal innervation of footpads in mice. The amount of epidermal nerves was significantly decreased in \( dt/+ \) mice than that in the wild type mice. To take an advantage of the behavioral hot plate test, we demonstrated that the retention time in \( dt/+ \) mice was longer than that in wild type mice. To sum up, these results indicate that the neuropathy of BPAG1 mutant became hypoalgesic.

On other hand, we also observed that sympathetic innervation of sweat gland was severely impaired in the hind paws of \( dt/dt \) mice. BPAG1 could be detected in DRG and sympathetic ganglia neurons during the embryonic stage (10). Disturbance in the cytoskeleton would alter the neuronal function when neural crest cells migrate (44). In \( dt \) embryos, cytoplasmic organelle accumulation was consistently observed (3), and this accumulation impaired axonal transport in neurons (42,45). It was revealed that sympathetic ganglia neurons that expressed BPAG1 were not appreciably affected by its absence (10). However, in our studies, axonal degeneration was evident not only in the epidermal sensory nerves but also in the sympathetic nerves of \( dt/dt \) mice. It will be interesting to answer why the neuronal loss in the PNS mostly occurred in the neurons derived from the neural crest in BPAG1 deficiency mice.

**Axonal dysfunction of DRG in \( dt/dt \) mutants**

It has been demonstrated that axonal swelling occurred more often after the postnatal stage than that during embryonic stage in \( dt/dt \) mutants (2). The disorganization of the neurofilament network and microtubules could be found in dorsal root axons of \( dt/dt \) mice (6). A significant reduction in the levels of microtubule-associated-protein, MAP2 and tau in \( dt/dt \) mice, indicates that BPAG1n
preferentially associates with microtubule and neuronal IF cytoskeletons in vivo (46). Other studies also showed that BPAG1 is one of the cytolinker proteins and that the C-terminal tail domain of BPAG1n isoform interacts with neuronal IFs (7,12,13). A key function of BPAG1n is to orchestrate the organization and stabilization of the microtubule network of sensory nerves to allow axonal transport over long distance (46). Recent studies revealed that BPAG1 is essential for retrograde axonal transport (45), and the anterograde and retrograde axonal transport in BPAG1 null mice is bidirectionally impaired (42). Our ultrastructural analysis of dorsal root axons from dt/dt mice revealed that cytoskeletal disturbance was more severe in the central processes of the DRG than that in the peripheral processes. We speculate that the axonal cytoarchitecture of the central processes may be different from that of the peripheral processes of DRG. One clue could be obtained from our observation that α-internexin is absent from the central processes and peripherin is present in the peripheral processes of BPAG1 deficient mice. Based on results from the present study, we suggest that the degeneration of central processes of DRG and subsequent loss of contact with central targets in the spinal cord could be one of reasons for the sensory impairment of dt/dt mutants. Nevertheless, the neuronal loss or the loss of peripheral axons and distal innervation may be also involved.

Roles of α-internexin in the degeneration of DRG in dt/dt mutants

Clinical studies showed that neurodegenerative disorders are morphologically characterized by progressive cell loss in neurons, associated typical cytoskeleton change (47-50). In the study of dt/dt mice, accumulation of swelling organelles and neuronal IFs within sensory axons are also observed (3). The earliest appearance of axonal swellings was found in processes of DRG neurons during perinatal stage, suggesting that the presence of neuronal IFs accumulation precedes
neurodegeneration. During the development of the nervous system, the expression of BPAG1n can be as early as E9.5 in differentiated neurons of mouse embryos (10). Among the neuronal IFs, the expression of α-internexin precedes that of neurofilament triplet proteins and reaches to the peak around E15 (19,26,28). As in the postnatal DRG development, the survival DRG neurons did not exhibit cytoplasmic and axonal aggregation of α-internexin. On the other hand, the embryonic DRG neurons with α-internexin aggregates were correlated with active caspase-3 expression in our culture system. These findings together with the fact that there is a neuronal loss in the DRG of postnatal dt/dt mice, suggested that α-internexin accumulation is sufficient to provoke cell death during development. Some of the survival neurons from dt mutant, there is no intermediate filament accumulation in the cell bodies which are TUNEL negative (Figure 7B). It is possible that those survival neurons found in the adult mutant may originally turn off the expression of α-internexin which could prevent the catastrophic accumulation. Nevertheless, we could not rule out the possibility that in the absence of α-internexin and BPAG1, other deregulated cytoskeletons may gradually participate in the postnatal neuronal degeneration. Besides, a failure of axonal transport may contribute to the abnormal cytoplasmic accumulation indicating a primary cause of cell death. This hypothesis is supported by studies of the neurons in transgenic mice and also in the PC12 cells that overexpression of α-internexin caused abnormal NF accumulation (51,52). Our results may be also relevant to the neuronal intermediate inclusion disease (NIFID), in which IF protein aggregation plays an important role in the mechanism of neuronal degeneration (53-55).

Apoptosis of DRG neurons in the development of dt/dt mutant

The caspase family of protease has been implicated in neuronal cell death in
some diseases (56-58). Caspase-3 is a ubiquitous cell death effector molecule, responsible at least in part for apoptotic cell death and is activated by initiator caspases (eg. caspase-8 and caspase-9). It was reported that caspase-3 is required for apoptosis-associated DNA fragmentation (59). In present study, we have shown that the neuronal death of BPAG1 mutant is correlated with the accumulation of cytoskeleton α-internexin and this apoptotic death is through caspase-3 pathway. Besides, the positive TUNEL staining could be identified in the eccentric nuclei of some cultured DRG cells from dt/dt embryos. In addition, the DNA fragmentation with oligonucleosomal “ladders” can occur from the detached cells of dt/dt embryos (data not shown). Taking together all these results, we suggest that the mechanism of neuronal death in the developing DRG from dt/dt mutant may be correlated with apoptotic caspase pathway.

In conclusion, our novel findings are: (1) the cell death of DRG neurons in BPAG1 mutant mice was associated with abnormal accumulation of α-internexin prior to the activation of caspase-3, (2) α-internexin was expressed in DRG neurons of wild type mice, but not in survival DRG neurons of postnatal dt/dt mutant, and (3) abnormal accumulation of α-internexin impairs the axonal transport and subsequently turns on the cascade of neuronal apoptosis in the development dt/dt mice.

**Acknowledgements**

We are grateful to Drs. Conrad Leung and Ronald Liem (Columbia University) for valuable suggestions and for the gift of antibodies. This work was supported by grants to C.-L. Chien (NSC 93-2320-B-002-101) and to K.-S Lu (NSC 92-2320-B-002-099) from the National Science Council, Taiwan. Facilities provided by grants from the Ministry of Education, Taiwan to J.-Y. Lin (Program for Promoting
Academic Excellence of Universities 89-B-FA01-1-4) and to the Center for Genomic Medicine in National Taiwan University (93-K0027) are also acknowledged.
References


11. Sun D, Leung CL, Liem RK. Characterization of the microtubule binding domain


FIGURE LEGENDS:

Fig. 1. RT-PCR and in situ hybridization analysis of BPAG1n, α-internexin, and peripherin mRNAs of DRG from wild type and dt/dt mice

RNA was extracted from tissues, and BPAG1n cDNA was generated. PCR amplification of BPAG1n was carried out using the primers described in Materials and Methods. ǃ-actin primers were used as a positive control. BPAG1n and α-internexin mRNAs transcripts are detected in the DRG of wild type and dt/+ mice but not in dt/dt mice. BPAG1n mRNA expression in the DRG is less in the heterozygous (dt/+)) than in the wild type mice. Peripherin mRNA transcripts are present in all of the mice (A). Antisense cRNA probes of BPAG1n, α-internexin, and peripherin were used to detect mRNA expression in wild type (B1-B3) and dt/dt mice (B4-B6). The DRG of dt/dt mice (B4-B6) are smaller than those of the wild type. Peripherin mRNA is detected in the DRG neurons of wild type (B3) and dt/dt mice (B6). BPAG1n and α-internexin mRNAs are expressed in the DRG neurons of wild type (B1, B2) but not dt/dt mice (B4, B5). Scale bar = 40 μm.

Fig. 2. α-Internexin, peripherin and NF-M immunoreactivity in the central process and peripheral process nerves of DRG in wild type and dt/dt mice

Peripheral and central processes as indicated in the upper panel were examined from DRG of wild type (A-H) and dt/dt mice (I-P). Serial sections were double labeled with antibodies to α-internexin (A, C, I and K) and NF-M (B, D, J and L), or peripherin (E, G, M and O) and NF-M (F, H, N and P). α-Internexin is detected in the central process nerves (C), and peripherin is present in the peripheral process nerves of wild type mice (E). Surprising, α-internexin is not found in the central process nerves (K), but peripherin is still detected in the peripheral process nerves of dt/dt mice (M). NF-M is used as a positive control for both peripheral and central processes. Scale bar = 40
Fig. 3. Sensory and autonomic nerves stained with anti-PGP 9.5 antibody and performance of dt/+ mice and wild type mice onto the hot plate test

In wild type mouse skin (A and B), the epidermal nerves (arrow) and numerous autonomic nerves that innervate sweat glands have typical continuous profiles of PGP 9.5-immunoreactivity. In dt/+ mouse skin (B and C), it is examined the progressive loss of cutaneous innervation (arrow). Conversely, in dt/dt mouse skin (E and F), only some residual epidermal (arrow) and autonomic nerves are visible. Dermal nerve trunks (double arrowheads) are visible in the skin of wild type mouse (B) and the less epidermal nerves are observed in the skin of dt/+ mouse (D), but only fragmented dermal and epidermal nerves are evident in dt/dt mouse skin (F). In the skin of wild type mouse (A), strong PGP 9.5-immunoreactivity is evident in autonomic nerves that innervate sweat glands. Almost all of the autonomic nerves have disappeared in dt/dt mouse (F). Scale bar = 40 µm. The histogram shows the comparison of epidermal nerve densities among the wild type, dt/+ and dt/dt mice (G). In wild type mice, the epidermal nerve density is 52.8 ± 4.2 fibers/mm. The epidermal nerve density is significantly reduced by 22.1% in dt/+ mice (41.2 ± 3.1 fibers/mm, P < 0.001) and by 65.7% in dt/dt mice (15.8 ± 2.4 fibers/mm, P < 0.001). For differentiation of phenotypes between wild type and dt/+, mice were placed on a hot plate at 48 ± 1 °C with a clear cylinder protection. The time between placement and licking of the fore paws was recorded as the index of response latency (seconds). For the same latent period, the wild type mouse licked its fore paws (H), but the dt/+ mouse had no response (I). The paw-withdrawal latency was significantly longer in the dt/+ mice than in the wild type mice (J). **P<0.001.
Fig. 4. Semi-thin sections and ultrastructural elements present in the central and peripheral processes from wild type and dt/dt mice

Myelinated axons are abundant in the peripheral (A) and central processes (B) of DRG from wild type mice. Degenerated fibers and fewer myelinated axons could be observed in the peripheral (C) and central processes (D) of dt/dt mutants. The distribution of the IFs and microtubules were oriented parallel to the long axis of the axon (E and F) and the distribution of IFs in the sensory axons of dt/dt mice (G, H, I and J) were disordered compared to that in axons of wild type mice. At higher magnification, note the disorganization of intermediate filaments was more severe in axons of the central processes (J) than that of the peripheral processes (I). Scale bars: 20 µm(A, B, C, and D); 1µm(E, F, G, H, I, and J).

Fig. 5. Immunoreactivity of neuronal IFs and ultrastructural patterns in cultured DRG neurons

DRG neurons were isolated from E 15.5 wild type (A and C) and dt/dt (B and D) embryos. Cultured DRG neurons were double labeled with antibodies to α-internexin (green) and peripherin (red). All the cell nuclei were stained with Hoechst 33342 (blue). α-Internexin and peripherin could be found in the growing processes of DRG neurons from wild type embryos at 3 DIV (A). α-Internexin was formed aggregates in the cytoplasm, yet peripherin protein was widely distributed in all processes of DRG neurons from dt/dt mutants (B). After 5 DIV, both α-internexin and peripherin could be found in all processes of DRG neurons from wild type embryos (C), but abnormal accumulations of α-internexin were predominant observed in the proximal region of axons and within cell bodies of cultured DRG neurons from dt/dt embryos (D). From ultrastructural observations of dt/dt embryos at 3 DIV, IF aggregate was found in perikaryon of cultured DRG neurons (arrowhead, E). Besides, randomly orientated
IFs and axonal organelles were found in the swelling processes of \( dt/dt \) DRG neurons (F). Scale bars: 40 µm (A, B, C, and D); 500 nm (E and F).

**Fig. 6. Immunoreactivity of \( \alpha \)-internexin and active caspase-3 in the DRG neurons in vitro and in vivo**

DRG neurons were cultured from E15.5 wild type (A and C) and \( dt/dt \) (B and D) embryos. Tissues sections of DRG were collected from E15.5 and P1 of wild type (E and G) and \( dt/dt \) (F and H) mice. Cultured DRG neurons and tissue sections were double labeled with antibodies to \( \alpha \)-internexin (green) and active caspase-3 (red) and their nuclei were stained with Hoechst 33342 (blue). At 3 DIV, immunopositive DRG neuron with active caspase-3 was hard to find from wild type and \( dt/dt \) embryos (A and B). At 5 DIV, only limited immunopositive cell could be detected in cultured neurons from wild type embryos (arrowhead, C), whereas a number of neurons with \( \alpha \)-internexin accumulations were labeled with the antibody to the active caspase-3 (D). At E15.5, no active caspase-3 immunoreactivity could be detected from DRG neurons in wild type and \( dt/dt \) embryos in vivo (E and F). At P1, the active caspase-3 immunoreactivity was hardly found in the neuron of DRG from wild type (G). Nevertheless, some neurons with cytosolic \( \alpha \)-internexin accumulations (green) were labeled with the antibody to the active caspase-3 (red) in the developing stage of \( dt/dt \) mutant (H). Scale bar = 40 µm.

**Fig. 7. TUNEL assays and ultrastructural observations on the cell death of cultured DRG neurons at 5DIV from the wild type and \( dt \) mutant mice**

No TUNEL-positive cell could be found from the cultured DRG cells of wild type embryos (A), but some TUNEL-positive cells could be detected from those of \( dt/dt \) embryos (B). At ultrastructural level, the cell nucleus of DRG neuron from wild type
embryos exhibited typical nuclear morphology with a nucleolus (C), while the nucleus of DRG neuron from $dt/dt$ embryos exhibited apoptotic characteristic of chromatin condensation with intact nuclear envelope (D). Scale bars: 40 µm (A and B); 1 µm (C and D).
### A

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT</th>
<th>dt/+</th>
<th>dt/dt</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPAG1n</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-internexin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripherin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### WT

1. **BPAG1n**
2. **α-internexin**
3. **Peripherin**

#### dt/+

4. **BPAG1n**
5. **α-internexin**
6. **Peripherin**

#### dt/dt

7. **BPAG1n**
8. **α-internexin**
9. **Peripherin**

**Image B1**: WT
**Image B2**: BPAG1n
**Image B3**: α-internexin
**Image B4**: dt/dt
**Image B5**: α-internexin
**Image B6**: Peripherin
Table 1. Quantitative analysis (%) of cultured DRG neurons with α-internexin accumulation

<table>
<thead>
<tr>
<th></th>
<th>3 day in vitro</th>
<th></th>
<th>5 day in vitro</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soma</td>
<td>Process</td>
<td>Soma</td>
<td>Process</td>
</tr>
<tr>
<td>WT</td>
<td>2.1 ± 0.8</td>
<td>2.9 ± 1.2</td>
<td>0.7 ± 0.6</td>
<td>4.0 ± 1.7</td>
</tr>
<tr>
<td>dt/dt</td>
<td>8.6 ± 4.2 **</td>
<td>5.2 ± 2.7</td>
<td>3.1 ± 1.5 **</td>
<td>28.6 ± 2.5**</td>
</tr>
</tbody>
</table>

Percentage of cultured neurons with α-internexin aggregations were calculated from cultured DRG prepared from wild type (WT) (n=12) and dt/dt (n=22) embryos.

Values were obtained by the random selection of 100 neurons from the cell population of each embryo. Results are expressed as mean ± SD and ** indicates a value that is statistically different (t-test, P<0.001) from the wild type control. (n: number of embryos).
Table 2. Percentages of cultured DRG neurons expressing active caspase-3 and accumulation of α-internexin at 5 DIV revealed by the immunocytochemical analysis.

<table>
<thead>
<tr>
<th></th>
<th>Active caspase-3 immuno-positive neurons</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With accumulation in neuron</td>
<td>Without accumulation in neuron</td>
</tr>
<tr>
<td>WT (n=12)</td>
<td>3.8 ± 1.4</td>
<td>5.5 ± 2.8</td>
</tr>
<tr>
<td>dt/dt (n=22)</td>
<td>22.3 ± 3.1 **</td>
<td>6.6 ± 2.1</td>
</tr>
</tbody>
</table>

Percentages of cultured neurons with active caspase-3 and α-internexin aggregations were calculated from cultured DRG prepared from the wild type (WT) (n=12) and dt/dt (n=22) embryos. Values were obtained by the random selection of 100 neurons from the cell population of each embryo. Values are expressed as mean ± SD and ** indicates a value that is statistically different (t-test, P<0.001) from the wild type control. (n: number of embryos).