Gap junctional communication is required to maintain mouse cortical neural progenitor cells in a proliferative state

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Abstract

The mechanisms that determine whether neural stem cells remain in a proliferative state or differentiate into neurons or glia are largely unknown. Here we establish a pivotal role for gap junction-mediated intercellular communication in determining the proliferation and survival of mouse neural progenitor cells (NPCs). When cultured in the presence of basic fibroblast growth factor (bFGF), NPCs express the gap junction protein connexin 43 and are dye-coupled. Upon withdrawal of bFGF, levels of connexin 43 and dye coupling decrease, and the cells cease proliferating and differentiate into neurons; the induction of gap junctions by bFGF is mediated by p42/p44 mitogen-activated protein kinases. Inhibition of gap junctions abolishes the ability of bFGF to maintain NPCs in a proliferative state resulting in cell differentiation or cell death, while overexpression of connexin 43 promotes NPC self-renewal in the absence of bFGF. In addition to promoting their proliferation, gap junctions are required for the survival of NPCs. Gap junctional communication is therefore both necessary and sufficient to maintain NPCs in a self-renewing state.

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Keywords: Gap junctional communication; Neural progenitor cells; Basic fibroblast growth factor

Introduction

During development of the cerebral cortex, neurons arise from neural progenitor cells (NPCs) located in the telencephalic ventricular zone (VZ) (Rakic, 1995; Takahashi et al., 1995) and then migrate away from the ventricular zone into the cortical plate and differentiate into neurons (McConnell, 1995; Takahashi et al., 1993). The signaling mechanisms that determine whether NPCs remain in a proliferative state or differentiate into neurons are largely unknown, although the Notch signaling pathway (Gaiano and Fishell, 2002), neurotrophic factors, such as basic fibroblast growth factor (bFGF) and epidermal growth factor (Cameron et al., 1998), and the diffusible gas nitric oxide (Cheng et al., 2003) appear to play important roles. bFGF may play a particularly important role in maintaining NPCs in a self-renewing state.

As evidence, treatment of cultured rodent and human NPCs with bFGF promotes proliferation of NPCs (Murphy et al., 1994). In addition, analyses of NPCs and neurogenesis in mice lacking bFGF and in normal mice administered exogenous bFGF suggest that bFGF is a critical signal regulating neurogenesis in vivo (Cheng et al., 2002). Cell surface receptors for bFGF possess intrinsic tyrosine kinase activity that initiates an intracellular signaling cascade involving p42/p44 mitogen-activated protein (MAP) kinases, also known as extracellular signal regulated kinases (ERK1 and ERK2) (Abe and Saito, 2000; Kinkl et al., 2001; Olson et al., 2000). Although the latter signaling pathway is presumed to be involved in maintaining NPCs in a proliferative state, this has not been established and the specific mechanism whereby this signaling pathway regulates NPCs fate is unknown.

One property of several types of stem cell populations, including those in the developing neocortex, is that proliferating NPCs are connected by gap junction channels (Bittman et al., 1997; LoTurco and Kriegstein, 1991). Gap junctions are formed by two hemichannels or connexons

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contributed by each of the coupled cells and provide conduits for the rapid transfer of ions, and low molecular weight messengers and metabolites between the coupled cells (Spray and Bennett, 1985; Veenstra, 1996). The proteins that form the gap junction channels are called connexins which are integral membrane proteins with four-transmembrane domains (Bruzzone et al., 1996; Good- enough et al., 1996). At least 20 rodent connexins have been identified (Evans and Martin, 2002; Sohl and Wil- lecke, 2003); in the developing and adult brain, connexins 43 (Cx43), 36 and 32 are the major isoforms (Al-Ubaidi et al., 2000; Belluardo et al., 2000; Condorelli et al., 2000; Dermietzel and Spray, 1993; Kumar and Gilula, 1996; Nadarajah et al., 1996, 1997) and connexins 43 and 45 (but not connexin 26) are found to be major isoforms expressed in E10.5 multipotent neural stem cells (Cai et al., 2002). Previous studies found that Cx43 is expressed at high levels in proliferating NPCs in the developing rodent brain (Bittman and LoTurco, 1999) and Cx43 levels decrease as NPCs differentiate into neurons in vivo (Leung et al., 2002) and in cell culture (Rozental et al., 1998). Previous studies (Nadarajah et al., 1998) also showed that treatment of bFGF in E16 neural progenitor cells can promote Cx43 expression and gap junctional coupling. It was suggested that gap junctions might promote the synchrony of clonal cell proliferation and contiguity of clonally related cells in the VZ (Cai et al., 1997), suggesting a role for gap junctions in regulating NPC proliferation and neurogenesis. However, the mechanisms that regulate gap junction coupling and the function of such coupling in regulating the fate of NPCs in the VZ are unknown. We now provide direct evidence that up-regulation of gap junction expression is both necessary and sufficient for the mitogenic effect of bFGF in NPCs. By promoting their proliferation and survival, gap junctional communication maintains the NPCs in a self-renewing state.

Materials and methods

E12 cortical progenitor cell cultures

Pregnant C57BL/6 mice were euthanized on gestational day 12 by cervical dislocation, embryos were harvested and their brains were removed. Meninges were removed from the brain and the cortical neuroepithelium was dissected, collected in cold calcium/magnesium-free Hank’s balanced saline solution (HBSS; Gibco/BRL) and then transferred to culture medium [DMEM/F12 supplemented with B27 and bFGF (30 ng/ml; Gibco/BRL)]. The cells were dissociated by gentle trituration with a fire-polished Pasteur pipette to yield suspensions of single cells or small cell clusters (30–50 cells/cluster). The dissociated cells or cell clusters were diluted with the culture medium and plated onto poly-L-lysine-coated glass coverslips or plastic culture dishes at desired cell densities.

E9.5 neuroepithelial precursor cell cultures

We isolated fetal multipotent neuroepithelial progenitor (NEP) cells from E9.5 rat embryos as described previously (Cai et al., 2002). The truncated segments of the E9.5 embryos were dissected and incubated in an enzyme solution containing collagenase type I (1 mg/ml; Worthington Biochemical) and dispase II (2 mg/ml; Roche) in HBSS at room temperature for approximately 10 min. The enzyme solution was then replaced with NEP basal medium (Kalyani et al., 1997). The segments were gently triturated with a Pasteur pipette to release neural tubes from surrounding somites and connective tissue. Cells in isolated neural tubes were dissociated using trypsin-EDTA solution, and NEP cells were grown in NEP basal medium with 10% chicken embryo extract and bFGF (20 ng/ml, Peprotech).

Primary neocortical neuron cultures

Brains of embryonic day 18 Sprague–Dawley rats were used as a source of cortical tissue. Cells were dissociated by mild trypsinization and trituration and were seeded onto polyethyleneimine-coated glass coverslips in MEM supplemented 10% with heat-inactivated fetal bovine serum. After a 3- to 5-h incubation period to allow for cell attachment, the medium was replaced with Neurobasal medium containing B27 supplements, 2 mM l-glutamine, 25 mg/ml genta- micin and 1 mM Hepes (Gibco/BRL). Analyses were performed on cells that had been in culture for 5–7 days.

Immunocytochemistry

Cells were fixed in a solution of 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 20 min and were then washed with PBS, and incubated for 5 min in a solution of 0.2% Triton X-100 and 5% normal goat serum in 0.1 M PBS containing 5% normal goat serum. Cells were then incubated in the same solution containing primary antibody at 4°C overnight, followed by washing with PBS and a 2-h incubation at room temperature in PBS containing FITC-conjugated secondary antibody. For bromodeoxyuridine (BrdU) immu- nocytochemistry, cells were incubated for 45 min in a solution of 2 N HCl. The primary antibodies and their dilutions were: mouse anti-nestin IgG1, 1:200 (Chemicon); mouse anti-MAP2 IgG1, 1:200 (Sigma); mouse anti-BrdU, 1:100 (Becton-Dickinson); mouse anti Cx43 IgG1, 1:500 (Chemicon). Images were acquired with a confocal laser scanning microscope (LSM 510) using a 40× objective in dual scanning mode for simultaneous detection of fluores- cein (488 nm excitation and 510 nm emission) and propi- dium iodide (545 nm excitation and 590 nm emission).

Cell proliferation and cell death assays

To evaluate cell proliferation, the cell cultures were exposed to experimental treatments for designated time
periods and BrdU was then added to cultures at a final concentration of 10 μM. After 16 h, the cells were fixed in 4% paraformaldehyde in PBS, and then processed for BrdU immunocytochemistry. The nuclei of cells were counterstained with propidium iodide and confocal images were acquired. Images from five random fields per coverslip were acquired (40× objective; NA = 1.3) and the percentage of cells that were BrdU-positive in each field was determined. A minimum of three coverslips for each condition was used in each experiment, and data presented are the results of three to four separate experiments. To evaluate cell survival, cells were exposed to experimental treatments for designated time periods and were then fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were then stained with the DNA-binding dye Hoechst 33258 and coverslips were mounted onto glass slides and examined under epifluorescence illumination using a 32× objective lens. Cells were considered “apoptotic” if their nuclear chromatin was condensed or fragmented, whereas cells were considered viable if their chromatin was diffusely and evenly distributed throughout the nucleus. 18-α-Glycyrrhetinic (Sigma) was prepared as a 10-mM stock in chloroform/methanol (2:3 vol/vol). Glycyrrhizic acid and PD98059 (Cell Signaling Technology) were prepared as a 10-mM stock in dimethylsulfoxide.

Scrape-loading dye transfer assay for gap junctional intercellular communication

The presence of intercellular gap junctions was assessed as described previously (Blanc et al., 1998; El-Fouly et al., 1987; Le and Musil, 1998). Briefly, the culture medium from a confluent monolayer of cells was removed and saved. The cells were rinsed three times with HBSS containing 1% bovine serum albumin (BSA), and then were incubated in Dulbecco’s phosphate-buffered saline containing 1% Lucifer yellow (LY) with or without 1% rhodamine–dextran. A 27-gauge needle was used to create two longitudinal scratches through the cell monolayer, and 1 min later, the culture was quickly rinsed three times with HBSS containing 1% BSA and then incubated for an additional 8 min in saved culture medium to allow the loaded dye to transfer to adjoining cells. The culture was then rinsed three times with PBS and fixed. The LY-loaded cells were counterstained with propidium iodide in PBS containing 0.2% Triton X-100 and 1% RNase. Dual channel confocal images of LY and either rhodamine–dextran or PI fluorescence were acquired using a 25× water immersion objective (NA = 1.3). Images were acquired of cells adjacent to the scrape in regions where the cell monolayer was complete. The relative extent of cell coupling was determined by counting the total number of LY-positive cells along a 1-mm longitudinal distance along the scrape line. At least three images were taken per dish with four dishes per condition; three separate experiments were performed.

Immunoblot analysis

After exposure to experimental treatments, the cells were solubilized in SDS-PAGE sample buffer, and the protein concentration in each sample was determined using a Bio-Rad protein assay kit with BSA as the standard. Proteins (50 μg per lane) were then separated in a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and immunostained as follows. Membranes were blocked with 4% nonfat milk in TBST (Tris–HCl buffer with 0.2% Tween 20, pH 7.5), and then incubated for 2 h in the presence of primary antibody. Membranes were then incubated for 1 h in the presence of a 1:5000 dilution of secondary antibody (IgG) conjugated to horseradish peroxidase. Reaction product was visualized using an Enhanced Chemiluminescence (ECL) Western blot detection kit (Amersham Pharmacia Biotech). The primary antibodies included anti-Cx43 (mouse, 1:1000, Temecula, CA), anti-phospho-Erk1, 2 (mouse, 1:1000, New England Biolabs) and anti-Erk1, 2 (rabbit, 1:1000, New England Biolabs).

Retroviral vector production

pBabe-cx43 cDNA (a generous gift of A.F. Lau, Molecular Carcinogenesis Section, Cancer Research Center of Hawaii), or pBabe-puro or pBabe-GFP were transfected into the Phoenix packaging cell line (G.P. Nolan, Stanford University). It produces MLV Gag and Pol proteins constitutively. The day before transfection, Phoenix cells were plated at 10⁵ cells per well in a six-well plate, with 2 ml of medium per well. Cells were grown to 70–80% confluency in each experiment, and data presented are the results of three to four separate experiments. To evaluate cell survival, cells that were BrdU-positive in each field was determined. Images from five random fields per coverslip were acquired. Images from five random fields per coverslip were acquired (40× objective lens). Cells were considered “apoptotic” if their nuclear chromatin was condensed or fragmented, whereas cells were considered viable if their chromatin was diffusely and evenly distributed throughout the nucleus. 18-α-Glycyrrhetinic (Sigma) was prepared as a 10-mM stock in chloroform/methanol (2:3 vol/vol). Glycyrrhizic acid and PD98059 (Cell Signaling Technology) were prepared as a 10-mM stock in dimethylsulfoxide.

RT-PCR

Reagents for RT-PCR were purchased from Invitrogen Life Technologies (Carlsbad, CA). Total RNA was extracted from cultured cells using TRizol reagent following the manufacturer’s instruction. Following digestion of genomic DNA with DNase treatment, the RNA samples were subjected to a reverse transcription (RT) reaction to synthesize single-strand cDNAs using M-MLV reverse transcriptase. Polymerization reactions were performed in a thermocycler in a 50-μl reaction volume containing 3 μl of cDNA (60 ng total RNA equivalents), Taq PCR buffer, 60 μM of each
dNTP, 1.25 mM MgCl2 and 2.5 units of Taq DNA polymerase. To ensure that there was no contamination with genomic DNA, the samples were tested without reverse transcriptase in a PCR reaction. The oligonucleotide primers and cycle numbers used for multiplex PCR were as follows. Cx43 (GenBank accession number: M63801): (forward) 5'-TAC CAC GCC ACC ACT GGC CCA-3'; (reverse) 5'-ATT CTG GTT GTC GTC GGG GAA ATC-3' 30 cycles; actin (GenBank accession number: AF122902); (forward) TGT CAC CAA CTG GGA CGA TA; (reverse) TCT CCG GAG TCC ATC ACA AT, 28 cycles. The thermal cycle profile employed a 4-min denaturing step at 94°C followed by the number of amplification cycles (30 s of denaturation at 94°C, 30 s of annealing at 55°C and 45 s of extension at 72°C), and an extension step of 7 min at 72°C. The cycle number of each primer pair was chosen within the expo-

Fig. 1. Characterization of E12 dissociated mouse cortical progenitor cell cultures. Cultures were established from mouse ventricular zone (VZ) neuroepithelium at E12, and were cultured at a density of 100,000 cells/cm² of culture surface in bFGF-containing medium. After 3 days in culture, the cells were incubated with 10 μM BrdU for 16 h and then fixed and immunostained with antibodies against BrdU (A), nestin (B) or MAP2 (C). All the cells were counterstained with propidium iodide (red). Note that vast majority of cells are proliferating (BrdU-positive: 73.2 ± 5.4%) and express the neural progenitor cell protein nestin (83.2 ± 6.3%), whereas a small portion of cells express the neuronal marker MAP2 (20.4 ± 5.4%). Bar, 50 μm.

Fig. 2. bFGF stimulates the proliferation of cortical progenitor cells. NPCs were cultured as either dissociated cells (A) or as small cell clusters (30–50 cells per cluster) (C) in the presence or absence of bFGF. The dissociated cells were plated at a density of 5 × 10³ to 100 × 10³/cm² and the cell clusters were plated at a density of 500 cell clusters/cm². After 3 days in culture, the cells were incubated with 10 μM BrdU for 16 h and then fixed for BrdU immunocytochemistry (A, C). All of the cells were counterstained with propidium iodide (red). Bar, 100 μm. (B). When the cells were grown in small clusters, their proliferation rate increased compared to cells not in contact with each other (D). Values are the mean and SD of three separate experiments. ** P < 0.01 (ANOVA with Scheffe post hoc tests).
Results

Stimulation of cortical progenitor cell proliferation by bFGF requires functional gap junctions

We employed NPC cultures established from E12 cerebral neuroepithelium, a time point when the onset of neurogenesis occurs (Cheng et al., 2003). When NPCs were plated at a high cell density and maintained for 3 days in the presence of bFGF, the majority of cells in the dissociated cell cultures were proliferating as demonstrated by their ability to incorporate BrdU and their expression of the neural progenitor cell protein nestin (Fig. 1). Approximately 20% of the cells in the cultures extended neurites and exhibited immunoreactivity with an antibody against microtubule-associated protein-2 (MAP2) indicating a neuronal phenotype (Fig. 1). When cultured in the absence of bFGF, the proliferation of the NPCs was significantly decreased (Fig. 2) and the percentage of cells that exhibited a neuronal phenotype was significantly increased (data not shown). However, when NPCs were plated at higher cell densities or in cluster cell cultures, the percentage of cells remaining in a proliferative state was significantly greater compared to low cell density dissociated cell cultures (Figs. 2B, C, D).

The above findings suggested that treatment with bFGF and physical association of NPCs in clusters can promote maintenance of NPCs in a proliferative state and also indicated a relationship between cell–cell contacts and bFGF mitogenic activity. A number of studies established that proliferating NPCs are connected by gap junction channels (Bittman et al., 1997; Cai et al., 2002; LoTurco and Kriegstein, 1991). Because high-density cell cultures or cluster cultures enhanced the possibility that gap junctional couplings are maintained to be intact in NPCs, we hypothesized that gap junctional coupling might play an important role in regulating mitogenic effect of bFGF. When dissociated NPC cultures were maintained in the absence of bFGF, many cells differentiated and lost im-

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Fig. 3. Gap junctional coupling is required for the mitogenic effect of bFGF. E12 neural progenitor cells were cultured at a density of 100,000 cells/cm² of culture surface in medium containing (+bFGF) or lacking (−bFGF) bFGF. After 3 days in cultures, the cells were fixed and immunostained with an antibody against Cx43. (A) The left panel (−bFGF) and middle panel (+bFGF) show confocal images of Cx43 immunoreactivity (green) overlayed with the phase-contrast images of the cells. Note that in the absence of bFGF, the majority of cells lack Cx43 immunoreactivity (arrowheads) and the Cx43⁺ cells exhibit intracellular staining. In the presence of bFGF, the majority of cells are Cx43 immunoreactive and exhibit punctate labeling on the cell surface characteristic of gap junctional complexes (arrows). The right panel is a confocal image showing a negative control in which the cells were processed without the primary antibody. Scale bars, 50 μm. (B) Dissociated NPCs were cultured at a density of 100 × 10⁴ cells/cm² in the absence or presence of bFGF for 2 days and then treated with 10 μM 18-α-glycyrrhetinic acid (GA), a gap junction uncoupler. GA treatment blocked the mitogenic effect of bFGF. (C) Concentration–response curve of the effect of GA on blocking the proliferation of NPC cultured in the presence of bFGF in comparison with cultures exposed to equal concentration of the inactive analog glycyrrhizic acid (GZA). Values are means ± SD of three separate experiments. ** P < 0.01 (ANOVA with Scheffe post hoc tests).
munoreactivity with an antibody against Cx43, a major connexin isoform in NPCs (Cai et al., 2002; arrowheads, Fig. 3A). In the cells that retained Cx43 immunoreactivity, the staining pattern indicated intracellular localization. In contrast, in the presence of bFGF, the majority of cells were Cx43 immunoreactive and the Cx43+ cells exhibited punctate labeling on the cell surface characteristic of gap junctional complexes (arrows, Fig. 3A). To determine whether gap junctions play a role in regulating the proliferation of NPC, we incubated bFGF-treated cells in the presence of increasing concentrations of 18-α-glycyrrhetinic acid (GA), an agent that uncouples gap junctions (Blanc et al., 1998; Goldberg et al., 1996). GA treatment decreased the number of NPCs that incorporated BrdU in a concentration-dependent manner, whereas cell proliferation was unaffected by treatment with the inactive analog glycyrrhyzic acid (GZA) (Fig. 3C). A concentration of 10 µM GA inhibited proliferation by 40% without any evidence of cytotoxicity, while a higher concentration of 20 µM significantly increased cell death (Fig. 8). GA had no significant effect on NPC proliferation in cultures maintained in the absence of bFGF (Fig. 3B).

bFGF induces connexin 43 expression and dye coupling in cortical progenitor cells

If an increase in gap junctions plays a key role in the mechanism whereby bFGF promotes the self-renewal of NPCs, then bFGF should increase the expression and/or functional activity of gap junction proteins. To address the latter question, we first measured relative levels of Cx43 mRNA and protein in NPCs that had been maintained in the absence or presence of bFGF. The amounts of Cx43 mRNA and protein were significantly greater in NPCs that had been treated for 24 h with bFGF compared to Cx43 mRNA and protein levels in parallel control cultures (Fig. 4). The slower migrating Cx43 immunoreactive bands (Fig. 4B) are likely phosphorylated forms of Cx43 as previously reported (Musil et al., 1990). Treatment with bFGF increased the levels of these phosphorylated forms of Cx43 (Fig. 4C). We next evaluated the functional coupling of NPCs using a dye scrape-loading method. In the experiment shown in Fig. 5A, cells were scrape-loaded with gap junction impermeable high molecular weight fluorescent dye rhodamine–dextran (RD, red) together with the gap junction permeable low molecular weight fluorescent dye Lucifer yellow (LY, green). We observed many RD+/LY cells adjacent to the scrape border (arrows, Fig. 5A), indicating that rhodamine–dextran was confined to the wounded cells into which dye had been directly introduced during the scrape-loading process, whereas LY was transferred to adjacent cells through gap junction channels. By scraping loading with LY, we next determined the effects of GA and bFGF on gap junction coupling. When maintained in the presence of bFGF, the transfer of LY between NPCs was considerable, and

**Fig. 4. Basic FGF increases Cx43 mRNA and protein levels in cortical progenitor cells. Dissociated NPCs were cultured at a density of 100 × 10^3/cm^2 in the absence of bFGF for 2 days, and were then stimulated with bFGF (30 ng/ml) for 24 h. (A) Cx43 mRNA was measured by semi-quantitative RT-PCR. (B) Cx43 proteins were measured by immunoblot analysis. The corresponding nonphosphorylated form (Cx43-NP) and two more slowly migrating phosphorylated forms are indicated (arrows) as previously described (Musil et al., 1990). Levels of Cx43 mRNA and protein (both Cx43-P and Cx43-NP) were significantly increased in cells treated with bFGF. (C) Densitometric analysis of immunoblots. Values are the means ± SD of determinations made in three separate experiments. ** P < 0.01 (unpaired t test).**
treatment with GA largely abolished the transfer of LY between cells in a dose-dependent manner (Fig. 5C). After 20 μM GA treatment for 6 h, the number of LY+ cells was similar to that of RD+ cells, whereas 20 μM GZA had no effect on the number of LY+ cells, indicating that GA blocks gap junction-mediated transfer of LY (Figs. 5B, C).

Following withdrawal of bFGF, the transfer of the dye between cells was significantly decreased (Fig. 5D). When taken together with the data showing that bFGF induces Cx43 expression and membrane localization, the dye transfer data demonstrate that bFGF increases functional gap junction coupling between NPCs.

Fig. 5. bFGF increases gap junction coupling in cortical progenitor cells. Dissociated NPCs were cultured at a density of 100 × 10^3 cells/cm² in the presence of bFGF for 3 days. (A) Cells were scrape-loaded with rhodamine–dextran (RD, red) mixed with Lucifer yellow (LY, green). Representative confocal images showing that gap junction impermeable dye rhodamine–dextran (Mr = 10 kDa) is confined to the wounded cells into which dye had been directly introduced during the scrape-loading process. In contrast, LY was transferred to adjacent cells. The arrows show RD ‘LY’ cells indicating gap junction-coupled cells. (B) Representative confocal images of cells that were scrape-loaded with LY after withdrawal of bFGF for different times or after 6 h of treatment with 18-α-glycyrrhetinic (GA), a gap junction blocker or glycyrrhizic acid (GZA) as a control. After 8 min in the presence of LY, the cells were fixed and counterstained with propidium iodide and images were acquired by confocal laser scanning microscopy. (C and D) The average number of LY-positive cells or RD-positive cells per 1 mm longitudinal distance along the site of the scrape was determined. Both withdrawal of bFGF and treatment with GA attenuated the intercellular transfer of the LY. Scale bars = 100 μM. Values are means ± SD of four separate experiments. ** P < 0.01 (ANOVA with Scheffe post hoc tests).
ERK activation mediates bFGF-induced gap junctional coupling and cell proliferation

Previous studies have shown that p42/p44 mitogen-activated protein (MAP) kinases, also known as extracellular signal regulated kinases (ERK1 and ERK2), mediate several biological responses to bFGF in different cell types (Abe and Saito, 2000; Kinkl et al., 2001; Olson et al., 2000). We therefore sought to determine whether ERKs mediate the proliferative effects of bFGF in NPCs, and whether this occurs by an effect of ERKs on gap junctions. An antibody that selectively binds to phosphorylated (activated) ERK1 and ERK2 was employed in immunoblot analyses of cell lysates from NPCs that had been exposed to bFGF for increasing time periods from 0.5 to 48 hours. ERK phosphorylation was greatest at the 0.5 h time point, then decreased through 24 hours, but remained elevated above the level of phospho-ERK in cells not exposed to bFGF (Fig. 6A). The ability of bFGF to increase ERK phosphorylation was largely eliminated in NPC cultures that had been incubated in the presence of the ERK inhibitor PD98059 during exposure to bFGF (Fig. 6D). The ability of bFGF to activate ERKs was unchanged in cells treated with GA at concentrations that uncoupled gap junction (Fig. 6D). When NPCs were treated with PD98059, the abilities of bFGF to stimulate cell proliferation and to increase gap junction-mediated dye coupling were abolished (Fig. 6B), demonstrating an essential role for ERKs in bFGF-induced gap junctions and cell proliferation. Collectively, these findings suggest that ERKs mediate the effects of bFGF on gap junctions and NPC self-renewal.

Overexpression of connexin 43 stimulates NPC proliferation in the absence of bFGF

To determine whether the expression of gap junctions is sufficient to account for the proliferative effects of bFGF on NPCs, we employed a retroviral vector to overexpress Cx43 in cultured NPC. Cells maintained in the absence of

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Fig. 6. Activation of ERKs is required for bFGF-induced gap junctional coupling and cell proliferation. (A) NPCs (at a density of 100 × 10^3/cm^2) were stimulated with bFGF for 0.5, 4, 8, 24 and 48 h. The relative levels of activation of ERK1, 2 were assessed by immunoblot analysis using antibodies that selectively recognize phospho-ERKs (p-Erks). The same membranes were then stripped and re-probed with antibodies against total ERKs (phosphorylation-independent antibodies). (B) Treatment of NPC with the ERK inhibitor PD98059 (15 μM) blocked the mitogenic effect of bFGF. (C) The scrape loading/dye transfer assay was performed on cells that had been exposed to the indicated treatments; note that the ERK inhibitor blocked gap junctional coupling. (D) NPCs were pretreated for 2 h with either vehicle, GA (10 μM) or PD98059 (15 μM) and were then stimulated with bFGF for 30 min. Levels of activated and total ERKs were determined by immunoblot analysis using antibodies against phospho-specific and total ERKs, respectively. Pretreatment with GA did not block the ability of bFGF to activate ERKs.
Fig. 7. Overexpression of connexin 43 stimulates the proliferation of cortical progenitor cells in the absence of bFGF. (A) Confocal image of GFP fluorescence 2 days after infection with retrovirus containing GFP cDNA (infected on culture day 1). Note that approximately 50% of the cells were infected. (B) Immunoblot analysis showing that the levels of Cx43 protein was greatly increased after 48 h of infection with retro-Cx43. (C) Confocal images of Lucifer yellow-positive cells along the scrape/load line in different cultures treated as indicated. Scale bar = 100 μm. (D) Quantitative analysis of Lucifer yellow-positive cells per 1 mm longitudinal distance along the scrape line. Note that overexpression of Cx43 significantly enhanced intercellular dye transfer in either the absence or presence of bFGF. (E) Two days after infection with retro-Cx43 or control retrovirus containing no GFP, the cells were incubated with 10 μM BrdU for 16 h, and the cells were then fixed and immunostained with BrdU antibody. The proliferation index was quantified and plotted. Values are means ± SD of three separate experiments. ** P < 0.01 (ANOVA with Scheffe post hoc tests).
bFGF were infected with retroviral vectors containing a cDNA encoding either green fluorescent protein (GFP) or Cx43. Two days after infection, approximately 50% of the cells exhibited fluorescence, indicating that they had been infected and were expressing GFP (Fig. 7A). Immunoblot analyses of cell lysates demonstrated that Cx43 levels were very low in cells infected with retro-GFP, and that Cx43 levels were increased 6- to 10-fold in cells infected with retro-Cx43 (Fig. 7B). Dye transfer analysis revealed that overexpression of Cx43 in NPCs maintained in the absence of bFGF resulted in a highly significant increase in cell coupling (Figs. 7C, D). The extent of coupling was equivalent to that in cells maintained in the presence of bFGF. When Cx43 was overexpressed in NPCs that were maintained in the presence of bFGF, cell coupling was further increased above the level seen in bFGF-treated NPCs infected with control retro-puro (Fig. 7C). We found that in absence of bFGF, overexpression of connexin 43 promoted NPC proliferation, mimicking the mitogenic effect of bFGF (Fig. 7D). A major role for up-regulation of Cx43 expression in the mitogenic action of bFGF is further suggested by the finding that bFGF was not able to further enhance the proliferation of NPCs overexpressing Cx43.

The survival of E12 NPC and E9.5 NEP cells requires functional gap junctions

The ability of neural stem cells to self-renewal depends, not only on their continued proliferation, but also upon their survival. A form of programmed cell death called apoptosis occurs in neural stem cells in both the developing and adult brain (Brand and van Roessel, 2003; Rakic and Zecevic, 2000). To determine whether gap junctions play a role in regulating the survival of neural stem cells, we compared the effects of GA on the survival of E12 NPCs with its effects on E9.5 neuroepithelial cells (NEP) (a highly proliferative population of neural stem cells) (Cai et al., 2002), and mature cortical neurons. In preliminary studies, we found that NEP cells contain higher amounts of Cx43 and exhibit more dye coupling than do NPCs, whereas Cx43 expression and dye coupling is absent in mature neurons (data not shown). We found that NEP cells were significantly more sensitive to being killed by GA than were NPCs (Fig. 8). On the other hand, mature neurons were completely resistant to being killed by GA. These findings suggest that the survival of highly proliferative neural stem cells is dependent upon gap junctions.

Fig. 8. Embryonic cortical stem cells are highly sensitive to death induced by uncoupling gap junctions. Primary E9.5 and E12 cortical neural stem cells and differentiated neocortical neurons were cultured as described in the Materials and methods. Cultures were treated with GA at the indicated concentrations. After 24 h, cells were fixed and stained with Hoechst 33258. (A) Representative photomicrographs illustrating the morphology of NEP cells and the nuclei of the cells subjected to the indicated treatments. (B) The percentage of cells displaying nuclear apoptotic morphology was determined for each culture; values are the means ± SD of three independent experiments. Note that E9.5 neural stem cells were significantly more vulnerable to death induced by GA compared to E12 NPCs which, in turn were more sensitive to GA than were differentiated neurons. Values are means ± SD of three separate experiments. ** P < 0.01 (ANOVA with Scheffe post hoc tests).
Discussion

Previous studies showed that proliferating NPCs in VZ of developing murine neocortex form small clusters in which cells are coupled by gap junctions (Bittman et al., 1997; LoTurco and Kriegstein, 1991). Gap junctions may promote the synchrony of clonal cell proliferation in the VZ (Cai et al., 1997), because uncoupling reagents such as halothane and octanol can decrease the number of cells in S phase (Bittman et al., 1997; Goto et al., 2002). Although the latter studies suggested a relationship between mitotic activity of NPCs and gap junctions, the present findings provide the first direct evidence that up-regulation of gap junctions is both necessary and sufficient for bFGF to maintain NPCs in a proliferative state. The stimulation of NPC proliferation by bFGF was associated with high levels of Cx43 expression and intercellular dye coupling, and treatment of NPCs with an agent that uncouples gap junctions blocked the ability of bFGF to stimulate cell proliferation, resulting in neuronal differentiation of the NPCs. The ability of bFGF to promote self-renewal of NPCs has been well documented in previous studies (Gritti et al., 1999; Qian et al., 1997; Vicario-Abejon et al., 1995), but the requirement of gap junctions for this action of bFGF in the NPCs is a novel finding. Increased Cx43 expression, resulting in gap junction formation, is sufficient to account for stimulation of NPC proliferation by bFGF because blockade of gap junction channels abolished the mitogenic effect of bFGF, and because over-expression of Cx43 stimulated NPC proliferation in the absence of bFGF. Although the present data from cell culture studies do not allow a firm conclusion as to whether gap junctions play a similar role in the self-renewal of NPCs in vivo, proliferating NPCs in the VZ of developing murine neocortex couple together into clusters, and the developmental profile of expression of Cx43 in proliferating NPCs and its down-regulation as neurogenesis proceeds (Duval et al., 2002) strongly suggests that gap junctions promote NPC proliferation in vivo. Cx43 is expressed at high levels in proliferating NPCs in the developing rodent brain (Bittman and LoTurco, 1999) and Cx43 levels decrease as cells differentiate into neurons (Leung et al., 2002). It has been determined that intercellular coupling in the VZ is a dynamic process involving proliferating cells but not migrating and postmitotic neurons (Bittman et al., 1997). Studies of stem cell populations from non-neural tissues have also suggested roles for gap junctions in regulating stem cell fate. For example, analyses of Cx43-deficient mice have demonstrated an important role for this gap junction protein in hematopoiesis (Montecino-Rodriguez and Dorshkind, 2001). A germline-specific gap junction protein was shown to be required for the survival of differentiating early germ cells (Tazuke et al., 2002). Studies of Cx32-deficient mice suggest a role for this gap junction protein in regulating the survival of oligodendrocyte progenitor cells (Melanson-Drapeau et al., 2003). Cx43-deficient mice exhibit developmental defects in the heart consistent with a role for gap junctions in regulating the proliferation and survival of cardiac myocytes (Reaume et al., 1995). Our data suggest that the up-regulation of connexin expression and the formation of functional intercellular channels is critical for the maintenance of NPCs in a self-renewing state. When bFGF signaling is decreased, gap junctions decrease and the cells are no longer maintained in a proliferative state; and therefore differentiate into neurons.

We found that an inhibitor of ERKs also blocked bFGF-induced proliferation of NPCs, suggesting a pivotal role for ERKs in the self-renewal of these neural stem cells. Our findings are consistent with the results of another study in which it was shown that exposure of subventricular zone neural progenitor cells to bFGF and EGF results in activation of ERKs and cell proliferation, and the ability of the growth factors to stimulate cell proliferation is blocked by an inhibitor of MEK1, the kinase responsible for phosphorylation and activation of ERKs (Learish et al., 2000). Interestingly, we found that an uncoupler of gap junctions blocked the mitogenic effect of bFGF, but did not block the activation of ERKs induced by bFGF, indicating that activation of ERKs is upstream of gap junctions in the mitogenic action of bFGF. We found that bFGF induced increases in the levels of Cx43 mRNA and protein in NPCs which was associated with increased dye coupling of the cells, suggesting that bFGF increases the number of functional gap junctions in the membranes of interacting NPC. These findings are consistent with a previous report that bFGF increases the synthesis of Cx43 in cultured endothelial cells, leading to enhanced gap junction assembly and intercellular coupling (Pepper and Meda, 1993). Ghosh and Greenberg (1995) have shown that application of bFGF to embryonic cortical cells leads to rapid activation of MAP kinase and increased c-fos expression. Other studies have shown that that activator protein-1 (AP-1) sites that may bind to the transcription factor Fos exist in the promoter regions of murine and human Cx43 genes (Geimonen et al., 1996; Lefebvre et al., 1995). Therefore, it is possible that activation of MAP kinases would induce the AP-1-mediated transcription of Cx43 genes as shown by Geimonen et al. (1996). Previous work (Le and Musil, 1998, 2001) also clearly showed that bFGF increases coupling of lens cells in the eye through sustained ERKs activation, but did not increase the expression of Cx43 or other connexins expression. It is therefore possible that ERK-mediated phosphorylation of Cx43 contributes to the increased coupling of NPCs induced by bFGF. However, other findings suggest that ERK-mediated phosphorylation of Cx43 either does not affect or inhibits gap junctions in other cell types (Brandes et al., 2002; Hill et al., 1994; Ruch et al., 2001; Warn-Cramer et al., 1998; Zhou et al., 1999). Our
additional observation that overexpression of Cx43 can enhance NPC proliferation in the absence of bFGF is consistent with the possibility that transcriptional activation of the Cx43 gene is a key mechanism whereby bFGF increases gap junction formation and promotes the proliferation and survival of neural stem cells. A reduction in ERK activation, resulting from decreased levels of bFGF or other factors, decreases gap junctions resulting in cell differentiation or death.

We found that E9.5 NEP cells are significantly more sensitive to be killed by the gap junctional uncoupler GA than are E12 NPC. Cortical neurons are generated from proliferating neural stem/progenitor cells located in ventricular zone, which lines the lateral ventricle. During brain development, VZ cells also undergo lineage restriction from multipotent neural stem cell to intermediate neural progenitor cells with restricted fate and deceased self-renewal ability. Before the onset of neurogenesis, neural stem cells undergo a series of symmetric divisions, serving to expand the population of progenitor cells. During the period of neurogenesis, a second type of division is observed in which cells divide asymmetrically such that one daughter differentiates while the other reenters the cell cycle (McConnell, 1995). Difference in sensitivity to gap junction uncoupler between E9.5 NEP cells and E12.5 NPCs indicates that gap junction plays an essential role in maintaining the survival of early, highly proliferative, neural stem cells. As neurogenesis proceeds, a gradual decrease in gap junctions might play a critical role in the daughter cells exiting the cell cycle. Also at this stage, uncoupling of gap junctions would promote the progression of neural progenitor cells from a proliferative mode to a postmitotic differentiated state.

Because gap junctions provide a direct conduit for the transfer of molecules between neural stem cells, it is possible that the survival factors or mitogens induced by bFGF pass through the gap junction to promote the survival factors or mitogens induced by bFGF pass through the gap junction to promote survival of NPCs and to keep them in a proliferative state. The identity of the molecules transferred through gap junction that are critical for maintaining NPCs in a proliferative state and for promoting cell survival is an important area for future investigation.

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References

Duval, N., Gomes, D., Calaora, V., Calabrese, A., Meda, P., Bruzzone, R., 1994; Goldberg et al., 2002). Signals that stimulate Ca2+ influx or release from intracellular stores have been shown to stimulate cortical progenitor cell proliferation (Ma et al., 2000), and Ca2+ is implicated as a signal regulating the differentiation and survival of hippocampal neural stem cells (Haughey et al., 2002). Passage of ATP through gap junction could allow metabolic coupling of neural stem cells which might be important for maintaining the cells in a proliferative state. The identification of molecules transferred through gap junction channels that are critical for maintaining stem cells in a proliferative state and for promoting cell survival is an important area for future investigation.


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