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Nerve Growth Factor (NGF) Differentially Regulates the Chemosensitivity of Adult Rat Cultured Sensory Neurons

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We have studied the effects of NGF on the chemosensitivity of adult rat DRG neurons over a 1–2 week period *in vitro*, using voltage-clamp and radioactive ion flux methods. A sustained proton evoked current was reversibly lost in NGF-free medium after 1 week. Proton-evoked efflux of radioactive $^{86}Rb^+$ ions was also depressed in NGF deprived cultures, although depolarization with 40 mM potassium still evoked a large $^{86}Rb^+$ efflux. A similar reversible loss of capsaicin sensitivity was noted. The response to GABA and a second, transient proton evoked current were also regulated by NGF, but over a longer time course. In contrast, the sensitivity to ATP was not influenced by the presence or absence of NGF. These data show that NGF regulates some, but not all, chemosensitivities of DRG neurons and that loss of sensitivity occurs at different rates for different agonists. The precise co-regulation of the response to capsaicin and the sustained response to protons provides further evidence that protons activate capsaicin-operated ion channels.

[Key words: dorsal root ganglion neurons, NGF, culture, capsaicin, protons, GABA, ATP]

NGF is required for the survival of the majority of embryonic sensory neurons (Johnson et al., 1980; Lindsay, 1988a; Crowley et al., 1994) and in the early postnatal rat can influence the differentiation of Aδ (Ritter et al., 1991, 1993; Lewin et al., 1992a) and C nociceptive afferent fibers (Lewin and Mendell, 1992). In the adult rat, NGF deprivation does not cause significant sensory neuron death *in vivo* (Gorin and Johnson 1980; Ritter et al., 1991) and dorsal root ganglion (DRG) neurons cultured from adult animals do not need NGF for survival (Lindsay, 1988a,b). NGF can, however, increase the expression of substance P and calcitonin gene related peptide (CGRP) in adult rat DRG neurons both *in vivo* (e.g., Wong and Oblinger, 1991) and in culture (Lindsay and Harmar, 1989; Lindsay et al., 1989). Furthermore, TTX resistant action potentials and responsiveness to capsaicin are lost when adult rat DRG neurons are cultured for 1–2 weeks in the absence of NGF (Winter et al., 1988, 1993; Aguayo and White, 1992).

Elevated levels of NGF are found in inflammatory conditions and this may be functionally important as systemic treatment of adult rats with NGF produces mechanical and thermal hyperalgesia (Lewin et al., 1993). Thermal hyperalgesia develops within minutes of exogenous NGF administration and could involve a direct sensitization of mechanoheat-sensitive fibers. Mechanical hyperalgesia develops over a period of hours and may be due to changes within the spinal cord, such as increased substance P release from central afferent terminals (Lewin et al., 1992b). Moreover, NGF neutralizing antibodies greatly attenuate the thermal and mechanical hyperalgesia induced by complete Freund's adjuvant (Woolf et al., 1994). Conversely, sensory neurons may be exposed to lower than normal levels of NGF in certain neuropathic conditions and this may contribute to the pathophysiology (Brewster et al., 1994).

DRG neurons are sensitive to a range of chemical agents (Rang et al., 1991), including ATP, bradykinin, 5-HT, ACh, and protons (low pH), which excite sensory neurons and evoke the sensation of pain, as well as GABA, which is not painful. We have therefore examined if the modulation of chemosensitivity by NGF was restricted to capsaicin or extended to other chemical activators. This investigation also allowed us examine whether or not exposure to elevated levels of NGF, which occur in inflammation, would increase chemosensitivity and thereby contribute to the hyperalgesia. In this article we show that (1) the expression of two types of proton-activated currents is regulated by NGF with different time courses, (2) GABA sensitivity is reduced slowly in the absence of NGF, and (3) ATP sensitivity is NGF independent. Thus, sensitivity to different chemical agents appears to be regulated by NGF in a selective manner.

Materials and Methods

Adult DRG neuron cultures. Neurons were isolated and grown by methods that have been described in detail previously (Lindsay, 1988b; Wood et al., 1988) with slight modifications. Spinal columns were removed aseptically from CO_2-asphyxiated adult Sprague–Dawley rats (>8 weeks old, 180–200 gm) and DRG were dissected from all spinal levels. Ganglia were digested with 0.125% collagenase (Boehringer Mannheim UK) in Ham's F-14 medium (Imperial Laboratories UK) containing 4% US-G (Ultraser-G serum substitute from GIBCO/Bethesda Research Labs, UK) for 90 min then for a further 90 min in fresh collagenase solution at 37°C. After being washed with US-G-containing medium, ganglia were triturated through a flame-polished Pasteur pipette to form a single-cell suspension. DRG neurons were then plated either on 35 mm tissue culture plastic petri dishes (approximate density 20,000 neurons/dish) or on 13 mm glass coverslips (10,000 neurons/coverslip). Dishes and coverslips had been previously coated with polyornithine and laminin (GIBCO/Bethesda Research Labs, UK). Cultures were treated with the mitotic inhibitor cytosine arabinoside to reduce the non-neuronal cell population and grown in F14 plus 4% US-G. Two protocols were used to study the effects of NGF deprivation. In the first, the culture medium was supplemented with either rat monoclonal 23c4 anti-NGF (Weskamp and Otten, 1983) 50 μg/ml (sufficient to block 200 ng/ml NGF) or with 200 ng/ml 2.5S NGF purified from mouse submaxillary gland plus a control rat monoclonal antibody (Y13-238, anti-ras21; Furth et al., 1982) of the same

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Table 1. Comparison of the chemosensitivities of freshly dissociated and cultured (200 ng/ml NGF) DRG neurons

		Freshly dissociated	1 week (all expts)	2 weeks (all expts)
Capsaicin (500 nM)	*P(R)*	48% (23/48)	48% (65/135)	49% (17/35)
	A(tot) (nA)	0.63 ± 0.20	0.67 ± 0.10	0.71 ± 0.22
	A(R) (nA)	1.32 ± 0.37	1.40 ± 0.18	1.47 ± 0.39
pH 5.8, sustained	*P(R)*	47% (36/76)	49% (67/138)	49% (17/35)
	A(tot) (nA)	0.66 ± 0.16	0.83 ± 0.13	0.90 ± 0.32
	A(R) (nA)	1.41 ± 0.28	1.70 ± 0.22	1.57 ± 0.57
pH 5.8, transient	*P(R)*	53% (46/87)	67% (92/138)	65% (111/172)
	A(tot) (nA)	1.36 ± 0.27	1.08 ± 0.16	1.24 ± 0.16
	A(R) (nA)	2.57 ± 0.45	1.62 ± 0.23	1.94 ± 0.22
GABA (100 μM)	*P(R)*	92% (45/49)	82% (22/27)	92% (84/91)
	A(tot) (nA)	1.67 ± 0.32	1.20 ± 0.23	0.78 ± 0.09*
	A(R) (nA)	1.82 ± 0.34	1.47 ± 0.24	0.85 ± 0.10**
ATP (10 μM)	*P(R)*	78% (32/41)	75% (18/24)	90% (115/128)
	A(tot) (nA)	1.56 ± 0.36	1.18 ± 0.27	0.75 ± 0.08
	A(R) (nA)	2.00 ± 0.43	1.57 ± 0.33	0.83 ± 0.09†

No statistically significant changes in P(R), A(tot), and A(R) were noted for capsaicin- and both transient and sustained proton-evoked responses ($P > 0.05$). A(R) was significantly reduced for both GABA and ATP responses ($P < 0.01$). Probabilities of differences between cultured and freshly dissociated neurons: *, < 0.05; **, < 0.01; †, < 0.001. In some experiments, neurons were challenged with brief (2.5 sec) applications of low pH medium. In these cases the amplitude of any abbreviated sustained response was not measured.

subtype as 23c4. In the second set of experiments, 50 ng/ml NGF (Promega) was added to both sets of cultures but one received the neutralizing (23c4) and the other the control (Y13-238) antibody. Both antibodies were purified from ascites fluid from nude mice that had been injected with the appropriate hybridomas. Some neurons were grown initially in NGF-deprived conditions (no added NGF plus neutralizing antibody), then switched into NGF-containing medium for a further period. Medium was changed every 3–4 d.

Electrophysiology. Acutely dissociated DRG neurons were maintained in medium without added NGF and studied electrophysiologically 6–18 hr after dissociation. In other experiments designed to test the effects of NGF, neurons were grown with or without NGF for 6–7 d (grouped as 1 week) or 12–16 d (grouped as 2 weeks). For one part of the study neurons were grown without NGF for 1 week and switched into NGF containing medium for a further week. In the early experiments, cells were removed from the petri dishes by trituration through a Pasteur pipette after a short incubation in trypsin (0.05%) They were then replated on 13 mm glass coverslips that had been previously coated with polyornithine. In later experiments, no enzymes were used and cells were removed mechanically from the petri dish, triturated and replated as before. For each experiment one batch of neurons were used as an "age matched" control and were cultured with NGF for the entire period. This provided an internal control for any possible difference in measured chemosensitivity between different DRG neuron preparations and experiments. Results from at least three independent experiments were obtained for each part of the investigation.

Cell diameters were measured microscopically. Two orthogonal measurements were made for each cell studied electrophysiologically and the mean calculated. No attempt was made to select for any particular cell size. The neuronal population, however, showed very few large diameter (>50 μm) cells; these may have been selectively lost in the cell preparation and replating procedures used. The mean diameter of cells cultured with NGF (24.25 μm) is very similar to the mean diameter (24.53 μm) of the major neuronal population (>93% of cells) measured in fixed cultured DRG neurons (Winter, 1987).

Recordings from cultured neurons were made between 16 and 24 hours after the neurons were replated. Before 8 hr the responses to capsaicin and low pH showed a great variability in their occurrence, but not their time course, and some preparations failed to respond to these agents even though the neurons responded before replating (data not shown). A likely explanation for this is that these receptors are located preferentially on the neurites of cultured cells and that after replating some time is required for the new receptors to be expressed on the surface of the soma. After 16–24 hr, the neurons elaborated few, if any, neurites and could still be studied easily. At longer times neurites were extended and the neurons could not be adequately voltage clamped.

Whole cell "tight seal" recordings were made from neurons with wide bore patch pipettes (2–5 MΩ). The methods used are described in detail in Bevan and Yeats (1991); experiments were carried out at 21–23°C. The basic external solution contained (mM) 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 40 sucrose, 5 glucose and was usually buffered to pH 7.4 with 10 mM HEPES titrated with NaOH. A 10 mM HEPES buffer was also used for solutions of pH 6.8–8.2. More acidic solutions used either 10 mM MES (pH 5.0- 5.8) or 10 mM PIPES (pH 6.6) as the buffer. Drugs or low pH solutions were rapidly applied from a "U-tube" (Fenwick et al., 1984) for either 2.5 or 5–7.5 sec. DMSO (1%) was included in both the bath and drug solutions when capsaicin sensitivity was tested to ensure that capsaicin remained in solution. Currents were usually recorded at a relatively low gain (1–2 mV/pA) and the limit of detection for an agonist induced current was 20–30 pA. Neurons with evoked currents less than this value were classed as nonresponsive. Sequential challenges of single DRG neurons with different agents were made with intervals of 2 min. With this protocol we avoided any potentiation of the capsaicin response by acidic external media (Petersen and LaMotte, 1993)

$^{86}Rb^+$ efflux experiments. Adult DRG neurons were grown at a density of approximately 10,000 per 13 mm coverslip, for 1 or 2 weeks, with or without NGF as described above. Cells were then incubated with 2 mCi/ml of ^{86}RbCl (300 mCi/mmol; Amersham UK) for 2–4 hr, placed in a perfusion chamber (volume 0.5 ml), and washed for 30 min with Hanks's BSS 5 mM HEPES (pH 7.4) at a perfusion rate of 1 ml/min. Perfusate fractions (1 ml) were collected directly into scintillation vials, and cells were challenged sequentially with low pH buffer (pH 5.4, 5 mM MES), high KCl (40 mM) and 10 mM capsaicin, each for 3 min, with a wash in pH 7.4 BSS after each challenge. Cultures were then dissolved with 0.2% SDS (sodium dodecyl sulfate) to measure $^{86}Rb^+$ remaining in the cells. Experiments were carried out at room temperature (21–23°C). The radioactivity in the perfusate fractions and the cells was measured in a liquid scintillation counter. Four coverslips were sampled for each point.

Statistics. Changes in resting potential, cell capacitance and size were analyzed with Student's *t* test. The current amplitudes were not normally distributed and so nonparametric tests were used, although values are expressed as mean ± SEM to give some indication of the scatter in the values. Differences in current amplitudes were usually analyzed by the Mann-Whitney *U* test. Paired observations in single neurons were compared with the Wilcoxon matched pairs test. Proportions of respond-

ing and non-responding cells were examined with the χ^2 test with Yates's correction for continuity except when the expected number in any category was 5 or less when Fisher's exact test was employed.

Results

Changes in chemosensitivity of neurons in culture may result from a change in the proportion of neurons that is responsive [$P(R)$], and a change in the mean response amplitude measured for the total population [A(tot)] or for the responsive population [$A(R)$]. We have studied the effects of NGF on DRG neuron chemosensitivity in terms of these three parameters.

Sensitivity of freshly isolated DRG neurons

The chemosensitivity of DRG neurons was measured within 18 hr of isolation and provided a comparison for any subsequent changes in neuronal properties. The types of currents evoked by the various chemical agents were identical to those seen after 1 or 2 weeks in culture. Acidification of the external medium to pH 5.8 evoked either one or two types of inward current in responsive neurons at −80 mV (fast, transient, and slow, sustained currents). Inward currents were also evoked in some, but not all, neurons by capsaicin (500 nM), ATP (10 μM), and GABA (100 μM). Table 1 shows the chemosensitivity of freshly isolated neurons as well as neurons cultured for either 1 or 2 weeks in the presence of NGF (50–200 ng/ml). The sensitivity to capsaicin and pH 5.8 medium (both currents) was not altered by up to 2 weeks in culture ($P > 0.5$ for A(tot), $A(R)$, and $P(R)$ in all cases). Changes were noted in the sensitivity to ATP and GABA with time in culture. Although $P(R)$ did not differ significantly ($P > 0.05$), $A(R)$ decreased over the 2 week culture period to 42% (ATP) and 47% (GABA) of the values seen in freshly isolated neurons ($P < 0.01$, Kruskall-Wallis test). In view of these time-dependent changes in chemosensitivity and the possibility of small differences in responses between different preparations of DRG neurons, we have studied the effects of NGF by comparing the sensitivity of approximately equal numbers of NGF-treated (NGF^+) and NGF-deprived (NGF^-) neurons from a given preparation after the same time in culture. In all cases, at least three preparations were used to obtain any given result.

Effects of NGF deprivation

Two experimental protocols were used to investigate the effects of NGF on DRG neuron properties. In the first series of experiments, two sets of sister cultures were studied. NGF was omitted from one set and a neutralizing anti-NGF monoclonal antibody added to eliminate any effects of NGF produced by cells in the culture. The other set received 200 ng/ml of NGF plus a control monoclonal antibody of the same immunoglobulin subclass. In the second series of experiments, both sets of cultures were treated with 50 ng/ml NGF; however, neutralizing antibody was added to one set and control antibody added to the other set. The results obtained with the two protocols were indistinguishable and have been pooled to aid presentation. Neurons exposed to NGF have been designated NGF^+ and those cultured either without added NGF or with NGF neutralized termed NGF^-.

Low pH

A typical response to a challenge with an acidic solution (pH 5.8) is illustrated in Figure 1*a* for a neuron that had been grown for 1 week in the presence of NGF. The two components of the response (transient and sustained) can be clearly seen. $P(R)$ was always greater for the transient current than for the sustained current so that some neurons displayed only the transient current (see Fig. 3*a,b*). Although recent studies have shown that the transient current in rat trigeminal ganglion neurons can be separated into three kinetic components (Pidoplichko, 1992), we found no clear evidence for multiple transient currents in our studies and have used the total transient current for our analyses.

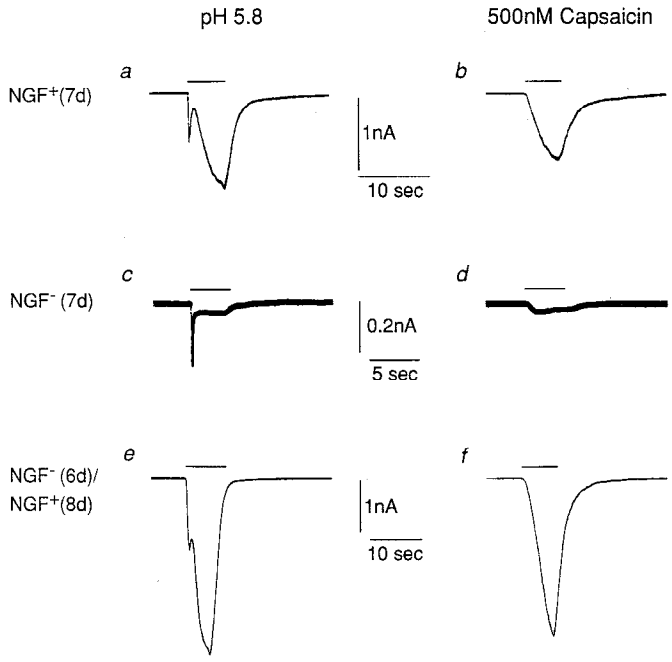

Figure 1. Current responses to pH 5.8 (*a, c, e*) and 500 nM capsaicin (*b, d, f*) in DRG neurons cultured for 1 week with either NGF (*a, b*) present or no added NGF plus neutralizing antibody to NGF (*c, d*). Traces in *e* and *f* show the recovery of the capsaicin and sustained, low pH responses when neurons were first grown without NGF for 1 week and then re-exposed to NGF for a further week. The transient proton evoked currents can also be seen in traces *a, c,* and *e*. Traces *a* and *b, c* and *d,* and *e* and *f* were obtained from individual cells sequentially challenged at 2 min intervals. *Horizontal bars* represent the time of agonist application.

Sustained proton-evoked current. The sustained response was influenced by the presence of NGF as illustrated by the results shown in Table 2. $P(R)$ was 48.6% for NGF^+ neurons but only 14.9% for NGF^- neurons after 1 week in culture. The mean amplitude of the response was also diminished in the NGF^- cultures (Figs. 1*c*, 2). A(tot) and $A(R)$ were reduced to 16.9% and 48.4% of the values seen in NGF^+ neurons.

(1) NGF does not alter pH activation. One possible explanation for the smaller number of cells responding to pH 5.8 medium in NGF^- cultures was that the pH sensitivity of the cells had been shifted in the direction of more acidic solutions. This was tested by challenging neurons with pH 5.2 medium as well as pH 5.8 medium. In this experiment, 7 out of 9 NGF^+ neurons responded to the low pH solutions. $A(R)$ was greater ($P < 0.05$) with pH 5.2 medium (0.85 ± 0.23 nA) than with pH 5.8 medium (0.38 ± 0.12 nA). In contrast, significantly fewer (1 out of 12, $P < 0.01$) NGF^- neurons showed a detectable sustained response to pH 5.2. Thus, the stronger pH stimulus did not succeed in causing responses in NGF^- neurons.

(2) NGF effects are reversible. Experiments were carried out to determine if the reduction in proton sensitivity was reversible,

Table 2. Effect of NGF deprivation for 1 week on the sustained, low pH- and capsaicin-evoked currents

		NGF^+ (1 week)	NGF^- (1 week)	Ratio NGF^-: NGF^+
pH-sustained	$P(R)$	48.6% (67/138)	14.9% (17/114)†	
	A(tot) (nA)	0.83 ± 0.13	0.14 ± 0.05†	16.9%
	$A(R)$ (nA)	1.70 ± 0.22	0.92 ± 0.26*	48.4%
Capsaicin	$P(R)$	48.1% (65/135)	14.1% (16/113)†	
	A(tot) (nA)	0.67 ± 0.10	0.11 ± 0.04†	16.4%
	$A(R)$ (nA)	1.40 ± 0.18	0.81 ± 0.24	57.9%

Values for P(R), A(tot), and A(R) shown for 1 week NGF^+ and NGF^- cultures. Probabilities of difference between NGF^+ and NGF^- cultures; *, < 0.05; †, < 0.01. No symbol indicates $P > 0.05$.

as previously reported for the effects of NGF on capsaicin sensitivity (Winter et al., 1988). DRG neurons were cultured first for 1 week in medium without NGF and then for a further week with NGF ($NGF^{-/+}$). The response to pH 5.8 solution was compared to that of NGF^+ neurons cultured for 2 weeks. $NGF^{-/+}$ neurons regained at least part of their proton sensitivity and large current responses were recorded in some neurons (Fig. 1*e*). This increase in sensitivity was reflected by changes in both the proportion of responsive neurons and the sizes of the responses (Table 3). 59% of the $NGF^{-/+}$ neurons showed the sustained proton-activated current, which is a significantly larger proportion than observed in 1 week NGF^- cultures (14.9%, see Table 2, $P < 0.0001$) and similar to the $P(R)$ seen in 1 week NGF^+ cultures (49%, see Table 2, $P > 0.3$) and 2 week NGF^+ cultures (49%, Table 3, $P > 0.4$). A(tot) was significantly larger in $NGF^{-/+}$ neurons (0.45 ± 0.17 nA, $n = 29$) than in 1 week NGF^- neurons (0.14 ± 0.05 nA, $P < 0.0001$), but not significantly different from the value seen in 2 week NGF^+ cultures (0.90 ± 0.32 nA, $P > 0.1$).

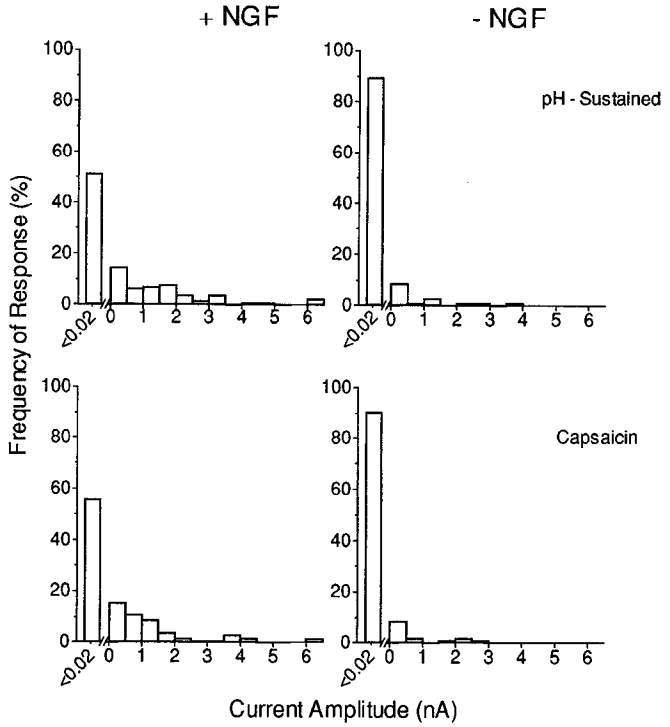

Figure 2. Frequency distributions of amplitudes of the sustained , low pH- (*top*) and capsaicin-evoked currents (*bottom*) after 1 week in culture either with NGF (*left*) or without NGF (*right*). Left-hand bars show percentage of neurons with no measurable current (<20–30 pA). Results correspond to amplitudes given in Table 2.

Transient proton-evoked current. The transient current evoked by acidic solutions (Figs. 1*a,c*; 3*a,b*) was also modulated when the neurons were deprived of NGF (Table 4). $P(R)$ was not significantly altered after either 1 or 2 weeks culture in NGF^- medium ($P > 0.05$), but the amplitude of the current response was reduced progressively in NGF^- neurons (see Figs. 3*a,b*; 4). After 1 week, A(tot) and $A(R)$ were 72.2% and 71.6% of the values found in NGF^+ neurons and these percentages were significantly reduced to 25.5% and 28.6% after 2 weeks in culture (Table 4).

(1) NGF does not influence activation or inactivation. The transient proton activated current in chick DRG neurons has been shown to inactivate at acidic or even neutral pH (Konnerth et al., 1987) and the number of available ion channels is maximum at pH > 7.8. One possible explanation for the reduced size of the transient current in NGF^- neurons was a greater degree of inactivation at pH 7.4. However, there was no obvious difference in the pH inactivation curves when 2 week NGF^+ and NGF^- neurons were challenged with pH 5.8 medium from different resting pH levels (pH 7.0, 7.8, 8.2; see Fig. 5). In both cases half-inactivation was observed at about pH 7.1 and currents were nearly maximal at pH 7.4. In other experiments neurons were bathed in pH 7.8 medium to activate the current maximally and the external pH was stepped to pH 5.8. Transient currents were evoked in a similar proportion of 2 week NGF^+ and NGF^- neurons [$P(R)$: 75% NGF^+, $n = 20$ and 59% NGF^-, $n = 17$; $P > 0.4$] and once again $A(R)$ in NGF^+ neurons (2.25 ± 0.55 pA) was larger than in NGF^- neurons (0.31 ± 0.07 pA, $P < 0.01$).

Neurons were also challenged with pH 5.2 as well as the pH 5.8 solution to test if the reduced transient response was due to a shift in the pH-response curve. After 1 week in NGF^+ medium, $A(R)$ was similar with pH 5.8 (0.95 ± 0.26 nA) and pH 5.2 solutions (1.11 ± 0.25 nA, $n = 8$, $P > 0.05$). Although the $P(R)$ value for responses to pH 5.2 medium was not significantly reduced in the NGF^- neurons (45%, $n = 11$ $P > 0.05$), $A(R)$ (0.36 ± 0.07 nA) was reduced compared to the NGF^+ cells ($P < 0.05$).

Capsaicin sensitivity

The sensitivity of DRG neurons to capsaicin is known to be regulated by NGF and was used as a control for the effects of NGF on other chemosensitivities. Responses to 500 nM capsa-

Table 3. Reversible effects of NGF on capsaicin-evoked currents and the sustained low pH-evoked current

		NGF^+ (2 weeks)	$NGF^{+/-}$ (1 week/1 week)	Ratio $NGF^{-/+}$: NGF^+
pH-sustained	$P(R)$	49% (17/35)	59% (17/29)	
	A(tot) (nA)	0.90 ± 0.32	0.45 ± 0.17	50.0%
	$A(R)$ (nA)	1.57 ± 0.57	0.97 ± 0.33nA	61.8%
Capsaicin	$P(R)$	49% (17/35)	50% (15/30)	
	A(tot) (nA)	0.71 ± 0.22	0.39 ± 0.14	54.9%
	$A(R)$ (nA)	1.47 ± 0.39	1.00 ± 0.31	68.0%

Responses of 2 week NGF^+ neurons are compared with the responses of neurons from the same cell preparation deprived of NGF for 1 week and then re-exposed to NGF for 1 week ($NGF^{-/+}$). No statistical differences ($P > 0.05$) in P(R), A(tot), and A(R) were seen between NGF^+ and $NGF^{-/+}$ neurons.

icin were monophasic (Fig. 1) and we found no evidence for the multiple currents reported by Lui and Simon (1994) in rat trigeminal ganglion neurons. In 1 week NGF^+ cultures, 48.1% of the neurons responded to 500 nM capsaicin with a detectable inward current (Table 2). In contrast, $P(R)$ was only 14.1% in 1 week NGF^- cultures ($P < 0.001$). The mean amplitude of the current was reduced in NGF^- neurons with A(tot) only 16.4% of the amplitude observed in NGF^+ neurons. Although the currents in the responsive NGF^- neurons were in general smaller than in NGF^+ neurons (Fig. 2), some NGF^- neurons showed a robust response to capsaicin. The presence of these large responses was reflected in the $A(R)$ value which was 57.9% of that in NGF^+ neurons ($P = 0.06$; see Table 2). A further observation was that all the NGF^- neurons that responded to the low pH solution also responded to capsaicin with similar current amplitudes (Fig. 2). This is reflected in the similar $P(R)$ and $A(R)$ values for the two agonists (see Table 2).

Capsaicin sensitivity was regained when NGF was added back to the cultures (Table 3) and many neurons showed large current responses (Fig. 1*f*). $P(R)$ increased to 59% in $NGF^{-/+}$ cultures, which is similar to the value in control, 2 week NGF^+ cultures (49%). A(tot) was significantly greater in $NGF^{-/+}$ (0.39 ± 0.14

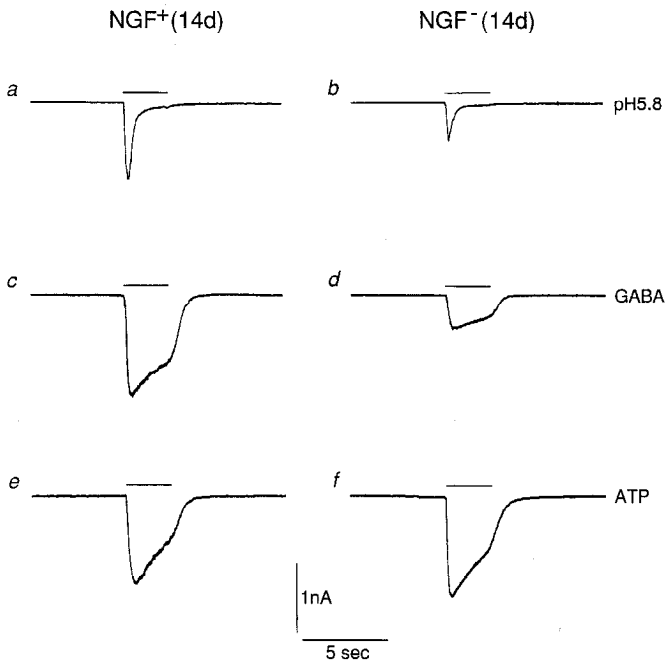

Figure 3. Transient low pH evoked currents in the absence of the sustained proton evoked response (*a, b*) and responses to GABA (*c, d*) and ATP (*e, f*). Neurons cultured for 2 weeks with NGF (*a, c, e*) or in NGF-free media (*b, d, f*). The responses in *a* and *b* are examples of neurons that show only one type of proton chemosensitivity. *Horizontal bars* represent the time of agonist application.

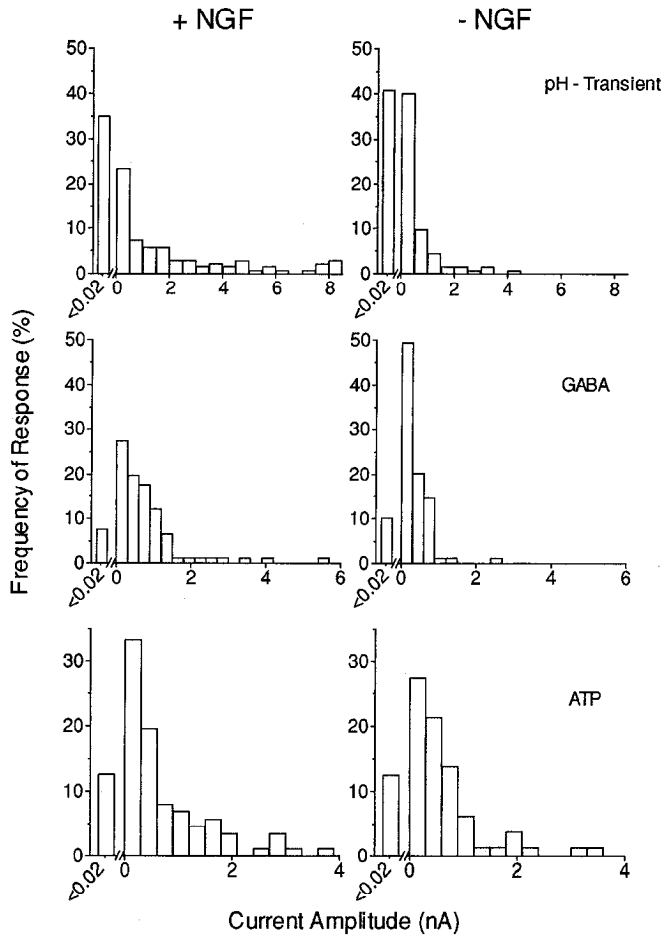

Figure 4. Frequency distributions of amplitudes of the transient, low pH- (*top*), GABA- (*middle*), and ATP-evoked currents (*bottom*). Left-hand bars show percentage of neurons with no measurable current (<20–30 pA). Neurons were cultured for 2 weeks in either NGF or NGF-free medium.

Table 4. Comparison of GABA, ATP, and low pH (transient) sensitivity after 1 and 2 weeks in culture with or without NGF

		1 week			2 weeks		
		NGF+	NGF−	Ratio NGF−: NGF+	NGF+	NGF−	Ratio NGF−: NGF+
GABA	*P*(*R*)	82% (22/27)	78% (31/40)		92% (84/91)	90% (80/89)	
	A(tot) (nA)	1.20 ± 0.23	0.83 ± 0.17	69.2%	0.78 ± 0.09	0.36 ± 0.04†	46.2%
	A(*R*) (nA)	1.47 ± 0.24	1.07 ± 0.20	72.8%	0.85 ± 0.10	0.40 ± 0.04†	47.1%
ATP	*P*(*R*)	75% (18/24)	82% (32/39)		87% (76/87)	88% (70/80)	
	A(tot) (nA)	1.18 ± 0.27	1.13 ± 0.18	95.8%	0.68 ± 0.08	0.67 ± 0.09	98.5%
	A(*R*) (nA)	1.57 ± 0.33	1.31 ± 0.20	83.4%	0.76 ± 0.09	0.76 ± 0.10	100%
pH-transient	*P*(*R*)	67% (92/138)	67% (83/123)		65% (89/137)	59% (80/135)	
	A(tot) (nA)	1.08 ± 0.16	0.78 ± 0.14	72.2%	1.41 ± 0.19	0.36 ± 0.06†	25.5%
	A(*R*) (nA)	1.62 ± 0.23	1.16 ± 0.19	71.6%	2.17 ± 0.26	0.62 ± 0.09†	28.6%

The data for each time point are from groups of sister cultures from the same batches of neurons. Probabilities of differences between NGF+ and NGF− cultures: *, < 0.05; †, < 0.001. No symbol indicates $P > 0.05$.

nA) than in 1 week NGF− neurons (0.11 ± 0.04 nA, Table 2, $P < 0.001$) and although smaller than in 2 week NGF+ neurons (0.71 ± 0.22 nA, Table 3) the difference was not significant ($P > 0.2$). The recovery of proton sensitivity paralleled that for capsaicin sensitivity. Again while some NGF−/+ neurons responded to both agents others were unresponsive.

GABA sensitivity

The presence or absence of NGF made no difference to the proportion of GABA (100 μM) sensitive DRG neurons, and *P*(*R*) values of 78–92% were found in NGF+ and NGF− neurons after 1 or 2 weeks in culture (see Table 4). However, the relative amplitude of the responses to GABA declined progressively in NGF− cultures. *A*(*R*) in 1 week NGF− neurons was 72.8% of the value in 1 week NGF+ sister cultures, although this change was not statistically significant ($P = 0.096$). A further reduction in GABA sensitivity was noted after another week in culture (Fig. 3*c*,*d*; Table 4) when *A*(*R*) in the 2 week NGF− neurons was significantly reduced to 47.1% of the value in NGF+ neurons ($P < 0.001$) and the distribution of current amplitudes was skewed further towards the smaller values (Fig. 4). This reduction in GABA sensitivity was unrelated to any difference in neuronal size (see below), as *A*(*R*) was still less in the 2 week NGF− neurons when the currents were normalized for cell capacitance (NGF+ 27.2 ± 3.8 pA/pF, $n = 84$; NGF− 13.0 ± 1.3 pA/pF, $n = 79$).

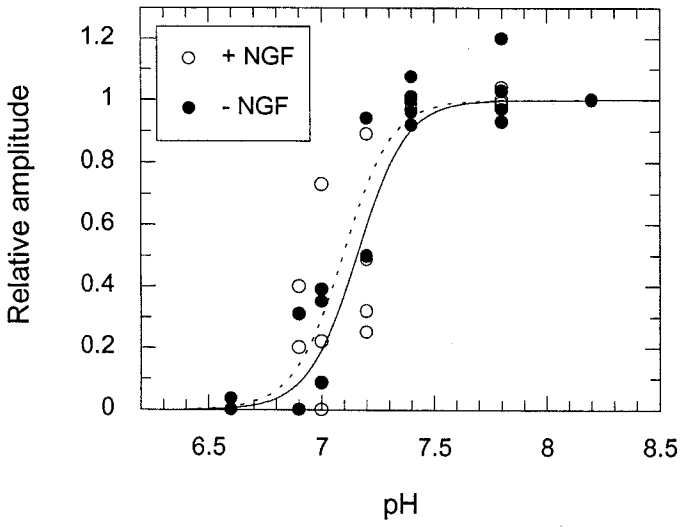

Figure 5. pH dependence of inactivation for the transient, low pH evoked current. Neurons were challenged with pH 5.8 medium after equilibration in solutions of varying pH (abscissa). Responses were normalized to the response measured after stepping from pH 8.2 to 5.8. Lines show sigmoidal plots fitted assuming a stoichiometry of $4H^+$ to 1 "binding site" (Konnerth et al., 1987); estimated half-maximal inactivations were at pH 7.15 (NGF+, *solid line*) and pH 7.09 (NGF−, *dotted line*).

ATP sensitivity

Ten micromolar ATP evoked a depolarizing inward current in 85.6% (267/312) of neurons tested (Fig. 3*e*,*f*). ATP sensitivity appeared to be unaffected by the presence or absence of NGF over a 2 week culture period (see Figs. 3, 4). *P*(*R*) was not decreased in NGF− cultures compared with NGF+ cultures after 1 or weeks (Table 4) and *A*(*R*) was not significantly different in the two conditions even after 2 weeks in culture. The absence of any decrease in ATP sensitivity in NGF− cultures was supported by calculation of the current density normalized for either cell capacitance or cell size (e.g., 2 weeks NGF+ neurons 2.21 ± 0.26 pA/pF, 2 weeks NGF− neurons 2.47 ± 0.28 pA/pF).

General neuronal membrane properties

The presence or absence of NGF had no obvious effect on membrane potential. Measured resting potentials after either one (NGF+ −49.4 ± 1.0 mV, $n = 83$, NGF− −48.8 ± 1.6 mV, $n = 55$) or 2 weeks in culture (NGF+ −48.8 ± 0.7 mV $n = 146$, NGF− −48.0 ± 0.1 mV $n = 140$) were not significantly different ($P > 0.2$). Cell capacitance was also similar for neurons cultured for 1 week in the two conditions (NGF+ 32.2 ± 1.4 pF, $n = 85$; NGF− 31.1 ± 1.8pF, $n = 54$, $P = 0.6$), whereas after 2 weeks a difference in capacitance of the sampled neurons was noted (NGF+ 34.6 ± 1.1 pF, $n = 143$; NGF− 29.9 ± 0.2 pF, $n = 139$, $P < 0.01$). This difference can be accounted for by the smaller size and therefore smaller surface area of neurons studied electrophysiologically in the NGF− cultures (diameter, NGF+ 24.25 ± 0.28 μm, NGF− 22.61 ± 0.29 μm; $P < 0.01$). A small decrease in capacitance of mouse cultured DRG neuron following NGF withdrawal was also noted by Coan et al., 1994).

$^{86}Rb^+$ efflux experiments

$^{86}Rb^+$efflux was used to test the responses of populations of DRG neurons to various stimuli. $^{86}Rb^+$ efflux was measured in

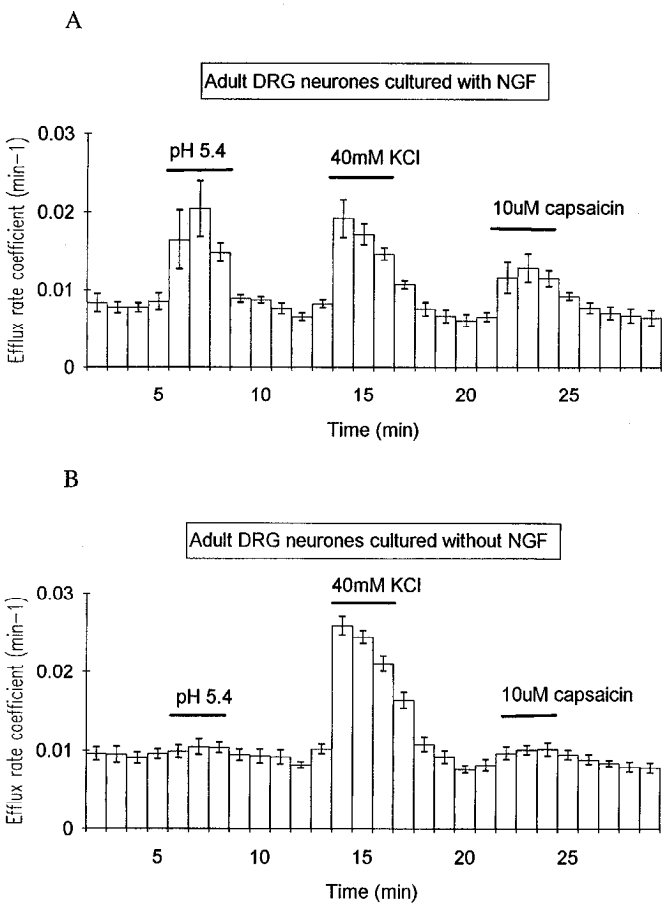

Figure 6. $^{86}Rb^+$ efflux from DRG neurons evoked by sequential 3 min challenges with pH 5.4 medium, high (40 mM) K^+ and 10 μM capsaicin. Neurons were cultured for 2 weeks either with or without NGF.

response to sequential 3 minute challenges with low pH (5.4), high potassium (40 μM KCl) and a high concentration of capsaicin (10 mM). We have shown elsewhere (Bevan et al., 1992) that the $^{86}Rb^+$ efflux response to 10 μM capsaicin is inhibited by the competitive capsaicin antagonist, capsazepine, and so represents a selective effect on DRG neurons. All three stimuli evoked large increases in the rate of efflux in 1–2 week NGF^+ cultures. The efflux responses to both low pH solutions and capsaicin were substantially reduced in 1 week NGF^- cultures, whereas the efflux produced by the high potassium solution was unchanged, or slightly increased. In 2 week NGF^- cultures, the low pH and capsaicin-induced effluxes were almost completely lost while high potassium solutions could still evoke a large efflux (Fig. 6). The results of these efflux studies agree well with our electrophysiological findings and indicate that the loss of capsaicin evoked and sustained proton evoked currents is unlikely to be due to any effect of replating the neurons.

Discussion

The sensitivity of DRG neurons to capsaicin and protons did not vary with time in culture in medium containing NGF whereas GABA and ATP sensitivity declined. For all agonists tested, chemosensitivity did not increase when the DRG neurons were cultured with a high concentration (200 ng/ml) of NGF. This was shown by the comparison of responses from cultured neurons and acutely dissociated neurons. Any change in chemosensitivity when cells were deprived of NGF can therefore be attributed to a loss of sensitivity rather than an NGF-mediated increase in sensitivity in the "control," NGF-treated group.

Our data show that NGF can regulate two types of proton-activated currents as well as GABA sensitivity in adult cultured rat DRG neurons. The effects are similar to those previously described for the capsaicin-activated conductance (Winter et al., 1988) although the responses to the various agents were reduced at different rates in NGF-free medium. Not all membrane properties are NGF regulated. ATP sensitivity, resting potential and the depolarization evoked release of $^{86}Rb^+$, which reflects the activity of voltage gated K^+ channels, were not obviously influenced by the presence or absence of NGF.

The effects of NGF were most pronounced for the capsaicin and sustained proton evoked currents. For the sustained proton evoked current, NGF deprivation resulted in a reduction in both the number of responsive neurons [$P(R)$] and the amplitudes of the currents [$A(R)$ and A(tot)]. This was reflected in the almost total abolition of proton (pH 5.4) evoked ^{86}Rb efflux in NGF^- cultures. The loss of proton sensitivity was reversible as $P(R)$, $A(R)$, and A(tot) were increased when NGF^- neurons were exposed to NGF for a further week. The reduced amplitude of the response of NGF^- neurons was not due to any obvious change in the pH threshold for activation. Similarly, Winter et al. (1988) showed that the loss of capsaicin responses was not due to any shift in the concentration–response curve to higher agonist concentrations.

The capsaicin induced currents and the sustained proton evoked currents were rapidly lost when NGF was removed and were restored to a similar degree when NGF was added back to the culture medium. Bevan and Yeats (1991) found that DRG neurons had either both types of conductance or neither, which raised the possibility that both protons and capsaicin open the same channel. Their co-regulation by NGF supports this idea, which is also consistent with the preliminary results from single channel recordings (Bevan et al., 1993).

The transient current evoked by exposure to low pH solutions (Krishtal and Pidoplichko, 1980, 1981; Konnerth et al., 1987) was also reduced when neurons were cultured in the absence of NGF. In this case the loss of sensitivity was slower than for both the sustained proton evoked current and the response to capsaicin. The smaller size of the transient current in NGF^- neurons cannot be ascribed to a difference in channel inactivation as NGF^+ and NGF^- neurons showed similar pH-current amplitude relationships (half inactivation at pH 7.1). Furthermore a reduced proton sensitivity in NGF deprived neurons was still seen after maximizing the amplitude of the response by alkalinization to pH 7.8 before challenge with the acid solution. We also found no evidence that removal of NGF shifted the pH activation curve to more acidic values. Together these results indicate that the reduction in transient current amplitude represents a genuine loss of functional membrane ion channels rather than a modification in their pH activation/inactivation characteristics.

Removal of NGF also caused a progressive slow loss of GABA sensitivity and $A(R)$ declined to 47% of the control NGF^+ value over 2 weeks. We have no data on the reversibility of this effect. Aguayo and White (1992) did not find any significant NGF-dependent change in GABA sensitivity in similar experiments with adult rat DRG neurons in culture. This discrepancy may be due to the lower concentrations of NGF and the small sample sizes used in their experiments. Previous studies have shown that GABA sensitivity of dorsal roots was attenuated *in vivo* when the distal nerve was transected (Kingery

et al., 1988; Bhisitkul et al., 1990) and the authors postulated that a reduced supply of NGF could account for this change in chemosensitivity. No obvious decrease was noted in the first week, but GABA induced depolarizations were reduced by 40–50% 12–21 d after surgery. The loss of GABA sensitivity could be ameliorated if the nerve was allowed to regenerate, possibly because Schwann cells were now able to provide NGF to the regenerating nerve fibers (Bhisitkul et al., 1990). Our results showing a relatively slow loss of GABA sensitivity when DRG neurons are deprived of NGF are consistent with these *in vivo* data.

Only 40% of adult rat DRG neurons in culture express mRNA for trkA (De Felipe and Hunt, 1994), which is a component of the high affinity NGF receptor (see, e.g., Meakin and Shooter, 1992). This is similar to the percentage of neurons that are sensitive to capsaicin and show the sustained proton evoked current. Furthermore, NGF regulated these chemosensitivities in most neurons which suggests an almost complete correspondence between the populations. Larger proportions of neurons showed the transient proton evoked current (53–67%) and responded to GABA (78–92%). In these cases, it is unclear if the chemosensitivities of all neurons are reduced in NGF-free medium or whether the changes are restricted to the trkA expressing neurons.

Our data show that NGF regulates the sensitivity of DRG neurons to capsaicin, protons, and GABA and that the NGF-regulated reduction in sensitivity occurs at different rates (capsaicin = sustained proton > transient proton > GABA). In contrast, ATP sensitivity was not regulated by NGF. Estimated half-times for the loss of chemosensitivities are, for capsaicin and sustained proton, 2–3 d; transient proton, ~9 d; GABA, 12–13 d. The electrophysiological estimates for the rate of loss of capsaicin sensitivity agree well with the more detailed time course shown by ^{45}Ca uptake experiments (half-time 3 d, Winter et al., 1988).

NGF belongs to a family of related molecules that is important in the development and maintenance of sensory neurons. Although NGF is not required for the survival of the majority of adult DRG neurons, it can affect the expression of substance P and CGRP (Lindsay and Harmar, 1989; Lindsay et al., 1989), two neuropeptides that may be involved in pain and neurogenic inflammation. Studies on rat nodose ganglion neurons in culture have illustrated that NGF promotes the expression of acetylcholine sensitivity by increasing the size of the currents but not the proportion of responsive neurons (Mandelzys and Cooper, 1992). Thus our data and other studies show that neuronal membrane chemosensitivity as well as neuropeptide content can be influenced by NGF. Other experiments have illustrated that NGF can also regulate the expression of ion channels involved in action potential generation (Chalazonitis et al., 1987; Aguayo et al., 1991; Aguayo and White 1992Ritter and Mendell, 1992). Voltage-clamp studies of adult rat cultured DRG neurons have shown that expression of TTX insensitive sodium currents requires NGF (Aguayo et al., 1991; Aguayo and White 1992), although the same experiments suggest that the TTX-sensitive sodium current is not downregulated by NGF deprivation. This finding, together with our observation that the responses to ATP were not regulated by NGF in cultured DRG neurons, indicates that not all ion channels are regulated in the same way.

In vivo, NGF is made in tissues that are innervated by peripheral neurons, and is retrogradely transported back to the DRG cell bodies (Goedert et al., 1981; Gordon, 1983) where it acts to regulate cellular phenotype. NGF levels can be greatly elevated in inflamed tissue (Otten, 1991; Woolf et al., 1994) and increased availability of NGF may upregulate production of neuropeptides in neurons innervating that tissue (Donnerer et al., 1992; Woolf et al., 1994). Moreover, when NGF is supplied to peripheral nerve *in vivo*, it dramatically increases the responses of dorsal horn neurons to a standard C-fiber stimulus of the NGF treated nerve (Lewin et al., 1992). Recent studies have shown that systemic application of NGF can cause thermal and mechanical hyperalgesia (Lewin et al., 1993), and that antisera that neutralize NGF can prevent the development of hyperalgesia in adjuvant-injected rat paws (Lewin and Mendell, 1992; Woolf et al., 1994). Changes in chemosensitivity in inflammation have not been reported. We found no evidence that any of the chemosensitivities studied were increased by exposure to high levels of NGF *in vitro*. Furthermore capsaicin sensitivity, as judged by $^{45}Ca^{2+}$ uptake, is not increased in DRG neurons innervating inflamed tissues (unpublished observations). These findings do not rule out the possibility that raised concentrations of NGF can upregulate the chemosensitivity of a subpopulation of neurons which innervate tissues that normally produce very low levels of NGF.

The sensory nerves may be deprived of an adequate supply of NGF in certain neuropathic conditions, some of which involve nerve damage. Changes in neuronal phenotype consistent with NGF-deprivation have been observed in experimentally induced diabetic rats (Brewster et al., 1994). In such conditions the neurons may display reduced chemosensitivity. Nerve section has been shown to decrease DRG neuron capsaicin sensitivity as judged by $^{45}Ca^{2+}$ uptake assays (unpublished observations). The sensitivity to the inhibitory transmitter GABA is also decreased by nerve section (Kingery et al., 1988) and NGF deprivation. This change in GABA sensitivity may augment the excitatory synaptic transmission between these primary afferents and spinal cord neurons. These observations, taken together, imply that NGF plays an important role in determining the chemosensitivity and "connectivity" of sensory neurons in hyperalgesic and neuropathic states.

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