Regulation of Aberrant Neurofilament Phosphorylation in Neuronal Perikarya. 
I. Production Following Colchicine Application to the Sciatic Nerve

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Abstract. Neurofilament (NF) triplet proteins are normally poorly phosphorylated 
in neuronal perikarya, the two high molecular weight polypeptides becoming exten-
sively phosphorylated once the NF enters the axon. Abnormal expression of phos-
phorylated NF (pNF) epitopes in neuronal perikarya has been revealed using mono-
elonal antibodies in a variety of human and experimental conditions. In the present 
study, we asked whether pNF epitopes are expressed in sensory neurons in the L4 
and L5 dorsal root ganglia (DRG) following blockade of fast axonal transport in a 
model producing few (<1%) degenerating fibers. Colchicine (5 mM) was briefly (45 
minutes) applied to the sciatic nerve at mid-thigh twice (once weekly) and the animals 
studied two weeks following the first colchicine application; contralateral nerves were 
either treated with saline or crushed. Modest to intense immunoreactivity was found 
with antibody 07-05 (directed against NF epitopes on the two high molecular weight 
NF polypeptides) in 30.4% and 45.1% of DRG neurons from colchicine-treated and 
crushed nerves, respectively; only a rare cell body demonstrated immunostaining 
from the contralateral saline-treated nerves. Immunoreactivity was not observed with 
antibody 07-05 at two and five days following single colchicine application. In a 
separate study, colchicine or saline was applied (as above) 1–2 cm proximal to a 
nerve crush. Colchicine application did not influence the extent of DRG neurons 
expressing pNF epitopes; immunostaining with antibody 07-05 was present in 44.7% 
and 43.8% of DRG neurons from saline-treated and colchicine-treated crushed nerves, 
respectively. The results indicate that structural interruption of nerve–target contact 
is not necessary to induce aberrant NF phosphorylation in neuronal perikarya. It is 
suggested that loss of a retrogradely transported "trophic" signal(s) triggers this re-
sponse.

Key Words: Axon reaction; Axotomy; Colchicine; Dorsal root ganglia; Neurofila-
ment epitopes; Neurofilament, nonphosphorylated; Neurofilament, phosphorylated.

INTRODUCTION

Phosphorylated neurofilament (pNF) epitopes, while abundant in axons, are nor-

mally present in very low levels in neuronal perikarya (1, 2). Neuronal perikarya 
demonstrating aberrant expression of pNF epitopes has been observed in a variety 
of human and animal diseases, including motor neuron disease (3–5); progressive 
supranuclear palsy (6); Creutzfeldt-Jakob disease (7); the disorder in copper metab-
olism, swayback, observed in lambs (5); and Alzheimer’s disease, where they are 
observed in neurofibrillary tangles, (8–14). The significance of this aberrant NF 
phosphorylation in the pathogenesis of any of these diseases is unclear. However, 
studies of experimental models in laboratory animals provide a means to address

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this issue. For example, the observation that abnormal expression of pNF epitopes in neuronal perikarya can be produced by nerve transection (axotomy) (15–17) suggests that, in at least some situations, this alteration may arise secondary to axonal injury.

Axotomy of peripheral nerve fibers results in a stereotypic pattern of structural changes as well as a reordering of protein synthesis in the neuronal perikaryon, termed the axon reaction (for reviews, see 18–20). Coincident with an overall increase in protein synthesis, there is a selective decrease in NF synthesis (21–24). Consequently, there is a reduction in axonal area (25, 26) reflecting the linear relationship between total numbers of NFs and cross-sectional area (25, 27). Atrophy begins near the neuronal perikaryon, advancing in a somatofugal fashion down the nerve at the rate of slow (i.e. NF) transport (somatofugal axonal atrophy). Reestablishment of normal caliber depends upon reconnection of the axon with its target tissue (25). Moreover, recent studies from our laboratory using colchicine to inhibit retrograde axonal transport (28) indicate that structural interruption of nerve–muscle contact is not necessary to initiate somatofugal axonal atrophy. Taken together, these observations suggest that a retrogradely transported target-derived “trophic” signal functions to regulate at least one component of the axon reaction (i.e. somatofugal axonal atrophy).

In the present study, we have asked whether inhibition of retrograde axonal transport by colchicine is sufficient to induce abnormal expression of pNF epitopes in neuronal perikarya of sensory neurons in the L4 and L5 dorsal root ganglia (DRG) of the rat. Colchicine was applied to the sciatic nerve using a procedure previously shown to block retrograde axonal transport in the majority of sensory neurons while producing axonal degeneration in only a few (<1%) fibers (28). Thus, using this protocol it was possible to determine if aberrant NF phosphorylation, like somatofugal axonal atrophy, can be produced in intact nerve fibers. The results suggest that aberrant neurofilament phosphorylation represents a component of the axon reaction, expression of which is also regulated by loss of a retrogradely transported “trophic” signal.

MATERIALS AND METHODS

Animals and Colchicine Application

Thirteen six week (wk)-old, two 44 day-old, and two 47 day-old Sprague-Dawley male rats were used in this study. Eight six wk-old rats had their left sciatic nerve treated with colchicine using the procedure described by Riley and Fahlman (29), as modified by Gold and Dark (28). Briefly, rats were anesthetized with an intraperitoneal (IP) injection of chloral hydrate (400 mg/kg), the sciatic nerve exposed at the mid-sciatic region, isolated from the underlying musculature using a small strip of paraffin, and a small cotton pledget soaked in 0.2 ml of a 5 mM solution of colchicine (Sigma, St. Louis, MO) gently placed on top of the nerve for 20–25 minutes (min). The area was washed with saline, a second cotton pledget soaked in colchicine (0.2 ml) was applied for an additional 20–25 min (total colchicine exposure, 45 min), and the area was again washed with saline and the wound closed. The contralateral sciatic nerve was exposed as above and either treated with saline alone (contralateral controls) (n = 2) or crushed (n = 6) twice for 30 seconds using fine watchmaker’s forceps. Two additional six wk-old rats were anesthetized and the left sciatic nerve treated with saline and served as normal controls (n = 2), the right sciatic nerves served as normal, unoperated control nerves (n = 2). At one wk, the animals were reanesthetized with chloral hydrate and the nerves again treated (as above) with colchicine, saline, or crushed.

Two 47 day-old and two 44 day-old rats were anesthetized with chloral hydrate and the
left sciatic nerve was treated with colchicine (5 mM), as described above; the contralateral side was treated with saline. These animals were studied at two and five days, respectively.

In a separate study, three six wk-old rats were anesthetized with chloral hydrate, the left nerve was crushed and colchicine (5 mM) applied 1–2 cm proximal to the crush site, as described above; the right sciatic nerve was crushed and treated with saline. One wk later, the animals were reanesthetized with chloral hydrate and the nerves again were treated with colchicine or saline.

**Tissue Fixation and Preparation**

At eight weeks of age, the animals were heparinized, anesthetized with chloral hydrate and perfused through the ascending aorta with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.6). The L4 and L5 DRG were dissected following overnight fixation in situ (4°C), dehydrated in a graded series of alcohols, and embedded in paraffin.

**Immunocytochemistry**

The immunocytochemical procedures using the present series of antibodies has been described previously (30). Briefly, sections (10 μm) were cut and mounted on chrom-alum subbed slides, deparaffinized in xylene and ethyl alcohol, incubated in 3% normal goat serum for one hour (h), and incubated overnight with one of the following primary antibodies (1:1,000 dilution in 1% normal goat serum): antibody 2-135 (directed against a nonphosphorylated epitope of the 200 kilodalton [kDa] NF polypeptide); or antibodies 06-17 and 07-05 (directed against phosphorylated epitopes shared by the 200 and 145 kDa polypeptides) (1, 31, 32). Sections were incubated for one h in goat-antimouse secondary antibody (1:20), washed, incubated for one h in mouse peroxidase-antiperoxidase (1:200), the immunoreactivity visualized with 0.05% diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxidase (eight min), and examined by light microscopy.

**Assessment of Immunostaining**

Immunoreactivity was quantitated as previously described (17). Immunoreactive cells were identified by counting all cells containing immunoreactivity above background; background staining was minimal in all sections. The proportion of cells demonstrating pNF epitopes was determined for each DRG by counting the total number of neuronal perikarya stained with antibody 07-05 and dividing this value by the total number of neuronal perikarya in the DRG (determined by counting the total number of neuronal perikarya stained with antibody 2-135 and lightly counterstained with cresyl violet). For each treatment side (i.e. colchicine, crush, saline, colchicine/crush, and saline/crush), values from L4 and L5 DRG were averaged to give one mean data point for each sciatic nerve.

**Statistical Analysis**

For purposes of further statistical analysis, saline-treated sciatic nerves contralateral to colchicine treatment (contralateral controls) (n = 2) and from normal animals (normal controls) (n = 2) were combined into one saline-treated control group (n = 4); DRG from the normal, unoperated nerves were not included in this analysis. In the colchicine study, a one-way analysis of variance (ANOVA) was performed followed by Scheffe's post-hoc analysis to test for differences between individual groups (i.e. colchicine, crush and saline). A paired t-test was used to test for differences between the colchicine/crush and saline/crush groups.

All values are mean ± SEM.

**RESULTS**

**Controls**

The present findings are in agreement with those previously reported in lumbar sensory neurons of the adult rat (16, 17, 30). In the L4 and L5 DRG of normal,
unoperated (n = 2) and saline-treated control nerves from normal rats (normal controls) (n = 2), intense immunoreactivity was observed in neuronal perikarya and axons to antibody 2-135 directed against nonphosphorylated NF epitopes (Fig. 1A). Immunoreactivity was not present, however, in neuronal perikarya with antibodies 06-17 and 07-05 directed against pNF epitopes (Fig. 1B; Table 1).
TABLE 1
Incidence of Phosphorylated NF Epitopes in Sensory Neuronal Perikarya

<table>
<thead>
<tr>
<th>Group</th>
<th>% Phosphorylated</th>
</tr>
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<tbody>
<tr>
<td>Saline (n = 4)</td>
<td>0.3 ± 0.32</td>
</tr>
<tr>
<td>Normal control (n = 2)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Contralateral control (n = 2)</td>
<td>0.6 ± 0.004</td>
</tr>
<tr>
<td>Colchicine (n = 8)</td>
<td>30.4 ± 2.07*</td>
</tr>
<tr>
<td>Crush (n = 6)</td>
<td>45.1 ± 3.76††</td>
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Percentage (mean ± SEM) of neuronal perikarya stained with antibody 07-05 directed against phosphorylated NF epitopes (see Materials and Methods). The saline group is subdivided into normal controls and contralateral controls from the colchicine group (see text).

n = Number of animals.

* p < 0.001; compared to saline and crush (one-way analysis of variance).

†† p < 0.001; compared to saline and colchicine (one-way analysis of variance).

Colchicine Studies

Immunoreactivity of neuronal perikarya to nonphosphorylated NF epitopes (2-135) from the colchicine-treated (Fig. 2A) or crushed (Fig. 2E) nerves did not differ from the saline-treated contralateral (Fig. 2C) and normal (Fig. 1A) controls at 14 days. Similarly, no alteration in immunostaining was observed in neuronal perikarya from colchicine-treated nerves at two and five days (not shown).

At 14 days, neuronal perikarya in the L4 and L5 DRG from colchicine-treated nerves demonstrated modest to intense immunostaining with antibodies 06-17 (not shown) and 07-05 (Fig. 2B). These antibodies gave similar results, although the intensity of staining was consistently greater with antibody 07-05. Thus, we quantitated the proportion of cells showing immunoreactivity against pNF epitopes using antibody 07-05 (see Materials and Methods). Immunoreactivity to pNF epitopes was present in 30.4 ± 2.07% (range 27.3–31.7%) of neuronal perikarya in the L4 and L5 DRG (Fig. 3; Table 1). Neuronal perikarya from the saline-treated contralateral side (contralateral controls) demonstrated only a rare (1–2/DRG) immunoreactive cell using antibodies against pNF epitopes (Fig. 2D; Table 1). A significant (p < 0.001) difference was observed in the incidence of neuronal perikarya demonstrating immunoreactivity to pNF epitopes between the colchicine-treated and combined saline-treated (see Materials and Methods) nerves (Fig. 3; Table 1).

In the contralateral DRG from the crushed sciatic nerves (n = 6), similar modest to intense staining was observed at 14 days in neuronal perikarya with antibodies 06-17 (not shown) and 07-05 (Fig. 2F). Quantitation of immunoreactive cells revealed staining against pNF epitopes in 45.1 ± 3.76% (range 40.9–48.7%) of neuronal perikarya in the L4 and L5 DRG (Fig. 3; Table 1). A significantly (p < 0.001) greater number of DRG demonstrated immunoreactivity against pNF epitopes in crushed compared to colchicine-treated sciatic nerves (Fig. 3; Table 1).

In contrast, immunoreactivity to pNF epitopes was not observed in neuronal perikarya from colchicine-treated nerves at two and five days (not shown); contralateral saline-treated nerves showed no staining to pNF epitopes at these time-points (not shown).

Colchicine/Crush Studies

Modest to intense immunoreactivity to pNF epitopes was observed in neuronal perikarya in the L4 and L5 DRG from the saline-treated/crushed nerves (saline/
Fig. 2. Peroxidase-antiperoxidase staining of L5 DRG from rats in which the sciatic nerve was treated with colchicine (A & B), saline (C & D), or crushed (E & F) at six and seven wk of age (see Material and Methods). A, C & E: Antibody 2-135, against nonphosphorylated NF epitopes, shows identical pattern of staining as in age-matched normal controls (see Fig. 1A). B, D & F: Antibody 07-05, against pNF epitopes, demonstrated intense homogeneous immunoreactivity in many neuronal cell bodies from colchicine-treated (B) and crushed (F) nerves, whereas the saline-treated nerve shows a lack of immunoreactivity in neuronal perikarya (D). Some perikarya from the colchicine-treated (B) and crushed (F) nerves demonstrate eccentric nuclei. Insets—High power views of stained cell bodies showing intense immunoreactivity in cells from the colchicine-treated (B) and crushed (F) nerves, and a rare, lightly stained cell body in the saline-treated nerve (D). ×58; Insets: ×230.

crush group) (Fig. 4A). The intensity and extent of immunostaining to pNF epitopes appeared similar in neuronal perikarya from the colchicine-treated/crushed nerves (colchicine/crush group) (Fig. 4B). Quantitation of immunoreactive cells revealed no significant (p > 0.05) difference in degree of staining against pNF epitopes between
Fig. 3. Percentage of neuronal perikarya in the L4 and L5 DRG stained with antibody 07-05 directed against pNF epitopes from rats in which the sciatic nerve was treated with saline, colchicine, or crushed and examined at two wk following the first treatment, as in Figure 2. * = p < 0.001, compared to saline and crush, † = p < 0.001, compared to saline and colchicine (see Table 1).

the saline/crush and colchicine/crush groups; immunoreactivity against pNF epitopes was present in 44.7 ± 0.81% (range 43.8–46.1%) and 43.8 ± 9.7% (range 40.1–48.2%) of neuronal perikarya in the L4 and L5 DRG from the saline/crush and colchicine/crush groups, respectively (Fig. 5).

DISCUSSION

Monoclonal antibodies directed against phosphorylated NF epitopes reveal aberrant phosphorylation of the two high molecular weight polypeptides comprising the mammalian NF triplet proteins in neuronal perikarya in a number of human diseases and experimental conditions (see Introduction). The wide variety of situations in which aberrant NF phosphorylation has been observed suggests that it represents a non-specific response of the neuron to injury (5). The demonstration that abnormal expression of pNF epitopes in neuronal perikarya is also produced by axotomy (15–17) supports this hypothesis. Thus, aberrant NF phosphorylation appears to be a component of the axon reaction.

The present study supports and extends this hypothesis by demonstrating that blockade of fast axonal transport by colchicine is sufficient to induce abnormal expression of pNF epitopes in neuronal perikarya of sensory neurons in rat lumbar DRG. Previous studies (28) have shown that the majority of fibers do not undergo axonal degeneration using the present protocol for application of colchicine to the sciatic nerve. Taken together, these findings suggest that structural interruption of the neuron from its target tissue is not necessary to elicit aberrant NF phosphorylation (as revealed by monoclonal antibodies 06-17 and 07-05) in sensory neuronal perikarya.

Our finding that axotomy results in production of aberrant NF phosphorylation in approximately 40–50% of sensory neurons in the rat lumbar DRG at 14 days is consistent with previously published data (16, 17). The fact that one-third fewer DRG neurons demonstrated immunoreactivity following colchicine application
Fig. 4. Peroxidase-antiperoxidase staining of L4 DRG with antibody 07-05 directed against pNF epitopes from rats in which the sciatic nerve was crushed and the proximal stump was treated with either saline (A) or colchicine (B). The proportion of cell bodies demonstrating immunoreactivity and the intensity of staining appears similar in the two groups (see Fig. 5). In each section, some perikarya demonstrate eccentric nuclei (arrows). ×165.

compared to axotomized nerves is most likely due to a failure of colchicine application to block fast axonal transport in all axons in the sciatic nerve (28). In addition, the time course for the development of aberrant NF phosphorylation may differ following axotomy and colchicine treatment. In this context, we have not measured the duration of the retrograde transport block produced by colchicine application; although blockade is apparent 24 h following colchicine application (28), it seems unlikely that the transport defect is maintained during the entire two wk period of study considering the lack of appreciable axonal degeneration distal to colchicine application (28). Thus, differences in absolute numbers of immunoreactive cells from axotomized and colchicine-treated nerves should not be interpreted as an indication of different underlying mechanisms leading to production of this perikaryal response.

What process common to axotomy and colchicine application could underlie the development of this perikaryal alteration? One possibility is suggested from previous studies (28, 33–35) on the development of another perikaryal response to injury; i.e. the reduction in NF synthesis and axonal caliber in the proximal axon observed following axotomy (21–24). Results from these studies indicate that loss of a retrogradely transported "trophic" signal(s) is responsible for initiation of the axotomy-induced reduction in NF synthesis (33) and axonal caliber (28). Moreover, nerve growth factor (NGF) appears to be one factor whose loss is responsible for the decrease in NF synthesis (34) and axonal caliber (33) in DRG neurons following axotomy.
The present results suggest that a similar process may function in the production of aberrant NF phosphorylation in sensory neuronal perikarya. Colchicine, which impairs retrograde axonal transport (36–38), appears to produce an axotomy-like response in the neuronal perikaryon (28, 39–45). Thus, colchicine may also elicit aberrant NF phosphorylation by preventing the retrograde transport of a "trophic" signal to the neuronal perikaryon. Future studies will determine whether loss of NGF is also responsible for production of abnormal expression of pNF epitopes in DRG neurons.

The extent of neuronal perikarya expressing pNF epitopes in axotomized nerves was not altered by colchicine application proximal to the site of crush. The lack of an increase in the number of neurons expressing pNF epitopes in colchicine-treated/ crushed nerves does not refute our hypothesis that these procedures (i.e. axotomy and colchicine application) act via similar mechanisms; in fact, this study cannot address this issue since the maximal extent of aberrant NF phosphorylation in DRG neurons appears to be produced by axotomy alone. However, taken together with the observation that abnormal expression of pNF epitopes can be induced in intact sensory neurons, the failure of colchicine application to prevent the development of aberrant NF phosphorylation provides further evidence (28) against the hypothesis (46) that delivery of a retrogradely transported "trophic" signal to the neuronal perikaryon produced in the distal stump of a transected nerve initiates the axon reaction.

Finally, a rare neuronal perikaryon demonstrated immunoreactivity to pNF epitopes in the saline-treated nerves contralateral to colchicine application (contralateral controls). The lack of any immunostaining to pNF epitopes in the saline-treated normal animals (normal controls) argues against an effect associated with surgical manipulation (i.e. mechanical injury). It remains possible that some colchicine may leak into the circulation producing a systemic effect. However, the present findings
indicate that the contribution of any systemic toxicity to the development of aberrant NF phosphorylation in the colchicine-treated nerves is minimal.

In summary, the present study demonstrates that aberrant expression of pNF epitopes in neuronal perikarya can be produced in intact DRG neurons. It is suggested that this perikaryal alteration is initiated by the loss of a retrogradely transported "trophic" signal(s) from the periphery. However, these results do not rule out the possibility that events taking place in the distal stump (e.g. degenerating/regenerating process) lead to production of another signal which serves to modulate aberrant NF phosphorylation in the neuronal perikaryon once it has been induced.

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REFERENCES


43. Purves D. Functional and structural changes in mammalian sympathetic neurons following colchicine application to post-ganglionic nerves. J Physiol (London) 1976;259:159-75
44. Richardson PM, Issa VM, Riopelle RJ. Distribution of neuronal receptors for nerve growth factor in the rat. J Neurosci 1986;6:2312-21
46. Singer PA, Mehtler S, Fernandez HL. Blockade of retrograde axonal transport delays the onset of metabolic and morphological changes induced by axotomy. J Neurosci 1982;2:1299-1306

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