Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 283 (2005) 437 – 445

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Neuregulin-1 increases the proliferation of neuronal progenitors from embryonic neural stem cells

Yun Liu[a], Byron D. Ford[b,*], Mary Anne Mann[c], Gerald D. Fischbach[c]

[a]*Sanofi-Aventis Pharmaceuticals, Inc., Bridgewater, NJ 08807, USA*
[b]*Department of Anatomy and Neurobiology, Morehouse School of Medicine, 720 Westview Drive, SW; MRC 223, Atlanta, GA 30310, USA*
[c]*Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA*

Received for publication 20 January 2005, revised 5 April 2005, accepted 27 April 2005
Available online 8 June 2005

Abstract

Neuregulins are a family of proteins expressed in the developing brain and in brain regions that continue to undergo neurogenesis in adult animals. We investigated the effects of neuregulins on embryonic neural stem cells (NSCs) isolated from E11 mouse telencephalon. Treatment of basic fibroblast growth factor (bFGF)-expanded neurosphere cultures with the EGF-like domain of neuregulin1-β1 (NRG-$1_{177-244}$) resulted in a 4-fold increase of bromodeoxyuridine (BrDU)-labeled cells, suggesting that NRG-1 stimulated proliferation. The majority of the BrdU-positive cells co-labeled with an antibody against MAP2, indicating that the proliferating cells were neuronal. No BrDU labeling was seen in GFAP- or O4-positive cells. In NRG-1-treated cultures, many of the MAP2-positive cells co-labeled with an anti-nestin antibody, suggesting that these cells are neuron-restricted progenitors (NRPs). Few MAP2/nestin-positive cells were seen in control cultures. The increase in the number of neuronal cells in NRG-1-treated cultures was due to increased proliferation of MAP2-positive cells rather than the regulation of cell survival or fate determination. These results suggest that neuregulins are mitogenic to NRPs, thus endogenous neuregulins may play important roles during CNS neurogenesis.

Keywords: Glial growth factor; Heregulin; Neurogenesis; Progenitor; Stem cell

Introduction

Neural stem cells (NSCs) of the CNS are patterned in vivo to generate neurons, oligodendrocytes, and astrocytes (McKay, 1997; Ostenfeld and Svendsen, 2003; Panchision and McKay, 2002; Qian et al., 2000; Sauvageot and Stiles, 2002). In vitro, pluripotent NSCs generate lineage-restricted, self-renewing neuron-restricted progenitors (NRPs), and glial-restricted progenitors (GRPs), which subsequently develop into fully differentiated neuron and glial cells, respectively. NRPs are mitotically active and electrically immature, and they express only a subset of neuronal markers. NRPs undergo additional changes to develop into mature, functional neurons (Kalyani et al., 1997; Mayer-Proschel et al., 1997; Mujtaba et al., 1999; Svendsen et al., 1999). NSCs, NRPs, and GRPs that undergo self-renewal in defined medium, and differentiate into multiple neural phenotypes in mass culture previously have been isolated from mouse neural tubes (Mujtaba et al., 1999).

When isolated, neuroepithelial NSCs are maintained in culture in the absence of a substrate that supports adhesion, cells form neurospheres (Kalyani et al., 1997; Mayer-Proschel et al., 1997; Mujtaba et al., 1999; Svendsen et al., 1999). Neurospheres contain a relatively homogeneous population of NSCs that undergo self-renewal in response to either bFGF or EGF. It was observed, however, that after mitogen withdrawal, the NSCs were unable to undergo neuronal differentiation directly (Ostenfeld and Svendsen, 2004). These cells emerged from the neurosphere as NRPs. These NRPs were required to go through one or more rounds

* Corresponding author. Fax: +1 404 752 1041.
E-mail address: bford@msm.edu (B.D. Ford).

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.04.038

of cell division before neurogenesis could proceed to generate neurons. The initiation of neurogenesis is modulated by multiple environmental signals, including peptide growth factors (McKay, 1997; Ostenfeld and Svendsen, 2003; Panchision and McKay, 2002).

Neuregulins and their receptors are expressed early in the developing brain in regions undergoing proliferation or neurogenesis (Corfas et al., 1995). This suggests a role for neuregulins in the regulation of neurogenesis in the developing central nervous system (CNS). Moreover, there is continued expression of neuregulin-1 (NRG-1) and its receptors in adult animals in regions that continue to undergo neurogenesis, such as the hippocampal dentate gyrus and the subventricular zone (SVZ) (Pinkas-Kramarski et al., 1997; Steiner et al., 1999). Therefore, neuregulins may play similar roles in neurogenesis in the mature nervous system. Targeted null mutations of the gene for NRG-1 or its receptors (erbB2 and erbB4) show disruptions in CNS development at embryonic day 10.5 (E10.5) (Kramer et al., 1996; Meyer and Birchmeier, 1995). This unfortunately limits the study of the functions of neuregulins in neural development since death of the knockouts precedes neurogenesis.

It has been reported that NSCs alter their properties over time and undergo distinct phases of development. In vivo, neurogenesis precedes the differentiation of oligodendrocytes and astrocytes (Abney et al., 1981; Frederiksen and McKay, 1988; Hirano and Goldman, 1988); therefore, NSCs isolated from early stage embryos mainly contained NSCs that developed into neurons while NSCs from later-stage embryos generate both neurons and glia (Qian et al., 2000; Sauvageot and Stiles, 2002). Although a few studies have demonstrated a role for neuregulins in the regulation of multipotent E14 mouse NSC cultures and NSCs from adult hippocampus (Calaora et al., 2001; Kornblum et al., 2000), the involvement of NRG-1 in the control of NRP that have committed to a neuronal lineage has not been reported. In the present study, we examined the effects of NRG-1 on NSCs and NRPs generated from E11 mouse telencephalon. Our results provide evidence that neuregulins regulate the proliferation of NRPs, suggesting an important role for neuregulins in the early stages of brain development.

Materials and methods

Neuregulin-1β EGF-like domain (NRG-1; residues 177–244) was purchased from R&D Systems (Minneapolis, MN). Dulbecco's Modified Eagle's Medium with F12 supplement (DMEM/F-12) was obtained from Life Technologies (Gaithersburg, MD). Transferrin, progesterone, selenium, putrescine, polyornithine, EGF, and FGF were obtained from Sigma (St. Louis, MO). Secondary antibodies conjugated to the fluorophore indocarbocyanine (Cy3 1:500), fluorescein isothiocyanate (FITC 1:200), or AMCA (1:50) were purchased from Jackson Immunochemicals (Westgrove, PA).

Primary neurosphere cultures

Embryos obtained from timed pregnant Swiss Webster mice (Taconic Farms, Germantown NY) at embryonic day 11 (E11) were placed in ice-cold Hanks' balanced salt solution (HBSS). Under a dissection microscope, skin and meninges were removed, and the telencephalon dissected and placed in ice-cold HBSS. Tissues were then incubated in 0.1% trypsin at 37°C for 5–10 min and washed thoroughly in HBSS. The generation of NSCs is similar to that described by Reynolds (Reynolds and Weiss, 1996). Briefly, tissues were triturated using a 200-μl pipette in serum-free growth medium (GM) containing DMEM/F12 including HEPES (5mM), glucose (0.6%), glutamine (2.5mM), sodium bicarbonate (3 mM), insulin (25μg/ml), transferrin (100 μg/ml), progesterone (20 nM), putrescine (100μM), and sodium selenite (30 nM). Cells were washed and the pellets were resuspended in 25-ml flasks to a cell density of 1×10^6 cell/10 ml in serum-free GM containing 20 ng/ml bFGF. Cultures were maintained in 37°C incubators with 5% CO_2 and 95% O_2. bFGF was added to the cultures every 2 days. In the absence of a substrate that supported adhesion, cells formed neurospheres after 5 to 7 days in bFGF-supplemented medium. Free-floating neurospheres in each culture were passaged at 7 days. For passaging, culture medium containing floating neurospheres was collected in 15-ml tubes and centrifuged at 1000 rpm. The tissue pellet was incubated in 0.1% trypsin for 5–10 min at 37°C, and then washed and triturated using 200-μl pipettes in GM. The dissociated cell suspensions were then plated in fresh GM containing bFGF to generate new neurospheres (secondary neurosphere). All neurospheres used for examining the actions of NRG-1 were passaged at least once.

Plating spheres

Sterile German glass coverslips in 24-well plates (Nunclon) were incubated overnight at 37°C in a solution of poly-L-ornithine (15 μg/ml) in phosphate-buffered saline (PBS). The coverslips were rinsed with PBS and then coated with fibronectin (1 μg/ml) for at least 6 h. Neurospheres, after 1–2 passages, were transferred onto the coated coverslips, and incubated in serum-free GM in the presence of bFGF. Cells continued to divide and migrate out of neurospheres to form a monolayer. Neurospheres were then treated with NRG-1 by rinsing free of bFGF and adding fresh GM with NRG-1 for an additional 4–5 days. The quantification was performed in the sphere outgrowth areas.

Single clone cultures

Single clone cultures were obtained after the first passage. Following dissociation of neurospheres, cells at very low density were transferred to a 100-mm petri dish. Under the dissection microscope, single cells were transferred directly onto glass coverslips (one cell/coverslip) by using a microcapillary pipette connected to an aspirator tube. The glass

coverslips were pre-coated with poly-L-ornithine and fibronectin in 24-well plates. Cells were cultured in serum-free medium with 20 ng/ml bFGF. More than 90% of cells survived and 5–15% of them could generate clones under these culture conditions. After 5–7 days, clones were treated by rinsing free of bFGF and adding fresh medium with NRG-1 for an additional 5 days.

Immunohistochemistry

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed three times in PBS buffer. Cultures were subsequently processed for immunocytochemical staining using various antibodies. A monoclonal antibody against $MAP2_{abc}$ (1:500 dilution; Sigma), polyclonal antiserum against GFAP (1:100 dilution; Chemicon, Temecula, CA), and monoclonal antibody against O4 (1:10, Chemicon) were used to identify neurons, astrocytes, and oligodendrocytes, respectively. Monoclonal or polyclonal antibodies against nestin (1:50 Developmental Studies Hybridoma Bank, Iowa City, IA) were used to identify NSCs and NRPs. Briefly, fixed cultures were blocked by incubation in PBS with 5% normal goat serum and 0.2% Triton X-100 at room temperature for 1 h. Cells were then incubated with primary antibody for either 2 h at room temperature or overnight at 4°C. Cells were washed four times with PBS, then incubated with secondary antibody for 1 h at room temperature. When labeling oligodendrocytes, Triton X-100 was excluded from the assay buffer.

Evaluation of cell viability

Neurospheres were dissociated by 0.025% trypsin-EDTA. Single-cell suspensions were plated on glass coverslips pre-coated with poly-L-ornithine and fibronectin at a density of 25,000 cells/cm^2. Cells were grown in serum-free culture medium in the absence or presence of 5 nM NRG-1. Analysis was performed after 2, 4, 6, 8, and 10 days. We assessed cell survival by using a Viability Assay Kit (Molecular Probes, Eugene, OR). The kit comprised two probes: calcein AM and ethidium homodimer-1. Calcein AM is a fluorogenic esterase substrate that is hydrolyzed to a green fluorescent product. Thus, green fluorescence is an indicator of cells that have esterase activity as well as an intact membrane to retain the esterase products. Ethidium homodimer-1 is a high-affinity, red fluorescent nucleic acid stain that is only able to pass through the compromised membranes of dead cells. The reagents were simultaneously added to the cell cultures, which were then incubated for 30–45 min. Results were analyzed by fluorescence microscopy to count the cells with green or red fluorescence labels.

BrDU labeling and detection

To label dividing cells with 5′-bromodeoxyuridine (BrDU), cultures were incubated with 100 μM BrDU for 24 h prior to fixation. After fixing with 4% paraformaldehyde for 30 min, cells were incubated with 2 N HCl for 20 min at room temperature to denature the double-stranded DNA and increase the access of BrDU antiserum. Following this, the cultures were neutralized with 0.1 M sodium borate buffer for 10 min, rinsed in PBS, and incubated with FITC-conjugated anti-BrDU antibody for 1 h at room temperature. For dual-staining experiments, cells were first stained with either anti-MAP2 or GFAP, and then processed for BrDU staining. Sections were mounted with an antifade medium containing the nuclear counterstain DAPI (4′,6-diamidino-2-phenylindole).

[^{3}H]Thymidine incorporation assay

Cell suspensions from dissociated neurospheres were plated onto poly-L-lysine- and laminin-coated 48-well Nunclon culture dishes at a density of 80,000 cells/well. After 4–5 days in the presence of bFGF, cells were rinsed free of bFGF and incubated with 5 nM NRG-1 for an additional 4–5 days. Solutions were removed and medium-containing [^{3}H]thymidine (1 μCi/ml) was added for 4 h at 37°C. At the end of the incubation, cells were fixed with methanol at −20°C for 10 min. Cells were then washed three times with 10% trichloroacetic acid (TCA) and lysed with 0.1 M NaOH/0.1% sodium deoxycholate. Lysates were transferred to vials containing scintillant and vigorously vortexed. Radioactivity was measured by liquid scintillation counting.

Results

The effect of NRG-1 on NSCs isolated from E11 mouse telencephalon

To investigate the effects of NRG-1 in multipotent NSCs, we isolated the telencephalon of E11 mouse embryos and cultured the dissociated cells as neurosphere cultures. In the present study, we treated cultures with the EGF-like domain of neuregulin-1β (NRG-1). The EGF-like domain contains the receptor-binding portion of the molecule and has been shown to display all the known biological activities of the full-length neuregulins (Fischbach and Rosen, 1997; Holmes et al., 1992). After 5–7 days in bFGF GM, the cells formed neurospheres and expressed nestin, an intermediate filament protein present in NSCs and NRPs in the developing CNS. We first examined whether the addition of NRG-1 to cell suspensions obtained from E11 mouse cortical tissue would generate neurospheres in the absence of bFGF. After 7 days in culture, we did not see a significant difference in the total number or size of neurospheres in the NRG-1-treated group compared with the untreated group (data not shown). This result demonstrated that NRG-1 alone, unlike bFGF, could not generate neurospheres. When bFGF-generated neurospheres were plated onto coated coverslips in the presence of bFGF, cells continued to divide and migrate out of the sphere

to form a monolayer (Fig. 1a). Upon withdrawal of bFGF, migrating cells differentiated into cells expressing neuronal, astrocyte, and oligodendrocyte markers. Neuronal cells were identified by labeling with the anti-MAP2 antibody (red, Figs. 1b, c). Oligodendrocytes were identified with an antibody directed against O4 (green, Fig. 1b) and astrocytes were identified with an antibody directed against GFAP (green, Fig. 1c). Morphologically, these MAP2-positive cells appeared neuronal and showed: (i) a spherical, ovoid, or pyramidal shaped soma; (ii) phase-bright appearance; (iii) branching processes (presumably dendrites) arising from the soma (Fig. 1d; same field as Fig. 1c).

NRG-1 increases the proliferation of MAP2-positive cells in neurosphere cultures

The actions of NRG-1 on bFGF-generated neurospheres were examined by plating neurospheres on coverslips as described above. Neurospheres were cultured in the absence or presence of 5 nM NRG-1 for 5 days, then co-labeled with BrDU and MAP2 or GFAP antibodies. After 5 days of treatment with 5 nM NRG-1, a dramatic increase in the number of cells surrounding the core of the neurosphere was observed in NRG-1-treated cultures (Figs. 2b, d) as compared to control (Figs. 2a, c). A 44 ± 3.3% increase in [^{3}H]thymidine incorporation was seen in NRG-1-treated cultures (Fig. 2e) that paralleled the increase in the total number of cells (Fig. 2f). More MAP2 and BrDU co-labeled cells (yellow) were found both in the central core and peripheral area of NRG-1-treated neurospheres, but few double-labeled cells were seen in the control. There was a 2.5-fold increase in MAP2-positive cells, but no increase in MAP2-negative cells, suggesting that the majority of additional cells in NRG-1-treated cultures were neuronal (Fig. 2f).

To further characterize the effect of NRG-1 on NSCs, neurospheres were cultured in the absence or presence of 1 or 5 nM NRG-1, then co-labeled with BrDU and MAP2 or GFAP antibodies. After 5 days, there was a 4-fold increase in the number BrDU-labeled cells (green) in the neurosphere outgrowth area in 5 nM NRG-1-treated group (Figs. 3b, c, and d) compared to control (Fig. 3a). A smaller, but significant increase was also observed with 1 nM NRG-1 treatment demonstrating a dose-dependent response of cells to NRG-1 (Fig. 3d). Most of the BrDU-positive cells co-labeled with the MAP2 (red, Fig. 3b), but not the GFAP antibody (red, Fig. 3c). Therefore, the increased proliferation was specific for neuronal cells and not in GFAP-positive astrocytes. The increase in number of MAP2-positive cells that co-labeled with BrDU was parallel to the increase of BrDU-positive cells, suggesting that most of the cells proliferating in response to NRG-1 were neuronal. In cultures maintained for 8 days after withdrawal of bFGF, virtually no cells showed BrDU incorporation in control cultures. By that time point, most cells had differentiated and lost the ability to proliferate. However, numerous BrDU-positive neurons were present in the NRG-1-treated group, suggesting that NRG-1

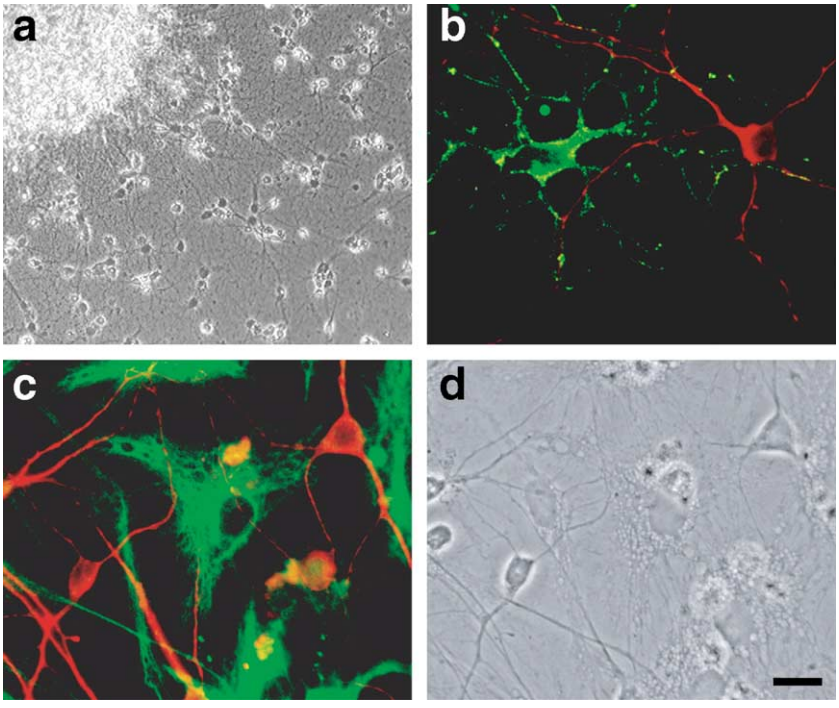

Fig. 1. NSCs migrate out from plated bFGF-generated neurospheres and differentiate into neurons, astrocytes, and oligodendrocytes. Phase contrast image shows cells migrating from attached neurosphere (a). Neurons were identified by staining with an antibody directed against MAP2 (b and c, red). Oligodendrocytes were identified with an antibody directed against O4 (b; green) and astrocytes were identified with an antibody directed against GFAP (c; green). (d) Phase image of field shown in panel c. Scale bar is 50 μm in panel a and 10 μm in panels b–d.

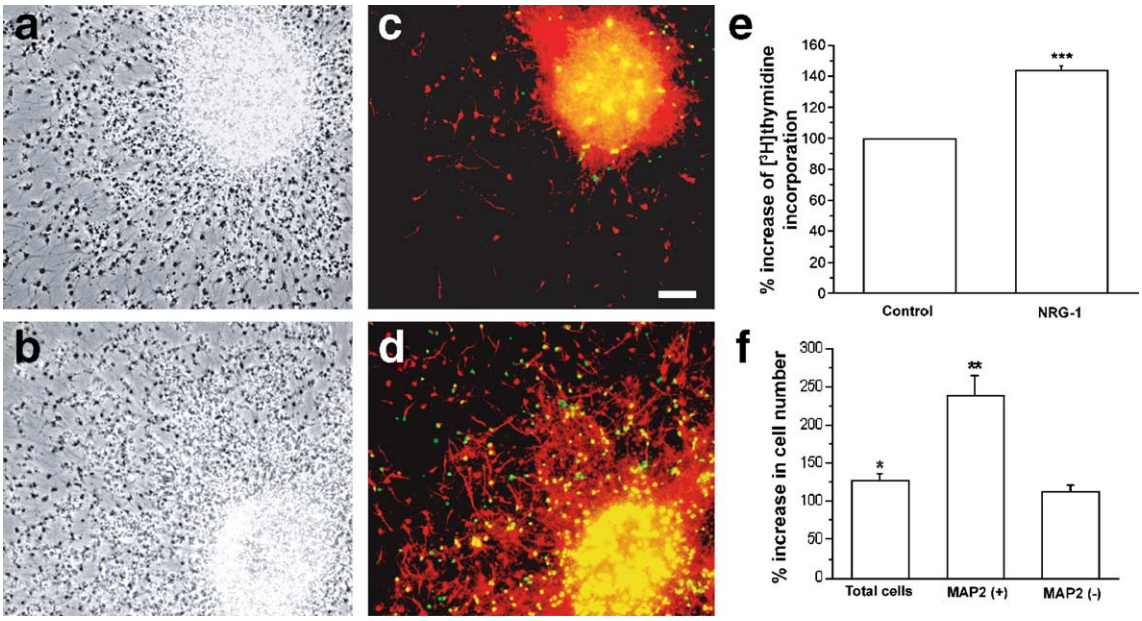

Fig. 2. NRG-1 increases the number of MAP2-positive cells. Five days after plating, neurospheres were rinsed free of bFGF and cultured for additional 5 days in the presence (b, d) or absence of 5 nM NRG-1 (a, c). Treatment with NRG-1 resulted in an increase in the number of MAP2- (red), BrDU- (green), and double-labeled (yellow) cells in the central core and outgrowth area. Few double-labeled cells were seen in the control. Scale bar is 100 μm. An increase in cell proliferation also was seen when measuring [^{3}H]thymidine incorporation (e). The data graphed in panel f show the percent increase (mean ± SEM) in the number of cells from 20 random fields in the peripheral area of neurospheres (10–15 neurospheres), in control and NRG-1-treated groups. Data show a representative result from one culture preparation of 4 independent experiments. The statistical significance of differences was tested by two-sample *t* test.

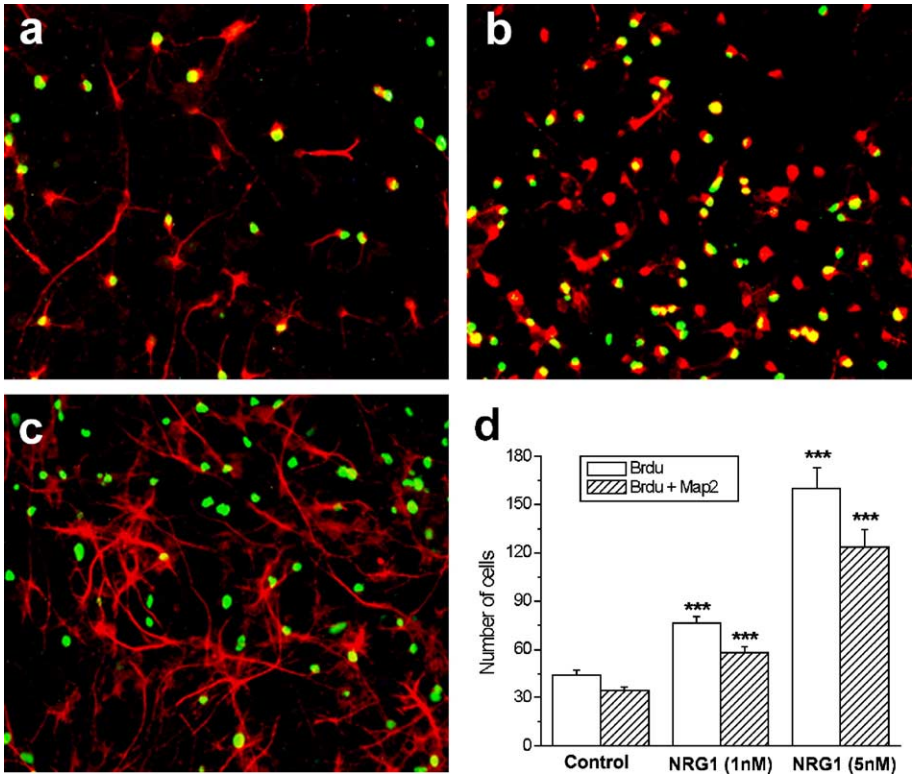

Fig. 3. NRG-1 increases the number of proliferating MAP2-positive cells. Most BrDU-positive cells (green) are double-labeled with MAP2 antibody (red; areas of co-localization are yellow) in the NRG-1-treated group (b), but not in controls (a). BrDU-positive nuclei were not seen in cells labeled with an anti-GFAP antibody (c). Scale bar is 50 μm. The total number of BrDU-positive and BrDU + MAP2 co-labeled cells increased in a dose-dependent manner in cultures treated with both 1 nM and 5 nM NRG-1 (d). The asterisks indicate that the values are significantly different from control ($P < 0.001$).

prolongs the proliferation of immature neuronal cells (data not shown). Under our culture conditions, few cells were labeled with GFAP and O4 in control cultures, or after treatment with NRG-1; therefore, the cells that labeled with BrDU alone were likely undifferentiated NSCs.

BrDU-positive neurons in NRG-1-treated cultures appeared less mature than in control cultures. These cells displayed round cell bodies with a few, short processes (Fig. 4a). We double labeled those cells with antibodies against MAP2 and nestin and found that most of the MAP2-positive neurons were also labeled by nestin in NRG-1 treated culture (Fig. 4b). In contrast, neurons in the control group were pyramidal in morphology and displayed long neurites (Fig. 4c). These neuronal cells did not co-express nestin as seen with NRG-1 treatment. Thus, it appears that the increased number of MAP2-positive cells after NRG-1 treatment was due to an increase in the number of NRPs.

NRG-1 increases proliferation rather than survival

The increased production of neurons could be altered (1) by affecting the proliferation of NRPs, (2) by affecting the differentiation of NSCs into neurons, or (3) by altering the survival of neuronal cells. Increased proliferation might result in an increase in the total number of cells as well as in the number of proliferating MAP2-positive cells; increased differentiation might result in an increase in the number of MAP2-positive cells within the same total population of cells; increased survival might result in an increase in MAP2-positive cells, but not necessarily cells co-labeled with BrDU or nestin. To determine whether the increase in the number of NRPs induced by NRG-1 was due to the increase of cell survival, we evaluated cell viability by using a Viability Assay Kit (Molecular probes). Our results showed that the total number of cells increased after 4 days in the cultures (Fig. 5a). The number of live cells was greater in NRG-1-treated group compared to control. Twice as many live cells were present in the NRG-1 group after 8 days. There was no difference in the number of dead cells in control or NRG-1-treated cultures at most time points (Fig. 5b). This result suggests that NRG-1 stimulated proliferation rather than cell survival.

NRG-1 increases the proliferation of MAP2-positive cells in clonal cultures

In order to study whether NRG-1 influenced cell lineage or promoted the differentiation of NSCs into neurons, the effects of NRG-1 were studied in single clone cultures. Single NSCs were dissociated from bFGF-generated neurospheres and plated on poly-L-ornithine- and fibronectin-coated glass coverslips in serum-free medium including bFGF. The cells divided and generated clones (Figs. 6a–c). A total of 52 clones in control and 72 clones in NRG-1 treated groups were quantified from 3 separate experiments. A significant increase in total number of cells was observed after treatment with NRG-1 (Fig. 6f) compared to control (Fig. 6d). The average number of cells generated from each clone was 69 ± 7 in control and 92 ± 9 in NRG-1-treated groups. Under our culture conditions, all clones produced more than one type of cell based on morphology and immunolabeling. Labeling of clones with antibodies against neuronal and glial cell markers revealed that the clones mainly contained MAP2-positive cells (Figs. 6e, g). GFAP- and O4-positive cells were rarely seen. The total number of MAP2-positive cells per clone was increased after treatment with NRG-1 by nearly 2-fold (36 ± 4) in the NRG-1

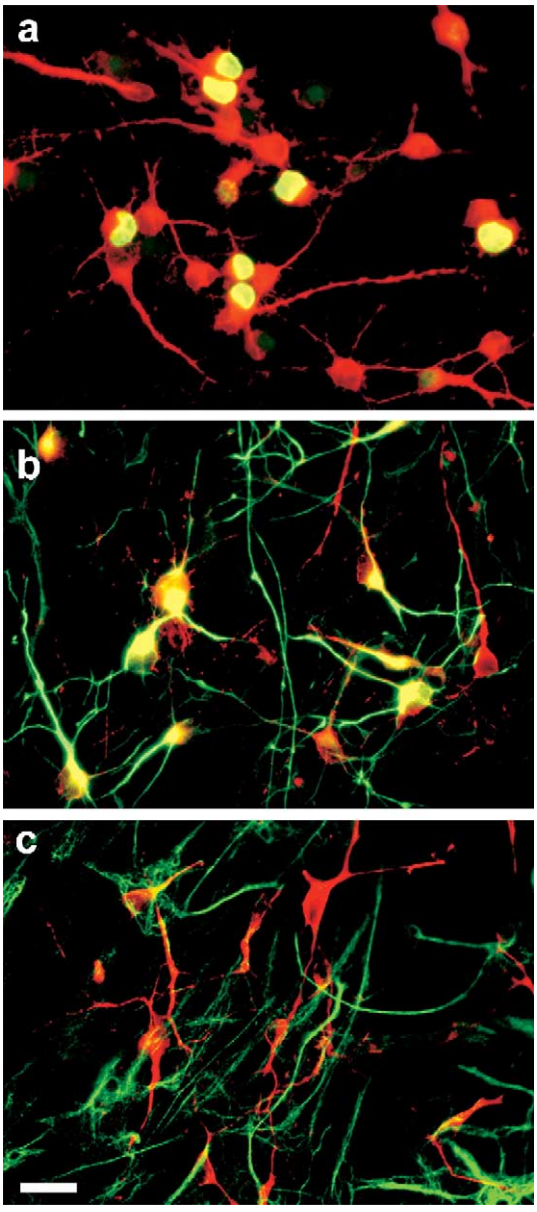

Fig. 4. NRG-1 increases the proliferation of NRPs. BrDU-positive neurons in NRG-1 treated cultures displayed round cell bodies with a few, short processes (a). Most of the MAP2-positive neurons (red) were also labeled by nestin (green, double labeled cells are yellow) in NRG-1-treated culture (b). Neurons in the control group were pyramidal in morphology and displayed long neurites and did not co-express nestin (c). Scale bar is 20 μm.

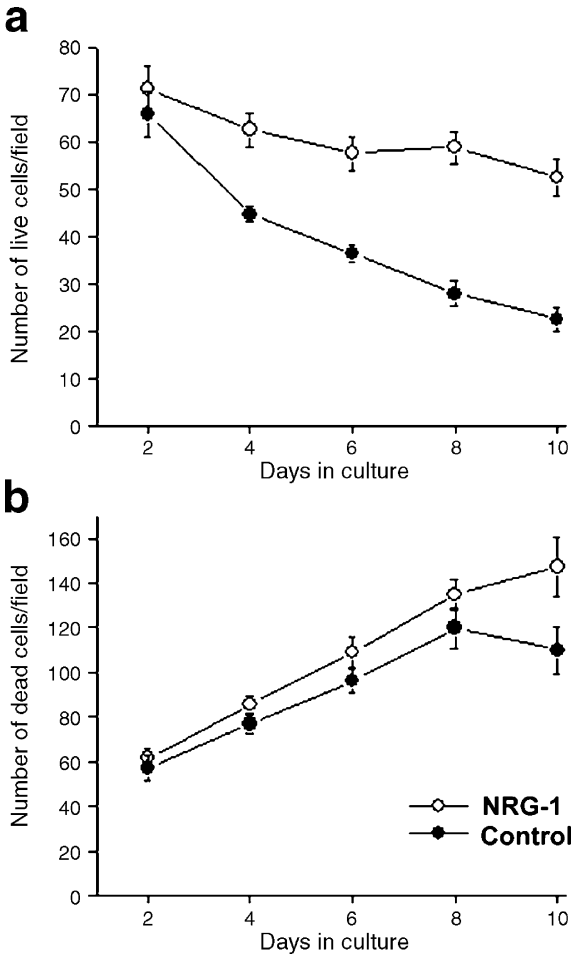

Fig. 5. NRG-1 increases cell proliferation but not cell survival. NSCs were dissociated from bFGF-generated neurospheres and cultured at low density (see Materials and methods). Analysis was performed after 2, 4, 6, 8, and 10 days after treatment with NRG-1. By day 4, the total number of live cells was greater in the NRG-1 group than in control (a). There is no decrease in the total number of dead cells (b). Experiments were performed twice; data show the mean ± SEM of 20 fields obtained from duplicate coverslips in control and NRG-1-treated cultures.

group (Fig. 6g), as compared to 19 ± 3 in control (Fig. 6e). No significant difference in the number of MAP2-negative cells was observed. The ratio of neurons per clone increased from 25.9 ± 1.7% in control to 36.2 ± 1.8% in NRG-1 treated group, suggesting that NRG-1 increased the proliferation of MAP2-positive cells and did not direct MAP2-negative NSCs to a neuronal lineage.

Discussion

NRG-1 is one of many growth factors that influence neural development (Buonanno and Fischbach, 2001; Lemke, 1996; Ma et al., 1998; Meyer and Birchmeier, 1995). Multiple roles of neuregulins have been reported, such as in the development of the neural crest (Kramer et al., 1996; Meyer and Birchmeier, 1995; Shah et al., 1994), myocardium(Kramer et al., 1996; Meyer and Birchmeier, 1995; Shah et al., 1994), Schwann cells (Garratt et al., 2000a), and oligodendrocytes (Canoll et al., 1999; Vartanian et al., 1999). In this study, we demonstrated a role for NRG-1 in the regulation of embryonic NRP cells. These results suggest that neuregulins may play additional roles in the early development of the nervous system by regulating the formation of neurons in the brain.

In the developing CNS, NSCs comprise a self-renewing cell population able to generate neurons, astrocytes, and oligodendrocytes (McKay, 1997; Ostenfeld and Svendsen, 2003; Panchision and McKay, 2002; Qian et al., 2000; Sauvageot and Stiles, 2002). A continuous supply of bFGF is necessary to repress differentiation and to maintain a population of rapidly dividing, nestin-positive NSCs. Withdrawal of the bFGF mitogen initiates differentiation within 24 h and results in a marked decrease in the number of nestin-positive cells (Ostenfeld and Svendsen, 2004; Vicario-Abejon et al., 1995). In our study, the strong mitogenic effect of NRG-1 was only obvious after bFGF withdrawal, suggesting the emergence of NRPs is necessary for NRG-1 action. The addition of NRG-1 leads to a significant increase in the number of cells co-expressing MAP2 and nestin, indicating that NRG-1 either directly induces proliferation of NRPs and/or prevents the differentiation of NRPs into mature neurons and allows them to continue proliferating in response to extrinsic or intrinsic mitogenic signals. The latter is consistent with a previous report that endogenous NDF/NRG-1, acting through the erbB4 receptor, inhibits neuronal differentiation and neurite outgrowth in E17 rat brain cultures and neuronal P19 teratocarcinoma cells (Pinkas-Kramarski et al., 1997).

As previously stated, mouse or rat NSCs isolated from early developmental periods differentiate into neurons, whereas those isolated from late prenatal and early postnatal stages differentiate into glia as wells as neurons (Qian et al., 2000; Sauvageot and Stiles, 2002). Therefore, it was not unexpected that under our culture conditions, we found few cells labeled by GFAP and O4. We assume that cells isolated from the E11 mouse telencephalon mainly contained NSCs that developed into NRPs and neurons. Previous studies of NRG-1 on NSCs found that NRG-1 did not generate neurospheres from E17 or P1 rat striatum (Kornblum et al., 2000). We saw a similar result with E11 mouse NSCs. NRG-1 also increased the size and number of neurospheres formed from P0 rat hippocampus (Lai and Feng, 2004) and an inhibitor of NRG-1 (soluble erbB3) decreased the size and number of neurospheres formed from E14 mouse striatum (Calaora, 2001). This is further evidence of an effect of NRG1 on neural stem cells. However, it is difficult to specify the type of stem cell within the neurosphere that responded to NRG-1 since the NSCs were from mice or rats at very different developmental stages. Neither group observed an increase in the proliferation of NRPs following NRG-1 treatment.

Several recent studies indicate, using mutant mice and soluble inhibitors of NRG-1, that endogenous NRG-1 can

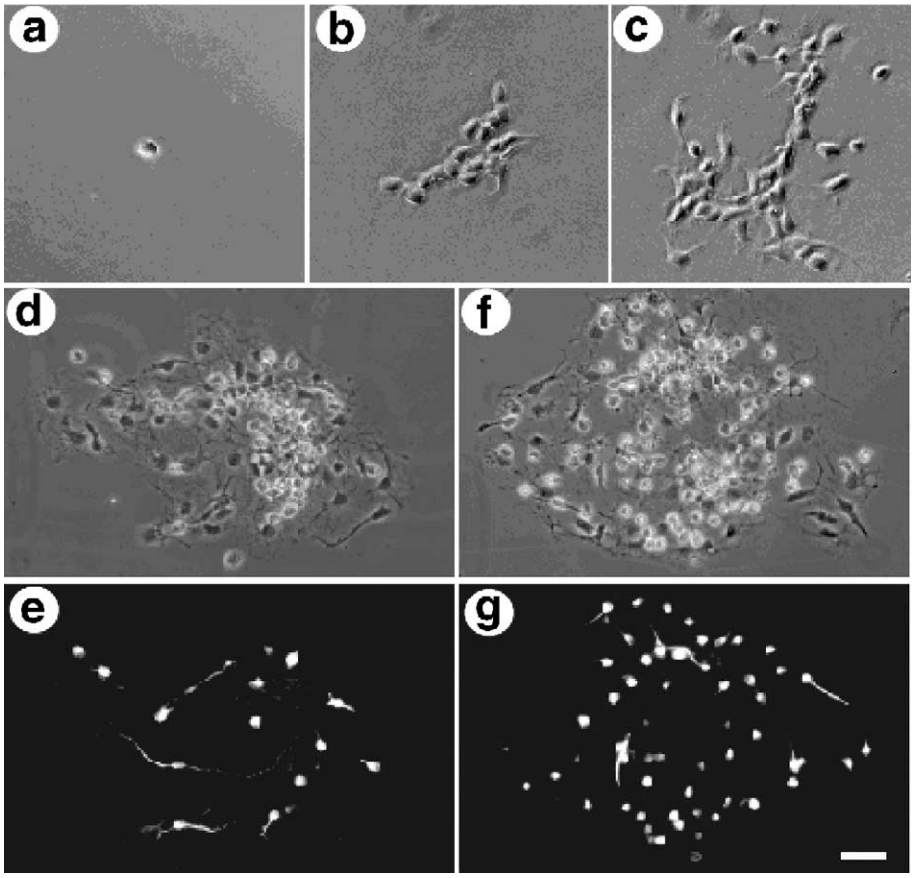

Fig. 6. NRG-1 increases the number of MAP2-positive cells in single clone cultures. Single NSCs were dissociated from bFGF-generated neurospheres and plated on glass coverslips in serum-free medium with 20 ng/ml bFGF (a). Clones are shown after 4 (b) and 7 (c) days in culture. After withdrawal of bFGF, control medium or NRG-1 was added to the medium for additional 5 days. In controls (d, e), there are few MAP2-positive neurons (red) compared to the NRG-1-treated group (f, g). Scale bar is 20 μm.

regulate the development of neuronal and glial precursors in vivo (Anton et al., 1997; Calaora et al., 2001; Garratt et al., 2000b; Kornblum et al., 2000; Park et al., 2001; Rio et al., 1997; Schmid et al., 2003). NRG-1 is endogenously present in neurogenic regions, and it is well known that neuregulins are highly expressed in cholinergic systems and in the hippocampus (Corfas et al., 1995; Kramer et al., 1996; Marchionni et al., 1993; Meyer and Birchmeier, 1995; Meyer et al., 1997). In addition, expression of NRG-1 and its receptors is upregulated after brain injury (Erlich et al., 2000; Tokita et al., 2001) and stroke in vivo (Parker et al., 2002). Interestingly, proliferation in the adult hippocampal dentate gyrus has been reported to be influenced by factors associated with stress and neural activity. Seizure activity induced by stimulation of the perforant pathway leads to a dramatic increase in cell proliferation in the dentate gyrus and the formation of new granule neurons in adult rats (Parent et al., 1997). Epileptic seizures induced by kainic acid results in increased NRG-1 expression in limbic cortical areas, hippocampus, and amygdala (Eilam et al., 1998), raising the possibility that NRG-1 is an endogenous regulator for NRP function. It will be interesting to determine whether NRG-1 induced by neural activity or brain injury influences the proliferation of NRPs in vivo in an attempt to repair the injury.

In summary, these data demonstrate that NRG-1 acts as a mitogen specifically for neuron-restricted progenitor cells. The involvement of neuregulins in the neurogenesis or proliferation of NRPs is of critical interest, and resolution of this issue may suggest mechanisms of controlling stem cell states during development and in adult animals after transplantation (Memberg and Hall, 1995). The mechanism by which NRG-1 modulates neuronal proliferation remains to be determined. Future studies will further characterize the role of endogenous neuregulins in neurogenesis in development and after neuronal injury.

References

Abney, E.R., Bartlett, P.P., Raff, M.C., 1981. Astrocytes, ependymal cells, and oligodendrocytes develop on schedule in dissociated cell cultures of embryonic rat brain. Dev. Biol. 83, 301–310.

Anton, E.S., Marchionni, M.A., Lee, K.F., Rakic, P., 1997. Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. Development 124, 3501–3510.

Buonanno, A., Fischbach, G.D., 2001. Neuregulin and ErbB receptor signaling pathways in the nervous system. Curr. Opin. Neurobiol. 11, 287–296.

Calaora, V., Rogister, B., Bismuth, K., Murray, K., Brandt, H., Leprince, P., Marchionni, M., Dubois-Dalcq, M., 2001. Neuregulin signaling regulates neural precursor growth and the generation of oligodendrocytes in vitro. J. Neurosci. 21, 4740–4751.

Canoll, P.D., Kraemer, R., Teng, K.K., Marchionni, M.A., Salzer, J.L., 1999. GGF/neuregulin induces a phenotypic reversion of oligodendrocytes. Mol. Cell. Neurosci. 13, 79–94.

Corfas, G., Rosen, K.M., Aratake, H., Krauss, R., Fischbach, G.D., 1995. Differential expression of ARIA isoforms in the rat brain. Neuron 14, 103–115.

Eilam, R., Pinkas-Kramarski, R., Ratzkin, B.J., Segal, M., Yarden, Y., 1998. Activity-dependent regulation of Neu differentiation factor/neuregulin expression in rat brain. Proc. Natl. Acad. Sci. U. S. A. 95, 1888–1893.

Erlich, S., Shohami, E., Pinkas-Kramarski, R., 2000. Closed head injury induces up-regulation of ErbB-4 receptor at the site of injury. Mol. Cell. Neurosci. 16, 597–608.

Fischbach, G.D., Rosen, K.M., 1997. ARIA: a neuromuscular junction neuregulin. Annu. Rev. Neurosci. 20, 429–458.

Frederiksen, K., McKay, R.D., 1988. Proliferation and differentiation of rat neuroepithelial precursor cells in vivo. J. Neurosci. 8, 1144–1151.

Garratt, A.N., Britsch, S., Birchmeier, C., 2000a. Neuregulin, a factor with many functions in the life of a schwann cell. BioEssays 22, 987–996.

Garratt, A.N., Voiculescu, O., Topilko, P., Charnay, P., Birchmeier, C., 2000b. A dual role of erbB2 in myelination and in expansion of the schwann cell precursor pool. J. Cell. Biol. 148, 1035–1046.

Hirano, M., Goldman, J.E., 1988. Gliogenesis in rat spinal cord: evidence for origin of astrocytes and oligodendrocytes from radial precursors. J. Neurosci. Res. 21, 155–167.

Holmes, W.E., Sliwkowski, M.X., Akita, R.W., Henzel, W.J., Lee, J., Park, J.W., Yansura, D., Abadi, N., Raab, H., Lewis, G.D, et al., 1992. Identification of heregulin, a specific activator of p185erbB2. Science 256, 1205–1210.

Kalyani, A., Hobson, K., Rao, M.S., 1997. Neuroepithelial stem cells from the embryonic spinal cord: isolation, characterization, and clonal analysis. Dev. Biol. 186, 202–223.

Kornblum, H.I., Yanni, D.S., Easterday, M.C., Seroogy, K.B., 2000. Expression of the EGF receptor family members ErbB2, ErbB3, and ErbB4 in germinal zones of the developing brain and in neurosphere cultures containing CNS stem cells. Dev. Neurosci. 22, 16–24.

Kramer, R., Bucay, N., Kane, D.J., Martin, L.E., Tarpley, J.E., Theill, L.E., 1996. Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. Proc. Natl. Acad. Sci. U. S. A. 93, 4833–4838.

Lai, C., Feng, L., 2004. Neuregulin induces proliferation of neural progenitor cells via PLC/PKC pathway. Biochem. Biophys. Res. Commun. 319, 603–611.

Lemke, G., 1996. Neuregulins in development. Mol. Cell. Neurosci. 7, 247–262.

Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J.L., Anderson, D.J., 1998. neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. Neuron 20, 469–482.

Marchionni, M., Goodearl, A., Chen, M., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., et al., 1993. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature 362, 312–318.

Mayer-Proschel, M., Kalyani, A.J., Mujtaba, T., Rao, M.S., 1997. Isolation of lineage-restricted neuronal precursors from multipotent neuroepithelial stem cells. Neuron 19, 773–785.

McKay, R., 1997. Stem cells in the central nervous system. Science 276, 66–71.

Memberg, S.P., Hall, A.K., 1995. Proliferation, differentiation, and survival of rat sensory neuron precursors in vitro require specific trophic factors. Mol. Cell. Neurosci. 6, 323–335.

Meyer, D., Birchmeier, C., 1995. Multiple essential functions of neuregulin in development. Nature 378, 386–390.

Meyer, D., Yamaai, T., Garratt, A., Riethmacher-Sonnenberg, E., Kane, D., Theill, L.E., Birchmeier, C., 1997. Isoform-specific expression and function of neuregulin. Development 124, 3575–3586.

Mujtaba, T., Piper, D.R., Kalyani, A., Groves, A.K., Lucero, M.T., Rao, M.S., 1999. Lineage-restricted neural precursors can be isolated from both the mouse neural tube and cultured ES cells. Dev. Biol. 214, 113–127.

Ostenfeld, T., Svendsen, C.N., 2003. Recent advances in stem cell neurobiology. Adv. Tech. Stand. Neurosurg. 28, 3–89.

Ostenfeld, T., Svendsen, C.N., 2004. Requirement for neurogenesis to proceed through the division of neuronal progenitors following differentiation of epidermal growth factor and fibroblast growth factor-2-responsive human neural stem cells. Stem Cells 22, 798–811.

Panchision, D.M., McKay, R.D., 2002. The control of neural stem cells by morphogenic signals. Curr. Opin. Genet. Dev. 12, 478–487.

Parent, J.M., Yu, T.W., Leibowitz, R.T., Geschwind, D.H., Sloviter, R.S., Lowenstein, D.H., 1997. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J. Neurosci. 17, 3727–3738.

Park, S.K., Miller, R., Krane, I., Vartanian, T., 2001. The erbB2 gene is required for the development of terminally differentiated spinal cord oligodendrocytes. J. Cell. Biol. 154, 1245–1258.

Parker, M.W., Chen, Y., Hallenbeck, J.M., Ford, B.D., 2002. Neuregulin expression after focal stroke in the rat. Neurosci. Lett. 334, 169–172.

Pinkas-Kramarski, R., Eilam, R., Alroy, I., Levkowitz, G., Lonai, P., Yarden, Y., 1997. Differential expression of NDF/neuregulin receptors ErbB-3 and ErbB-4 and involvement in inhibition of neuronal differentiation. Oncogene 15, 2803–2815.

Qian, X., Shen, Q., Goderie, S.K., He, W., Capela, A., Davis, A.A., Temple, S., 2000. Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron 28, 69–80.

Reynolds, B.A., Weiss, S., 1996. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev. Biol. 175, 1–13.

Rio, C., Rieff, H.I., Qi, P., Khurana, T.S., Corfas, G., 1997. Neuregulin and erbB receptors play a critical role in neuronal migration. Neuron 19, 39–50.

Sauvageot, C.M., Stiles, C.D., 2002. Molecular mechanisms controlling cortical gliogenesis. Curr. Opin. Neurobiol. 12, 244–249.

Schmid, R.S., McGrath, B., Berechid, B.E., Boyles, B., Marchionni, M., Sestan, N., Anton, E.S., 2003. Neuregulin 1-erbB2 signaling is required for the establishment of radial glia and their transformation into astrocytes in cerebral cortex. Proc. Natl. Acad. Sci. U. S. A. 100, 4251–4256.

Shah, N.M., Marchionni, M.A., Isaacs, I., Stroobant, P., Anderson, D.J., 1994. Glial growth factor restricts mammalian neural crest stem cells to a glial fate. Cell 77, 349–360.

Steiner, H., Blum, M., Kitai, S.T., Fedi, P., 1999. Differential expression of ErbB3 and ErbB4 neuregulin receptors in dopamine neurons and forebrain areas of the adult rat. Exp. Neurol. 159, 494–503.

Svendsen, C.N., Caldwell, M.A., Ostenfeld, T., 1999. Human neural stem cells: isolation, expansion and transplantation. Brain Pathol. 9, 499–513.

Tokita, Y., Keino, H., Matsui, F., Aono, S., Ishiguro, H., Higashiyama, S., Oohira, A., 2001. Regulation of neuregulin expression in the injured rat brain and cultured astrocytes. J. Neurosci. 21, 1257–1264.

Vartanian, T., Fischbach, G., Miller, R., 1999. Failure of spinal cord oligodendrocyte development in mice lacking neuregulin. Proc. Natl. Acad. Sci. U. S. A. 96, 731–735.

Vicario-Abejon, C., Johe, K.K., Hazel, T.G., Collazo, D., McKay, R.D., 1995. Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. Neuron 15, 105–114.