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# Crucial role of TrkB ligands in the survival and phenotypic differentiation of developing locus coeruleus noradrenergic neurons

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## SUMMARY

**The role of glial cell-line derived neurotrophic factor (GDNF) and neurotrophins in the development of locus coeruleus noradrenergic neurons was evaluated. We found that two neurotrophic factors previously reported to prevent the degeneration of lesioned adult central noradrenergic neurons, GDNF and neurotrophin 3 (NT3), do not play significant roles in the prenatal development of locus coeruleus noradrenergic neurons, as demonstrated by: (1) the lack of alterations in double *Gdnf*/*Nt3* null mutant mice; and (2) the lack of survival-promoting effects of GDNF and/or NT3 in rat E13.5 primary cultures. In contrast, null mutant mice for TrkB, the tyrosine kinase receptor for brain-derived neurotrophic factor and neurotrophin 4, displayed a clear loss of locus coeruleus noradrenergic neurons. In accordance with this, treatment of rat E13.5 primary cultures with TrkB ligands prevented the early loss of noradrenergic neurons and maintained their survival for up to 6 days in vitro. Moreover, an additional 5-10-fold increase in the number of tyrosine hydroxylase positive noradrenergic neurons was detected after 12 hours in culture. This second effect of TrkB ligands involved neither proliferation nor survival, because the number of BrdU- or TUNEL-positive noradrenergic neurons did not change and the effect was elicited by delayed administration of either factor. Because TrkB ligands increased the number of tyrosine hydroxylase-positive cells expressing Phox2a, a paired homeodomain protein required for the development of locus coeruleus noradrenergic neurons, but did not affect the number of Phox2a-positive tyrosine hydroxylase-negative cells, our results suggest that the second effect of TrkB ligands may involve promoting or inducing a noradrenergic phenotype. In summary, our findings suggest that, unlike NT3 and GDNF, TrkB ligands are required and sufficient to promote the development of central noradrenergic neurons.**

Key words: TrkB, TrkC, BDNF, NT4, NT3, GDNF, Locus coeruleus, Neurotrophins, Rat primary cultures

## INTRODUCTION

More than half of central noradrenergic (NA) neurons reside in the locus coeruleus (LC). Innervation from LC reaches target regions throughout the entire brain and regulates a variety of functions, such as mood, vigilance, attention and memory acquisition (Barnes and Pompeiano, 1991; Cirelli and Tononi, 2000). Interestingly, NA neurons are particularly vulnerable and are lost in most neurodegenerative disorders affecting the brain, including Parkinson's disease (Hassler, 1938), Pick's disease (Arima and Akashi, 1990), Huntington's disease (Zweig et al., 1992), and Alzheimer's disease (Nyberg et al., 1985; Zweig et al., 1988). Two neurotrophic factors, glial cell-line derived neurotrophic factor (GDNF) and neurotrophin 3 (NT3; NTF3 – Mouse Genome Informatics), have been found to be particularly efficient at preventing the degeneration of adult LC NA neurons in adult 6-hydroxydopamine lesion models (Arenas and Persson, 1994; Arenas et al., 1995). However, the function of these neurotrophic factors during prenatal LC development is unclear because neither *Gdnf* null mutant mice (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), nor *Nt3* null mutant mice (Ernfors et al., 1994b; Fariñas et al., 1994) displayed clear developmental abnormalities in that region. In contrast with this finding, the development of LC NA neurons is severely affected by reductions in the availability of bone morphogenetic proteins (BMPs) in vivo (Guo et al., 1999; Vogel-Hopker and Rohrer, 2002), and by the lack of the basic helix loop helix, Mash1, or the Phox2a or Phox2b homeodomain proteins, as shown in null mutant mice (Hirsch et al., 1998; Morin et al., 1997; Yang et al., 1998).

There are, however, indications that LC NA neurons could respond to neurotrophic factors of the GDNF family during development because GDNF receptors are expressed by LC

neurons and primary cultures of E13.5 LC NA neurons respond to GDNF and to a second neurotrophic factor of the GDNF family, neurturin (NTN) (Holm et al., 2002). Interestingly, GDNF and NTN promoted the neuritogenesis of NA neurons in serum-free cultures (Holm et al., 2002), suggesting that GDNF and NTN might regulate this aspect of LC neuron development. However, when LC cultures were grown in the presence of BMP2 or serum, GDNF or NTN enhanced the survival-promoting effects of BMP2 on LC NA neurons (Reiriz et al., 2002). Thus, provided that the LC is exposed to BMPs in vivo (Guo et al., 1999; Vogel-Hopker and Rohrer, 2002), it is conceivable that GDNF and NTN may also regulate the survival of LC NA neurons during development.

With regard to the neurotrophins, their high affinity tyrosine kinase receptors TrkB (NTRK2 – Mouse Genome Informatics) and TrkC (NTRK3 – Mouse Genome Informatics) are expressed in the adult rat LC (King et al., 1999; Merlio et al., 1992; Smith et al., 1995; Tetzlaff et al., 1994), but it is not known whether they are also expressed in the developing LC NA neurons. Primary LC cultures grown in the presence of serum and treated with brain-derived neurotrophic factor (BDNF), NT3 or NT4 (NTF5 – Mouse Genome Informatics), but not NGF, have shown increased survival of NA neurons and norepinephrine uptake (Friedman et al., 1993; Sklair-Tavron and Nestler, 1995; Sklair-Tavron and Segal, 1993), suggesting a function of neurotrophins in the developing LC. However, we have recently reported that NT3 per se, in serum-free culture conditions, does not increase the survival of LC neurons, and that NT3 and BMP2 or NT3 and serum promote survival of LC NA neurons (Reiriz et al., 2002). It thus remains to be determined whether other neurotrophins play a role per se and whether they regulate the development of LC NA neurons in vivo.

In the present study we re-evaluated the function of BDNF, NT3, NT4 and GDNF in the development of LC NA neurons both in vitro and in vivo. We found that mice with null mutations for both *Gdnf* and *Nt3* displayed intact LC cell number, neuropeptide expression and target innervation. This independence of GDNF and NT3 for proper LC development was further emphasized by the lack of effect of these molecules in rat primary NA cultures. We also found that LC neurons express higher levels of *TrkB* mRNA than *TrkC* mRNA through development, and *TrkB*, but not *TrkC*, null mutant mice showed a 25-30% loss of LC neurons at P0. Moreover, addition of either of the two TrkB ligands, BDNF or NT4, to primary cultures resulted in a tenfold increase in number of TH-positive cells by mechanisms involving both survival of NA neurons and induction or promotion of a NA phenotype.

## MATERIALS AND METHODS

### Generation of double null mutant mice

Because all individual null mutant mice required for this study, *Gdnf* (Pichel et al., 1996), *Nt3* (Ernfors et al., 1994b), *TrkB* (Klein et al., 1993) and *TrkC* (Klein et al., 1994), die within 2 weeks of birth, heterozygous mice were bred to generate double heterozygous mice that were mated and gave rise to double null mutant mice. Genotyping was performed by PCR analysis of tail DNA, as described (Ernfors et al., 1994b; Pichel et al., 1996).

### In situ hybridization

In situ hybridization with $^{35}$S-labeled TrkB and TrkC riboprobes (Funakoshi et al., 1993) and Ret, GFRα1 and GFRα2 riboprobes (Trupp et al., 1997) was performed as previously described, on fresh frozen sections (Trupp et al., 1997). In brief, sections were fixed for 15 minutes in refrigerated 4% PFA and rinsed three times in PBS. The tissue was deproteinated in 0.2 M HCl for 10 minutes, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 20 minutes, and dehydrated in increasing concentrations of ethanol. Slides were incubated for 16 hours in a humidified chamber at 53°C with $1\times10^6$ cpm of probe in 200 μl of hybridization cocktail. Washes were performed at 62°C. First, 2×15 minutes in 1×SSC, 30 minutes in 50% formamide/0.5×SSC and 15 minutes in 1×SSC. Then the slides were subjected to a 30-minute RNase treatment (40 μg/ml) at 37°C before washing 2×15 minutes in 1xSSC with DTT and 1x5 minutes in room temperature 1×SSC. Finally, the slides were dehydrated in ethanol, air-dried, dipped in NTB-2 photoemulsion diluted 1:1 in water (Eastman Kodak), exposed at 4°C for 6 weeks, developed with D19 (Eastman Kodak), fixed with AL-4 (Agfa Gevaert), and counterstained with Cresyl Violet.

Digoxigenin (DIG) in situ hybridization was performed using the same protocol as for the $^{35}$S-labeled probe until the last washing step, but with UTP labeled with DIG instead of $^{35}$S. Thereafter the tissue was washed 2×30minutes in PBS with 0.1% Tween 20 and 2×15 minutes in PBT (PBS with 0.1% Triton X-100 and 2 mg/ml BSA). Blocking was performed for 3-5 hours in PBT with 20% Horse Serum (HS) (GIBCO). The sections were then incubated with an α-DIG alcaline phosphatase antibody 1:2000 (Boeringer Manheim) in PBT with 20% HS at 4°C overnight. After 3×30-minute washes in PBT, the alcaline phosphatase antibody was visualized by incubating the slides in an alcaline phosphatase buffer containing levamisole, nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (BCIP) for 1-2 days at room temperature. The buffer was changed every 12 hours. The reaction was stopped with EDTA and the slides were postfixed in 4% PFA for 15 minutes and washed 3×15 minutes in PBS before let dry. After 5 minutes in xylene the slides were mounted with Pertex (Histolab Products AB) and coverslipped.

In situ hybridization with $^{35}$S-labeled oligoprobes was performed as previously described (Kresse et al., 1995). Probes used were DBH (Nakano et al., 1992) (nucleotides 481-528), NPY (Larhammar et al., 1987) (nucleotides 1671-1714), galanin (Vrontakis et al., 1987) (nucleotides 152-199), BDNF (Maisonpierre et al., 1991) (nucleotides 645-694), and CGRP (Amara et al., 1982) (aminoacids 5-23).

### Immunohistochemistry

Pups were perfused with cold 4% PFA in PBS and the heads were left in PFA for two hours before they were sequentially immersed in 10% sucrose (1 day) and 20% sucrose the following day. Serial cryostat sections (14-μm thick) were obtained covering the entire hindbrain, including the LC. Immunohistochemistry was performed by first blocking with 20% HS in PBT for 30 minutes before incubating the slides in PBT at 4°C overnight with the following primary antibodies: 1:300 mouse anti-TH (DiaSorin), 1:500 rabbit anti-NOS (kind gift from Gerhard Skofitsch) and 1:300 mouse anti-ki67 (Abcam). After washing with PBS, the slides were incubated with 1:200 donkey anti-mouse rhodamine (Jackson) or 1:200 goat anti-rabbit rhodamine (Jackson) for 1-2 hours, washed with PBS again and finally mounted with Vectashield anti-fading solution and coverslipped.

### Cell counts in brain sections

Sections every 30 or 100 μm, throughout the entire LC, were processed for DBH or BDNF in situ hybridization and counterstained with cresyl violet. Cell counts were performed at ×100 magnification in bright field. Clearly identified cresyl-violet-positive cells covered by silver grains were counted as positive, as described (Kresse et al., 1995). Similarly, sections processed for TH or NOS

immunohistochemistry were analyzed. Locus coeruleus cells showing a clear NOS-positive cytoplasm around a non-stained nuclei, were counted as positive in blind determinations in a Zeiss microscope, at ×100, as described (Arenas et al., 1995).

### DiI tracing

The analysis of the projections from the LC into different parts of the central nervous system in P0-GDNF/NT3 double null mutant mice was performed by placing crystals of carbocyanine DiI (Molecular Probes) into perfusion-fixed brains of null mutant and wild-type littermates. DiI crystals were placed with glass micropipettes into the dorsal horn and intermediolateral column of the cervical spinal cord as well as the area of the nucleus accumbens in the basal ganglia. The brains were stored for 6 weeks in phosphate-buffered 4% paraformaldehyde (PFA) pH 7.0 at 37°C to allow for retrograde transport of the dye. After cryoprotection for 48 hours in 20% sucrose, 50 μm sections were cut in a cryostat, mounted on glass slides, coverslipped with a fluorescence-protecting mounting medium (Vectashield, Vector Laboratories), and photographed under a microscope with epi-fluorescence illumination using rhodamine optics.

### Cell culture and treatments

Pregnant Sprague-Dawley rats (B&K Universal AB) were killed at E13.5 by exposure to $CO_2$, and then soaked in 70% ethanol for a minute. Embryos were removed under sterile conditions and kept in ice-cooled PBS during dissection. The whole proximal rhombencephalic ring (rhombomeres 1 and 2), between the distal part of the mesencephalic flexure and the proximal part of the pontine flexure (Specht et al., 1981), was dissected. The tissue was gently dissociated with a fire-polished Pasteur pipette and plated on poly-D-lysine coated 12-well plates (BD Falcon) in N2 media at a density of $1.25\times10^5$ cells per $cm^2$ ($5\times10^5$ cells/well). Time elapsed between the death of the mothers and plating of the cells was ≤1.5 hours. Trophic factors were added to the wells just before plating the cells (if not stated differently), at a concentration of 30 ng/ml. BDNF, NT4, GDNF, NT3 and bFGF were all obtained from R&D Systems. Solutions used in tissue culture were sterilized by filtration through 0.22 μm filters (Millipore). Cultures were grown at 37°C in a water-saturated 5% $CO_2$ and 95% air incubator without changing media or supplementing the factors.

### Immunocytochemistry and cell counts of primary cultures

Cultures were fixed in 4% PFA in phosphate buffer with pH 7.4 (PBS) for 50 minutes and then washed in PBS and incubated overnight with the following primary antibodies: 1:1000 mouse anti-TH (DiaSorin), 1:50 mouse anti-5-bromodeoxyuridine (BrdU) (DAKO), and 1:1000 rabbit anti-Phox2a (generous gift from Dr Christo Goridis), diluted in PBS containing 1% BSA and 0.3% Triton X-100 (PBT), at 4°C. After washing, cultures were incubated for 1-2 hours with biotinylated horse anti-mouse, or goat anti-rabbit, IgG (Vector Laboratory), 1:500, in PBT. Immunostaining was visualized with the Vector Laboratories ABC immunoperoxidase kit, using either gray (SG) or red (AEC) substrates.

### BrdU incorporation assay

To determine whether the cells in the primary cultures synthesized DNA, cultures were incubated in 10 μM BrdU for a period of 6 hours (if not stated differently) prior to fixation and then treated with 2M HCl for 20 minutes and visualized with a mouse anti-BrdU antibody (1/50, DAKO).

### BDNF and NT4 ELISA

The production of BDNF or NT4 protein was analyzed in conditioned media from LCr cultures grown in N2 medium. Conditioned media was collected after 12 and 24 hours in vitro and analyzed with a BDNF and NT4 ELISA kits ($E_{max}$® ImmunoAssay System, Promega) according to the manufacturer's recommendations. A standard curve of pure BDNF and NT4 proteins provided in the kits were used to quantify the levels of neurotrophic factors in the media. The detection limit of the ELISA assays was below 0.1 ng/ml of BDNF or NT4. BDNF- and NT4-treated wells were used as positive controls.

### Detection of nuclear DNA fragmentation by TUNEL staining

DNA fragmentation in primary cell cultures was examined by using the DeadEnd™ Colorimetric TUNEL System (Promega). Paraformaldehyde fixed cultures were permeabilized with 0.2% Triton® X-100 solution in PBS, washed with PBS, equilibrated and exposed to a reaction mix containing 1% terminal deoxynucleotidyl transferase and 1% biotinylated nucleotide in equilibration buffer for 60 minutes at 37°C. The reaction was terminated with 2×SSC and the cells were washed with PBS. Endogenous peroxidases were blocked with 0.3% hydrogen peroxide for 5 minutes at room temperature. Following PBS wash, the cells were covered with Streptavidin horseradish peroxidase solution 1:500 in PBS for 30 minutes at room temperature. The cells were washed with PBS again and stained with gray (SG) chromogen (Vector Laboratories).

## RESULTS

### Developmental regulation of TrkB, TrkC, GFRα1, GFRα2 and Ret receptors in the mouse LC

In order to examine the possible roles of neurotrophic factors of the GDNF and neurotrophin families during development, we first examined the expression of neurotrophic factor receptors in the LC. TrkB, TrkC, GFRα1, GFRα2 and Ret mRNA expression was analyzed in the mouse LC by in situ hybridization at four developmental stages (E13, E15, E17 and P0). In situ hybridization was performed with a specific TH riboprobe labeled with DIG to identify the LC, and adjacent sections were hybridized with TrkB, TrkC, GFRα1, GFRα2 or Ret $^{35}$S-labelled riboprobes. Our results indicate that TrkB mRNA expression is high at E13 and then declines (Fig. 1A). The expression of TrkC mRNA was close to the detection limit at all stages, being higher at E15 (Fig. 1A). Expression of Ret mRNA was very high at E13 and stabilized at high level at E17 and P0 (Fig. 1B). GFRα1 and GFRα2 expression was low through development but GFRα1 expression increased at P0 (Fig. 1B). The presence of these receptors in the LC and their regulation during development suggested that their respective ligands may play a physiological role. However, analysis of null mutant mice for individual neurotrophic factors, including GDNF (Granholm et al., 1997; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996), NT3 (Ernfors et al., 1994b; Fariñas et al., 1996) and BDNF (Ernfors et al., 1994a; Jones et al., 1994), did not reveal any developmental abnormality in the LC. Provided that both GDNF and NT3 have been reported to prevent the degeneration of adult LC NA neurons in lesion models (Arenas and Persson, 1994; Arenas et al., 1995), we decided to test whether GDNF or NT3 are required for LC development and analyzed double null mutant mice for *Gdnf* and *Nt3*.

### Absence of detectable phenotype in the LC of *Gdnf*/*Nt3* double null mutant mice

P0 double null mutant brains were analyzed in parallel with wild-type, heterozygotes and compound null mutant littermate brains. First we examined by in situ hybridization the expression of dopamine β-hydroxylase (DBH) mRNA, which

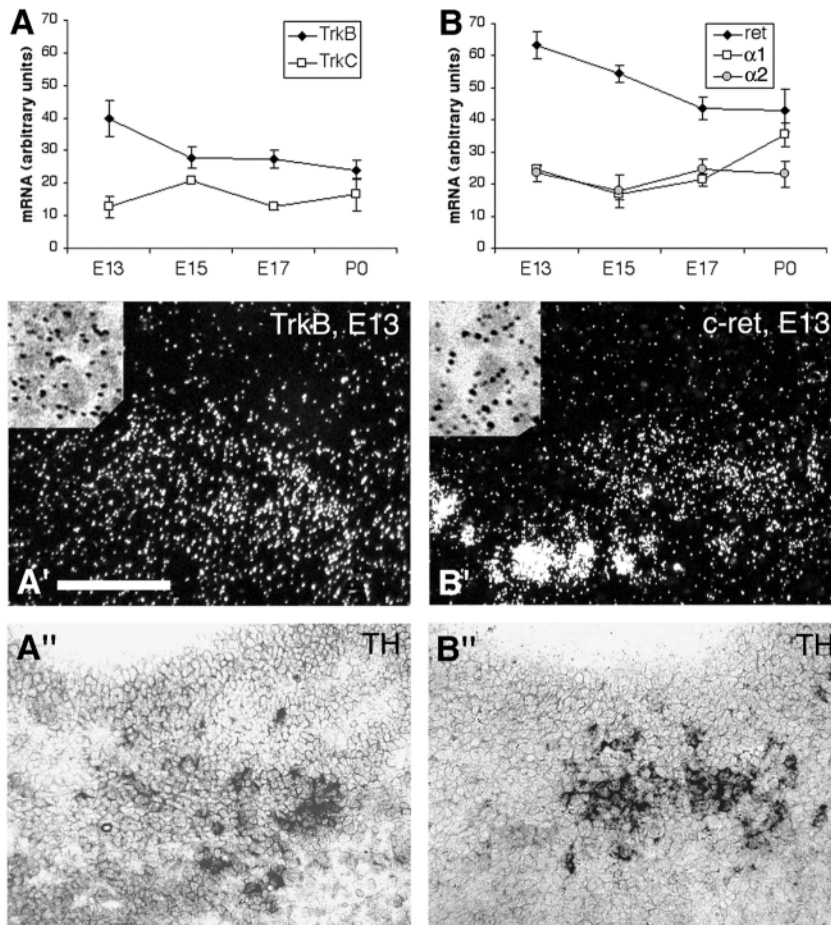



**Fig. 1.** Developmental regulation of TrkB (A-A′), TrkC (A), Ret (B-B′), GFRα1 (B) and GFRα2 (B) mRNA expression in the mouse LC, as assessed by in situ hybridization with $^{35}$S-labeled riboprobes from embryonic day (E) 13 to postnatal day (P) 0. Dark-field pictures in A′ and B′ show TrkB and Ret hybridization, respectively. Note the intense Ret signal in the mesencephalic nucleus of the trigeminus, in the lower-left corner of B′. High magnification bright field pictures of representative cells of the main dark field pictures are presented in the upper-left corner of A′ and B′. Adjacent sections hybridized with a DIG-labeled TH riboprobe were used to identify the position of the LC (bright field pictures in A″ and B″). The hybridization signal for each of the $^{35}$S-labeled probes was measured using NIH Image in: (1) the area occupied by the LC; (2) in the vicinity of the LC to control for background signal in the tissue; (3) in the tissue-free area of the fourth ventricle. The background value of the tissue-free area was subtracted from the labeling values in the LC area and the non-labeled control tissue, before statistical comparison and graph plotting. Values represent the mean±s.e.m. of 4 measures per section in 3 sections per animal in 3 animals per condition. Statistical analysis of the in situ signal showed that all time points in A and B, except for the GFRα1 signal at E15 and the TrkC signal at P0, signals were significantly higher than the tissue background signal ($P<0.05$, paired *t*-test). Scale bar: 100 μm.

encodes the enzyme that catalyses hydroxylation of dopamine to NA. Positive LC cells were counted every 98 μm (5-6 sections counted per LC) in wild-type (Fig. 2A), *Gdnf* null mutant (Fig. 2B), *Nt3* null mutant (Fig. 2C), and *Gdnf*/*Nt3* double null mutant LC (Fig. 2D). The number of DBH-positive LC cells was indistinguishable in these four conditions [mean±s.e.m. (*n*=8): 415.125±26.89, 420.5±28.10, 417.875±33.85, 417.125±42.95, respectively]. This result was confirmed by cell counts of TH-positive cells in the LC (not shown) after TH immunohistochemistry (Fig. 2E-E'). Moreover, the number of NOS immunoreactive LC cells (approximately 10% of the LC cells, that are not NA) did not differ in wild-type and in *Gdnf*/*Nt3* double null mutant animals (37.75±7.81, *n*=8 and 36.25±7.87, *n*=8, respectively) (Fig. 2F-F'). Cell counts of BDNF in situ hybridization positive LC cells (Fig. 2G-G') averaged 296.75±34.30 (*n*=8) in the double null mutant and 290.875±42.31 (*n*=8) in the wild type. The expression of several neuropeptides, including Neuropeptide Y (NPY) (Fig. 2H-H'), Galanin (Fig. 2I-I') and Calcitonin gene related peptide (CGRP) (Fig. 2J-J') was similar in wild-type and *Gdnf*/*Nt3* double null mutant LC, as assessed by in situ hybridization. Finally, we analyzed the innervation of two target structures of LC NA neurons, the spinal cord and the nucleus accumbens, in *Gdnf*/*Nt3* double null mutant and wild-type mice. Retrograde DiI-tracing labeled both LC and SubLC neurons projecting to either the spinal cord (Fig. 2K-K') or the nucleus accumbens (Fig. 2L-L') in both wild-type and double null mutant littermates. Thus, our findings indicate that GDNF and NT3 are dispensable during embryonic development and are not required for survival, phenotypic maturation and target innervation before birth.

### GDNF and/or NT3 do not promote the survival of LC NA neurons in serum-free primary cultures

In our experiments we have considered the LC region (LCr) as rhombomeres 1 and 2 and therefore our cultures include the LC and subcoeruleus (A6 dorsal and ventral), and the A4, A5 and A7 NA groups. It has been proposed in the past that all of these nuclei could be considered as part of the LC complex (provided their proximity and similarities) (Smeets and Reiner, 1994). Moreover, from a developmental perspective, NA neurons in these nuclei have in common that they are deleted in the *Phox2b* null mutant mice (Pattyn et al., 2000).

To further confirm the function of GDNF and NT3 on developing LC NA neurons, primary cultures of rat E13.5 LCr were treated with GDNF, NT3 or both factors (Fig. 3) in serum-free conditions for 6 days in vitro (DIV). Counts of TH immunoreactive cells in these conditions revealed no difference between treated and untreated wells in neuronal survival (Fig. 3), indicating that NT3 and/or GDNF are not sufficient to promote the survival of LCr NA neurons or to induce a NA fate. However, consistent with a previous report

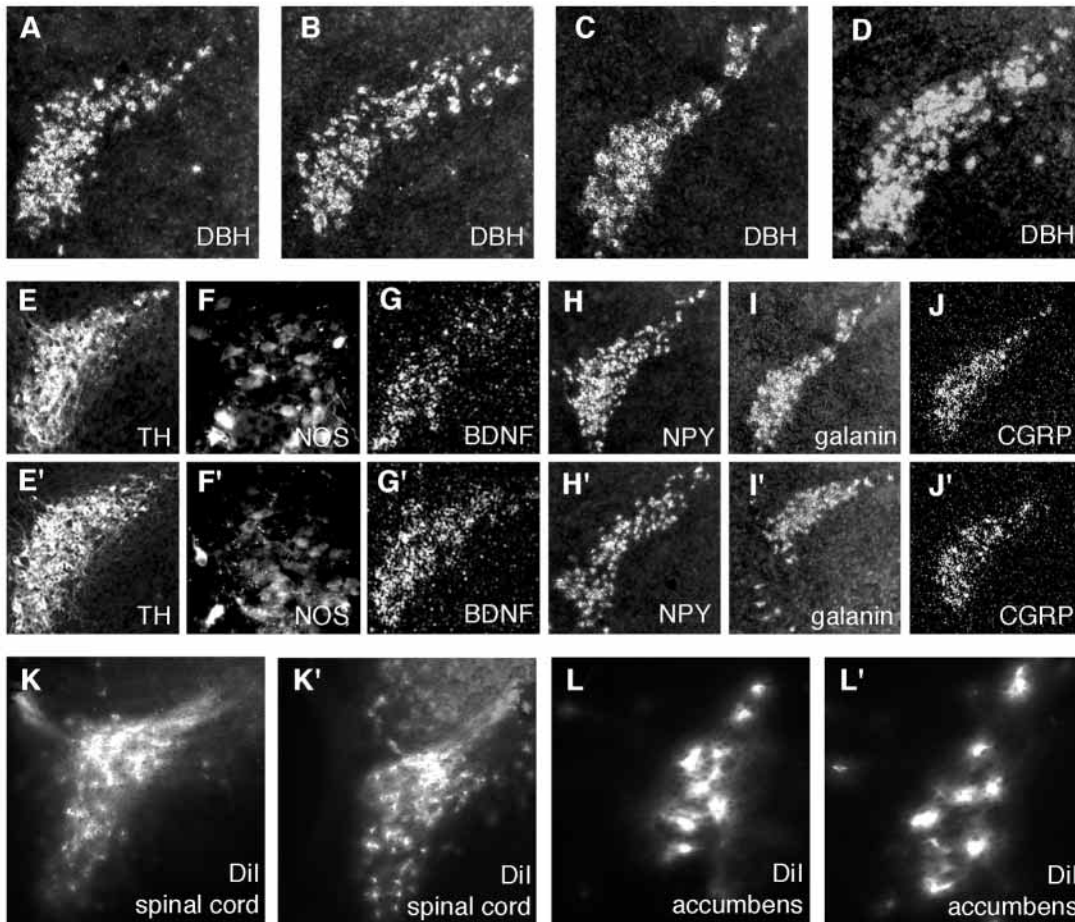



**Fig. 2.** *Gdnf/Nt3* double null mutant mice do not differ from wild-type mice in LC cell numbers, expression of phenotypic markers or target innervation. The LC of wild-type (A,E-L), *Gdnf* null mutant (B), *Nt3* null mutant (C) and *Gdnf/Nt3* double null mutant (D,E'-L') were analyzed at P0. The number of LC NA neurons, assessed by in situ hybridization with riboprobes against DBH mRNA in wild-type, *Gdnf* $^{-/-}$, *Nt3* $^{-/-}$ and *Gdnf/Nt3* double null mutants, did not differ (A-D). Moreover, immunohistochemistry for TH (E,E') and NOS (F,F') and in situ hybridization mRNA detection of BDNF (G,G'), NPY (H,H'), Galanin (I,I') and CGRP (J,J') did not differ in wild-type and *Gdnf/Nt3* double null mutant mice. Finally, retrograde DiI tracing from the spinal cord (K,K') and nucleus accumbens (L,L') showed that the innervation of target structures in double null mutant mice does not differ from that in the wild-type mice.

(Holm et al., 2002), we found that GDNF promoted robust neurite extension of an axon-like process, an activity that was neither observed in control cultures nor induced by NT3 (not shown). Provided that GDNF and NT3 are neither sufficient nor required to promote the survival of LC NA neurons, we next set out to examine whether other neurotrophins could be involved in regulating the survival of LC NA neurons and analyzed individual and double *TrkB* and *TrkC* mutant mice.

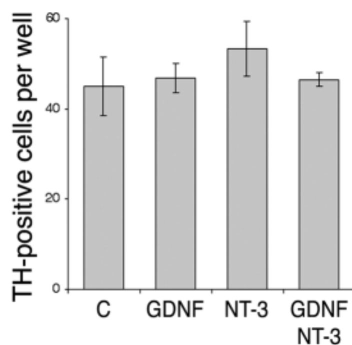



**Fig. 3.** Neither GDNF nor NT3 increases the number of TH-immunoreactive noradrenergic neurons in E13.5 rat LCr primary cultures. Cells were treated with 30 ng/ml GDNF and/or 30 ng/ml NT3 and were analyzed after 6 days in vitro. Cell counts revealed no significant differences in the number of TH-positive cells between control wells and wells treated with GDNF, NT3 or GDNF+NT3.

## Loss of NA LC neurons in the *TrkB* null mutant mice

Offspring of compound TrkB and TrkC heterozygous mutant mice crosses were genotyped, perfused, and their brains were analyzed. TH-immunoreactive LC cells were counted every 98 μm in serial sections (Fig. 4). The average number of TH-positive cells in the wild-type LC at P0 was 182.6±36.43, $n$=10. This number was not found to be significantly different in *TrkB* $^{+/-}$ or *TrkC* $^{-/-}$ mice. However, *TrkB* null mutant mice showed a significant decrease ($P<0.05$, unpaired $t$-test) in the number of TH-positive neurons (137.5±33.57, $n$=8) compared to the wild-type mice (25% decrease) or the *TrkB* $^{+/-}$ mice (37% decrease). Homozygous double null mutant mice were not obtained during the course of the experiments because only few pups survive at birth (Silos-Santiago et al., 1997). However, analysis of *TrkB* $^{-/-}$ and *TrkC* $^{+/-}$ mutant mice showed no further decrease in the number of TH-positive neurons (Fig. 4D,E), indicating that TrkB, but not TrkC, regulates the number of LC NA cells.

In order to determine whether the decrease of TH-positive cells in the LC of P0 *TrkB* null mutant mice resulted from the loss of neurons and not a downregulation of TH expression in *TrkB* null mutant mice, we examined the LC in further detail. Adjacent sections were respectively stained with TH antibodies

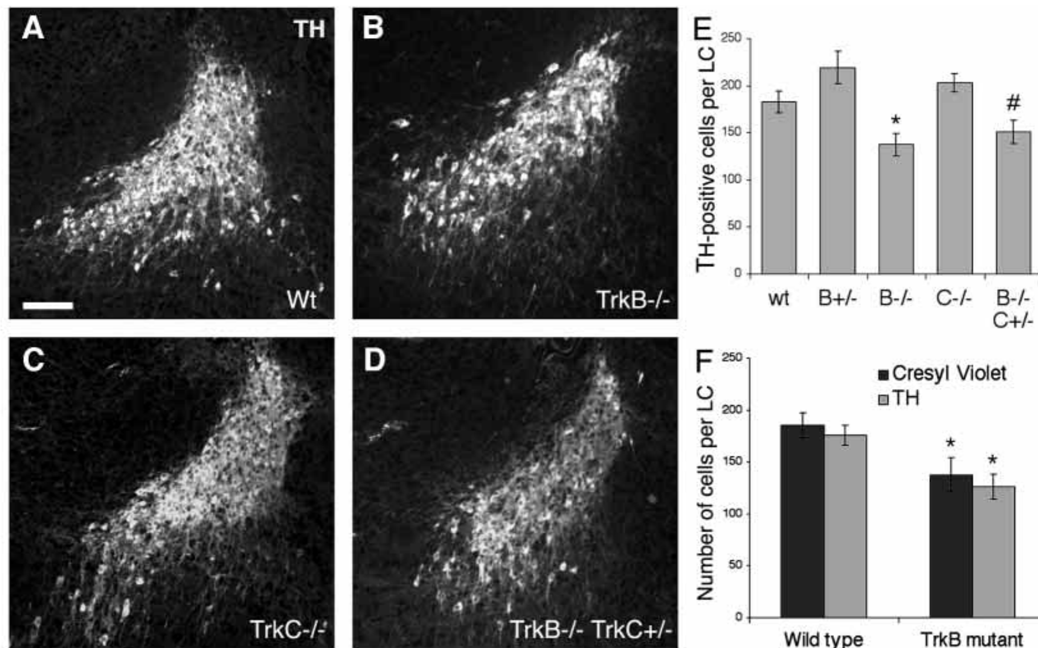



**Fig. 4.** Loss of TH-positive and CV-positive LC NA neurons in the *TrkB* null mutant mice. A-D show representative sections through the LC of wild-type (A), $TrkB^{-/-}$ (B), $TrkC^{-/-}$ (C) and $TrkB^{-/-}$ $TrkC^{+/-}$ (D) at P0 after TH immunohistochemistry. The graph in E describes the average number of TH-positive cells (±s.d.) in the LC of wild-type, $TrkB^{+/-}$, $TrkB^{-/-}$, $TrkC^{-/-}$ and $TrkB^{-/-}$ $TrkC^{+/-}$ mice. $TrkB^{-/-}$ was significantly different from wild-type and $TrkB^{+/-}$ (*$P$<0.05), and $TrkB^{-/-}$ $TrkC^{+/-}$ was different from $TrkB^{+/-}$ ($^{\#}P$<0.05) as assessed by unpaired *t*-test (*n*=4-10). The number of TH-positive and CV-positive neurons in the LC was examined in adjacent sections of wild-type and *TrkB* null mutant mice at P0 (F). A decrease of 28.3% and 25.7% in the number of TH-positive and CV-positive neurons, respectively, was detected in the $TrkB^{-/-}$ mice. (*$P$<0.05, paired *t*-test). Scale bar: 100 μm.

and cresyl violet (CV) and the number of TH-positive or pigmented cells in the LC were counted. In wild-type LC, 185.33±12.01 (*n*=3) CV-positive and 175.67±9.29 (*n*=3) TH-positive neurons were detected, whereas 137,67±16.50 (*n*=3) CV-positive and 126±12.124 (*n*=3) TH-positive cells were present in the mutant mice. The finding that TH- and CV-positive cells decreased to the same extent (28%, Fig. 4F) indicated that deletion of the TrkB receptor results in the true loss of LC NA neurons (Fig. 4F). Moreover, analysis of the few *TrkB* null mutant mice that remained alive by P12 (*n*=2) revealed a more pronounced loss of TH-positive cells in the LC at this stage. Although the reduced number of animals at P12 did not allow a qualified study, all our findings suggest that TrkB is required for the development of LC NA neurons, at least during embryonic stages. We next set to determine whether activation of the TrkB receptor by its ligands, BDNF and NT4, is also sufficient to promote the development of LC NA neurons.

## BDNF or NT4 increases the number of TH-positive neurons in E13.5 rat primary cultures of the LC region

Serum-free E13.5 LCr primary cultures were treated with either BDNF (Fig. 5A,C) or NT4 (Fig. 5A,D) alone or in combination with other factors, including GDNF and NT3. The cultures were fixed after 6 days and immunostained for detection of TH (Fig. 5B-D). Control wells (Fig. 5A,B) contained an average of 53±18.14 (*n*=6) TH-positive cells, whereas BDNF- and NT4-treated wells contained an average of 517.75±135.00 (*n*=8) and 541.43±119.597 (*n*=8) TH-positive cells, respectively. This corresponds to an increase of 971% in the number of TH-positive neurons in BDNF-treated wells and of 1015% in NT4-treated wells (Fig. 5A). Combinations of BDNF and NT4 with each other or with GDNF or NT3 (Fig. 5A) did not induce any further significant increase in the number of TH-positive cells compared to BDNF or NT4 alone. Thus our findings clearly show that either BDNF or NT4 is sufficient to increase the number of LCr NA neurons in vitro by tenfold. We next set out to determine the possible mechanism by which TrkB receptor and ligands regulate the number of TH-positive cells in the LC.

## TrkB ligands do not promote the proliferation of cells in the LC

We first examined the possibility that TrkB ligands could regulate the proliferation of progenitors in the *TrkB* null mutant mice. However, immunostaining with ki67, which detects cells in late $G_1$, S, $G_2$, and M phase of the cell cycle, labeled very few cells in the LC at P0, the stage at which the cell loss was detected, and no difference was observed between *TrkB* mutant and wild-type littermates (data not shown).

We next examined whether the increase in TH-positive neurons in rat LCr primary culture at E13.5 resulted from increased proliferation induced by BDNF or NT4. To address this possibility BrdU was added to the culture at the following time points: (1) At the time of plating the cells, in wells that were fixed 6, 24 or 48 hours later. (2) At the time of plating, with a change of media after 6 hours, in wells that were fixed after 24 or 48 hours. (3) Six hours before fixation in cultures that were kept for 24 or 48 hours. In all the conditions and time

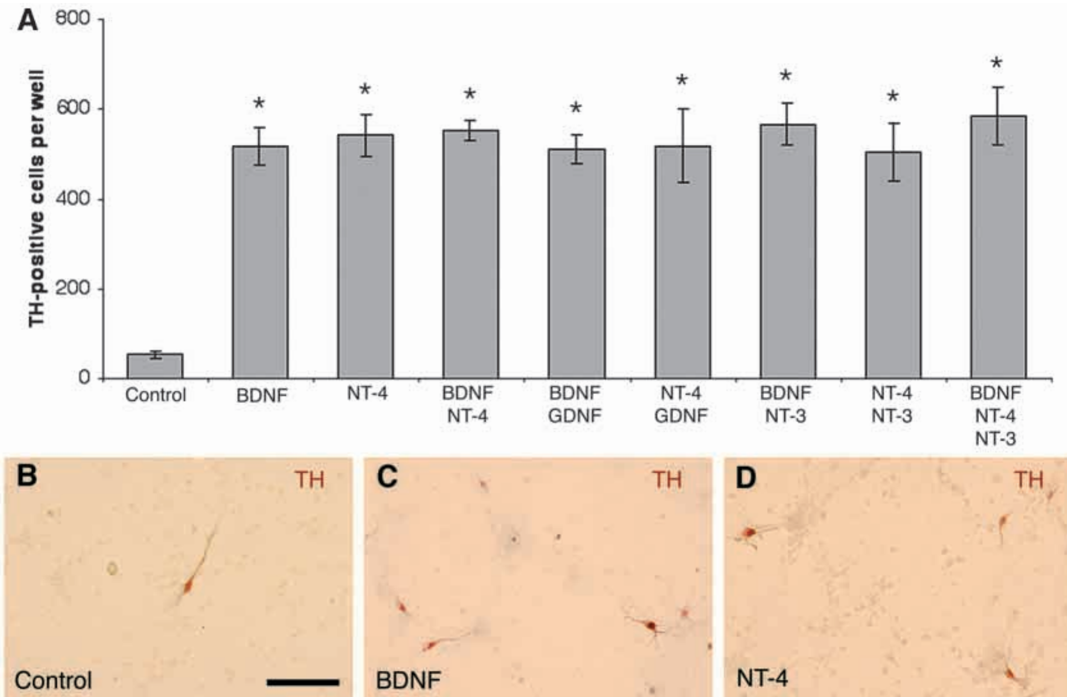
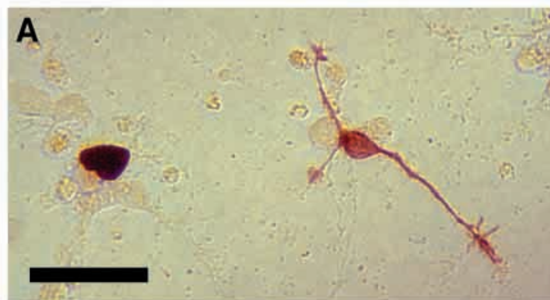
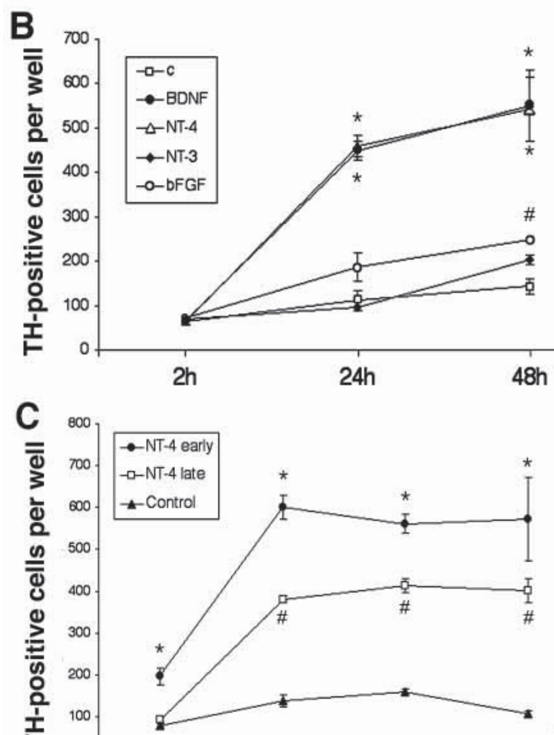



**Fig. 5.** BDNF or NT4 increases the number of LCr NA neurons after 6 days in vitro. Dissociated rat E13.5 LCr primary cultures were treated as indicated on the *x*-axis. (A) Cell counts showed a tenfold increase in number of TH-positive cells for all conditions in which a TrkB ligand is present (*$P$<0.001, unpaired *t*-test). B-D show representative fields of LCr cultures with TH-positive cells after treatment with control media (B, N2) or N2 with BDNF (C) or NT4 (D). Scale bar: 100 µm.




points mentioned above, less than 5 TH-positive cells per well (0-2%) were found to be BrdU-positive (Fig. 6A) (data not shown). Moreover, comparison of the effects of BDNF and NT4 at 24 and 48 hours with those of NT3 or basic fibroblast growth factor (bFGF), two factors with mitogenic activity, revealed that the kinetics of the increase in the number of TH-positive LCr neurons in the cultures were completely different. Thus, despite the fact that proliferating cells were present in the cultures, our results suggested that the increase in numbers of TH-positive cells was not because of increased proliferation.

**Fig. 6.** The increase in the number of TH-positive cells in LC cultures does not involve proliferation but rather survival and a second mechanism. In A, a representative TH-positive cell (red cytoplasm) and a BrdU-positive cell (dark nucleus) from an E13.5 LCr culture treated with BrdU and BDNF for 24 hours is shown. TH-positive cells in the culture were negative for BrdU and the number of BrdU-positive cells did not increase by BDNF or NT4 treatment. The graph in B displays the number of TH-positive cells after treatment with BDNF, NT4, NT3, bFGF or control media for 2, 24 or 48 hours. A steep increase in the number of TH-positive cells was detected between 2 and 24 hours in BDNF and NT4-treated cultures, whereas bFGF only induced a significant increase in cell number after 48 hours (*$P$<0.01, #$P$<0.05, unpaired *t*-test). (C) When NT4 was added to E13.5 LC cells before tissue dissociation (NT4 early), a constant higher number of TH-positive cells were detected in the wells, at all stages analyzed, as compared to wells in which NT4 was added at the time of plating (NT4 late), suggesting that early administration of NT4 prevents the loss of a population of TH-positive cells that would otherwise die shortly after dissociation. Between 2 and 12 hours a parallel increase in the number of noradrenergic neurons was detected in both early (at the time of dissociation) and late (at the time of plating) NT4 treatment, indicating that an additional mechanism is involved. *$P$<0.01 with respect to control and $P$<0.05 with respect to late NT4 treatment, #$P$<0.01 with respect to control, by unpaired *t*-test. Scale bar: 50 µm.

## TrkB ligands promote the survival of TH-positive cells in early LCr cultures

In order to determine whether TrkB ligands also had survival-promoting effects on rat LCr NA neurons, we examined the ability of BDNF and NT4 to prevent the loss of TH-positive neurons in culture. Because the number of TH-positive cells in control cultures rapidly decreased during the first two hours in vitro, but not thereafter (Fig. 6B and not shown), the effects of NT4 (30 ng/ml) on survival were examined as early as 2 hours after plating. In this experiment, for every brain dissected out, one block of tissue containing one LCr was placed in control N2 media and the contralateral LCr in N2 media supplemented with 30 ng/ml NT4. After dissociation the cells were plated in wells with N2 media (control) or with a final concentration of 30 ng/ml NT4. In this preparation, the levels of endogenous BDNF or NT4 in control cultures were below the detection limit in our ELISA assay (<0.1 ng/ml, respectively). The cultures were fixed at 2, 12, 24 and 48 hours after plating and the number of TH-positive LCr neurons was counted. Interestingly, 2 hours after plating, the cells that were treated with NT4 from tissue dissociation (early treatment) displayed a 2-fold increase in number of TH immunoreactive cells, as compared to the cells treated with NT4 at the time of plating (Fig. 6C). Moreover, the difference in number of TH-positive cells between these two groups remained constant through all culture times, suggesting that TrkB ligands work from an early phase of the culture as survival factors, to prevent the loss of TH-positive LCr NA neurons and to maintain their survival.

We next examined whether the late effect of TrkB ligands (between 2 and 12 hours, Fig. 6C) could also be related to survival, but several lines of evidence indicated that this was not the case: (1) The number of TH-positive cells did not decrease between 2 and 48 hours in untreated control cultures (Fig. 6C). (2) The number of TH-positive/TUNEL-positive neurons was not different in control and BDNF treated cultures after 3, 6, 12 or 24 hours (whereas no double positive cells were detected at 3 and 6 hours, the percentage of double positive cells in control and BDNF treated cultures at 12 hours were 0.52±0.06 and 0.49±0.07 respectively, and at 24 hours: 0.38±0.1 and 0.40±0.05 respectively). (3) Administration of NT4 with a 24-hour delay still increased the number of TH-positive neurons in as much as cultures treated with the TrkB ligand from the time of plating (Fig. 7). Interestingly, this late effect was not detected after a 48-hour delayed administration, suggesting that there is a window of time of approximately 24 hours during which delayed administration of TrkB ligands increase the numbers of TH-positive neurons in LCr cultures. Thus, combined, our results suggest that the increase in the number of LCr NA TH-positive cells between 2 and 12 hours results from an additional mechanism that does not involve proliferation or survival. We therefore examined whether *Bdnf* could work as an inductive signal for LCr NA neurons.

## TrkB ligands increase the number of neurons expressing both TH and Phox2a in primary LCr cultures

In order to investigate whether this second mechanism involved the induction of a NA phenotype, and to verify that the new TH-positive cells emerging in the cultures between 2 and 12 hours in vitro were indeed LCr NA neurons, we examined the expression of Phox2a, a homeobox protein expressed by LC NA neurons (Lo et al., 1999; Morin et al., 1997; Yang et al., 1998). In control conditions, approximately half of the Phox2a immunoreactive cells in the E13.5 LCr cultures were TH-positive (Fig. 8B). Interestingly, BDNF or NT4 treatment did not significantly modify the number of Phox2a-positive/TH-negative cells, a population that could correspond to committed precursors (Brunet and Pattyn, 2002). However, TrkB ligands clearly increased the number of Phox2a and TH-positive neurons (Fig. 8B) starting as early as 6 hours after treatment (Fig. 8A). Interestingly, the effect was complete after 6 hours and no further increases were detected for up to 6 DIV (Fig. 8B). Thus, our results suggest that both Phox2a and TH are simultaneously and rapidly induced by TrkB ligands in LCr cultures between 3 and 6 hours after treatment.

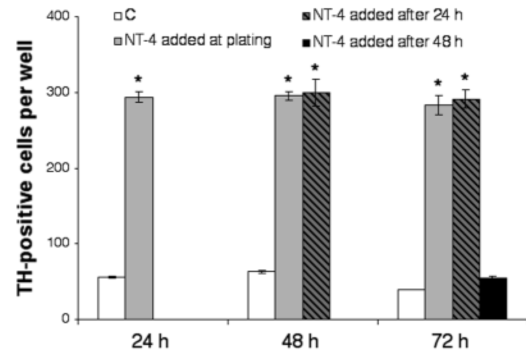



**Fig. 7.** Delayed administration of NT4 for 24 hours but not 48 hours increases the number of TH-positive cells in LCr cultures. NT4 was added to E13.5 LCr cultures at the time of plating or after 24 or 48 hours and then the cultures were fixed after 24, 48 or 72 hours. Counts of TH immunoreactive cells revealed that addition of NT4 after 24 hours gave the same number of TH-positive cells than at the time of plating, suggesting a second effect of NT4 distinct from the early survival-promoting effect. *$P$<0.01, unpaired $t$-test.

In summary, our data indicates that TrkB ligands, in addition to promoting the survival of LC NA neurons, are capable of promoting the phenotypic maturation and/or the induction of a NA phenotype in primary LCr cultures.

## DISCUSSION

In the present study we report that TrkB is required for the survival of a subpopulation of mouse LC neurons during development in vivo and that BDNF and NT4, the two TrkB ligands, promote both the survival and the NA phenotype of rat LCr NA neurons in vitro. Previous results from our group indicated that GDNF and NT3 were good survival-promoting factors for adult LC NA neurons in lesion models (Arenas and Persson, 1994; Arenas et al., 1995). However, analysis of individual *Gdnf* and *Nt3* null mutant mice showed no developmental alteration (see references in Introduction), suggesting that the function of GDNF and NT3 was either redundant or that trophic requirements during development and in adult lesion models could be different. To address this question, double null mutant mice for *Gdnf* and *Nt3* were generated and analyzed in detail. The results of this experiment reinforce the notion that trophic requirements during

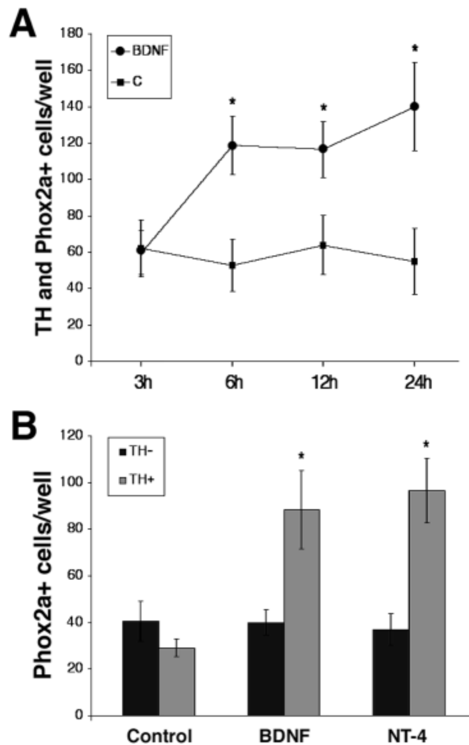



**Fig. 8.** BDNF or NT4 selectively increased the number of Phox2a-positive cells that co-express TH. (A) BDNF increased the number of TH and Phox2a double positive cells between 3 and 6 hours in culture. This effect was maintained for up to 6 days in vitro (B). (B) Administration of either BDNF or NT4 selectively increased the pool of Phox2a-positive/TH-positive cells after 6 days in vitro, leaving the pool of Phox2a-positive/TH-negative cells unaffected. *$P<0.05$, unpaired *t*-test.

development differ from those in lesion models because no alteration on survival, neurite extension, neuropeptide expression, BDNF expression or innervation of target structures could be detected at birth in the double *Gdnf*/*Nt3* null mutant mice. Moreover, administration of either GDNF and/or NT3 to LCr NA neurons in serum-free primary cultures did not promote survival of NA neurons. Thus, our results suggest that GDNF and NT3 are not required for the embryonic development of LCr NA neurons. Interestingly, GDNF, but not NT3, induced a strong neuritogenic effect both in vitro (Holm et al., 2002) (this study) and in vivo (Arenas et al., 1995; Holm et al., 2002), and the survival of LCr NA neurons could be promoted by co-treatment of primary cultures with BMP2 and GDNF or NT3 (Reiriz et al., 2002). These biological activities in vitro suggest that although the double *Gdnf*/*Nt3* null mutant did not show any alteration at birth, GDNF and/or NT3 may play a role in LC development in combination with other factors at postnatal stages. However, because all *Gdnf*/*Nt3* double null mutant mice die at birth, a detailed study of postnatal stages could not be performed. Conditional mutant mice may allow for the determination of the physiological role of GDNF or NT3 in the postnatal development of adult LC NA neurons in vivo.

We also examined whether other neurotrophic factors of the neurotrophin family, in particular BDNF and NT4, were required and sufficient for the development of LC NA neurons. The 30% decrease in the number of TH-positive LC neurons in the *TrkB*$^{-/-}$ mice or the *TrkB*$^{-/-}$ and *TrkC*$^{+/-}$, but not in *TrkC*$^{-/-}$ or *TrkB*$^{+/-}$ mice at P0, indicated that the TrkB receptor was required for LC development. Moreover, because the decrease of TH-positive LC cells in *TrkB* null mutants was accompanied by a decrease in CV-positive LC cells, our results indicated that LC NA neurons are lost by birth and suggest a physiological role of BDNF and NT4 in LC development. In agreement with this possibility, addition of either of these factors to E13.5 rat primary LCr cultures prevented the loss of NA neurons that takes place during the first 2 hours in vitro and maintained their survival for upto 6 days in vitro (the latest time-point tested). Despite the fact that the function of TrkB ligands on NA neurons was analyzed in both mutant mice and primary cultures, our results do not allow for the determination of whether the effects of the TrkB ligands are direct or indirect on LC NA neurons because cell compositions both in vitro and in vivo are heterogeneous. However, the presence of TrkB receptors in LC neurons during development, the rapid kinetics in the biological effects of BDNF and NT4 in vitro, and the availability of TrkB ligands in the LC suggest that a direct action is very probable. Interestingly, NA neurons are tightly packed in the LC and produce high levels of BDNF (Numan et al., 1998) (Fig. 2G). Moreover, overexpression of BDNF under the DBH promoter does not affect LC NA neurons (Fawcett et al., 1998), suggesting that they are normally exposed to sufficiently high levels of BDNF in vivo. In contrast, NA neurons in LCr primary cultures responded to the administration of either NT4 or BDNF with increased survival after damage by tissue dissociation and reduction in the availability of endogenous BDNF levels by dilution in the culture media. This effect persisted for the duration of the culture (6 days), indicating that the survival-promoting effect is not transient and that either BDNF or NT4 maintained the survival of LCr NA neurons. Thus, in vivo and in vitro data suggests that TrkB ligands are both required and sufficient, respectively, to promote the survival of a subset LC NA neurons.

In addition to the survival-promoting effects of BDNF and NT4, treatment of LCr NA neurons with either of these factors resulted in a massive 6-10-fold increase in the number of TH-positive LCr NA neurons between 2 and 12-24 hours in vitro. This effect did not involve proliferation, which was not increased in BDNF- or NT4-treated cultures, or survival, because no reduction in the number of TUNEL-positive cells was detected in BDNF-treated cultures and the increase in TH-positive cells in the culture could be induced by delayed administration of TrkB ligand. Interestingly, the effect of TrkB ligands involved an increase in the number of neurons expressing both Phox2a and TH. Surprisingly, the upregulation in both TH and Phox2a protein by TrkB ligand was simultaneous and had a very fast kinetic, because the effects were not detected at 3 hours and they were already maximal by 6 hours. Thus, our results indicate that TrkB ligands increase the number of LCr NA neurons by two different mechanisms that follow very different kinetics: (1) A very fast survival-promoting mechanism (first detected at 2 hours in vitro) which is sustained for 6 days in vitro and that is only elicited by administration of TrkB ligand with a short time window (<30 minutes) at the time of dissection. (2) A slower effect on the induction or maturation of a NA phenotype (first detected at 6 hours in vitro), which is sustained for upto 6 days

in vitro and could be elicited by administration of TrkB ligand in a broad time window (from the time of dissection to upto 24 hours after plating).

The simultaneous increase in Phox2a/TH double positive cells in the cultures also has interesting implications with regard to the mechanism by which the NA phenotype is regulated. It is known that activation of the TrkB receptor leads to an increase in cAMP response element-binding protein (CREB) (Feng et al., 1999; Pizzorusso et al., 2000), which in turn regulates the expression of TH (Kim et al., 1993; Lane-Ladd et al., 1997; Piech-Dumas and Tank, 1999). Our results suggest that in addition to this pathway, activation of TrkB receptor by BDNF may regulate the expression of Phox2a. This is interesting because Phox2a is known to be part of a NA developmental pathway independent from cAMP-CREB but involving BMPs, Mash1 and Phox2a/b (Lo et al., 1999). Thus, our results suggest that there might be a link between the two pathways allowing a regulation of Phox2a by signaling components downstream of TrkB. Interestingly, the converse type of regulation, that BMP2 induces the expression of BDNF has also been described in striatal cultures (Gratacos et al., 2001). Thus, it is probable that both BMPs and BDNF cooperate by regulating the expression of critical components of each other's signaling pathways.

In summary, the findings presented here show that TrkB is required for the development of mice LC NA neurons in vivo and that BDNF or NT4 are sufficient to promote both the survival and the NA phenotype of rat primary LCr neurons in vitro.

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