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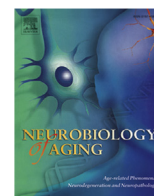

Neuronal degeneration associated with sympathosensory plexuses in the trigeminal ganglia of aged mice that overexpress nerve growth factor

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A B S T R A C T

Aberrant sympathetic sprouting is seen in the uninjured trigeminal ganglia of transgenic mice that ectopically express nerve growth factor under the control of the glial fibrillary acidic protein promoter. These sympathetic axons form perineuronal plexuses around a subset of sensory somata in 2- to 3-month-old transgenic mice. Here, we show that aged transgenic mice (i.e., 11–14 and 16–18 months old) have dystrophic sympathetic plexuses (i.e., increased densities of swollen axons), and that satellite glial cells, specifically those in contact with dystrophic plexuses in the aged mice display strong immunostaining for tumor necrosis factor alpha. The colocalization of dystrophic plexuses and reactive satellite glial cells in the aged mice coincides with degenerative features in the enveloped sensory somata. Collectively, these novel results show that, with advancing age, sympathetic plexuses undergo dystrophic changes that heighten satellite glial cell reactivity and that together these cellular events coincide with neuronal degeneration.

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1. Introduction

The ability of nerve growth factor (NGF) to induce directional growth of peripheral fibers *in vivo* and outgrowth of neurites *in vitro* was first demonstrated by Rita Levi-Montalcini and colleagues in the 1950s and 1960s (Levi-Montalcini and Booker, 1960; Levi-Montalcini and Hamburger, 1951; Levi-Montalcini et al., 1954). Levi-Montalcini et al. (1954) showed that chick sympathetic ganglia formed halos of neurites in response to co-explants of NGF-releasing mouse sarcomas in culture. The robust outgrowth of ganglionic fibers, as well as hyperplastic and hypertrophic changes in the associated ganglia has all been attributed to elevated levels of NGF. Because of those seminal studies, the generation of many different lines of NGF transgenic mice have collectively confirmed that this neurotrophin stimulates the directional growth of postganglionic sympathetic axons, as well as increases neuronal numbers and sizes *in vivo* (Albers et al., 1994; Davis et al., 1994; Edwards et al., 1989; Elliott et al., 2009; Hassankhani et al., 1995; Hoyle et al., 1998; Kawaja and Crutcher, 1997). One curious manifestation of NGF-induced sympathetic sprouting in transgenic mice is the appearance of basket-like structures in the uninjured sensory ganglia (Albers et al., 1994; Davis et al., 1994; Petrie et al., 2013; Walsh and Kawaja, 1998; Walsh et al., 1999). This phenomenon, referred to as sympathosensory plexuses, involves the abnormal growth of postganglionic sympathetic axons into the sensory ganglia where these fibers converge on a subpopulation of NGF-responsive somata and form perineuronal plexuses. Importantly, injury to the peripheral nerves can also induce the formation of sympathosensory plexuses in the affected sensory ganglia of adult rats (Chung et al., 1993; McLachlan et al., 1993), adult mice (Ramer and Bisby, 1997, 1998a), and humans (Shinder et al., 1999).

In our laboratory, we have examined perineuronal plexuses in the uninjured trigeminal ganglia of 2- to 3-month-old transgenic mice that ectopically express NGF under the control of the glial fibrillary acidic protein (GFAP) promoter (Kawaja and Crutcher, 1997). That is, those cell types that produce GFAP (e.g., astrocytes, Schwann cells) will abnormally overexpress NGF. These aberrant sympathetic axons are in immediate proximity to the satellite glial cells that surround a subpopulation of

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trkA-immunopositive somata. Despite this close association between the plexuses and the satellite glial cells and/or sensory somata, no obvious signs of reactivity have been detected in these glial cells. In this study, our objective was to determine the overall consequence of sympathosensory plexuses in the trigeminal ganglia of aged NGF transgenic mice. This is the first study to show that, with age, perineuronal plexuses of sympathetic axons are associated with both glial reactivity and neuronal degeneration and/or loss in NGF transgenic mice. From these data, we can infer that sympathosensory plexuses in the injured ganglia of humans may have long-term detrimental effects on sensory function that is compounded by the process of aging.

2. Methods

2.1. Animals

We have generated transgenic mice that ectopically express NGF under the control of the GFAP promoter (Kawaja and Crutcher, 1997); these animals display sympathetic perineuronal plexuses in the uninjured trigeminal ganglia (Walsh and Kawaja, 1998; Walsh et al., 1999). Genotyping was conducted by polymerase chain reaction. Briefly, ear punches were taken from each animal at 1 month of age; the isolated DNA was digested with EcoRI and amplified with the following primers: NGF (5′-CTAGAATTCTGCAGGCAACTCAGCC; 5′-CCTGAATTCTAGTGAACATGCTGTGCC). All amplifications were carried out in an Eppendorf Mastercycler (Hamburg, Germany). For this study, we used both male and female NGF transgenic mice from 3 age ranges: 2–3, 11–14, and 16–18 months old. Careful examination revealed no qualitative differences in the density, morphology, or neurochemical features of plexuses between male and female transgenic mice. Importantly, there are no wild-type controls for this phenomenon as sympathosensory plexuses do not occur in wild-type mice in the absence of nerve injury. It is the overexpression of NGF that induces the formation of these plexuses in the sensory ganglia of adult mice. All experiments were performed according to the guidelines of the Canadian Council for the Use and Care of Animals Guidelines and were approved by the Queen's University Animal Welfare Committee.

2.2. Tissue preparation and fluorescent immunostaining

Under deep anesthesia (sodium pentobarbital at 8.2 mg/mouse was delivered intraperitoneally) NGF transgenic mice (n = 15–20 per age group, n = 9–10 female) were perfused transcardially with 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.4); lower concentration of fixative improves immunostaining for tyrosine hydroxylase (TH). The trigeminal ganglia were removed from each animal, postfixed in perfusion fixative for 2 days and then cryoprotected in 30% phosphate-buffered sucrose for 2 days. All tissues were sectioned at 10 μm thickness using a cryostat and directly mounted onto Superfrost slides (Fisher Scientific) and then stored at −80 °C. Slides were allowed to thaw at room temperature for 5 minutes and postfixed in cold 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Slides were washed in 0.1M Tris buffer saline (TBS) for 5 minutes (performed after each step) and then blocked for 1 hour in 10% normal donkey serum (NDS) plus 0.25% triton-X100 (TBX) at room temperature in a humidified chamber. Sections of trigeminal ganglia were incubated with one (or doubled labeled with TH) of the following antibodies diluted in 3% NDS plus 0.25% TBX for 2 days at room temperature: sheep anti-TH IgG (1:1000; Millipore, Temecula, CA, USA), rabbit anti-p75 neurotrophin receptor (p75NTR) IgG (9651 IgG; 1:2000; kindly provided by Dr M. Chao, NYU Langone Medical Center, New York, NY, USA), rabbit anti-cFos IgG (1: 1000; Abcam, Cambridge, MA, USA), rabbit anti-ionizing calcium adapter binding molecule 1 (Iba1) IgG (1:1000; Wako, Richmond, VA, USA), rabbit anti-tumor necrosis factor alpha (TNFα) IgG (1:1000; a kind gift from Dr C. Cahill, University of California, Irvine; this antibody can be purchased from Millipore), rabbit anti-growth associated protein-43 (GAP-43) IgG (1:1000; Millipore, Billerica, MA, USA), rabbit anti-GFAP IgG (1:1000; DakoCytomation, Glostrup, Denmark), goat anti-tumor necrosis factor receptor 1 (TNFR1) IgG (1.25 μg/mL, R&D Systems, Minneapolis, MN, USA), and rabbit anti-inward rectifying potassium channel (Kir4.1) IgG (1:1000, Alomone Labs, Jerusalem, Israel). See Section 2.4 for more information. Slides were incubated for 2 hours with appropriate secondary antibodies (in 3% NDS plus 0.25% TBX) tagged with fluorophores for visualization: FITC-conjugated AffiniPure donkey anti-sheep (for TH), DyLight 594-conjugated AffiniPure donkey anti-rabbit (for p75NTR, cFos, Iba1, GFAP, TNFα, and Kir4.1, all secondary antibodies from Jackson ImmunoResearch, West Grove, PA, USA). All appropriate controls including the absence of primary or secondary antibodies revealing no autofluorescence or nonspecific staining have been performed on sections of trigeminal ganglia from adult NGF transgenic mice. Slides were cover slipped with DAPI-mounting media (Vector Laboratories) and were viewed under a Zeiss Axioskop 2 MOT microscope with Zeiss axiocam.

2.3. Chromogenic immunostaining

For double labeling with TNFα and TNFR1, we used the chromogens 3,3′-diaminobenzidine (DAB) and benzidine dihydrochloride (BDHC) for visualization of positive immunostaining (Levey et al., 1986). For the DAB and BDHC reactions, slides were first blocked in 0.3% hydrogen peroxide and 10% bovine serum albumin (BSA) and then incubated for 48 hours with TNFα IgG (1:1000 in 3% BSA), after which the sections were incubated in biotinylated goat anti-rabbit (1:200 in 3% BSA) for 2 hours, followed by a 2-hour incubation in avidin and/or biotin complex (ABC, Vector Laboratories). Positive immunostaining for TNFα was revealed by the DAB reaction, which results in a brown reaction product. The sections were then rinsed in buffer and incubated for 48 hours with TNFR1 IgG (1.25 μg/mL in 3% BSA), after which the sections were incubated in biotinylated rabbit anti-goat (1:200 in 3% BSA) for 2 hours, followed by a 2-hour incubation in ABC (Vector Laboratories). Positive immunostaining for TNFR1 was revealed by the BDHC reaction, which results in a blue crystalline reaction product. All appropriate controls including the absence of primary or secondary antibodies revealing no autofluorescence or nonspecific staining have been performed on sections of trigeminal ganglia from NGF mice. Slides were cover slipped with Permount (Fischer Scientific) and were viewed under bright field using a Zeiss Axioskop 2 MOT microscope with Zeiss axiocam.

2.4. Antibody characterization

1. Anti-cFos IgG (catalog # ab7963; Abcam). This rabbit polyclonal IgG was raised against the synthetic peptide, MMFSGFNA-DYEASS, which corresponds to the N-terminus amino acid 1–14 of human cFos. The cFos antibody recognizes a single band of 60 kDa in Western blots (manufacturer's datasheet).
2. Polyclonal anti-bovine GFAP IgG was purchased from DakoCytomation (catalog # Z0334). This antibody, developed in rabbit, was raised against GFAP isolated from cow's spinal cord. It stains a single band of 50 kDa molecular weight on Western blot (unpublished observations from our laboratory; Jahed et al., 2007), and staining of sections through the olfactory

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