CNTF/LIF/gp130 receptor complex signaling maintains a VZ precursor differentiation gradient in the developing ventral forebrain

Christopher Gregg and Samuel Weiss*

Genes and Development Research Group, Hotchkiss Brain Institute, University of Calgary Faculty of Medicine, 3330 Hospital Drive NW, Calgary, Alberta T2N 4N1, Canada
*Author for correspondence (e-mail: weiss@ucalgary.ca)

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Summary

The extrinsic signaling pathways responsible for the formation and maintenance of the unique laminar organization of the forebrain germinal zones are largely unknown. In the present study, we asked whether ciliary neurotrophic factor (CNTF)/leukemia inhibitory factor (LIF)/gp130 signaling plays a role in the development of the germinal layers in the lateral ganglionic eminence. We found that CNTF/LIF/gp130 receptor signaling promotes the self-renewal/expansion of a subpopulation of fibroblast growth factor-responsive ventricular zone (VZ) precursors in the ventral forebrain. Analysis of *Lifr*$^{-/-}$ mice suggests that CNTF/LIF/gp130 signaling maintains a subpopulation of GSH2+ VZ precursors, which are necessary for normal growth of the early ventral forebrain and for maintaining a gradient of VZ precursor differentiation in the lateral ganglionic eminence, as defined by GSH2, MASH1 and DLX2 expression. Furthermore, addition of exogenous CNTF to embryonic forebrain explant cultures deprived of choroid plexus-derived CNTF, was sufficient to promote a VZ differentiation gradient. In contrast to the forebrain, CNTF/LIF/gp130 signaling reduced, rather than enhanced, precursor self-renewal/expansion in the spinal cord. These results demonstrate a novel region-specific role for CNTF/LIF/gp130 signaling in the development of the germinal layers of the embryonic telencephalon.

Key words: Ventricular zone, Subventricular zone, Self-renewal, Ciliary neurotrophic factor, Leukemia inhibitory factor, Mouse

Introduction

The lateral ganglionic eminence (LGE) of the embryonic forebrain is the principle source of neurons that comprise the mature striatum (Deacon et al., 1994; Olsson et al., 1998; Olsson et al., 1995; Wichterle et al., 2001), and also contributes interneurons to the olfactory bulb and perhaps the neocortex (Corbin et al., 2001; Marin and Rubenstein, 2001). Like other regions of the telencephalon, two precursor cell populations reside within the LGE. The ventricular zone (VZ) forms first and VZ precursors undergo interkinetic nuclear migration during the cell cycle and mitotic division at the ventricular surface (Bhide, 1996; Sheth and Bhide, 1997; Smart, 1976). At embryonic day (E) 11, the VZ gives rise to a secondary proliferative population that undergoes mitosis away from the ventricular surface and resides within the subventricular zone (SVZ) (Bhide, 1996; Halliday and Cepko, 1992; Sheth and Bhide, 1997; Smart, 1976). Precursors in the VZ are thought to produce the majority of the neurons generated from within the LGE, while SVZ precursors generate neurons (Anderson et al., 1997; Brazel et al., 2003; Sheth and Bhide, 1997) and glial cells (Brazel et al., 2003; Levison and Goldman, 1993; Luskin and McDermott, 1994). The presence of two germinal layers probably supports forebrain growth by overcoming congestion at the ventricular surface, allowing for expansion of the precursor pool and a prolonged period of proliferation by cells exiting the VZ (Smart, 1972; Smart, 1976). Indeed, in smaller structures such as the spinal cord, cells are derived from only a VZ during development (Smart, 1972; Smart, 1976). The signals responsible for the formation and maintenance of these two distinct germinal layers are largely unknown.

VZ precursors of the LGE express the homeobox transcription factor GSH2 (Hsieh-Li et al., 1995; Yun et al., 2003), while more committed precursors in the SVZ are distinguished by increased expression of the proneural basic helix-loop-helix transcription factor, MASH1 (Ascl1 – Mouse Genome Informatics) (Casarosa et al., 1999; Fode et al., 2000; Porteus et al., 1994; Torii et al., 1999) and the Distal-less related homeobox genes, DLX1 and DLX2 (Anderson et al., 1997; Eisenstat et al., 1999; Porteus et al., 1994; Yun et al., 2002). It has been suggested that MASH1 modulates Notch signaling in the germinal zone by regulating expression of the Notch ligands *Delta1/3*, and in the absence MASH1 (and hence Notch signaling) VZ precursors take on SVZ characteristics (Casarosa et al., 1999; Yun et al., 2002). Furthermore, overexpression of a proneural gene functionally related to MASH1, neurogenin 2 (NGN2), has been demonstrated to promote the acquisition of a SVZ fate by cells born in the cortical VZ (Miyata et al., 2004). These studies strongly suggest major roles for Notch signaling and proneural gene activity in the establishment and/or maintenance of the VZ and SVZ germinal compartments in the telencephalon.

We have previously reported that ciliary neurotrophic factor (CNTF) promotes the self-renewal of embryonic forebrain epidermal growth factor (EGF)-responsive neural stem cells (NSCs) in vitro (Shimazaki et al., 2001) by increasing the expression of NOTCH1 and inhibiting the expression of MASH1 (Chojnacki et al., 2003), potentially conferring a more VZ like phenotype. CNTF signaling is transduced by a heterotrimer composed of the CNTFRα/LIFRβ/gp130 receptor complex, whereas LIF signaling is mediated by the LIFRβ/gp130 heterodimer, both of which can activate the JAK-STAT signaling cascade (Taga and Kishimoto, 1997). Others have found that LIF and gp130-mediated signaling can regulate the self-renewal of cortical NSCs in vivo (Hatta et al., 2002). In the present study, we tested the hypothesis that CNTF/LIF/gp130 receptor signaling contributes to the formation and maintenance of the germinal layers in the LGE during early stages of development. Our results suggest CNTF/LIF/gp130 receptor signaling is both necessary and sufficient to promote the self-renewal/expansion of a subpopulation of VZ precursors, establishing a VZ precursor differentiation gradient that is required for the normal growth of the ventral forebrain.

Materials and methods

Animals and genotyping

CD-1 mouse stocks were maintained in the University of Calgary Animal Resources Center (Calgary, Alberta, Canada). Breeding and genotyping of LIFRβ mutant mice has previously been described (Shimazaki et al., 2001). Morning of vaginal plug was considered embryonic day 0.5 (E0.5). All mice were sacrificed by cervical dislocation.

Neural stem cell culture and growth factors

The generation and differentiation of neurospheres derived from the E14 CNS was performed as previously described with minor modifications (Represa et al., 2001; Reynolds et al., 1992; Shimazaki et al., 2001). Briefly, the lateral and medial ganglionic eminences or cervical/thoracic regions of the spinal cord were dissected from E14 mouse embryos, mechanically dissociated and plated as primary cultures at a density of 100,000 cells/ml in the presence of FGF2 (20 ng/ml; R&D Systems, Minneapolis, MN) and heparin sulfate (2 μg/ml; Sigma, St Louis, MO). After 7 days in vitro (DIV), the resulting primary neurospheres were mechanically dissociated and plated as pass 1 cells at 50,000 cells/ml. Additional cytokines used were CNTF (20 ng/ml; a generous gift from Dr Robert Dunn, Montreal General Hospital Research Institute) and LIF (20 ng/ml; Chemicon, CA, USA). To dissociate pass 1 neurospheres, we used a 3-minute treatment of 3 ml trypsin-EDTA (Invitrogen, Canada) at 37°C, followed by 5 ml trypsin inhibitor (Sigma). Cells were centrifuged at 600 rpm for 10 minutes and resuspended in 5 ml of trypsin inhibitor and mechanically dissociated with a fire-polished pipette. Cells were then suspended in 8 ml of basal media, centrifuged, resuspended in 2 ml of basal media and then used for experiments. For quantification of nestin+ cells, pass 1 neurospheres were dissociated, plated at 200,000 cells/ml on poly-l-ornithine coated coverslips and fixed after 30 minutes for processing for immunocytochemistry. For quantification of β-tubulin III+ cells, pass 1 neurospheres were dissociated, plated at 200,000 cells/ml for 5 DIV and fixed for immunocytochemical analysis. For quantification of NSC self-renewal, dissociated pass 1 neurospheres were plated in 96-well plates at a density of 10,000 cells/ml and the number of secondary neurospheres was quantified after 10 DIV.

Area measurements of the LGE and cortex

The forebrains of E12.5 *Lifr*$^{-/-}$, *Lifr*$^{+/-}$ and *Lifr*$^{+/+}$ embryos were serially sectioned and collected onto two rounds of six slides with eight sections per slide and the slides were stained with Hoechst (Sigma). Slides were randomly sampled from each embryo and sections of the LGE and cortex were photographed beginning at the most rostral appearance of the LGE and extending caudally to the first appearance of the dorsal thalamus. The LGE was outlined and the area was calculated using Image J software (NIMH, MD, USA). The areas of the *Lifr*$^{-/-}$ LGE and cortex were normalized to the averaged areas of *Lifr*$^{+/-}$ and *Lifr*$^{+/+}$ littermates for statistical analysis.

Bromodeoxyuridine labeling, detection and quantification

Pregnant dams received a single injection of bromodeoxyuridine on E12.5 (BrdU; 120 mg/kg, ip; dissolved in 0.007% NaOH in phosphate buffer; Sigma) and were sacrificed thirty minutes post-injection. The brain and upper thoracic spinal cord was removed from E12.5 embryos and processed for immunohistochemistry as described below. Brains and spinal cords were sectioned serially onto two rounds of six slides with eight 14 μm sections per slide. Sections were pretreated with 1 M HCl for 30 minutes at 60°C to denature the DNA. Rat monoclonal anti-BrdU (1:50; Sera-Lab, Sussex, UK) and biotinylated donkey anti-rat (1:200; Jackson ImmunoResearch, West Grove, PA) with Streptavidin-Cy3 (1:2000; Jackson ImmunoResearch) were used for BrdU detection. The number of BrdU-expressing cells in the LGE or cortex was quantified on randomly sampled slides, beginning at the first appearance of the dorsal thalamus and extending rostrally for five sections for each animal. The same methodology was used for GSH2, MASH1 and pHH3 quantification.

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL)

Apoptosis was detected in tissue sections using the In Situ Cell Death Labeling Kit (Roche; Indianapolis, IN) according to the manufacturer's instructions with the following modifications. Sections were incubated in 100 μl Proteinase K (diluted 1:1000 from a stock solution of 20 μg/μl in 10 mM Tris-HCl pH 8.0) for 6 minutes at room temperature and then washed extensively in PBS. The slides were incubated for 2 hours at 37°C in the presence of the TUNEL reaction mixture supplied by the kit, were washed in PBS, and then incubated overnight in the presence of mouse anti-FITC (1:400; Sigma). Detection was performed using biotinylated goat anti-mouse (1:200; Jackson ImmunoResearch) and Streptavidin-FITC (1:1000; Jackson ImmunoResearch). We quantified only those cells that were positive on the FITC channel and therefore not autoflourescent. Quantification was carried out in the same manner as for BrdU and the number of TUNEL-positive cells was normalized to the calculated area of the LGE, as described above.

Explant cultures

Explant cultures were performed as described previously (Kingsbury et al., 2003) with modifications. Each cerebral hemisphere was dissected away from the developing E14.5 CNS in PBS. Next, the choroid plexus and regions of the septum and medial cortex were carefully dissected away to allow the culture media access to the ganglionic eminences. One hemisphere from each brain was placed in basal media (Opti-MEM I; Gibco, NY, USA) and the other was placed in basal media containing 50 or 100 ng/ml CNTF (either concentration was effective) in six-well plates (Nunc). The explants were placed on a rocker in a tissue culture incubator at 37°C, 5% CO_2 in air, for 18.5 hours, fixed in 4% paraformaldehyde at 4°C for 2 hours and cryoprotected in 25% sucrose overnight. Tissue was cryosectioned and processed for immunohistochemistry and quantification as described above for embryonic tissue. Explants that appeared damaged or unhealthy were excluded from analysis.

RT-PCR analysis

The choroid plexus was dissected from the lateral ventricles of 10-12 E14.5 CD-1 embryos and pooled for each round of RNA extraction. RNA was isolated using the RNeasy Protect Mini Kit (Qiagen, ON, Canada) and reverse transcribed using the Superscript III First-Strand Synthesis Kit (Invitrogen, CA, USA) with random hexamer primers, all according to the manufacturers instructions. PCR analysis of the resulting cDNA was performed to determine the expression of LIF using methods previously described (Bauer et al., 2003). CNTF cDNA was amplified by PCR for 35 cycles of 95°C for 45 seconds, 55°C for 45 seconds and 72°C for 45 seconds using 5′-ATG-GCTTTCGCAGAGCAA-3′ as sense primer and 5′-CTACAT-TTGCTTGGCCC-3′ as the antisense primer (Subang et al., 1997), which amplified a 596 bp fragment. CNTF and LIF RT-PCR products were sequenced by the University of Calgary DNA Sequencing Laboratory and checked for accurate identification by a BLAST search of the mouse genome.

Antibodies and immunohistochemistry

The primary antibodies used in this study were as follows: rabbit anti-NOTCH1, anti-FGFR1, anti-FGFR2, anti-LIFRβ, anti-gp130, anti-CNTF and anti-LIF (1:50, Santa Cruz Biotechnology); goat anti-CNTFRα (1:50, Santa Cruz Biotechnology); mouse anti-Ki67 (1:100; NovoCastra Laboratories, Newcastle, UK); mouse anti-β-III tubulin (1:100; Sigma); rabbit anti-GSH2 (generous gift from Dr Kenneth Campbell, Children's Hospital Research Foundation, Cincinnati); mouse anti-MASH1 (generous gift from Dr David Anderson, Caltech); rabbit anti-DLX1 and rabbit anti-DLX2 (1:100; generous gifts from Dr David Eisenstat; University of Manitoba); mouse anti-GAD65 (1:100; BD Pharmingen); and rabbit anti-phospho Histone H3 (pHH3; 1:100; Upstate Biotechnologies).

For immunohistochemical analysis on embryonic tissue sections, timed pregnant mice were killed, the brain or upper thoracic spinal cord was removed from the embryos, fixed in 4% paraformaldehyde for 2 hours at 4°C and then cryoprotected by 10% sucrose and then 25% sucrose solutions overnight. All tissue was embedded in Tissue Tek OCT compound (Sakura Finetek, Torrance, CA) and cryosectioned at 14 μm. For receptor staining, tissue was post-fixed in methanol at –20°C for 8 minutes. Primary antibodies were followed by incubation with fluorescein- or rhodamine-conjugated secondary antibodies against mouse, rabbit or goat IgG, or by using biotin-conjugated secondary antibodies (all 1:200) followed by Streptavidin-CY3 (1:2000; Jackson ImmunoResearch). For nuclear counterstaining, Hoechst was used (1:100; Sigma).

Statistical analysis

Values are mean±s.e.m. Statistical significance between groups was assessed using ANOVA and the Tukey Honest Significant Difference Test, or paired *t*-test where noted.

Results

CNTF/LIF/gp130 signaling promotes the self-renewal/expansion of FGF-responsive ventral forebrain NSCs within the VZ

To determine whether CNTF/LIF/gp130 signaling acts upon VZ and/or SVZ LGE precursors during development, we analyzed the expression pattern of the receptors in the LGE. At early stages of forebrain development, the majority of VZ precursor cells proliferate in response to fibroblast growth factor (FGF), rather than EGF (Martens et al., 2000; Raballo et al., 2000; Tropepe et al., 1999; Vaccarino et al., 1999). As expected, we observed both FGFR1 and FGFR2 immunoreactivity within the neuroepithelium of the ventral forebrain as early as E9.5 (Fig. 1A,B). At this stage, CNTFRα expression is absent (Fig. 1C); however, the expression of LIFRβ is robust throughout the ventral forebrain neuroepithelium (Fig. 1D). By E11.5, with the first appearance of both a VZ and SVZ (Smart, 1976), FGFR1, FGFR2, CNTFRα and LIFRβ are all expressed within the germinal zone of the LGE (Fig. 1E-H).

To determine whether the CNTF/LIF/gp130 and FGF signaling pathways might be regulating VZ precursors and/or SVZ precursors, we performed a series of double labeling experiments with Ki67, a marker specific for dividing cells, at E14.5 when both the VZ and SVZ are prominent (Smart, 1976). Double labeling for FGFR2 and Ki67 at E14.5 revealed co-expression in dividing VZ precursors, but not by those in the SVZ (Fig. 1I,J). Similarly, LIFRβ is expressed by VZ precursors, but not by Ki67-positive cells within the SVZ (Fig. 1K,L). These results suggest that CNTF/LIF/gp130 receptor signaling might act in coordination with FGF signaling to maintain precursors within the VZ, rather than those in the SVZ of the developing LGE.

The relationship between CNTF/LIF/gp130-mediated signaling and FGF signaling with respect to NSC regulation is not known. To assess whether CNTF or LIF signaling modulates FGF-responsive NSC proliferation, NSCs residing within the E14.5 ganglionic eminences were isolated and grown as neurospheres in FGF2. These neurospheres were dissociated, reseeded at 50,000 cells/ml in FGF2 and the presence or absence of either CNTF or LIF, and the total number of cells generated in each growth condition was assessed after 7 DIV. Remarkably, the number of cells generated by ventral forebrain NSCs in the presence of FGF-2 (Fig. 1M; $2.9\pm0.3\times10^6$) doubled in response to the addition of either CNTF ($6.0\pm0.8\times10^6$; P=0.009; n=5) or LIF ($5.4\pm0.5\times10^6$; P=0.03; n=5). To determine whether or not this increase might be due to an increase in the total number of neurospheres generated, primary FGF2 neurospheres were dissociated and replated in 96-well plates in FGF2 and the presence or absence of CNTF or LIF. After 10 DIV, we found no difference in the number of neurospheres generated in the presence of CNTF or LIF relative to FGF2 alone (Fig. 1N); however, the size of the neurospheres increased in CNTF- and LIF-treated conditions (Fig. 1O). Quantification revealed over a two-fold increase in the number of large neurospheres (>200 μm in diameter) in the CNTF and LIF growth conditions compared with FGF2 alone (Fig. 1P; CNTF, P=0.0006; LIF, P=0.005; n=3).

One possible explanation for the increased size of the CNTF or LIF treated neurospheres is that CNTF/LIF/gp130 signaling promotes NSC self-renewal and expansion, as we suggested previously for EGF-responsive NSCs (Shimazaki et al., 2001). To determine the number of precursors within the neurospheres cultured in the absence or presence of CNTF or LIF, we counted (in each growth condition) the percentage of cells that expressed nestin, a marker of undifferentiated cells (Frederiksen and McKay, 1988). In the CNTF/LIF-treated cultures, we observed a ~50% increase in the percentage of cells expressing nestin relative to cultures generated in FGF2 alone (Fig. 1Q; CNTF P=0.03; LIF P=0.04; n=3). Additionally, when neurospheres generated in either FGF2, FGF2+CNTF or FGF2+LIF were dissociated and replated in 96-well plates in the presence of FGF2, the number of secondary neurospheres generated after 10 DIV doubled in the CNTF- and LIF-treated

cultures (Fig. 1R; CNTF, P=0.02; LIF, P=0.02; n=3). These results suggest that CNTF/LIF/gp130 signaling promotes the self-renewal/expansion of FGF-responsive NSCs residing within the VZ.

CNTF/LIF/gp130 signaling does not promote the self-renewal/expansion of spinal cord NSCs

Unlike the forebrain, the developing spinal cord has only a single germinal layer, called the VZ. We therefore sought to

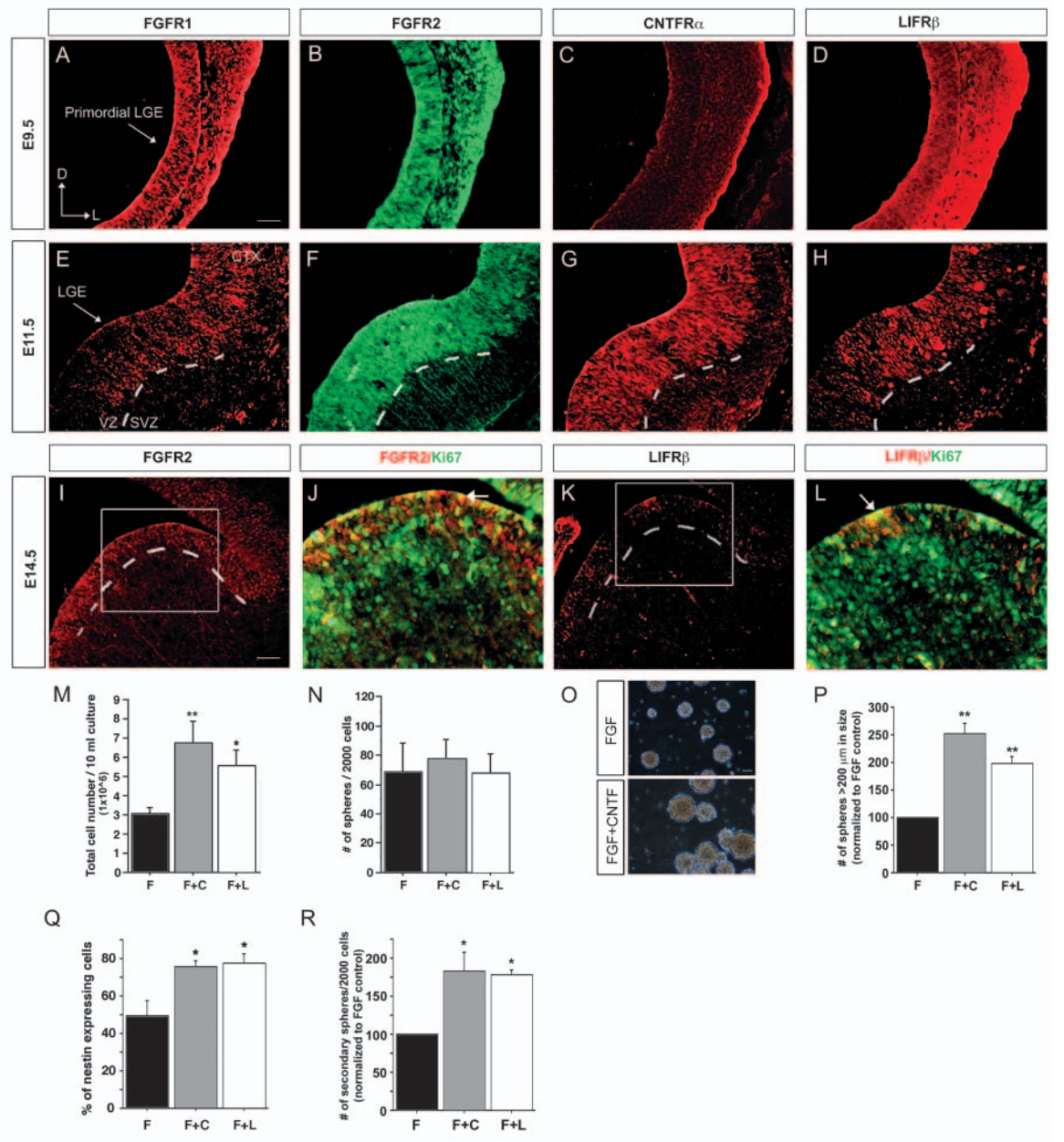


Fig. 1. CNTF/LIF/gp130 receptor signaling promotes the self-renewal/expansion of FGF-responsive neural stem cells (NSCs) from the ventricular zone (VZ) of the ventral forebrain. E9.5 ventral neuroepithelial cells expressed the FGF receptors FGFR1 (A) and FGFR2 (B). CNTFRα expression (C) was not observed; however, LIFRβ (D) was expressed. At E11.5, FGFR1 (E), FGFR2 (F), CNTFRα (G) and LIFRβ (H) were expressed within the LGE VZ. Double-labeling of the E14.5 LGE for FGFR2 (I) and Ki67 (J), as well as LIFRβ (K) and Ki67 (L), revealed receptor expression by VZ precursors, not SVZ precursors (arrows in J,L indicate examples of co-expressing cells). (M) The total number of cells generated in pass 1 FGF-2 neurosphere cultures doubled in the presence of either CNTF (F+C) (**P=0.009; n=5) or LIF (F+L) (*P=0.026; n=5). (N) The total number of neurospheres generated in pass 1 FGF-2 neurosphere cultures was not changed by the addition of either CNTF or LIF (n=3). (O,P) The number of neurospheres that were 200 μm or greater in diameter was doubled by the addition of either CNTF (**P=0.0003) or LIF (**P=0.005; results are normalized to FGF control; n=3). (Q) CNTF (*P=0.03) and LIF (*P=0.04) significantly increased the percentage of cells that expressed the undifferentiated cell marker nestin in pass 1 FGF2 neurosphere cultures (n=3). (R) The number of secondary neurospheres formed in FGF-2 alone per 2000 cells plated was significantly increased by either CNTF (*P=0.02) or LIF (*P=0.02; results are normalized to FGF control; n=3). Scale bars: in A, 50 μm for A-H; in I, 50 μm (25 μm in enlarged images) for I-L; in O, 100 μm.

determine whether CNTF/LIF/gp130 signaling might play a different role in the regulation of spinal cord NSCs compared with forebrain NSCs. At E11.5, FGFR2 expression is clearly present in the spinal cord VZ (Fig. 2A). However, CNTFRα expression is primarily in the mantle zone (Fig. 2B), the region of neuronal maturation as shown by β-tubulin III expression (Fig. 2C). By E14.5, the spinal cord germinal zone is reduced in size, as shown by FGFR2 expression (Fig. 2D). The expression of CNTFRα (Fig. 2E) continued to predominate in the mantle zone (Fig. 2F), although low levels of expression are evident in the VZ. These results suggest that CNTF/LIF/gp130-mediated signaling might primarily influence cells of the neuronal lineage in the mantle zone rather than NSCs within the VZ of the spinal cord.

To examine the actions of CNTF/LIF upon the self-renewal/expansion of spinal cord NSCs, pass 1 neurospheres were generated from the spinal cord of E14.5 embryos in FGF2 and the absence or presence of CNTF or LIF. These neurospheres were dissociated and replated in 96-well plates in the presence of FGF2 alone, as was done to examine the self-renewal/expansion of ventral forebrain NSCs. Remarkably, the addition of either CNTF or LIF significantly inhibited the self-renewal of spinal cord NSCs, resulting in a 50% reduction in the number of secondary neurospheres formed (Fig. 2G; CNTF, P=0.003; LIF, P=0.007; n=4). This was the opposite effect to that observed with ventral forebrain NSCs.

Previous work has demonstrated a role for CNTFRα and LIFRβ signaling in regulating the differentiation/survival of spinal motoneurons (Li et al., 1995; Murphy et al., 1997; Nakashima et al., 1999; Richards et al., 1992). Given that we observed the majority of CNTFRα expression in the mantle zone, we asked whether the addition of CNTF/LIF to spinal cord neurosphere cultures affected neurogenesis by quantifying the number of β-tubulin III+ neurons. The addition of either CNTF (**P=0.007; n=4) or LIF (**P=0.0001; n=4) to E14.5 pass 1 spinal cord neurosphere cultures resulted in a doubling of the number of neurons generated by spinal cord NSCs, compared with FGF2 alone (Fig. 2H). By contrast, there was no change in the number of neurons generated by E14.5 ventral forebrain neurosphere cultures treated with either CNTF or LIF (Fig. 2I; n=3).

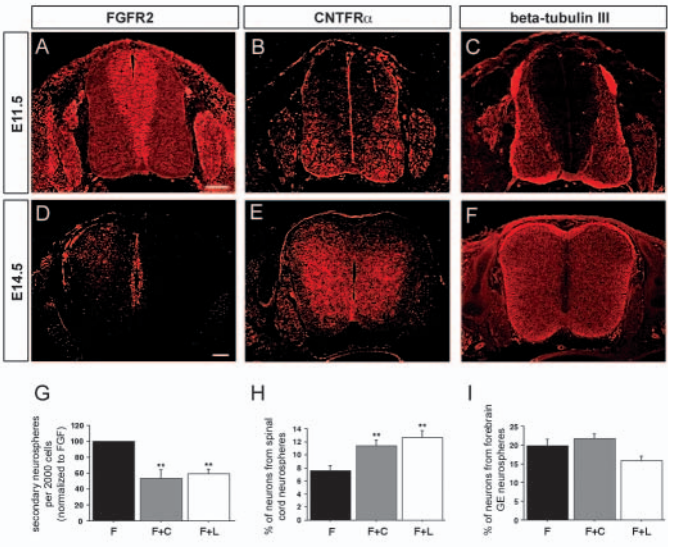


Fig. 2. CNTF/LIF/gp130 receptor signaling does not promote spinal cord neural stem cell (NSC) self-renewal/expansion in vitro. FGFR2 (A) was robustly expressed in the E11.5 spinal cord VZ. By contrast, the expression of CNTFRα (B) was largely restricted to the mantle zone, demonstrated by β-tubulin III staining (C). By E14.5, the FGFR2-expressing VZ precursor population (D) was reduced. CNTFRα (E) continued to be expressed in the mantle zone, indicated by β-tubulin III (F), though low levels were present in the VZ. (G) CNTF- (**P=0.003) or LIF- (**P=0.007) treated E14.5 spinal cord neurospheres generated significantly fewer secondary neurospheres compared with FGF2 alone (results are normalized to FGF2 control; n=4). (H) CNTF (**P=0.007) or LIF (**P=0.0002) treatment significantly increased the number of β-tubulin III neurons generated by E14.5 spinal cord NSCs (n=4). (I) Neither CNTF nor LIF significantly increased the number of β-tubulin III-expressing neurons generated by ventral forebrain NSCs. Scale bars: in A, 50 μm for A-C; in D, 100 μm for D-F.

CNTF/LIF/gp130 signaling is required *in vivo* for normal growth of the developing forebrain

To determine whether CNTF/LIF/gp130 signaling was required in vivo for the normal growth of the forebrain during development, we analyzed *Lifr* null mutant mice, in which both CNTF and LIF signaling are disrupted. The forebrains of E12.5 $Lifr^{+/+}$, $Lifr^{+/-}$ and $Lifr^{-/-}$ littermate embryos were serially sectioned and the gross morphologies of the forebrains were compared. No obvious difference in forebrain growth was observed between the $Lifr^{+/+}$ and $Lifr^{+/-}$ littermates (data not shown). On the other hand, although an obvious difference in overall brain size was not noted, a histological comparison of sections counterstained with the nuclear marker Hoechst from rostral to caudal between $Lifr^{+/+}/Lifr^{+/-}$ and $Lifr^{-/-}$ mice revealed gross morphological differences that were suggestive of impaired growth in the mutant forebrains (Fig. 3). At rostral levels, the most obvious difference was a decrease in the size of the LGE in the $Lifr^{-/-}$ mice (Fig. 3A,B). At more caudal regions of the forebrain, we observed that the cortex appeared thinner compared with $Lifr^{+/+}/Lifr^{+/-}$ controls, and the LGE, MGE and caudal ganglionic eminence were also reduced in size (Fig. 3C-H). This phenotype was partially penetrant with 56% (n=9) of the $Lifr^{-/-}$ embryos displaying gross morphological changes relative to littermate $Lifr^{+/+}/Lifr^{+/-}$ controls.

Further analysis was focused on the LGE at E12.5, a stage when the LGE is clearly distinguishable from the MGE and VZ and SVZ germinal layers are present (Smart, 1976). Measurements of the LGE (see Materials and methods) in $Lifr^{+/+}/Lifr^{+/-}$ versus $Lifr^{-/-}$ embryos revealed a 22% reduction in the size of the LGE in affected $Lifr^{-/-}$ embryos (paired t-test **P=0.008; n=5; Fig. 3I,J). Immunohistochemical analysis for β-tubulin III demonstrated a concurrent decrease in the size of the mantle zone (Fig. 3K,L), which suggested either a decrease in overall growth or an increase in cell death. The number of TUNEL+ cells in the LGE was quantified and normalized to the area. No difference in the number of cells undergoing cell death between the $Lifr^{-/-}$ (0.5±0.1 cells/unit area) and $Lifr^{+/+}/Lifr^{+/-}$ embryos (0.5±0.1 cells/unit area; n=4) was observed. To determine whether the defect might instead be related to fewer precursor cells in the germinal zone, proliferating precursors were labeled by injecting dams with

BrdU. The number of BrdU+ cells in the VZ/SVZ of the LGE of *Lifr*$^{-/-}$ embryos (608±15; Fig. 3O) was reduced by 30% relative to *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ embryos (865±41; P=0.009; paired t-test; n=5; Fig. 3P). These observations were not limited to the LGE and nearly identical defects were observed in the developing cortex (see Fig. S1A-H in the supplementary material). By contrast, however, the number of BrdU positive cells in the E12.5 spinal cord VZ was not significantly decreased in *Lifr*$^{-/-}$ relative to *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ embryos, and no obvious morphological changes related to growth were observed (see Fig. S2A,B in the supplementary material). These initial in vivo analyses suggested a region specific role for CNTF/LIF/gp130 signaling in regulating the self-renewal/expansion of telencephalic precursors and justified further investigations into a potential role for this pathway in establishing the VZ and SVZ precursor populations of the LGE.

GSH2, MASH1 and DLX2 expression define a gradient of VZ precursor differentiation in the LGE

To investigate the role of CNTF/LIF/gp130 signaling in the formation and maintenance of the ventral forebrain germinal layers, it was necessary to establish criteria to distinguish undifferentiated VZ precursor cells from more differentiated precursors. Previous studies have suggested that the transcription factors GSH2, MASH1 and DLX2 might be useful as markers of VZ precursor differentiation (Eisenstat et al., 1999; Porteus et al., 1994; Yun et al., 2002; Yun et al., 2003), prompting us to examine their expression in more detail.

At E8.5, neither GSH2 nor MASH1 were expressed in the VZ (data not shown), in agreement with previous studies (Yun et al., 2002), and we have designated these cells rudimentary precursor cells (RPs). At E9.5, when the germinal zone is composed of a VZ only, a large number of precursor cells expressed GSH2 in the LGE, and we have designated these as P1 precursor cells (Fig. 4A). A subpopulation of VZ precursors also expressed MASH1 (Fig. 4B), and all the MASH1+ cells co-expressed GSH2 (Fig. 4C). Very few DLX2+ cells were detected at the pial surface of the E9.5 LGE VZ (Fig. 4D), and these cells co-expressed MASH1 (Fig. 4E,F). The DLX2+ subpopulation was presumed to express GSH2, as all MASH1+ cells at this stage appeared to express GSH2 (the triple labeling

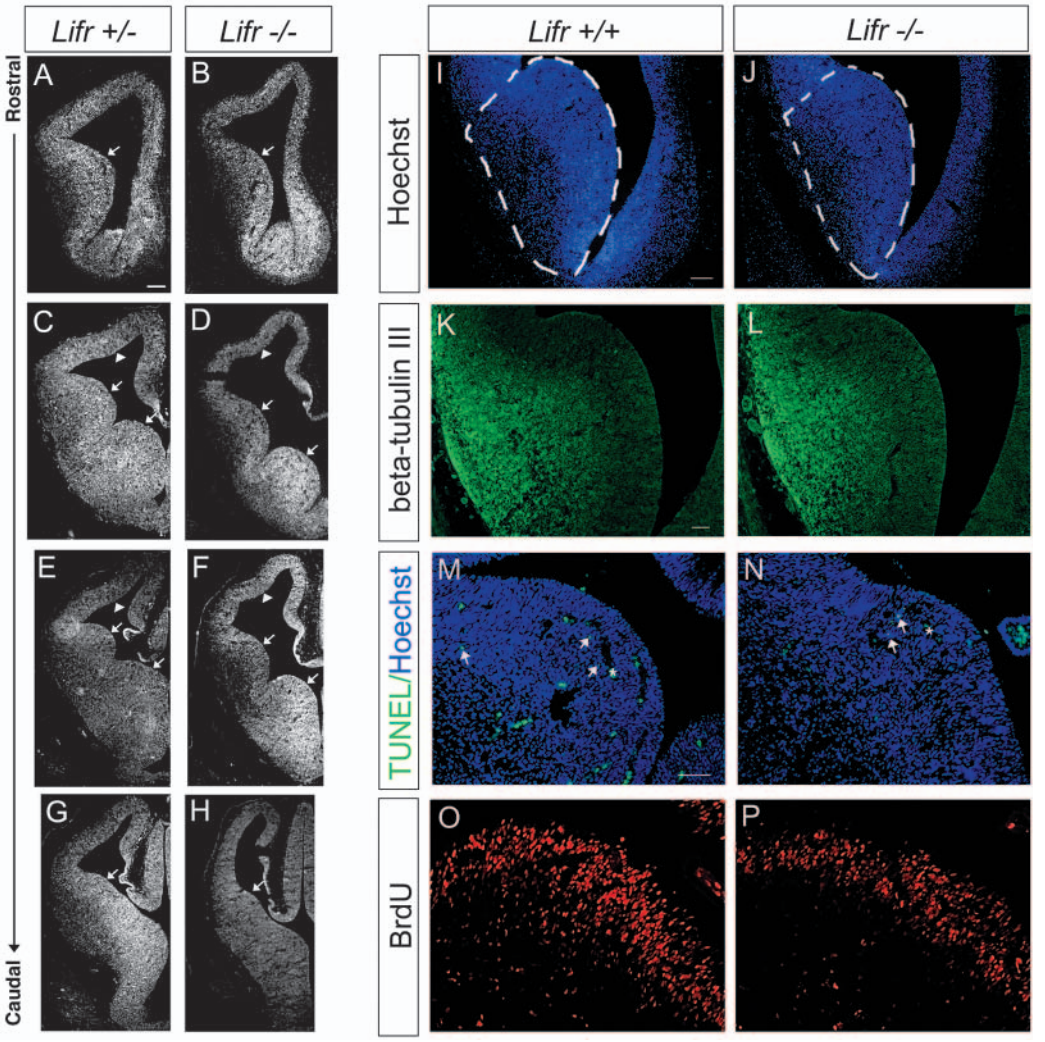
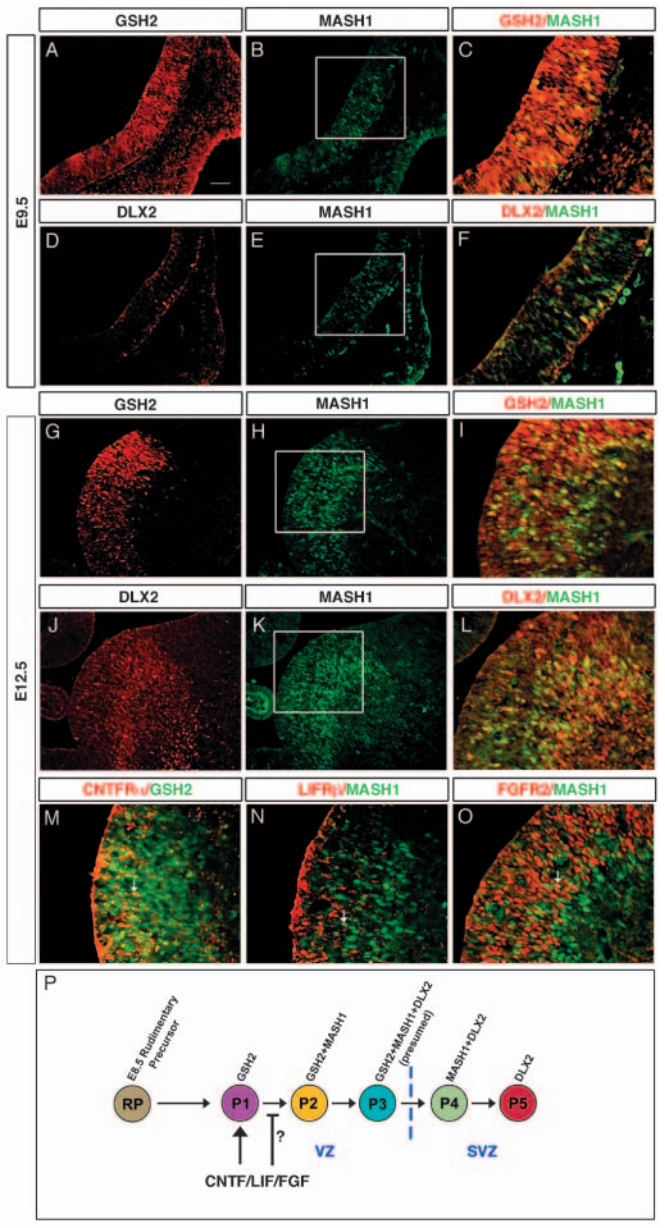


Fig. 3. Gross morphological changes in the forebrains of E12.5 *Lifr*$^{-/-}$ embryos relative to control littermates are suggestive of defects related to growth. At rostral regions of the forebrain, the LGE (arrow) in *Lifr*$^{-/-}$ embryos (B) was smaller relative to *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ (A) littermates. At more caudal regions, decreased thickness of the cerebral wall was observed (arrowhead), as well as decreased size of the LGE and MGE (arrows) in *Lifr*$^{-/-}$ (D,F) relative to *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ embryos (C,D). At the most caudal regions analyzed (G,H), a reduced caudal ganglionic eminence (arrow) was clearly observed (56% penetrant; *Lifr*$^{-/-}$ n=9; *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ n=9). Measurements of the area of the LGE revealed a 22% decrease in size in *Lifr*$^{-/-}$ embryos (I,J; paired t-test **P=0.008; n=5). A concurrent decrease in the mantle zone was also observed, as shown by β-tubulin III staining (K,L). The number of dying cells in the LGE of *Lifr*$^{-/-}$ embryos indicated by TUNEL labeling was normalized to the area of the LGE (M; 0.5±0.1 cells/unit area) and no difference was observed relative to *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ embryos (N; 0.5±0.1 cells/unit area; n=4; arrows indicate positive cells; stars indicate examples of autoflorescence). The number of BrdU+ precursors in *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ (O; 865±41) was significantly higher than *Lifr*$^{-/-}$ embryos (P; 608±15; paired t-test **P=0.009; n=5). Scale bar: in A, 100 μm for A-H; in I, 100 μm for I,J; in K, 50 μm for K,L; in M, 50 μm for M-P.

experiment was not possible). This analysis, at a stage when the SVZ is absent (Smart, 1976), suggests that the LGE VZ is composed of P1, P2 and P3 precursors, as depicted in Fig. 4P.

By E12.5, the LGE germinal zone is composed of both VZ and SVZ precursors (Sheth and Bhide, 1997; Smart, 1976). GSH2 expression continued to be heavily expressed by VZ precursors (Fig. 4G), whereas MASH1+ cells were organized in a gradient, with fewer precursors expressing low levels in the VZ, to more precursors expressing higher levels in the SVZ (Fig. 4H). GSH2/MASH1 double-labeling revealed a VZ to SVZ molecular gradient from GSH2+ only precursors (P1 precursors) at the ventricular surface, to GSH2+MASH1+ precursors and then MASH1+ only precursors in SVZ (Fig. 4I). Analysis of DLX2 and MASH1 expression revealed a molecular gradient of low DLX2 and MASH1 at the ventricular surface to increased numbers of MASH1+ precursors, MASH1+DLX2+ precursors, and, finally, DLX2+ precursors at the superficial edge of the SVZ and in the mantle zone (Fig. 4J-L). This analysis expands on previous studies (Yun et al., 2002) and suggests that a gradient of LGE precursor differentiation exists that can be defined by GSH2, MASH1 and DLX2 expression, as summarized in the schematic in Fig. 4P.

To understand how CNTF, LIF and FGF signaling might regulate this differentiation gradient, we performed a series of double-labeling experiments. Analysis of the expression of CNTFRα and GSH2 revealed that a subpopulation of GSH2+ VZ precursors located close to the ventricular surface express CNTFRα (Fig. 4M; arrow indicates a double-labeled cell). LIFRβ, required for both CNTF and LIF signaling, is also expressed by VZ precursors close to the ventricular surface. Double labeling revealed that very few LIFRβ+ cells co-express MASH1 (Fig. 4N; arrow indicates an example of a double-labeled cell). Similarly, only a small subpopulation of MASH1+ cells co-expressed FGFR2 (Fig. 4O). These results suggest that CNTF, LIF and FGF signaling might act primarily to regulate the self-renewal/expansion of the VZ P1 precursor (GSH2+MASH1–) cell population, perhaps inhibiting progression to more differentiated precursor fates (P2-P5), as depicted in the schematic in Fig. 4P.



CNTF/LIF/gp130 signaling is required in vivo to maintain the VZ precursor differentiated gradient

We hypothesized that impaired self-renewal/expansion of P1 precursor cells, owing to lost CNTF/LIF/gp130 signaling, results in the precocious differentiation of VZ precursors. To test this, we analyzed the number of GSH2+ VZ precursors in the LGE of E12.5 *Lifr*$^{-/-}$ animals relative to *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ embryos. *Lifr*$^{-/-}$ embryos had ~30% fewer GSH2+ precursors (Fig. 5B; 911±23) relative to *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ controls (Fig. 5A;

Fig. 4. A gradient of VZ precursor differentiation is defined in the LGE by the expression of GSH2, MASH1 and DLX2. At E9.5, GSH2 (A) was expressed by precursors within the LGE (P1 precursors). (B) MASH1 was expressed in a small population of VZ precursors, and all MASH1+ cells co-expressed GSH2 in the primordial LGE (P2 precursors; C). (D) A few DLX2+ cells were present at E9.5, primarily near the pial surface of the neuroepithelium. All DLX2+ cells co-expressed MASH1 at this stage (E,F; presumptive GSH2+MASH1+DLX2-expressing P3 precursors). At E12.5, GSH2 expression appeared within the VZ (G), while MASH1 expression was relatively low in the VZ, but increased towards the SVZ (H). GSH2 and MASH1 double labeling revealed a molecular gradient from GSH2 (P1 precursors) to GSH2/MASH1 (P2 and P3 precursors) and MASH1 alone (presumptive P4 precursors) expressing precursors (I). DLX2 was also expressed in a VZ to SVZ gradient (J). MASH1 expression demonstrated a similar gradient of expression (K) and double-labeling (L), suggesting that farther from the ventricular surface precursors increasingly expressed MASH1, MASH1/DLX2 (P3 and P4 precursors) and (finally) DLX2 alone (P5 precursors). CNTFRα was expressed by a subpopulation of GSH2+ cells close to the ventricular surface (M; arrow indicates example of a double-positive cell). LIFRβ expression was similar to CNTFRα, and very few MASH1+ cells co-expressed LIFRβ (N; arrow indicates a double-positive cell). FGFR2 and MASH1 double labeling also revealed only a very small population of co-expressing cells (O; arrow indicates double-labeled cell). (P) The proposed gradient of VZ precursor differentiation and a potential role for CNTF/LIF/gp130 signaling in maintaining the P1 precursor population within the gradient (see Results and Discussion for details). Scale bar: 50 μm and 100 μm in enlarged images.

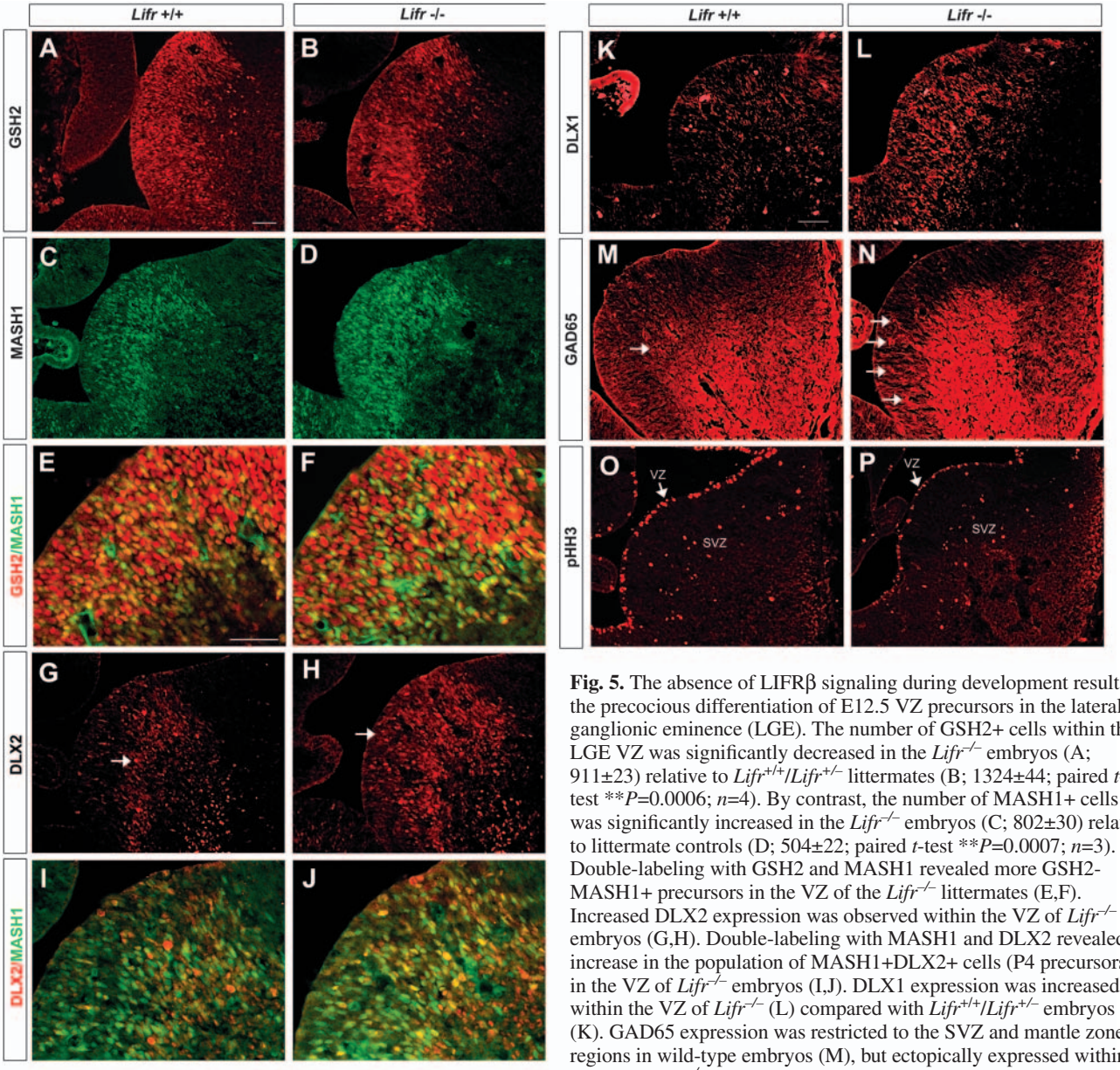


Fig. 5. The absence of LIFRβ signaling during development results in the precocious differentiation of E12.5 VZ precursors in the lateral ganglionic eminence (LGE). The number of GSH2+ cells within the LGE VZ was significantly decreased in the *Lifr*$^{-/-}$ embryos (A; 911±23) relative to *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ littermates (B; 1324±44; paired *t*-test ***P*=0.0006; *n*=4). By contrast, the number of MASH1+ cells was significantly increased in the *Lifr*$^{-/-}$ embryos (C; 802±30) relative to littermate controls (D; 504±22; paired *t*-test ***P*=0.0007; *n*=3). Double-labeling with GSH2 and MASH1 revealed more GSH2-MASH1+ precursors in the VZ of the *Lifr*$^{-/-}$ littermates (E,F). Increased DLX2 expression was observed within the VZ of *Lifr*$^{-/-}$ embryos (G,H). Double-labeling with MASH1 and DLX2 revealed an increase in the population of MASH1+DLX2+ cells (P4 precursors) in the VZ of *Lifr*$^{-/-}$ embryos (I,J). DLX1 expression was increased within the VZ of *Lifr*$^{-/-}$ (L) compared with *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ embryos (K). GAD65 expression was restricted to the SVZ and mantle zone regions in wild-type embryos (M), but ectopically expressed within the VZ of *Lifr*$^{-/-}$ embryos (N; arrows indicate GAD65 staining). The number of surface pHH3+ cells (VZ precursors) was reduced by 40% in the LGE of the *Lifr*$^{-/-}$ embryos compared with littermate *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ embryos (O,P; paired *t*-test ***P*=0.0005; *n*=4). The number of non-surface pHH3+ cells (SVZ precursors) was not significantly different between the *Lifr*$^{-/-}$ and *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ littermates. For all experiments, *n*≥3. Scale bars: in A, 100 μm for A-D,G,H,K-N; in E, 50 μm for E,F,I,J; in M, 100 μm for M-P.

1324±44; paired *t*-test ***P*=0.0006; *n*=4). When normalized to the decreased area of the LGE, the overall decrease in the *Lifr*$^{-/-}$ GSH2+ population was 15% relative to *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ littermates (paired *t*-test **P*=0.04). By contrast, the number of MASH1+ precursors within the germinal zone of the *Lifr*$^{-/-}$ embryos (Fig. 5D; 802±30) was increased by 37% compared with *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ embryos (Fig. 5C; 504±22; paired *t*-test ***P*=0.0007; *n*=3). Double-labeling of MASH1 and GSH2 demonstrated a dramatic increase in the MASH1+GSH2– cell population within VZ of the *Lifr*$^{-/-}$ embryos (Fig. 5E,F). Analysis of DLX2 also revealed increased expression by VZ precursors within the *Lifr*$^{-/-}$ LGE (Fig. 5G,H). Double labeling with MASH1 and DLX2 demonstrated that the majority of the DLX2+ cells within the VZ of the *Lifr*$^{-/-}$ embryos co-expressed MASH1 (Fig. 5I,J).

To confirm that VZ precursors within the LGE of *Lifr*$^{-/-}$ embryos precociously differentiate, we analyzed the expression of the transcription factor DLX1 (Fig. 5K,L), which is normally largely restricted to the SVZ (Eisenstat et al., 1999), as well as the enzyme GAD65 (Fig. 5M,N), which is expressed exclusively in the SVZ and mantle zone (Stuhmer et al., 2002). DLX1 expression was clearly upregulated within the VZ of the *Lifr*$^{-/-}$ embryos (Fig. 5K,L). Furthermore, GAD65 expression, which normally was restricted to the SVZ and mantle zone (Fig. 5M), was ectopically expressed within the VZ of the *Lifr*$^{-/-}$ embryos (Fig. 5N; arrow indicates ectopic VZ expression in *Lifr*$^{-/-}$ embryos). Finally, we took advantage of the fact that VZ precursors undergo mitosis at the ventricular

surface and SVZ precursors undergo non-surface mitosis. VZ and SVZ precursors were quantified using pHH3, which marks cells in m-phase. The number of surface pHH3+ cells (VZ precursors) was reduced by 40% in the LGE of the *Lifr*$^{-/-}$ embryos compared with littermate *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ embryos (Fig. 5O,P; paired *t*-test ***P*=0.0005; *n*=4). However, the number of non-surface pHH3+ cells (SVZ precursors) was not significantly different between the *Lifr*$^{-/-}$ and *Lifr*$^{+/+}$/*Lifr*$^{+/-}$ littermates. Therefore, the ratio of SVZ precursors to VZ precursors was increased in the *Lifr*$^{-/-}$ embryos (similar results were attained for the cortex as shown in see Fig. S1I,J in the supplementary material). These results suggest that in the absence of CNTF/LIF/gp130 signaling, VZ precursors downregulate GSH2 expression and precociously differentiate to a more committed cell fate.

CNTF/LIF/gp130 signaling is sufficient to maintain a gradient of VZ precursor differentiation within the LGE

To understand better how CNTF/LIF/gp130 signaling might operate to maintain VZ precursors, we attempted to determine the likely source of CNTF and LIF acting upon the VZ during forebrain development. Immunostaining for CNTF expression in the forebrain of E14.5 embryos revealed robust expression within the choroid plexus, as well as at the base of the choroid plexus (Fig. 6A,B; arrow and arrowheads indicate staining). Immunoreactive cells were also observed within the mantle zone of the LGE (Fig. 6B); however, it is unlikely that these cells would greatly influence precursors within the VZ. LIF expression was also robust in the choroid plexus (Fig. 6C,D). We further confirmed the expression of both CNTF and LIF in the E14.5 choroid plexus by RT-PCR (Fig. 6E). The choroid plexus generates the cerebrospinal fluid (CSF) that bathes the interior of the embryonic and adult brain, and it is known to secrete many different growth factors and cytokines (Chodobski and Szmydynger-Chodobska, 2001; Dziegielewska et al., 2001; Gard et al., 2004; Speake et al., 2001; Sturrock, 1979). Our results suggest that the embryonic choroid plexus also generates CNTF and LIF. The presence of these molecules in the CSF would place them in an ideal position to influence precursors at the ventricular surface and establish a gradient of VZ precursor differentiation, as depicted in the model in Fig. 6F.

In order to test the role of the choroid plexus, and the sufficiency of CNTF/LIF/gp130-mediated signaling to maintain the VZ differentiation gradient, we performed the following experiment (see schematic in Fig. 7A). Cerebral hemispheres were dissected from E14.5 embryos and the medial cortex, septum and choroid plexus were removed. The hemispheres were then cultured as explants in either basal media, thereby removing the CSF as a source of CNTF and LIF, or basal media containing CNTF for 18.5 hours. Explants were first analyzed for changes in the level of proliferation within the LGE by staining for pHH3. The number of proliferating cells in the LGE germinal zone of CNTF-treated explants (235±27) was increased by 79% relative to the number observed in the explants cultured in basal media alone (Fig. 7B-D; 131±19; paired *t*-test ***P*=0.009; *n*=5). Therefore, CNTF treatment was sufficient to increase the number of proliferating cells in germinal zone of the LGE.

We next sought to determine whether this increased number

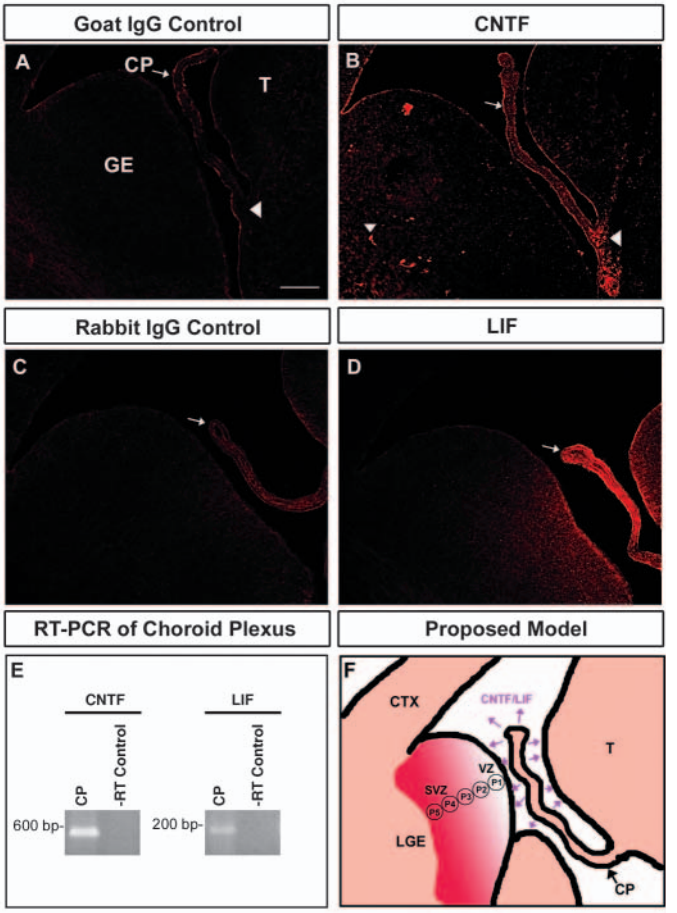


Fig. 6. The choroid plexus is the likely source of CNTF and LIF that acts upon VZ precursors in the LGE of the developing forebrain. (A,B) Immunostaining for CNTF in the E14.5 forebrain revealed strong expression by the choroid plexus (CP; arrow in B) and at the base of the choroid plexus (large arrowhead in B). Immunoreactive cells were also observed in the mantle zone (small arrowhead in B). Similar staining was not observed in control sections labeled with goat IgG (A). (C,D) LIF immunoreactivity was observed in the choroid plexus (arrow in D), but not other regions of the E14.5 ventral forebrain. Similar staining was not observed in control sections labeled with rabbit IgG (C). (E) The expression of both CNTF and LIF in the E14.5 choroid plexus was confirmed by RT-PCR analysis (*n*=3). (F) These findings suggest a model in which the choroid plexus secretes CNTF and LIF, establishing a gradient of VZ to SVZ precursor cell differentiation from P1 to P5 precursors within the LGE germinal zone. GE, ganglionic eminence; T, thalamus; LGE, lateral ganglionic eminence; CTX, cortex. Scale bar: 100 μm.

of proliferating cells in response to CNTF treatment might be related to maintenance of the graded differentiation of VZ precursors. The number of GSH2+ cells within the germinal zone of the CNTF-treated explants (1107±166) increased by 51% relative to the basal media-treated explants (729±81; paired *t*-test **P*=0.03; *n*=4), though this population appeared diminished in both conditions relative to the normal E14 LGE (Fig. 7E-G). MASH1 was clearly expressed in a gradient from VZ to SVZ in the normal E14.5 LGE (Fig. 7H). However, in the basal media-treated explants, the expression gradient was completely disrupted and MASH1 was expressed by VZ precursors abutting the ventricular surface (Fig. 7I; *n*=5),

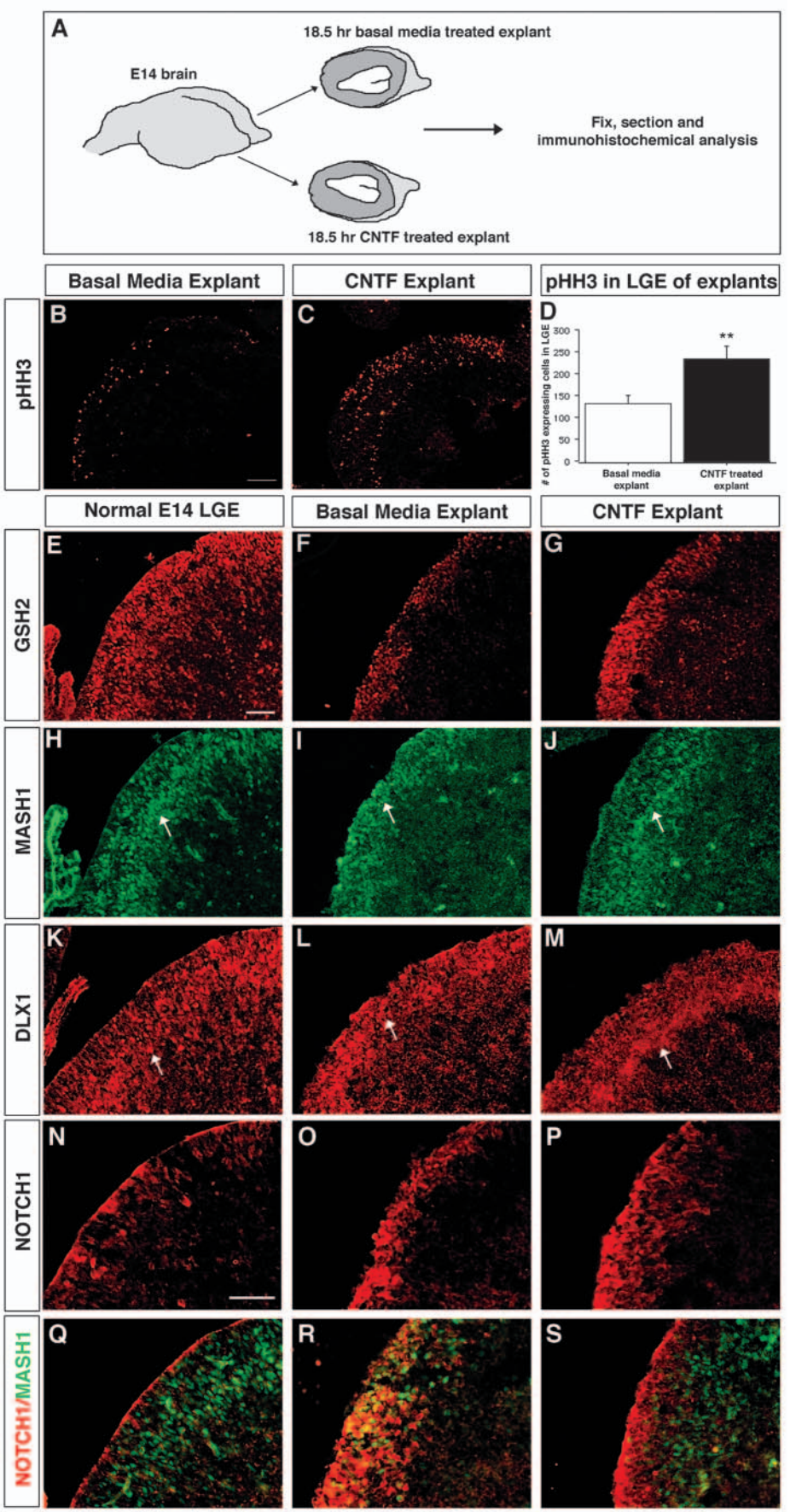


Fig. 7. CNTF is sufficient to promote the formation and maintenance of the VZ precursor differentiation gradient in the LGE. (A) Schematic overview of the E14.5 explant culture experiment (see Results for details). (B-D) The number of mitotically active precursors, indicated by pHH3 expression, in the LGE of CNTF-treated explants (C,D*;* 234±27) was significantly increased relative to basal media explants (B,D*;* 132±19; paired *t*-test ***P*=0.009; *n*=5). (E-G) GSH2 expression in normal E14.5 embryos (E) was expressed throughout the VZ. The number of GSH2+ cells was 51% higher in the VZ of the CNTF treated explants (G) relative to the basal media condition (F; paired *t*-test **P*=0.03; *n*=4). (H-J) A gradient of increasing MASH1 expression from VZ to SVZ was present in normal E14.5 embryos (H). This MASH1 gradient was absent in the basal media-treated explants, in which MASH1 expression was localized in the VZ region (I; *n*=5). The MASH1 VZ to SVZ gradient was restored in CNTF-treated explants (J; *n*=5). (K-M) A gradient of increasing DLX1 expression from VZ to SVZ was observed in normal E14.5 embryos (K). The DLX1 gradient was absent in basal media-treated explants (L; *n*=5) and this defect was rescued in the CNTF-treated explants (M; *n*=5). A NOTCH1 gradient was present at E14.5 where the highest expressing cells were at the ventricular surface (N). The gradient was lost and replaced with clustered NOTCH1+ cells in basal media explants (O), but restored in CNTF-treated explants (P; *n*=5). MASH1 expression was normally excluded from the highest NOTCH1-expressing cells in the gradient at the ventricular surface (Q). CNTF treatment maintained high NOTCH1- and MASH1-expressing cells as largely separate populations in opposing gradients (R,S). Arrows indicate regions of robust immunoreactivity. Scale bars: in B, 100 μm for B,C; in E, 50 μm for E-M; in N, 50 μm for N-S.

similar to the *Lifr*$^{-/-}$ phenotype. Remarkably, the addition of CNTF to the explant condition was sufficient to rescue this defect, as these explants clearly retained a VZ to SVZ gradient of MASH1 expression (Fig. 7J; *n*=5). Similarly, while DLX1 expression was present in a VZ to SVZ gradient in the normal E14.5 LGE (Fig. 7K), this expression pattern was disrupted in the basal media-treated explants in a manner similar to MASH1, whereby DLX+ cells were located near the ventricular surface (Fig. 7L). The gradient of DLX1 expression was also restored in the CNTF-treated explants (Fig. 7M).

Previously, we have reported that CNTF signaling can regulate the expression of NOTCH1 (Chojnacki et al., 2003) and a disruption of Notch signaling has been suggested to result in the precocious expression of SVZ characteristics by VZ precursors (Casarosa et al., 1999; Yun et al., 2002). Therefore, we examined the expression of the NOTCH1 receptor in our explant culture conditions. In the normal E14.5 LGE, NOTCH1 expression appeared in a gradient opposite to MASH1, being highest at the ventricular surface and decreasing in expression away from the surface (Fig. 7N). In the basal media explant condition NOTCH1 expression was sustained; however, the gradient was disrupted and replaced by a cluster of NOTCH1 immunoreactivity along the ventricular surface (Fig. 7O; *n*=5). The gradient was restored in the CNTF-treated explants (Fig. 7P; *n*=5). Double-labeling with NOTCH1 and MASH1 in the normal E14.5 LGE revealed that MASH1 expression was restricted to cells expressing low levels of NOTCH1 at superficial levels of the NOTCH1 gradient or by cells that did not express NOTCH1 in the SVZ (Fig. 7Q). Remarkably, in the basal media explants many of the clustered NOTCH1-expressing cells co-expressed MASH1 (Fig. 7R; *n*=5), while CNTF treatment maintained the graded separation of these two cell populations (Fig. 7S; *n*=5).

Discussion

In this study, we tested the hypothesis that CNTF/LIF/gp130 signaling plays a key role in the formation and maintenance of the unique laminar organization of the ventral forebrain germinal zone. Our results suggest that CNTF/LIF/gp130 receptor complex signaling regulates the self-renewal/expansion of a subpopulation of VZ precursors and that this process is both necessary and sufficient to maintain the gradient of VZ precursor differentiation in the LGE. These findings clarify how the ventral forebrain is able to establish the germinal layers that support the growth of the developing striatum. In addition, our findings reveal how the CNTF/LIF/gp130 receptor complex signaling may operate regionally to regulate precursor cells in the developing CNS.

CNTF/LIF/gp130 signaling maintains a gradient of VZ precursor differentiation

The signals that form and maintain the germinal layers of the forebrain are poorly understood. Building on the work of Yun et al. (Yun et al., 2002), we have proposed that a gradient of VZ precursor differentiation can be defined by GSH2, MASH1 and DLX1/2 expression. On this basis, precursor differentiation in the LGE can be considered to occur in three major steps: (1) VZ precursors are initially specified by expression of GSH1 and GSH2, which inhibit dorsal cell fate characteristics by repressing Pax6 (Corbin et al., 2000; Schuurmans and Guillemot, 2002; Toresson et al., 2000; Yun et al., 2003; Yun et al., 2001); (2) Precursors become initially committed to the neuronal lineage by the expression of MASH1 (Casarosa et al., 1999; Fode et al., 2000; Nieto et al., 2001; Torii et al., 1999; Yun et al., 2002); and (3) MASH1 initiates the expression of the Dlx genes (Fode et al., 2000), which induce glutamic acid decarboxylase expression and regulate the generation of later born neuronal populations (Anderson et al., 1997; Eisenstat et al., 1999; Stuhmer et al., 2002; Yun et al., 2002). Recent time-lapse studies of cortical precursors have demonstrated that the SVZ arises from asymmetric divisions of precursors in the VZ (Miyata et al., 2004; Noctor et al., 2004), and a similar relationship probably exists between the VZ and SVZ in the LGE (Halliday and Cepko, 1992). Therefore, the differentiation gradient we have described in the LGE (summarized in Fig. 4P) may represent differentiating daughter cells, asymmetrically born at the ventricular surface from P1 precursors, trafficking through the VZ out to the SVZ.

A role for Notch signaling and proneural gene expression in maintaining the separate VZ and SVZ layers has been suggested. NGN2 promotes the commitment of VZ daughter cells to the SVZ in the cortex (Miyata et al., 2004). It is likely that MASH1 plays a similar role in the ventral telencephalon, given its known role in promoting precursor commitment and that NGN2 is sufficient to rescue neurogenesis defects in the ventral telencephalon of MASH1 mutant mice (Parras et al., 2002). By contrast, NOTCH1 signaling has been suggested to play a role in maintaining the VZ population of the ventral forebrain (Casarosa et al., 1999; Yun et al., 2002). We have previously demonstrated that CNTF/LIF/gp130 signaling supports embryonic NSC self-renewal in vitro (Shimazaki et al., 2001) by promoting NOTCH1 and repressing MASH1 expression (Chojnacki et al., 2003). Our present results suggest that FGF-responsive P1 precursors located at the ventricular surface are the primary targets of CNTF/LIF/gp130 signaling in the developing LGE. First, components of the CNTF/LIF/gp130 signaling pathway are expressed primarily by GSH2+MASH1– precursors at the ventricular surface. Second, this pathway promotes FGF-responsive NSC self-renewal/expansion in vitro and promotes the P1 precursor fate in forebrain explant cultures. Finally, in the absence of this signaling pathway VZ precursors precociously differentiate and acquire characteristics of more differentiated cells at the expense of the GSH2+ cell population. CNTF/LIF/gp130 signaling thus appears to maintain an undifferentiated P1 precursor population in the VZ, which contributes to the normal formation of two germinal layers having distinctly different degrees of cellular commitment.

It is reasonable to suggest that CNTF/LIF/gp130 signaling may be acting upstream of NOTCH1, as we have suggested previously (Chojnacki et al., 2003), or in coordination with NOTCH1 signaling (Kamakura et al., 2004) to maintain the P1 precursor population. Our explant culture experiments reveal that CNTF/LIF/gp130 signaling maintains NOTCH1- and MASH1-expressing cells largely as separate populations. It may be that in the absence of this signaling pathway fewer NOTCH1 receptors are expressed per P1 precursor cell, resulting in decreased Notch signaling and the precocious expression of MASH1 and other more differentiated cellular characteristics. Alternatively, the activation of STAT3 by

CNTF/LIF/gp130 signaling may mediate important crosstalk between the Notch and STAT signaling pathways [as recently suggested by Kamakura et al. (Kamakura et al., 2004)], which is required to maintain the undifferentiated state of P1 precursors.

A role for the choroid plexus in the maintenance of a stem cell niche in the developing forebrain

Our results suggest that the primary source of CNTF and LIF acting upon VZ precursors may be the choroid plexus, a secretory structure that gives rise to the CSF that bathes the interior of the developing brain (Dziegielewska et al., 2001; Speake et al., 2001). The choroid plexus is known to secrete many different growth factors and cytokines into the CSF for delivery to the brain tissue (Chodobski and Szmydynger-Chodobska, 2001), appears as early as E11 in the forebrain (Sturrock, 1979), and is believed to produce CSF throughout development (Dziegielewska et al., 2001). The CSF of the developing embryonic forebrain is ideally positioned to influence precursor cells within the germinal zone such that cells closest to ventricular surface would be closest to the source of CNTF/LIF, while those positioned farther away from this source would be in an environment increasingly permissive for differentiation (see Fig. 6F). In support of this model, our results demonstrate that the removal of both the choroid plexus and the CSF in the basal media explant condition results in the disruption of the VZ precursor differentiation gradient. This defect was entirely rescued by the addition of CNTF to the media bathing the explant and concurrently increased the number of proliferating cells within the germinal zone.

It has been suggested that the adult choroid plexus does not express CNTF and LIF (Gard et al., 2004), which may be significant given that only the SVZ (and not the VZ) is maintained into adulthood. Perhaps the production of CNTF and LIF by the choroid plexus is required to maintain levels of these molecules sufficient for the formation of both the VZ and SVZ germinal layers. This is an area for future investigation that may be important for strategies aimed at the intrinsic repair of the forebrain.

Distinct roles for CNTF/LIF/gp130 signaling in the ventral forebrain and spinal cord during development

Virtually all previously identified pathways that promote NSC self-renewal/expansion are known to function in both the forebrain and spinal cord. For example, FGF signaling promotes NSC self-renewal/expansion in both the forebrain (Martens et al., 2000; Raballo et al., 2000; Tropepe et al., 1999; Vaccarino et al., 1999) and spinal cord (Del Corral and Storey, 2004; Represa et al., 2001). Similarly, Notch signaling promotes NSC self-renewal/expansion in both forebrain (Chojnacki et al., 2003; Hitoshi et al., 2002; Schuurmans and Guillemot, 2002; Yun et al., 2002) and spinal cord regions (Appel et al., 2001; le Roux et al., 2003; Lindsell et al., 1996), as do the membrane-associated proteins Numb and Numblike (Petersen et al., 2002; Petersen et al., 2004). Our review of the literature suggests that a specific signaling pathway that promotes VZ precursor self-renewal/expansion within the forebrain, but not the spinal cord, has not been previously identified.

We found that the components of the CNTF/LIF/gp130 receptor complex were robustly expressed by VZ precursors in the developing LGE, contrasting with CNTFRα and LIFRβ expression in the spinal cord (Fig. 2) (C.G. and S.W., unpublished), which was largely restricted to the mantle zone. A similar pattern of CNTFRα expression was previously observed in the developing rat spinal cord (MacLennan et al., 1996). This suggests that CNTF/LIF/gp130 signaling might have a different role in the spinal cord compared with the forebrain. Indeed, CNTF and LIF inhibited spinal cord NSC self-renewal/expansion in vitro, instead promoting the generation/survival of neurons from FGF-responsive spinal cord NSCs. Furthermore, we observed no impairment in the growth or number of proliferating precursors in the developing spinal cord of *Lifr*$^{-/-}$ mice and the addition of CNTF to spinal cord explant cultures decreased the number of proliferating precursors in the spinal cord VZ (see Fig. S3A,B in the supplementary material). Taken together, these findings suggest that CNTF/LIF/gp130 receptor signaling specifically contributes to growth of the forebrain (but not the spinal cord) during development, by contributing to the formation and maintenance of the ventral forebrain VZ.

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Supplementary material

Supplementary material for this article is available at
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