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The Role of gp130 in Cerebral Cortical Development: *In Vivo* Functional Analysis in a Mouse *Exo Utero* System

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The role of gp130 in cerebral cortical histogenesis remains unknown. Mice lacking gp130 showed a hypoplastic cortical plate and decreased incorporation of 5-bromo-2′-deoxyuridine (BrdU) in progenitor cells of the developing cerebrum. In contrast, injection of leukemia inhibitory factor (LIF), a gp130 ligand, into the lateral cerebral ventricle of wild-type embryos *exo utero* induced hyperplasia of the cerebral cortex and increased the incorporation of BrdU in progenitor cells. Furthermore, chronologically controlled injection of LIF followed or preceded by BrdU revealed that gp130-mediated signals promote the progenitor cells to reenter the stem cell cycle without affecting the duration of cell cycle and enhance the migration of postmitotic neurons in the developing cerebrum.

Key words: gp130; leukemia inhibitory factor; cerebral cortex; neurogenesis; exo utero *development; knock-out mouse*

gp130 is a common signal transducer acting in association with ligand-specific receptors for the interleukin-6 (IL-6) family of cytokines, including IL-6, IL-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin-M (OSM), and cardiotrophin-1 (CT-1). Signals of IL-6 and IL-11 are transduced via a homodimeric complex of gp130, and those of LIF, CNTF, OSM, and CT-1 are transduced via a heterodimeric complex of gp130 and the LIF receptor (LIFR) (Taga et al., 1989; Hibi et al., 1990; Yin et al., 1993; Kishimoto et al., 1994; Pennica et al., 1995; Taga and Kishimoto 1997). gp130 thus acts as a common transducer and causes some redundancy in the functions of these cytokines.

gp130-mediated signals play roles in a variety of cells (Taga et al., 1992; Kishimoto et al., 1994; Taga and Kishimoto, 1997). LIF and CNTF contribute to the survival and/or differentiation of neurons and glial cells *in vitro* (Ernsberger et al., 1989; Murphy et al., 1991, 1993; Ip et al., 1992; Mayer et al., 1994). In addition, their involvement in the survival of developing neurons in the brainstem and spinal cord has been suggested in knock-out mice lacking the LIFR (Li et al., 1995; Ware et al., 1995) or the CNTF receptor (CNTFR) (DeChiara et al., 1995). Disruption of *gp130* resulted in prenatal death with disorders of myocardial and hematological development. Development of cerebral cortex could not be examined because prenatal death occurred before neural histogenesis in the cerebral cortex of gp130 (−/−) in the genetic background of a mixture of 129 and C57BL/6 (Yoshida et al., 1996). However, by generating mice that harbor a cardiac ventricular restricted knock-out of gp130 via Cre-*loxP*-mediated recombination, it was revealed that conditional mutant mice had normal cardiac structure and function and that the cardiac defect seen in conventional gp130 (−/−) embryos likely represents a secondary effect of gp130 deficiency (Hirota et al., 1999). Recently, some gp130 null mutants have managed to be survived until birth after a change in the genetic background from a mixture of 129 and C57BL/6 (Yoshida et al., 1996) to ICR (Kawasaki et al., 1997). Using this strain, we reported that the survival of motor neurons in the brainstem and spinal cord and sensory neurons in the dorsal root ganglia (DRG), as well as the differentiation of astrocytes, were impaired in gp130-deficient mice (Nakashima et al., 1999a), which is consistent with findings in knock-out mice lacking the LIFR or CNTFR. In addition, we showed that gp130, LIFR, and LIF are expressed in cultured progenitor cells from embryonic day 14.5 (E14.5) wild-type cerebrum, and gp130-mediated signal transduction is stimulated by exogenously added LIF *in vitro* (Nakashima et al., 1999a,b). However, the exact function of gp130 in the neurogenesis of the developing cerebral cortex remains unclear.

In the present study, we observed histologically that the cerebral cortex is hypoplastic and that 5-bromo-2′-deoxyuridine (BrdU) incorporation decreased in the gp130-deficient embryos. We then analyzed the role of gp130-mediated signals in cerebral cortical development by injecting LIF as an exogenous ligand into the embryonic cerebral ventricle and observed effects on progenitor cells in the ventricular zone (VZ), as well as on postmitotic neurons in the cortical plate (CP). The experiment was based on the mouse *exo utero* development system (Muneoka et al., 1986; Hatta et al., 1994; Zhang et al., 1998). This experimental system can set "gain of function" conditions in a spatiotemporally controlled and readily modifiable manner, which is difficult to attain using the conventional transgenic or even the latest Cre-*loxP* strategy (Hirota et al., 1999; Li et al., 2000). Thus, we could functionally dissect and analyze the effects of LIF/gp130-mediated signals on the developing cerebrum.

MATERIALS AND METHODS

Animals

Jcl:ICR female mice aged 8–20 weeks old (CLEA Japan, Tokyo, Japan) were used. A female mouse was housed with a potent male overnight, and

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the day a vaginal plug was recognized was designated E0. For *exo utero* surgery, dams were anesthetized with pentobarbital. All treatments involving experimental animals were performed in accordance with the guidelines for animal experiments of Shimane Medical University (Izumo, Japan). The gp130-deficient mice used in this study had the genetic background of ICR, and some of the null embryos survived until birth: the original strain, whose genetic background is a mixture of 129 and C57BL/6, is embryonic lethal (Yoshida et al., 1996; Kawasaki et al., 1997).

Exo utero *surgery and microinjection of LIF*

On E14, *exo utero* surgery was performed as described previously (Hatta et al., 1994). Briefly, after making a longitudinal incision along the myometrium on the opposite side of the placenta, we injected 1 μl of LIF solution into the left cerebral ventricle of the embryos with a fine glass needle through the embryonic membrane. Recombinant murine LIF (R & D Systems, Minneapolis, MN) was dissolved at 10 ng/μl in sterilized saline with 0.1% bovine serum albumin as a carrier and injected into embryos in the left uterine horn, whereas control embryos in the right horn of the same dam were injected with the vehicle. The dose of LIF (10 ng/embryo, 40–50 μg/kg body weight) was determined based on a pilot study and a previous report (Akita et al., 1996). The embryos together with the uterus were placed back in the abdominal cavity of the dams. On E14, E15, E16, and E18, dams were killed by an overdose of diethyl ether, and the embryos were obtained.

Semiquantitative study on cerebral cortical neurons

For the morphometric study of the number of cortical neurons in gp130 (−/−) embryos on E15 and LIF-injected embryos on E18, we applied a nonstereological but semiquantitative analysis (Satriotomo et al., 2000) with a random and systematic sampling. In brief, the left cerebral cortex was divided into three parts: anterior, intermediate, and posterior parts. The initial section of the anterior part was determined as the most rostral one in which the pallidal fork of the lateral ventricle clearly divided the elevated ganglionic eminence (GE) into medial (pallidum) and lateral (striatum) parts on E15 and the anterior commissure crossing the midline on E18. The initial section of the intermediate part was determined as the most rostral one in which the interventricular foramen of Monroe appeared on both E15 and E18. The initial section of the posterior segment was determined as the caudal end of GE, at which the elevation of GE into the lateral ventricle was flattened. Each initial section was numbered as 1, with all subsequent sections being numbered sequentially rostral to caudal. In each embryo, a number between 1 and 5 was selected at random to determine the first section. Every third section for E15 brains and fifth section for E18 was chosen for semiquantitative analysis (for example, sections 2, 5, 8, etc. for E15 were selected and sections 2, 7, 12, etc. for E18). Five and six sections at E15 and E18, respectively, were chosen from each of the anterior, intermediate, and posterior parts and used for the subsequent analysis. Photographs of the left hemisphere were taken with a light microscope (model BX50; Olympus Optical, Tokyo, Japan) at 1–4× magnification depending on the size of the area, using a CCD camera (model CS520MD; Olympus Optical). The boundary of the cerebral cortex was drawn on the captured images (Vaccarino et al., 1999), and the area of the left cerebral cortex per section was measured using the Scion Image software (Beta 4.0.2; Scion, Frederick, MD). The cell density of the cerebral cortex in each section was estimated as follows. Several regions of the left cerebral cortex, the margins of which should overlap, were captured at 20 or 40× magnification. The transformed images were used to reconstruct the whole left cortex on the section using Adobe Photoshop (version 5.0; Adobe Systems, San Jose, CA). Then the grid was superimposed on the reconstructed images using the grid function of the software. The distance between adjacent cross points of the grid lines was 25 μm, so that the area of a square (counting box) determined by the grid line was $25 \times 25\ \mu m^2$. The counting box located in the most medial and most dorsal region was numbered 1. The other boxes were then numbered serially medial to lateral in the dorsoventral dimension. Every 15th box was selected for cell counting, and ~20 boxes per section on E15 and 50 boxes on E18 were used (in total, ~300 boxes at E15 and 750 boxes at E18 per brain). The average number of cells in a counting box was obtained, and the estimated density of cells per $1\ \mu m^2$ was calculated. The total number of cells was calculated by multiplying the mean cell density by area in each section, and then the average of the total number of cells per section was calculated in the anterior, central, and posterior parts in the cerebral cortex as a representative value for each part. Three embryos were used in the LIF-injected and control groups on E18, and three and six embryos were used in gp130 (−/−) and (+/+) on E15, respectively.

BrdU injection schedules

Dams were injected intraperitoneally with 50 mg/kg body weight of BrdU (Sigma, St. Louis, MO) in distilled water. The schedules of BrdU injections are as follows.

The effects on the proliferative activity of progenitor cells in the VZ. Progenitor cells at S phase in the VZ of gp130 (−/−) [(−/−), $n = 3$; (+/+), $n = 6$)], and LIF-injected embryos were labeled with an intraperitoneal injection of BrdU given to the dams on E15 (24 hr after LIF injection) (see Fig. 3*c*) (LIF, $n = 3$; control, $n = 3$), and embryos were removed 2 hr after BrdU injection.

The effects on the nuclear translocation of progenitor cells. BrdU was injected 2 hr before LIF, and embryos were killed 6, 10, and 14 hr later on E14 (see Fig. 4*j*). In the *exo utero* system on E14, the inner subset of the labeled cell population (distributed around bin 4) reached the ventricular surface (bin 1) ~6 hr after BrdU injection. Most of the labeled cells accumulated there (bin 1–2) at 10 hr after BrdU injection, exited M phase, and moved upward ~14 hr after BrdU injection (Hatta et al., 1994). Three embryos were used for each time point in the LIF-injected and control groups.

The effects on the migration of the postmitotic neurons. BrdU was injected 12 hr before LIF on E14 (see Fig. 5*a*). When LIF was injected 12 hr after BrdU on E14, the labeled cell population had finished M phase and was migrating upward from the ventricular surface to the cerebral cortex (Fig. 4*h*,*i*) (for details, see Results). Thus, the initial number of labeled progenitor cells was the same between the LIF-injected and control groups. At 60 hr after BrdU injection (48 hr after LIF injection), BrdU-labeled cells appeared in the CP. In the control embryos, labeled neurons derived from the first cell division after BrdU injection had entered the CP but were still migrating to the pial surface across the CP, as was determined in a pilot study performed *exo utero*. In the present time range, whereas the first generation of labeled daughter cells reached the CP, the later (second or third) generation of labeled cells had not yet reached the CP. Therefore, the number of BrdU-labeled cells in the CP was equal between LIF-injected and control groups (for details, see Results), and the number and distribution of BrdU-labeled cells along the ventrodorsal dimension in the CP should reflect the migration of first generation of postmitotic neurons. They were examined semiquantitatively as described below in three embryos each for the LIF-injected and control groups.

Tissue preparation

Whole embryos on E11–E13 were fixed by immersion for 6–12 hr at 4°C in a mixture of 4% formaldehyde and 70% methanol. On E14 and E15, embryos were decapitated and fixed in the manner described above. On E16 and E18, the brains were dissected out in physiological saline at 4°C under a dissecting microscope and fixed as described above. Specimens were embedded in paraffin and sectioned at 5 μm coronally or sagittally. The brains of gp130 (−/−) and (+/+) embryos on E15 and of LIF-treated and control embryos on E18 were sectioned in the coronal plane. In the first several sections through the olfactory bulb and the anterior part of the cerebral ventricle in the coronal plane, the cutting angle was adjusted right–left symmetrically and as perpendicular as possible by adjusting the microtome. Then serial sections were prepared throughout the cerebrum and stained with hematoxylin–eosin (HE) for general histology and cresyl violet for the counting of cerebral cortical neurons. For BrdU analysis, serial paramedian sagittal sections of the left cerebrum were prepared at 5 μm.

Semiquantitative study on BrdU-labeled cells

The number and distribution of BrdU-labeled cells were analyzed semiquantitatively based on the bin method used by Takahashi et al. (1993, 1995, 1996a,b) with some modifications. The paramedian sagittal section in which the olfactory bulb had disappeared was designated as the first section, and subsequent sections were numbered serially in the mediolateral dimension. The first and every third sections were selected, and five sections per brain were examined. The distribution of the BrdU-labeled cells was analyzed in the sector, which was settled in the dorsomedial cerebral wall, corresponding to the intermediate cortex in the coronal plane described above. The region of analysis was 360 μm in its rostrocaudal dimension and was composed of 18 sectors, 20 μm in width. Sectors were numbered in the rostrocaudal dimension, and every second sector (nine sectors per section) was selected. Sectors were then divided

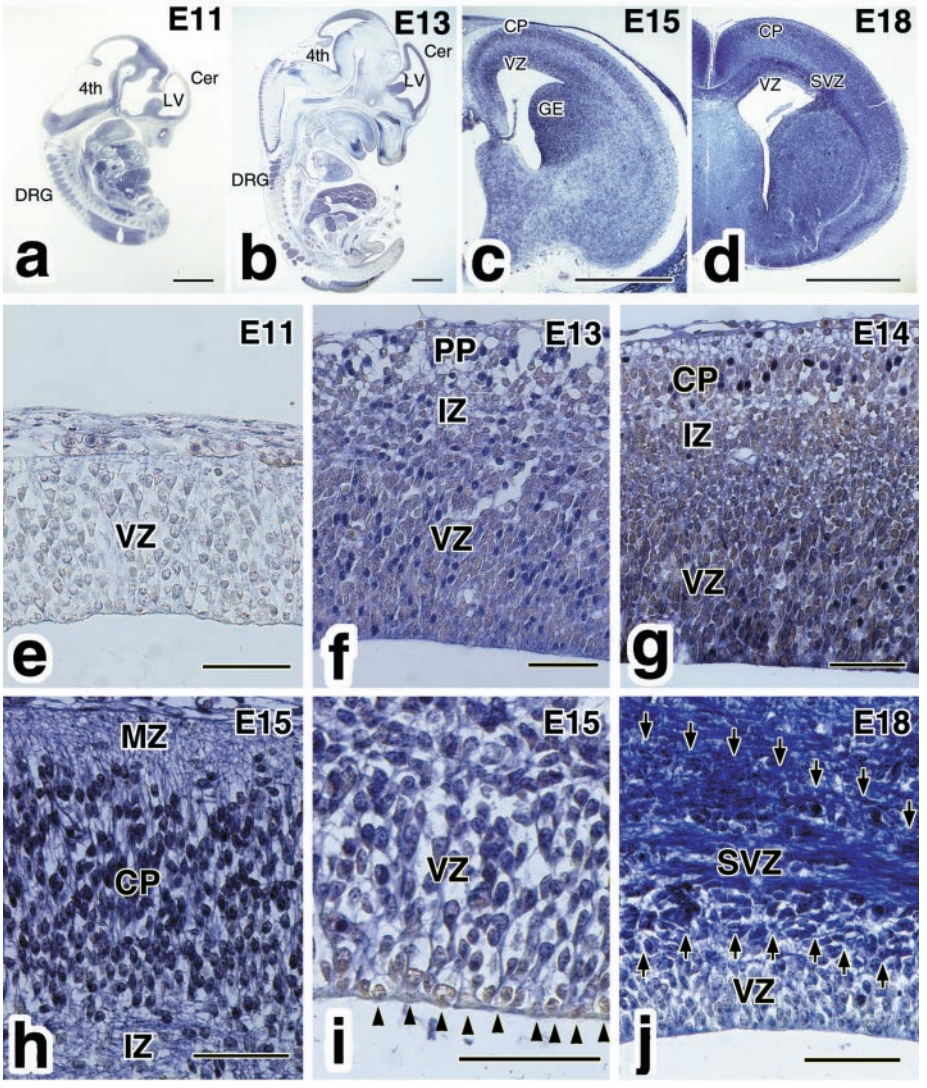


Figure 1. Expression of gp130 in the developing cerebral wall. On E11, the staining intensity is faint in the cerebral wall (*Cer*) (*a*, *e*). On E13, gp130 staining is generally positive in the cerebral wall (*b*). Some heavily stained cells appear among the many lightly stained cells in the VZ, IZ, and PP (*f*). On E14, the staining of the progenitor cells became more intense in the VZ, and the number of heavily stained neurons increased in the CP (*g*). On E15, cells and fibers in the CP, IZ, and VZ are stained heavily (*c*, *h*, *i*). Note that the mitotic cells on the ventricular surface are not stained for gp130 (*arrowheads* in *i*). On E18, gp130 staining is strongly positive in cells and fibers in the CP and SVZ (*d*, *arrows* in *j*); in contrast, the staining intensity was decreased in the VZ (*j*). Negative controls were incubated with normal rabbit IgG and showed no staining (data not shown). *MZ*, Marginal zone; *LV*, lateral ventricle; *fourth*, fourth ventricle. Scale bars: *a–d*, 1 mm; *e–j*, 100 μm.

into bins, 20 μm in height in the ventrodorsal dimension. The bins were numbered 1, 2, 3, and so on, from the ventricular surface outward for analyzing the number and distribution of BrdU-labeled cells in the VZ and from the pial surface inward for those in the CP (Takahashi et al., 1993, 1995, 1996a,b). The number of BrdU-positive cells per bin per sector was obtained in each experiment and was shown by a histogram.

Immunohistochemistry

Sections were incubated with 10% normal goat serum (Dako, Glostrup, Denmark) in Tris-buffered saline (TBS) (100 mM Tris-Cl and 150 mM NaCl, pH 7.5) for 30 min at room temperature, the rabbit polyclonal anti-human gp130 antibody (1:500; 8.5 μg/ml; Upstate Biotechnology, Lake Placid, NY) in TBS at 4°C overnight, and the alkaline phosphatase-conjugated anti-rabbit IgG (1:500; Vector Laboratories, Burlingame, CA) in TBS for 30 min at room temperature. Sections were visualized with a mixture of 4-nitroblue tetrazolium chloride (Boehringer Mannheim, Mannheim, Germany) and 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer Mannheim) containing 1 mM levamisole (Sigma) as an inhibitor for nonspecific phosphatase reactions. Non-immunized normal rabbit IgG (8.5 μg/ml; Zymed, San Francisco, CA) was used as an immunohistochemical control for gp130.

To detect the BrdU-labeled cells, sections were digested with 0.1% trypsin in 0.1% $CaCl_2$ for 8–10 min at 37°C, denatured with 2N HCl for 30 min, and neutralized with 0.1 M borax-borate buffer, pH 8.8, for 5 min at room temperature. Sections were pretreated with a mixture of 0.3% hydrogen peroxide and 99% methanol for 15 min. They were incubated in the 10% normal goat serum for 30 min, in anti-BrdU antibody (1: 50) (Dako) overnight at 4°C, and then in Dako Envision polymer (Dako, Carpinteria, CA) for 1 hr at room temperature. Sections were visualized with 0.05% 3,3′-diaminobenzidine-tetrahydrochloride (Wako, Osaka, Japan) and 0.01% hydrogen peroxide. Adjacent sections were incubated in TBS without primary antibody as a negative control of BrdU immunostaining.

Statistical analysis

All data in this study are presented as the mean ± SD. An ANOVA was used for comparisons of groups and a *post hoc* pairwise comparison (Fisher's *post hoc* test) for each factor between groups. The difference in the total number of cells per sector between groups was analyzed by Student's *t* test.

RESULTS

Expression of gp130 in the developing cerebrum of the wild-type embryo

On E11, the expression of gp130 in the cerebral wall was faint compared with that in the other parts of the CNS and DRG (Fig. 1*a*,*e*) in the wild-type embryo. On E13, gp130 was expressed throughout the cerebral wall (Fig. 1*b*). Most cells showed staining of moderate intensity, but some cells in the VZ, intermediate zone (IZ), and preplate (PP) were heavily stained (Fig. 1*f*). On E14, the staining of the progenitor cells became more intense in the VZ, and the number of heavily stained neurons increased in the CP (Fig. 1*g*). On E15, the number of neurons in the CP and

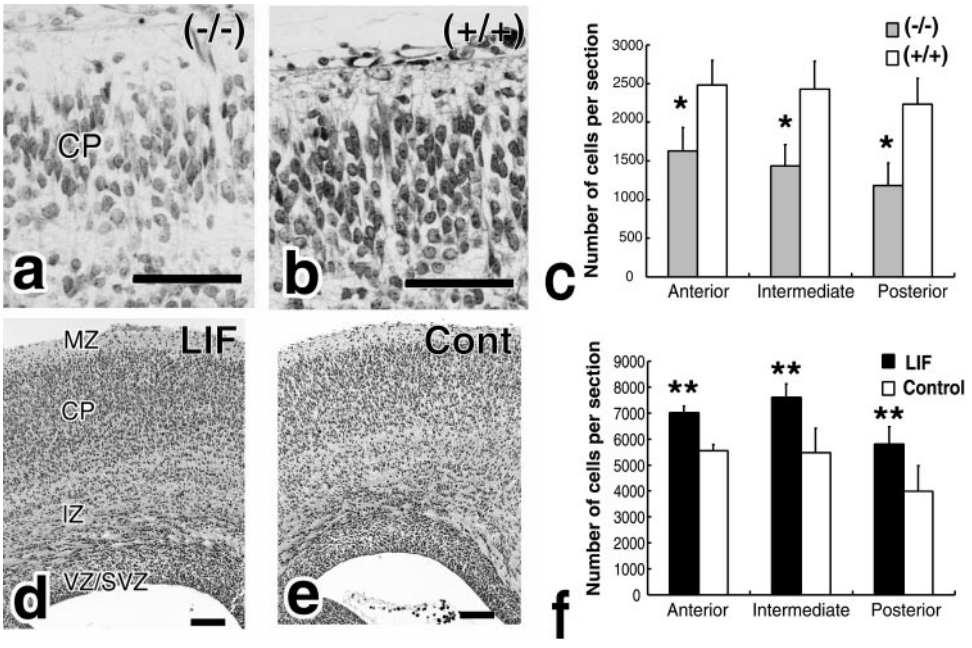


Figure 2. Histological changes of the cortical plate in LIF-injected or gp130-deficient embryos. *a–c*, The cortical plate in gp130 (−/−) and (+/+) embryos on E15. Coronal sections of the dorsal region of the left anterior cortex in gp130 (−/−) (*a*) and (+/+) (*b*) (cresyl violet staining). *c*, Semiquantitative analysis revealed that the gp130 (−/−) embryos differed significantly from gp130 (+/+) embryos in the number of cells in the cerebral cortex ($p < 0.001$; $F = 52.9$; ANOVA). The number of cells per section in the anterior, intermediate, and posterior parts of the cortex was significantly decreased by disruption of gp130 ($^*p < 0.01$; Fisher's *post hoc* test). *d–f*, LIF-injected and control brains on E18. The cellularity of the CP is greater in the LIF group (*d*) than in the control group (*e*) (anterior part of the cerebral cortex in the coronal plane on E18, HE staining). *f*, Semiquantitative analysis revealed that LIF-treated animals differed significantly from controls in the number of cells in the cerebral cortex per section ($p < 0.001$; $F = 32.7$; ANOVA). The number of cells per section in the anterior, intermediate, and posterior parts of the cortex was significantly increased by the LIF-treatment ($^{**}p < 0.05$; Fisher's *post hoc* test). *MZ* , Marginal zone. Scale bars, 100 μm.

progenitor cells that were heavily stained increased, and most cells were strongly positive for gp130 (Fig. 1*c*,*h*,*i*). Mitotic cells on the ventricular surface were not stained for gp130 (Fig. 1*i*, *arrowheads*). On E18, cells in the CP and subventricular zone (SVZ) and fibers in the SVZ were heavily stained, whereas the staining intensity was decreased in the VZ (Fig. 1*d*,*j*). Negative controls were incubated with non-immunized rabbit IgG and showed no staining (data not shown).

Characteristic phenotypes in the cerebrum of gp130 (−/−) and LIF-injected embryos

Cellularity in the CP was characteristically lower in gp130 (−/−) than gp130 (+/+) embryos on E15 (Fig. 2, compare *a*, *b*). Semiquantitative analysis revealed that gp130 (−/−) embryos differed significantly from gp130 (+/+) embryos in the number of cortical neurons per section in the left hemisphere (ANOVA; $p < 0.001$; $F = 52.9$). *Post hoc* test (Fisher's *post hoc* test) revealed significant effects of genotype on the number of neurons per section in the anterior, intermediate, and posterior parts of the cerebral cortex in the left hemisphere ($p < 0.01$) (Fig. 2*c*). In contrast, histological observation revealed that wild-type embryos injected with LIF on E14 showed higher cellularity in the CP than controls on E18 (Fig. 2, compare *d*, *e*). Semiquantitative analysis revealed that LIF-injected embryos differed significantly from controls in the number of cortical neurons per section in the left hemisphere (ANOVA; $p < 0.001$; $F = 32.7$). *Post hoc* test (Fisher's *post hoc* test) revealed significant effects of LIF on the number of neurons per section in the anterior, intermediate, and posterior parts of the cortex in the left hemisphere ($p < 0.05$) (Fig. 2*f*). Glial fibrillary acidic protein-immunostaining for astrocytes was negative in the CP on E15 and E16 (data not shown). Apoptotic cells were rare in the cerebral wall and indistinguishable between gp130 (−/−) and (+/+) embryos or between LIF-injected and control groups stained by HE or cresyl violet or by the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling method (Gavrieli et al., 1992) (data not shown).

Proliferation of progenitor cells in the cerebral ventricular zone of gp130 (−/−) and LIF-injected embryos

On E15, gp130 (−/−) embryos differed significantly from gp130 (+/+) embryos in the number of BrdU-positive cells per sector (ANOVA; $p < 0.001$; $F = 33.5$) (Fig. 3*a*). The total number of BrdU-positive cells per sector was significantly decreased by the gp130 disruption [(+/+), 12.6 ± 3.56; (−/−), 7.0 ± 1.21; $p < 0.05$; Student's *t* test], indicating a reduction in the proliferation of progenitor cells on disruption of gp130. We then examined the changes of BrdU incorporation in progenitor cells after LIF injection into the cerebral ventricle of wild-type embryos. BrdU was injected into dams on E15, 24 hr after LIF treatment (Fig. 3*c*), and embryos were analyzed 2 hr after the injection. The LIF-injected group differed significantly from the control in the number of BrdU-positive cells per sector (ANOVA; $p < 0.001$; $F = 61.0$) (Fig. 3*b*). The total number of labeled cells per sector was significantly increased by LIF injection (LIF, 20.1 ± 3.71; control, 10.0 ± 0.71; $p < 0.01$; Student's *t* test), indicating that the LIF induced progenitor cells to reenter the cell cycle, and then, as a result, the number of progenitor cells at S phase increased.

Effects of LIF on the elevator movement of progenitor cells

To clarify the mechanisms underlying the changes described above, we further analyzed the effects of LIF on the progenitor cell kinetics known as elevator movement. Elevator movement and associated production of neurons is divided into the following phases: (1) downward nuclear translocation to the ventricular surface (S to G_2/M phase and mitosis), (2) upward nuclear translocation (G_1 phase) of a daughter cell, which reenters the stem cell cycle as described above and also later in the next section, and (3) migration of another daughter cell, which exits the stem cell cycle (G_0), up to the CP as a postmitotic neuron (Fujita, 1963; Jakobson, 1991).

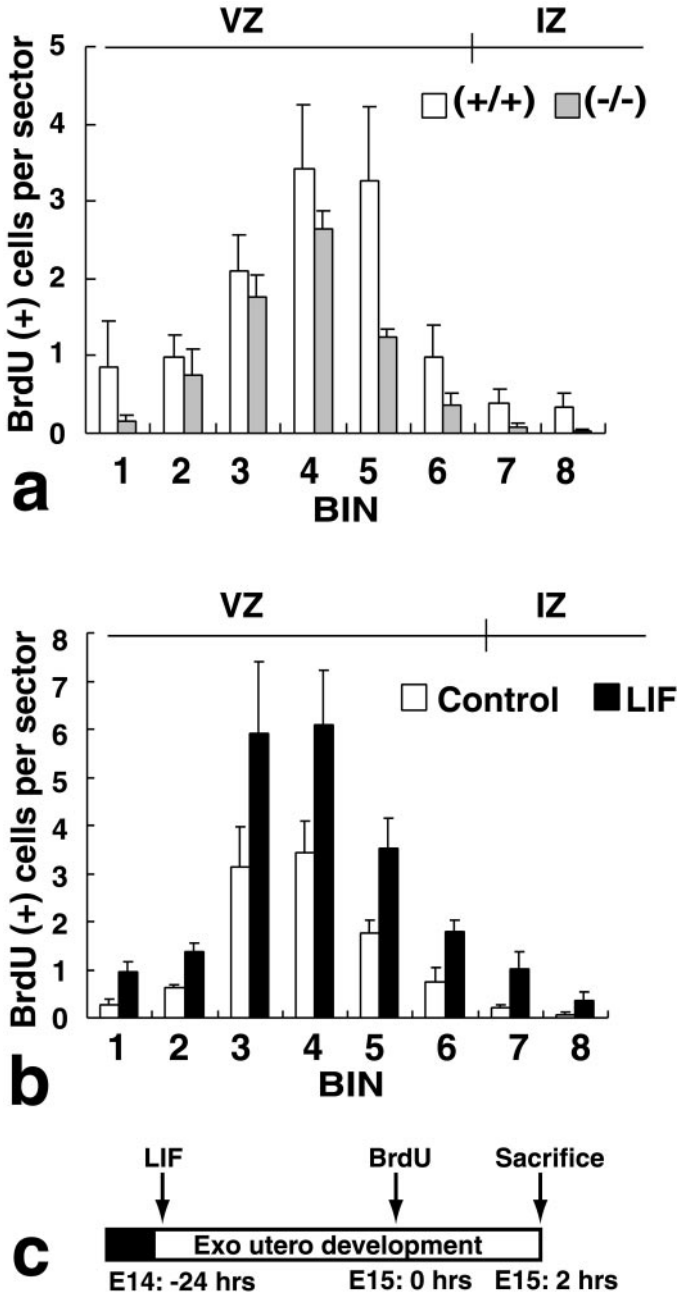


Figure 3. Mitotic labeling in the ventricular zone after gp130 deletion and activation. *a*, Progenitor cells were labeled by single injection of 50 mg/kg BrdU in gp130 (−/−) embryos and gp130 (+/+) littermates on E15. Embryos were analyzed 2 hr after the injection. Semiquantitative analysis revealed that gp130 (−/−) differed significantly from gp130 (+/+) in the number of labeled cells ($p < 0.001$; $F = 33.5$; ANOVA). Total number of BrdU-positive cells per sector was significantly decreased in gp130 (−/−) compared with gp130 (+/+) [(−/−), 7.0 ± 1.21; (+/+), 12.6 ± 3.56; $p < 0.05$; Student's *t* test]. *b*, Progenitor cells were labeled by single injection of 50 mg/kg BrdU in LIF-injected and control embryos on E15. BrdU was injected into dams 24 hr after LIF, and embryos were analyzed at 2 hr after BrdU injection, as shown in *c*. Semiquantitative analysis revealed that the LIF-injected embryos differed significantly from the controls in the number of labeled cells ($p < 0.001$; $F = 61.0$; ANOVA). The total number of BrdU-positive cells per sector was significantly increased in the LIF-injected group compared with the control (LIF, 20.1 ± 3.71; control, 10.0 ± 0.71; $p < 0.01$; Student's *t* test).

We first analyzed the effects of LIF on the downward nuclear translocation of progenitor cells (duration of S to G_2/M phase). We injected BrdU into dams 2 hr before LIF to eliminate possible effects of LIF on the labeling efficiency and then evaluated the number and localization of BrdU-positive nuclei of progenitor cells at 6, 10, and 14 hr after BrdU injection (Fig. 4*j*). The interpeak of the BrdU-positive cell distribution in both LIF-treated and control groups moved from bin 4 (60–80 μm from the ventricular surface) to bin 1 (0–20 μm from the ventricular surface) at 6 hr (Fig. 4*a*,*d*,*g*), and a majority of the labeled cells had accumulated on the ventricular surface (bin 1) at 10 hr after BrdU injection (Fig. 4*b*,*e*,*h*). At 14 hr after BrdU injection, most of the labeled cells were distributed through bin 1 to bin 3, and the interpeak of the distribution was in bin 2 for both groups. When observed closely, the inner half of bin 1 was found to be occupied by unlabeled cells, indicating that the labeled cells had already passed through M phase and changed position to the deeper area of the VZ at 14 hr after BrdU injection in both groups (Fig. 4*c*,*f*,*i*). Semiquantitative analysis revealed that there were no significant interactions between treatment (LIF injection or control) and bin in the number of labeled cells per sector or no significant differences in the number of BrdU-positive cells for any bin between LIF-injected and control groups (Fig. 4*g–i*).

Then the total number of BrdU-positive cells per sector was plotted for each time point (Fig. 5*a*). The number increased at 2 hr after BrdU injection and was twice the initial value at 14 hr in the LIF-injected and control groups (not significantly different between the groups by ANOVA). The percentage of increased ratio in BrdU-positive cells relative to the initial value (2 hr after BrdU) was calculated for each time point (Fig. 5*b*). There were no significant differences between LIF-injected and control embryos in the percentage of increase in BrdU-positive cells and no significant interactions between treatment and time point (ANOVA). When the labeled population had finished mitosis, the number of labeled cells was twice the initial value (represented as 100%). The labeled population finished mitosis at 10.5 and 10.7 hr in the LIF-injected group and control group, respectively, as estimated from a simple regression (LIF, $r^2 = 0.95$; control, $r^2 = 0.95$) (Fig. 5*b*). The percentage of labeled nuclei at M phase reached 100% at 6 hr after BrdU injection in both LIF-injected and control groups. The duration of G_2 + M + 1/2S in both groups can, therefore, be estimated as ~6 hr (Jakobson, 1991), which is in the range of that established by Takahashi et al. (1993, 1995).

These findings suggest that LIF does not affect the downward nuclear translocation or duration of the 1/2S–G_2–M phases in progenitor cells. The upward nuclear translocation could not be examined because of a lack of appropriate markers for differentiating between overlapping BrdU-positive progenitor cells and postmitotic neurons in the VZ.

The effect of LIF on the postmitotic migration of the BrdU-positive cells

We further examined the effects of LIF on the postmitotic migration of the BrdU-positive cells. Dams were injected with BrdU on E13, at 12 hr later on E14, they were injected with LIF, and then the distribution of BrdU-positive neurons was analyzed in the cerebral wall of the wild-type embryos on E16 (at 60 hr after BrdU injection, 48 hr after LIF injection) (Fig. 6*a*). The total number of BrdU-positive cells in the VZ, SVZ/IZ, and CP per

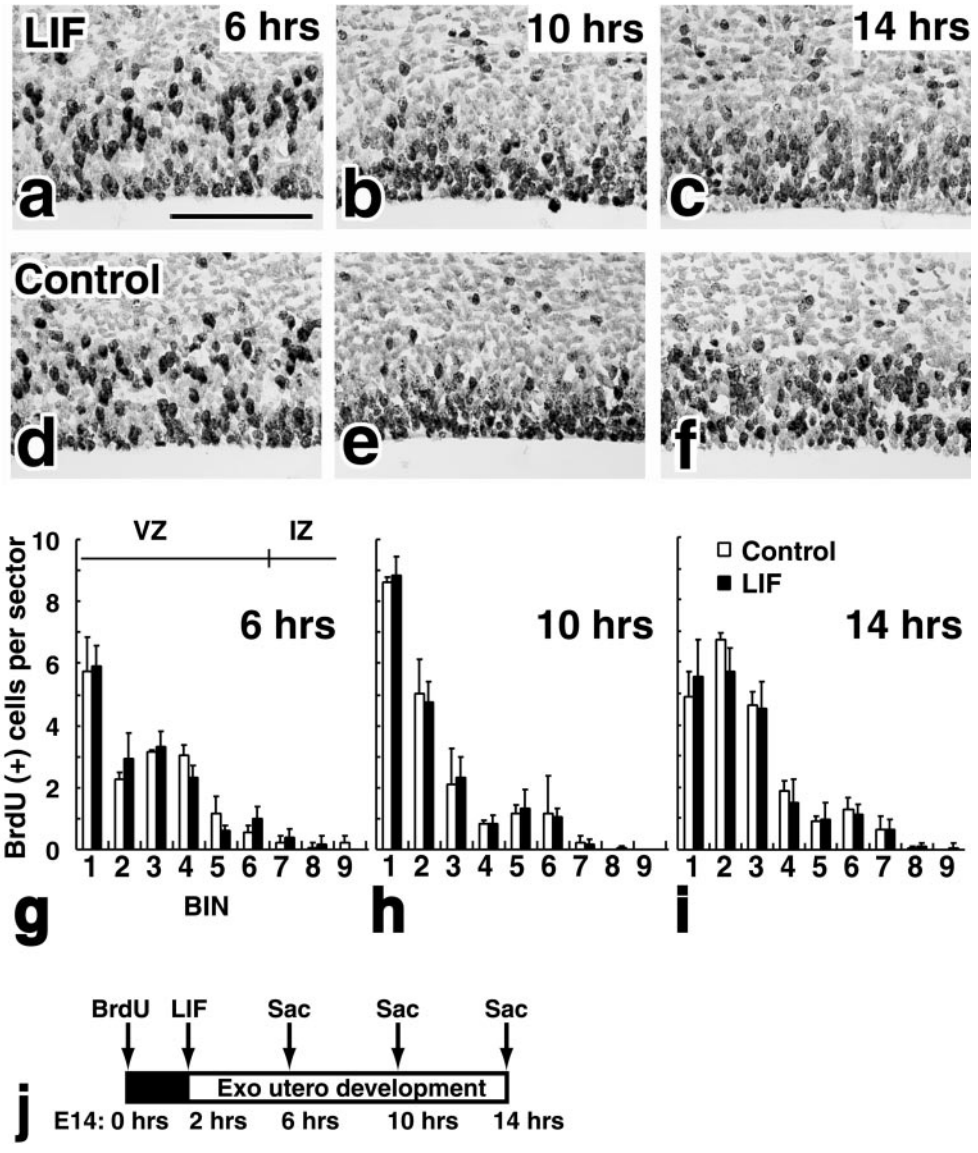


Figure 4. Effect of LIF on the downward nuclear translocation of the progenitor cells. Progenitor cell cells were labeled with BrdU 2 hr before injection of LIF on E14, and the distribution of BrdU-positive nuclei was analyzed 6, 10, and 14 hr after BrdU injection (*j*). The interpeak of the BrdU-positive cell distribution in both groups shifted from bin 4 (60–80 μm from the ventricular surface) (see Fig. 3) to bin 1 (0–20 μm from the ventricular surface) at 6 hr (*a*, *d*, *g*). Most cells had accumulated on the ventricular surface (bin 1) at 10 hr after BrdU injection (*b*, *e*, *h*), when the cells had entered or just finished M phase. At 14 hr after BrdU injection, the interpeak of the labeled cells shifted from bin 1 to bin 2. When observed closely, the inner half of bin 1 was found to be occupied by unlabeled cells in both groups, indicating that the labeled cell population had already passed through M phase and was changing position to a deeper area of VZ (*c*, *f*, *i*). Statistical analysis revealed that there were no significant differences between LIF-injected and control embryos in the number of labeled cells (ANOVA) and no significant interactions between treatment (LIF injection or control) and bin in the number of labeled cells for each time point (ANOVA) (*g–i*). *Sac*, Sacrifice. Scale bar, 100 μm.

sector was calculated by accumulating the number of cells per bin per sector in each zone and revealed that the BrdU-positive cells in the LIF-injected group significantly differed from those in controls in the VZ (LIF, 16.9 ± 0.59; control, 8.8 ± 0.68) and SVZ/IZ (LIF, 20.8 ± 1.06; control, 11.3 ± 0.55) (Fisher's *post hoc* test; $p < 0.01$). However, there was no significant difference in the CP between LIF-injected and control groups (LIF, 12.1 ± 0.60; control, 11.3 ± 0.55) (Fig. 6*b*), indicating that, at 60 hr after BrdU injection, the first generation of labeled neurons had reached the CP, but the next generation of labeled neurons had not yet arrived.

The distribution pattern of the BrdU-positive cells in the CP was analyzed, as represented in the histograms of the BrdU-positive cells per sector in the bins (bins 17–22) (Fig. 6*c*). It was demonstrated that the BrdU-positive cells in the CP were localized more in pial side (bin 22 and bin 21; $p < 0.05$) and less in bin 17 ($p < 0.1$) and bin 18 ($p < 0.05$) in the LIF-injected embryos than in the control (Fisher's *post hoc* test), suggesting that LIF promoted the migration of postmitotic neurons to the CP.

In contrast to the CP, the number of BrdU-positive cells significantly increased in the VZ and SVZ/IZ (Fig. 6*b*,*c*) in the LIF-injected embryos than that in the control, indicating that LIF induced progenitor cells to reenter the stem cell cycle as described above (Fig. 3*b*).

DISCUSSION

Do gp130-mediated signals play roles in the developing brain?

In contrast to the mild changes in the CNS induced by gene disruption of *LIF* (Escary et al., 1993) and/or *CNTF* (Masu et al., 1993), knock-out mice lacking LIFR, CNTFR, or gp130 showed hypoplasia of motor nuclei in the brainstem and spinal cord, as well as a loss of astrocytes (DeChiara et al., 1995; Li et al., 1995; Nakashima et al., 1999a). Therefore, it has been suggested that gp130-mediated signals contribute to the survival of postmitotic neurons and differentiation of astrocytes during development. However, the function of gp130 in neurogenesis and cerebral cortical histogenesis remains unknown.

The present results regarding the expression pattern of gp130 suggest a contribution of gp130 to neuronal production. The expression of gp130 in the VZ of the wild-type embryos was detected on E11, when the production of neurons had started

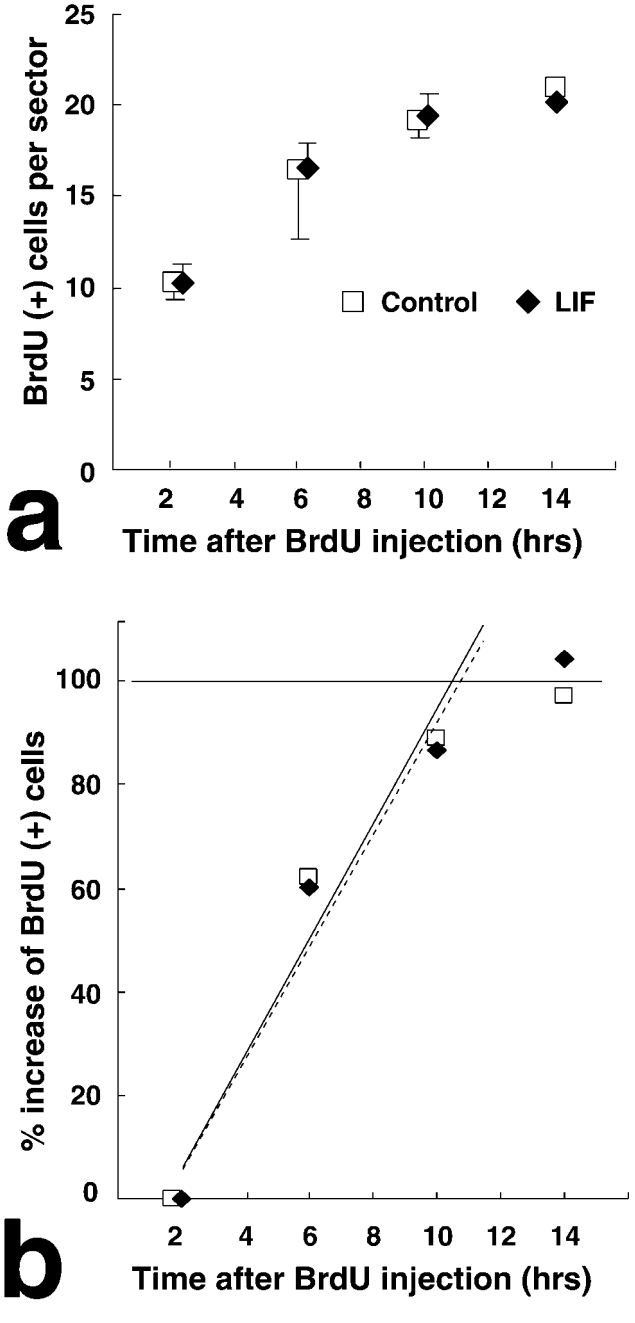
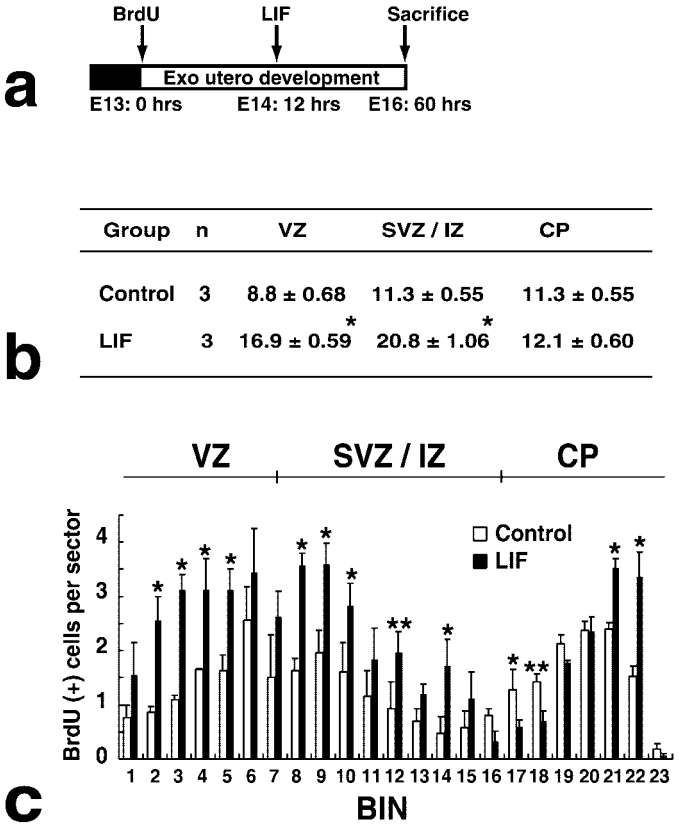


Figure 5. Effects of LIF on the cell cycle of progenitor cells. The total number of BrdU-positive cells on E14 was calculated from data obtained in the analysis of downward nuclear translocation. *a*, The number of labeled cells per sector was plotted for each time point: 6, 10, and 14 hr after BrdU injection. The number of labeled cells at 2 hr after BrdU injection without LIF was used as the initial value. In this experiment, the number of initially labeled cells was not affected by LIF injection, as shown in Figure 4*j*. The initial number of cells at 2 hr was ~10 per sector, increased, and then doubled between 10 and 14 hr in both groups. There were no significant differences between LIF-injected and control embryos (ANOVA) and no significant interaction between treatment and time point (ANOVA). *b*, The percentage of increase in BrdU-positive cells relative to the initial value was calculated for each time point. When the population completed mitosis, the number of labeled cells was twice the initial value (represented as 100%). There were no significant differences between LIF-injected and control embryos in the percentage of increase of BrdU-positive cells and no significant interaction between treatment and time point (ANOVA). The labeled population finished mitosis at 10.5 and 10.7 hr after BrdU injection in the LIF-injected and control groups, respectively. The values were obtained using linear models (simple regression; LIF, $r^2 = 0.95$; control, $r^2 = 0.95$).



b

Group	n	VZ	SVZ / IZ	CP
Control	3	8.8 ± 0.68	11.3 ± 0.55	11.3 ± 0.55
LIF	3	16.9 ± 0.59*	20.8 ± 1.06*	12.1 ± 0.60

Figure 6. Change of the number and distribution of BrdU-labeled cells at 60 hr elapse. Dams were injected with BrdU on E13 (12 hr before LIF injection), and the number and distribution of BrdU-positive cells was analyzed in the cerebral wall of the wild-type embryos 60 hr after BrdU on E16 (*a*). The total number of BrdU-labeled cells per sector in the cerebral wall was significantly increased in the LIF-injected group than that in the control (ANOVA; $p < 0.01$) (*b*). *Post hoc* analysis revealed that the number of labeled cells in the VZ and SVZ/IZ significantly increased in the LIF-injected group ($p < 0.01$; Fisher's *post hoc* test) but that, in the CP, did not significantly differ between the LIF-injected and control groups (*b*). This result indicates that the labeled neurons derived from the first cell division after BrdU labeling had reached to the CP, but the later generation had not yet arrived. It is also suggested that LIF directed the postmitotic labeled daughter cells to reenter the cell cycle to increase the stem cell pool in the VZ and SVZ, because the number of labeled migrating neurons in SVZ/IZ increased. The histogram demonstrates the distribution pattern of BrdU-positive cells per sector in each bin (*c*). Note that, although the total number of BrdU-positive cells in the CP did not differ between LIF-injected and control groups (*b*), BrdU-positive cells were localized significantly more in bins 21 and 23 ($p < 0.05$) and less in bins 17 ($p < 0.05$) and 18 ($p < 0.1$) in the LIF-injected group than in the control (Fisher's *post hoc* test) (*c*). This finding indicates that the first generation of the BrdU-positive neurons was localized more in the outer and less in the inner region of the CP in the LIF-injected group than in the control group, suggesting that LIF promoted the migration of postmitotic neurons. $^*p < 0.05$; $^{**}p < 0.1$.

(Angevine and Sidman, 1961), increased as development proceeded, peaked at approximately E15, and decreased in the VZ but increased in the SVZ on E18. This chronological change in the expression of gp130 well corresponds to the change in the distribution of cells with neuronal production in the VZ/SVZ and suggests that gp130-mediated signals are involved in the production in the VZ for cerebral cortical histogenesis. These results further suggest that gp130 plays roles in the progenitor cells of the cerebral wall at approximately E14, when LIF was used to

stimulate the gp130 signal transduction in the developing cerebral wall in the present study.

gp130 signals affect the proliferation of the progenitor cell

In the present study, gp130 (−/−) showed a hypoplastic CP, and BrdU-labeling experiments revealed that neuronal production was impaired among progenitor cells in gp130 (−/−). We next examined the *in vivo* effects of the gp130-mediated signals on the neurogenesis of the cerebral cortex using mouse *exo utero* system. Consequently, it was clearly revealed that hyperplasia of the CP is induced by injection of LIF, allowing additional detailed analyses of the effects of LIF on the neuronal production.

First, the effect of LIF was analyzed by injecting LIF on E14, BrdU at 24 hr later on E15, and examining the number of BrdU-positive cells at 2 hr after BrdU injection. In the VZ, the number of BrdU-positive cells significantly increased in the LIF-injected group than in the control. Furthermore, when BrdU was injected 12 hr before the LIF injection and the number of labeled cells was analyzed 60 hr after BrdU (48 hr after LIF) injection, the number of BrdU-positive cells in the VZ significantly increased in the LIF-injected group compared with that in the control. In this experimental model, the initial frequency of BrdU incorporation was equal between the LIF and control groups. The cell cycle of progenitor cells was not affected by LIF and was in the range of that established in normal mouse embryos by Takahashi et al. (1993, 1995, 1996a,b, 1999). These findings suggest that LIF increased the stem cell pool by directing the postmitotic cells, which normally exit the cycle, to reenter S phase of the stem cell cycle.

We could not detect any significant differences in the number of apoptotic cells in the CP between LIF-injected and control groups or between gp130 (−/−) and gp130 (+/+) embryos. Thus, it is suggested that the present changes in the number of cerebral cortical neurons were induced by the LIF effect on neuron production rather than on cell survival.

LIF enhances the migration of postmitotic neurons

For analyzing the migration of postmitotic neurons, LIF was injected 12 hr after BrdU, when the labeled progenitor cells had already finished the first M phase. Therefore, the injection of LIF did not affect the number of the labeled postmitotic neurons derived from the first cell division after BrdU-labeling. In fact, the number of BrdU-positive cells per sector in the CP 60 hr after BrdU injection, when only the first generation of BrdU-positive cells reached the CP, did not differ significantly between the LIF-injected and control groups. However, in the CP, the number of BrdU-positive cells was significantly larger on the pial side (bins 21 and 22) and smaller on the ventricular side (bins 17 and 18) in the LIF-injected group than that in the control. This indicates that the labeled cortical neurons at 60 hr after BrdU injection were derived from the initial cell division after BrdU labeling and did not include the second wave of migrating neurons from the later generation and that the BrdU-positive neurons reached the upper part of the CP faster in the LIF-injected group than in the control. The second wave of BrdU-positive neurons was observed in the SVZ/IZ, which were increased in number in the LIF-injected group than in the control, but had not yet reached the CP. These findings suggest that the migration of postmitotic neurons to the CP was accelerated by the LIF-triggered activation of gp130. The migration of postmitotic neurons is closely related to their differentiation, and therefore gp130 signals may also contribute to the differentiation of cortical neurons *in vivo*, as reported in the sensory neurons of embryonic DRG (Murphy et al., 1991, 1993) or sympathetic neurons (Yamamori et al., 1989) *in vitro*.

In conclusion, we revealed that the LIF/gp130 signal plays important roles in producing neurons and also in the migration of postmitotic neurons in the present study.

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