Non-neuronal cells inhibit catecholaminergic differentiation of primary sensory neurons: role of leukemia inhibitory factor

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SUMMARY

Although some sensory ganglion cells in mature animals are catecholaminergic, most mammalian sensory neurons that express the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH) do so only transiently during early gangliogenesis in vivo. The lack of TH expression at later stages appears to be due to modulation of this catecholaminergic potential. A previous study showed that the phenotype reappears, for example, when E16.5 and older sensory ganglia are dissociated in culture into single cells, suggesting that extracellular influences can modulate TH expression. Moreover, TH expression in dissociate cultures is cell-density dependent, as a four-fold increase in plating density led to a 30% decrease in the percentage of TH neurons. The present study demonstrates that inhibition of TH expression in high density cultures is mediated by ganglionic non-neuronal cells (NNC), as removal of NNC abolished density-dependent inhibition. Moreover, plating E16.5 trigeminal neurons at low density on top of NNC monolayers resulted in an 85% decrease in the percentage of TH neurons. Treatment of cultures with non-neuronal cell conditioned medium (NNC-CM) reproduced the effect of coculture with NNC, suggesting that diffusible factors from NNC were involved in the inhibition of TH. The inhibitory effect of NNC-CM was mimicked by treatment of dissociate cultures with ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF). However, immunoprecipitation of NNC-CM with antibodies against LIF or CNTF showed that only anti-LIF antibodies were able partially to remove the TH inhibitory activity of NNC-CM. Therefore, LIF is one, but not the only, factor mediating NNC inhibition of TH expression in cultured sensory neurons. In summary, these data indicate that ganglionic NNC can regulate sensory transmitter phenotype in culture by inhibiting expression of specific molecular traits. The finding that LIF can partially account for the inhibitory effect of ganglionic NNC on TH expression suggests a novel role for this cytokine in regulating differentiation of catecholaminergic properties in sensory neurons.

Key words: transmitter phenotype, ciliary neurotrophic factor, LIF, tyrosine hydroxylase, sensory ganglia, rat embryo

INTRODUCTION

Primary sensory neurons exhibit markedly diverse neurochemical properties, including multiple neuroactive peptides, monoamines and amino acid transmitters (Hökfelt et al., 1976; Katz and Karten, 1980; Katz et al., 1987; Dodd et al., 1983; Hökfelt, 1991; Price, 1985; Battaglia and Rustioni, 1988; Kai-kai, 1989). Despite considerable progress in documenting transmitter diversity in mature sensory neurons, relatively little is known about mechanisms that generate phenotypic heterogeneity in these cells. Recent studies, however, suggest that epigenetic factors, including target tissues and ganglionic non-neuronal cells, may play an important role. Studies in vivo, for example, indicate that in some populations of mature sensory neurons, transmitter phenotype is correlated with patterns of peripheral target innervation (Katz and Black, 1986; McMahon and Gibson, 1987; McMahon and Moore, 1988; Horgan et al., 1990, Horgan and van der Kooy, 1992; Philippe et al., 1988). Moreover, forcing sensory neurons to innervate tissues other than their normal targets can alter patterns of peptide expression to some degree (McMahon and Gibson, 1987; McMahon and Moore, 1988; Horgan and van der Kooy, 1992). These observations suggest that target-derived signals can influence sensory transmitter properties under experimental conditions in vivo.

Recent studies indicate that factors intrinsic to the developing ganglionic microenvironment may also be important in regulating sensory transmitter development (Mudge, 1981a,b; Katz, 1991). For example, previous work in this and other laboratories demonstrated that many sensory neurons only transiently express the catecholamine synthesiz-
ing enzyme tyrosine hydroxylase (TH) during early stages of gangliogenesis (embryonic day (E)10.5-15.5) in vivo (Jonakait et al., 1984; Katz and Erb, 1990). However, the ability to express this phenotype persists at later stages, indicating that the loss of expression in vivo is due to modulation of this catecholaminergic potential (Katz, 1991). Moreover, the phenotype reappears when E16.5 and older ganglia are dissociated in culture into single cells, suggesting that factors associated with cell aggregation modulate TH expression (Katz, 1991). In support of this hypothesis, we found that TH expression was inversely proportional to cell density; specifically, a fourfold increase in plating density led to a significant 30% decrease in the number of TH neurons without a drop in overall neuronal survival (Katz, 1991). These findings raised the possibility that cell-cell interactions play a role in inhibiting TH expression in sensory neurons after the period of transient expression in vivo. The present study was undertaken, therefore, to begin defining molecular mechanisms that may be responsible for modulating catecholaminergic phenotypic expression during sensory gangliogenesis.

MATERIALS AND METHODS

Dissociated cell cultures

E16.5 trigeminal ganglia (TG) from Sprague-Dawley rat embryos (Zivic-Miller) were grown in Leibovitz’s L-15/CO2 medium containing 10% NuSerum (Collaborative Research), 7.5% heat-inactivated fetal bovine serum (Gibco), fresh vitamin mixture (Mains and Patterson, 1973), nerve growth factor (NGF; 5 ng/ml) and penicillin/streptomycin (50 IU/ml; 50 µg/ml) in plastic 24-well tissue culture plates (Corning). Cells were plated onto acid-washed glass coverslips coated with polylysine (0.1 mg/ml) and laminin (0.3 µg/ml) in 24-well tissue culture plates (Corning). NGF was prepared according to the protocols of Smith et al. (1969) and Stach et al. (1977) and was generously provided by Dr Kenneth Neet (Chicago Medical College). Plating efficiency, that is, the percentage of neurons plated that survived during the 24 hour culture period, was determined by comparing the number of neurons in control cultures 6 and 24 hours after plating. 6 hours was the earliest point at which the round, phase-bright neuronal cell bodies could be readily distinguished from the flat, phase-dark non-neuronal cells and was therefore used to estimate the number of neurons initially plated. The same fields were counted at both time points. On average, over 95% of the neurons seen at 6 hours were present after 24 hours [522.8±95.1 (6 hours) versus 514.2±104.85 (24 hours), n=5, P<0.05]. For experiments involving mitotic labeling, dissociated cell cultures were incubated for 24 hours with bromodeoxyuridine (BrdU; 1:100; Collaborative Research). Recombinant CNTF was supplied by Regeneron Pharmaceuticals, Inc. and Synergen. Recombinant human LIF was purchased from Collaborative Research.

Neuron-enriched cultures

Neuron-enriched cultures were obtained using the preplating technique of McCarthy and Partlow (1976) with minor modifications. In brief, dissociated ganglion cells were plated into 60 mm plastic Petri dishes and left for 3-4 hours in the tissue culture incubator. During this period, most non-neuronal cells and a small number of neurons attached firmly to the Petri dish, leaving the majority of neurons and some non-neuronal cells loosely attached or floating in the medium. The suspended and loosely attached cells were removed by aspirating the medium which was then spun to produce a cell pellet. The pellet was then resuspended in the appropriate media for culture.

Immunocytochemistry

After appropriate times in culture, cells were rinsed with serum-free culture medium and then fixed sequentially in 4% paraformaldehyde in 0.1 M sodium acetate buffer, pH 6.5, for 10 minutes followed by 4% paraformaldehyde in 0.1 M sodium borate buffer, pH 10.5 for 20 minutes. Cultures were then rinsed in several changes of phosphate-buffered saline (PBS) and processed for TH and neurofilament protein (NF) double-immunostaining as previously described (Katz, 1991). The following immunocytochemical reagents were used: polyclonal anti-TH (1:200; Pel-Freez), monoclonal anti-NF160,68 (1:40; Sigma), monoclonal anti-BrdU (1:50; Becton Dickinson), goat anti-rabbit IgG-FITC (1:200; Boehringer Mannheim) and sheep anti-mouse IgG-Rhodamine (1:200; Cappel). The proportion of TH neurons (TH+/NF+) was determined by direct cell counts using 20x and 40x objectives.

Western blotting

Cultures were harvested by trypsinization and pelleted. The cell pellets were suspended in 35 µl Laemmli buffer (Laemmli, 1970), vortexed and boiled for 10 minutes. Supernatants were then fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (10%) using Rainbow molecular weight markers (Collaborative Research) as standards. Each lane received supernatant from equivalent numbers of cultured ganglia. Fractionated proteins were electrotransferred to nitrocellulose membranes and probed with monoclonal anti-TH (1:1000; Incstar) and secondary goat anti-mouse-HRP (1:3000; Cappel). Bound antibody-HRP was reacted with ECL reagents (Amersham) and the immunoblots were then exposed to Hyperfilm (Amersham). To verify that equal numbers of neurons were loaded per lane, blots were reprobed after anti-TH staining with monoclonal anti-neurofilament 68 (NF-68) antibody. Briefly, after TH staining, blots were stripped with Tris (62.5 mM) buffer containing SDS (2%) and beta-mercaptoethanol (100 mM) (50°C for 30 minutes) and reprobed with monoclonal anti-Neurofilament-68 (NF-68; 1:300; Sigma). The amount of immunoreactive TH and NF was quantified by densitometric scanning (SciScan 5000, USB).

Immunoprecipitation

(a) Anti-CNTF

Non-neuronal cell conditioned medium (NNC-CCM) was first concentrated 5- to 10-fold by 10x10³ M Cutoff Centricon and Centricron filters. The concentrated CM was diluted in buffer containing PBS, pH 7.3 with 0.2% BSA and 0.02% polyethylene glycol 6000. A rabbit polyclonal antiserum (Regeneron Pharmaceuticals, Inc.) generated against recombinant CNTF was added to concentrated NNC-CM at a final dilution of 1:4. After overnight incubation at room temperature, the antigen-antibody complex was absorbed to 1/10 volume of protein A-Sepharose (Sigma) for a further 2 hours at room temperature. The complexes were separated by centrifugation and the supernatant was used for cell culture assays. As a negative control, normal L-15 medium was processed in parallel by the same immunoprecipitation procedure, with or without anti-CNTF. Medium containing CNTF (100 ng/ml) was used as a positive control.

(b) Anti-LIF

A rabbit polyclonal antiserum generated against the N terminus of LIF (P.H. Patterson, Caltech) was added to NNC-CM at a final
dilution of 1:100 or 1:20. After overnight incubation at room temperature, the antigen-antibody complex was absorbed to protein A-Sepharose. The complexes were separated by centrifugation and the supernatant used for cell culture assays. Preimmune serum was used as a control for the specificity of anti-LIF.

RESULTS

TH expression in E16.5 trigeminal ganglion dissociates

Previous studies in our laboratory demonstrated that approximately 40% of sensory neurons in the E16.5 jugular-superior sensory ganglia (JSG) express TH immunoreactivity in dissociate culture, despite the fact that virtually no cells exhibit this phenotype in vivo at this age (Katz, 1991). Growth at high cell density reduced the percentage of TH neurons, suggesting that factors associated with cell aggregation may play a role in regulating expression of the enzyme. The utility of the JSG for further studies was limited, however, by its small size; consequently, we sought to determine whether cells in the larger trigeminal ganglion exhibited similar patterns of TH expression and regulation in culture. Dissociate cultures of embryonic day E16.5 rat trigeminal ganglia (TG) were grown as described in Experimental Procedures. At low plating density (0.5 TG/well), 24 hour cultures contained approximately 35% TH+ neurons (Fig. 1). The TH+ cells exhibited typical sensory neuron profiles, including large, rounded bipolar cell bodies and smooth neurites, and they were indistinguishable morphologically from other neurons in the cultures (Fig. 1). The specificity of the TH immunofluorescence was established by (1) the abolition of staining in the absence of primary antibody and (2) Western blots of TH protein in homogenates of TG dissociate cultures (Fig. 7). These initial data demonstrated that, as in the vagal sensory ganglia, 24 hour dissociate cultures of E16.5 TG contain a large proportion of neurons that express TH in vitro, despite the fact that this phenotype is not seen in the E16.5 ganglion in vivo. To define the time course of TH expression in vitro, cultures were examined 6, 10 and 24 hours after plating. These studies revealed that TH was already detectable in approximately 10% of neurons after 6 hours of culture and reached a plateau of approximately 35% by 10 hours (Fig. 2).

Is the increase in TH expression in ganglion dissociates due to neuronal proliferation?

The marked increase in the proportion of TH neurons during the first 24 hours of culture could have been due to proliferation of cells with catecholaminergic potential or to an increase in TH levels per cell, or both. To distinguish among these possibilities, cultures were grown in the presence of bromodeoxyuridine (BrdU), a uridine analog that

Fig. 1. Comparison of tyrosine hydroxylase (TH) immunoreactivity in E16.5 trigeminal neurons in situ and after 24 hours in low density cultures. (A,B) Neurofilament [NF; (A)] and TH (B) immunoreactivity in a 10 µm section through an E16.5 TG. Only rare, faintly TH+ neurons can be seen in the ganglion in vivo at this age (arrow). (C,D) NF (C) and TH (D) staining in a 24 hour low density culture of E16.5 TG dissociates. Approximately 35% of ganglion neurons exhibit strong TH immunoreactivity in culture (see also Fig. 2). Scale bar equals 34 µm.
is incorporated into cells undergoing DNA synthesis. Cultures were then stained for BrdU and TH and examined for the presence of cells exhibiting both markers. Although many non-neuronal cells, which are known to divide in culture, were BrdU+, neither TH+ nor TH− neurons showed mitotic labeling. Cell division cannot, therefore, account for the rapid rise in TH cell number observed during the first 24 hours of culture. Instead, these data indicate that TH levels per cell had increased, above the threshold for detectability, in a subset of postmitotic trigeminal neurons during the 24 hour culture period.

**Is TH expression in trigeminal dissociates regulated by cell density?**

To determine whether the level of TH expression in TG neurons was dependent on cell density, dissociates were plated at 0.5 and 2.0 ganglia per well and monitored for TH immunoreactivity. These experiments revealed a significant 50% decrease in the percentage of TH neurons in high density cultures compared to low density controls. Thus, as previously described for vagal ganglion cells (Katz, 1991), TH expression in trigeminal dissociates was sensitive to changes in plating density.

**Are density effects on TH expression mediated by non-neuronal cells?**

To determine whether density effects on TH expression were mediated by cues of neuronal or non-neuronal cell origin, neuron-enriched cultures of E16.5 TG were grown at low (equivalent to 0.5 TG/well) and high (equivalent to 2.0 TG/well) densities and monitored for TH expression. Non-neuronal cells were removed by the pre-plating technique of McCarthy and Partlow (1976) with minor modifications (see Materials and Methods). This procedure produced an approximate 9-fold enrichment in the proportion of neurons to non-neuronal cells. Under these conditions, there was no difference in the percentage of TH neurons at low and high plating densities after 24 hours in culture (Fig. 3), suggesting that non-neuronal cells were responsible for density-dependent modulation. To examine this possibility directly, TG dissociates were plated at low density onto a monolayer of ganglionic non-neuronal cells. Non-neuronal cells were obtained by plating ganglion dissociates for 3-5 days in the absence of NGF; under these conditions, neurons die, leaving only Schwann and satellite cells and fibroblasts. In low density neuron cultures growing on top of the non-neuronal cell monolayer, TH cell numbers were reduced by 85% compared to low density cultures alone (Figs 4 and 5). Total neuronal survival was not decreased by coculture with non-neuronal cells, ruling out the possibility that the drop in TH cell number was due to a selective loss of cells with catecholaminergic potential. These data demonstrated that cues of non-neuronal cell origin could account for the inhibition of TH seen in high density mixed cultures of trigeminal ganglion cells.

**Is contact required for non-neuronal cell inhibition of TH?**

To determine whether cell-cell contact was necessary for the inhibition of TH expression seen in the presence of non-neuronal cells, neurons were grown at low density in the presence of non-neuronal cell conditioned medium (NNC-CM). In these experiments, non-neuronal cell layers were obtained as before (see above); medium from these cultures was then collected between days 1 and 3 or days 4 and 5.
Fig. 4. Non-neuronal cell (NNC) layers inhibit TH expression in low density TG dissociates. The percentage of TH neurons in 24 hour dissociate cultures of E16.5 trigeminal ganglia were counted in the absence (Control) or presence (+NNC) of a layer of ganglionic non-neuronal cells. The increased number of neurons in the cocultures appeared to be due to better attachment during the immunostaining procedures. Each value represents the mean ± s.e.m. of at least nine determinations from more than three independent experiments. *$P<0.0001$ (unpaired t-test).

Fig. 5. TH/NF double-staining of E16.5 trigeminal sensory neurons grown at low density on a layer of ganglionic non-neuronal cells. (A) NF-immunoreactive neurons growing on a background of ganglionic non-neuronal cells. (B) The same cells double-stained for TH; arrow indicates one faintly stained neuron. Scale bar equals 34 µm.

Fig. 6. (A) Effect of non-neuronal cell conditioned medium on TH expression in E16.5 trigeminal dissociates. TG non-neuronal cell conditioned medium (NNC-CM) was collected between days 1 and 3 (1-3d CM) and days 4 and 5 (4-5d CM) of culture and used at a dilution of 1:1 with fresh medium (see text for details). Control = 24 hour low density cultures. Each value represents the mean ± s.e.m of at least eight determinations from at least three independent experiments. *$P<0.001$ (one-way ANOVA).

(B) Dose-response curve for NNC-CM. NNC-CM was concentrated approximately 10-fold with $10^3 M_r$-cutoff Centriprep filters. Concentrated NNC-CM (+NNC-CM) or concentrated control medium (Control) was then added to low density TG cultures at concentrations of 0.2%, 5% and 15%. TH inhibition produced by 5% concentrated CM was equivalent to that seen with 50% unconcentrated CM. Each point represents the mean ± s.e.m of six determinations.
of culture and used at a dilution of 1:1 with fresh medium. In the presence of 50% NNC-CM, the percentage of TH neurons in low density TG cultures was significantly reduced to approximately 50% of controls without any change in overall neuronal survival (Fig. 6A). Moreover, the effect of NNC-CM was dose-dependent (Fig. 6B). To determine if our counts of TH cell number faithfully reflected a quantitative reduction in TH expression, low density cultures grown in the presence or absence of 50% NNC-CM were assayed by TH Western blotting. These experiments demonstrated that treatment with NNC-CM caused an average 60% reduction in the level of TH protein per culture compared to controls (Fig. 7). These data suggested that ganglionic non-neuronal cells released a diffusible factor (or factors) that could mimick the TH inhibitory effect of direct co-culture.

To determine whether cell-cell contact could also inhibit TH expression in the absence of secreted factors, non-neuronal cell layers were killed by exposure to 2 N acetic acid for five minutes, washed extensively with medium, and then used as a culture substratum. In contrast to the effect of NNC-CM, or living non-neuronal cells, growth of TG neurons on top of killed non-neuronal cells had no significant effect on the percentage of TH neurons. Moreover, total cell survival was not significantly different from controls (Fig. 8).

**Molecular mechanisms of TH inhibition by non-neuronal cell CM**

To begin characterizing potential molecular mechanisms underlying non-neuronal cell effects on TH expression in sensory neurons, we next sought to mimic the response with factors known to modulate TH levels in other systems. In sympathetic neurons, for example, coculture with ganglionic non-neuronal cells also inhibits TH; this effect is mediated by LIF (Fukada, 1985; Yamamori et al., 1989). Another peptide, Ciliary Neurotrophic Factor (CNTF), also reduces TH levels and increases ChAT activity in cultures of sympathetic neurons (Saadat et al., 1989). To determine whether TH in sensory neurons was regulated by similar mechanisms, low density TG cultures were grown in the presence or absence of 50% NNC-CM. Virtually no neurons survived either in control cultures (without CM) or in the presence of 50% CM (sufficient to produce 50% inhibition of TH in E16.5 TG dissociate cultures), whereas most neurons survived in the presence of added CNTF (>1 ng/ml; Fig. 11). These findings suggested either that CNTF was absent in NNC-CM, or alternatively, that CNTF was present at concentrations lower than 2 ng/ml (1 ng/ml CNTF was the minimum concentration required for CG neuron survival; data not shown). To distinguish among these possibilities, we examined the effect of immunoprecipitation with anti-CNTF antibodies on the TH inhibitory activity in NNC-CM. In controls, anti-CNTF removed greater than 90% of CNTF activity from medium containing 100 ng/ml added CNTF. Under the same conditions, however, immunoprecipitation with anti-CNTF antibodies had no effect on the TH inhibitory activity in NNC-CM. (Fig. 12A,B).

To examine whether LIF was present in NNC-CM, immunoprecipitation was also performed with anti-LIF antibodies. As shown in Fig. 12C, anti-LIF, diluted 1:100, was able to remove a significant fraction of the TH inhibitory activity found in NNC-CM. To determine if this partial effect was due to a low concentration of antibody, the dilution of anti-LIF was increased five-fold to 1:20. neuronal survival (Fig. 9A). This corresponded to a 65% decrease in TH protein per culture as determined by Western blot (Fig. 10). Similar effects were obtained with recombinant CNTF (10 ng/ml; Fig. 9B).

In view of the effects of exogenous CNTF and LIF, we investigated whether either peptide was present in NNC-CM. To approach this issue, we first examined whether non-neuronal cell CM exhibited CNTF biologic activity. For this purpose, survival of E8 chicken ciliary ganglion neurons, which are CNTF-dependent in culture (Adler et al., 1979; Barbin et al., 1984), was assayed in the presence or absence of TG NNC-CM. Virtually no neurons survived either in control cultures (without CM) or in the presence of 50% CM (sufficient to produce 50% inhibition of TH in E16.5 TG dissociate cultures), whereas most neurons survived in the presence of added CNTF (>1 ng/ml; Fig. 11). These findings suggested either that CNTF was absent in NNC-CM, or alternatively, that CNTF was present at concentrations lower than 2 ng/ml (1 ng/ml CNTF was the minimum concentration required for CG neuron survival; data not shown). To distinguish among these possibilities, we examined the effect of immunoprecipitation with anti-CNTF antibodies on the TH inhibitory activity in NNC-CM. In controls, anti-CNTF removed greater than 90% of CNTF activity from medium containing 100 ng/ml added CNTF. Under the same conditions, however, immunoprecipitation with anti-CNTF antibodies had no effect on the TH inhibitory activity in NNC-CM. (Fig. 12A,B).

![Western blot analysis of TH protein levels in E16.5 TG dissociates grown in the presence or absence of non-neuronal cell conditioned medium. Lanes (a) liver (negative control); (b) E16.5 superior cervical ganglion (positive control); (c) E16.5 TG, freshly dissociated; (d) E16.5 TG after 24 hours in low density dissociate culture; (e) E16.5 TG after 24 hours in low density dissociate culture in the presence of 50% NNC-CM. Densitometric scanning revealed a 60% decrease in TH protein in the presence of NNC-CM.](image-url)
Under these conditions, immunoprecipitation reduced the level of TH inhibitory activity to the same extent as was seen with the lower concentration of antibody. Taken together, these data indicated that LIF, or a LIF-like molecule, could partially account for the TH inhibitory activity in TG NNC-CM and suggested that other inhibitory factor(s) were present as well.

**DISCUSSION**

Our findings indicate that diffusible cue(s) derived from ganglionic non-neuronal cells, including LIF or a LIF-like molecule, can inhibit TH expression in E16.5 trigeminal sensory neurons in vitro. In view of the fact that trigeminal neurons with catecholaminergic potential do not exhibit detectable TH on E16.5 in vivo, our data raise the possibility that influences of non-neuronal cell origin normally inhibit expression of this phenotype in the intact ganglion.

The appearance of TH⁺ neurons in dissociate cultures of trigeminal sensory neurons is consistent with our previous finding that other cranial ganglia in the embryonic rat con-
tain sensory neurons with catecholaminergic potential (Katz, 1991). These cells exhibit structural features characteristic of embryonic sensory neurons and are distinguished from catecholaminergic autonomic neurons by immunocytochemical criteria, morphology and growth factor responsiveness (Katz, 1991). The fact that trigeminal neurons exhibit detectable levels of TH after 24 hours in vitro, and not in vivo, indicates that the catecholaminergic phenotype expressed by these cells is extremely labile and can be regulated by changes in the extracellular environment.

In the presence of large numbers of ganglionic non-neuronal cells or conditioned medium, trigeminal neurons did not exhibit the marked increase in TH expression observed in low density control cultures. By itself, this observation could be explained either by inhibition of the catecholaminergic phenotype or by selective death of neurons with catecholaminergic potential in the cocultures. However, the fact that the number of TH neurons declined in cocultures is in the absence of any decrease in overall neuronal survival argues against the possibility of selective catecholaminergic cell death. If, on the other hand, death of the catecholaminergic population was accompanied by selective survival of an equivalent number of non-catecholaminergic neurons (i.e., approximately 35% of all neurons), overall neuron survival would also have been the same in the presence and absence of non-neuronal cell influences. This scenario requires, however, that any neurons selectively supported by the non-neuronal cells died in control cultures. This seems unlikely, given that neuron plating efficiency, that is, the percentage of neurons plated that survived after 24 hours in vitro, was over 95% in controls (see Materials and Methods). We conclude, therefore, that the low percentage of TH neurons in the presence of non-neuronal cells or conditioned medium was due to inhibition of catecholaminergic phenotypic expression in an otherwise stable population of cells.

In vivo, TH is normally undetectable in the trigeminal ganglion after E13.5 (Jonakait et al., 1984), despite the presence of sensory neurons with catecholaminergic potential as late as E16.5 (Katz, 1991 and present study). Therefore, the low level of TH expression observed in our cultures, in the presence of non-neuronal cells, closely mimics the in vivo situation. We hypothesize, therefore, that ganglionic non-neuronal cells may normally inhibit TH expression in the E16.5 ganglion in vivo. If this is true, the appearance of large numbers of TH neurons in TG dissociates would indicate that inhibitory influences present in the intact ganglion were below the threshold for detectable biologic activity in low density cultures.

Our findings are similar to the observations of Patterson and colleagues (Patterson and Chun, 1974, 1977a,b) and Bunge and colleagues (Johnson et al., 1976; Bunge et al., 1978) that transmitter phenotype in sympathetic autonomic neurons can be altered by coculture with non-neuronal cells. Specifically, these workers found that non-neuronal cells derived from various tissues, including sympathetic ganglia, could inhibit expression of catecholaminergic properties and stimulate cholinergic differentiation in cultured sympathetic neurons (Patterson and Chun, 1974, 1977a,b). This effect was mediated by a soluble factor (CDF) that was subsequently isolated, sequenced and cloned and found to be identical to LIF (Fukada, 1985; Yamamori et al., 1989). The effect of LIF in vitro was, however, paradoxical, in that most sympathetic neurons exhibit catecholaminergic properties in vivo, despite the presence of non-neuronal cells in sympathetic ganglia. Recently, however, Yamamori demonstrated that LIF mRNA was undetectable in sympathetic ganglia in situ, indicating that non-neuronal cells in this tissue do not normally synthesize the factor in vivo (Yamamori, 1991).

Our data indicate that LIF, or a LIF-like molecule, can partially account for NNC-mediated inhibition of TH expression in cultured TG neurons. This finding indicates that regulatory pathways linking LIF to inhibition of TH expression are not a unique feature of sympathetic neurons.
but rather, shared by sensory neurons as well. LIF has previously been shown to regulate other transmitter properties in cultured DRG neurons. Nawa and colleagues, for example, found that treatment with LIF increased VIP levels and choline acetyltransferase activity and decreased levels of Substance P (Nawa et al. 1991). Thus, LIF appears capable of regulating multiple transmitter phenotypes in responsive populations of sensory neurons in vitro. Our observations raise the possibility that LIF normally inhibits expression of catecholaminergic traits in trigeminal sensory neurons in vivo. In newborn rats, LIF mRNA expression appears to be highly restricted to brain and non-neuronal tissues in the hindlimb footpad (Yamamori, 1991); to our knowledge, expression in sensory ganglia has not been examined. Preliminary analyses of the non-neuronal cells present in our trigeminal ganglion dissociates indicate the presence of both Schwann cells and fibroblasts. Fibroblastic cells from diverse tissues and cell lines are known to secrete LIF in culture (Tomida et al., 1984; Rathjen et al., 1990; Lubbert et al., 1991; Wetzler et al., 1991), suggesting that fibroblasts may be the source of LIF in our cultures.

At present, we cannot exclude the possibility that the inhibitory influence of trigeminal non-neuronal cells on TH expression in sensory neurons is a function of growth in vitro and does not necessarily account for TH inhibition in vivo. However, preliminary data from our laboratory are consistent with a role for LIF in regulating ganglion cell development in vivo as well. Using reverse transcriptase polymerase chain reaction, we have recently detected LIF messenger RNA (mRNA) in the E16.5 TG, suggesting LIF can be produced in the intact ganglion. Moreover, the ganglion contains high levels of mRNA coding for the LIF receptor (LIFR) subunits LIFRB and gp130 (Ip et al., 1992), suggesting that TG cells are LIF-responsive in vivo (Fan, Ip and Katz, unpublished observations).

In contrast to the TH-inhibitory action of LIF, the peptide had no effect on TG neuron survival in our experiments. LIF has, however, recently been shown to support survival of embryonic DRG neurons in culture (Murphy et al., 1991). Thus, it is possible that the survival-promoting effect of LIF is not shared between DRG and TG neurons. Alternatively, the failure of LIF to increase TG neuron survival may have been due to the fact that NGF was always present in our culture medium. Neurons that express TH in E16.5 TG cultures are already dependent on NGF for survival at this age (data not shown; see also Katz, 1991). We cannot, therefore, exclude the possibility that, in the absence of NGF, some TG neurons would be supported by LIF in culture.

We found no evidence that CNTF was present in NNC-CM; however, addition of exogenous peptide decreased TH expression to a level similar to that seen with LIF. This is perhaps not surprising in view of recent evidence that CNTF and LIF share common signalling pathways in some cells (Ip et al., 1992). CNTF has previously been shown to support survival of a subset of dorsal root ganglion (DRG) cells (Barbin et al., 1984). However, the present findings are, to our knowledge, the first evidence that CNTF can also alter transmitter phenotype in sensory neurons. For example, Rao et al. (1992) found that CNTF had no effect on levels of neuropeptide Y, vasoactive intestinal peptide, Substance P or somatostatin in cultured rat DRG neurons. These observations, in conjunction with the present results, indicate that CNTF can selectively alter expression of particular sensory transmitter phenotypes, possibly by acting on only restricted subsets of ganglion cells.

Although the conditioned medium effect that we observed indicates a role for diffusible factor(s), including LIF, in the inhibitory action of non-neuronal cells, NNC-CM alone did not reduce TH levels to the same extent as coculture with non-neuronal cell monolayers. The maximum decrease that we observed with concentrated NNC-CM, for example, was approximately 50% (Fig. 6B) compared to 85% in cocultures. These data may indicate that the inhibitory effect of soluble cues can be facilitated by direct cell contact. This possibility is consistent with reports of membrane-associated activities (Adler and Black, 1986; Kessler et al., 1986; Lee et al., 1990), some of which may be of non-neuronal cell origin (Kessler et al., 1986) that can alter transmitter phenotype in cultured sympathetic neurons.

That ganglionic non-neuronal cells might regulate sensory transmitter properties was first proposed by Mudge and colleagues (Mudge, 1981a,b) based on studies of peptide expression in cultured DRG neurons. These workers found that coculture of chick dorsal root ganglion neurons with nonneuronal cells resulted in a 50-fold increase in somatostatin levels compared to neuron-enriched controls (Mudge, 1981b). Subsequently, however, studies by LeDouarin and colleagues (Xue et al., 1985, 1987, 1988; Ziller et al., 1987) demonstrated that chick DRG contain a population of precursor cells, capable of giving rise to neurons with autonomic phenotypes, in vitro. Moreover, New and Mudge found that peripheral autonomic neurons in chick transiently express somatostatin in vivo (New and Mudge, 1986). Consequently, New and Mudge concluded that non-neuronal cell effects on somatostatin levels in DRG cultures actually reflected peptide expression in latent autonomic precursors (New and Mudge, 1986). Therefore, it is presently unknown whether, in addition to their TH-inhibitory activity, non-neuronal cells also play a role in regulating differentiation of peptidergic traits in sensory neurons.

Our hypothesis that non-neuronal cells inhibit TH expression in sensory neurons in vivo must take into account the fact that some sensory ganglion cells exhibit a catecholaminergic phenotype even in mature animals (Katz et al., 1983; Price and Mudge, 1983). For example, approximately 10% of neurons in the glossoharyngeal petrosal ganglion, as well as neurons in the nodose and geniculate ganglia, contain TH (Katz et al., 1983; Katz and Black, 1986; Katz, 1991). Detailed studies of TH cells in the petrosal ganglion revealed that these are dopaminergic afferent neurons (Finley et al., 1992). The presence of such cells could indicate that either (1) in contrast to the trigeminal ganglion, non-neuronal cells in the nodose and geniculate ganglia do not inhibit TH expression or (2) the neurons in these ganglia are either insensitive to inhibitory cues or receive additional signals that override inhibition by non-neuronal cells. Preliminary experiments indicate that non-neuronal cells from the nodose and trigeminal gan-
glia are equally effective in inhibiting TH expression in trigeminal sensory neurons in vitro. In contrast, TH levels were unchanged in newborn nodose neurons cultured in the presence or absence of TG non-neuronal cells or CM (Fan and Katz, unpublished observations). These data suggest that differences in the ability of neurons to respond to non-neuronal cell influences may contribute to ganglion-specific patterns of TH expression.

In summary, our data indicate that many embryonic trigeminal sensory neurons are capable of expressing a catecholaminergic phenotype even though such traits are not normally seen after early stages of gangliogenesis in vivo. These cells exhibit marked phenotypic plasticity, as evidenced by the mutability of TH expression in the presence or absence of non-neuronal cell influences in culture. Moreover, diffusible signals, including LIF or a LIF-like molecule, mediate non-neuronal cell effects on catecholaminergic expression in embryonic ganglion cell cultures. More generally, our findings suggest that ganglionic non-neuronal cells can influence transmitter diversity in developing sensory ganglia by inhibiting expression of specific neuronal phenotypic traits.

The authors would like to acknowledge the technical assistance of Elizabeth Myers in preparing Western blots. We thank Regeneron Pharmaceuticals, Inc. for generously providing us with CNTF and anti-CNTF antibodies and Synergen for also providing CNTF. We are grateful to Paul Patterson for providing anti-LIF antibodies. This work was supported by NIH grant HL-42131 (D. M. K.) and a Dysautonomia Foundation award (D. M. K.).

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(Accepted 27 January 1993)