Transganglionic Neuropeptide Y Response to Sciatic Nerve Injury in Young and Aged Rats

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Abstract. Gracile neuroaxonal dystrophy (NAD) is a hallmark of the aging human and rodent sensory nervous systems which may represent an abnormal transganglionic response to peripheral axonal injury. To examine the structural plasticity of central dorsal root ganglia (DRG)-derived axons in the gracile nucleus, we evaluated the response of the lumbar DRG and their central projections to sciatic nerve injury in young and old rats. In uninjured rats neither the DRG nor its central projections contained histochemical immunoreactivity for neuropeptide Y (NPY). However, within 1 week of sciatic nerve crush or transection injury, NPY immunoreactivity appeared in the lumbar DRG and its central projections, reaching an apparent maximum in number and intensity of processes at 28 days. Neuropeptide Y immunoreactivity was more intense and sustained in response to transection compared to crush injury, results supported by NPY radioimmunoassay. Neuropeptide Y-immunoreactive processes in the gracile nuclei of axotomized young animals consisted of delicate axons or slightly enlarged profiles that may represent regenerative elements. Lumbar dorsal rhizotomy performed simultaneously with sciatic nerve transection prevented the transganglionic NPY response. Dystrophic axons in the gracile nucleus of non-lesioned aged animals were not NPY-immunoreactive; however, after sciatic nerve transection, NPY immunoreactivity developed in both delicate axons and markedly swollen dystrophic elements, a finding confirmed by ultrastructural immunolocalization. These results establish that despite the presence of NAD in DRG projections to aged gracile nuclei these elements remain capable of a plastic NPY response to peripheral nerve injury.

Key Words: Aging; Axotomy; Gracile nucleus; Neuroaxonal dystrophy; Neuropeptide Y.

INTRODUCTION

Neuroaxonal dystrophy (NAD) is a distinctive poorly understood form of axonopathy which is characterized by the development of swollen preterminal axons and synapses containing a variety of unusual subcellular organelles (reviewed in 1). Dystrophic axonopathy, a common neuropathologic process of unknown pathogenesis, is characteristic of a variety of toxic, heredodegenerative, age-related and metabolic human and experimental conditions and may involve various neuronal populations in the central and peripheral nervous systems (1). Sensory nerve terminals in the human and rodent gracile nuclei develop NAD as a function of age (2, 3), vitamin E deficiency (4, 5), genetic disorders (1, 6) and, in experimental animals, diabetes (7). Gracile NAD has been generally regarded as an age-related change of no obvious clinical significance, although it may contribute to the loss of vibration sensitivity in the lower extremities which is a common feature of aging in the human sensory nervous system (8). The ultrastructural appearance of gracile NAD is shared with NAD occurring in other parts of the central and autonomic nervous systems in various pathological conditions. Although the pathogenesis of NAD remains unknown, it has been proposed to represent frustrated axonal regeneration or an abnormality of the normal process underlying synaptic remodeling or plasticity (9–11). Hachisuka and coworkers (12) have demonstrated significantly increased numbers of dystrophic axons in the ipsilateral gracile nucleus following unilateral hindlimb amputation in the cat, a procedure which generated axotomized axons whose peripheral regenerative progress was chronically frustrated. The sequence of structural changes in presynaptic central terminals (13–15) which develop in response to peripheral nerve injury, collectively known as transganglionic effects (16), and, in particular, those involving the subpopulation of dorsal root ganglia (DRG) neurons projecting to the gracile nuclei, may be relevant to the pathogenesis of gracile NAD.

Recent studies of the response of DRG neurons and their central axonal terminals to peripheral axotomy have revealed a previously unrecognized diversity of cellular responses among subpopulations of DRG neurons (17–20). Neuropeptides such as substance P, somatostatin, and calcitonin gene-related peptide (CGRP), which are found in a population of small to medium-sized DRG neurons including those which subserves nociception and temperature sensation, are downregulated following peripheral axotomy (20, 21). In contrast, certain neuropeptides such as galanin, VIP and neuropeptide Y (NPY) are upregulated in DRG neurons following peripheral axotomy (17–20). Neuropeptide Y immunoreactivity is increased after axotomy predominantly in medium to large DRG neurons, i.e. those neurons whose central projections include afferents to the gracile nucleus (22). Axotomy-induced changes in neuropeptide expression in DRG neurons re-
sults in altered transganglionic expression in their central terminals in the spinal cord and gracile nuclei. Thus, CGRP and substance P are depleted in the substantia gelatinosa following sciatric axotomy, while NPY-immunoactive fibers appear in the spinal dorsal horn (laminae III and IV) and the gracile nuclei, sites in which they are absent in uninjured animals. Subpopulations of neurons which contain different neuropeptides may, therefore, differ in their mode of response to peripheral injury. In addition, approximately 25% of DRG neurons will die within 6 weeks of axotomy (16). Axonal profiles immunoreactive for GAP-43, the 43 kDa “growth-associated protein” which is a constituent of the axonal growth cone in axonal development and regeneration, increase in the substantia gelatinosa following sciatic nerve transection (23–25). A regenerative or plastic response of central DRG projections may coexist, therefore, with degenerative alterations as part of a complex transganglionic response to peripheral nerve injury.

Neuropeptide Y is of particular interest in our study of transganglionic responses of DRG since it is not immunohistologically detectable in the DRG and its central connections under normal conditions and is upregulated in medium to large DRG neurons following peripheral axotomy (22, 26, 27). These features make NPY immunohistochemistry a particularly useful tool in studying the dynamic nature and morphology of transganglionic changes of large DRG neurons which contribute to the innervation of the dorsal column nuclei (28) and are targeted by NAD in this site.

We set out to examine the plasticity of central synapses in response to sciatic nerve injury in order, ultimately, to investigate the mechanisms underlying the development of NAD in the DRG projection to the gracile nucleus. Our study has confirmed and extended the initial observations of upregulation of NPY in axotomized DRG using immunohistochemistry and radioimmunoassay and systematically studied upregulation of NPY in the central processes of DRG neurons of young and aged rats at various times after sciatic nerve injury. The comparison of the effect of sciatic nerve crush, in which regeneration is facilitated, to transection injury, in which regeneration is intentionally prevented, was used to investigate the role of axonal regeneration in the normalization of upregulated NPY immunoreactivity. Finally, we have compared the NPY response with that of GAP-43, a marker of axonal growth and plasticity, and CGRP, which identifies a different subpopulation of DRG neurons than NPY.

MATERIALS AND METHODS

Animals

Adult young (3–4 months) and aged (14–26 months) male Sprague-Dawley rats were obtained from Sasco (O’Fallon, MO). Several 16 month old Fischer 344 rats were obtained from the National Institutes of Aging colony at the Charles Rivers Co. (Wilmington, MA).

Surgical Methods

Rats were anesthetized with chloral hydrate (350 mg/kg, i.p.) and the right sciatic nerve was exposed and either crushed with watchmaker’s forceps or transected at a level below the branch to the biceps femoris. Transected sciatic nerves were ligated with 3-0 silk at the same level, cut distally, and the distal stump removed to prevent regeneration. The left sciatic nerve was left untreated. Young (3–4 months) animals were sacrificed 1, 2, 4, 8, and 16 weeks after surgery (n = 2–5 rats at each time point). Similarly, one sciatic nerve was transected and the other left untreated for 4 weeks in 14 (n = 2), 21 (n = 2) and 26 (n = 1) month old Sprague-Dawley and 16 (n = 3) month old Fischer 344 rats. In a few young animals (n = 3), one sciatic nerve was transected; simultaneously the other sciatic nerve was crushed in order to directly compare the differences between the two types of peripheral nerve injury in the same animal.

Two animals received bilateral sciatic nerve transection 3–4 days prior to dorsal rhizotomy of the sensory roots arising from the L3 to L6 DRG on one side, thereby abolishing most of the primary afferent input to the ipsilateral gracile nucleus from the sciatic nerve. The animals were allowed to survive for 28 days before sacrifice.

Histologic Methods

Animals were anesthetized with chloral hydrate (350 mg/kg, i.p.) and subsequently perfused with heparinized physiological saline followed by freshly prepared and filtered 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.3. The brain and spinal cord with attached lumbar DRG were removed and postfixed overnight in the same fixative at 4°C. Samples of the medulla at the level of the gracile nuclei and the L4–5 spinal cord were cryoprotected with 30% sucrose in PBS until the tissue samples sank. Blocks of medulla and spinal cord were embedded in OCT, 20 μm thick sections were cut on a cryotome, collected as floating sections, and stored in PBS at 4°C until used. L4 and/or L5 DRG were cut as 10 μm thick frozen sections on a cryostat, mounted on gelatin-coated slides, and stored at −20°C.

Immunohistochemical Methods

Light Microscopy: Sections were stained using an immunogold silver method or by fluorescence immunohistochemistry. The primary antisera used in this study were NPY (rabbit anti-porcine NPY, 1:1,000; Peninsula Labs, Belmont, CA); CGRP (rabbit anti-CGRP, 1:1,000; Peninsula Labs); and GAP-43 (rabbit anti-GAP-43 polyclonal antisera, 1:400; kindly provided by Dr. Mark Willard). Antisera were applied to the sections overnight at 4°C. In some cases gold-conjugated anti-rabbit IgG serum (1:40) was next added to the sections for 1 hour at room temperature which were subsequently silver-enhanced according to the manufacturer’s protocol (Amersham, Arlington Heights, IL). In other cases the second step was replaced by incubation with fluorescein-conjugated sheep anti-rabbit IgG (diluted 1:200), washed and examined with a fluorescence microscope. We found the fluorescence method helpful in visu-
TABLE 1
Effect of Sciatic Nerve Transection or Crush Injury on L4 + L5 DRG NPY Content Determined by Radioimmunoassay

<table>
<thead>
<tr>
<th>Group</th>
<th>Right (fmol/L4 + 5DRG)</th>
<th>Left (fmol/L4 + 5DRG)</th>
<th>R/L ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoperated controls</td>
<td>70 ± 5</td>
<td>75 ± 6</td>
<td>0.96 ± 0.09</td>
</tr>
<tr>
<td>Transection (n = 4)</td>
<td>273 ± 43*</td>
<td>118 ± 17†</td>
<td>2.37 ± 0.28*</td>
</tr>
<tr>
<td>Crush injury (n = 3)</td>
<td>130 ± 18‡</td>
<td>93 ± 13</td>
<td>1.40 ± 0.05*</td>
</tr>
</tbody>
</table>

In this experiment right sciatic nerves were either transected or crushed 28 days prior to sacrifice; left sciatic nerves were not injured. A few unoperated control animals were also sacrificed. Values represent the mean ± SEM of the numbers of animals in parentheses.

* = p ≤ 0.01 versus unoperated control DRG.  
† = p ≤ 0.05 versus unoperated control DRG.  
‡ = p ≤ 0.05 versus right transected DRG.

Analyzing fine axonal varicosities and their arborizations. Stained sections were mounted on slides and overslipped with 1:1 PBS/glycerol. Immunohistochemical controls included replacement of the primary antiserum by normal rabbit serum, which resulted in the absence of specific staining in the gracile nuclei, dorsal horn of the spinal cord and DRG.

Ultrastructural Immunohistochemistry: Rats (3–4 months, n = 2; 16 months, n = 3) to be used for ultrastructural immunohistochemistry were perfused transcardially with heparinized saline at 4°C followed by 4% paraformaldehyde containing 0.1% glutaraldehyde, pH 7.3. The brain was removed and post-fixed overnight at 4°C. A 2 mm thick block of medulla containing the gracile nuclei was frozen in liquid nitrogen, thawed, and 50 µm sections were cut on a vibratome. The thick sections were immunostained with NPY using the same antibodies and concentrations as for light microscopic sections. The reaction products were visualized with an avidin-biotin method using a commercial kit (Vectastain, Vector Labs, Burlingame, CA). The sections were then osmicated with 1% OsO₄ and ultrathin sections were cut. Alternate sections were stained with lead citrate and uranyl acetate or left unstained, and examined with a JEOL 1200 electron microscope.

NPY Radioimmunoassay

Young (3–4 months old) rats were subjected to either right sciatic nerve transection (n = 4) or crush (n = 3) injury; the left sciatic nerve was left intact. A few animals (n = 5) formed an age-matched nonsurgical control group. Twenty-eight days after the surgery, the rats were anesthetized, perfused with saline at 4°C and decapitated. The L4 and L5 DRG from both operated and unoperated sides were removed within 15 minutes, immediately frozen on dry ice and stored at –20°C until assay. The protein extraction and subsequent radioimmunoassay were performed at the same time for samples from controls and operated animals using previously reported methods (29). Tissues were sonicated in acidic acetone and centrifuged. The supernatants were first dried and then resuspended in the assay buffer (0.1 M sodium phosphate/saline, pH 7.4, containing 1% Triton X-100 and 1% gelatin). Pairs of DRG, buffer alone, or buffer containing NPY standards (Amersham) were mixed with trace levels of ¹²⁵I-NPY (porcine NPY, Amersham) and primary antibody (rabbit anti-porcine NPY, RAS 7180, Peninsula Labs) and incubated overnight at 4°C. A second antibody (goat anti-rabbit serum, Antibodies Inc., Davis, CA) was subsequently added, allowed to react for 30 minutes at room temperature before the solution was centrifuged and the precipitate counted in a Micromedic gamma counter. Each individual tissue extract was measured in duplicate. The within-assay coefficient of variation was 5% or less. The Ic50 in this assay was 50 pM. Neuropeptide Y concentrations were expressed as femtomoles of NPY for each combined pair of L4 and L5 DRG.

RESULTS

Alteration of NPY Immunoreactivity after Sciatic Nerve Injury in Young Rats

Dorsal Root Ganglia (DRG): No NPY-immunoreactive neurons were observed in the L4 and L5 DRG of young uninjured control rats. As early as 7 days after sciatic nerve crush injury, DRG neurons became NPY-immunoreactive and increased in intensity to an apparent maximum at 4 weeks (Fig. 1A, B) compared to the uninjured contralateral DRG (not shown). Neuropeptide Y-immunoreactive cells were predominantly large diameter neurons (arrows, Fig. 1B) in comparison to smaller neurons (arrowheads, Fig. 1B) which remained unlabeled. Transection injury resulted in comparable NPY upregulation at early times. After extended periods (up to 16 weeks) the DRG of transected sciatic nerve contained many NPY-positive neurons in contrast to crush injury, in which the number of NPY-positive neurons progressively declined after an apparent maximum 4 weeks after injury with only a few positive neurons maintained for 16 weeks.

In order to confirm and quantitate the NPY immunohistochemical alteration in the axotomized DRG, the NPY content of combined L4 and L5 DRG was determined by radioimmunoassay (Table 1). In accordance with our immunohistochemical observations, the results showed a significant increase of radioimmunoassayable

NPY in the DRG following sciatic nerve axotomy; however, in contrast to the immunohistochemical results, ganglia from uninjured control animals did contain some NPY by radioimmunoassay. The effect of transection on the induction of upregulation of NPY in the ipsilateral ganglia was greater than that of crush injury when compared 28 days postinjury. The DRG contralateral to sciatic nerve crush injury showed a small but statistically significant increase in NPY content compared with unoperated controls, although only rare immunohistochemically reactive neurons were observed. Thus, the relative magnitude of induction of NPY in axotomized DRG neurons was greater when compared to uninjured DRG than DRG contralateral to crush injury.

Spinal Cord: In uninjured control L4–L5 spinal cord, NPY fluorescence was recognized as a dense band outlining laminae I and II (arrowheads, Fig. 1C) and was also found as scattered delicate processes in the central gray matter. Dense NPY fibers appeared in ipsilateral laminae III–IV (arrows, Fig. 1C) beginning 7 days after sciatic axotomy and reached an apparent maximum at 28 days. The density and extent of distribution of NPY-immunoreactive fibers in the dorsal horn were increased in individual animals with ipsilateral transection injury (arrow, Fig. 1D) compared to simultaneous contralateral crush injury (arrowhead, Fig. 1D). By day 28 after crush injury, NPY-immunoreactive fibers in the ipsilateral laminae III–IV could no longer be recognized; however, after transection of the sciatic nerve, laminae III–IV of the dorsal spinal cord remained NPY-positive for at least 16 weeks after the injury, the latest time point examined. Swollen NPY-containing dystrophic axons were never seen in the dorsal horn of non-surgical control animals of any age or after either crush or transection injury.

Gracile Nucleus: The gracile nuclei of unoperated animals failed to show detectable NPY immunoreactivity. As early as 7 days after sciatic nerve crush or transection injury, NPY-immunoreactive fibers were recognized in the ipsilateral gracile nucleus. The density and immunoreactivity of NPY-containing axons in the ipsilateral gracile nucleus (arrow, Fig. 1E), which extended into the preterminal axons of the gracile fasciculus (arrowhead), were progressively increased with time compared to the uninjured contralateral side (Fig. 1F), reaching an apparent maximum at 28 days after sciatic axotomy. Predominantly delicate axons as well as a few coarse, slightly enlarged (≤5 μm) NPY-containing axons (arrow, Fig. 1G) were identified in the ipsilateral gracile nuclei of young animals following sciatic nerve injury, although markedly enlarged (10–30 μm) axons comparable to dystrophic axons found in aged animals were not encountered. Rare NPY-containing neurites could also be seen in some contralateral gracile nuclei at 28 days, most likely involving the few DRG-derived axons known to project to the contralateral gracile nucleus or the result of upregulation of NPY in the contralateral DRG (30). Intrinsic neurons comprising the gracile nucleus consistently failed to show NPY immunoreactivity in uninjured controls or at any time after crush or transection injury.

The pattern and extent of gracile NPY upregulation was indistinguishable for the first 14 days after sciatic crush or transection but differed thereafter. Specifically, NPY-containing processes were maintained in the ipsilateral gracile nuclei without developing markedly enlarged NPY-containing dystrophic axons as long as 8–16 weeks (Fig. 1H) after transection injury in which axonal regeneration was prevented. However, the density of NPY-positive fibers in the gracile nucleus after crush injury progressively decreased after day 28, presumably reflecting regeneration of the injured peripheral sensory axon, and resulted in only a few fibers of weak immunofluorescence in the gracile nucleus 8 weeks after axotomy and none after 16 weeks. This observation was further confirmed in an experiment in which transection (arrow, Fig. 1I) and crush (arrowhead, Fig. 1I) injuries

Fig. 1. Immunolocalization of NPY in young adult (3–5 months) rat DRG and its central projections in response to sciatic nerve injury. A, B) NPY immunoreactivity in DRG 28 days after ipsilateral sciatic nerve transection injury. NPY immunoreactivity was increased in medium to large diameter neurons (arrows, B) compared to unlabeled small neurons (arrowheads, B) (NPY immunofluorescence, A: 100×; B: 250×). C) NPY immunoreactivity in the dorsal horn of the spinal cord formed a dense band in laminae I and II (arrowheads) on the uninjured left side and expanded into laminae III and IV on the right (arrows), shown 14 days after transection (NPY immunofluorescence, 100×). D) Combined transection and crush injury to the sciatic nerve. Prominent NPY immunoreactivity in laminae III and IV in the spinal dorsal horn was maintained 28 days after sciatic nerve transection injury (arrow = right) which has largely resolved in laminae III and IV after simultaneous crush injury (arrowhead = left) (NPY immunofluorescence, 100×). E, F) NPY immunoreactivity of parasagittal sections of the medullary gracile nucleus (arrow) and rostral gracile fasciculus (arrowhead) ipsilateral (E) and contralateral (F) to sciatic nerve transection performed 28 days previously (NPY immunofluorescence, 50×). G) Many delicate and a few coarse NPY-containing fibers and rare minute swellings (arrow) are shown at high magnification in the gracile nucleus 28 days after ipsilateral sciatic transection (NPY immunofluorescence, 400×). H) A coronal section of the gracile nuclei showed marked NPY immunoreactivity ipsilateral to 8 week sciatic transection injury (arrow) as well as a few NPY-containing processes in the uninjured contralateral gracile nucleus (NPY immunofluorescence, 100×). I) Immunoreactivity of NPY in the gracile nuclei was maintained 8 weeks after transection (arrow) of the right sciatic nerve but was diminished after simultaneous crush (arrowhead) injury to the left sciatic nerve (NPY immunofluorescence, 100×).
Fig. 2. Immunolocalization of selected neuropeptides and GAP-43 in dorsal horn and gracile nucleus in response to sciatic nerve injury. A, B) CGRP immunoreactivity was depleted 14 days after axotomy in the medial aspects of laminae I and II (arrow, A) which was largely restored 28 days after crush injury in simultaneous crush and transection injuries (B, arrow = transection; arrowhead = crush) (CGRP immunofluorescence, 100×). C) Increased GAP-43 immunoreactivity in the dorsal horn of the spinal cord was greater in transection than crush injury shown 28 days after axotomy (arrow = transection; arrowhead = crush) (GAP-43 immunolocalization, silver intensification of immunogold method, 100×). D) GAP-43 immunoreactivity was increased in the gracile nucleus ipsilateral (arrow) to a 28 day sciatic nerve transection injury (GAP-43 immunolocalization, silver intensification of immunogold method, 100×). E, F) L3–6 rhizotomy prevented the increase in NPY immunoreactivity which developed with transection injury in the gracile nucleus (E, arrow = transection; arrowhead = transection + rhizotomy) and laminae III and IV of the dorsal horn of the spinal cord (F, arrow = transection; arrowhead = transection + rhizotomy) (NPY immunofluorescence, 100×). G) CGRP immunoreactivity was decreased in transected sciatic nerve after 28 days and further decreased with superimposed L3–6 rhizotomy (arrow = transection; arrowhead = transection + rhizotomy) (CGRP immunofluorescence, 100×).
were made to the right and left sciatic nerves, respectively, in the same animal.

Comparison of Altered NPY Immunoreactivity to CGRP and GAP-43 in Response to Sciatic Axotomy in Young Rats

A marker of small caliber primary sensory afferents, CGRP showed only a few positive fibers in uninjured control gracile nuclei and no substantive morphological changes in amount or distribution could be elicited either by sciatic nerve crush or transection injury. Dystrophic axonopathy involving CGRP-containing gracile axons was never observed. In the ipsilateral dorsal horn of the lumbar spinal cord the medial aspects of laminae I and II showed depletion of CGRP immunoreactivity (arrow, Fig. 2A) by 14 days following sciatic nerve axotomy, which was greater in transection than crush injury. Restoration of CGRP immunoreactivity in the dorsal horn of the spinal cord was demonstrated by 28 days after crush injury (arrowhead, Fig. 2B) but remained depleted for the entire time course examined in animals with sciatic transection injury (arrow, Fig. 2B).

In order to examine the possible association of upregulated NPY expression with a marker of axonal growth, immunolocalization of the 43 kDa growth-associated protein (GAP-43) and NPY was performed on adjacent sections. GAP-43 immunoreactivity increased in laminae I and II, but not laminae III and IV, of the ipsilateral lumbar dorsal horn from 14 days on following sciatic nerve axotomy. GAP-43 immunoreactivity was increased in intensity and prolonged after transection compared to axonal crush injury, which is well shown in animals with combined sciatic transection (arrow, Fig. 2C) and crush (arrowhead, Fig. 2C) injuries. The overall intensity of GAP-43 immunoreactivity in the ipsilateral gracile nucleus (arrow, Fig. 2D) following sciatic nerve injury was increased in comparison to unoperated controls and the contralateral side. The increase in GAP-43 immunoreactivity was apparent in the ipsilateral gracile nucleus 28 days after sciatic nerve transection but was inconstant after sciatic nerve crush. Unlike the dorsal horn of the spinal cord where upregulated NPY but no obvious GAP-43 immunoreactivity was seen in laminae III–IV, the topographical distribution of GAP-43 coincided with that of induced NPY in the gracile nuclei. The pattern of upregulated gracile GAP-43 differed from that of NPY, however, in that it was diffuse and did not show an obvious neuritic pattern. In addition, 8 weeks after sciatic nerve transection, GAP-43 immunoreactivity was equivalent on both injured and control sides, while NPY remained strongly positive on the injured side.

Effect of Dorsal Rhizotomy on Transganglionic NPY Expression in Young Rats

Upregulated gracile NPY immunoreactivity may involve the central nerve terminals of neurons whose peripheral axons were injured in the sciatic nerve or, alternatively, may represent sprouting of nerve terminals of adjacent uninjured DRG neurons (i.e. those arising at different segmental levels not contributing peripheral axons to the injured level of the sciatic nerve) within the dorsal horn of the spinal cord and medullary gracile nucleus. To directly address the source of injury-induced NPY immunoreactivity in the gracile nucleus and dorsal horn of lumbar spinal cord, bilateral sciatic nerve transection was performed followed a few days later by unilateral rhizotomy of the L3 to L6 dorsal roots. Sciatic transection with dorsal rhizotomy completely prevented the development of upregulated NPY immunoreactivity in the ipsilateral gracile nucleus (arrowhead, Fig. 2E) compared to transection without rhizotomy (arrow, Fig. 2E) and induced an astrocytic reaction. In the lumbar spinal cord, dorsal rhizotomy prevented the development of NPY immunoreactivity in laminae III and IV (arrowhead, Fig. 2F) compared to transection without rhizotomy (arrow, Fig. 2F) but did not affect laminae I and II.

The NPY result was in striking contrast to changes in the distribution of CGRP-positive terminals following simultaneous transection and dorsal rhizotomy. CGRP immunoreactivity in spinal cord originates from DRG and the medial two-thirds of laminae I and II showed moderate loss of CGRP-positive materials following sciatic nerve transection (arrow, Fig. 2G), which was further depleted by simultaneous sciatic transection and dorsal rhizotomy (arrowhead, Fig. 2G).

Ultrastructural Immunolocalization of Gracile NPY in Young Rats

Electron microscopic immunolocalization of NPY was performed 28 days after sciatic transection injury, i.e. at the time of maximal NPY immunoreactivity by light microscopy. Neuropeptide Y-stained elements consisted of minute axonal processes, many surrounded by myelin sheaths (arrows, Fig. 3A) and other, somewhat larger,

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Fig. 3. Ultrastructural immunolocalization of NPY in the gracile nucleus of young rats 28 days following sciatic transection injury. A) NPY immunoreactivity was prominent in a subpopulation of small myelinated processes (arrows) surrounded by unlabelled axons (Osmium/NPY ultrastructural immunolocalization, ABC method, 18,210×). B, C) Scattered NPY-immunoreactive processes contained a variety of mitochondria and vacuoles, some with occasional small “filopodial” projections (arrow, C) (B: Osmium/NPY ultrastructural immunolocalization, ABC method; C: Osmium/lead citrate/uranyl acetate, NPY ultrastructural immunolocalization, ABC method; B: 30,000×; C: 40,000×).

processes containing significantly more cytoplasm and organelles (Fig. 3B), some with delicate “filopodial” processes (arrow, Fig. 3C), which are reminiscent of the growing tips or growth cones of regenerating axons. Although a few mildly enlarged NPY-containing processes were demonstrated, none had the ultrastructural features of classical NAD. Occasional NPY-containing processes exhibited synaptic specializations.

Neuropeptide Y Immunohistochemistry in Aged Control Animals and Its Response to Sciatic Nerve Transection

In unoperated middle-aged and aged (14–26 months) rats, no NPY-immunoreactive fibers were detected in the gracile nuclei, although at these ages numerous markedly swollen (10–30 μm) dystrophic axons are seen in one μm thick plastic sections (arrows, Fig. 4A). In the lumbar spinal cord of unoperated aged rats, the expected NPY-containing normal-appearing fiber network was seen in the substantia gelatinsosa, where no dystrophic axonal swellings could be found. Neuropeptide Y-immunoreactive neurons were not found in the lumbar DRG of unoperated aged control rats.

Four weeks after sciatic nerve transection, ipsilateral gracile nuclei from 14–26 month old rats showed a network of delicate to coarse NPY-positive fibers (Fig. 4B), whose density varied from that comparable to young rats (Fig. 4B–D) to a more sparse distribution (Fig. 4E) in the eldest (26 months) animal. In addition, many 10–30 μm swollen dystrophic axons were densely NPY-immunoreactive (arrows, Fig. 4B, C). Occasionally, tangled aggregates of NPY-immunoreactive fibers intermingled with dystrophic axons were found in the gracile nuclei (arrow, Fig. 4D).

The L4 and L5 DRG of 14–22 month old rats sampled 28 days after sciatic nerve transection showed many large NPY-immunoreactive neurons in the ipsilateral DRG (Fig. 4F) as well as rare individual NPY-immunopositive neurons in the contralateral unoperated DRG (not shown). The DRG from a single 26 month old rat showed rare NPY-immunopositive neurons only in the ipsilateral ganglia.

Transverse sections of the L4 lumbar spinal cord of aged rats sampled 28 days after sciatic nerve transection demonstrated delicate NPY-immunoreactive fibers, although no dystrophic axons, in laminae III and IV.

Ultrastructural Immunolocalization of NPY in Aged Gracile Nucleus

The ultrastructural appearance of the gracile nucleus of aged uninjured rats consisted of numerous enlarged dystrophic axons (arrows, Fig. 5A), typically surrounding the cell bodies of intrinsic gracile nucleus neurons, containing a variety of subcellular structures including large compact membranous aggregates (arrows, Fig. 5B),lucent clefts, and dense unstructured axoplasm with scattered dense core and agranular vesicles. Ultrastructural localization of NPY immunoreactivity in the gracile nucleus of 16 month old rats was examined 17–28 days after sciatic nerve transection and showed several patterns of immunolocalization. Neuropeptide Y immunoreactivity was found in small axons (arrows, Fig. 5C, D) comparable to those seen in young rats. In addition, several patterns of NPY immunoreactivity were demonstrated in dystrophic axons including diffuse positivity (arrowheads, Fig. 5C, D) in granular axons, patchy deposition within accumulated membranous axonal contents (arrows, Fig. 5E), and label confined to a subplasmalemmal rim (arrows, Fig. 5F–H) surrounding largely unlabeled membranous aggregates.

Immunolocalization of GAP-43 and CGRP in Aged Rats

Immunolocalization of GAP-43 revealed increased activity in the ipsilateral gracile nuclei (arrow, Fig. 4G) and dorsal horn (arrow, Fig. 4H) in all aged rats examined 28 days after sciatic nerve transection. In the gracile nuclei, dystrophic axons were not immunoreactive for GAP-43 in all aged non-surgical control rats and did not develop GAP-43 immunopositivity with transection injury. Similarly, antisera against CGRP failed to stain gracile NAD in either aged non-surgical controls or aged rats 28 days after sciatic nerve transection.

DISCUSSION

Alterations in the central axonal terminals of primary sensory neurons following peripheral axotomy (16, 19, 22, 26, 31, 32), a process which has been variously called

Fig. 4. Alterations in the DRG and its central projections in aged rats. A) Large numbers of dystrophic axons (arrows), ranging in diameter from 10–30 μm, are shown in a one μm thick plastic section of the gracile nucleus of a 21 month old rat (Toluidine blue, 360X). B–E) Intensely NPY-immunoreactive axons developed ipsilateral to 28 day sciatic transection injury in 14–26 month old rats including numerous dystrophic axons (arrows, B, C). Unusual aggregates of coarse processes were also encountered (arrow, D). Dystrophic axons showed diminished NPY immunoreactivity (E) in a 26 month old rat, the oldest animal examined (NPY immunofluorescence, B: 100X; C–E: 250X). F) NPY immunolocalization in the DRG of a 21 month old rat ipsilateral to a 28 day transected sciatic nerve (NPY immunofluorescence, 100X). G, H) GAP-43 immunoreactivity was increased in the ipsilateral gracile nucleus (arrow, G, 21 month old rat) and dorsal horn of the spinal cord (arrow, H, 14 month old rat) 28 days after sciatic transection injury (NPY immunolocalization, silver intensification of immunogold method, 100X).
transganglionic degeneration, synaptogenesis or atrophy, has been most extensively studied in the substantia gelatinosa of the spinal cord, a site in which predominantly nociceptive unmyelinated fibers (C fibers) terminate. These terminals, derived from small to medium-sized DRG neurons richly endowed with substance P and CGRP, are depleted after permanent peripheral axotomy. However, synaptogenesis of CGRP-containing terminals (33) and the appearance of GAP-43-immunoreactive axon sprouts reported in substantia gelatinosa following sciatic axotomy (23, 24, 31, 32) suggest the occurrence of a concomitant regenerative effort. The response of dorsal column sensory afferents to peripheral axotomy may differ from those to the substantia gelatinosa since they are composed of myelinated axons derived predominantly from large diameter DRG neurons (20, 28).

Our results have confirmed and extended the previous studies (19, 22, 26) of axotomy-induced increased NPY immunoreactivity predominantly in medium to large DRG neurons and in laminae III and IV of the ipsilateral dorsal horn. Dorsal lumbar rhizotomy abolished upregulation of NPY immunoreactivity in the gracile nuclei which is most consistent with its origin from the same DRG neurons whose peripheral processes were axotomized and against the idea that the gracile NPY alterations were the result of collateral sprouting and NPY upregulation in central terminals arising from adjacent uninjured DRG or from neurons intrinsic to the gracile nucleus. In the spinal cord, transection followed by dorsal rhizotomy resulted in depletion of NPY immunoreactivity in laminae III and IV, but not that in the substantia gelatinosa, which is known to receive terminals from intrinsic spinal cord neurons (34, 35). The role of NPY upregulation in the response to injury is unknown; how-

Fig. 5. Ultrastructural immunolocalization of NPY in dystrophic nerve terminals in aged rats. A, B) Multiple dystrophic axons (arrows, A) which have not been immunostained for NPY vary in subcellular contents and cluster around a gracile neuron in a 14 month old rat. At higher magnification (B), tightly compacted membranous aggregates (arrows) and dense axoplasm with scattered vacuoles and mitochondria are demonstrated (Osmium/lead citrate/uranyl acetate stain, A: 3,210×; B: 25,710×). C) NPY immunoreactivity in gracile nucleus of a 14 month old rat was found in non-dystrophic delicate axons (arrows) as well as enlarged swellings (arrowheads) with granular axoplasm, scattered mitochondria and clefts (Osmium/ NPY ultrastructural immunolocalization, ABC method, C: 6,430×; D: 16,070×). E, F) Markedly enlarged dystrophic axons showed patchy (arrows, E) or subplasmalemmal (arrows, F) NPY immunoreactivity (Osmium/NPY ultrastructural immunolocalization, ABC method, 7,860×). G, H) A dystrophic axon with predominantly subplasmalemmal NPY immunoreactivity (arrows) is shown at higher magnification in H (Osmium/ NPY ultrastructural immunolocalization, ABC method, G: 4,290×; H: 12,290×).
ever, it has been recently demonstrated (36) that large DRG neurons, but not intrinsic gracile nucleus neurons, also upregulate NPY receptor mRNA in response to axotomy, a result which suggests a potential autoreceptor function for upregulated NPY receptors and NPY. In the normal dorsal horn of the cat NPY has been shown to decrease stimulus-evoked substance P release (37). Cultured DRG neurons treated with NPY exhibit diminished Ca$^{2+}$ influx via voltage-sensitive Ca$^{2+}$ channels (38) while increasing cytoplasmic free Ca$^{2+}$ (39) and stimulating the synthesis of selected phosphoinositides (39). Neuropeptide Y upregulation may, therefore, function to modulate excitability of sensory neurons and diminish phantom sensory dysfunction after peripheral axotomy.

Subpopulations of DRG neurons differ in size, trophic dependence during development (40), target and neuro-
peptide content. Recent advances in the understanding of the role of trophic factors in the sensory nervous system have suggested, at least during development, that large diameter DRG neurons are most dependent on neurotrophin-3 (NT-3), while small DRG neurons depend on nerve growth factor (NGF) for their survival and differentiation (40–43). Similarly, only certain subpopulations display injury-induced increased NPY immunoreactivity. It is likely that transganglionic changes of primary sensory neurons are more diverse than previously considered, reflecting the diversity of cell size, neuropptide expression and potential for regeneration.

Immunohistochemical and ultrastructural changes in the gracile axon terminals of axotomized DRG neurons of young rats consist of the appearance of NPY-immunoreactive coarse and delicate myelinated and non-myelinated processes, which occasionally show an apparently abrupt increase in diameter with the development of an irregular fan-shaped terminus with scattered subcellular organelles that is reminiscent of the regenerative growth cone. Previous studies of transganglionic changes in rat gracile nucleus have described an increase in microtubules and smooth endoplasmic reticulum in enlarged nerve terminals as early as 10–15 days after crush injury, resolving soon thereafter (21), or as late as 32 weeks (44) after sciatic nerve transection. It is likely that the pre-existing central synaptic contacts of axotomized neurons would be subjected to considerable dynamic structural changes or remodeling which may be associated with the degeneration and removal of ineffective terminals followed by a regenerative phase. Alternatively, degeneration and regeneration may occur in different subsets of axotomized neurons. These morphological changes could represent the earliest stages of NAD. However, these axons, which rarely reach diameters of 5–10 μm, contain amorphous material, neurofilaments and a variety of normal and degenerating subcellular organelles (44) but fail to develop the size or gamin of distinctive ultrastructural features (e.g. anastomosing tubulovesicular elements or ring, fingerprint and paracrystalline membranous bodies, etc. [1]) of mature gracile NAD (2–6). The few dystrophic-appearing profiles in long-term studies may reflect the development of age-dependent NAD. Nonetheless, some of the “reactive” processes we observed in young animals may be, in fact, the first stages of NAD which may resolve or be transformed by superimposed insults such as age, vitamin E deficiency, or diabetes into frankly dystrophic elements. Our ultrastructural immunolocalization studies continued to show similar reactive processes accompanying NPY upregulation in frankly dystrophic axons.

**Transaction vs Crush Injury:** Our results showed clear differences between transection and crush injury of the peripheral nerve on the extent and time course of NPY upregulation in DRG neurons and their central processes. Thus, both in the gracile nuclei and dorsal horn laminae III–IV, the NPY-immunoreactive fibers were typically more numerous and intense in transection than in crush injury at any time points examined after 14 days and by DRG NPY radioimmunoassay performed on day 28. In crush injury, NPY immunoreactivity rapidly declined in DRG and in their central projections, which most likely represents the effect of ongoing regeneration of the peripheral axon. These results differ from those of CGRP, which remains depleted in the substantia gelatinosa after sciatic nerve transection but is largely restored 14 days after sciatic nerve crush, a time at which target reinnervation is not completed. These results are similar to the response of substance P in the substantia gelatinosa (21) in which sciatic crush injury consistently failed to induce the same changes as transection. The coordinate changes in substance P and CGRP may reflect the role of CGRP as a modulator of substance P activity (20). Individual peptidergic DRG neurons may restore their normal level of peptide expression if successful regeneration follows peripheral axotomy. However, the signal(s) to control peptide production in axotomized sensory neurons following peripheral axotomy may not only be derived from reinnervated targets but through interactions between regenerating axons and Schwann cell tubes forming bands of Bungner. In support of this idea, the marked upregulation of NGF and other trophic substances has been described in the distal segment of axotomized nerve which is normalized during axonal regeneration (43). Transganglionic changes of central terminals of primary sensory neurons may vary greatly depending on the presence or absence of peripherally derived signals.

**Upregulation of GAP-43:** The 43 kDa “growth-associated protein” (GAP-43), a constituent of the axonal growth cone, is found in a variety of neuronal growth states ranging from embryonic development and axonal regeneration to more subtle degrees of synaptic plasticity (reviewed in 45). GAP-43 is known to increase in DRG neurons and their central connections following peripheral nerve injury (25, 46). Unlike the strong immunoreactivity of GAP-43 in the substantia gelatinosa ipsilateral to sciatic axotomy, laminae III and IV of the dorsal horn of the spinal cord were immunoreactive for NPY but not substantially for GAP-43. In the same animals, immunoreactive GAP-43 was only modestly increased in the ipsilateral gracile nuclei. In addition, while NPY immunohistochemistry showed a fiber network with occasional dilated axons in the gracile nuclei, GAP-43 immunoreactivity consisted of delicate threads or a fine granular pattern suggesting their presence in the unmyelinated or small myelinated axons. In our study GAP-43 immunoreactivity remained robust in the substantia gelatinosa after transection and extirpation of the distal sciatic segment, but declined with time after crush injury.
in which peripheral regeneration of the sciatic nerve routinely followed.

Transganglionic Responses in Aged Animals: In all 14–26 month old rats examined 28 days following unilateral sciatic nerve transaction, transganglionic alterations of NPY immunoreactivity developed in ipsilateral DRG neurons and their central axonal terminals. Although both small delicate and coarse NPY-containing processes developed as in young animals, the most striking alteration in aged animals was the development of NPY-immunoreactive dystrophic axons. Our results establish the likely origin of many dystrophic axons in the gracile nucleus from neurons in the lumbar DRG and, most likely, from medium to large diameter primary sensory neurons which develop NPY immunoreactivity with axotomy. Other studies of anterograde axonal transport from lumbar DRG have demonstrated labeling of terminals in gracile nuclei, including early dystrophic forms (47). Aged animals may respond to sciatic axotomy with transganglionic changes characterized from the outset by NAD, i.e., axotomy may induce dystrophic changes de novo in aged animals rather than fill pre-existent dystrophic endings with NPY, mechanisms which are not discriminated by our experiments. Nonetheless, clearly the neurons whose gracile terminals show dystrophic changes are capable of reacting to peripheral axotomy by appropriately upregulating NPY and anterogradely transporting it to the dystrophic terminals. Our limited observation that only a few DRG neurons, gracile axons, and gracile NAD were NPY-immunoreactive in a 26 month old rat, the eldest examined in this study, suggests these functions may eventually decline with further aging which could also reflect age-related loss of DRG neurons. In addition, DRG neurons which do not express NPY could also contribute to gracile NAD. Cholecystokinin-like immunoreactive dystrophic axons have been described in the gracile nuclei of aged rats which progressively declined with advanced aging (48). It is known that occasional CGRP-containing unmyelinated axons also ascend in the dorsal column (49); however, CGRP-containing dystrophic axons were not observed in our studies.

In aged animals GAP-43 stained only small neuritic processes while NPY labeled both small neurites and dystrophic elements. Subpopulations of projections may show differences in transganglionic alterations, e.g. small regenerative elements may label with GAP-43 in comparison to dystrophic elements. These results are consistent with the report that in the spinal cord GAP-43 mainly occurs in unmyelinated and small myelinated axons (50) or that signals which alter GAP-43 expression in unmyelinated and myelinated afferents operate with a different time course following peripheral axotomy (51). Dystrophic axons have been variously reported to be GAP-43-immunoreactive in cortical neurites of Alzheimer's disease (52) or lacking GAP-43 immunoreactivity in rat and human aged sympathetic ganglia (53). Alternatively, the cytoplasmic expansion of dystrophic terminals may simply dilute GAP immunoreactivity.

The pathogenesis of NAD is unknown, although a variety of mechanisms have been proposed (1, 47, 54). Although occasionally considered evidence of a "degenerative" axonal change, frank degeneration of dystrophic axons with phagocytosis by macrophages or association with significant amounts of extracellular debris are not typically seen in NAD. Dystrophic axons may persist stably for extended periods without degenerative sprouting or degeneration. Unilateral NAD which develops in the gracile nucleus ipsilateral to lower limb amputation in the cat (12) suggests that injury to the peripheral portion of the DRG neuron or failure to complete peripheral regeneration may produce central transganglionic changes which, without resolution, eventually become dystrophic. Our limited observations up to 16 weeks in young animals did not reproduce this finding in the rat; however, the superimposition of age-related changes in the older cats used in that study (12) may have provided additional elements critical to the development of gracile NAD. The chronic turnover of nerve terminals, which appears to represent a normal phenomenon underlying synaptic plasticity in many sites within the central and peripheral nervous systems (55, 56), may provide the baseline substrate for the development of NAD as the result of additional insults which may include aging (2, 3), vitamin E deficiency (4, 5) and diabetes (7). Interestingly, in the spinal dorsal and ventral horns in which some central processes of NPY-immunoreactive sensory neurons terminated, NPY-immunoreactive dystrophic axons did not develop, even though classical neuroanatomical studies suggest branching of large diameter sensory axons to ascend in the dorsal column as well as to project locally within the spinal cord segment subserving various reflexes. This may be a function of length of the central nerve segment as seen, for example, in central–peripheral distal axonopathies. The lesser degree of NAD involving the shorter central projections of cervical DRG to the cuneate nucleus in aged animals may also represent a similar function of length of the central nerve process. Although clearly speculative at this time, it is possible that central connections of branches from a single parent axon may develop NAD in the gracile nucleus and fail to do so in its collateral branches into the local dorsal and ventral horns of the spinal cord, i.e. all branches may not be equally involved. Postsynaptic elements unique to the gracile nuclei may also contribute to the development of gracile NAD. Neuroaxonal dystrophy is, however, not specific for a single experimental or clinical disease entity and may be seen in a wide variety of experimental and clinical conditions in which multiple pathogenetic mechanisms may contribute. The prevertebral sympathetic
ganglia of aged human subjects reproducibly develop a form of NAD in which dystrophic axons are consistently labeled by antisera to NPY and are accompanied by an increased number of small diameter NPY-containing axonal profiles (57), which is reminiscent of the injury response of sensory projections to the gracile nucleus described in the current study.

In conclusion, disorders of synaptic plasticity may represent a common theme in neuropathology resulting in subtle or overt protein neuronal system dysfunction. The use of basic neurobiologic techniques to begin to understand the mechanisms underlying such “synaptic dysplasia” in relatively simple primary sensory pathways may be applicable to some of the most enigmatic human neurodegenerative diseases.

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