Role of Growth Factor Receptors in Neural Stem Cells Differentiation and Dopaminergic Neurons Generation

Lucía Calatrava, Rafael Gonzalo-Gobernado, Antonio S. Herranz, Diana Reimers, Maria J. Asensio, Cristina Miranda and Eulalia Bazán
Department of Neurobiology, Ramón y Cajal Institute for Health Research, Madrid Spain

1. Introduction

Neural stem cells (NSCs) are defined as clonogenic cells with self-renewal capacity and multilineage potential (Bazán et al., 2004). Cells with these characteristics have been isolated from the embryonic and adult Central Nervous System (CNS) (Gil-Perotín et al., 2009; Merkle & Alvarez-Buylla, 2006; Weiss et al., 1996). Under specific conditions, these cells proliferate in culture as cell clusters, called neurospheres, and differentiate into neurons, glia, and non-neural cell types (Kennea & Mehmet, 2002; Lobo et al., 2003; Reynolds & Weiss, 1992; Vescovi et al., 2002; Arias-Carrión & Yuan, 2009). Moreover, these cultures represent a potential source for cell replacement therapies in neurological diseases such as Parkinson’s disease (PD) (Bjugstad et al., 2008; Pluchino et al., 2005; Reimers et al., 2011; Zhu et al., 2009).

Both basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) promote the proliferation of NSCs (Ciccolini & Svendsen, 1998; Gritti et al., 1996, 1999; Reynolds et al., 1992; Palmer et al., 1999). Moreover, growth factors (GFs) and intracellular mechanisms have been reported to influence or even determine NSCs phenotypic choice “in vivo” and “in vitro” (Daadi & Weiss, 1999; Hagg, 2005, 2009; Ninkovic & Götz, 2007; Redondo et al., 2007; Reimers et al., 2001, 2008). Thus, bFGF in combination with agents that increase cAMP levels and/or protein kinase C (PKC) activators induced the expression of tyrosine hydroxylase (TH), which is the rate-limiting enzyme involved in the synthesis of catecholaminergic neurotransmitters (Lopez-Toledano et al., 2004).

Growth factors exert their action through their interaction with specific receptors. A subset of FGFRs have been described in NSCs and their progeny (Reimers et al., 2001, Lobo et al., 2003), but to present nothing is known regarding the role played by these different FGFRs subtypes in the TH-inductive effect of bFGF above described. NSCs also express a 170 kD protein corresponding to the EGF receptor (EGFR) (Lobo et al., 2003). Since bFGF modulates EGF responsiveness in striatal precursors (Ciccolini & Svendsen, 1998), we wonderer whether TH induction in NSCs progeny could be associated with changes in EGFR protein expression and/or cellular localization.
2. Material and methods

2.1 Isolation of neural stem cells from the embryonic rat striatum

Striatal primordia from E15 Sprague-Dawley rat embryos were dissected and mechanically dissociated. Cells were grown in suspension in a defined medium (DF12) composed of Dulbecco's modified Eagle's medium and Ham's F-12 (1:1), 2 mM L-glutamine, 1 mM sodium piruvate (all from Gibco BRL, Life Technologies Inc, Grand Island, NY), 0.6% glucose, 25 μg/ml insulin, 20 nM progesterone, 60 μM putrescine, and 30 nM sodium selenite (all from Sigma Chemical Co, St Louis, MO), 100 μg/ml human transferrin (Boehringer Mannheim GmbH, Germany) and 20 ng/ml human recombinant EGF (PreproTech EC Ltd., London, England). After 48-72 hr in vitro, the cells grew as free-floating neurospheres and were passaged by mechanical dissociation every 2-3 days (Lobo et al., 2003; Reimers et al., 2001).

After a minimum of 4 and a maximum of 5 passages, neurospheres were dissociated and plated at a density of 20,000–30,000 cells/cm² on 15μg/ml poly l-ornithine (Sigma)-coated round glass cover slips (ø12 mm) or plastic dishes (ø 35 mm). Cultures were maintained in DF12 and 20 ng/ml EGF for 3 days and then switched to DF12 without EGF for longer culture periods (control group). At 7 days postplating (dpp), parallel cultures were treated with 10 ng/ml human recombinant bFGF (Boehringer Mannheim) and 1 mM dibutryryladenosine 3,5-cyclic monophosphate (bFGF + dbcAMP) in the absence (vehicle group), or presence of 20μM PD98058 or 10⁻⁷M staurosporin. Cellular phenotypes were determined immunocytochemically 24 hr later using antibodies to β-tubulin isotype III (β-tubulin III) for neurons, glial fibrillary acidic protein (GFAP) for astrocytes, A2B5, which stains bipotential O2A glial progenitors (Raff et al. 1984; Schnitzer & Schachner 1982) as well as subsets of neurons (Schnitzer & Schachner 1982), O4 for immature oligodendrocytes, O1 for mature oligodendrocytes, and TH for catecholaminergic phenotypes.

2.2 Immunocytochemical staining

The polyclonal antibodies used in this study were anti-β-tubulin III (BabCO; Richmond, CA), anti-TH (Chemicon International;Temecula, CA), anti-DOPA-decarboxilase (Sigma, Missouri, USA), anti-dopamine transporter (DAT, Chemicon), anti-vesicular monoamine transporter 2 (VMAT2, Pel-Freez, Arkansas LLC, USA) and anti-GFAP (Dako; Glostrup, Denmark). The antibodies used for the detection of FGF receptors (FGFR1, FGFR2, FGFR3), and EGF receptor (EGFR) were from Santa Cruz Biotechnology Inc., Burlingame, CA, USA. Monoclonal antibodies against β-tubulin III were obtained from Sigma, anti-TH was obtained from Chemicon, and anti-nestin (clone Rat 401) was from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IO). Monoclonal antibodies against A2B5, O4, and O1 were obtained in our laboratory as hybridoma supernatants. The secondary antibodies used were: biotinylated goat anti-mouse IgG (Zymed Laboratories; South San Francisco, CA), streptavidin-biotin-peroxidase complex (DakoCytomation), diaminobenzidine (DAB) + substrate–chromogen system (both from DakoCytomation), Alexa Fluor-568 goat anti-mouse IgG, Alexa Fluor-488 donkey anti-rat IgG, and Alexa Fluor-488 goat anti-rabbit IgG (1:400; all from Molecular Probes; Eugene, OR), Fluorescein-conjugated goat anti-mouse IgG (1:25; Jackson ImmunoResearch Laboratories Inc, West Grove, PA), Cy3-conjugated donkey anti-
guinea pig IgG (1:500, Jackson ImmunoResearch Laboratories Inc.), and Rhodamine-conjugated goat anti-rabbit IgG (1:100, Chemicon International Inc.).

For immunocytochemical studies, cells were fixed with 4% paraformaldehyde for 10 min and immunostained for A2B5 (1:10), O4 (1:10), O1 (1:10), FGFR1 (1:100), FGFR2 (1:100), FGFR3 (1:50) and EGFR (1:50) as previously described (Reimers et al., 2001). Permeabilization for GFAP (1:500), β-tubulin III (1:200 for monoclonal and 1:3000 for polyclonal anti-β-tubulin III, and TH (1:500) was achieved by treating cultures with 0.05% Triton X-100 at 4°C for 5 min. Immunofluorescent procedures were applied for neural antigens and FGFR3 detection, and immunoperoxidase methods for FGFR1 and FGFR2 visualization. Cover slips were mounted in a medium containing p-phenylenediamine and bis-Benzimide (Hoechst 33342; Sigma).

2.3 Western blot protein analysis

NSCs progeny were treated for 24 hr with TH inducers, in the absence or presence of 20 μM PD98058 or 10⁻⁷M staurosporin, and proteins were processed for Western blot analysis to determine the relative levels of growth factor receptors (GFRs) and neural antigens. Cells were lysed with 0.5 M Tris-HCl buffer (pH 7.4) containing 0.24% Triton X-100, 10 mg/ml leupeptin, and 0.5 mM PMSF, all from Sigma. After 1 hr at 4°C, samples were centrifuged at 12,000g for 30 min. Total protein content was quantified using a BCA kit (Pierce; Rockford, IL). Aliquots of 30 μg of protein were separated by electrophoresis on 10% SDS-polyacrylamide minigels and transferred to nitrocellulose filters. Membranes were soaked in blocking solution (0.2 M Tris-HCl, 137 mM NaCl, and 3–5% dry skimmed milk, pH 7.6) and incubated with primary antibodies diluted in the same blocking solution: anti-FGFR1 (1:200), anti-FGFR3 (1:200), anti-EGFR (1:250), anti-TH (1:10,000), and anti-GFAP (1:1000), anti-β-tubulin III (1:10,000), and anti-CNPasa (1:1000). After extensive washing membranes were incubated with the peroxidase-conjugated secondary antibodies diluted 1:1000 in blocking solution. The filters were developed with enhanced chemiluminescence Western blotting analysis, following the procedure described by the manufacturer (Amersham, Buckinghamshire, England). Membranes were immunolabeled for control charge using mouse anti-β actine (1:5000; Sigma Aldrich). Autoradiograms were quantified by computer-assisted videodensitometry.

2.4 Data analysis and cell counting

For Western blot analysis, results are expressed as mean ± SEM from two to four independent experiments. Where indicated, data represent the mean ± SEM of several cover slips. For each cover slip, stereological sampling of 25 visual fields (magnification of 200x or 400x) was performed by fluorescence microscopy. The number of cells was corrected for cover slip area. Statistical analyses were performed using Student’s t-test or one-way ANOVA followed by Newman-Keuls multiple comparison test, and differences were considered significant at p ≤ 0.05.

3. Results and discussion

3.1 Acquisition of a dopaminergic phenotype in the progeny of neural stem cells

Previously, we demonstrated that bFGF in combination with the PKA activator dbcAMP induced TH immunoreactivity in a subset of neurons and A2B5-positive progenitors
derived from striatal EGF-expanded NSCs (striatal EGF-NSCs) (Lopez-Toledano et al., 2004). However, to present nothing is known regarding the ability of bFGF + dbcAMP to promote the expression of other features of dopaminergic mature neurons in these cells. As shown in Fig. 1A, bFGF + dbcAMP treatment increased by 1.5-fold TH protein expression in the progeny derived from striatal EGF-NSCs.

**Fig. 1. Induction of a dopaminergic phenotype in the progeny derived from striatal EGF-NSCs.** As shown in A-C, bFGF + dbcAMP treatment (black bars) increased the expression of TH (A), VMAT2 (B) and DAT (C). Note how 20 µM PD98059 (vertical line bars) and 10^-7 M staurosporin (horizontal line bars) prevented TH protein overexpression (A). Line 1, control cultures, line 2, bFGF + dbcAMP treated cultures, line 3, bFGF + dbcAMP in the presence of PD98059, line 4, bFGF + dbcAMP in the presence of staurosporin. Results represent the mean ± SEM of 2 independent experiments. **p ≤ 0.01 vs control.** D shows how in bFGF + dbcAMP treated cultures DOPA-decarboxilase immunoreactivity (D, green) associates with TH-positive cells (D, red, white arrows).

In the presence of 20 µM PD98059 or 10^-7 M staurosporin, which are inhibitors of the mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK1/2) and PKC, respectively, the raise in TH protein levels promoted by bFGF + dbcAMP treatment was prevented (Fig. 1A). These results are in agreement with our previous studies showing that the activation of the MAPK/ERK1/2 signaling pathway and PKC activity were required for the generation of TH-positive cells in striatal EGF-NSCs progeny (Lopez-Toledano et al., 2004). Besides TH, bFGF + dbcAMP treated cultures also showed DOPA-decarboxilase immunoreactivity that in some cases was associated with TH-positive cells (Fig. 1D). This enzyme catalyzes the conversion of levodopa (L-DOPA) in dopamine, so we may consider that under TH-inductive conditions striatal EGF-NSCs progeny is able to
synthesize this neurotransmitter which deficit is involved in the progression of PD (Aron et al., 2011). As a matter of fact, our preliminary studies indicate that bFGF + dbcAMP treatment significantly increases L-DOPA levels by 1.7-fold (p \leq 0.01 vs control). Moreover, in the presence of the DOPA-decarboxilase inhibitor NSD-1015, L-DOPA levels were significantly higher than those observed under control conditions (p \leq 0.001), or bFGF + dbcAMP treatment (p \leq 0.01). Altogether, these results strongly suggest that under our experimental conditions TH and DOPA-decarboxilase are active enzymes.

We also analyzed the effect of the TH-inductive treatment in the expression of other dopaminergic markers such as VMAT2 and DAT. Thus, bFGF + dbcAMP increased by 2-fold VMAT2 and DAT protein levels (Fig. 1B, C). The expression of both proteins was not regulated by the MAPK/ERK1/2 signaling pathway or PKC activity because neither PD98059, nor staurosporin prevented VMAT2 or DAT upregulation (Fig. 1C, D). In our cultures, bFGF + dbcAMP treatment increased the phosphorylation of the cyclic AMP response element binding protein (CREB) probably through the activation of PKA by dbcAMP (Lopez-Toledano et al., 2004). Because this transcription factor regulates the expression of catecholamine biosynthetic enzymes and transporters (Lewis-Tuffin et al., 2004; Lim et al., 2000; Watson et al., 2001), its activation could be responsible for the increase in VMAT2 and DAT protein levels observed in this study. Besides, bFGF + dbcAMP could stimulate striatal EGF-NSCs to release neurotrophins that are able to increase the expression of TH and monoamine transporters in neural precursors (Maciacyk et al., 2008; Sun et al., 2004), and in the damaged brain (Emborg et al., 2008).

### 3.2 Dopaminergic inductors modulate the expression and cellular localization of fibroblast growth factor receptors in the progeny of neural stem cells

Striatal EGF-NSCs and their progeny express a subset of FGFRs, so we were interested in to determine the role played by the different FGFR subtypes in the acquisition of dopaminergic features in these cells. FGFR1 is expressed in nestin-positive neural precursors (Lobo et al., 2003), and was down-regulated during their differentiation in neurons and glial cells (Reimers et al., 2001). Under dopaminergic-inductive conditions, FGFR1 immunoreactivity was higher than in controls (Fig.2A, C). Similarly, FGFR1 protein expression was significantly raised by more than 10-fold in bFGF + dbcAMP treated cultures (Fig. 2B). FGFR1 up-regulation was not affected in the presence of MAPK/ERK1/2 or PKC inhibitors (Fig. 2B, D), indicating that other signaling pathways are involved in FGFR1 over-expression. Growth factors are able to stimulate the phosphatidylinositol 3-kinase (PI3K)/Akt signaling transduction pathway in NSCs to promote their proliferation, survival and differentiation (Lim et al., 2007; Meng et al., 2011; Nguyen et al., 2009; Torroglosa et al., 2007). As mentioned above, FGFR1 is expressed in nestin-positive neural precursors. In our cultures, bFGF + dbcAMP treatment significantly increased nestin protein expression by 2-fold (p \leq 0.05 vs control), and neither PD98059 nor staurosporin were able to abolish this effect. Because similar results were observed under bFGF treatment (Reimers et al., 2001), our results strongly suggest that the GF promotes the survival and/or proliferation of resting FGFR1-/nestin-positive neural precursors probably through the stimulation of PI3K/Akt signaling pathway.

Dopaminergic-inductive conditions modulate FGFR3 protein expression and cellular localization. Under control conditions, FGFR3 was localized in the membrane of GFAP-positive astrocytes (Fig. 3A), in the cell bodies of β-tubulin III-positive neurons (Fig. 3B), and in the nuclei of O4-positive preoligodendrocytes (Fig. 3C). Western blot analysis revealed
that FGFR3 levels were significantly up-regulated by bFGF + dbcAMP treatment (Fig. 3D). This experimental condition changed the morphology of GFAP-positive cells that showed longer and thinner processes where FGFR3 immunoreactivity was observed (Fig. 3E). Moreover, FGFR3 immunostaining was also detected in their nuclei (Fig. 3E). bFGF + dbcAMP treatment also affected the morphology of O4-positive cells and increased FGFR3 immunoreactivity in their nuclei (Fig. 3G). Besides, some TH-positive cells showing a neuronal morphology co-expressed FGFR3 in their cell bodies (Fig. 3F). Other authors have shown the presence of two forms of FGFR3 in the nucleus of malignant and non-malignant epithelial cells (Johnston et al., 1995; Zammit et al., 2001). However, to our knowledge this is the first study reporting the nuclear localization of FGFR3 in neural cells. From our results, it is difficult to determine whether the higher levels of FGFR3 nuclear staining observed in GFAP-positive cells are due to the increase in protein expression, or to the translocation of FGFR3 to perinuclear localization.

Fig. 2. Tyrosine hydroxylase inductors upregulate FGFR1 protein expression in the progeny derived from striatal EGF-NSCs. A, C and D show FGFR1 immunocytochemical staining in control conditions (A), and in cultures treated with bFGF + dbcAMP (T) in the absence (C) or presence of 20 µM PD98059 (D). Note how in the presence of TH-inductors FGFR1 immunoreactivity is increased (C). bFGF + dbcAMP treatment also upregulates FGFR1 protein expression (B, black bar), and this effect is not prevented by 20 µM PD98059 (B, vertical lines bar) or 10⁻⁷M staurosporin (B, horizontal lines bar). Line 1, control cultures, line 2, bFGF + dbcAMP treated cultures, line 3, bFGF + dbcAMP in the presence of PD98059, line 4, bFGF + dbcAMP in the presence of staurosporin. Results represent the mean ± SEM of 2 independent experiments. **p ≤ 0.01 vs control.
Fig. 3. Tyrosine hydroxylase inductors modulate FGFR3 protein expression and cellular localization in the progeny derived from striatal EGF-NSCs. A-C show how under control conditions FGFR3 immunoreactivity (green) is localized in the membranes of GFAP-positive astrocytes (A, white arrows), cell bodies of β-tubulin III-positive neurons (B, yellow), and nuclei of O4-positive preoligodendrocytes (C). E-I show FGFR3 immunostaining in basic FGF + dbcAMP treated cultures (T). Under TH-inductive conditions, FGFR3 is localized in the nuclei of GFAP-positive astrocytes (E, white arrowheads) and preoligodendrocytes (G), and in the cell bodies of TH-positive neurons (F, yellow). Note, how astrocytes (E, red) and O4-positive cells (G, red) show longer and thinner extensions in bFGF + dbcAMP treated cultures, and how FGFR3 immunoreactivity is decreased in the presence of 20 μM PD98059 (H, green) and 10^{-7}M staurosporin (I, green). TH inductors also upregulate FGFR3 protein expression (D, black bar), and this effect is prevented by 20 μM PD98059 (D, vertical lines bar) or 10^{-7}M staurosporin (D, horizontal lines bar). Line 1, control cultures, line 2, bFGF + dbcAMP treated cultures, line 3, bFGF + dbcAMP in the presence of PD98059, line 4, bFGF + dbcAMP in the presence of staurosporin. Results represent the mean ± SEM of 4 independent experiments. *p ≤ 0.05 vs control, +p ≤ 0.05 vs vehicle.

Interestingly, the nuclear translocation of FGFRs in response to bFGF has been reported in reactive astrocytes (Clarke et al., 2001) and Swiss 3T3 fibroblasts (Maher, 1996). Moreover, the antibody used in this study recognizes a 135 kDa form of FGFR3 that showed a mix of nuclear and cytoplasmic localization in epithelial cells that depends on its degree of activation by different members of the FGF family (Zammit et al., 2001).

Similarly to TH protein expression, FGFR3 up-regulation was not observed in the presence of 20 μM PD98059 or 10^{-7}M staurosporin (Fig. 3D). FGFR3 immunoreactivity was also reduced in both experimental conditions (Fig. 3H, I). Moreover, PKC inhibition prevented the morphological changes promoted by bFGF + dbcAMP treatment (Fig. 3I). These results suggest that the modulation of FGFR3 and TH induction are two events closely associated. As a matter of fact, FGFRs nuclear localization has been related with the differentiation of neural progenitor cells (Fang et al., 2005; Stachowiak et al., 2003), and TH gene expression...
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(Peng et al., 2002). We should comment that not all TH-positive cells were FGFR3 immunoreactive (our unpublished observations), so it seems that they are not direct targets for bFGF. Under our TH-inductive conditions, GFAP protein expression was significantly raised by more than 1.6-fold (p ≤ 0.05 vs control). GFAP over-expression and morphological changes are features of reactive glia which is able to synthesize trophic factors involved in neuronal survival and differentiation (Barreto et al., 2011). Because glial derived factors are also involved in the differentiation of NSCs in TH-immunoreactive dopaminergic neurons (Anwar et al., 2008; Maciaczyk et al., 2008; Sun et al., 2004), from our results we propose that stimulation of FGFR3 localized in glial cells mediate the release of several unknown factors that in combination with PKA activators stimulate TH-induction in the target cells. In fact, glial conditioned medium in combination with PKA activators elicits the expression of TH in the progeny of striatal EGF-NSCs (Reimers et al., 2008).

We have also analyzed the effects of bFGF + dbcAMP treatment in FGFR2 expression. Under the experimental conditions presented in this study, FGFR2 nuclear localization and FGFR2 protein expression were not affected (data not shown), indicating that probably this FGFR is not involved in the acquisition of a dopaminergic phenotype in striatal EGF-NSCs progeny.

3.3 Tyrosine hydroxylase-inducing cues trigger nuclear epidermal growth factor receptor accumulation in the progeny of neural stem cells

EGFR stimulation is essential for the proliferation of striatal EGF-NSCs (Bazán et al., 1998, 2006; Reimers et al., 2001). EGFR protein expression has been detected during the differentiation of these cells in neurons and glia (Lobo et al., 2003); however, to present nothing is known regarding its cellular localization, and the role, if any, played by this receptor in the differentiation of striatal EGF-NSCs to TH-immunopositive cells. As shown in Fig. 4A, EGFR-positive cells were observed in 8 dpp control cultures. At this experimental time, EGFR immunoreactivity was localized in the cell bodies of β-tubulin III- (Fig. 4B) and O4-positive cells (Fig. 4D). Moreover, EGFR immunostaining was also observed in the membrane of GFAP-positive astrocytes (Fig. 4C). TH-inductive conditions did not affect EGFR protein expression (Fig. 4K), but promoted the translocation of EGFR to the nuclei in many cells (Fig. 4E). Nuclear EGFR immunoreactivity was observed in small spots, suggesting that the EGFR could be localized in the nucleolar compartment. Functional nuclear EGFR have been described in normal and tumoral cells (Jaganathan et al., 2011; Lo & Hung, 2007; Xu et al., 2009), but to our knowledge this is the first study reporting the nuclear localization of EGFR in NSCs and its derived progeny. Interestingly, a recent report discusses the possibility that proliferation and differentiation of NSCs could be controlled by nuclear receptors (Katayama et al., 2005). Moreover, NSCs proliferation seems to be regulated by a nucleolar mechanism that involves the interaction of proteins located in their nucleoli (Tsai & McKay, 2002). Neither EGFR protein expression (Fig. 4K), nor nuclear localization (Fig. 4I) were affected in the presence of PD98059. However, both parameters were significantly reduced in the presence of the PKC inhibitor staurosporin (Fig. 4J, K). As a matter of fact, a recent report demonstrates that PKC activation triggers nuclear EGFR accumulation in a bronchial carcinoma cell line (Wanner et al., 2008). Besides, the nuclear translocalization of EGFR may require its phosphorylation at Ser-229 by Akt (Huang et al., 2011). Further experiments are warranted to determine whether the PI3K/Akt signaling pathway mediates bFGF + dbcAMP-induced EGFR nuclear translocalization in striatal EGF-NSCs.
Fig. 4. Tyrosine hydroxylase inducers elicit nuclear translocalization of EGFR in the progeny derived from striatal EGF-NSCs. In control cultures EGFR immunoreactivity is localized in the cell bodies (A, red), and in membranes (A, white arrows). Under this condition, EGFR is expressed in the cell bodies of neurons (B, yellow) and O1-positive oligodendrocytes (D), and in the membranes of GFAP-positive astrocytes (C, open triangle). Note how bFGF + dbcAMP (T) translocates EGFR to the nuclear compartment (E, white arrowheads), and how nuclear EGFR is observed in a few β-tubulin III-positive neurons (F, white star), a few astrocytes (G, open arrow), and in many A2B5-positive progenitors (H). Neither EGFR immunoreactivity nor nuclear EGFR localization are affected in the presence of 20 μM PD98059 (I, red), but 10^{-7} M staurosporine significantly decreases both parameters (J, red). bFGF + dbcAMP treatment in the absence (K, black bar) or presence of 20 μM PD98059 (K, vertical lines bar) does not affect EGFR protein expression; however, 10^{-7} M staurosporin significantly reduces EGFR levels (K, horizontal lines bar). Line 1, control cultures, line 2, bFGF + dbcAMP treated cultures, line 3, bFGF + dbcAMP in the presence of PD98059, line 4, bFGF + dbcAMP in the presence of staurosporin. Results represent the mean ± SEM of 2 independent experiments. +p ≤ 0.05 vs vehicle.
Double immunostaining showed that some neurons (Fig. 4F) and a few GFAP-positive astrocytes (Fig. 4G) presented nuclear EGFR immunoreactivity. Besides, a population of cells showing EGFR nuclear localization colabeled with A2B5-positive cells (Fig. 4H). Emerging evidence suggest that EGFR nuclear translocalization regulates gene expression and mediate other cellular processes such as DNA repair (Chen & Nirodi, 2007; Dittmann et al., 2010; Lo, 2010). Its transcriptional activity depends on its C-terminal transactivation domain, and its physical and functional interaction with other transcription factors that lead the activation of genes (Lo & Hung, 2006). Under our experimental conditions TH is expressed in 4% of the total population of β-tubulin III-positive neurons, and 20% of the TH-positive cells colabel with A2B5 (Lopez-Toledano et al., 2004), so we may hypothesize that nuclear EGFR could regulate TH gene expression in both cell types.

![Diagram](https://via.placeholder.com/150)

**Fig. 5.** Schematic diagram illustrating how bFGF + dbcAMP treatment induces the expression of specific features of dopaminergic neurons in the progeny derived from striatal EGF-NSCs through the modulation of GFRs expression and cellular localization.

### 4. Conclusions

1. In striatal EGF-NSCs, bFGF + dbcAMP treatment up-regulates the expression of the specific dopaminergic markers TH, DAT and VMAT2.
2. FGFR1 and nestin protein levels are significantly raised in bFGF + dbcAMP treated cultures, suggesting the survival and/or proliferation of undifferentiated neural precursors under this experimental condition.
3. bFGF + dbcAMP treatment increases FGFR3 protein expression and FGFR3 immunoreactivity in the glial progeny derived from striatal-EGF-NSCs. Moreover, this experimental condition up-regulates GFAP protein levels, and elicits the translocalization of FGFR3 to the nucleus in reactive GFAP-positive astrocytes.

4. In the presence of MAPK/ERK1/2 or PKC inhibitors, TH protein expression, FGFR3 up-regulation, and glial reactivity are partially prevented, suggesting that in bFGF + dbcAMP treated cultures, the modulation of FGFR3 in glial cells and TH induction are two events closely associated.

5. Under bFGF + dbcAMP treatment, EGFR immunostaining shows a nuclear localization in β-tubulin III-positive neurons and in A2B5-positive precursors derived from striatal EGF-NSCs. Because both cell types are able to express TH, we hypothesize that the nuclear translocalization of EGFR is necessary for the induction of TH in these cells.

As summarize in Fig. 5, our results demonstrate that in striatal EGF-NSCs, bFGF and dbcAMP treatment induces TH protein expression through the stimulation of FGFR3 expressed in glia, and nuclear translocalization of EGFR in the target cells. Under these TH-inductive conditions, striatal EGF-NSCs progeny acquire other specific features of mature dopaminergic neurons, so they can be considered as an excellent tool for stem cell-based replacement therapies in PD.

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6. References


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This book is a collective work of international experts in the neural stem cell field. The book incorporates the characterization of embryonic and adult neural stem cells in both invertebrates and vertebrates. It highlights the history and the most advanced discoveries in neural stem cells, and summarizes the mechanisms of neural stem cell development. In particular, this book provides strategies and discusses the challenges of utilizing neural stem cells for therapy of neurological disorders and brain and spinal cord injuries. It is suitable for general readers, students, doctors and researchers who are interested in understanding the principles of and new discoveries in neural stem cells and therapy.

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