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Localization of cholinergic differentiation factor/leukemia inhibitory factor mRNA in the rat brain and peripheral tissues

(sympathetic neuron/sweat gland/phenotypic choice/cytokine)

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ABSTRACT **Sympathetic neurons display considerable plasticity in the neurotransmitter and neuropeptide phenotypes they express *in vitro* and *in vivo*. The cholinergic differentiation factor (CDF, also known as leukemia inhibitory factor, LIF) induces cultured rat sympathetic neurons to become cholinergic, without affecting their survival or growth. To understand the role of this factor in normal development, it is essential to determine where it is produced *in situ*. To localize CDF/LIF mRNA, a semiquantitative, reverse transcription–polymerase chain reaction method was employed. Actin and tubulin mRNA were used as internal controls, and two different sets of CDF/LIF primers were compared. In postnatal rat peripheral tissues, CDF/LIF mRNA was selectively localized in the target area of developing, sympathetic cholinergic neurons; the mRNA was not detected in the targets of sympathetic noradrenergic neurons. This finding supports the hypothesis that CDF/LIF is a target-derived neuronal differentiation factor. In postnatal rat brain, CDF/LIF mRNA is localized selectively in two parts of the visual system, visual cortex and superior colliculus. Thus, CDF/LIF may play a role in this system as well.**

Neuronal phenotype can be influenced by environmental factors (1–3), and culture assays have been used to identify molecules that control phenotypic decisions. One such factor, cholinergic differentiation factor (CDF, also known as leukemia inhibitory factor, LIF; ref. 4), is produced by certain cultured non-neuronal cells and switches the phenotype of cultured rat sympathetic neurons from noradrenergic to cholinergic (5). This protein is known to affect the proliferation and differentiation of many cell types derived from the earliest embryo to the adult (6). The pleiotropic effects of this cytokine revealed *in vitro* raise the question of how and where it acts *in vivo*. Moreover, since a number of distinct proteins can induce cultured sympathetic neurons to become cholinergic (7–10), it is essential to determine where and when CDF/LIF and the other factors are produced *in situ*.

The sweat glands of the rat footpad are well-known targets for cholinergic sympathetic neurons. During normal development, noradrenergic sympathetic neurons are converted to the cholinergic phenotype at the time their axons innervate the glands (11). Moreover, footpads containing sweat glands can induce noradrenergic sympathetic neurons that normally innervate other targets in hairy skin to become cholinergic, when the pads are transplanted to an ectopic site in the hairy skin (12, 13). In addition, extracts of footpads contain an activity that converts cultured sympathetic neurons from noradrenergic to cholinergic (14). Therefore, sweat glands represent a target tissue in which CDF/LIF could be localized *in vivo*. Furthermore, since CDF/LIF can also enhance the cholinergic properties of cultured central nervous system neurons (15), it is also important to study the distribution of this factor in the brain.

While it is possible to detect a CDF/LIF mRNA band in total RNA from cultured heart cells (the source of CDF for the original purification; ref. 16) by Northern hybridization and RNase protection assays (unpublished results), it has been difficult to detect a CDF/LIF signal in tissues such as the rat footpad. Presumably this is due to the low abundance of the CDF/LIF mRNA *in vivo* and the small amounts of tissue available. These findings necessitated the use of the more sensitive reverse transcription–polymerase chain reaction (RT–PCR) method. Indeed, this method was recently used to identify CDF/LIF transcripts in preimplantation blastocysts (17).

MATERIALS AND METHODS

RNA Preparation and PCR. Total RNA was extracted by the acid/phenol method (18) from the tissues described. The amount of total RNA was estimated by the DipStick system (Invitrogen); 0.5 μg of total RNA was used for the cDNA reactions. The volume of the cDNA reaction mixture, containing random primer (pN_6; ref. 19), was 20 μl. One to five microliters of each cDNA reaction mixture was added to make a final 25 or 50 μl of PCR mixture containing 100 ng of either CDF/LIF primers, α-tubulin primers, or β-actin primers. Reaction mixtures were subjected to 40 cycles of PCR (each consisting of 95°C for 1 min, 57°C for 2 min, 72°C for 3 min). Five microliters of each PCR mixture was applied to an agarose (NuSieve GTG, FMC) gel, stained with ethidium bromide, and visualized with UV light. The PCR products subjected to analysis were therefore 1/200th (1/20 × 5/50) of the total PCR products generated in each sample.

In an experiment to calibrate the method for detecting CDF/LIF transcripts, an authentic CDF/LIF transcript was made by T7 RNA polymerase *in vitro*. Various amounts of this transcript were added to 1 μg of total RNA extracted from adult rat heart (Fig. 1).

Primers. The CDF/LIF primers used were 5′-CAATGCCCTCTTTATTTCCTATTACACAGC-3′ and 5′-GGGGACACAGGGCACATCCACATGGCCCAC-3′. The first, 5′ primer covered the junction between the second and third exons (20). This choice was made to avoid a possible PCR contamination from genomic template. The second, 3′ primer covered part of the third exon. The expected size of the PCR product with these CDF/LIF primers is 333 base pairs (bp), denoted by the arrowheads in the upper gels in Figs. 1 and 2. An alternative 5′ primer was also used (5′-CCGTGTCACGGCAACCTCATGAACCAGATC-3′) that covered part of the second exon; the expected size of the PCR product with this primer and the 3′ primer above is 396 bp. The tubulin primers were 5′-CCTGGAACCCACAGTTATTGATGAAGT-

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Abbreviations: CDF, cholinergic differentiation factor; LIF, leukemia inhibitory factor; RT–PCR, reverse transcription–polymerase chain reaction; P*n*, postnatal day *n*.

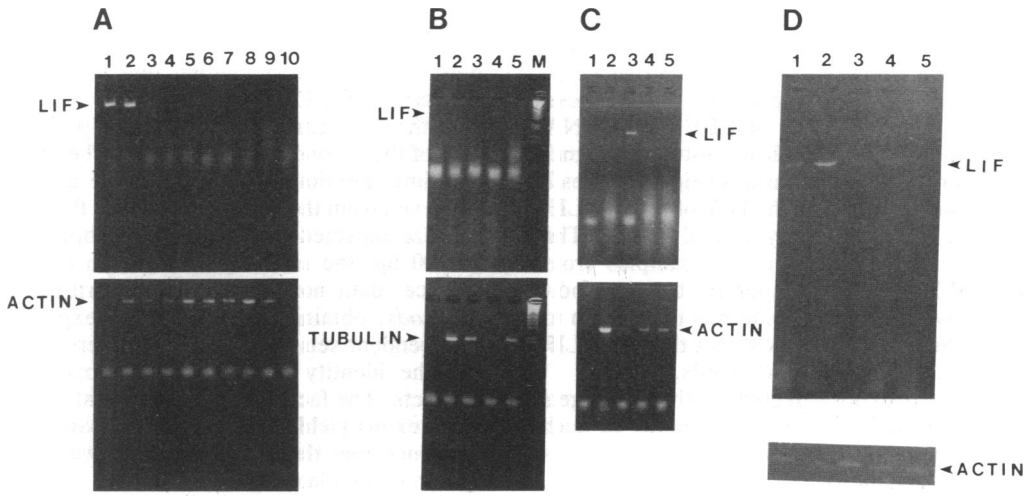

FIG. 1. CDF/LIF mRNA can be selectively detected in rat footpads. PCR used CDF/LIF primers (*Upper*) or control β-actin or α-tubulin primers (*Lower*) (see *Materials and Methods*). The experiments shown in *A–D* were independently conducted at different times. (*A*) Reaction products from PCR amplification of serial dilutions of a CDF/LIF transcript, plus 1 μg of total RNA extracted from adult rat heart. The following amounts of CDF/LIF template were used for lanes 1–10, respectively: 400 pg, 20 pg, 1 pg, 0.05 pg, 2.5 fg, 125 ag, 6.25 ag, 0.3 ag, 0.015 ag, none. (*B*) RNA for PCR amplification was extracted from postnatal day 8 (P8) subcutaneous connective tissue (hairy skin area) (lane 2), P8 hairy skin (lane 3), P8 footpad (lane 4), or P16 footpad (lane 5). Lane 1, no template; lane M, molecular size markers. (*C*) RNA for PCR amplification was extracted from the lacrimal gland (lane 1), gut (lane 2), footpad (lane 3), skeletal muscle (lane 4), and submaxillary gland (lane 5) of P8 rats. (*D*) RNA for PCR amplification was extracted from footpads of P4 (lane 1), P8 (lane 2), P16 (lane 3), P30 (lane 4), and 2-month-old (lane 5) rats. cDNA for the actin assay was diluted 100-fold before use in the PCR amplification.

TCG-3′ and 5′-AGAAGCCCTGGAGACCCGTGCACTG-GTCAG-3′ (21), and the size of the product is 200 bp. The primers used in the actin assay were 5′-TCATGAAGTGT-GACGTTGACATCCGTAAAG-3′ and 5′-CCTAGAAG-CATTTGCGGTGCACGATGGAGG-3′ (22), and the size of the product is 285 bp.

RESULTS

Specificity of the Assay. Conditions were established in which the RT–PCR assay yielded increasing amounts of product with addition of increasing amounts of known CDF/LIF mRNA in the presence of tissue RNA that did not display a CDF/LIF signal of its own (Fig. 1*A*). A band of the correct size (upper arrowhead) was detected within the range of about 1–400 pg CDF/LIF mRNA (lanes 1–3), in the presence of total RNA from adult rat heart tissue, added before cDNA synthesis. This dose–response curve was quite similar to that obtained in the absence of added heart RNA (data not shown). Thus the detection limit of the assay is between 0.05 and 1 pg per sample under these conditions. Additional dilution experiments indicate that the limit is usually 0.2 pg. These results also demonstrate that the absence of a positive CDF/LIF mRNA signal in adult heart (lane 10) is not likely to be due to an inhibitor of the PCR process; that is, the result is not a false negative.

CDF/LIF Is Specifically Expressed in Footpads. A positive CDF/LIF mRNA signal was obtained from the rat footpad, a target of cholinergic sympathetic neurons. Total RNA from P8 and P16 footpads (Fig. 1*B*, lanes 4 and 5) yielded a single band of the size expected for CDF/LIF mRNA. While these bands are weak in the reproduction, the signal was quite clear

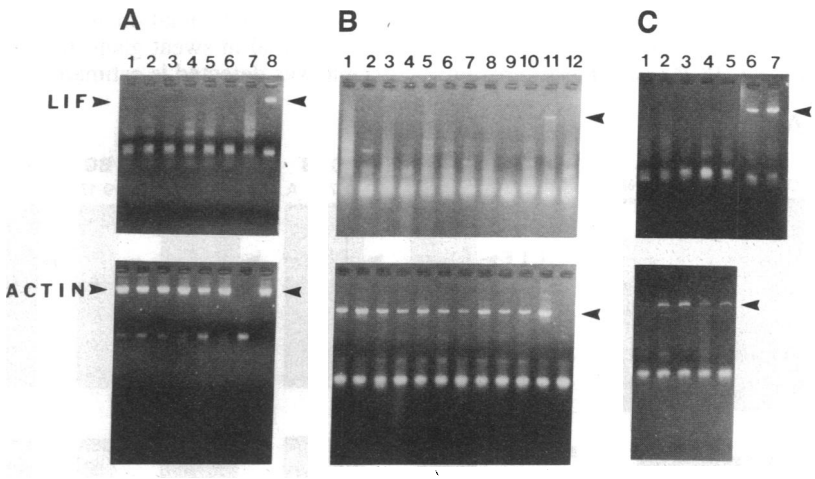

FIG. 2. CDF/LIF mRNA is not detected in a number of peripheral tissues. PCR used CDF/LIF (*Upper*) or actin (*Lower*) primers. Total RNA (0.5 μg) was extracted from various tissues and subjected to PCR amplification. (*A*) Lanes: 1, P7 heart; 2, P18 heart; 3, P9 hippocampus; 4, P19 hippocampus; 5, P18 liver; 6, P16 brain; 7, no template; 8, cultured heart cells [0.02 μg of poly(A)$^+$ RNA was used in this case instead of 0.5 μg of total RNA]. (*B*) Lanes: 1, P8 lacrimal gland; 2, P8 skin; 3, P8 gut; 4, P8 liver; 5, P8 skeletal muscle; 6, P8 submaxillary gland; 7, P11 liver; 8, adult liver; 9, adult skeletal muscle; 10, adult liver (experiment independent from that in lane 8); 11, cultured heart cells; 12, no template. (*C*) Lanes: 1, P8 parotid; 2, adult subcutaneous connective tissue; 3, adult heart; 4, adult liver; 5, adult hairy skin; 6, 1 pg of CDF/LIF RNA produced *in vitro*; 7, 400 pg of CDF/LIF RNA.

in the original gel. Moreover, strong signals were seen in other experiments, as shown below. The CDF/LIF signal was selectively localized, since P8 hairy skin and subcutaneous connective tissue did not display this band; these samples therefore contained <0.2 pg of CDF/LIF mRNA (Fig. 1*B*, lanes 2 and 3). The significant α-tubulin signal generated from the same RNA preparations (Fig. 1*B*, lanes 2 and 3, lower arrowhead) argues that the lack of a CDF/LIF band was not due to a gross deficiency in total mRNA. The lack of a CDF/LIF mRNA signal in the samples from connective tissue and hairy skin is important because footpads contain connective tissue and epidermis in addition to sweat glands. Thus, these results suggest that the CDF/LIF mRNA is indeed localized to the sweat glands.

CDF/LIF mRNA was also not detected in other P8 targets of noradrenergic sympathetic neurons. Examples of such tissues are lacrimal and submaxillary glands (Fig. 1*C*, lanes 1 and 5; Fig. 2*B*, lanes 1 and 6), liver (Fig. 2*A*, lane 5; Fig. 2*B*, lanes 4, 7, 8, and 10; Fig. 2*C*, lane 4), parotid gland (Fig. 2*C*, lane 1), and *in vivo* heart tissue (Fig. 1*A*, lane 10; Fig. 2*A*, lanes 1 and 2; Fig. 2*C*, lane 3). In addition, no CDF/LIF signal was detected in P8 gut (Fig. 1*C*, lane 2; Fig. 2*B*, lane 3) or skeletal muscle (Fig. 1*C*, lanes 2 and 4; Fig. 2*B*, lanes 5 and 9). In most of these samples, strong actin bands confirmed the integrity of the RNA and the PCR procedures. Positive controls in these experiments included the clear CDF/LIF signal in the footpad (Fig. 1*B*, lane 4; Fig. 1*C*, lane 3; Fig. 1*D*), and in cultured heart cells (the original source of CDF isolation and purification; ref. 16) (Fig. 2*A*, lane 8; Fig. 2*B*, lane 11), as well as *in vitro* transcribed CDF/LIF mRNA (Fig. 1*A*, lanes 1–3; Fig. 2*C*, lanes 6 and 7). Therefore, these results indicate that the targets of noradrenergic sympathetic neurons and the targets of other cholinergic neurons (enteric and spinal motor) do not contain detectable CDF/LIF mRNA, at least at P8.

The sympathetic neurons that innervate the sweat glands undergo the noradrenergic-to-cholinergic conversion after their axons reach the footpads, beginning in the second postnatal week (11). To determine whether CDF/LIF mRNA is present in the footpads during this critical time, extracts were made at several ages (Fig. 1*D*). The relative intensity of the CDF/LIF bands (upper gel) compared with those of β-actin (lower gel) generated from the same samples indicates that CDF/LIF mRNA levels are at their highest during the time of initial innervation (P8, lane 2). The level prior to innervation (P4, lane 1) appears to be less than at P8 and the signal appears to decline following P8 (lanes 3–5). Given the semiquantitative nature of the RT–PCR assay, these apparent changes in CDF/LIF mRNA levels are necessarily tentative. Nonetheless, similar results were obtained in three independent experiments, including one using a different set of primers for the CDF/LIF mRNA.

In the latter case, an alternative 3′ primer that covers only part of the second exon was used. When this primer plus the 5′ primer previously employed were used, two bands were observed from the PCR with footpad RNA. One band was of the size expected for CDF/LIF (396 bp). The other band was ≈1100 bp, the size expected for an exon–intron–exon sequence (data not shown). As described in *Materials and Methods*, obtaining a band of the expected size with two independent sets of CDF/LIF primers is important support for the identity of the RT–PCR band from sweat-gland extracts. The fact that the primer that spans the second and third exons yielded only the lower band provides additional evidence that the CDF/LIF band was not generated from genomic templates.

CDF/LIF Expression in Brain. Since a CDF/LIF mRNA band could be reliably detected in select peripheral tissues, the same method was employed for a number of brain areas. While a clear CDF/LIF mRNA band was found in extracts from adult visual cortex and P17 superior colliculus (Fig. 3*A*), no signal was detected in samples from various postnatal ages of hippocampus (Fig. 2*A*, lanes 3 and 4), frontal cortex, olfactory bulb, cerebellum, basal forebrain, and ventral brainstem (Fig. 3*B*). The data in Fig. 3 *A* and *B* come from the same experiment, and actin mRNA was used as the internal control to normalize the relative amounts of RNA in the tissue samples. Since there were several bands present in some of the samples from the CDF/LIF PCR, a second CDF/LIF primer (5′-CAATGCCCTCTTTATTTCCTATTACACAGC-3′) was used in further experiments on these various brain areas. No bands were detected in the size range expected for CDF/LIF in the brain areas represented in Fig. 3*B*, whereas the CDF/LIF band was again observed in extracts of adult visual cortex (data not shown).

DISCUSSION

A positive CDF/LIF signal was detected in footpad RNA and not in a number of other tissues from postnatal rat. This indicates that the RT–PCR method has not simply amplified an extremely rare mRNA that is uniformly present in all tissues, nor is it likely that the positive signal in footpads represents contamination or a PCR artifact (23–26). The ratio of the signal in sweat gland to that in the tissues where no signal was detected is estimated to be at least 20:1 (5–10 pg

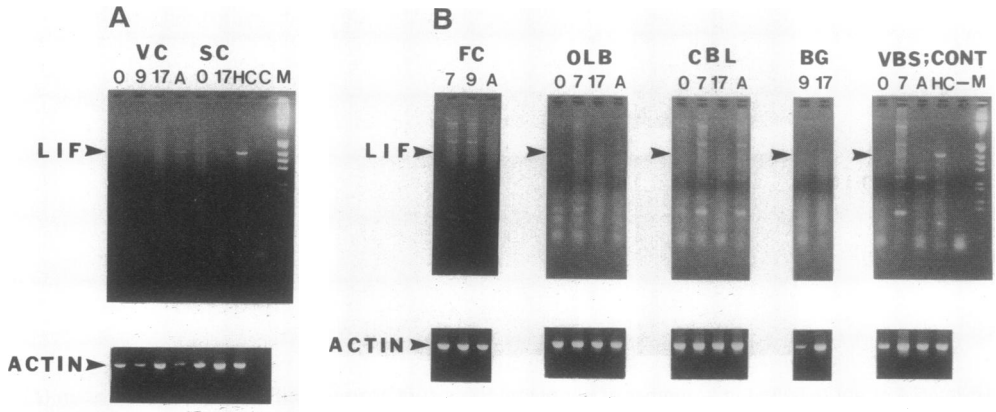

FIG. 3. CDF/LIF mRNA is detected in particular brain areas, at specific stages. For the CDF/LIF assays (*Upper*), these primers were used: 5′-CAATGCCCTCTTTATTTCCTATTACACAGC-3′ and 5′-GGGGACACAGGGCACATCCACATGGCCCAC-3′. Actin primers were used for control assays (*Lower*). (*A*) VC (visual cortex): 0, neonatal; 9, P9; 17, P17; A, adult. SC (superior colliculus): 0, neonatal; 17, P17. HC, cultured heart cells. C, no template. M, size markers. (*B*) FC (frontal cortex), OLB (olfactory bulb), CBL (cerebellum), BG (basal forebrain), VBS (ventral brainstem): 0, neonatal; 7, P7; 9, P9; 17, P17; A, adult. CONT (controls): HC, cultured heart cells; −, no template; M, size markers.

in P8 footpads and a detection limit of 0.2 pg). It is of interest that postnatal heart tissue RNA does not contain detectable CDF/LIF mRNA, whereas cultured heart cell RNA does. It is known that production of CDF/LIF by heart cells is under hormonal control (27). The result with heart tissue *in vivo* is consistent with the fact that sympathetic neurons that innervate the heart are noradrenergic. All of the other targets of noradrenergic sympathetic neurons tested were also negative for CDF/LIF mRNA (lacrimal, parotid and submaxillary glands, liver, and hairy skin; the level of RNA in the parotid sample was low, however). Thus, there is a strong correlation between the presence of CDF/LIF in peripheral tissues and the phenotype of the sympathetic neurons innervating these tissues (Table 1). In addition, the lack of detectable CDF/LIF mRNA in heart and gut may indicate that this factor is not responsible for the cholinergic phenotype of the parasympathetic neurons that are found in these tissues. These conclusions must remain tentative, however, until samples from embryos are tested. The apparent developmental regulation of the signal in footpads (Fig. 1*D*) illustrates the importance of this qualification. Moreover, the cholinergic innervation of the other muscles and glands tested occurs during embryonic development, in contrast to the postnatal innervation of the sweat gland.

The present results should also be integrated with those of Rao and Landis (14). They have found that footpad homogenates contain a factor(s) that induces cholinergic differentiation in cultured sympathetic neurons. Most of this cholinergic activity is not, however, precipitated with an antiserum generated against the N-terminal region of CDF, nor is the chromatographic behavior of the activity in the homogenate identical to that of CDF purified from cultured heart cells. These results suggest that the cholinergic factor in the footpads may not be CDF/LIF (14), raising the possibility that the CDF/LIF mRNA in this tissue is not translated into protein. However, a negative posttranslational control of this type would render the highly selective expression of the CDF/LIF mRNA pointless. Alternatively, it is possible that much of the cholinergic activity in the footpad homogenate is due to another protein with cholinergic activity [likely to be ciliary neurotrophic factor (CNTF); ref. 14; M. S. Rao, P. H. Patterson, and S. C. Landis, personal communication], one that is released by homogenization but may not normally be secreted by the sweat glands and would therefore not be a true target-derived factor. Because of its lack of a conventional signal sequence, CNTF may not be secreted; thus it has been proposed that CNTF is a factor used in response to nerve damage (28). According to this hypothesis, CDF/LIF protein would also be present in the homogenate, but as a minor cholinergic component. This possibility is supported by the recent finding that antibodies that directly block the activity of recombinant CDF/LIF are able to block the activity of a minor fraction of the cholinergic activity in the footpad extract (M. S. Rao, P. H. Patterson, and S. C. Landis, personal communication).

Table 1. CDF/LIF mRNA expression

Tissue	P0–4	P7–9	P16–19	Adult
Peripheral				
Footpad (sweat gland)	+	++	+	+
Connective tissue		−		−
Skin		−		−
Parotid gland		−		
Submaxillary gland		−		
Lacrimal gland		−		
Heart		−	−	−
Liver		−		−
Skeletal muscle		−		−
Gut		−		
Brain				
Visual cortex	−	−	±	+
Superior colliculus	−		+	
Olfactory bulb	−	−	−	−
Cerebellum	−	−	−	−
Basal forebrain	−	−	−	−
Ventral brainstem	−	−		
Frontal cortex		−		
Hippocampus		−	−	

++, Significant band (>1 pg of CDF/LIF mRNA per μg of total RNA) of the correct size; +, ≈1 pg/μg; ±, in the range 0.2–1 pg/μg; −, no detectable band (<0.2 pg/μg). These are semiquantitative estimates, as described for Fig. 1. Blank space means that the tissue of that age was not tested.

Since CDF/LIF can influence the phenotype of other types of cultured neurons besides sympathetics (29, 30), it was of interest to determine whether its mRNA could be detected in the brain. Since the factor can alter the expression of many neuropeptides and transmitters, and the particular genes affected depend on the neurons being tested (30), it was important to assay many brain areas, not just those receiving projections from the cholinergic basal forebrain (31). Summarizing the results obtained thus far with brain, Table 1 shows that CDF/LIF mRNA levels are much higher in visual cortex and superior colliculus than in other brain areas. Therefore, the presence of the factor in postnatal brain does not directly correlate with the presence of a cholinergic projection from the basal forebrain. This does not preclude that such a correlation may be found in embryonic brain, however. The relatively late appearance of detectable CDF/LIF mRNA in the visual system is unexplained at present. Perhaps this factor is involved in an as yet undescribed postnatal phenotypic plasticity. It is also possible that the factor plays an entirely different role, unrelated to that established in the peripheral nervous system. Phenotypic plasticity has, however, been demonstrated for neurons from various brain areas (e.g., refs. 32 and 33). While it is also possible that the presence of the mRNA does not connote the production of the CDF/LIF protein, the strong correlation of the CDF/LIF mRNA with a particular neuronal phenotype in the sympathetic system suggests that the presence of this message in the visual system is indeed meaningful.

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