Molecular Mechanisms Regulating Embryonic Cerebral Cortex Development

by

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ABSTRACT

Cerebral cortex development is a complex process that integrates both extrinsic and intrinsic mechanisms. The surrounding cellular environment triggers receptor activation, which in turn initiates components of different signalling cascades and subsequently gene transcription, influencing cell survival, proliferation, and differentiation. Genetic mutations causing a loss-of-function or gain-of-function of signalling pathways elements can lead to cortical abnormalities and result in cognitive dysfunctions. In this thesis, I examined the receptor tyrosine kinase (RTK) TrkB and TrkC, the small GTPase Ras, and the C/EBP family of transcription factors, investigating their roles during cerebral cortex development. First, I looked at the role of C/EBPs during cortical cell fate determination. I determined that inhibition of C/EBPs decrease neurogenesis, keeping precursors in an undifferentiated state and later promoting their differentiation into astrocytes, while expression of an activated form of C/EBP promoted neurogenesis and reduced astrogenesis. Moreover, the inhibition of MEK, a mediator of C/EBPβ phosphorylation, also caused a decrease in neurogenesis. Thus, activation of the MEK-C/EBP pathway biases precursor cells to become neurons rather than astrocytes, thereby acting as a differentiation switch. Second I examined the involvement of Trk signalling during cortical development. I showed that genetic knockdown using shRNA, or inhibition using dominant negative of TrkB and TrkC lead to a decrease in proliferation and later to postnatal precursor cells depletion. Moreover, it caused a reduction in number of neurons combined with mislocalization of the generated neurons.
to the different cortical layers. Thus, Trk signalling plays an essential role in the regulation of cortical precursor cell proliferation and differentiation during embryonic development. Third, I elucidated the effect of Costello syndrome H-Ras mutations during cerebral cortex formation. I determined that these mutations promoted cell proliferation and astrogenesis, while reducing neurogenesis. Together, these data support a model where proper Trks/Ras/MEK/C/EBP signalling is essential for normal genesis of neurons and astrocytes and show that cortical development perturbations can ultimately lead to cognitive dysfunction as seen in Costello syndrome patients.
ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Freda Miller, for believing in me from the start, and for her support and encouragement during those challenging years as a graduate student. She has been more than a mentor to me, and in my opinion, serves as an inspirational role model for women in science. I would also like to acknowledge Dr. David Kaplan for guidance and supervision, and Dr. Cindi Morshead, who as a member of my committee has given me insightful advices. Special thanks to Dr. Ryoichiro Kageyama and his laboratory, specifically Goichi Miyoshi and Masami Sakamoto, for hosting me and a fellow student in Japan, to teach us the in utero electroporation technique. Furthermore, I would like to thank the past and present students and post-doctoral fellows of the Miller/Kaplan laboratory, especially Greg Walsh, Karun Singh, Jeff Biernaskie, Karl Fernandes, Olivia Furstoss, Christine Laliberté, Jean-François Lavoie, Ian Weaver, Monica Wetzel, Kristen Smith, Dan Lin, Smitha Paul, Mark Zander, John Vessey, and Denis Gallagher. Particular thanks to Ian McKenzie, Catherine Ménard and Fanie Barnabé-Heider, who have been excellent teachers, and Katarzyna Bartkowska, Masashi Fujitani, Julie Wasylinka, and Christian Hordo with whom I have collaborated. I am grateful to Andrée Gauthier-Fisher and Frédéric Sweeney for scientific advices, as well as strong friendships which kept me sane along those exciting, but sometimes difficult years. Finally, special thanks to Sagar Dugani for many insightful discussions and great collaborations.

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« Comme il est profond, ce mystère de l’Invisible! Nous ne le pouvons sonder avec nos sens misérables, avec nos yeux qui ne savent apercevoir ni le trop petit, ni le trop grand, ni le trop près, ni le trop loin, ni les habitants d’une étoile, ni les étoiles d’une goutte d’eau... avec nos oreilles qui nous trompent, car elles nous transmettent les vibrations de l’air en notes sonores. Elles sont des fées qui font ce miracle de changer en bruit ce mouvement et par cette métamorphose donnent naissance à la musique, qui rend chantante l’agitation muette de la nature... avec notre odorat, plus faible que celui du chien... avec notre goût, qui peut a peine discerner l’âge d’un vin! » Le Horla - Guy de Maupassant
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LIST OF ABBREVIATIONS

A-C/EBP   acidic C/EBP
AD        activation domain
ATF       activating transcription factor
BDNF      brain-derived neurotrophic factor
bHLH      basic helix-loop-helix
BLBP      brain lipid-binding protein
BP        basal precursor
BSA       bovine serum albumin
bZIP      basic region leucine zipper
CA-C/EBP  constitutively active C/EBP
CaMKIV    Ca2+/calmodulin-dependent kinase IV
cAMP      cyclic adenosine monophosphate
CAT       chloramphenicol acetyl-transferase
CBP       CREB-binding protein
CC3       cleaved caspase 3
CDKs      cyclin-dependent kinases
C/EBP     CCAAT/enhancer binding protein
CFC       cardio-facio-cutaneous
CNS       central nervous system
CNTF      ciliary neurotrophic factor
CP        cortical plate
CREB      cAMP response element binding
CS        Costello syndrome
CT-1      cardiotrophin-1
C-terminal carboxy-terminal
Cux       transcription factor cut-like
Cx43      connexion 43
DBD       DNA binding domain
DIV       days in vitro
Dlx       distal-less homeobox gene
DN        dominant negative
DNA       deoxyribonucleic acid
E12       embryonic day 12
E2F       E2 promoter binding factor
EGF       epidermal growth factor
EGFP      enhanced GFP
EGFR      epidermal growth factor receptor
Emx       empty spiracles homeobox gene
ER        endoplasmic reticulum
ERK       extracellular signal regulated kinase
FGF       fibroblast growth factor
FGFR      fibroblast growth factor receptor
FRS2      fibroblast growth factor receptor substrate 2
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Gab1</td>
<td>Grb2-associated binding protein 1</td>
</tr>
<tr>
<td>GABAergic</td>
<td>gamma-aminobutiric acid-ergic</td>
</tr>
<tr>
<td>GAPs</td>
<td>GTPase activating proteins</td>
</tr>
<tr>
<td>GCN5</td>
<td>general control nondepressible 5</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GEFs</td>
<td>guanosine exchange factors</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
</tr>
<tr>
<td>gp130</td>
<td>glycoprotein 130</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>Grb2</td>
<td>growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>Gsh</td>
<td>genetic screen homeobox gene</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HAC</td>
<td>histone acetylase</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>heparin-binding EGF</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES buffer solution</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balance salt solution</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HEK293</td>
<td>human embryonic kidney 293</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HuD</td>
<td>Hu antigen D</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Id</td>
<td>inhibitor of differentiation</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin growth factor</td>
</tr>
<tr>
<td>ILK</td>
<td>integrin-linked kinase</td>
</tr>
<tr>
<td>IP</td>
<td>intermediate precursor</td>
</tr>
<tr>
<td>IZ</td>
<td>intermediate zone</td>
</tr>
<tr>
<td>JAK</td>
<td>janus activated kinase</td>
</tr>
<tr>
<td>JMML</td>
<td>juvenile myelomonocytic leukemia</td>
</tr>
<tr>
<td>JNK</td>
<td>jun N-terminal kinase</td>
</tr>
<tr>
<td>LacZ</td>
<td>Lactose operon Z</td>
</tr>
<tr>
<td>LAP</td>
<td>liver-enriched transcriptional activator protein</td>
</tr>
<tr>
<td>LEOPARD</td>
<td>lentigines, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormal genitalia, retarded growth, deafness</td>
</tr>
<tr>
<td>Lfc</td>
<td>Lsc’s first cousin</td>
</tr>
<tr>
<td>LGE</td>
<td>lateral ganglionic eminence</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor</td>
</tr>
<tr>
<td>LIFR</td>
<td>leukemia inhibitory factor receptor</td>
</tr>
<tr>
<td>LIP</td>
<td>liver-enriched transcriptional inhibitory protein</td>
</tr>
<tr>
<td>LZ</td>
<td>leucine zipper</td>
</tr>
<tr>
<td>MAP2K</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MAP3K</td>
<td>MAPK kinase kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mash</td>
<td>mammalian achaete-scute homolog</td>
</tr>
</tbody>
</table>
MEK  mitogen activated Erk kinase
MGE  medial ganglionic eminence
MKs  MAPK-activated kinases
mRNA messenger RNA
MZ  marginal zone
NCFC neuro-cranio-facial cutaneous
NeuN neuronal nuclei
NeuroD neurogenic differentiation
NF1 neurofibromatosis protein 1
NGF nerve growth factor
Ngn neurogenin
NGS normal goat serum
Nkx2.1 NK2 homoebox 1 (also known as TTF1, thyroid transcription factor 1)
NRAMP1 natural resistance-associated macrophage protein 1
Nrg neuregulin
NS Noonan syndrome
NT-3 neurotrophin-3
NT-4 neurotrophin-4
N-terminal amino-terminal
P3 postnatal day 3
p75NTR neurotrophin receptor
PACAP pituitary adenyl cyclise activating protein
PBS phosphate buffer solution
PC12 rat pheochromocytoma cell line
PDGF platelet-derived growth factor
PDGFR platelet-derived growth factor receptor
PDK phosphoinositide-dependent kinase
PFA paraformaldehyde
PGC-α PPAR-γ coactivator-1-alpha
PI3K phosphatidylinositol-3 kinase
PKB protein kinase B
PKC protein kinase C
PLCγ phospholipase C gamma
PNS peripheral nervous system
PPAR-γ peroxisome proliferator activated receptor-gamma
pRb product of retinoblastoma gene
PTP protein tyrosine phosphatase
Rap1 ras-proximate-1
RG radial glia
RIPA radioimmunoprecipitation assay
RNA ribonucleic acid
RNAi RNA interference
RSK ribosomal S6 kinase
Rnd2 rho-related GTP-binding protein RhoN precursor
RTK receptor tyrosine kinase
Runx runt domain factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCD1</td>
<td>stearoyl-CoA desaturase 1</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Shc</td>
<td>src homology 2 domain-containing</td>
</tr>
<tr>
<td>SHP-2</td>
<td>src homology domain protein tyrosine phosphatase-2</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>SIRT1</td>
<td>sirtuin1</td>
</tr>
<tr>
<td>SOS</td>
<td>son of sevenless</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>Svet1</td>
<td>subventricular expressed transcript 1</td>
</tr>
<tr>
<td>SVZ</td>
<td>subventricular zone</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>switch/sucrose nonfermentable</td>
</tr>
<tr>
<td>Tbr</td>
<td>T-box transcription factor</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris-buffered saline tween-20</td>
</tr>
<tr>
<td>Trk</td>
<td>tyrosine kinase</td>
</tr>
<tr>
<td>UCP1</td>
<td>uncoupling protein 1</td>
</tr>
<tr>
<td>vGlut2</td>
<td>vesicular glutamate transporter2</td>
</tr>
<tr>
<td>VZ</td>
<td>ventricular zone</td>
</tr>
<tr>
<td>ZVAD</td>
<td>inhibitor of sequence Z-V-A-D(OMe)-FMK</td>
</tr>
</tbody>
</table>
CHAPTER 1: LITERATURE REVIEW

The human cerebral cortex plays key roles in memory, language, thought processing, awareness, and behaviour. It can be divided into four anatomically distinct lobes: frontal, parietal, temporal, and occipital lobes, and comprises approximately 20 billion neurons (Pakkenberg and Gundersen, 1997). A good and accessible model to study mammalian cortical development is the mouse. Although the mouse cortex may represent a simplified version of the human cortex, many genes and developmental stages are conserved between these two species. Deciphering the molecular mechanisms regulating cortical development is crucial to a better understanding of developmental disorders and neurodegenerative diseases. Therefore, this literature review will focus on the complex interplay between extracellular signals and intracellular machinery regulating cortical cell fate determination, using the mouse as a mammalian model.

1.1- Mammalian embryonic cerebral development

1.1.1-Murine cortical development

Development of the mammalian cerebral cortex is a highly complex and ordered process determined by intrinsic genetic mechanisms that are influenced by extrinsic cues such as growth factors. The right proportions of neurons, astrocytes or oligodendrocytes have to be generated at the appropriate time and cells have to migrate and localize to the right position to form a functional network. These cells originate from a single layer of rapidly proliferating precursor cells lining the neuroepithelium of the telencephalon. The
telencephalon consists of the anterior portion of the forebrain and gives rise to the cerebral hemispheres, the basal ganglia and the olfactory bulbs. In the mouse, the generation of neurons, astrocytes and oligodendrocytes can be divided into three separate waves of differentiation (Fig. 1A). Neurogenesis starts at embryonic day 12 (E12), peaks at E15, and finishes around E18. Astrogenesis starts at E18 and continues during the first weeks of neonatal life, while oligodendrogenesis only starts during the postnatal period and proceeds up to early adulthood (Qian et al., 2000; Shen et al., 2006). At a later stage, the remaining cortical precursor cells will contribute to the generation of ependymal cells present in the adult brain (Spassky et al., 2005; Willaime-Morawek et al., 2006).

The ventricular zone (VZ) accounts for the first layer of proliferative cells. These cells are radially oriented and attached to the ventricular lumen and the pial surface of the brain. They proliferate throughout embryonic development to generate neurons, astrocytes, and oligodendrocytes. Since they were first thought to produce glial cells and simply support the migration of newly born neurons, they had been termed radial glial (RG) cells. Recently, radial glia have been implicated in a variety of developmental processes including an important role as neural precursors. Here, these cortical precursor cells will be termed radial precursor cells, or simply precursor cells. The expansion of the initial pool of precursors, and the generation of the different cell types depends on cell division. According to many recent studies, three types of cell division can occur (reviewed in Casanova and Trippe, 2005; Kriegstein et al., 2006). Initially, symmetric cell division takes place to expend the pool of precursors, where one cortical precursor generates two daughter cortical precursors. Later on, asymmetric cell division occurs, where one cortical precursor generates one daughter cortical
Figure 1. Mammalian cerebral cortex development. (A) The sequential generation of different cell types during development. The three different cell types, neurons, astrocytes, and oligodendrocytes, are generated during three separate waves of differentiation. Neurogenesis starts at embryonic day 12.5 (E12.5), peak at E15.5, and then decreases. Astrogenesis starts at E18.5, while oligodendrogenesis occurs in the first few weeks postnatally. Common markers for cortical precursors, neurons, astrocytes, and oligodendrocytes are nestin, MAP2, GFAP, and O4, respectively. (B) In utero electroporation technique. The lateral ventricle of E13.5 embryos are injected with plasmids encoding mutants of the protein of interest. Following electroporation, plasmids are incorporated into cortical precursor cells lining the neuroepithelium. Brain coronal sections can later be analyzed for different markers by immunostaining.
precursor and one neuron, or one daughter cortical precursor and one intermediate precursor cell (IP, also called basal progenitor, BP). These IP cells are more restricted in terms of cell fate and they localize to the subventricular zone (SVZ), a second zone of proliferating cells localized between the VZ and the cortical layers. These cells express markers such as Svet1 (Tarabykin 2001), Cux1 and Cux2 (Nieto et al., 2004; Zimmer et al., 2004), and Tbr2 (Englund et al., 2005). Eventually, IP cells divide symmetrically to give rise to two daughter neurons (Haubensak et al., 2004; Noctor et al., 2004). It is thought that these IP cell symmetric divisions serve to increase the cell number within the same cortical layer. These cells are loosely arranged and not radially oriented. It is believed that one of the factors influencing symmetric versus asymmetric cell division is cell cycle length. The progression of cortical precursor cells from proliferative to neurogenic is generally associated with an increase in cell cycle length (Takahashi et al., 1995; Calegari and Huttner, 2003; Hodge et al., 2004; Calegari et al., 2005). Interestingly, our group has recently investigated the role of Lfc, a Rho-GEF that interacts with spindle microtubules, as well as Tctex-1, its negative regulator, during cortical cell fate determination (Gauthier et al., 2009). Genetic knockdown of Lfc was shown to maintain precursor cells in a proliferative state, while genetic knockdown of Tctex-1 increased neuronal differentiation. Moreover, Lfc knockdown was shown to increase the percentage of vertical symmetric cell division, which resulted in this increase in proliferative cells. Therefore, by regulating symmetric versus asymmetric cell division Lfc can dictate cortical cell fate determination.

The mammalian cortex is comprised of six layers that are formed from proliferating precursor cells within the germinal zones (VZ and SVZ) mentioned above and consist of the
marginal zone (MZ, layer I) and the cortical plate (CP, layer II-VI). The six different layers composing the cortical cortex are generated in an inside-out manner, with the deeper-inner layer generated first and the most outer layer generated last. Therefore, the laminar position of each neuron is mainly determined by their birth date, and temporal differentiation perturbations can lead to mislocations of these neurons (Mizutani and Saito, 2005).

Moreover, transplantation experiments where precursors from inner layers (layer IV or V) were transplanted into older brain generating outer layers (layer II and III) revealed that differentiating neurons could adapt to the new laminar fate and properly integrate into the cortical architecture (Desai and McConnell, 2000, Shen et al., 2006).

1.1.2- The origin of neurons and glia in the cortex.

The embryonic telencephalon can be divided into two distinct structures: the dorsal telencephalon, also called the pallium, and the ventral telencephalon, also called the subpallium. Both contribute to the generation of neurons, astrocytes and oligodendrocytes that populate the murine cerebral cortex. However, both structures are specialized in the subtype of neurons they can produce and the pattern of migration through which the cells reach their final destination. The neuroepithelium of the dorsal telencephalon gives rise to glutamatergic projection pyramidal neurons, astrocytes, and some oligodendrocytes. Differentiated cells originating from the VZ/SVZ migrate radially to the cortical plate. As mentioned in the previous section, the cortical plate is built in an inside-out manner, therefore the first cells migrate radially to the inner-most layer while the later-born neurons migrate radially past the first-generated cortical layers and to the outer-most portion of the
cortical plate. Brain structures generated by the dorsal telencephalon include the hippocampus, the olfactory cortex, and the neocortex.

Radial migration has long been recognized as the primary mechanism by which newly-born neurons reach their final position in the cortex. However, a major proportion of the GABA(\(\gamma\)-aminobutiric acid)ergic inhibitory interneurons populating the cerebral cortex originate from the ventral telencephalon and have to travel tangentially to the cortical plate (Nadarajah and Parnavelas, 2002). The ventral telencephalon consists of the medial and lateral ganglionic eminences (MGE and LGE, respectively) which will subsequently make up the basal ganglia (Parnavelas, 2000; Kessaris et al., 2006). At mid-embryonic stages (E12.5-14.5) GABAergic interneurons from the MGE migrate tangentially to the SVZ and intermediate zone (IZ), which is situated between the SVZ and the cortical plate, to then reach their final destination and populate the cortical plate by radial migration (Anderson et al., 2001; Wichterle et al., 2001). Conversely, the GABAergic interneurons originating from the LGE migrate tangentially all the way to the olfactory bulbs (Stenman et al., 2003; Tucker et al., 2006).

During neurogenesis, neuronal precursor cells express a panel of different neuronal genes over time, which dictate what type of neuron will be generated. This mechanism is responsible for the neuronal diversity observed in the brain. A regional or temporal change in the expression of these cell identity genes will affect the neuronal subtype of these neuronal precursors, the boundaries within which those cells reside, and the dorso-ventral patterning of telencephalon. For example, in the dorsal telencephalon, differentiating cortical precursors express Tbr1, Tbr2, Emx1, and Emx2, which regulate neuronal identity towards a cortical
projection neuron phenotype (Hevner et al., 2001; Cecci and Boncinelli, 2000; Muzio and Mallamaci, 2003; Englund et al., 2005). In addition, neurogenic proteins such as neurogenin1 (Ngn1) and neurogenin2 (Ngn2) play a role by promoting this same cell fate and repressing ventral telencephalic identity. The absence of Ngn2 or Ngn1 and Ngn2 results in perturbations of projection neuron differentiation and causes them to adopt a GABAergic interneuron phenotype (Schuurmans et al., 2004). Moreover, a recent study has also revealed a role for Ngn2 in migration by inducing the expression of Rnd2, a small GTP-binding protein (Heng et al., 2008).

In the ventral telencephalon, Nkx2.1, and Gsh2 are transcription factors known to regulate regional specification and migration of GABAergic interneurons (Fogarty et al., 2007; Nobrega-Pereira et al., 2008). The loss or overexpression of these genes will affect the boundaries between the LGE and MGE as well as the pool of neuronal precursors in each region. As a consequence, the number of interneurons migrating to the dorsal telencephalon or olfactory bulbs can be affected (Sussel et al., 1999; Stoykova et al., 2000; Yun et al., 2001). Moreover, cells from the MGE express Dlx genes and Mash1, which are essential for interneuron cell identity (Anderson et al., 1997; Marin et al., 2000). These genes both regulate the timing of neurogenesis and induce the GABAergic interneuron phenotype in the ventral telencephalon.

Another ventrally derived cell population that migrates tangentially to the developing dorsal telencephalon is oligodendrocytes. In vivo, most of the oligodendrocytes populating the cortex originate from the ventral telencephalon (Spassky et al., 1998), and they are generated in separate waves depending on their regional origin within the ventral
telencephalon (Ivanova et al., 2003; Kessaris et al., 2006). Interestingly, these 
oligodendrocytes are generated from a common pool of precursors that produced neurons 
beforehand (He and al., 2001; Hack et al., 2004). This switch in cell fate determination is 
regulated by the combination of expression of different genes. For example, to promote the 
formation of GABAergic interneurons Dlx1 and Dlx2 negatively regulate the expression of 
Olig2, an important pro-oligodendrocyte gene. As mentioned previously, the proneuronal 
gene Mash1 plays a critical role during neurogenesis. Conversely, it allows for the formation 
of oligodendrocyte precursors by restricting the expression of Dlx1 and Dlx2 and therefore 
allowing Olig2 expression later on during development (Parras et al., 2007; Petryniak et al., 
2007). In a similar way, mice deficient in Ngn2 and Mash1 show a reduction in neurogenesis 
and a propensity to generate glial cells prematurely (Nieto et al., 2001).

The radial or tangential migration of differentiating cells is a process that depends on 
different molecular mechanisms regulated by physical contact with surrounding cells and 
extrinsic molecular cues (Ayala et al., 2007). Physical contact with the cells already 
composing the developing embryonic cortex provides support for the new differentiating 
cells during migration. Radial precursor cells serve this purpose since their extended 
processes connect the pial ventricular surface and the cortical plate. They guide the migration 
of cells to their appropriate location in the different cortical layers. Furthermore, this journey 
along the radial fibers is mediated by connexins, which provide adhesive contact between 
newly-born neurons and precursors during migration (Elias et al., 2007; Wiencken-Barger et 
al., 2007). More specifically, recent work has examined neuronal migration in a conditional 
mouse model where connexin 43 (Cx43) has been knocked down in cortical precursors (Cina 
et al., 2008). In these mice, newly-born neurons mislocalized to the intermediate zone and
did not migrate to the cortical plate, exhibiting a significant decrease in neuronal migration compared to control mice, and revealing a critical role for Cx43 in neuronal development. Interestingly, recent studies showed that blood vessels can serve as scaffolds for neuronal migration as well (Bovetti et al., 2007; Honda et al., 2007). However, the mechanisms governing these interactions remain to be elucidated.

Besides physical interactions, cell migration is influenced by extrinsic molecular cues. For example, Cajal-Retzius cells located in the marginal zone secrete reelin, a large extracellular matrix glycoprotein that attracts cortical neurons and enable them to pass their predecessors (reviewed in Rice and Curran, 2001). In the reelin knockout mouse, termed reeler mouse, the cortex is built in an outside-in manner, which suggest a critical role for reelin in migration and the establishment of the proper inside-out patterning in wild type mice. Neuregulins are another molecular cue that influences cell migration by guiding the neurons along the the radial precursor cells and to their final destination in the cortical plate (Anton et al., 1997; Anton et al., 2004).

1.1.3- Models to study cortical development.

To study the molecular mechanisms regulating mammalian cortical development in vitro, our laboratory utilizes murine primary cell culture of cortical precursor cells. Cortices of E12-13 mouse embryos are dissected out, dissociated and plated in a culture dish. This technique has been previously described, and has demonstrated that cortical precursor cells follow the same patterns of differentiation seen in vivo (Davis and Temple, 1994; Ghosh and Greenberg, 1995; Qian et al., 1997). Upon plating, the precursor cells are proliferating, and
they generate neurons for the following five days. After this period of neurogenesis, astrocytes start to differentiate and later on oligodendrocytes are produced. The in vitro protocols developed in our laboratory are well established, widely used and serve as a powerful tool for investigating the mechanisms which regulate cortical development.

Classical techniques to study development in vivo have been to generate knockout or transgenic mouse models. These are usually great in vivo models, but they are costly and time-consuming to generate. Moreover, in some cases, the knockdown of ubiquitously expressed proteins causes lethality during embryogenesis, making the knockout mouse an inadequate tool. Mosaic mouse models can now be generated with tissue-specific protein expression knockdown. The Cre-lox recombination system allows the targeting of genes in a tissue- and time-specific manner (reviewed in Sauer, 1998). Cre is a site specific recombinase that recognizes and excises the DNA between two loxP sites, which can conveniently be inserted in the targeted gene. A common approach is to crossbreed a transgenic mouse expressing Cre under a tissue specific promoter with a mutant mouse genetically manipulated to contain loxP sites in the gene of interest. For example, some groups created a mutant mouse that expresses Cre under the control of the nestin promoter, a gene expressed in dividing and proliferating cortical cells (Lendahl et al., 1990). This transgenic mouse can then be crossed with another containing loxP sites inside the gene of interest. This loss-of-function experiment allows the study of genes during cortical development or even postnatally (Knoepfler et al., 2002; Balordi and Fishell, 2007; Ishii et al, 2008).
A useful technique to alter gene expression in a cell autonomous manner is the injection of retroviruses during embryonic development (Burrows et al., 1997; Stott et al., 2006). Similarly, DNA plasmids encoding mutant proteins and siRNA oligos can also be injected into the ventricles of embryonic brain and electroporated into precursor cells located in the ventricular zone (Ohtsuka et al. 1999; Young-Pearse et al., 2007; Fig. 1B). RNA interference (RNAi) technology has gained a lot of interest and is used routinely to recognize homologous mRNA sequences and target them for degradation (Hannon, 2002; Tijsterman and Plasterk, 2004). These in utero injections and electroporations make for a powerful technique since they are relatively cost- and time-effective allowing the study of different stages of cortical development.

1.2- The extracellular signals regulating cortical precursor cells survival, proliferation, and differentiation.

During embryogenesis, the integration of different signals received at the cell membrane is crucial for modulation of precursor cell biology. Although cell intrinsic mechanisms clearly have a significant role in determining cellular fate, various growth factors and ligands regulate cell survival, proliferation, and differentiation by binding to their specific receptors and initiating signal transduction. These factors can act in an autocrine manner, where the cell expresses both the ligand and the receptor, or in a paracrine manner, where a cell expresses the ligand and the adjacent cell expresses the receptor. Alternatively, ligands can be expressed at a distance from cells expressing the receptors, at times in a gradient fashion, to act as morphogens and influence cell fate determination. These external
signals are crucial for the spatio-temporal regulation of cellular response. In this section, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), neurotrophins, and cytokines implication during mammalian cortex development will be reviewed.

1.2.1- FGF and PDGF

The fibroblast growth factor (FGF) family of growth factors is composed of more than 20 members that signal through tyrosine kinase FGF receptors (FGFRs). FGF signalling plays a role in many events during mammalian development including proliferation, migration, morphogenesis, and patterning (Yamaguchi et al., 1994; Reuss and von Bohlen und Halbach, 2003; Cayuso and Marti, 2005; Rhinn et al., 2006). Specifically with regard to cortical precursors, FGF2 (also known as basic FGF, bFGF) is endogenously expressed in the developing telencephalon. It has been shown to promote cell cycle renewal and maintain cortical precursors in an undifferentiated state (Ghosh and Greenberg, 1995; Qian et al., 1997; Vaccarino et al., 1999; Raballo et al., 2000; Zheng et al., 2004). However, FGF signalling can have pleiotropic effects depending on i) the other growth factors present in the cellular environment and ii) the temporal expression of its ligands and receptors. In contrast to stem cell pool maintenance, in vitro studies also reported that FGF2 can increase neuronal differentiation when in presence of other growth factors such as BDNF and NT-3 (Murphy et al., 1990; Vicario-Abejon et al., 1995). Furthermore, studies revealed that brain infusion with FGF2 can increase the number of neurons or astrocytes depending on the time of injection (Vaccarino et al., 1999). When the injection was done during neurogenesis (E15.5), the number of neurons was increased, whereas when the injection was done later during
development (E20.5), it was the number of astrocytes that was increased. After injury, FGF2 was shown to promote proliferation and differentiation of precursors into neurons in the hippocampus (Yoshimura et al., 2001). Together, these experiments suggest a role for FGF2 in the expansion of neuronal or astroglial precursor pools.

FGF2 knockouts are viable but present cerebral cortex defects. Their brains are smaller than wild type littermates, they have a decreased number of dividing progenitors in the ventricular zone, and a reduced neuronal density with no noticeable difference in survival (Dono et al., 1998; Ortega et al., 1998; Vaccarino et al., 1999; Raballo et al., 2000). Interestingly, they revealed a decrease in cortical pyramidal neurons, but no change in GABA revealing neuronal subtype specificity (Korada et al., 2002). This concurs with experiments done by Vaccarino and colleagues where strong expression of FGF2 in the dorsal telencephalon and very low levels in the ganglionic eminence was observed (Raballo et al., 2000). In addition, the FGF2 null animals present a decrease in the number of cortical astrocytes and a more permeable blood brain barrier (Reuss et al., 2003). They also display an increase in the number of oligodendrocytes, which would suggest a role for FGF2 in the inhibition of oligodendrocyte differentiation (Murtie et al., 2005). This indicates once more the pleiotropic potential of FGF2 on different cell types. More recently, a FGF2 knockout mouse specifically deleting the alternative exon 3 revealed similar phenotypes with decreased numbers of neurons and astrocytes, and deficits in the proliferation of precursors (Chen et al., 2008). Furthermore, these mice exhibited a decrease in proliferation of adult cortical precursors, a phenotype that has not been previously observed in the FGF2 null animals. Following injury, the absence of FGF2 promotes oligodendrogenesis and remyelination (Armstrong et al., 2002; Jungnickel et al., 2004).
Figure 2. Platelet-derived growth factor (PDGF), neurotrophins, cytokines and their receptors (A-B) PDGF ligands and their affinity to the PDGFR isoforms, in vitro (A) and in vivo (B). (C) Neurotrophins and their affinity to Trks receptors. (D) Cytokines and their affinity to LIFR, gp130, and CNTFR.
FGF2 can signal through FGF receptor1 (FGFR1), FGF receptor2 (FGFR2), and FGF receptor3 (FGFR3), which are expressed in the developing cortex by precursors and neurons (Orr-Urtreger et al., 1991; Peters et al., 1993; Raballo et al., 2000). FGFR1 or FGFR2 knockout is embryonically lethal due to early neural defects (Yamaguchi et al., 1994; Arman et al., 1998), while FGFR3-/− mice are viable but exhibit inner ear and bone defects (Deng et al., 1996; Colvin et al., 1996). Interestingly, a transgenic mouse model of FGFR3, where a specific mutation on the receptor causes constitutive tyrosine kinase activity was generated. The animals present larger brains and increase cortical thickness due to an increase in survival and proliferation (Inglis-Broadgate et al., 2005). Together with the studies done on FGF2 knockout mice, these data confirm the crucial mitogenic role for FGF signalling during mammalian cortical development.

The platelet-derived growth factor (PDGF) family of growth factors was identified over 25 years ago. It comprises four different ligands: PDGF-A, PDGF-B, PDGF-C, and PDGF-D and two different receptors: PDGFR-α and PDGFR-β. Ligands bind the receptors as dimers and signal transduction is initialized when receptors dimerize and autophosphorylate. Many in vitro studies have shown the interaction between the different ligands and receptors (summarized in Fig. 2A). In vitro, PDGF-A and PDGF-B can homo- and heterodimerize, while PDGF-C and PDGF-D can only homodimerize. With regard to the receptors, PDGFR-α and PDGFR-β can both homo- or heterodimeraze. The ligand PDGF-AA can only bind PDGFR-α, PDGF-BB can bind all three combination of receptors: PDGFR-αα, PDGFR-ββ, and PDGFR-αβ, while PDGF-AB binds to PDGFR-αα and
PDGFR-αβ. Similarly, PDGF-CC interacts with PDGFR-αα and PDGFR-αβ, while PDGF-DD binds PDGFR-αβ and PDGFR-ββ. However, because the spatio-temporal regulation of PDGF expression pattern of the different ligands in tissues is usually not overlapping, this suggests that homodimers appear to dominate during development and that heterodimerization is a rare event in vivo (Hoch and Soriano, 2003). This results in known interactions between PDGF-AA and PDGFR-αα, PDGF-BB and PDGFR-ββ, PDGF-CC and PDGFR-αα, while PDGF-DD pattern of expression and interaction in vivo still remains to be elucidated (Fig. 2B).

In the developing mammalian cortex, precursor cells express PDGFR-α receptor, PDGF-A, and PDGF-C, while neurons express both PDGF receptors, PDGF-A, PDGF-B, and PDGF-C. With regard to astrocytes and oligodendrocytes, they both express PDGFR-α and PDGF-A (Pringle et al., 1992; Schattman et al., 1992; Sasahara et al., 1992; Omesmar et al., 1997; Fruttiger et al., 2000). To determine the role of PDGF signalling in vivo, several experiments have been conducted including the generation of knockout mouse models. It was demonstrated that disrupting any of the genes encoding for PDGF ligands or PDGF receptors resulted in embryonic or early postnatal lethality. More specifically, PDGFR-α and PDGF-A null animals showed defects in the neural tube closure, reduced numbers of oligodendrocytes, hypomyelinated brains and optic nerves as well as defects in oligodendrocyte migration (Fruttigier et al., 1999; Joosten et al., 2001; Klinghoffer et al., 2002). Surprisingly, PDGFR-β and PDGF-B mice knockout analysis did not reveal neurogenesis defects even if precursors and neurons express these ligands and receptors in vivo (Fruttiger et al., 1999; Enge et al., 2003). These mutants were shown to mostly die from haematological and cardiovascular anomalies (Leveen et al., 1994; Soriano, 1994). Neuron-
specific ablation of PDGF-B did not show any phenotype besides cellular susceptibility to injuries, suggesting a role for PDGF in neuroprotection (Enge et al., 2003; Egawa-Tsuzuki et al., 2004; Ishii et al., 2006). More recently, studies done with PDGFR-β -/- neurospheres revealed a higher propensity to apoptosis, indicating a requirement for PDGF signalling for cell survival (Ishii et al., 2008). Generation of a knockout mouse for PDGF-C revealed craniofacial and neural tube defects (Ding et al., 2004). Moreover, double knockout of PDGF-A and PDGF-C showed similar phenotypes to the PDGFR-α knockout mice, suggesting that both of these ligands are able to signal through the PDGFR-α during early development.

A knock in experiment of PDGFR-β at the PDGFR-α locus showed that PDGFR-β signalling can substitute for PDGFR-α (Klinghoffer et al., 2001). The reverse experiment also revealed a partial rescue of PDGF signalling suggesting a possible compensation mechanism between isoforms. Interestingly, while PDGFR-β and PDGF-B knockout phenotypes are similar, PDGFR-α and PDGF-A knockouts differ in the severity of their phenotypes. The PDGFR-α null embryos do not survive past E15, while PDGF-A mutant mice survive during the first few weeks postnataly (Bostrom et al., 1996; Tallquist and Soriano, 2003). Together with the studies done on the PDGF-C null mice, this would indicate a role for PDGF-C signalling through PDGFR-α and potential isoform compensation in vivo.

Pioneering experiments performed by Raff and Noble two decades ago revealed that PDGF ligands are playing a role in the timing of oligodendrocyte generation in vivo (Raff et al., 1988). They cultured rat embryonic optic nerve cells in the presence of PDGF or astrocytes, and looked at the number of oligodendrocyte precursors. They were able to show
that astrocytes can secrete PDGF and that this was the signal that drove the generation of oligodendrocyte precursors. This provided strong evidence for sequential cellular differentiation regulated by growth factors endogenously secreted. More recently, treatment of oligodendrocyte precursors with PDGF-A revealed that PDGFR-α signalling promotes proliferation, but also inhibit oligodendrocyte maturation. Oligodendrocyte precursors can differentiate into myelinating oligodendrocytes only once the PDGF-A ligand concentration is reduced (van Heyningen et al., 2001). It has also been demonstrated that overexpression of PDGF-B in neural progenitor can induce the formation of oligodendrogliomas and oligodendrocytomas (Dai et al., 2001).

PDGF signalling is primarily paracrine. Under biological stress and in cancers, PDGF has been shown to signal in an autocrine fashion as well. Dimerization of the receptor is key to its activation, followed by autophosphorylation that later leads to initiation of signalling cascades including MAPK, PI3K, and PLCγ. Cellular responses to PDGF take from seconds to minutes after PDGFR activation and the duration is limited by dephosphorylation of the cytoplasmic domain of the receptor, or internalization and targeting of the receptor for lysosomal degradation.

1.2.2- Neurotrophins

The neurotrophins are growth factors that are well-known for regulating the biology of neural stem cells. They can regulate survival, proliferation, and differentiation (Gao et al., 1995; Huang and Reichardt, 2003; Kaplan and Miller, 2000), as well as influence neuronal neurite outgrowth (Segal et al., 1995; Miller and Kaplan, 2003). The neurotrophins
include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4), all of which are expressed throughout the developing nervous system (Maisonpierre et al., 1990; Behar et al., 1997; Fukumitsu et al., 1998; Barnabé-Heider and Miller, 2003; Fukumitsu et al., 2006). Neurotrophins bind with high affinity to different receptors: TrkA, TrkB, TrkC, and p75NTR (Fig. 2C). Here, the role of neurotrophins in the nervous system will be discussed, while their receptors and the signalling cascades they initiate will be reviewed in the next section.

In the past 20 years, extensive work has been done to understand the role of neurotrophins during mammalian development. NGF was the first to be identified and found to specifically bind the TrkA receptor (Kaplan et al., 1991). Initial experiments showed a critical role for NGF for neuronal survival and growth (Hamburger and Levi-Montalcini, 1949). Generation of knockout mice confirmed these early studies and revealed a loss of sympathetic ganglia neurons and dorsal root ganglia sensory neurons. Moreover, these animals also displayed insensitivity to pain due to loss of nociceptive sensory neurons (Crowley et al., 1994; Smeyne et al., 1994). Interestingly, human patients showing lower sensitivity to pain present mutations in the trkA gene (Indo et al., 1996). BDNF specifically binds to TrkB and promotes survival of cortical precursors and neurons (Ghosh and Greenberg, 1995; Barnabé-Heider and Miller 2003). BDNF -/- newborns die shortly after birth, and display neuronal deficiencies (Jones et al., 1994; Ernfors et al., 1994). They experience trouble with body balance explained by increased death of cerebellar granule cells (Schwartz et al., 1997) and defects in the vestibular system (Ernfors et al., 1994; Johns et al., 1994; Schimmang et al., 1995). BDNF not only promotes neuronal survival, but also enhances axonal elongation (Segal et al., 1995). Moreover, in adult animal living in an
enriched environment, BDNF has been shown to be required for hippocampal neurogenesis (Rossi et al., 2006).

NT-3 is known to regulate cell cycle exit and enhances neuronal differentiation of cortical precursor cells (Ghosh and Greenberg, 1995; Lukaszewicz et al., 2002). It mainly signals through TrkC, but can also bind to TrkA and TrkB with lower affinity (reviewed in Lewin and Barde, 1996). NT-3 knockout mice also die within a few weeks of birth. They present loss of muscle sensory neurons, and deficiencies in movement and posture (Tessarollo et al., 1994). However, the CNS of these mice appears normal anatomically, which may be due to redundancy of BDNF, NT-3, and NT-4 acting on Trk receptors (Farinas et al., 1994; Ernfors et al., 1994). Interestingly, a recent study where the lateral ventricles of E13 embryos were injected with NT-3 showed an increase in neurogenesis, due to both increased proliferation of precursors and increased neurogenesis (Ohtsuka et al., 2008), suggesting a role for neurotrophins in proliferation and differentiation in vivo. NT-4 binds to TrkB receptor and has been shown to be expressed in the dorsal root ganglia and the brain stem (Katoh-Semba et al., 2003). NT-4 null mice are viable, but revealed a decrease in the number of neurons following birth, suggesting a role in the maintenance and survival of neurons (Liu et al., 1995; Conover et al., 1995). In agreement with these findings, other groups determined neuroprotective properties for NT-4 following brain injury and suggested using it for treatment of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), to slow down neuronal cell death (van Westerlaak et al., 2001; Royo et al., 2006).

With specific regard to cortical precursors, our laboratory has investigated the roles of BDNF and NT-3 during cortical development (Barnabé-Heider and Miller, 2003). It was
first determined that precursor cells both express and are responsive to BDNF and NT-3, which result in the activation of Akt and ERK. Moreover, using inhibitors of PI3K and MAPK signalling cascades, we were able to show that cortical precursor survival and proliferation was specifically dependent on PI3K signalling, while neuronal differentiation was dependent on MAPK signalling. Together, these data strongly suggest that endogenously secreted BDNF and NT-3 promote survival and neurogenesis of cortical precursor cells, and they do so via two distinct signalling pathways; PI3K and MAPK, respectively.

Other functional roles for neurotrophins include the regulation of survival and regrowth of neurons following injury or degeneration of neighboring neurons (reviewed in Lindvall et al., 1994; Oppenheim, 1996; Wang et al., 1998) and synaptic plasticity (Lo, 1995; Thoenen, 1995). For example, NT-3 was shown to increase neurite fasciculation and induces structural and functional modification of synapses through different signalling cascades such as the mitogen-activated protein kinase (MAPK) and Ca\(^{2+}\)/calmodulin-dependent kinase IV (CaMKIV) pathways (Segal et al., 1995; Je et al., 2006). The various biological roles of the neurotrophins have been reviewed extensively and will not be further discussed here except with reference to the developing cortex, which is reviewed below.

1.2.3- Cytokines

Cytokines are small signalling molecules involved in normal developmental processes as well as immune responses to injury. They signal through the gp130 receptor complex which activates the Janus-activated kinase-signal transducer activator of transcription (JAK-STAT) and the mitogen-activated protein kinase (MAPK) signal
transduction pathways. Here, I will focus on three specific cytokines involved in mammalian
cortical development: leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF),
and cardiotrophin-1 (CT-1).

During early development, LIF is well known for promoting totipotent embryonic
stem cell self-renewal (Williams et al., 1988). In cultures, it is endogenously secreted by
neurospheres, and expressed by astrocytes (Murphy et al., 1995; Chang et al., 2004). In vivo,
LIF has been shown to be expressed postnatally, including by adult neurons (Lemke et al.,
1996; Barnabé-Heider et al., 2005). The analysis of LIF null mice revealed a decrease in the
number of cortical astrocytes in the adult and a decreased proportion of adult neural stem
cells (Bugga et al., 1998; Koblar et al., 1998; Shimazaki et al., 2001; Barnabé-Heider et al.,
2005). In vitro, LIF-/- cells showed impaired capacity in generating secondary neurospheres
(Pitman et al., 2004). Moreover, studies done on cortical precursor cells where LIF was
injected in the murine developing lateral ventricles suggest a role for LIF in regulating cell
cycle re-entry by acting through gp130 receptor (Hatta et al., 2002).

Exogenous addition of CNTF can induce premature generation of astrocytes in vitro
and CNTF injections in the embryonic ventricle lead to cortical hyperplasia and increased
proliferation (Bonni et al., 1997; Hatta et al., 2002; Gregg and Weiss, 2005). However,
CNTF has also been shown to inhibit glial cell fate determination of uncommitted
neurospheres, while promoting the differentiation of cells already committed to a gliogenic
fate (Shimazaki et al., 2001). Similarly to LIF, in vivo studies have revealed that CNTF is
mainly expressed after birth (Stockli et al., 1991; Barnabé-Heider et al., 2005). In contrast,
CT-1 is the one cytokine endogenously expressed during murine cerebral cortex
development (Barnabé-Heider et al., 2005). The knockout animals exhibit a loss in motoneurons and a strong deficit in the number of cortical astrocytes (Oppenheim et al., 2001; Barnabé-Heider et al., 2005). This loss in astrocytes indicates an essential role for CT-1 during embryonic cortical development, while LIF and CNTF play important roles postnatally.

The disruption of individual cytokines did not produce critical phenotypes and animals were all viable indicating possible redundancy between the cytokine family members. To initiate signal transduction, LIF, CNTF, and CT-1 all bind to gp130 receptor and LIF-receptor (LIFR) heterodimers. Moreover, CNTF binds to a third subunit, the CNTF-receptor (CNTFR) that together forms a trimeric receptor (Fig. 2D). Once the cytokines bind to their respective membrane spanning receptors, this relays the signal to JAK-STAT and MAPK signalling cascades as previously mentioned. When the gp130 subunit of the receptor complex is deleted, the mice die shortly after birth (Nakashima et al., 1999; Hatta et al., 2002). In addition, gp130 receptor-/- animals showed hypoplastic cortical plate, decrease proliferation and marked deficit in astrocytes. These studies confirm the importance of cytokine signalling during normal cortical development, and the severity of the gp130 -/- phenotype suggests that there is compensation between different cytokines.

Recently, our laboratory investigated the role of endogenous cytokines during cortical precursor cell differentiation (Banabé-Heider et al., 2005). We showed that cortical precursors are responsive to exogenous cytokines such as CNTF, LIF, and CT-1, both in culture and in vivo, influencing them to differentiate into astrocytes. Moreover, while CNTF and LIF are not endogenously produced during early embryonic stages, CT-1 is synthesized
by embryonic cortical neurons. Cell culture experiments using 4 day culture-conditioned medium or cortical neuron-conditioned medium, on freshly-plated cortical precursors resulted in premature astrogenesis. The addition of CT-1 function-blocking antibodies to these conditioned media prevented premature generation of astrocytes. Together, these data strongly support the idea that CT-1 produced by cortical neurons regulates astrogenesis in culture. Careful examination of CT-1 knockout murine cortex at P3 revealed a deficit in astrocytes when compared to control littermates. Furthermore, CT-1-mediated astrogenesis was found to act via the JAK-STAT signalling pathway, and disruption of this signalling pathway in vivo also resulted in a decrease in the generation of astrocytes. Thus, neuron-derived CT-1 is essential for appropriate astrogenesis during cortical development.

Cytokines are important survival signalling factors during development and following injury in the adulthood. It is noteworthy that in the adult, these same cytokines can have different effects. For example, brain infusion of CNTF can increase proliferation of subventricular zone-derived neurospheres (Shimazaki et al., 2001). Interestingly, CNTF addition also stimulated neurogenesis in the adult hippocampus and hypothalamus (Emsley and Hagg, 2003; Kokoeva et al., 2005). Moreover, different experiments using LIF and CNTF have demonstrated a role in promoting survival and maintenance of developing motor neurons postnatally (Sendtner et al., 1990; Cheema et al., 1994; Ikeda et al., 1996).

1.3- The role of Trk receptors and signalling cascades during cortical development

The transmission of signals from the outside of the cell to the cytoplasm and eventually to DNA requires several steps. From the receptor response to ligand binding to the
activation of key proteins responsible for the activation of specific signalling cascades, every step is crucial to the proper cellular response. Here, we will take a closer look at Trk receptor activation, and the role of adaptor proteins and different kinases that lead to the activation of two signalling pathways in particular: the mitogen-activated protein kinase (MAPK) and the phosphoinositide-3 kinase (PI3K) pathways during nervous system development (summarized in Fig. 3).

1.3.1- The Trk receptors

The Trk tyrosine kinase receptors play a critical role in proliferation, survival, and cell fate in the nervous system. Neurotrophins bind to Trk receptors (TrkA, TrkB, and TrkC), resulting in receptor dimerization, autophosphorylation, and tyrosine phosphorylation of downstream signalling proteins. Extensive studies done by many groups revealed Trk receptors function primarily in the nervous system. TrkA is expressed in peripheral sensory neurons, including dorsal root ganglia and sympathetic ganglia and in cholinergic neurons of the basal forebrain in the CNS (Martin-Zanca et al., 1990; Holtzman et al., 1992). TrkA null mice exhibit similar phenotypes to NGF knockouts: a loss of neurons in the dorsal root and sympathetic ganglia. They also show deficiencies in the cholinergic projections connecting the basal forebrain neurons to the hippocampus and cerebral cortex (Smeyne et al., 1994), TrkA has also been determined essential for sympathetic neurons and maturation of cholinergic neurons (Fagan et al., 1996; Fagan et al., 1997).

TrkB is found throughout the developing nervous system, including the brain, the spinal cord and cranial and spinal ganglia (Klein et al., 1990; Yan et al., 1997). TrkB-/- mice
Figure 3. Receptor tyrosine kinase (RTK) activation of PI3K and MAPK. RTKs can activate components of the PI3K and MAPK signaling pathways resulting in cell survival, proliferation, or differentiation.
die within the first week following birth due to an inability to feed. They present deficiencies in coordination and balance as well as defects in the vestibular system (Ernfors et al., 1994; Jones et al., 1994; Schimmang et al., 1995). They exhibit increased neuronal cell loss in the motor facial nucleus (Klein et al., 1993), and an elevated levels of apoptotic cell death in the dentate gyrus, cerebral cortex, striatum, and thalamus (Alcantara et al., 1997). Interestingly, TrkB signalling via PI3K may play a significant role in interneuron migration from the MGE to the cerebral cortex (Polleux et al., 2002).

TrkC has a similar pattern of expression to TrkB, and is found in the developing brain, spinal cord and cranial and spinal ganglia (Lamballe et al., 1991; Merlio et al., 1992; Tessarollo et al., 1993). TrkC null animals display deficits in proprioception and limb postures. Unlike NT-3 null mice that die early postnatally, TrkC-/- were able to survive up to six months of age (Klein et al., 1994). They presented with sensory defects and fewer large myelinated axons in the dorsal root. The lack of TrkC signalling also led to deficiencies in the generation of glial cells in the CNS (Kahn et al., 1999).

With specific regard to cortical precursor cells, both TrkB and TrkC are expressed (Tessarollo et al., 1993; Barnabé-Heider and Miller, 2003) and perturbations of Trk signalling have been shown to effect their development (Jones et al., 1994; Alacantara et al., 1997; Ringstedt et al., 1998; Xu et al., 2000; Lotto et al., 2001; Medina et al., 2004). Previous studies have investigated the role of neurotrophic factor signalling, downstream of TrkB and TrkC, in vitro. Detailed analysis of perturbations resulting from genetic inactivation of TrkB and TrkC in vivo have not been described. In Chapter 4, a the role for TrkB and TrkC receptors in cortical precursor biology in vivo is elucidated.
1.3.2- The adaptor proteins

The adaptor proteins are responsible for the relay of the signal from the activated receptor to the signalling proteins present in the cytoplasm. Once the receptor dimerizes, this activates the receptor-intrinsic tyrosine kinase, resulting in autophosphorylation of several tyrosine residues within the cytoplasmic domain of the receptor, and creating docking sites for adaptor proteins such as Src homology 2 domain-containing (Shc), growth factor receptor-bound protein 2 (Grb2), fibroblast growth factor receptor substrate 2 (FRS2), and son of the sevenless (SOS). She protein does not have catalytic function itself, but mediates the association of additional proteins with the receptor. Once it is phosphorylated by the receptor, it binds to another protein, Grb2. Grb2 then recognizes and binds SOS. SOS is brought to the plasma membrane to activate Ras and initiate signalling cascades including the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways. Moreover, other adaptor proteins that do not bind receptor tyrosine kinases and that regulate receptor-mediated MAPK and PI3K activities are GAB1, IRS1, and IRS2.

1.3.3- Ras

Ras proteins have been intensively studied in a variety of tissues and have been shown to be involved in cell proliferation, survival, and differentiation. They exist in three different isoforms: K-Ras, H-Ras, and N-Ras, that are encoded by three different genes. These small GTPases cycle between an active GTP-bound form and an inactive GDP-bound form to integrate and regulate a wide range of cellular pathways. Ras isoforms undergo different post-translational changes such as farnesylation which regulate subcellular
localization and activity (Casey 1995; Rocks et al., 2005). In general, H-Ras is primarily associated with Golgi and endoplasmic reticulum (ER) membranes, while K-Ras is predominantly associated with the plasma membrane. They have a slow intrinsic GTPase activity that can be catalyzed by GTPase activating proteins (GAPs), while the replacement of the GDP for GTP is mediated by guanine nucleotide exchange factors (GEFs). Once the GDP molecule is removed, a GTP molecule passively binds to Ras and initiates its activation. Son of the sevenless (SOS) is one of the major Ras-GEF and acts as a positive regulator of Ras activity by increasing the rate of guanine nucleotide exchange (Margarit et al., 2003). It exists in two different isoforms, SOS-1 and SOS-2, and while the embryonic knockdown of SOS-1 was shown to be lethal, SOS-2 null mice were viable (Esteban et al., 2000; Qian et al., 2000). Neurofibromin is an important Ras-GAP and negative regulator of Ras. It can control proliferation, survival, and astrocyte differentiation of neural precursors and neurofibromin gene (NF1) disruption is embryonically lethal (Jacks et al., 1994; Dasgupta and Gutmann, 2005). The loss of only one allele in the mouse or in the human leads to learning disabilities, macrocephaly, brain tumors, and an increased in number of oligodendrocyte precursors in the spinal cord (Lynch and Gutmann, 2002; Bennett et al., 2003). Moreover, mice genetically engineered to lack NF1 in the cortical precursors or neurons leads to abnormal development resulting from a variety of deficits in glial cells, including globally reactive astrogliosis and increased proliferation of glial precursor cells (Bajenaru et al., 2001; Zhu et al., 2001; Zhu et al., 2005; Hegedus et al., 2007).

The three Ras isoforms seem to play different physiological roles. For example, the generation of knockout animals showed that N-Ras and H-Ras null mice are viable whereas K-Ras deletion was lethal indicating its essential role in mouse embryogenesis (Johnson et
al., 1997; Koera et al., 1997). N-Ras -/- revealed defects in immune response and H-Ras -/- showed decrease predisposition to tumor formation (Umanoff et al., 1995; Ise et al., 2000; Esteban et al., 2001; Peres de Castro et al., 2003). Mutations in Ras are a common cause of human cancers, however the purpose of this review will be to focus on its role during embryonic nervous system development.

In sympathetic neurons, Ras activation or inhibition achieved using different mutants was shown to be directly related to their survival. While inhibition of Ras activity seemed to increase sympathetic neuronal cell death, the activation of Ras promoted survival of these neurons (Mazzoni et al., 1999; Xue et al., 2000). Moreover, the use of an inhibitor of farnesyl transferase, an enzyme responsible for Ras and other small GTP-binding proteins post-translational modifications, which is an essential step for proper cellular localization, was shown to induce neuroprotection (Ruocco et al., 2007). During synaptogenesis, ectopic expression of activated Ras promotes the formation of synapses and help in their maintenance (Seeger et al., 2005). In cortical precursor cells, little work has been done to investigate the role of Ras during development. It has been suggested that Ras signalling plays a role in survival of these cells, and that it can regulate the size of the cerebral cortex through the Rap1 guanine nucleotide exchange factor C3G (Bonni et al., 1999; Voss et al., 2006). Recently, Vallejo and colleagues have demonstrated that partial inhibition of Ras or its family member Rap1, reduced PACAP-induced glial fibrillary protein (GFAP) expression (Lastres-Becker et al., 2008). In Chapter 5, we investigated the effect of two activated H-Ras mutations that are reported to cause neurodevelopmental defects in human patients. This syndrome, called Costello syndrome, will also be reviewed in depth below.
Ras can activate a variety of proteins and functions by translating and directing growth factor-mediated response to different signalling pathways (reviewed in Mitin et al., 2005). Here, I will review two major cascades triggered by Ras: mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K).

1.3.4- MAPK pathway

The mitogen-activated protein kinase (MAPK) pathway was one of the first signalling cascades identified. It is highly conserved between organism and cell types, and plays an important role in growth factor and cytokine signalling. Aberrant functions of MAPKs have been identified and linked to diseases ranging from cancer to inflammatory disease to developmental disorders. Activation of receptors such as receptor tyrosine kinases (RTKs), G protein-coupled receptors (GPCRs), cytokine receptors, and serine/threonine receptors can stimulate MAPK pathway, which then coordinate diverse stimuli and trigger the context appropriate response. The diverse cellular activities of MAPK signalling include cell proliferation, cell cytoskeleton rearrangement, cell migration, survival and differentiation. In a general way, a MAPK kinase kinase (MAP3K) activates a MAPK kinase (MAP2K) by phosphorylation of serine/threonine residues, which then in turn phosphorylates tyrosine/threonine residues on a MAPK. The most studied MAPKs are extracellular regulated kinase1 (ERK1) and ERK2, Jun N-terminal kinases (JNKs), and p38 kinases. Here, I will focus on ERK1 and ERK2 and their activating proteins, where MAP3K are members of the RAF family, and MAP2K are members of the MEK family of proteins. This specific signalling cascade is of great interest since mutations in these different proteins
have been identified in a series of human syndromes termed neuro-cardio-facio-cutaneous (NCFC) syndromes, which will be discussed in more details below.

RAF is a serine/threonine kinase that interacts with small GTP proteins such as Ras. Once bound to Ras at the membrane, it gets phosphorylated and activated. Three homologs of RAF have been identified: A-RAF, B-RAF, and RAF-1 (or C-RAF). These proteins are highly regulated and contain several serines and threonines that can be phosphorylated resulting in activation or inactivation (Wellbrock et al., 2004). The generation of knockout animals showed that disruption of A-RAF is lethal early postnatally with newborn exhibiting neurological and intestinal defects (Pritchard et al., 1996). When B-RAF or RAF-1 are knocked down, embryos died in utero due to endothelial and neural defects indicating a predominant role for these homologs during development (Wojnowski et al., 1997; Galabova-Kovacs et al., 2006). To investigate the effect of B-RAF disruption during brain development, a conditional knockout mouse was generated and revealed deficits in learning and memory (Chen et al., 2006). Moreover, deletion of B-RAF in neuronal precursors showed a reduced ERK activation and deficits in oligodendrocyte differentiation (Galabova-Kovacs et al., 2008). To validate if the expression of a homolog could compensate for the loss of B-RAF, A-RAF was expressed in the B-RAF locus. Results showed that although these transgenic animals were viable, they exhibited decreased cellular proliferation and impaired neuronal migration (Camarero et al., 2006).

MEK is a MAPK kinase and can be activated by RAF. Three main isoforms are known to be expressed in the brain: MEK1, MEK2, and MEK5 (DiBenedetto et al., 2007). MEK deletion is lethal during development due in part from placental defects (Giroux et al.,
Kuhn and colleagues generated a mouse model expressing MEK shRNA under the nestin promoter to investigate the role of MEK during neural development. They found that these mice suffered from dwarfism, displayed anxiety behaviour and died prematurely (Hitz et al., 2007). Similarly, mice expressing a dominant negative form of MEK under the Tα1 α-tubulin gene, an early neuronal gene, presented fear conditioning impairments (Shalin et al., 2004). Other groups used pharmacological inhibitors of MEK and demonstrated that MEK is required for long-term recognition memory (Kelly et al., 2003), but also that this inhibition can protect against oxidative stress and ischemia (Satoh et al., 2000; Namura et al., 2001). In terms of neuronal differentiation, dominant negative forms of MEK were shown to inhibit neurogenesis in both cortical and hippocampal precursors, while a constitutively active form promoted neurogenesis (Ménard et al., 2002; Kim and Son, 2006).

Extracellular signal-regulated proteins (ERKs) come in eight different isoforms, and are major mitogen-activated protein kinase (MAPK). The most extensively studied are ERK1 and ERK2, and both are expressed throughout the brain (Selcher et al., 2001; Mazzucchelli et al., 2002; DiBenedetto et al., 2007). Generation of knockout mice demonstrated that ERK1-/- animals are viable without obvious phenotypes, whereas ERK2-/- animals died from trophoblast formation defects in utero (Pages et al., 1999; Selcher et al., 2001; Saba-El-Leil et al., 2003; Aouadi et al., 2006). Landreth and colleagues generated a conditional knockout of ERK2 under the GFAP promoter. These mice displayed reduced cortical thickness, a decrease in neurogenesis, and an increase in astrogenesis (Samuels et al., 2008). Moreover, these ERK2 conditional knockouts exhibited impaired learning and long-term memory, data that have also been corroborated by other groups (Cohen-Matsliah et al., 2007, 2008). Interestingly, studies done on another ERK isoform, ERK5, revealed that a dominant
negative form of ERK5 inhibited neurogenesis, while constitutive expression of ERK5 promoted the formation of neurons in culture (Liu et al., 2006). Furthermore, it was demonstrated that ERK5 plays a role in neuronal survival (Liu et al., 2003).

Depending on which receptor triggers the activation of the signalling cascade, different responses can result. For example, acetylcholine activation of MAPK promotes the survival of cortical precursor cells, whereas PDGF stimulation of MAPK promotes the generation of neurons from both dorsal and ventral precursor cells in culture (Ma et al., 2000; Ménard et al., 2002; Barnabé-Heider and Miller, 2003). Upon stimulation, ERKs can further amplify the signal by activating MAPK-activated kinases (MKs). Among MKs, p90 ribosomal S6 kisases (RSKs) are critical effectors phosphorylated by ERKs (Richards et al., 1999; Richards et al., 2001). Once activated, RSKs can translocate to the nucleus and independently activate shared or different substrates by phosphorylation.

1.3.5- PI3K pathway

The phosphoinositide 3-kinase pathway is another signalling cascade that can be activated by Ras (Rodriguez-Viciana et al., 1994; Rodriguez-Viciana et al., 1996). It is composed of two subunits, a 85kD SH2 domain containing a regulatory region (p85) and a 110kD catalytic subunit (p110) (Carpenter et al., 1990). In cortical precursor cell cultures, pharmalogical inhibition of PI3K suppressed survival (Barnabé-Heider and Miller, 2003).

Particularly, the PI3K/PDK1/Akt cascade has been recognized as a major cellular survival signal. Phosphoinisotide-dependent kinase 1 (PDK1) was first identified as an
activator of Akt in vitro (Alessi et al., 1997; Stokoe et al., 1997). Work on embryonic stem cells also showed that lack of PDK1 caused an inability to activate downstream proteins including Akt (Williams et al., 2000). PDK1-/- embryos die at around embryonic day 9 due to developmental anomalies such as lack of somites, forebrain, and neural crest derived tissue. Hypomorphic PDK mice, which express very low levels of PDK1, are smaller than wild type due to a smaller cellular size and lower cellular number (Lawlor et al., 2002). Moreover, the knockdown of PDK1 was shown to reduce cell proliferation and cell cycle progression in immortalized mouse fibroblasts (Nakamura et al., 2008). In term of cell survival, PDK1 has been shown essential for BDNF-mediated neuronal survival (Kharebava et al., 2008).

Akt (also known as protein kinase B, PKB) is phosphorylated by PDK1 as mentioned above, but can also be directly activated by PI3K. Akt exists in three different isoforms: Akt1, Akt2, and Akt3 encoded by three different genes. Most Akt null animals show growth retardation, have smaller brains and die prematurely (Chen et al., 2001; Easton et al., 2005; Tschopp et al., 2005). Brain analysis revealed atypical dendritic architecture of pyramidal neurons causing memory deficits (Lai et al., 2006). The generation of single, double, or triple knockout animals showed that Akt1 functions as the prominent isoform and that only the expression of one allele (Akt1+/Akt2-/-,Akt3-/-) can rescue most knockout phenotypes (Dummler et al., 2006). In cultured cells, Akt is a major contributor to neuronal survival. IGF or neurotrophin-mediated PI3K activation of Akt has been shown to be essential for cerebellar or sympathetic neuronal survival, respectively (Dudek et al., 1997; Vaillant et al., 1999). Moreover, the overexpression of Akt has anti-apoptotic effects on many different cell
types (Downward, 1998). In cortical precursor cell, constitutively activated Akt1 promoted survival, proliferation and impaired cellular migration in vivo (Sinor and Lilien, 2004).

1.4- The C/EBP family of transcription factors

1.4.1- The C/EBP family members and their role in differentiation of tissues

CCAAT/enhancer binding proteins (C/EBPs) are a family of basic region leucine zipper (bZIP) transcription factors that are encoded by six genes to generate six different members, C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ. They are composed of related sequences and obtained their name from their affinity binding to CCAAT sequence and enhancer core elements (Johnson et al., 1987). Their structure includes an activation domain at the N-terminus, a regulatory domain region, and a basic region responsible for DNA binding next to a leucine zipper at the C-terminus which characterizes the family of transcription factor (Landschulz et al., 1989; Friedman and McKnight, 1990; Umek et al., 1991). Homo- or hetero-dimerization between family members is required for DNA binding and for gene transactivation. C/EBPγ is the only family member that lacks the activation domain and can therefore act as an inhibitor of C/EBP function (Cooper et al., 1995). Moreover, C/EBPα, C/EBPβ, and C/EBPε also exist in different isoforms caused by alternative translation or differential splicing (Descombes and Schibler, 1991; Ossipow et al., 1993; Lin et al., 1993; Yamanaka et al., 1997). Some of these truncated versions of C/EBP also lack the activation domain and act as inhibitors. Since the C/EBP family of transcription factors includes so many different isoforms, they will further be referred as the C/EBPs for simplicity purpose. However, most studies have involved specific isoforms, and therefore
examples mentioned in this review of literature will indicate which isoform in particular was investigated in the cited study.

C/EBPs are ubiquitously expressed in the vertebrae, but are at particularly high levels in the liver, adipose tissues, intestine, lung, and myeloid cells. More specifically related to the field of this study, they are highly expressed in the developing nervous system and adult brain (Sterneck and Johnson, 1998; Sterneck et al., 1998; Yukawa et al., 1998; Ménard et al., 2002; Nadeau et al., 2005). Their expression level changes with physiological and pathophysiological conditions including access to nutrients, hormonal changes, stress, and infections.

How are C/EBPs regulated in the cell? C/EBP expression and activation can be controlled at four different levels. First, they are regulated at the transcriptional level by family members or other proteins. For example, C/EBPβ and C/EBPδ are expressed in early stages of differentiation of adipocytes and they themselves later induce the transcription of C/EBPα gene (Cao et al., 1991; Christy et al., 1991). Once C/EBPα activity is induced, it is maintained by auto-activation for terminal differentiation. Other proteins such as CREB and STAT3 are also able to control C/EBP transcription by binding to their promoters (Niehof et al, 1997; Yamada et al., 1997; Cantwell et al., 1998; Hutt et al., 2000; Zhang et al., 2007). Second, C/EBPs can autoregulate themselves at the translational level by producing activating or inhibitory isoforms. For example, C/EBPβ mRNA can produce two isoforms, the liver-enriched transcriptional activator protein (LAP, 35kDa) and the liver-enriched transcriptional inhibitory protein (LIP, 21kDa), by alternative use of translational initiation codons or by proteolytic cleavage (Descombes and Schibler, 1991; Welm et al., 1999). By
changing the relative ratio of activating versus inhibitory isoforms, C/EBPs can control and fine-tune their degree of activation (Tanabe et al., 2000). Third, C/EBPs can be regulated by post-translational modifications. C/EBP proteins can be altered by lysine sumoylation or acetylation. Lysine sumoylation happens at the regulatory domain motif of the protein and, in most cases, negatively regulates C/EBP function (Kim et al., 2002; Sato et al., 2006). Moreover, sumoylation can limit transcriptional synergy, when promoters harbour multiple copies of a response element (Subramanian et al., 2003). C/EBP acetylation of lysine residues can modulate protein-protein interaction and DNA binding. For example, C/EBPβ acetylation by the histone acetyltransferase GCN5, decreases its association with histone deacetylase-1 (HDAC1) interfering with differentiation of preadipocytes (Wiper-Bergeron et al., 2007). Finally, C/EBP activation can be controlled by phosphorylation. Phosphorylation of C/EBPs changes their conformation from inactive to active by exposing their activation domain and acquiring DNA-binding activity. Many C/EBP residues can be phosphorylated, and these are discussed in more details below. Homo- or hetero-dimerization of C/EBPs is a pre-requisite for DNA binding.

Several C/EBP knockout mice have been generated and have helped in understanding the role of C/EBPs in development and formation of different organs. C/EBPα knockout mice show impaired glycogen storage, lower mass of brown adipose tissue, and die shortly after birth (Wang et al., 1995). They also present defects in hepatocyte differentiation and lung development (Flodby et al., 1996), as well as defects in hematopoietic system and macrophages (Zhang et al., 1997; Heath et al., 2004). Studies done on C/EBPβ knockout mice indicated that these animals are susceptible to infections (Screpanti et al., 1995; Tanaka et al., 1995), and revealed defects in mammary epithelial cells and ovary development.
C/EBPδ knockout animals are generally healthy, but are susceptible to genomic instability (Huang et al., 2004). C/EBPε knockout mice show impairments in macrophage maturation, cytokine production, and inflammatory responses (Lekstrom-Himes and Xanthopoulos, 1999; Tavor et al., 2002), while C/EBPζ knockout animals present defects in apoptosis (Zinzsner et al., 1998; Oyadomari et al., 2001). Finally, C/EBPγ knockout mice reveal defects in natural killer-cell cytotoxic activity and interferon-γ (IFN-γ) production (Kaisho et al., 1999).

Is there compensation between isoforms? Studies have shown that compensation between isoforms is possible, and that they can also functionally rescue each other in some tissues (Chen et al., 2000; Begay et al., 2004). Specifically pertaining to the brain, our laboratory has showed that cortical progenitors express C/EBPα, C/EBPβ, and C/EBPδ (Ménard et al., 2002). We also demonstrated that C/EBPβ is essential for neurogenesis of cortical precursor in culture. However, C/EBPβ knockout mice did not exhibit any cortical dysgenesis, suggesting that the other family members can compensate for the lack of the C/EBPβ isoform. Acute C/EBP knockdown experiments in vivo could overcome this issue, and we have used this approach in Chapter 3.

1.4.2- C/EBP phosphorylation and interaction with other proteins

Activation of C/EBPs is achieved by phosphorylation. When unphosphorylated the protein exists in a repressed form, and once it gets phosphorylated it changes conformation exposing its transactivation domain. All C/EBP isoforms possess different phosphorylation sites that are targets of different kinases. For example, C/EBPβ can be phosphorylated on
threonine 188 by Erk (T188) (Zhu et al., 2002), on T235 by Rsk (Nakajima et al., 1993), on serine 105 (S105) by PKC (Trautwein et al., 1993), and on S276 by Ca2+/calmodulin-dependent protein kinase (Wegner et al., 1992). Furthermore, to transactivate certain genes C/EBPs require binding to different cofactors, which is affected by these phosphorylations. The binding to these different cofactors will dictate the downstream effects, and ultimately lead to a change in proliferation, differentiation, apoptosis, or chromosomal remodelling.

C/EBPs have key roles in regulating cellular proliferation. They interact with cell cycle proteins including p21, retinoblastoma (Rb), E2F, p107, and cyclin dependent kinases (CDKs). Specifically, C/EBPα can act by repressing the E2F complex in a Rb-dependent manner and by interfering with CDK2 and CDK4 function (Iakova et al., 2003; Sebastian et al., 2005). C/EBPα is mainly antiproliferative and promotes differentiation, which explains its predominant expression in post-mitotic cells (Lekstrom-Himes and Xanthopoulos, 1998; Hendricks-Taylor and Darlington 1995). Conversely, C/EBPβ has been shown to be able to both inhibit and promote cell proliferation, depending on cellular context and other C/EBP isoforms present at the time (Greenbaum et al., 1998; Buck et al., 1999).

C/EBPs also function at the differentiation level. For example, peroxisome proliferator activated receptor-γ (PPAR-γ) and C/EBPα work together to induce cell cycle withdrawal and differentiation of pre-adipocytes (Rosen and Spiegelman, 2002). C/EBPβ and Runx2 interact and synergized the induction of bone-specific promoters during osteogenesis (Hata et al., 2005). In hematopoietic progenitors, the lack of, or the introduction of, GATA-2 and C/EBPα cofactors at different time during differentiation can alter the cellular fate (Iwasaki et al., 2006). In the brain, C/EBPs can interact with the proneuronal
basic helix-loop-helix (bHLH) NeuroD and induce neuronal terminal differentiation (Calella et al., 2007). The fact that C/EBPs can have different effects at different time is explained by collaboration with cofactors. The relative levels of C/EBPs or cofactor, or the competition with other protein for these cofactors, determine the eventual outcome.

In situations of stress such as hypoxia or neurotrophin deprivation, C/EBPs mediate cell apoptosis (Marshall et al., 2003; Tajiri et al., 2006; Haltermen et al., 2008). On the other hand, studies done on C/EBP null mice showed reduced brain damage compared to littermate following cerebral ischemia (Kapadia et al., 2006). C/EBP proteins do not possess chromatin remodelling or histone deacetylase properties per se, but they are able to bind and recruit proteins that exhibit those properties to different DNA promoters. For example, C/EBPs can bind HDAC1, or the SWI/SNF nucleosome remodelling complex to silence gene transcription (Kowenz-Leutz et al., 1999; Pederson et al., 2001; Wang et al., 2008;).

1.4.3- C/EBP activation of specific genes during development

After C/EBPs have been phosphorylated and formed complexes with family members or other cofactors, they bind DNA to transactivate specific genes. These genes can be involved in cell proliferation, differentiation, and survival. As previously mentioned, C/EBPs can have a proliferative or an anti-proliferative effect depending on which protein they are interacting with. Many studies have shown interaction between C/EBPs and CREB-binding protein (CBP)/p300 (Mink et al., 1997; Erickson et al., 2001; Wang et al., 2007). These transcriptional coactivator proteins enhance transcription of target promoters by histone acetylation (reviewed in Chan and La Thangue, 2001). Once C/EBPs bind CBP/p300, the
complex is recruited to the E2F promoter and initiates its transcription. This maintains cells in a proliferative state and allows for a proliferative effect of C/EBP. Another cell cycle key player is Id2; overexpression of Id2 in cortical precursor cells affects neuronal differentiation and keeps cells in a proliferative state (Toma et al., 2000). Studies revealed that C/EBPβ could activate the transcription of Id2 by binding to its promoter (Karaya et al., 2005). In contrast, C/EBPs can have an anti-proliferative effect. They can dimerize with HDAC and recruit this complex to the E2F promoter (Wang et al., 2008). Since this protein has histone deacetylase activity, this causes chromatin remodelling, blocks E2F gene transactivation, and drastically slows down cell proliferation. Furthermore, in myeloid cells, C/EBPα can negatively regulate c-myc and allows precursor cells to leave their undifferentiated state (Johansen et al., 2001).

C/EBPs play an important role during cell differentiation. For example, during adipocyte formation C/EBPs activates the transcription of genes such as the 422(aP2), stearoyl-CoA desaturase 1 (SCD1), fatty acid translocase/CD36, and SIRT1, all important for adipocyte differentiation and energy homoestasis maintenance (Christy et al., 1989; Qiao et al., 2006; Qiao et al., 2008). Moreover, as suggested by the mouse C/EBPβ knockout which revealed deficiency in brown adipose tissue (Wang et al., 1995), further experiments established that C/EBPbeta can activate the transcription of PPAR-γ coactivator-1α (PGC-1α) and uncoupling protein 1 (UCP1), genes that are essential for commitment of preadipocytes to brown adipose tissue (Karamanlidis et al., 2007). In other tissues, C/EBPs regulate gene expression in hepatocyte development by binding to the albumin promoter (Friedman and McKnight, 1990; Trautwein et al., 1996; Tan et al., 2007), in metabolism by binding to the nutrient deprivation-induced gene asparagine synthetase with the cofactor
ATF5 (Al Sarraj et al., 2005), and in myeloid cells development by acting on the NRAMP1 gene (Richer et al., 2008). This argues that C/EBPs can function at the level of transcription to influence differentiation of many cell types.

In the nervous system, C/EBPs have been shown to promote the transcription of the Tα1 α-tubulin gene (Ménard et al., 2002). Tα1 α-tubulin gene expression is induced as soon as cortical precursor cells differentiate into neurons (Miller et al., 1987; Gloster et al., 1994, 1999). Furthermore, C/EBPs appear to play an important role during brain injury. Specifically, they have been shown to regulate the expression of several genes involved in inflammatory processes, and C/EBPβ deficient animals revealed reduced brain damage after injury (Cortes-Canteli et al., 2004; Kapadia et al, 2006; Cortes-Canteli et al., 2008). In addition, C/EBPβ mediates the expression of Tα1 α-tubulin following facial motor neuron injury in the adult (Bamji and Miller, 1996; Wu et al., 1997; Nadeau et al., 2005). Taken together, these data suggest an important role for C/EBPs during development and inflammation of the central nervous system by direct regulation of gene expression.

Recently, our laboratory investigated the role of C/EBPs during cortical cell fate determination (Ménard et al., 2002). We used three different C/EBP mutants. Acidic-C/EBP (A-C/EBP) and dominant negative-C/EBP (DN-C/EBP) were used to assess the effect of C/EBP inhibition on neurogenesis, and constitutively active-C/EBP (CA-C/EBP) was used to determine the effect of activated C/EBP overexpression on neurogenesis. The A-C/EBP mutant featured a mutated DNA-binding domain, while DN-C/EBP lacked its transactivation domain. Subsequently, both of these mutants acted as dominant negative and were unable to activate gene transcription necessary for proper cellular differentiation. CA-C/EBP consisted
of a phosphorylation mimic mutant, where the RSK phosphorylation site on C/EBP was permanently functionally activated. Using A-C/EBP and DN-C/EBP mutants in culture, we were able to show that inhibition of C/EBPs resulted in a significant decrease in neurogenesis, while CA-C/EBP revealed an important increase in the number of neurons compared to controls. Moreover, we examine the effect of C/EBP inhibition on astrocyte formation by treating cortical precursor cells with CNTF. As previously mentioned, CNTF is known to promote premature gliogenesis in these cell cultures (Bonni et al., 1997; Nakashima et al., 1999). We infected cortical precursor cells with A-C/EBP and noted an increase in the number of astrocytes compare to control after 5 days in culture. Therefore, not only C/EBPs appear necessary for neurogenesis, but their inhibition also drives precursor cells to adopt an astrocytic fate.

To address the mechanism that regulates neurogenesis upstream of C/EBPs, we examined the effect of MEK inhibition on neurogenesis. MEK is known to regulate the C/EBP family of transcription factors in nonneural cells (Davis, 1995). Cortical precursor cells were transfected with a dominant negative-MEK (DN-MEK) and neuronal differentiation was assessed. We showed that MEK inhibition significantly decreased the percentage of neurons in culture, and therefore is essential for proper cortical neurogenesis. We then asked about possible downstream effectors of C/EBPs. We observed potential C/EBP binding sites in the Tα1 α-tubulin promoter sequence, which lead us to the idea that this might be one of C/EBPs direct targets. Using Tα1 α-tubulin promoter construct and conducting a chloramphenicol acetyl-transferase (CAT) assay, we detected a 10 to 35-fold increase in activity compared to control. The binding specificity of C/EBPs to this promoter was further confirmed by series of gel shift assays. Together, these data strongly support a
model where C/EBPs are activated through a MEK-RSK signalling cascade and directly activate transcription of neuron-specific genes including Tα1 α-tubulin in cortical precursor cell culture.

In conclusion, many studies have been published on C/EBPs and their role during proliferation and differentiation of different tissue. However, the specific roles and mechanisms of action of C/EBPs in the developing mammalian brain remain elusive. In Chapter 3, we have investigated the function of C/EBPβ phosphorylation during cortical precursor cell fate determination in vivo.

1.5- Costello Syndrome: an example of developmental dysregulation

1.5.1- The neuro-cardio-facial cutaneous family of syndromes

The neuro-cardio-facial cutaneous family of syndromes (NCFC) is a family of related disorders and includes Noonan syndrome (NS; MIM 163950), Costello syndrome (CS; MIM 218040), cardio-facio-cutaneous syndrome (CFC; MIM 115150), neurofibromatosis type 1 (NF-1; MIM 162200), and LEOPARD syndrome (LS; MIM 151100). They all present mutations in different proteins involved in the SHP-2/Ras/MAPK signalling cascade (Fig. 4) and were therefore recently also termed the Ras/MAPK syndromes (Aoki et al., 2008).

Patients suffering from NCFC generally show a combination of coarse facial features, short stature, mental retardation, heart defects, and skin abnormalities (Bentires-Alj et al., 2006). Although these syndromes are congenital disorders with significant clinical overlaps, each
Figure 4. NCFC family of syndromes. Mutations affecting components of the SHP-2/Ras/MAPK signaling pathways are responsible for Noonan syndrome (NS), LEOPARD syndrome (LS), neurofibromatosis-1 (NF1), cardio-facio-cutaneous syndrome (CFC), and Costello syndrome (CS).
syndrome also presents unique characteristics and skilled medical geneticists can distinguish between them for proper diagnosis.

1.5.2- Noonan syndrome

Noonan syndrome (NS) is an autosomal dominant disorder that affects 1:1000-1:2500 live births (Ferrero et al., 2008). About 50% of patients show missense mutations in the human \textit{ptpn11} gene encoding for the SHP-2 phosphatase (Tartaglia et al., 2002; Neel et al., 2003; Tartaglia and Gelb, 2005). These mutations scattered throughout the protein are gain-of-functions that impair the switch between the active and the inactive conformations of SHP-2, without altering its catalytic capability (Neel et al., 2003; Tartaglia et al., 2006). SHP-2 is required for the full activation of the Ras-RAF-MEK-ERK signalling pathway in most cell types and mutations found in NS showed an increase in ERK activation (Fragale et al., 2004; Mohi and Neel, 2007). Germline mutations in K-Ras and SOS-1 have also been associated with NS with an incidence of about 2% and 10%, respectively (Schubbert et al., 2007; Roberts et al., 2007; Tartaglia et al., 2007). Finally, gain-of-function mutations in RAF-1 have also been implicated in NS, with the most common mutation being S257L, showing a remarkable increase in kinase activity (Pandit et al., 2007; Razzaque et al., 2007). The distinguishing features of NS are cardiovascular defects including hypertrophic obstructive cardiomyopathies and pulmonary valve stenosis, webbed or short neck, pectus deformities, and hematologic abnormalities including easy bruising and juvenile myelomonocytic leukaemia (JMML) (Sharland et al., 1992; Gelb and Tartaglia, 2006; Ferrero et al., 2008).
Recently, our laboratory examined the role of SHP2 during brain development in the Noonan syndrome brain, focusing on the SHP-2 activated mutant D61G (Gauthier et al., 2007). Cortical precursor cells transfected with this mutant showed an increase in neurogenesis and inhibition of astrogenesis. Moreover, genetic knockdown of SHP-2 using RNAi showed that this phosphatase is essential for the genesis of cortical neurons and caused precocious astrocyte formation leading to an increase in the number of astrocytes postnatally. Neel and colleagues created a NS transgenic mouse model that constitutively expressed the D61G SHP-2 mutation (Araki et al., 2004). They demonstrated that this specific mutation was able to increase ERK activation in embryonic tissue, and that the transgenic mice presented craniofacial and cardiac abnormalities. Further analysis done by our laboratory showed that these animals also display increased cellular and neuronal density, as well as a decrease in the number of astrocytes in the dorsal cortex and hippocampus when compared to control littermates (Gauthier et al., 2007).

1.5.3- LEOPARD syndrome

LEOPARD syndrome (LS) is an autosomal dominant disorder that overlaps phenotypically with NS. In 90% of diagnosed cases, mutations also occur within the SHP-2 phosphatase (Digilio et al., 2002). However, unlike NS SHP-2 mutations which are found scattered along the protein, LS SHP-2 mutations are restricted to the PTP domain with the most common being Y279C and T468M (Sarkozy et al., 2004; Keren et al., 2004; Chan et al., 2005). These loss-of-function mutants have an impaired phosphatase activity, and recent studies have raised the possibility that they could exhibit dominant negative effects (Hanna et
al., 2006; Kontaridis et al., 2006). Other mutations found in LS patients are missense mutations in the serine threonine RAF-1, which showed an ability to increase ERK expression in cells (Pandit et al., 2007). LEOPARD syndrome’s acronym name is used to describe the specific phenotypes of the disorder: lentigines, ECG conduction abnormalities, ocular hypertelorism, pulmonary stenosis, abnormalities of genitalia, retardation of growth and sensorineural deafness (Gorlin et al., 1969; Gorlin et al., 1971). Patients also present café-au-lait spots and have a predisposition to cancers such as neuroblastomas and acute myelogenous leukaemia (Keren et al., 2004; Merks et al., 2005).

1.5.4- The cardio-facio-cutaneous syndrome

Cardio-facio-cutaneous (CFC) syndrome is a rare sporadic disorder with less than 300 cases diagnosed worldwide. It is caused by de novo germline missense mutations in one of four different genes: B-RAF, K-Ras, MEK1, and MEK2. B-RAF is highly expressed in the brain and is the most common affected gene (50-60%) with point mutations predominantly located in its kinase domain. In most cases, these amino acid substitutions increased B-RAF kinase activity, which create a constitutively active protein. However, recent evidence also suggests that some mutations produce proteins that are kinase impaired (Rodriguez-Viciana et al., 2006). Several studies revealed that the most common mutations are: Q257R, S467A, L485F, and K499E (Rodriguez-Viciana et al., 2006; Niihori et al., 2006; Narumi et al., 2007). In 1986, the first CFC cases were reported by Opitz and colleagues (Reynolds et al., 1986). CFC patients present high forehead, narrowed temples, and sparse hair. They show mild to severe mental retardation, failure to thrive due to feeding problems
after birth, delayed language and motor development, and seizures (Sabatino et al., 1997; Yoon et al., 2007). Their mental defects are likely due to cortex, brain stem and ventricular system dysgenesis including cerebral atrophy, frontal lobe hypoplasia, absence or hypoplasia of the corpus callosum, increased ventricular size and hydrocephalus, and brain stem atrophy (Reynolds et al., 1986; Wieczorek et al., 1997; Drolet et al., 2000; Grebe and Clericuzio, 2000; Raymond and Holmes, 1993; Sabatino et al., 1997; Roberts et al., 2005). Unlike the other NCFC syndromes, CFC individuals do not seem susceptible to cancers.

1.5.5- Neurofibromatosis type 1

Neurofibromatosis type 1 syndrome (NF1) is an autosomal dominant disorder that affects 1:4000 births (Kratz et al., 2006). NF-1 gene encodes for the tumor suppressor neurofibromin. It is expressed early during embryogenesis and at high levels in the brain suggesting a role during neuronal differentiation (Xu et al., 1990; Gutmann et al., 1995). Neurofibromin is part of the Ras-GTPase activating protein (Ras-GAP) family of proteins and functions as a negative regulator of Ras by catalyzing its GTPase activity. Patients diagnosed with NF1 syndrome have mutations that cause inactivation of the protein and lead to hyperactive Ras signalling. They have a high incidence of cognitive dysfunction and learning disabilities (Hyman et al., 2005). They can also develop scoliosis, multiple cutaneous neurofibromas, café-au-lait spots, and Lisch nodules (North, 1993). NF1 individuals are predisposed to malignant tumors including optic gliomas, neurofibromas, astrocytomas and malignant peripheral nerve-sheath tumors. Moreover, children with NF1
have a 200-fold increase in developing juvenile myelomonocytic leukemia (JMML) (Side et al., 1998).

Many studies have been done on NF1 and its role during development. The complete loss of neurofibromin is lethal to mice and humans (Jacks et al., 1994). Therefore, to investigate its role in vivo, heterozygous knockout mouse models have been generated. They revealed cognitive deficits including learning disabilities, behavioural abnormalities and impaired performance in spatial recognition tests (Jacks et al., 1994; Silva et al., 1997; Costa et al., 2002). Moreover, they showed astrogliaosis in several regions of the brain (Rizvi et al., 1999).

The gene responsible for NF1 encodes for a tumor suppressor that functions as a negative regulator of Ras. In mice, inactivation of NF1 gene expression increases proliferation of glial precursors and differentiation of precursors into astrocytes in vitro and in vivo (Bajenaru et al., 2001; Bajenaru et al., 2002). Consistent with the role of Ras in tumor development, 15–20% of children with NF1 develop low-grade glial cell neoplasms (Listernick et al., 1994, 1999). Moreover, mice genetically engineered to lack NF1 in the CNS exhibit a variety of deficits in glial cells, including global reactive astrogliosis and increased proliferation of glial precursor cells which result in the formation of optic gliomas (Bajenaru et al., 2003; Zhu et al., 2005). Dasgupta and Gutmann (2005) showed that NF1 inactivation in neural stem cell populations generated increased numbers of morphologically abnormal, immature astroglial cells in vitro. In a more recent study published by this same group, NF1 inactivation in BLBP-positive precursor was shown to promote gliogenesis in a Ras-dependent manner, and reduce neuronal maturation in a cAMP-dependent manner.
Hegedus et al., 2007). Pawson and colleagues also showed that the activation of Ras can mediate proliferation of human astrocytomas (Guha et al., 1997). Together, these data suggest an important role for NF1 during gliogenesis, specifically through Ras regulation.

1.5.6- Costello syndrome

Costello syndrome (CS) is a rare congenital disorder first described in the 70’s by Jack Costello, a New Zealand paediatrician (Costello, 1971; Costello, 1977). To this day, about 300 individuals have been diagnosed with CS worldwide. Recently, point mutations in H-Ras have been identified and found responsible for causing this condition (Aoki et al., 2005). In 80% of cases, the mutation is situated at position 12 in the guanine nucleotide binding site, and results in a decrease in the GTP hydrolysis capacity, and thus acts as a gain-of-function mutant. These are de novo germline mutations, and in most cases inheritance of the mutated H-Ras allele is exclusively from the father, confirming a paternal bias in the parental origin of the mutation (Aoki et al., 2005; Gripp et al., 2006; Estep et al., 2006). The most prevalent mutation is H-RasG12S, where the glycine residue at position 12 is switched to a serine residue. Other substitutions at position 12 include glycine to aspartic acid (G12D), glycine to valine (G12V), glycine to alanine (G12A), and at position 13 such as glycine to aspartic acid (G13D), and glycine to cysteine (G13C) (Aoki et al., 2005; Gripp et al., 2006; Estep et al., 2006). The H-RasG12V mutation is present in about 5% of CS cases, and is of great interest because of its oncogenic implication. Cancers occurs predominantly in patients diagnosed with CS, more than in any other neuro-cardio-facial-cutaneous (NCFC) syndromes, and these include neuroblastomas, rhabdomyosarcomas, and bladder carcinomas.
(Gripp et al., 2002; Hennekam, 2003; Gripp, 2005). Moreover, genetic analysis of tumors from CS patients revealed a loss or silencing of the normal H-RAS allele. Previous studies done on H-Ras comparing the activity of different mutations at codon 12 showed that G12V had the highest transformation potential (Fasano et al., 1984; Seeburg et al., 1984). However, this high predisposition to cancer could also lead to embryonic lethality and might explain why H-RasG12V mutations are not more prevalent in CS. In contrast, H-RasG12S mutations are milder germline lesions and seem tolerated during embryonic development.

Individuals diagnosed with CS have common facial trait, sparse and curly hair, short stature, and growth retardation. They have loose skin on hands and feet and tend to develop papillomatas. They present with heart defects, most often hypertrophic cardiomyopathies, and neurological conditions including ventricular dilatation that requires a ventriculo-peritoneal shunt, hydrocephaly, Chiari 1 malformation, and seizures (Delrue et al., 2003; Hennekam 2003; Gripp 2005; Zampino et al., 2007). These conditions result in developmental delays and mental retardation, and these cognitive dysfunctions are more severe in individuals with CS and CFC than with any of the other NCFC disorders (Zampino et al., 2007).

Interestingly, a recent study investigated the effect of H-RasG12V activation under the synapsin promoter, a protein that is turned on in neurons (Heumann et al., 2000). They showed that this aberrant activation of H-RasG12V did not change the number of neurons, but that it caused cell soma atrophy. Moreover, data revealed a decrease in the degeneration of motor neurons following facial nerve lesion, indicating that hyperactivation of H-Ras is neuroprotective.
In Chapter 5, we hypothesized that the expression of two of the Costello syndrome H-Ras alleles (H-RasG12S and H-RasG12V) in cortical precursor cells will affect their proliferation and differentiation in culture and in vivo. Moreover, we speculate that this disruption of cortical development might explain the cortical abnormalities and the cognitive dysfunctions seen in Costello syndrome individuals.
RESEARCH AIMS AND HYPOTHESES

The general aim of this study was to elucidate the roles of growth factor-mediated MAP kinase signaling cascade components during embryonic cerebral cortex development. Specifically, I hypothesized that dysregulation or misexpression of C/EBPs transcription factors, Trk receptor signaling, or Ras activity could have an impact on cellular proliferation, survival, and differentiation of cortical precursor cells. I therefore divided this work into three objectives:

**Objective 1:** To determine the role of C/EBP phosphorylation during cortical cell fate determination (Chapter 3).

**Objective 2:** To examine the involvement of Trk signaling during cortical development (Chapter 4).

**Objective 3:** To elucidate the effect of Costello syndrome H-Ras mutations during cerebral cortex formation (Chapter 5).
CHAPTER 2: EXPERIMENTAL METHODS

2.1- Cortical precursor cell cultures

Cortical precursor cells were cultured as previously described (Toma et al., 2000; Ménard et al., 2002; Barnabé-Heider and Miller, 2003). Cortices were dissected from embryonic day 12 (E12) to E13 CD1 mouse embryos in ice-cold HBSS (Invitrogen, Gaithersburg, MD) and transferred to neurobasal medium (Invitrogen) containing 500 µm L-glutamine (Cambrex Biosciences, Hopkinton, MA), 2% B27 supplement (Invitrogen), and 1% penicillin-streptomycin (Invitrogen). The medium was supplemented with 40 ng/ml FGF2 (Promega, Madison, WI). The tissue was mechanically tritured with a plastic pipette into single cells and cells were plated on two-well or four-well chambers slides (Nunc, Naperville, IL). Chamber slides were previously coated with 2% laminin and 1% poly-D-lysine (BD Biosciences, Bedford, MA) and cell density was 250,000 cells/well for two-well chamber slides and 125,000 cells/well for four-well chamber slides.

2.2- Treatments of cortical precursor cell cultures and transfection constructs

For transfections, 1 to 2 hours after plating, 1 µg of DNA and 1.5 µl of Fugene 6 (Roche, Welwyn Garden City, UK) mixed with 100 µl of Opti-MEM (Invitrogen) were incubated at room temperature for 45 minutes and then added to each well of four-well chamber slides (the amount was doubled for two-well chamber slides). For the C/EBP project, a plasmid expressing a nuclear localized GFP driven from the elongation factor 1 (EF1) promoter was used with either
an empty CMV500 vector, or plasmids encoding acidic C/EBP (A-C/EBP; Ménard et al., 2002), HA-tagged DN-MEK (Mansour et al., 1994; Shalin et al., 2004) or C/EBPβ mutants where threonine 217 was mutated to glutamate (the T217E phosphorylation mimic or CA-C/EBPβ) or where threonine 217 and threonine 188 were mutated to alanine (C/EBPβ T/A phosphorylation mutant; both were the kind gift of Dr. Peter Johnson). For the Trks project, the rat dnTrkB mutant consisted of a single mutation (K538N) in the ATP binding-site that rendered it kinase-dead (Atwal et al., 2000) while the rat dnTrkC mutant contained three mutated tyrosines (Y705N, Y709N, and Y710N) within catalytic subdomain VIII (the kind gift of Pantelis Tsoulfas, University of Miami). These Trk constructs were subcloned into the pEF-GM expression vector which drives expression from the EF1α promoter (the kind gift of Ryoichiro Kageyama, Kyoto University). The two TrkB shRNA constructs targeted two different regions on the TrkB mouse mRNA sequence. The sequence for TrkB shRNA1 was 5’TTGTGGATTCCGCTTAAATTAAGAGATTTAAGCCGGAATCCACAA3’, for TrkB shRNA2 was 5’CCTTGTAGGAGAAGATCAATTCAAGAGATTGATCTTCTCCTACAAGG3’, and for the control shRNA was 3’TTCTCCGAACGTCACGTTCCTTCAAGAGAACCAGTGACACGTTCCGGAGAA3’. This control shRNA was mismatched to known human and mouse genes (EZBiolab, Westfield, IN). The dnAkt mutant contains a point mutation (K179M) within its ATP binding site (Songyang et al., 1997). Finally, for the H-Ras project, H-RasG12V plasmid consists of a point mutation at codon 12 (GGC->GTC) which switches the glycine residue to a valine (Invitrogen) and H-RasG12S plasmid consist of point mutation at codon 12 (GGC->AGC) which switches the glycine residue to a serine. Both plasmids encodes for a constitutively active form of H-Ras. An
empty vector was used as a control (pEF-GM), and a plasmid encoding GFP under the EF1-promoter (pEF-GFP) was used as a marker for cotransfected precursor cells.

The day following plating, 50 ng/ml ciliary neurotrophic factor (CNTF; Peprotech, Rocky Hill, NJ) was added in some experiments (as specified in Results sections of each chapters), by changing one half of the medium. In some experiments precursors were cultured in the presence of 100µM pan-caspase inhibitor ZVAD-FMK (Calbiochem). For transfection of HEK 293 cells, 4µg of DNA and 10µl of Lipofectamine 2000 (Invitrogen) with 250µl of OPTI-MEM was added to 70% confluent cells in six-well plates.

2.3 - In utero electroporation

In utero electroporation was performed as previously described (Ohtsuka et al., 1999; Gauthier et al., 2007). E13/E14 or E14/15 CD1 pregnant mice were anesthesized with isoflurane, and a midline incision was performed to access the embryos. A total of 4 ug of DNA was injected in the lateral ventricle of each embryo with 0.05% trypan blue as a tracer. We used a nuclear EGFP expression plasmid driven from the EF1 promoter (pEF-GFP). This pEF-GFP was co-electroporated with pEF-GM (empty vector) or LacZ as controls, and with A-C/EBP, T/A-C/EBP, CA-C/EBP, dnMEK, dnTrkB, dnTrkC, dnTrkB/C, shTrkB, BDNF, dnAkt, H-RasG12V, or H-RasG12S in different experiments. After injection, electroporation was performed using a square electroporator CUY21 EDIT (TR Tech, Japan), delivering five 50 ms pulses of 40-50V with 950 ms intervals per embryo. Embryos were then reimplemented in utero and left to further develop for 3-8 days. For analysis, brains were fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA) at 4°C overnight, cryoprotected in 30%
sucrose at 4ºC overnight, and embedded in OCT compound (Sakura Finetek, Torrance, CA). The brains were kept at -80ºC until cryosectioned (16µm) and immunostained.

2.4- Immunocytochemistry

Immunocytochemistry of cultured cells and tissue sections was performed as described (Gauthier et al., 2007). For immunocytochemistry of cultured cells, cells were washed with HEPES-buffered saline (HBS) and fixed with 4% PFA for 15 minutes, permeabilized with 0.2% NP-40 (USB Corporation, Cleaveland, OH) in HBS, and blocked with buffer containing 6% normal goat serum (NGS) (Jackson ImmunoResearch, West Grove, PA) and 0.5% bovine serum albumin (BSA) (Jackson ImmunoResearch) for 1-2 hours at room temperature. Cells were then incubated with primary antibodies in HBS containing 3% NGS and 0.25% BSA at 4ºC overnight. After washing with HBS, cells were incubated with secondary antibodies prepared in HBS containing 3% NGS and 0.25% BSA at room temperature for 1 hour. Samples were then washed with HBS, counterstained with Hoechst 33258 (1:1000; Sigma, St-Louis, MO) for 2 minutes, and mounted with GelTol (Fisher Scientific, Houston, TX). For immunocytochemistry of tissue sections, sections were dried at 37ºC for 15 minutes, washed in phosphate buffer solution (PBS) (Hyclone, Logan, UT), and postfixed with 4% PFA for 10-15 minutes. They were then blocked and permeabilized with 10% BSA and 0.3% Triton X-100 (EMD Chemicals Inc., Gibbstown, NJ) for 1 hour. The M.O.M. blocking kit (Vector Laboratories, Burlingame, CA) was then used according to the manufacturer’s protocol. Sections were incubated with primary antibodies at 4ºC overnight, washed with PBS, and incubated with secondary antibodies at room temperature for 1 hour. They were then counterstained with Hoechst 33258 for 2 minutes and mounted with
GelTol. Primary antibodies used were mouse anti-GFP (1:1000; Invitrogen), rabbit anti-GFP (1:500; Chemicon, Temecula, CA), mouse anti-Ki67 (1:200; BD Biosciences), mouse anti-HuD (1:200; Invitrogen), mouse anti-βIII-tubulin (1:800; Covance, Princeton, NJ), and rabbit anti-GFAP (1:1000, Accurate Chemical & Scientific Corp., Westbury, NY), rabbit anti-pTrk-Y490 (1:100; Santa Cruz Biotechnology), rabbit anti-myc-Tag (1:200; Upstate, Lake Placid, NY), rabbit anti-cleaved caspase 3 (1:500; Cell Signaling Technology, Beverly, MA), mouse anti-NeuN (1:200; Chemicon), rabbit anti-GAD (1:200; Chemicon), rabbit anti-TrkB (1:500; Santa Cruz Biotechnology), mouse anti-HA (1:400; Boehringer Mannheim), rabbit anti-C/EBPβ (C-19) (1:800; Santa Cruz), mouse anti-MAP2 (1:400; Sigma-Aldrich), rabbit anti-pC/EBPβ (1:500; Cell Signaling), mouse anti-nestin (1:400; Chemicon), mouse anti-S100β (1:1000; Sigma), biotinylated anti-musashi (1:500; the kind gift of Dr. Hideyuki Okano), mouse anti-H-Ras (1:400; Calbiochem), rabbit anti-phosphoERK (1:500; Cell Signaling Technology), goat anti-doublecortin (1:100; Santa Cruz). Secondary antibodies used for immunocytochemistry were indocarbocyanine (Cy3)-conjugated goat anti-mouse, goat anti-rabbit IgG, donkey anti-goat IgG (1:400; 1:400; 1:1000; Jackson ImmunoResearch), FITC conjugated anti-mouse and anti-rabbit IgG (1:200; Jackson ImmunoResearch), dichlorotriazinyl amino fluorescein-conjugated streptavidin (1:1000; Jackson ImmunoResearch), Cy3-conjugated streptavidin (1: 1000; Jackson ImmunoResearch), Alexa Fluor 350 goat anti-mouse and anti-rabbit (1:500; Molecular Probes, Eugene, OR), and Alexa Fluor 647 goat anti-mouse (1:1000; Molecular Probes).

2.5- Western blots and immunoprecipitations
For biochemical analysis, 293 cells were washed with ice-cold HBSS and lysed directly in the dish with RIPA buffer (50mM Tris, pH7.2, 150mM NaCl, 2mM EDTA, 1% NP-40, 1% Na deoxycholate and 0.1% v/v SDS) supplemented with protease inhibitor mixture (Boehringer Mannheim) and 1.5mM sodium vanadate. Lysates were scraped into Eppendorf tubes, rocked for 10 min at 4°C, and cleared by centrifugation. Protein concentration was determined using the bicinchoninic acid assay (Pierce) and bovine serum albumin (BSA) as a standard. Equal amounts of protein (40-50μg) were boiled in sample buffer, separated by 10-15% SDS-PAGE gels, and transferred to 0.2 μm nitrocellulose membranes for 3hr at 0.75A at 4°C. Membranes were blocked in 5% skim milk powder in TBS-T (TBS and 0.5% Tween 20) for 2hr at room temperature and then incubated overnight at 4°C with primary antibodies. Primary antibodies used were mouse anti-HA (1:1000; Boehringer Mannheim), rabbit anti-GFP (1:1000; Chemicon), rabbit anti-pErk (1:5000; Promega), rabbit anti-Erk (K-23) (1:5000; Santa Cruz), rabbit anti-C/EBPβ (C-19) (1:500; Santa Cruz), mouse anti-phosphotyrosine (4G10; 1:100; Upstate Biotechnology), rabbit anti-TrkBout, and rabbit anti-TrkCout (1:5000; Knusel et al., 1994; Hoehner et al., 1995). After washing with TBS-T, membranes were incubated with secondary antibodies, HRP-conjugated goat anti-mouse or anti-rabbit (1:10,000; Boehringer Mannheim), in blocking solution for 2hr at room temperature. Detection was performed using the ECL chemiluminescence reagent (Amersham Biosciences) and XAR x-ray films (Eastman Kodak).

For immunoprecipitation, cortical precursors (0, 2, and 4 DIV) or cortices from E14.5, P3, and adult CD1 mouse were lysed as described above. Equal amount of protein were incubated with 10μl of the pan-C/EBP (Δ198) antibody (Santa Cruz), or with 3 μl of the pan-Trk antibody (203b) (Hempstead et al., 1992), overnight at 4°C and then incubated for 2hr with 30μl
of protein A-Sepharose (Sigma). The precipitated proteins were collected by centrifugation, washed three times with RIPA buffer, boiled with sample buffer, loaded on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane. Membrane was blocked with 3% BSA in TBS-T and processed as detailed above using primary antibody.

### 2.6- Microscopy and confocal analysis

For quantification of immunocytochemistry on cultured cells, approximately 300 cells per condition per experiment were counted and analyzed. Digital image acquisition was performed with Northern Eclipse software (Empix, Mississauga, Ontario, Canada) using a Sony (Tokyo, Japan) XC-75CE CCD video camera. For quantification of immunocytochemistry on tissue sections, brains were chosen with a similar anatomical distribution and level of GFP expression. After sectioning, three to four brain sections at the same anatomical level per embryo were analyzed using a Zeiss Pascal confocal microscope and the manufacturer’s software (Oberkochen, Germany). A mean of four scans taken with a 40x objective were computed for each image. In all graphs, error bars indicate Standard Error of the Mean (SEM), and the statistics were performed using the Student’s t test or one-way ANOVA with Mann-Whitney post hoc test, as appropriate.
CHAPTER 3: CCAAT/ENHANCER-BINDING PROTEIN PHOSPHORYLATION BIASES CORTICAL PRECURSORS TO GENERATE NEURONS RATHER THAN ASTROCYTES IN VIVO.

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CONTRIBUTION OF AUTHORS

A.P. performed all of the experiments in this chapter except for the dnMEK experiments, and co-wrote the manuscript with F.D.M. F.B.H. performed the dnMEK experiments and provided advices and guidance. R.K. kindly invited us to his laboratory to teach us the in utero electroporations and provided us with reagents. F.D.M. helped design experiments, and provided support, advices and guidance.
3.1 ABSTRACT

The intracellular mechanisms that bias mammalian neural precursors to generate neurons versus glial cells are not well-understood. We have previously demonstrated that the growth factor regulated kinase MEK and its downstream target, the C/EBP family of transcription factors are essential for neurogenesis in cultured cortical precursor cells (Ménard et al., 2002). Here, we have examined a role for this pathway during cortical cell fate determination in vivo using in utero electroporation of the embryonic cortex. These studies demonstrate that inhibition of the activity of either MEK or the C/EBPs inhibits the genesis of neurons in vivo. Moreover, the MEK pathway mediates phosphorylation of C/EBPβ in cortical precursors, and expression of a C/EBPβ construct where the MEK pathway phosphorylation sites are mutated inhibits neurogenesis. Conversely, expression of a C/EBPβ construct where the same sites are mutated to glutamate and therefore are "constitutively" phosphorylated, enhances neurogenesis in the early embryonic cortex. A subpopulation of precursors in which C/EBP activity is inhibited are maintained as cycling precursors in the ventricular/subventricular zone of the cortex until early in postnatal life, when they have an enhanced propensity to generate astrocytes, presumably in response to gliogenic signals in the neonatal environment. Thus, activation of a MEK-C/EBP pathway in cortical precursors in vivo biases them to become neurons and against becoming astrocytes, thereby acting as a growth factor-regulated switch.
3.2 INTRODUCTION

Development of the mammalian nervous system is determined by a complex interplay between intrinsic genetic mechanisms and extrinsic cues such as growth factors, an interplay perhaps best-studied in the developing cortex (Lillien, 1998; Shen et al., 1998; Ferguson and Slack, 2003). In vivo, cortical neurogenesis occurs early while gliogenesis occurs later, peaking postnatally. The same sequential genesis of neurons and then glia also occurs in embryonic cortical precursor cultures (Qian et al., 2000; Barnabé-Heider et al., 2005) and recent work indicates that this timed sequence is regulated by growth factors in the neural environment. For example, the gliogenic cytokine cardiotrophin-1 is expressed by newly-born neurons and acts as a feedback timer to regulate the onset of cortical astrocyte formation (Barnabé-Heider et al., 2005). Conversely, ligands that signal via receptor tyrosine kinases regulate cortical neurogenesis; FGF2 is necessary for neurogenesis (Raballo et al., 2000) and PDGF and the neurotrophins enhance the genesis of neurons in the presence of FGF2 (Ghosh and Greenberg, 1995; Williams et al., 1997; Park et al., 1999; Barnabé-Heider and Miller, 2003). However, the intracellular mechanisms that allow precursors to integrate these diverse signals to ultimately generate a neuron versus a glial cell are still not well-understood.

What are the intracellular growth factor-mediated signals that regulate neurogenesis? We recently demonstrated that MEK, which is downstream of many growth factor receptors, promotes neurogenesis from cultured cortical precursors without apparently regulating either survival or proliferation (Ménard et al., 2002; Barnabé-Heider and Miller, 2003). In addition, we showed that one of the targets of MEK, the C/EBP family of transcription factors, is essential for cortical precursors to generate neurons in culture (Ménard et al., 2002), where it directly promotes transcription of at least one early panneuronal gene, Tα1 α-tubulin (Miller et al., 1987;
Gloster et al., 1994; 1999). The C/EBP family is composed of basic leucine zipper DNA-binding proteins (C/EBPs alpha, beta, gamma, delta, epsilon, and zeta) that recognize a common DNA-binding sequence (Johnson and Williams, 1994), and that are expressed in virtually all tissues, including the developing and adult brain (Sterneck and Johnson, 1998; Sterneck et al., 1998; Nadeau et al., 2005).

Since previous work indicates that the neurogenic bHLHs are also essential for cortical neurogenesis (Nieto et al., 2001; Sun et al., 2001), then we have hypothesized that cortical precursors are biased to generate neurons by their expression of neurogenic bHLHs, and that growth factors in their immediate environment activate MEK, which in turn instructs those precursors to generate neurons via phosphorylation and activation of the C/EBP family. Here, we have directly tested this hypothesis in vivo, using in utero electroporation to genetically manipulate cortical precursors in the embryonic telencephalon. Our data indicate that MEK-mediated phosphorylation of the C/EBPs is essential for cortical precursors to generate neurons, and that at the same time activated C/EBP promotes neurogenesis, it inhibits astrocyte formation, thereby acting as a growth factor-regulated fate switch in vivo.

### 3.3 RESULTS

#### 3.3.1 MEK activity is important for cortical neurogenesis in vivo

We have previously demonstrated that the growth factor-regulated kinase MEK is not required for the survival or proliferation of cultured cortical precursors, but is instead essential for those precursors to generate neurons (Ménard et al., 2002; Barnabé-Heider and Miller, 2003). To ask whether MEK activation was also required for cortical neurogenesis in vivo, we
performed in utero electroporation with an HA-tagged dominant-negative form of MEK (DN-MEK) (Mansour et al., 1994; Shalin et al., 2004). Plasmids encoding a nuclear-targetted GFP and DN-MEK or empty vector were coelectroporated following injection into the E15 lateral ventricles, embryos were reimplemented and were then analyzed 1-3 days later. In some cases, a plasmid encoding a nuclear-targetted β-galactosidase (LacZ) was also used as a control. One day following electroporation, only cells in the ventricular zone/subventricular zone (VZ/SVZ) of the telencephalon were transfected (data not shown), as previously reported (Ohtsuka et al., 1999; Barnabé-Heider et al., 2005). Of these transfected cells, approximately 85% expressed the proliferation marker Ki67, indicating that they were dividing precursors. These electroporated cells maintained expression of the transfected construct until at least postnatal day 7 (data not shown). Importantly, immunocytochemistry revealed that at all timepoints analyzed the vast majority of cells were positive for both GFP and the HA-tagged DN-MEK in cortices coelectroporated with both of these plasmids (Fig. 5A). Biochemical confirmation that these plasmids were appropriately expressed was obtained by performing Western blot analysis on electroporated cortices where either a GFP-expressing plasmid or GFP plus HA-tagged DN-MEK-expressing plasmids were injected into one lateral ventricle (Fig. 5B).

Having established the efficacy of this procedure, we then asked whether DN-MEK inhibited neurogenesis. Analysis of control electroporated brains revealed that at E18, three days following electroporation, many GFP or LacZ-expressing control cells were still in the VZ/SVZ, but many had also migrated into the cortical plate, which contains newly-born neurons (Fig. 5C, left panels). Quantitation demonstrated that approximately 40-50% of the total control transfected cells had migrated into the cortical plate region (Fig. 5D). In contrast, many more DN-MEK-expressing cells remained in the VZ/SVZ, with only 15-30% migrating into the
Figure 1: Cellular analysis of P0 E18 mouse brains. (**), (†), * and ** are statistical significance at p<0.001, 0.01, 0.05 and 0.05 respectively. All data represent the mean ± S.D. of at least 3 independent experiments. 

A) Images of P0 E18 mouse brains with control and dnMEK conditions.

B) Western blot analysis of HA and GFP expression in sample 1 and sample 2.

C) Confocal images showing LacZ, GFP, and HuD expression under control and dnMEK conditions.

D) Bar graph showing the percentage of GFP+ cells within the cortical plate.

E) Bar graph showing the percentage of Ki67/GFP+ cells.

F) Bar graph showing the percentage of HuD/GFP+ cells.

G) Bar graph showing the percentage of NeuN/GFP+ cells.

H) Bar graph showing the percentage of HuD/GFP+ cells in the total GFP+ cells.

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Figure 5. *DN MEK inhibits cortical neurogenesis in vivo.* In utero electroporations of precursor cells in the E14-15 telencephalic ventricular and sub-ventricular zone (VZ/SVZ) with plasmids encoding GFP and HA-tagged DN MEK or GFP and the empty vector or a LacZ plasmid. Three days after electroporation, coronal sections of the embryonic telencephalon were analyzed by immunocytochemistry for GFP and several markers. (A) Photomicrographs of a section from an E18 brain that was cotransfected with plasmids encoding GFP and HA-tagged DN MEK, and then immunostained for GFP and HA. Scale bar = 200 microns. Note that the majority of the GFP-positive cells also express HA-tagged DN MEK. (B) Western blot analysis of cortices electroporated with GFP and HA-tagged DN MEK (sample 1) or with GFP and the empty vector (sample 2). The non-electroporated hemisphere from the same animal of each sample was run as a control. The membrane was probed for the HA epitope and GFP. (C) Photomicrographs taken on a light microscope of sections spanning the ventricle (V) to the meninges (M) through E18 cortices of embryos transfected with the LacZ vector (left 3 panels) or with the DN MEK plasmid (right 3 panels). In each set of 3 panels, the top shows the GFP immunostaining, the middle the neuron specific HuD immunostaining, and the bottom the merge. Scale bar = 100 microns. (D) Quantitation of sections similar to those shown in (c) for the percentage of migration of GFP positive cells within the cortical plate. Transfected cells within the cortex of three littermate pairs, three to four sections per embryo, were counted to obtain these numbers. *p<0.05, **p<0.01 relative to control-transfected sections (ANOVA or Student’s t-test). Error bars indicate SEM. (E) Quantitation of confocal microscopic analysis of GFP and the proliferation marker Ki67. Transfected cells within the cortex of three littermate pairs, four sections per embryo, were counted to obtain these numbers. *p<0.05, **p<0.01 relative to control-transfected sections (ANOVA or Student’s t-test). Error bars indicate SEM. (F) Quantitation of confocal microscopic analysis of GFP and the mature neuron specific marker NeuN, as described in (e). **p<0.01, ***p<0.001 relative to control-transfected sections (ANOVA or Student’s t-test). Error bars indicate SEM. (G-H) Quantitation of confocal microscopic analysis of GFP and the early neuron specific marker HuD expression, as described in (e). In (H), the left panel indicates all the GFP positive cells throughout the cortical section, while the right panel indicates only the GFP positive cells present in the cortical plate. **p<0.01, relative to control-transfected sections (ANOVA or Student’s t-test). Error bars indicate SEM.
cortical plate region (Fig. 5C, D). To ask whether this lack of migration indicated that DN-MEK had inhibited the genesis of neurons, we performed immunocytochemistry for two neuronal markers, HuD, an early marker (Fig. 5C), and NeuN, a somewhat later marker. Confocal microscopy and quantitation revealed that approximately 20-30% of the GFP-expressing control cells expressed HuD and approximately 10% NeuN (Fig. 5F, G, H). In contrast, 1-10% of the cells expressing DN-MEK were positive for HuD, and less than 1% for NeuN (Fig. 5F, G, H). More detailed spatial analysis revealed that, of the transfected cells that were in the cortical plate, more than 80% were positive for HuD, and this percentage was similar for both control and DN-MEK-transfected populations (Fig. 5H), indicating that some DN-MEK-expressing cells do become postmitotic neurons, and that it is these cells that migrate to the cortical plate. In contrast, no HuD-positive cells were observed in the VZ/SVZ, nor were any GFAP-positive astrocytes observed (data not shown). Instead, many of the DN-MEK expressing cells in the VZ/SVZ continued to proliferate, as indicated by immunocytochemical analysis for Ki67; approximately 15% of the total DN-MEK expressing cells were Ki67-positive, as compared to 5-8% of control cells (Fig. 5E). Thus, inhibition of MEK inhibits neurogenesis, and inappropriately maintains many cortical precursors as dividing cells within the VZ/SVZ.

3.3.2 C/EBPβ is phosphorylated by the MEK-ERK pathway during neurogenesis

One of the targets of the MEK pathway is C/EBPβ, which is phosphorylated at threonine-188 by ERK and threonine-217 by RSK, both of which are kinases downstream of MEK (Davis, 1995). To ask whether C/EBPβ phosphorylation by the MEK pathway occurred coincident with neurogenesis, we utilized an antibody specific for C/EBPβ phosphorylated at the ERK site,
threonine-188. Initially, we examined cultured cortical precursors isolated from E12.5 mouse cortex and plated in the presence of FGF2. We have previously shown that upon plating these cells are dividing, nestin-positive precursors that for the first 5 days generate only neurons, but then also generate astrocytes and oligodendrocytes (Slack et al., 1998; Toma et al., 2000; Ménard et al., 2002; Barnabé-Heider and Miller, 2003; Barnabé-Heider et al., 2005). Western blot analysis of pan-C/EBP immunoprecipitations showed that phosphoC/EBPβ could be detected in freshly-isolated cortical precursors, as well as in later cortical cultures that contained both precursors and neurons (Fig. 6D). Double-label immunocytochemical analysis of precursors cultured for five days revealed that a subpopulation of nestin-positive cells were immunoreactive for phosphoC/EBPβ, as were almost all MAP2-positive newly-born neurons (Fig. 6A). In contrast, phosphoC/EBPβ was not detectably expressed in cells that were positive for S100β, a marker of nonneuronal precursors and young astrocytes. To confirm that phosphoC/EBPβ was downstream of MEK in these cells, we transfected precursor cultures with GFP alone or GFP plus DN-MEK, and five days later, performed immunocytochemistry. This analysis revealed that DN-MEK significantly reduced the percentage of transfected cells expressing detectable levels of phosphoC/EBPβ (Fig. 6E). Thus, phosphoC/EBPβ levels are highest in a subset of neuronal precursors and in newly-born neurons, and this C/EBP phosphorylation is downstream of MEK, consistent with a potential role in neurogenesis. To ask if a similar pattern of C/EBPβ phosphorylation was seen in vivo, we cryosectioned E18 brains and performed double-label immunocytochemistry for phosphoC/EBPβ and the neuronal marker HuD or the precursor markers nestin (all precursors) or NG2 (oligodendrocyte-biased precursors). This analysis revealed that a subpopulation of nestin-positive precursors in the VZ/SVZ were positive for
Figure 6. **C/EBP is phosphorylated at the ERK site in newly-born neurons in culture and in vivo.** (A) Double-label immunocytochemical analysis of cortical precursor cells after 3 DIV for phospho-C/EBPβ (pC/EBPβ) (in red) and MAP2, Nestin, or S100β (in green). Arrows represent cells that coexpress both markers and arrowheads represent cells that are positive for pC/EBPβ only. Scale = 100 microns. (B-C) Double-label immunocytochemistry on sections of the E18 cortex for pC/EBPβ (red) and HuD (B) or nestin (C) (green). In both (B) and (C) the right panel is a higher magnification micrograph of sections similar to those in the left panel. Arrows represent cells that coexpress both markers and arrowheads represent cells that stained for pC/EBPβ only. (D) Western blot analysis for pC/EBPβ in cortical precursor cell lysates that were immunoprecipitated to an antibody specific for all C/EBP family members. Cortical precursors were analyzed immediately after plating (0DIV), and after 2 and 4 days of culture (2DIV, 4DIV). (E) Quantitation of the percentage of transfected, phospho-C/EBPβ-positive cells in cortical precursor cultures transfected with GFP alone or with GFP plus DN-MEK. *p<0.05; Student’s t-test. Scale bar, 100µm. Error bars indicate SEM.
phosphoC/EBPβ (Fig. 6C), as were many HuD-positive neurons in the cortical plate (Fig. 6B) while the large majority of NG2-positive cells did not express phosphoC/EBPβ (data not shown).

3.3.3 C/EBPβ phosphorylation is essential for neurogenesis in culture

We have previously shown that the C/EBP family is essential for cortical neurogenesis in culture using a dominant-negative, acidic form of C/EBP (A-C/EBP) where the basic DNA binding domain is mutated to acidic residues so that it, and any wildtype C/EBP with which it dimerizes, can no longer bind to DNA (Ménard et al., 2002). Moreover, we have shown that C/EBPβ mutated to glutamate at threonine-217 (thereby acting as a mimic of C/EBPβ phosphorylated at the Rsk site), was sufficient to enhance neurogenesis in culture (Ménard et al., 2002; Fig. 7D). To ask whether C/EBP phosphorylation was also necessary for C/EBPs neurogenic actions, we performed the converse experiment, utilizing a C/EBPβ mutant where both threonine-188 and threonine-217 were mutated to alanine, and could thus no longer be phosphorylated in response to MEK activation (C/EBP T/A mutant; Fig. 7A, B). E12.5 cortical precursors were cotransfected in culture with GFP and the C/EBP T/A mutant on the day of plating, and four days later, were analyzed immunocytochemically for neuron-specific βIII-tubulin (Fig. 7C). Quantitation demonstrated that the C/EBPβ T/A mutant decreased the number of neurons that were generated from cortical precursors by approximately two-fold (Fig. 7D). Thus, C/EBP phosphorylation via the MEK pathway is both necessary and sufficient for neurogenesis in cultured cortical precursors.

In addition to regulating neurogenesis, when the C/EBP family is completely inhibited using A-C/EBP, precocious cytokine-induced gliogenesis is enhanced (Ménard et al., 2002; Fig.
Figure 7. Expression of a C/EBP phosphorylation mutant at the ERK and RSK sites inhibits neurogenesis in culture. (A) Schematic of C/EBPβ wild type protein. Two activation domains (ADI and ADII) are situated to the amino-terminus (NH2) while the DNA-binding domain (DBD) and the leucine zipper dimerization domain (LZ) are located at the carboxyl-terminus (COOH). The two phosphorylation sites investigated in this study, threonine-188 (T188; a substrate for ERK) and threonine-217 (T217; a substrate for RSK), are also shown. (B) Western blot analysis for the different C/EBPβ mutants expressed in HEK 293 cells showing expression of proteins of the appropriate sizes. (C) Double label immunocytochemical analysis for GFP (green) and βIII-tubulin (red) at 4DIV on precursor cells that were transfected with either GFP plus the empty vector or with GFP and the C/EBPβ T/A phosphorylation mutant. Arrows represent cells that coexpress both markers and arrowheads represent cells that were transfected but that do not express βIII-tubulin. Cells were counterstained with Hoechst (blue) to show all nuclei in the field. Scale bar = 100 microns. (D) Quantitation of data similar to that shown in (C) for precursor cells transfected with either GFP plus empty vector, GFP and the C/EBPβ T/A mutant, or GFP and the CA-C/EBPβ phosphorylation mimic, cultured for 4 DIV and then immunostained for βIII-tubulin. *p<0.01, relative to control-transfected cultures (ANOVA or Student’s t-test). Error bars indicate SEM. (E) Quantitation of data similar to that shown in (C) for precursor cells stimulated with CNTF following transfection with plasmids encoding either GFP plus empty vector, GFP plus the C/EBP T/A mutant, or GFP plus A-C/EBP. Cells were immunostained for GFAP after 4DIV. *p<0.01, relative to control-transfected cultures (ANOVA or Student’s t-test). Error bars indicate SEM.
To ask whether phosphorylation of C/EBPs plays any role in this effect on gliogenesis, we transfected the C/EBPβ T/A mutant into cortical precursors, and exposed them to CNTF. In contrast to A-C/EBP, this phosphorylation mutant had no effect on astrocyte formation, as monitored by immunocytochemistry for the astrocyte protein GFAP (Fig. 7E). These data argue that phosphorylation by the MEK pathway is not important for C/EBP-mediated inhibition of astrocyte formation, consistent with our previous work showing that inhibition of MEK itself also does not affect cytokine-induced gliogenesis (Ménard et al., 2002; Barnabé-Heider and Miller, 2003; Barnabé-Heider et al., 2005). Thus, while C/EBPs promote neurogenesis and inhibit gliogenesis, the mechanisms by which they regulate these two events are dissociable.

3.3.4 C/EBP phosphorylation by the MEK pathway is essential for neurogenesis in vivo

To ask whether C/EBPs were essential for cortical neurogenesis in vivo, as suggested by our culture studies, we performed in utero electroporation experiments. The lateral ventricles of E15 embryos were injected with plasmids encoding A-C/EBP and GFP, electroporation was performed, and the brains were harvested three days later. Analysis of sections through the cortex of these brains demonstrated that the vast majority of the cells transfected with A-C/EBP remained in the VZ/SVZ and did not migrate to the cortical plate, results similar to those seen with DN-MEK (Fig. 5D). Specifically, in control brains 40-50% of the GFP-positive cells had migrated into the cortical plate over the three-day period, but only approximately 10% of the GFP-positive cells coexpressing A-C/EBP were in the cortical plate region (Fig. 8D). Immunocytochemistry for the neuronal markers NeuN and HuD, and quantitation by confocal microscopy (Fig. 8C, E, F) revealed that this deficit in migration was due to the fact that A-
Figure 8. Inhibition of C/EBPs decreases neurogenesis and maintains cells as proliferating precursors in vivo. In utero electroporation of precursor cells in the E14-15 telencephalic VZ/SVZ with plasmids encoding either GFP and A-C/EBP, GFP and the C/EBPβ T/A phosphorylation mutant (T/A), or with GFP and the empty vector. Three days after electroporation, coronal sections of the embryonic telencephalon were analyzed by immunocytochemistry for GFP and several markers. (A) Photomicrographs taken on a light microscope of sections spanning the ventricle (V) to the meninges (M) through E18 cortices of embryos cotransfected with GFP and the empty vector (top 3 panels) or with GFP and the C/EBPβ T/A mutant (bottom 3 panels). In each set of 3 panels, the left shows the GFP immunostaining, the middle the neuron specific NeuN immunostaining, and the right the merge. Scale bar = 100 microns. (B) Photomicrographs of E18 cortex sections from brains that were cotransfected with GFP and the empty vector (top panels) or GFP and A-C/EBP (bottom panels), and then immunostained for the proliferation marker Ki67. Scale bar = 100μm. (C) Confocal image of colocalization for NeuN (in red) and GFP (in green) in a section through the cortical plate region. Arrows represent cells that coexpress both markers and arrowheads represent cells that stain for GFP only. (D) Quantitation of sections similar to those shown in (A) for the percentage of GFP positive cells present in the cortical plate region. Transfected cells within the cortex of three littermate pairs, three to four sections per embryo, were counted to obtain these numbers. *p<0.05, relative to control-transfected sections (ANOVA or Student’s t-test). Error bars indicate SEM. (E-H) Quantitation of confocal microscopic analysis of GFP and the mature neuronal marker NeuN (E), GFP and the early neuronal marker HuD (F), GFP and the proliferation marker Ki67 (G), and GFP and the proliferation marker Musashi (H) in cortical sections similar to those shown in A-C. Transfected cells within the cortex of littermate pairs, four sections per embryo, were counted to obtain these numbers. *p<0.05, **p<0.01 relative to control-transfected sections (ANOVA or Student’s t-test). Error bars indicate SEM.
C/EBP inhibited cortical neurogenesis; 20-30% and 5-15% of the control cells expressed HuD and NeuN, respectively, while only approximately 5% and 1-2% of the A-C/EBP-expressing cells expressed these two markers. Moreover, as seen for DN-MEK, none of the A-C/EBP-expressing cells in the VZ/SVZ expressed NeuN or HuD, while transfected cells in the cortical plate region were almost all positive for HuD, whether they expressed GFP alone or GFP plus A-C/EBP. Thus, C/EBP activity is essential for cortical neurogenesis in vivo, and when it is inhibited cells remain in the VZ/SVZ and do not express neuronal genes.

To ask whether, as seen for DN-MEK, the A-C/EBP-expressing cells that did not become neurons were maintained as proliferating precursors, we performed immunocytochemistry for the proliferation marker Ki67 and for the neural precursor marker musashi (Kaneko et al., 2000). Quantitation by confocal microscopy revealed that a significantly greater percentage of A-C/EBP-expressing cells versus control cells were Ki67-positive (Fig. 8B, G). Moreover, approximately 40% of the A-C/EBP-expressing cells were positive for musashi, while only approximately 25-30% of control cells expressed this precursor marker (Fig. 8H). Analysis of adjacent sections for GFAP revealed that, as seen in culture (Ménard et al., 2002), none of the A-C/EBP-expressing cells became astrocytes (data not shown), presumably because of a lack of gliogenic signals in the neural environment at this timepoint (Barnabé-Heider et al., 2005). Thus, inhibition of the C/EBP family inhibits neurogenesis, and maintains cells in the VZ/SVZ as cycling precursor cells.

To ask whether MEK pathway-mediated phosphorylation of C/EBPs was important for the neurogenic actions of these transcription factors, we performed similar in utero electroporation experiments using the C/EBPβ T/A phosphorylation mutant. E15 cortices were electroporated with plasmids encoding either the C/EBPβ T/A mutant plus GFP or with empty
vector plus GFP, and embryos were then sacrificed 3 days later. Analysis of these experiments revealed that the C/EBPβ T/A mutant gave results similar to those seen with A-C/EBP with regard to all of the parameters measured. Specifically, the vast majority of the C/EBPβ T/A mutant-expressing cells remained in the VZ/SVZ (Fig. 8D), where many of them were proliferating, as monitored by expression of Ki67 (Fig. 8G). In addition, significantly fewer of the C/EBPβ T/A mutant-expressing cells were positive for NeuN, arguing that this mutant prevented precursors from adopting a neuronal phenotype (Fig. 8A, E). Finally, as seen for A-C/EBP, none of the C/EBP T/A mutant-expressing cells were positive for GFAP at this timepoint (data not shown). Thus, MEK-mediated phosphorylation of C/EBPs is essential to promote cortical neurogenesis in vivo as it is in culture.

3.3.5 Phosphorylation of C/EBPβ is sufficient to promote neurogenesis at early embryonic timepoints in vivo

Since our data indicated that C/EBP phosphorylation was essential for neurogenesis in vivo as it was in culture, we asked whether enhanced C/EBP phosphorylation was sufficient to promote neurogenesis in vivo. Initially, we performed in utero electroporations of E15 mouse embryos, cotransfecting GFP plus the phosphorylation mimic CA-C/EBP or GFP plus the empty vector control plasmid. Embryos were sacrificed three days later, and cortical sections were analyzed by immunocytochemistry. Initially, we noted that there was no difference in the migration of control versus CA-C/EBP-expressing cells; in both cases approximately 50% of the GFP-positive cells were in the cortical plate region (Fig. 9E). Confocal microscopic quantitation of sections immunostained for NeuN and Ki67 also revealed no differences between control and
Figure 9.  A C/EBPβ phosphorylation mimic promotes cortical neurogenesis in vivo at E13-E16, but not at E15-E18 when endogenous ERK activation is already maximal.  (A-E) In utero electroporation of precursor cells in the E15 cortex with plasmids encoding either GFP and the C/EBPβ phosphorylation mimic (CA-C/EBPβ) or GFP and the empty vector. Three days after electroporation, coronal sections of the embryonic telencephalon were analyzed by immunocytochemistry for GFP and several markers. (A,B) Photomicrographs taken on a light microscope of sections spanning the ventricle (V) to the meninges (M) through E18 cortices of embryos cotransfected with GFP and the empty vector (top 3 panels) or with GFP and CA-C/EBPβ (bottom 3 panels). Sections were immunostained for GFP and NeuN (A) or GFP and Ki67 (B). Scale bar = 100 microns. CP=cortical plate, VZ/SVZ=ventricular zone/subventricular zone. (C-D) Quantitation of confocal microscopic analysis of GFP and NeuN (C), or GFP and Ki67 (D) in sections similar to those shown in a and b. Transfected cells within the cortex of littermate pairs, four sections per embryo, were counted to obtain these numbers. Error bars indicate SEM. (E) Quantitation of sections similar to those shown in (A) for the percentage of GFP positive cells present within the cortical plate region. Transfected cells within the SVZ of two littermate pairs, three to four sections per embryo, were counted to obtain these numbers. Error bars indicate SEM. (F) Western blot analysis for phosphorylated ERKs (pERK) in equal amounts of protein isolated from the cortex at embryonic days 13 to 19. The blots were reprobed for total ERKs as a loading control. (G, H) In utero electroporation of precursor cells in the E13 cortex with plasmids as described for panels (A-E). Sections were analyzed at E16 by immunostaining and quantitated by confocal microscopy for GFP and NeuN (G), or GFP and HuD (H). Transfected cells within the cortex of littermate pairs, four sections per embryo, were counted to obtain these numbers. *p<0.05, relative to control-transfected sections (ANOVA or Student’s t-test). Error bars indicate SEM.
CA-C/EBP-expressing cells (Fig. 9A-D), arguing that at this developmental timepoint, enhanced C/EBP phosphorylation did not promote neurogenesis.

One potential explanation for these results is that during midgestation the environment is maximally proneurogenic, the MEK-C/EBP pathway is already fully activated, and thus enhanced phosphorylation of C/EBP cannot promote neurogenesis further. To ask if this was the case, we performed Western blot analysis for activated, phosphorylated ERKs, which are downstream of MEK and upstream of the C/EBPs. This analysis (Fig. 9F) revealed that phosphoERK levels were robustly increased between E13 and E16 and were then maintained until E19. We therefore predicted that enhanced C/EBP phosphorylation might promote neurogenesis between E13 and E16, before maximal activation of the MEK pathway occurred. To test this prediction, we electroporated the same constructs into the cortices of mouse embryos at E13, and analyzed them three days later. Analysis by immunocytochemistry and confocal microscopy revealed a modest but significant increase in the number of CA-C/EBP-expressing cells that were positive for the neuronal markers NeuN and HuD relative to controls (Fig. 9G, H). Thus, phosphorylation of C/EBPs is sufficient to enhance neurogenesis at early but not later embryonic timepoints, likely because activation of the MEK-C/EBP pathway is already maximal at these later timepoints.

3.3.6 C/EBPs inhibit gliogenesis in vivo as they do in culture

These in vivo data demonstrated that inhibition of the MEK-C/EBP pathway maintained increased numbers of undifferentiated precursors within the VZ/SVZ from E15 to E18. To ask about the ultimate fate of these cells, we performed similar in utero electroporation experiments
with A-C/EBP at E15, and analyzed the cortex at P3. At this stage, approximately 75% of the transfected cells were present in the cortical plate region, and this number was similar whether cells were transfected with A-C/EBP plus GFP or with GFP alone. Of these, more than 95% were neurons, as indicated by expression of HuD (Fig. 10A). These data therefore suggest that A-C/EBP delayed but apparently did not permanently inhibit precursors from becoming neurons, although the transient nature of the transfections also makes it possible that A-C/EBP levels had decreased sufficiently by these later timepoints to allow neurogenic C/EBP-mediated transcription to occur. If this interpretation is correct, then one prediction is that the delayed, later-differentiating neurons would be more superficially located in the neonatal cortex. To test this prediction, we analyzed the localization of GFP-positive neurons that had differentiated from precursors transfected with or without A-C/EBP at E15. In control transfected brains, by P3 all of the GFP-positive cells in the cortical plate were found within cortical layer II/III (Fig. 10B, left panel), with approximately 65% of the transfected neurons located in the upper half of this layer (Fig. 10C). In contrast, in A-C/EBP-transfected brains, almost all of the GFP-positive neurons were found within the upper half of layer II/III (Fig. 10B, right panel; Fig. 10C), indicating that they were, as predicted, more superficially located.

This analysis also revealed that, at P3, none of the transfected cells in the VZ/SVZ expressed HuD or NeuN. We therefore asked whether any of these cells were astrocytes, since (i) our previous culture data indicated that inhibition of C/EBPs enhanced astrocyte formation in response to a gliogenic, cytokine environment (Ménard et al., 2002), and (ii) the early neonatal cortex is a cytokine-rich, gliogenic environment (Morrow et al., 2001; Barnabé-Heider et al., 2005). Immunocytochemical analysis for GFAP revealed that a significantly higher percentage of A-C/EBP-transfected cells expressed GFAP relative to their control transfected counterparts.
Figure 10. *Inhibition of C/EBPs increases gliogenesis after birth.* In utero electroporation of precursor cells in the E15 cortex with plasmids encoding GFP and A-C/EBP, GFP and the C/EBPβ T/A mutant, or GFP and the empty vector. Animals were sacrificed at P3, and coronal sections through the cortex were analyzed by immunocytochemistry for GFP and the neuronal marker HuD (A) or the glial marker GFAP (B,C). (A) Quantitation of confocal microscopic analysis of GFP and HuD expression in the cortical plate region. (B) Photomicrographs of coronal sections through the P3 neocortex of brains that were transfected with GFP (in green) plus control plasmid (left panel) or A-C/EBP plasmid (right panel), and then counterstained with Hoechst (blue) to show cell nuclei. The cortical layers are denoted to the side of each panel. Layer II/III is demarcated with hatched lines, and the solid line represents the boundary between the upper and lower halves of this layer. Scale bar = 100 μm. (C) Percentage of GFP-positive cells in the upper half of neocortical layer II/III in P3 brains that were transfected at E15 with GFP plus control plasmid or A-C/EBP plasmid, quantitated from sections similar to those shown in (B). n = 3 control and 3 A-C/EBP-transfected brains. (D) Confocal image of colocalization for GFP (in green) and GFAP (in red). Arrows represent cells that coexpress both markers and the arrowhead represents a cell that stained for GFP only. (E) Quantitation of confocal microscopic analysis of GFP and GFAP expression in the VZ/SVZ. Transfected cells within the cortex of non-littermates, four sections per embryo, were counted to obtain these numbers. *p<0.05, relative to control-transfected sections (ANOVA or Student’s t-test). Error bars indicate SEM.
(Fig. 10D, E); an average of 25% and 43% of control versus A-C/EBP-transfected cells, respectively. Interestingly, transfection of the T/A C/EBPβ mutant had no effect on the percentage of VZ/SVZ cells that became astrocytes (Fig. 10E), findings similar to what we had observed in culture (Fig. 7E). These data therefore support the hypothesis that C/EBP activation promotes neurogenesis at the same time that it inhibits astrocyte formation in response to a gliogenic environment, and that the neurogenic and gliogenic mechanisms are dissociable.

3.4 DISCUSSION

The data presented here support three major conclusions. First, activation of the growth factor-regulated MEK pathway is essential for cortical precursors to generate neurons in vivo, and when this activation is inhibited, many precursors remain in the VZ/SVZ in an undifferentiated state. Second, MEK pathway activation leads to C/EBP phosphorylation in a subset of precursors, and this phosphorylation is necessary and sufficient to promote neurogenesis. Finally, in the absence of C/EBP family activation, precursors initially remain in the VZ/SVZ as cycling precursors, but then have an enhanced propensity to generate astrocytes in the late embryonic/early neonatal environment, presumably when they are exposed to extrinsic, gliogenic cues. Thus, growth factor-mediated activation of the MEK-C/EBP pathway promotes the genesis of neurons from cortical precursors, and, at the same time, C/EBP activation apparently inhibits the genesis of astrocytes when the same precursors are exposed to a gliogenic environment. The C/EBPs therefore act as growth factor-regulated switches that directly couple alterations in the neural environment to cell fate choices during embryogenesis.
Our experiments demonstrate that, even at E18, some precursors "escape" the effects of inhibiting MEK or C/EBPs, commit to being neurons, and migrate to the cortical plate, and that this "escape" is much more pronounced by P3. One explanation for this finding is that at E15 we are transfecting multiple populations of VZ/SVZ precursors, including biased neuronal progenitors that are undergoing terminal mitosis at the time of transfection, and thus no longer require MEK-C/EBP pathway activation. A second explanation is a technical one; these transfections are transient, and levels of expression and inhibition differ from cell to cell. Thus, it is likely that MEK-C/EBP pathway activity is still sufficient in some transfected cells to promote neurogenesis. This technical limitation would become more important at later timepoints following transfection. Finally, other pathways may be able to promote neurogenesis, albeit perhaps less efficiently, in the absence of MEK-C/EBP pathway activation. Interestingly, regardless of the explanation, our data demonstrate that these delayed precursors then generate neurons that are appropriately more superficially localized. These data are reminiscent of similar findings with cortical precursors that were transiently inhibited from differentiating by Notch pathway activation (Mizutani and Saito, 2005), and suggest that these delayed precursors can still ultimately respond appropriately to developmental cues.

We therefore propose a model where growth factors in the extrinsic environment play a key role in dictating the timing of neurogenesis versus gliogenesis in the embryonic cortex. Specifically, we propose that early embryonic cortical precursors are biased to become neurons by expression of positively-acting bHLHs (Nieto et al., 2001; Sun et al., 2001), but that these biased precursors do not generate neurons until tyrosine kinase receptor ligands in the neural environment signal via the MEK-C/EBP pathway. At the same time, the C/EBPs inhibit cortical precursors from generating astrocytes in response to low levels of embryonic neural cytokines.
such as neuropoietin and cardiotrophin-like cytokine (Uemura et al., 2002; Derouet et al., 2004). Such an inhibitory mechanism is necessary, since early cortical precursors are competent to generate astrocytes if cytokine levels in their environment are sufficiently high (Barnabé-Heider et al., 2005). Later in embryogenesis, the cortical environment alters to become increasingly gliogenic (Morrow et al., 2001), in large part because of synthesis of the cytokine cardiotrophin-1 by newly-born cortical neurons (Barnabé-Heider et al., 2005). Over this same time period, cortical precursors become sensitized to cytokines via a variety of mechanisms, including increased expression of EGFR (Burrows et al., 1997; Sun et al., 2005) and/or components of the gliogenic JAK-STAT pathway (He et al., 2005), and demethylation of astrocyte-specific genes (Takizawa et al., 2001). Ultimately, when neuron-derived cytokine levels become sufficiently high, these sensitized precursors respond by generating astrocytes instead of neurons.

Interestingly, gliogenic signaling via the gp130 cytokine receptor and STAT3 not only promotes the genesis of astrocytes, but also inhibits the genesis of neurons (Bonni et al., 1997; Barnabé-Heider et al., 2005), thereby ensuring the efficiency of the neurogenic to gliogenic switch. Thus, while intrinsic alterations in precursor cell bias and responsiveness are clearly important, we propose that it is growth factors in the developing environment that ultimately dictate when and how many of these different cell types are generated.

What are the growth factors that activate the MEK-C/EBP pathway and thereby promote neurogenesis? Previous work has identified a number of candidate receptor tyrosine kinase ligands, including FGF2, neurotrophins, HB-EGF, PDGF and IGF1. Specifically, FGF2 is mitogenic for cortical precursors (Lukaszewicz et al., 2002) and is essential for normal neurogenesis (Raballo et al., 2000). Cortical precursors express the neurotrophin receptors TrkB and TrkC, and ablation of either TrkB or the neurotrophins BDNF and/or NT-3 perturb the
development of these precursors (Barnabé-Heider and Miller, 2003; Medina et al., 2004). Heparin-binding EGF (HB-EGF) is expressed in the embryonic VZ/SVZ (Nakagawa et al., 1998), and promotes neurogenesis in the adult CNS (Jin et al., 2003). Cultured cortical precursors express PDGF receptor and respond to PDGF with enhanced neurogenesis (Johe et al., 1996; Williams et al., 1997; Park et al., 1999). Finally, overexpression of IGF1 from the nestin promoter causes increased proliferation and neuron number in the embryonic cortex (Popken et al., 2004). What are the sources of these growth factors during neurogenesis? Cortical precursors of the VZ/SVZ themselves express BDNF, NT-3 (Maisonpierre et al., 1990; Fukumitsu et al., 1998), FGF2 (Raballo et al., 2000), and HB-EGF (Nakagawa et al., 1998). Intriguingly, endothelial cells also express BDNF (Kim et al., 2004), FGF2 (Albuquerque et al., 1998) PDGF and HB-EGF (Arkonac et al., 1998), and embryonic endothelial cells have recently been shown to promote self-renewal and neurogenesis in neural stem cells (Shen et al., 2004). Thus, it is likely that cortical precursors are exposed to diverse ligands from multiple sources, and signaling via these growth factors could converge on the MEK-C/EBP pathway to ultimately regulate the genesis of neurons. It is also likely that these same growth factors regulate survival and proliferation of cortical precursors via other pathways such as the PI3K-Akt pathway (Barnabé-Heider and Miller, 2003; Sinor and Lillien, 2004), and thus the precise repertoire of responses is likely defined by the relative levels and/or timecourse of activation of these different pathways.

How do the C/EBPs promote neurogenesis? Precedent for the C/EBP family of transcription factors acting to regulate cellular differentiation in response to growth factors derives from studies on a number of developing systems, most notably during hematopoiesis (Yamanaka et al., 1998) and adipogenesis (Lane et al., 1999). In these systems, various
members of the C/EBP family act in concert with cell type-specific determination signals to promote or repress transcription of genes essential for terminal differentiation. We propose a similar mechanism for neurogenesis, where the C/EBPs collaborate with proneurogenic bHLHs to ultimately promote neurogenesis by transcription of neuron-specific genes such as Tα1 α-tubulin (Ménard et al., 2002). Interactions between these two families of transcription factors might be mediated by the transcriptional coactivators p300/CBP (Goodman and Smolik, 2000), which bind to both neurogenic bHLHs such as Neurogenin1 (Sun et al., 2001) and NeuroD/BETA2 (Mutoh et al., 1998), as well as to C/EBPs (Mink et al., 1997). C/EBPs and neurogenic bHLHs might also cooperate to transcriptionally activate a neuron-specific gene expression program by binding to separate consensus sites within the same genes. Alternatively, the C/EBPs might promote neurogenesis by sequestering ATF5, another transcription factor of the leucine zipper family that maintains neural precursors in an undifferentiated state (Angelastro et al., 2003; 2005). Previous work has shown that C/EBPs and ATFs can heterodimerize (Schuman et al., 1997), and that the heterodimers bind to different DNA binding sites than do either ATF or C/EBP homodimers (Haas et al., 1995). Thus, the relative levels and activation state of ATF5 versus C/EBPs may well determine whether or when a precursor differentiates into a neuron.

One of the interesting conclusions deriving from our work is that activation of the C/EBPs not only promotes neurogenesis, but also inhibits gliogenesis. Neurogenic bHLHs have a similar ability to bias precursors to become neurons while at the same time inhibiting them from becoming glial cells (Tomita et al., 2000; Nieto et al., 2001; Sun et al., 2001). For bHLHs such as Neurogenin1, these two effects are apparently mediated via different mechanisms, with the proneurogenic effects being transcriptionally-mediated and the antigliogenic effects resulting
from Neurogenin1-mediated sequestration of p300/CBP (Sun et al., 2001). For the C/EBPs, these two activities also appear to be dissociable, since inhibition of total C/EBP family activity promoted neurogenesis and inhibited gliogenesis, but inhibition of C/EBP phosphorylation by the MEK family only affected neurogenesis. However, although these two functions are dissociable, both the proneurogenic and antigliogenic effects may be transcriptionally-mediated, since C/EBPs both enhance and repress transcription (Lekstrom-Hines and Xanthopoulos, 1998), and phosphorylation differentially regulates these transcriptional actions.

In conclusion, our data support the hypothesis that activation of a MEK-C/EBP pathway is essential for cortical precursors to generate neurons in vivo, and that the C/EBPs act as switches to promote neurogenesis and inhibit gliogenesis. Such a growth factor-regulated intracellular switch provides a mechanism for integrating intrinsic cellular programs with the external neural environment, and thereby ensuring the genesis of appropriate numbers of neurons at the appropriate time in embryogenesis.
CHAPTER 4: TRK SIGNALING REGULATES NEURAL PRECURSOR CELL PROLIFERATION AND DIFFERENTIATION DURING CORTICAL DEVELOPMENT

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CONTRIBUTION OF AUTHORS

A.P. performed all of the in vivo experiments in this chapter except for some of the dnTrkB and dnTrkC experiments, and co-wrote the manuscript with K.B. and F.D.M. K.B. performed the dnTrkB and dnTrkC experiments in vitro and some of the dnTrkB and dnTrkC experiments in vivo. A.S.G. performed some of the experiments in vivo and provided advices. F.D.M. and D.R.K. helped design experiments, provided support, advices and guidance.
4.1 ABSTRACT

Increasing evidence indicates that development of embryonic CNS precursors is tightly regulated by extrinsic cues located in the local environment. Here, we have asked whether neurotrophin-mediated signaling through Trk tyrosine kinase receptors is important for embryonic cortical precursor cell development. These studies demonstrate that inhibition of TrkB and/or TrkC signaling using dominant-negative Trk receptors, or genetic knockdown of TrkB using shRNA, caused a decrease in embryonic precursor cell proliferation both in culture and in vivo. Inhibition of TrkB/C also caused a delay in the generation of neurons, but not astrocytes, and ultimately perturbed the postnatal localization of cortical neurons in vivo. Conversely, overexpression of BDNF in cortical precursors in vivo promoted proliferation and enhanced neurogenesis. Together, these results indicate that neurotrophin-mediated Trk signaling plays an essential, cell-autonomous role in regulating the proliferation and differentiation of embryonic cortical precursors and thus controls cortical development at earlier stages than previously thought.
4.2- INTRODUCTION

Development of the cerebral cortex is achieved through a common pool of precursor cells that sequentially generate neurons and glial cells. Emerging evidence indicates that while intrinsic cues are important in cortical precursor cell behavior, differences in the developmental availability of growth factors determine precursor cell survival, proliferation, and the appropriate timed genesis of neurons versus astrocytes (Miller and Gauthier, 2007). For example, FGF2 is an essential survival and proliferation factor for cortical precursors in vivo (Vaccarino et al., 1999; Raballo et al., 2000) and in vitro (Ghosh and Greenberg, 1995; Lukaszewicz et al., 2002), and cell-contact mediated signals, such as those involving Notch (Chambers et al., 2001; Shen et al., 2002; Mizutani and Saito, 2005) regulate precursor cell proliferation, maintenance and differentiation. Moreover, appropriate early neurogenesis requires extrinsic activation of a SHP-2-Ras-MEK-ERK-C/EBP pathway (Ménard et al., 2002; Paquin et al., 2005; Gauthier et al., 2007), while the later onset of astrocyte formation requires activation of the gp130-JAK-STAT pathway (Bonni et al., 1997; Johe et al., 1996) by neuron-derived cardiotrophin-1, a cytokine that is not expressed in the cortex until late embryonic timepoints (Barnabé-Heider et al., 2005).

The neurotrophins are growth factors that are well-known for regulating the biology of CNS neurons (Huang and Reichardt, 2003), but that might also play a role in regulating cortical precursor cell biology. At least two members of the neurotrophin family, BDNF and NT-3, along with their preferred tyrosine kinase receptors, TrkB and TrkC, are expressed in the cortical ventricular/subventricular zones (VZ/SVZ) during the period of cortical neurogenesis (Maisonpierre et al., 1990; Tessarollo et al., 1993; Behar et al., 1997; Fukumitsu et al., 1998; Fukumitsu et al., 2006). Moreover, culture work indicates that (i) NT-3 selectively regulates cell cycle exit and neuronal differentiation in cortical progenitors (Ghosh and Greenberg, 1995;
Lukaszewicz et al., 2002), and (ii) cortical precursors themselves synthesize and secrete the neurotrophins BDNF and NT-3 which promote their survival and differentiation in an autocrine/paracrine fashion by activating TrkB/TrkC receptors (Barnabé-Heider and Miller, 2003). However, an in vivo role for the Trk receptors in cortical precursor biology has not yet been established.

Here, we have asked whether Trk signaling is important for embryonic cortical precursor cell development in vivo, by performing in utero electroporation with dominant-negative TrkB and TrkC or with TrkB shRNA to acutely, and in a cell-autonomous fashion, disrupt Trk signaling. In this regard, the TrkB receptor can be activated by BDNF, NT-3, and NT-4 (Huang and Reichardt, 2003), and while previous work (Jones et al., 1994; Alcantara et al., 1997; Ringstedt et al., 1998; Xu et al., 2000; Lotto et al., 2001; Medina et al., 2004) has indicated that BDNF-mediated TrkB activation is important for cortical development in vivo, these studies concluded that the observed perturbations were a consequence of altered TrkB signaling in cortical neurons. The TrkC receptor is only activated by NT-3, and previous work on NT-3-/- and TrkC-/- mice has primarily focused upon the profound deficits observed in the peripheral nervous system (Emfors et al., 1994; Wilkinson et al., 1996; Klein et al., 1994; Tessarollo et al., 1994), or on perturbations in the biology of committed CNS glia or neurons (Minichiello and Klein, 1996; Martinez et al., 1998; Kahn et al., 1999; Ma et al., 2002; von Bohlen und Halbach et al., 2003). Since both BDNF and NT-3 are known to be expressed in precursor cells of the cortical neuroepithelium (Maisonpierre et al., 1990; Fukumitsu et al., 1998; Behar et al., 1997; Barnabé-Heider and Miller, 2003; Fukumitsu et al., 2006), and since cortical precursors express both of these receptors (Tessarollo et al., 1993; Behar et al., 1997; Barnabé-Heider and Miller, 2003), then we have chosen to disrupt signaling via these two receptors both individually and
together. These studies demonstrate that TrkB and TrkC receptor activation, presumably in response to BDNF and NT-3, are necessary for the appropriate proliferation and differentiation of embryonic cortical precursors. Thus, some of the cortical perturbations observed in BDNF-/- and TrkB-/- mice are likely due to altered precursor cell function, and indicate that neurotrophins play an earlier role in the embryonic cortical development.

4.3- RESULTS

4.3.1- TrkB and TrkC are essential for the development of cultured cortical precursors

To ask whether Trk receptors are important for the behavior of neural precursors, we examined primary murine E12.5 cortical precursor cells, a system we have previously characterized in detail (Toma et al., 2000; Ménard et al., 2002; Barnabé-Heider and Miller, 2003; Barnabé-Heider et al., 2005; Gauthier et al., 2007). Upon plating in FGF2, these cortical precursors are virtually all dividing, nestin-positive cells that first generate neurons at 1 day in vitro (DIV), and astrocytes and oligodendrocytes at 5-6 DIV. The increase in differentiated cells is accompanied by depletion of proliferating precursors.

We previously showed that freshly-isolated cortical precursors express TrkB and TrkC, and respond to endogenously produced BDNF and NT-3 in culture (Barnabé-Heider and Miller, 2003). To ask how these Trk receptors regulate cortical precursor biology, we inhibited their function using dominant-negative TrkB and TrkC receptors (dnTrkB and dnTrkC). The dnTrkB is a kinase-dead ATP-binding site mutant we previously characterized (Atwal et al., 2000). The dnTrkC has three mutated tyrosines within the catalytic domain and functions as a dominant-negative in TrkC-expressing PC12 cells (Pantelis Tsoufas, University of Miami, personal communication). We confirmed the efficacy of these dnTrks by cotransfecting them with EGFP
into freshly-plated cortical precursors and immunostaining 2 days later with an antibody for activated Trk phosphorylated at Y490. In cells transfected with dnTrkB and/or dnTrkC, only approximately 40% of the GFP-positive cells expressed readily-detectable phosphoTrk levels compared to 70% in controls.

We next asked whether Trk receptor inhibition affected survival. Precursors were cotransfected with EGFP and dnTrkB and/or dnTrkC, and survival was assessed at 2 DIV by counting EGFP-positive cells with condensed, apoptotic nuclei, or by immunostaining for cleaved caspase-3. Inhibition of TrkB, TrkC, or both caused an approximately 2-3-fold increase in condensed nuclei (Fig. 11A, B), and a 2-4-fold increase in cleaved caspase-3-positive cells (Fig. 11C). Thus, Trk signaling is important for cortical precursor survival in culture, consistent with our previous work showing that endogenously-produced neurotrophins in these cultures support cell survival (Barnabé-Heider and Miller, 2003).

We also asked whether Trk signaling was important for cell proliferation by performing similar culture experiments, and immunostaining cells at 2 DIV for the proliferation marker, Ki67 (Fig. 12A); inhibition of TrkB and/or TrkC caused a significant decrease in the number of proliferating cells (Fig. 12B). However, since this decrease could be a secondary effect of enhanced cell death rather than a direct effect on cell division, we performed similar experiments in the presence of the pan-caspase inhibitor, ZVAD. ZVAD completely abolished the enhanced death seen in cells transfected with dnTrkB and/or dnTrkC, and decreased cell death to levels below those seen in basal conditions (Fig. 12C, D). Immunostaining for Ki67 revealed that, even when cell death was prevented with ZVAD, inhibition of TrkB and/or TrkC caused a decrease in precursor proliferation (Fig. 12E). Thus, Trk signaling, presumably activated by endogenously produced BDNF and NT-3, directly promotes proliferation of cultured cortical precursors.
Figure 11. *TrkB and TrkC signaling are essential for the survival of cultured embryonic cortical precursor cells.* (A) Fluorescence micrographs of cortical precursor cultures cotransfected with plasmids encoding EGFP and the empty vector (control), EGFP and dnTrkB (dnTrkB), EGFP and dnTrkC (dnTrkC) or EGFP and both dnTrkB and dnTrkC (dnTrkB/C), and then analyzed immunocytochemically at 2DIV for EGFP (green, GFP), and cleaved caspase 3 (red, casp 3). Cell were also counterstained with Hoechst 33258 (blue) to show all of the nuclei in the field. Arrows denote double-labelled cells. Scale bar, 100µm. (B,C) Quantification of the percentage of transfected, apoptotic cells in experiments similar to that shown in (A), as monitored by (B) the percentage of EGFP-positive cells with condensed, fragmented Hoechst-stained nuclei, and (C) the percentage of double-labelled, EGFP-, cleaved caspase-3-positive cells. In each panel, two representative experiments of 4 are shown. Error bars denote SEM. *p<0.05, **<0.01, ***<0.001 relative to control-transfected cultures.
Figure 12. *TrkB and TrkC are essential for proliferation of cultured embryonic cortical precursor cells.* (A) Double-label immunocytochemical analysis for EGFP (green, GFP) and Ki67 (red) for precursor cells that were transfected with plasmids encoding EGFP plus empty vector (control), EGFP plus dnTrkB, EGFP plus dnTrkC, or EGFP plus dnTrkB and dnTrkC (dnTrkB/C) and then cultured for 2 days. Cells were counterstained with Hoechst 33258 to show all of the nuclei in the field. Arrows denote double-labelled cells. Scale bar, 100µm. (B) Quantification of the percentage of transfected, Ki67-positive cells in experiments similar to that shown in panel (A). Two representative experiments of 4 are shown. Error bars indicate the SEM *p<0.05, **p<0.01 relative to control-transfected cultures. (C, D) Quantification of the percentage of apoptotic cells in experiments similar to that shown in panel (A), except that after transfection, cells were cultured with or without the pan-caspase inhibitor, ZVAD for two days. Apoptosis was assessed both by analysis of condensed, fragmented nuclei by Hoechst counterstaining (C) and by double-label immunocytochemistry for EGFP and cleaved caspase-3 (D). In both panels, one representative experiment of 2 is shown. Error bars indicate the SEM *p<0.05, **p<0.01, ***p<0.001 relative to control-transfected cultures. Note that ZVAD reduced apoptosis to lower than baseline levels. (E) Quantification of the percentage of dividing, transfected cells as monitored by double-labelling for EGFP and Ki67, as shown in panel (A), except that after transfection, cells were cultured with or without the pan-caspase inhibitor ZVAD for two days. Error bars indicate SEM *p<0.05, **p<0.01, ***p<0.001 relative to control-transfected cultures.
4.3.2- Trk signaling in cortical precursors is essential for normal cortical development in vivo

To ask whether Trk signaling was necessary for cortical precursor development in vivo as well as in culture, we performed in utero electroporation to transfet precursors of the VZ/SVZ of the embryonic cortex (Barnabé-Heider et al., 2005; Paquin et al, 2005; Gauthier et al., 2007). We have previously demonstrated that one day following electroporation at E14/15, all of the transfected cells reside in the VZ/SVZ and most of them are proliferating (Paquin et al, 2005). Many of these transfected cells differentiate into neurons over the next few days, which migrate out of the VZ/SVZ and into the cortical plate, where they ultimately become principal neurons of layers 2 and 3. Later in development, at early postnatal periods, many of the transfected cells that remain in the VZ/SVZ adopt an astrocytic fate.

Initially, we confirmed that TrkB and TrkC were expressed in the E14.5 cortex in vivo, as previously reported (Tessarollo et al., 1993; Behar et al., 1997). Cortical tissue was isolated at E14.5, postnatal day 3 (P3) or from adults, and was triturated and exposed to either 100 ng/ml BDNF or 200 ng/ml NT-3 for 5 minutes to maximally activate Trk receptors. Full-length Trk receptors were then immunoprecipitated using a panTrk antibody, and visualized by Western blot analysis with anti-phosphotyrosine. Blots were then reprobed for total TrkB or TrkC. This analysis confirmed that full-length TrkB was present in the E14.5 cortex, albeit at lower levels than at P3 or in the adult (Fig. 13A), as we have previously shown (Knusel et al., 1994). In contrast to TrkB, full-length TrkC levels were constant from E14.5 to adulthood. Interestingly, the level of tyrosine phosphorylation of TrkB and TrkC were lower in the adult brain, likely as a
**A**

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**B**

**C**

**D**

**E**

**F**

**G**

% GFP +ve cells within the cortical mantle

control control control control
dnTrkB dnTrkB/CdnTrkC control control
dnTrkB dnTrkB/CdnTrkB

% GFP +ve cells with high levels of TrkB

control control shTrkB-1 shTrkB-2

% GFP +ve cells within the cortical mantle

control shTrkB-1 control shTrkB-2
Figure 13. **Trk signaling is necessary for development of neural precursors within the embryonic cortex.** (A) Western blot analysis for phosphotyrosine (pTyr) in immunoprecipitates of freshly-isolated cortical tissue from E14.5, P3 and adult mouse that was stimulated for 5 min with BDNF or NT-3, and immunoprecipitated with a panTrk antibody. Blots were then reprobed for TrkB (left panel) or TrkC (right panel). Arrowheads in upper panels indicate the pTyr-positive bands migrating at the level of full-length TrkB or TrkC. Arrowheads in lower panels indicate full-length TrkB and TrkC. (B) Fluorescence micrograph of a coronal section of an E16/17 mouse brain that was cotransfected with plasmids encoding EGFP and myc-tagged dnTrkB at E13/14, and then 3 days later immunostained for EGFP (green) and the myc-tag (red). Double-labelled cells are yellow in this merged image. Scale bar, 100µm. (C) Fluorescence micrographs of coronal sections through the cortex of mice cotransfected with plasmids encoding EGFP and the empty vector (control; top panels) or dnTrkB (bottom panels) at E13/14 and analyzed 3 days later at E16/17. Sections were double-labelled for the neuron-specific protein HuD (red, left panels), and EGFP (GFP, green, middle panels; the right panels are the merged image). CM = cortical mantle, VZ/SVZ = ventricular zone/subventricular zone, V = ventricle. Scale bar 100 µm. (D) Quantification of sections similar to those shown in (C) for the percentage of EGFP-positive transfected cells present within the cortical mantle. For each comparison, n = 10-12 each of control and experimental brains, four to five sections per embryo (a total of n=30 animals for controls, and 10-12 each for dnTrkB, dnTrkC and dnTrkB plus dnTrkC). (E) Quantification of the percentage of transfected precursor cells that express high levels of TrkB 3 days after cotransfection with GFP and either the control shRNA, or one of the two TrkB shRNAs (shTrkB-1, shTrkB-2). (F) Fluorescence micrographs of coronal sections through the cortex of mice cotransfected with plasmids encoding EGFP and the empty vector (control; top panels) or TrkB shRNA (shTrkB; bottom panels) at E13/14 and analyzed 3 days later at E16/17. Sections were double-labelled for the neuron-specific protein HuD (red, left panels), and EGFP (GFP, green, middle panels; the right panels are the merged image). CM = cortical mantle, VZ/SVZ = ventricular zone/subventricular zone, V = ventricle. Scale bar 100 µm. (G) Quantification of sections similar to those shown in (F) for the percentage of EGFP-positive transfected cells present within the cortical mantle. n = 6 controls and 4 TrkB shRNA brains, five to six sections per embryo. *p<0.05, **p<0.01, ***p<0.001 relative to control-transfected sections. Error bars indicate SEM.
consequence of increased truncated Trk receptors (Knusel et al., 1994). Thus, signal transducing full-length TrkB and TrkC are both present within the E14.5 cortex.

We therefore used in utero electroporation to ask whether Trk signaling was essential for cortical precursor development. We electroporated plasmids encoding a nuclear EGFP and dnTrkB, dnTrkC, or both at E13/14, and analyzed the embryos 3 days later at E16/17. To confirm that cells were appropriately cotransfected, we performed double-label immunocytochemistry for EGFP and the myc tag on dnTrkB (Atwal et al., 2000); 77 ± 6% (n = 5 brains) of the GFP-positive cells expressed detectable dnTrkB (Fig. 13B). We then analyzed cell migration by immunostaining for EGFP and the neuronal marker HuD to help define cortical morphology (Fig. 13C). This analysis revealed that, relative to control EGFP-positive cells, 30-50% fewer dnTrkB or dnTrkC-expressing cells had migrated to the cortical mantle (which includes both the intermediate zone of migrating neurons and the cortical plate) (Fig. 13D).

To confirm these findings, we also knocked-down TrkB mRNA using two different TrkB shRNAs. To establish the efficacy of these shRNAs in cortical precursors, we cotransfected them with EGFP into cultured precursors, and immunostained them for TrkB 3 days later. Approximately half of the control cells expressed TrkB at high levels, and the TrkB shRNAs reduced this percentage by 2-3-fold (Fig. 13E). We then utilized these shRNAs to knockdown TrkB in vivo; E13/14 cortices were coelectroporated with EGFP and one of the two TrkB shRNAs, and then were analyzed 3 days later for cell migration (Fig. 13F). As seen with the dnTrks, the TrkB shRNAs reduced the percentage of transfected cells in the cortical mantle by approximately 50% (Fig. 13G). Thus, TrkB and TrkC signaling are necessary for normal development of cortical precursors in vivo.
4.3.3- Trk signaling is essential for cortical precursor cell proliferation but not survival in the embryonic cortex

To determine the cellular basis for the perturbed cortical development observed when TrkB or TrkC were inhibited, we characterized cell survival and proliferation 1 and 3 days postelectroporation. Initially, we examined survival by immunostaining for cleaved caspase-3. Only very small numbers of EGFP-positive, cleaved caspase-3-positive cells were observed in cortices electroporated with the empty vector or with dnTrkB, dnTrkC, or both (Fig. 14A). Numbers ranged from 0 to 3 cells per section, with never more than 11 double-labelled cells within the 6 sections/brain that were analyzed. These numbers were similar in control and experimental brains at both timepoints. Moreover, the total numbers of EGFP-positive cells 3 days post-electroporation were similar between control and dnTrk groups (Fig. 14B; 1250 ± 202 EGFP-positive control cells and 1256 ± 146 dnTrk transfected cells when all groups were combined), arguing that there was no loss of dnTrk-expressing cells. Thus, unlike cultured precursors, cortical precursors in vivo do not require Trk signaling for survival, perhaps because there are alternative survival factors within the cortical neuroepithelium.

We next performed similar electroporations and asked whether Trk signaling was important for precursor cell proliferation. Confocal microscopy of sections immunostained for Ki67 revealed that virtually all transfected, Ki67-positive cells were located within the VZ/SVZ in all groups (Fig. 14C). At 1 and 3 days post-electroporation, approximately 70-80% and 8-13%, respectively, of control transfected cells within the VZ/SVZ were Ki67-positive (Fig. 14D, E). At both timepoints, inhibition of TrkB and/or TrkC signaling reduced precursor cell
A) Immunochemical detection of caspase-3 and GFP in different experimental conditions. Arrows indicate cells of interest.

B) Graph showing the average number of GFP+ve cells in the cortex across different conditions.

C) Detection of Ki67 and GFP in control and experimental conditions. Arrows indicate Ki67+ve cells.

D) Graph showing the percentage of Ki67+ve/GFP+ve cells in total GFP+ve cells in the SVZ.

E) Graph showing the percentage of Ki67+ve/GFP+ve cells in total GFP+ve cells in the SVZ across different conditions.

F) Graph showing the percentage of pHistone-H3+ve/GFP+ve cells in total GFP+ve cells in the SVZ across different conditions.

G) Graph showing the percentage of pHistone-H3+ve/GFP+ve cells in total GFP+ve cells in the SVZ across different conditions.
Figure 14. Trk receptor signaling regulates embryonic cortical precursor cell proliferation but not survival in vivo. (A-E) Precursor cells of the embryonic cortex were transfected by in utero electroporation at E13/14 with plasmids encoding EGFP and the empty vector (control), EGFP and dnTrkB, EGFP and dnTrkC, or EGFP and dnTrkB plus dnTrkC (dnTrkB/C), and then analyzed 1-3 days later. (A) Confocal micrographs of coronal sections through transfected cortices that were immunostained for EGFP (GFP, green) and cleaved caspase 3 (casp 3, red) 1 day after electroporation. The right panels show the merged images. Arrows indicate transfected cells that are positive for cleaved caspase-3, while arrowheads indicate cells that only express cleaved caspase-3. Scale bar, 100 µm. (B) Quantification of the number of transfected, EGFP-positive cells through the cortices of animals transfected at E13/14 and analyzed three days later at E16/17. Total EGFP-positive cells in six sections per brain of 9-15 animals each in control and experimental groups were determined to obtain these numbers. There is no significant difference between the control and experimental groups in any of the pairs. (C) Confocal micrographs of coronal sections through the VZ/SVZ of transfected cortices that were immunostained for EGFP (GFP, green) and the proliferation marker Ki67 (red) three days after electroporation. The right panels show the merged images. Arrows indicate transfected cells that coexpress Ki67. Scale bar 100 µm. (D, E) Quantification of the percentage of transfected, Ki67-positive cells in the VZ/SVZ (where all of the Ki67-positive cells were located) of mice transfected as in panel (B) and then analyzed 1 day (D, E14/15) or 3 days (E, E16/17) following electroporation. Numbers in (D) were obtained from analysis of 3-5 littermate pairs, four to five sections per embryo for each comparison between control and experimental groups (a total of n=11 animals for controls, and 3-5 each for dnTrkB, dnTrkC and dnTrkB plus dnTrkC at each timepoint). Numbers in (E) were obtained from analysis of three experiments, 3-4 littermate pairs, four to five sections per embryo for each comparison between control and experimental groups (a total of n=28 animals for controls, and 10-11 each for dnTrkB, dnTrkC and dnTrkB plus dnTrkC at each timepoint). (F, G) Quantification of the percentage of transfected, Ki67-positive cells (F) or phospho-histone-H3-positive cells (G) in the VZ/SVZ of mice transfected with EGFP and control shRNA (control) or one of two TrkB shRNAs (shTrkB-1, shTrkB-2) at E13/14 and analyzed 3 days following electroporation. n = 6 controls and 4 TrkB shRNA brains, five to six sections per embryo. *p<0.05, **p<0.01, ***p<0.001 relative to control-transfected sections. Error bars indicate SEM.
proliferation (Fig. 14D, E). For example, dnTrkC reduced proliferation at by 23% and 50% at 1 and 3 days, respectively.

We obtained similar results when we electroporated cortices with the TrkB shRNAs and analyzed them 3 days later. As seen with the dnTrks, only very small numbers of control or TrkB shRNA-expressing cells were positive for EGFP and cleaved caspase-3 (normally 0-2 cells/section, and never more than 7 total double-labelled cells within the 5-6 sections/brain that were quantified). In contrast, knockdown of TrkB reduced the number of proliferating, transfected precursors as monitored by immunostaining for either Ki67 or for the mitosis marker, phospho-histone H3 (Fig. 14F, G). The magnitude of this reduction was similar to that obtained with the dnTrks at 3 days (Fig. 14E).

These data indicate that Trk signaling is required in a cell-autonomous fashion for precursor proliferation. Two well-characterized Trk signaling cascades that might mediate this proliferative response are the MEK-ERK and the PI3K-Akt pathways. We previously demonstrated that electroporation of dnMEK into cortical precursors selectively inhibited neurogenesis and increased, rather than decreased the number of proliferating precursors (Paquin et al., 2005). We therefore asked whether Akt might be the relevant downstream effector protein. To do this, we generated an HA-tagged form of a previously-characterized dominant-negative Akt (Songyang et al., 1997). Transfection of this construct into 293 cells demonstrated expression of an HA-tagged protein of the appropriate size (Fig. 15A). We then coelectroporated plasmids encoding dnAkt and EGFP into E13/14 cortices, and analyzed survival and proliferation of transfected cells 2 days later. Only very small numbers of dnAkt-expressing, cleaved caspase-3-positive cells were observed (from 0-2 cells/section, with no more than 10 double-labelled cells within the 6 sections/brain that were quantified), indicating that this was not
Figure 15. (A, B) Akt is not essential for survival or proliferation of cortical precursors in vivo. (A) Western blot analysis of lysates of HEK 293 cells transfected with an HA-tagged dominant-negative Akt, or with GFP, and then probed with antibodies for the HA-tag or for GFP. The upper arrow denotes an HA-positive band of the appropriate molecular weight. (B) Quantification of the percentage of transfected, Ki67-positive cells in the VZ/SVZ of mice transfected with EGFP and empty vector (control) or with dominant-negative Akt (dnAkt) at E13/14 and analyzed 2 days following electroporation. n = 6 each of control and experimental brains, five to six sections per embryo. (C-F) Trk receptor signaling is necessary for appropriate embryonic neurogenesis. (C) Confocal micrographs of cortical sections from mice electroporated at E13/14 with plasmids encoding EGFP and dnTrkB and immunostained 3 days later for neuron-specific HuD (red) and EGFP (GFP, green). The upper panels are higher magnification images through the cortical plate (which contains neurons), and the lower panels are lower magnification images that encompass both the VZ/SVZ and part of the cortical mantle (CM). Arrows indicate double-labelled, transfected cells. Scale bar 100 µm. (D, E) Quantification of micrographs similar to those shown in (C) analyzing the percentage of transfected, EGFP-positive cells that coexpressed HuD within the cortical mantle (D) or in the entire cortex (E) of brains that were transfected with EGFP and the empty vector (control), dnTrkB, dnTrkC, or both (dnTrkB/C) at E13/14 and analyzed at E16/17. Numbers were obtained from analysis of three experiments, two-four littermate pairs, four to five sections per embryo for each comparison between control and experimental groups (a total of n=31 animals for controls, and 10-11 each for dnTrkB, dnTrkC and dnTrkB plus dnTrkC ). (F) Quantification of the percentage of transfected, HuD-positive cells in the cortex of mice transected with EGFP and control shRNA (control) or one of two TrkB shRNAs (shTrkB-1, shTrkB-2) at E13/14 and analyzed 3 days following electroporation. n = 6 controls and 4 TrkB shRNA brains, five to six sections per embryo. (G-I) Trk receptors function together to regulate precursor proliferation and neurogenesis. Quantification of the percentage of GFP-positive cells in the cortical mantle (G), proliferating, GFP-positive, Ki67-positive cells in the VZ/SVZ (H), and GFP-positive, HuD-positive neurons (I) in cortices coelectroporated with EGFP and 1.5 µg of dnTrkB or dnTrkC alone (1/2 dnTrkB, 1/2 dnTrkC), with 1.5 µg each of dnTrkB and dnTrkC together (dnTrkB+C) or with 3 µg each of dnTrkB and dnTrkC together (double B+C). Cortices were electroporated at E13/14 and analyzed 3 days later. n = 6 controls, 4 dnTrkB alone, 5 dnTrkC alone, 10 dnTrkB+C, and 10 double B+C, with an average of 4 sections analyzed per embryo. Error bars indicate SEM. NS = nonsignificant, *p<0.05, **p<0.01, ***p<0.001.
a major survival pathway. Moreover, similar numbers of transfected, Ki67-positive precursors were seen in control and dnAkt-transfected cortices (Fig. 15B), indicating that this was also not a major proliferative pathway. Thus, Trks likely signal via targets other than Akt and MEK to mediate cortical precursor proliferation.

4.3.4- Trk signaling is essential for appropriate neurogenesis

We previously showed that one downstream Trk pathway, the SHP-2-MEK-ERK pathway, is essential for differentiation of cortical precursors into neurons (Ménard et al. 2002, Barnabé-Heider and Miller, 2003; Paquin et al., 2005; Gauthier et al., 2007). Our finding (Fig. 13C, D, F, G) that dnTrks inhibited cells from moving from the VZ/SVZ into the cortical mantle suggested that they either (i) inhibited neurogenesis, or (ii) inhibited migration of newly-born cortical neurons to the cortical plate. To distinguish these two possibilities, we performed immunocytochemistry for the early neuronal markers HuD and βIII-tubulin to ask if newly-born neurons were trapped within the VZ/SVZ. Confocal microscopy demonstrated that this was not the case; only very small numbers of transfected, newly-born neurons were located within the VZ/SVZ in any of the conditions. Instead, virtually all of the transfected neurons were present within the cortical mantle (Fig. 15C). Thus, even when TrkB/C signaling is inhibited, cells that become neurons can migrate out of the VZ/SVZ, implying that the decreased percentage of cells within the cortical mantle reflects decreased neurogenesis.

To examine this idea further, we determined the percentage of EGFP-positive cells within the cortical mantle that expressed HuD; approximately 75% of control- or dnTrk-transfected cells that migrated to the cortical mantle expressed HuD (Fig. 15C, D). We then used these
numbers, together with the counts of numbers of cells within the cortical mantle versus the VZ/SVZ to calculate the percentage of neurons in each of these brains (Fig. 15E). This analysis demonstrated that approximately 17-25% of control transfected cells were neurons after 3 DIV, and that Trk inhibition significantly reduced this percentage (Fig. 15E). To confirm that Trk signaling was essential for appropriate neurogenesis, we also electroporated the TrkB shRNAs, and directly counted the percentage of total transfected cells that expressed HuD 3 days later. This analysis revealed a robust, 2-3-fold decrease in the percentage of TrkB shRNA-expressing versus control shRNA-expressing neurons (Fig. 15F). Since virtually no cleaved caspase-3-positive cells were observed within the cortical mantle following TrkB shRNA electroporation, then we conclude that TrkB is essential for normal cortical neurogenesis.

4.3.5- TrkB and TrkC collaborate to regulate proliferation and neurogenesis in the cortical neuroepithelium

The previous experiments did not show increased effects on cortical development when dnTrkB and TrkC were electroporated together as opposed to when they were electroporated individually. However, only half as much of each of these constructs was used when they were electroporated together versus when they were electroporated alone (2 μg versus 4 μg each), suggesting a possible technical reason for this result. We therefore performed similar electroporations where we used similar amounts of dnTrkB or dnTrkC DNA alone or together (1.5 μg of each in both cases) to ask whether TrkB and TrkC signaling cooperated to regulate precursor development. We also coelectroporated twice as much (3 μg) of each together to ensure that we obtained maximal inhibition of Trk signaling. Analysis of these experiments
showed that when 1.5 μg of dnTrkB or dnTrkC were electroporated individually, there were somewhat fewer GFP-positive cells in the cortical plate (Fig. 15G), and small decreases in proliferation (Fig. 15H), and neurogenesis (Fig. 15I), but that none of these reached significance. However, when we electroporated 1.5 μg of each together, the magnitude of all of these differences was increased and, in all cases, reached high significance (Fig. 15G-I; P<0.001). Similar, but even more pronounced, results were obtained when 3 ug of each were electroporated together (Fig. 15G-I). Thus, TrkB and TrkC signaling cooperate to promote cortical precursor proliferation and neurogenesis.

4.3.6- Overexpression of BDNF in cortical precursors promotes proliferation and enhances neurogenesis

These data support the idea that BDNF and/or NT-3 made within the cortical neuroepithelium regulate cortical precursor cell proliferation and neurogenesis. To directly test this idea, we overexpressed BDNF in cortical precursors in vivo by cotransfecting E13/14 cortices with plasmids encoding BDNF and EGFP, and performed immunocytochemistry 3 days later. This analysis revealed an increase in the percentage of transfected cells in the VZ/SVZ that expressed Ki67 (Fig. 16A), indicating that BDNF directly promoted proliferation. We then asked whether overexpression of BDNF also enhanced neurogenesis by immunostaining these sections for EGFP and HuD. This analysis revealed that the percentage of cells located within the cortical mantle and the percentage of HuD-positive neurons were both higher in cortices transfected with BDNF (Fig. 16B, C). Interestingly, we also observed clusters of HuD-positive neurons in the VZ/SVZ, something that was never seen in control brains (Fig. 16D). Some of
Increased BDNF expression in the cortical neuroepithelium promotes precursor proliferation and enhances neurogenesis. (A) Quantification of the percentage of transfected, Ki67-positive cells in the VZ/SVZ of mice transfected with EGFP and empty vector (control) or BDNF at E13/14 and analyzed 3 days following electroporation. n = 8 each of control and experimental brains, five to six sections per embryo. *p<0.05. Error bars indicate s.e.m. (B) Quantification of the percentage of transfected cells in the cortical mantle of mice transfected with EGFP and empty vector (control) or BDNF at E13/14 and analyzed 3 days following electroporation. n = 8 each of control and experimental brains, five to six sections per embryo. *p<0.05, ***p<0.001. Error bars indicate SEM. (C) Quantification of the percentage of transfected, HuD-positive cells in the cortex of mice transfected with EGFP and empty vector (control) or with BDNF at E13/14 and analyzed 3 days following electroporation. n = 8 each of control and experimental brains, five to six sections per embryo. *p<0.05. Error bars indicate SEM. (D) Confocal micrographs of cortical sections from mice electroporated at E13/14 with plasmids encoding EGFP and empty vector (control) or BDNF, and immunostained 3 days later for HuD (red) and EGFP (green). The left panels of each pair are HuD staining, and the right panels are merges showing both HuD and EGFP staining. Arrows indicate a cluster of EGFP-negative, HuD-positive cells. V = ventricle. Scale bar 100 µm.
these HuD-positive neurons were EGFP-positive, but many others were EGFP-negative and were located in close proximity to transfected cells (Fig. 16D, right panel), supporting the idea that BDNF was secreted from the transfected cells and promoted the premature genesis of neurons in their vicinity. Thus, BDNF levels are normally limiting, and increasing BDNF within the cortical epithelium promotes precursor cell proliferation and enhanced neurogenesis.

**4.3.7- Perturbation of Trk signaling does not alter astrocyte formation, but leads to a depletion of postnatal precursors within the VZ/SVZ**

These in vivo data demonstrated that inhibition of Trk signaling perturbed appropriate neurogenesis, with a higher percentage of undifferentiated, but less proliferative, cells remaining in the VZ/SVZ from E14 to E17. We therefore asked whether it also caused more longterm perturbations by examining neonatal brains at postnatal day 3 (P3). Immunocytochemical analysis for the neuronal markers HuD and NeuN, and the astrocyte marker GFAP (astrogenesis from transfected precursors occurs during the late embryonic/early neonatal period) revealed that in controls at this age all of the transfected neurons were present within the cortical layers (Fig. 18A), and all of the transfected, GFAP-positive astrocytes were located within the VZ/SVZ (Fig. 17A), as we have previously published (Barnabé-Heider et al., 2005; Paquin et al., 2005; Gauthier et al., 2007). A similar pattern of double-labelled, transfected cells was observed in brains transfected with dnTrkB and/or dnTrkC (Fig. 17A, 18A). However, quantification of the percentage of transfected cells present within the VZ/SVZ (which contains only undifferentiated precursors and newly-differentiated glial cells at this stage) revealed that while in controls
Figure 17. *Trk signaling in embryonic precursors is necessary for maintenance of normal numbers of precursors in the postnatal SVZ, but not for astrocyte formation.* Precursor cells of the embryonic cortex were transfected by in utero electroporation at E13/14 with plasmids encoding EGFP and the empty vector (control), EGFP and dnTrkB, EGFP and dnTrkC, or EGFP and dnTrkB plus dnTrkC (dnTrkB/C), and then analyzed after the animals were born at postnatal day 3. (A) Confocal micrographs of coronal sections through the VZ/SVZ of transfected cortices that were immunostained for EGFP (GFP, green) and the astrocyte protein GFAP (red). The right panels show the merged images. Arrows indicate transfected cells that coexpress GFAP. Scale bar, 100 µm. (B) Quantification of light micrographs of sections similar to those shown in (A) for the percentage of total transfected, EGFP-positive cells remaining within the VZ/SVZ at P3. Transfected cells within the brains of 5 each of control and transfected animals, five sections per animal were counted to obtain these numbers. Error bars indicate SEM. **p<0.01, ***p<0.001 relative to control-transfected sections. (C) Quantification of confocal micrographs similar to those shown in (A) to determine the percentage of transfected, GFAP-positive cells within the VZ/SVZ (where all of these cells are located). Transfected cells within the brains of 5 each control and transfected animals, five sections per animal were counted to obtain these numbers. There was no significant difference between groups. Error bars indicate SEM. p>0.05. (D) Quantification of confocal micrographs similar to those shown in (A) where brains were coelectroporated with EGFP and control shRNA (control) or TrkB shRNA (shTrkB) to determine the percentage of transfected, GFAP-positive cells within the VZ/SVZ. n = 4 control and 3 TrkB shRNA brains. Error bars indicate SEM. p>0.05.
approximately 30% of the transfected cells were located within the VZ/SVZ, for dnTrkB and/or dnTrkC-transfected cells, this number was only approximately 5-10% (Fig. 17B).

To confirm that this was a selective depletion of cells within the VZ/SVZ, we calculated the total number (as opposed to the proportion) of cells within the SVZ versus cortical layers of these same brains, recognizing that there would be some variability as a consequence of the differences in efficiency of electroporation from litter to litter. This analysis revealed that the total number of transfected, EGFP-positive cells within the control versus dnTrk-transfected brains was approximately similar for all groups (in the five sections/animal that were quantified, the means ± s.e.m. were control, 606 ± 110; dnTrkB, 664 ± 68; dnTrkC, 663 ± 68; dnTrkB/C, 739 ± 103; p>0.4 for all comparisons relative to the control; n = at least four animals per group). The total number of transfected cells within the cortical layers was also statistically similar (means ± s.e.m. were control, 458 ±102; dnTrkB, 594 ± 51; dnTrkC, 625 ± 66; dnTrkB/C, 682 ± 108; p>0.15 for all comparisons relative to the control). In contrast, the total number of transfected cells in the SVZ was significantly reduced in dnTrk-transfected brains (means ± s.e.m. were control, 148 ± 8; dnTrkB, 70 ± 24; dnTrkC, 38 ± 5; dnTrkB/C, 57 ± 9; p<0.002 for all comparisons relative to control). Thus, inhibition of Trk signaling led to a specific depletion of precursors within the SVZ of the postnatal cortex.

This selective depletion of dnTrk-expressing cells within the SVZ could be due to the decreased precursor cell proliferation observed during embryogenesis, and/or to a perturbation in astrocyte formation, which takes place in the VZ/SVZ. To distinguish these possibilities, we quantified the proportion of GFAP-positive cells within the VZ/SVZ using confocal microscopy. This analysis demonstrated that approximately 15% of the EGFP-positive cells in the VZ/SVZ coexpressed GFAP and that expression of dnTrkB and/or dnTrkC had no effect on this
proportion (Fig. 17A, C). We confirmed that Trk signaling was not required for normal astrogenesis by performing similar experiments with one of the two TrkB shRNAs, electroporating at E13/14 and analyzing the brains at P3/P4. Quantification of the percentage of transfected, GFAP-positive astrocytes in the SVZ demonstrated similar astrocyte numbers in control and experimental brains (Fig. 17D). Thus, inhibition of Trk receptor signaling depleted cells within the postnatal SVZ, not because of alterations in gliogenesis, but potentially because of decreased precursor cell proliferation.

4.3.8- Acute inhibition of Trk receptor signaling in cortical precursors leads to longterm effects on cortical neuron development

We also characterized the development of cortical neurons in these transfected, neonatal brains by immunostaining for HuD and for a second neuronal marker, NeuN (Fig. 18A). Confocal microscopy and quantification (Fig. 18B, C) revealed that approximately 60-70% and 75% of the GFP-positive cells within the cortical layers expressed NeuN and HuD, respectively, and that this did not differ between control and experimental groups. However, we did note a difference in the location of these transfected cells within the cortical layers (Fig. 18D). While virtually all of the control transfected cells (>90%) were located within the upper part of layers 2/3, approximately 30-40% of the dnTrkB and/or dnTrkC-transfected cells were instead located within the lower portion of layers 2/3 (Fig. 18D, E).

These data indicate (i) that inhibition of Trk signaling during embryogenesis delayed but apparently did not permanently inhibit precursors from becoming neurons (although the transient nature of the transfections also makes it possible that dnTrk levels had decreased sufficiently by
**A**

Images showing the expression of NeuN and GFP in different conditions. Arrows indicate the presence of GFP-positive cells.

**B**

Bar graph showing the percentage of NeuN+ GFP+ cells in total GFP+ cells within the cortical layers.

**C**

Bar graph showing the percentage of HuD+ GFP+ cells in total GFP+ cells within the cortical layers.

**D**

Images showing the distribution of GFP+ cells in control, dnTrkB, dnTrkC, and dnTrkB/C conditions. Scale bar indicates measurement.

**E**

Bar graph showing the percentage of GFP+ cells migrating within the cortical layers in different conditions.

**F**

Images showing the distribution of GFP+ cells in control, dnTrkB, dnTrkC, and dnTrkB/C conditions.
Figure 18. Inhibition of Trk signaling in embryonic cortical precursors leads to postnatal perturbations in cortical neurons. Precursor cells of the embryonic cortex were transfected by in utero electroporation at E13/14 with plasmids encoding EGFP and the empty vector (control), EGFP and dnTrkB, EGFP and dnTrkC, or EGFP and dnTrkB plus dnTrkC, and then analyzed after the animals were born at postnatal day 3. (A) Confocal micrographs of coronal sections through transfected cortices that were immunostained for EGFP (GFP, green) and the neuron-specific protein NeuN (red). The right panels show the merged images. Arrows indicate transfected cells that coexpress NeuN. Scale bar, 100µm. (B, C) Quantification of confocal micrographs similar to those shown in (A) to determine the percentage of transfected cells expressing NeuN (B) or HuD (C) within the cortical layers. Transfected cells within the brains of 5 each control and transfected animals, five sections per animal were counted to obtain these numbers. There was no significant difference between groups. Error bars indicate SEM. p>0.05. (D) Photomicrographs taken on the fluorescence microscope of coronal section through the P3 neocortex of brains immunostained for EGFP (green) and counterstained with Hoechst (blue) to show cell nuclei. The cortical layers are denoted on the left side of upper left photomicrograph, and the regions denoted as the upper part of layers II/III and the lower part of layers II/III are shown with the white lines. Scale bar, 200µm. (E) Quantification of the percentage of transfected, EGFP-positive cells in the upper one-half (white columns) and in the lower one-half (black column) of neocortical layers II/III in sections similar to those shown in (D). n=5 animals in each group, five sections per brain. Error bars indicate SEM. **p<0.01, ***<0.001 relative to control-transfected sections. (F) Photomicrographs of coronal sections through the P3 neocortex of brains immunostained for EGFP (green) and GAD67 (red) and counterstained for Hoechst (blue). The left and middle panels show low-magnification micrographs showing EGFP and Hoechst (left) or GAD67 and Hoechst (middle). The right panel is a higher-magnification view showing a rare, double-labelled cell (arrow), and several EGFP-positive, GAD-67-negative cells (arrowheads). Scale bar, 50 µm.
later timepoints to allow neurogenesis to proceed), and (ii) that these later-born neurons did not migrate to a location appropriate for their birthdate, suggesting that Trk signaling might also be essential for appropriate cortical neuron migration within the cortical layers. To ask whether this perturbation was due to a requirement for TrkB/TrkC signaling in cortical interneuron migration, as has been previously suggested (Polleux et al., 2002), we immunostained cortices for the GABAergic interneuron marker GAD67. This analysis demonstrated that only a very small number of transfected cortical precursors developed into GAD-67-positive interneurons (Fig. 18F), and that this number was unchanged by expression of dnTrkB, dnTrkC, or both (1-2 EGFP-positive/GAD-67-positive cells per section in all groups). This result is consistent with the finding that most rodent cortical interneurons derive from the medial ganglionic eminence, and not the cortical neuroepithelium (reviewed in Xu et al., 2003). Thus, the perturbations recorded here are likely due to a more general requirement for TrkB/TrkC signaling in migration of principal cortical neurons (Medina et al., 2004).

4.4- DISCUSSION

The studies described here support a number of major conclusions. First, experiments acutely inhibiting TrkB and TrkC and overexpressing BDNF in vivo demonstrate that neurotrophin-mediated activation of these two receptors is essential for the proliferation of embryonic cortical precursors, an unexpected role for Trk signaling within the cortical neuroepithelium. Second, we show that inhibition of TrkB and TrkC signaling in vivo leads to a delay in the generation of new neurons, and ultimately perturbs postnatal localization of principal cortical neurons. Conversely, BDNF overexpression enhances the generation neurons from
precursors, indicating that Trk signaling regulates the timing and potentially extent of cortical neurogenesis. In contrast, TrkB and TrkC receptor signaling is not required for cortical astrocyte formation, at least within the first few days postnatally. Finally, these studies demonstrate that inhibition of Trk signaling in embryonic cortical precursors leads to a decrease in the proportion of postnatal cortical precursors that are maintained within the SVZ, possibly as a direct consequence of the decrease in embryonic proliferation. Together, these results indicate that neurotrophin-mediated TrkB and TrkC activation regulates the proliferation, maintenance, and differentiation of embryonic cortical precursors, suggesting that this family of growth factors may play a more general role in the regulation of CNS neural precursor cells.

The neurotrophin receptors TrkB and TrkC are activated in response to their preferred ligands; BDNF, NT-3 and NT-4 in the case of TrkB and NT-3 in the case of TrkC (Huang and Reichardt, 2003). BDNF and NT-3 are both expressed within the developing cortical neuroepithelium (Maisonpierre et al., 1990; Behar et al., 1997; Fukumitsu et al., 1998; Fukumitsu et al., 2006), and TrkB and TrkC are coincidently expressed on embryonic cortical precursors (Tessarollo et al., 1993; Behar et al., 1997; Barnabé-Heider and Miller, 2003; data shown here). Moreover, we and others have shown that, in cultured cortical precursors, BDNF and/or NT-3 can regulate survival, proliferation, and differentiation (Ghosh and Greenberg, 1995; Lukaszewicz et al., 2002; Barnabé-Heider and Miller, 2003). However, although cortical perturbations have been observed in BDNF-/- and TrkB-/- mice (Jones et al., 1994; Alcantara et al., 1997; Ringstedt et al., 1998; Xu et al., 2000; Lotto et al., 2001; Medina et al., 2004), these changes were attributed to the lack of BDNF-mediated TrkB signaling in cortical neurons. Similarly, any CNS deficits observed in NT-3-/- and TrkC-/- mice have been attributed to perturbations in survival and connectivity of central, including cortical, neurons (Minichiello and
Klein, 1996; Martinez et al., 1998; Ma et al., 2002; von Bohlen und Halbach et al., 2003), or to alterations in glial cell development (Kahn et al., 1999). Here, we report that TrkB and TrkC are both necessary for the proliferation and differentiation of cortical precursor cells in vivo, and that they act together, presumably when activated in response to BDNF and NT-3 in the cortical neuroepithelium.

Results were similar for both receptors, and our DNA titration experiments indicate that the two receptors function together to promote proliferation and neurogenesis, playing complementary roles in cortical precursors during this developmental window. This, and the acute, cell-autonomous nature of the perturbations made here, may explain why previous studies examining single neurotrophin and/or receptor knockouts have not observed such changes. In this regard, a previous study (Medina et al., 2004) examining a nestin-driven conditional TrkB knockout demonstrated that a higher proportion of TrkB-/- cells were maintained within the embryonic VZ/SVZ, a phenotype very similar to that seen here with acute inhibition of TrkB. These TrkB-/- cells within the SVZ, which were not characterized phenotypically, were interpreted as being newly-born neurons that did not migrate. However, on the basis of data presented here, we propose that this perturbation at least partially reflected the necessity for TrkB in the appropriate timing/extent of cortical neurogenesis, as we have documented here using both dominant-negative Trk receptors and with an acute shRNA-mediated knockdown of TrkB.

While our studies indicate that neurotrophins, acting through TrkB and TrkC, are important for cortical precursor cell proliferation, our data showing that inhibition of Trk signaling only reduces proliferation by approximately 50% indicates that other proliferative cues are also important. In particular, other candidate proliferative growth factors include FGF2,
heparin-binding EGF (HB-EGF), and IGF-1. FGF2 is a major proliferative factor for cortical precursors (Ghosh and Greenberg, 1995; Vaccarino et al., 1999; Raballo et al., 2000), and deficits in FGF2 lead to reduced neurogenesis (Raballo et al., 2000). Heparin-binding EGF (HB-EGF) is expressed in the embryonic VZ/SVZ (Nakagawa et al., 1998), and promotes neurogenesis in the adult CNS (Jin et al., 2003). Finally, overexpression of IGF-1 from the nestin promoter in mice caused increased proliferation and neuron number in the embryonic cortex (Popken et al., 2004). What are the sources of these growth factors during neurogenesis? Cortical precursors of the VZ/SVZ themselves express BDNF, NT-3 (Behar et al., 1997; Maisonpierre et al., 1990; Fukumitsu et al., 1998; Fukumitsu et al., 2006), FGF2 (Raballo et al., 2000), and HB-EGF (Nakagawa et al., 1998). Intriguingly, endothelial cells also express BDNF (Kim et al., 2004), FGF2 (Albuquerque et al., 1998) and HB-EGF (Arkonac et al., 1998), and embryonic endothelial cells have recently been shown to promote self-renewal and neurogenesis in neural stem cells (Shen et al., 2004). Thus, it is likely that cortical precursors are exposed to diverse receptor tyrosine kinase ligands from multiple sources, and that signaling via these growth factors converge on to similar intracellular pathways to ultimately regulate their proliferation and differentiation.

How do receptor tyrosine kinases like the Trks signal to regulate the biology of cortical precursor cells? We have previously demonstrated (Barnabé-Heider and Miller, 2003) that the PI3K but not the MEK-ERK pathway is essential for survival of cultured cortical precursors, findings similar to those recently reported for embryonic stem cells (Pyle et al., 2006). Moreover, a second study demonstrated that overexpression of the PI3K target protein Akt-1 led to enhanced cortical precursor cell survival, proliferation and self-renewal (Sinor and Lillien, 2004). However, data presented here inhibiting Akt in cortical precursors in vivo showed that
Akt was not necessary for either the survival or proliferation of these cells, at least within the timeframe we examined. Perhaps PI3K mediates cortical precursor cell survival and potentially proliferation via other targets such as ILK (Hannigan et al., 2005) or PDK1 (Mora et al., 2004). With regard to cortical precursor differentiation, we previously demonstrated that the MEK-ERK pathway is essential for neurogenesis, acting to phosphorylate and activate the C/EBP family of transcription factors (Ménard et al., 2002; Paquin et al., 2005). In addition, we have recently shown that SHP-2, a protein tyrosine phosphatase that is necessary for maximal activation of the MEK-ERK pathway downstream of TrkB (Easton et al., 2006), is essential for the differentiation, but not survival or proliferation, of cortical precursor cells (Gauthier et al., 2007). Interestingly, evidence indicates that this same SHP-2-MEK-ERK-C/EBP pathway inhibits astrocyte formation while it promotes neurogenesis (Ménard et al., 2002; Paquin et al., 2005; Liu et al., 2006; Gauthier et al., 2007), providing a mechanism that would allow growth factors like the neurotrophins to bias precursors to make only neurons during the neurogenic period.

While our experiments in the embryonic cortex indicate that inhibition of Trk signaling functions to decrease and/or delay neurogenesis, this effect was no longer apparent by P3. One explanation for this finding is that these transfections were transient, and that the level of Trk inhibition decreased over time so that these precursors were ultimately able to generate equal numbers of neurons. A second explanation is that other extrinsic cues also promote neurogenesis, potentially via the same SHP-2-MEK-ERK-C/EBP pathway, and that ultimately neurogenesis "catches up", even when Trk signaling is inhibited. While our data do not distinguish between these possibilities, they do indicate that a perturbation of Trk signaling in embryonic precursors ultimately caused mislocalization of principal cortical neurons. If this was simply due to a delay in neurogenesis, then the delayed neurons should be resident in more
superficial cortical layers, as was previously seen with cortical precursors that were transiently inhibited from differentiating by Notch pathway activation (Mizutani and Saito, 2005). Instead, we document here that these later-born neurons are located in deeper cortical layers at this timepoint, providing further support for previous studies showing that neurotrophin signaling regulates cortical neuron migration (Gates et al., 2000; Polleux et al., 2002; Medina et al., 2004) and that BDNF can regulate the phenotype of newly-born cortical neurons (Fukumitsu et al., 2006).

Little is known about how environmental cues such as growth factors regulate the survival, proliferation and differentiation of multipotent neural precursors in the embryonic CNS. Here, we provide evidence that the neurotrophins, growth factors previously thought to primarily regulate the development of differentiated CNS neurons and glial cells, also regulate the proliferation and differentiation of cortical precursors in vivo. Moreover, we propose that since many other extrinsic cues converge on to the same signaling pathways, then this provides one way in which the complex extracellular environment of the neuroepithelium can be integrated to dynamically regulate precursor cell survival, proliferation, and ultimately cell genesis during development.
CHAPTER 5: COSTELLO SYNDROME H-RAS ALLELES REGULATE CORTICAL DEVELOPMENT

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CONTRIBUTION OF AUTHORS

A.P. performed most of the experiments in this chapter and co-wrote the manuscript with D.R.K. and F.D.M. C.H performed experiments shown in Fig. 19A, B and one of the experiments in Fig. 19F. D.R.K. and F.D.M. helped design experiments, provided support, advices and guidance.
5.1- ABSTRACT

Genetic mutations in H-Ras cause Costello Syndrome (CS), a complex developmental disorder associated with cortical abnormalities and profound mental retardation. Here, we have asked whether there are perturbations in precursor cell proliferation, differentiation, or survival as a consequence of expressing CS H-Ras alleles that could explain the cognitive deficits seen in this disorder. Two different H-Ras alleles encoding mutations present in CS patients, H-RasG12V and H-RasG12S were expressed in cortical progenitors in culture and in vivo by in utero electroporation and their effects on cortical precursor cell fate examined. Expression of both mutants in cultured precursors inhibited neurogenesis and promoted proliferation and astrogensis. In vivo, expression of either form of CS H-Ras promoted cell proliferation and inhibited neurogenesis. Moreover, these H-Ras mutants promoted premature gliogenesis, causing formation of astrocytes at a time when normal gliogenesis has not yet begun, ultimately leading to an increase in the number of astrocytes postnatally. Thus, aberrant H-Ras activation enhances neural precursor cell proliferation, and perturbs the normal genesis of neurons and glial cells, effects that likely contribute to the cortical abnormalities and cognitive dysfunction seen in CS.
5.2- INTRODUCTION

During mammalian development, the cerebral cortex is generated through a heterogeneous pool of precursor cells that can produce neurons and glial cells. Emerging evidence suggests that growth factors determine the precise timing and extent of the genesis of these two cell types (Miller and Gauthier, 2007). In vivo, cortical neurogenesis starts early in development at embryonic day 12 (E12) and ends at around E18, at which point astrogenesis starts and continues through the first weeks of life. These sequential waves of differentiation are regulated by both intrinsic mechanisms and growth factors in the neural environment. Many of these growth factors converge onto the Ras signaling pathway, which has been shown to play a central role in regulating neural precursor cell proliferation, survival, and cell fate decisions (Samuels et al., 2009). With specific regard to the developing cortex, aberrant H-Ras activation in postmitotic neurons causes neuronal cell bodies to hypertrophy and subsequent ventricular enlargement (Heumann et al., 2000; Hennekam, 2003). Moreover, components of the Ras signaling cascade, including SHP-2, MEK, ERK2 and C/EBP have been shown to be play important roles in cortical precursor cell neurogenesis (Gauthier et al., 2007; Ménard et al., 2002; Paquin et al., 2005; Samuels et al., 2008; 2009). In contrast, activation of the gp130-JAK-STAT pathway by cardiotrophin-1 has been shown to be required for astrocyte formation (Bonni et al., 1997; Barnabé-Heider et al., 2005).

Recent evidence suggests that germline mutations in Ras pathway proteins are the cause of the neuro-cardio-facial-cutaneous (NCFC) syndromes (Bentires-Alj et al., 2006). These syndromes include Noonan syndrome, Costello syndrome (CS), neurofibromatosis-1 (NF1), LEOPARD syndrome, and cardio-facio-cutaneous syndrome. Individuals with these disorders present with a combination of facial abnormalities and heart defects. Importantly, mental
retardation and cognitive dysfunction are also common. In this regard, we recently asked whether aberrant development of neural precursors might contribute to the cortical abnormalities seen in this family of disorders, and focused upon Noonan syndrome, where the SHP2 protein tyrosine phosphatase is aberrantly activated (Gauthier et al., 2007). These studies demonstrated that SHP2-mediated dysregulation of the MEK-ERK and gp130-JAK-STAT pathways caused aberrant genesis of neurons and glial cells in the embryonic cortex, and provided support for the idea that neural precursor dysfunction might be an underlying cause of perturbed neural development and the resultant cognitive deficits in this family of disorders.

Costello syndrome is one member of the NCFC family of syndrome where all of the patients show mental retardation (Costello 1971, 1977). Moreover, imaging studies of CS patients show that close to 50% show neuroanatomical perturbations including corpus collosum agenesis, enlarged ventricles, frontal and cerebellar atrophy, cerebellar malformations, hypodense white matter, and hydrocephalus (Delrue et al., 2003; Hennekam, 2003; Gripp et al., 2002, 2005; Zampino et al., 2007). Recently, point mutations in H-Ras have been identified as responsible for causing this disorder (Aoki et al., 2005). In about 80% of cases, the mutation is situated at position 12 in the guanine nucleotide binding site. These mutations result in decreased GTP hydrolysis, causing increased and sustained Ras activity, and thus act as gain of function mutations. These findings, together with previous work suggesting that components of the Ras pathway regulate cortical development, suggest that the cortical abnormalities seen in CS may well be a consequence of perturbed cortical cell genesis. Further support for this idea comes from work on the NF1 gene, which encodes a tumor suppressor that, amongst several activities, is a negative regulator of Ras (Hegedus et al, 2007). There is a high incidence of cognitive dysfunction in NF1 patients (Hyman et al., 2005), and mice where NF1 is genetically
ablated in CNS precursors exhibit a variety of deficits in glial cells, including globally reactive astrogliosis and increased proliferation of glial precursor cells (Zhu et al., 2005; Hegedus et al., 2007). On the basis of these findings, we have hypothesized that aberrant H-Ras activation causes cognitive dysfunction by perturbing the proliferation and the differentiation of neural precursor cells and their progeny.

Here, we provide evidence in support of this hypothesis, demonstrating that expression of two Costello syndrome H-Ras alleles in cortical precursors enhances cell proliferation and inhibits neurogenesis in vivo. We also demonstrate that they cause premature gliogenesis, which ultimately leads to an increase in astrocytes postnataally. These findings suggest that aberrant H-Ras activation disrupts normal cortical development and this may explain the cortical abnormalities seen in CS.

5.3- RESULTS

5.3.1- Costello syndrome H-ras mutants promote proliferation and astrogenesis in cultured cortical precursors

The hyperactivated H-ras mutations found in Costello syndrome cause cortical abnormalities (Gripp, 2005; Zampino et al., 2007). To ask if they might do this by perturbing neural precursor development, we examined cortical precursor cells that were isolated from the E12.5-E13.5 mouse cortex and plated in the presence of FGF2, which is essential for cortical precursor proliferation (Gosh and Greenberg, 1995; Raballo et al., 2000). We have shown previously that, upon plating, these cells are dividing, nestin-positive precursors (Slack et al., 1998; Ménard et al., 2002). Over the next 3-5 days in culture, many of these precursors exit the
cell cycle to become neurons, while after 5 days the same pool of precursors starts to generate astrocytes (Barnabé-Heider et al., 2005; Paquin et al., 2005; Gauthier et al., 2007), a temporally-regulated sequence of events that also occurs in vivo (Qian et al., 2000). We transfected these precursors with plasmids encoding one of two Costello syndrome H-Ras mutants that together comprise approximately 80% of patients with this syndrome; both of these mutations affect amino acid 12, and involve mutations from glycine to valine (H-RasG12V) or serine (H-RasG12S). To follow transfected precursors, we cotransfected them upon plating with plasmids encoding EGFP and one of the two CS mutants. In agreement with our previous work (Paquin et al., 2005; Bartkowska et al., 2007), more than 80% of the cells that expressed EGFP also expressed H-Ras, indicating the reliability of the cotransfection approach.

We first confirmed that the CS Ras mutants stimulated the activity of ERK1/2, a downstream target of Ras, in cortical precursors as they do in other cell types (Seeburg et al., 1984; Mazzoni et al., 1999). Plasmids encoding H-RasG12V or LacZ were cotransfected with the EGFP-expression plasmid into freshly-plated cortical precursor cells. Immunocytochemical analysis 5 days later for EGFP and the phosphorylated, activated ERK1/2 revealed that whereas fewer than 20% of the control LacZ-transfected cells expressed detectable levels of phospho-ERK, approximately 60% of the H-RasG12V-transfected cells were phospho-ERK-positive (Fig. 19A,B). Thus, CS H-Ras mutants are active in cortical precursors, as they are in other cells.

To determine whether CS H-Ras mutants promoted aberrant proliferation of precursors, similarly-transfected cells were analyzed after 3 days in culture by double-label immunocytochemistry for EGFP and Ki67, a marker for proliferating cells (Fig. 19C). The
Figure 19. Activated H-RasG12V and H-RasG12S increase proliferation and perturb differentiation of cultured cortical precursor cells. (A) Immunocytochemical analysis for EGFP (green) and activated, phosphorylated Erk (red) in cortical precursor cells cotransfected with plasmids encoding EGFP and LacZ, or EGFP and H-RasG12V. Cells were cultured for 5 days post-transfection. Cultures were counterstained with Hoechst 33258 (blue) to show all of the nuclei in the field. Arrows denote double-labeled cells. Scale bar = 20µm. (B) Quantification of transfected phosphoErk-positive cells as shown in panel (A) in 4 independent experiments. Error bars denote SEM. ***p<0.005. (C, E, G) Fluorescence photomicrographs of cultured cortical precursors cotransfected with plasmids encoding EGFP and an empty vector (control), EGFP and H-RasG12V, or EGFP and H-RasG12S, as indicated. Cultures were analyzed after 3 DIV (C, E) or 5 DIV (G) for EGFP (green) and Ki67 (C), βIII-tubulin (E), or GFAP (G) (all in red). Cells were counterstained with Hoechst 33258 (blue) to show nuclei. Arrows denote double-labelled cells, while arrowheads indicate transfected cells negative for the marker. Scale bar: 50µm. (D, F, H) Quantification of the percentage of EGFP-positive cells also positive for Ki67 (D), βIII-tubulin (F), or GFAP (H) in experiments similar to those shown in panels C, E, or G respectively. In panels D and H, the graphs represent combined data from 3 independent experiments, while in panel F, individual representative experiments (expt.) are shown. Error bars denote SEM. ***p<0.001, relative to control-transfected cultures.
proliferation of cells transfected with H-RasG12V or H-RasG12S was increased 1.5 to 2-fold relative to control plasmid-transfected cells (Fig. 19D).

To ask whether CS H-Ras mutants also perturbed differentiation, we performed the same cotransfections and immunostained for the neuron-specific protein βIII-tubulin (Fig. 19E), or the astrocyte protein GFAP (Fig. 19G). To ask about neurogenesis, we performed this analysis at 3 or 5 days post-transfection. Quantification showed that both H-RasG12V and H-RasG12S decreased the number of neurons that were generated in these cultures (Fig. 19F). To ask about astrogenesis, cultures were treated with ciliary neurotrophic factor (CNTF) upon transfection, and then were analyzed 5 days later. CNTF treatment of cortical precursor cell cultures induces astrocyte formation (Bonni et al., 1997; Barnabé-Heider et al., 2005), and thus allows us to study astrogenesis in vitro in a timely manner. Double-label immunocytochemistry for EGFP and GFAP revealed that both H-Ras mutants increased the number of astrocytes that were present relative to controls by approximately two-fold (Fig. 19H). Thus, sustained activation of H-Ras in cultured cortical precursors increases both cell proliferation and astrogenesis, while coincidently decreasing neurogenesis.

5.3.2- Sustained H-Ras activation promotes proliferation of cortical precursors in vivo

To ask whether the CS H-Ras mutants affected cortical precursors in the embryo as they did in culture, we performed in utero electroporation. We have previously shown that cortical precursors that are transfected at E13.5 in vivo proliferate within the ventricular and subventricular zones (VZ/SVZ) and that over time, these transfected precursors generate first neurons, and then glial cells, as do nontransfected cortical precursors (Paquin et al., 2005; Bartkowska et al., 2007; Gauthier et al., 2007). Plasmids encoding a nuclear EGFP and either...
the empty vector, or one of the two CS H-Ras mutants, were coelectroporated after injection into the E13-14 embryo lateral ventricles and these embryos were examined 3 days later. Coronal sections through the cortex of these electroporated embryos were double-labelled for EGFP and Ki67 (Fig. 20A) and confocal microscopy was used to determine the percentage of EGFP-positive cells that expressed Ki67 (Fig. 20B, C). We also determined the percentage of proliferating, transfected cells in the VZ/SVZ, which at this timepoint contains precursor cells and newly-born neurons, and the cortical plate, which is comprised of newly-born neurons postmigration. This analysis revealed that, at this timepoint, 7-11% of total control cells were Ki67-positive (Fig. 20B, C), as we have previously published (Paquin et al., 2005; Bartkowska et al., 2007; Gauthier et al. 2007). Virtually all of these proliferating cells were in the VZ/SVZ, consistent with a precursor cell phenotype, with no, or very few transfected, proliferating cells in the cortical plate, also consistent with the fact that newly-born neurons exit the cell cycle within the VZ/SVZ before they migrate to the cortical plate. In contrast, approximately 20-25% of the H-RasG12V and H-RasG12S transfected cells were Ki67-positive (Fig. 20B, C), a 2-to-3-fold increase relative to controls. Moreover, while much of this increase occurred in precursors in the VZ/SVZ (Fig. 20D-F), many EGFP-positive, Ki67-positive cells were also observed in the cortical plate (Fig. 20G-I), something that was rarely observed in controls. Thus, the increased H-Ras activity that occurs with CS mutations promotes aberrant proliferation of cortical precursor cells and potentially even newly-born neurons in vivo.

5.3.3- Costello syndrome H-Ras mutants decrease neurogenesis in vivo

To ask whether H-Ras sustained activation also affected cell genesis in the embryonic cortex, as it did in culture, we performed similar in utero electroporations with plasmids encoding nuclear EGFP and the empty vector, H-RasG12V, or H-RasG12S, and analyzed these
Figure 20. CS H-Ras mutants increase cortical precursor cell proliferation in vivo. Precursor cells of the embryonic cortex were transfected by in utero electroporation at E13/14 with plasmids encoding nuclear EGFP and an empty vector (control), EGFP and H-RasG12V, or EGFP and H-RasG12S, and then analyzed 3 days later at E16/17. (A) High magnification confocal image of cells in the VZ/SVZ that were electroporated with EGFP and H-RasG12V, and immunostained for EGFP (green) and Ki67 (red; colocalization is yellow, and is denoted by arrows; arrowheads denote singly-labeled cells). Scale bar, 20µm. (B, C) Quantification of the number of transfected, EGFP and Ki67-positive cells as a percentage of total transfected cells in the entire cortex. (D, G) Confocal micrographs of coronal sections through the ventricular zone/subventricular zone (VZ/SVZ; D) or cortical plate (CP; G) of transfected cortices that were immunostained for EGFP (green) and the proliferation marker Ki67 (red). The red boxes in the low magnification images on the left indicate the areas that are shown in the higher magnification images. The right panels show the merged images. Arrows indicate transfected cells that are positive for both EGFP and Ki67, while arrowheads denote cells that are positive for EGFP or Ki67 only. In (D), the hatched lines outline the VZ/SVZ, while in (G) the hatched lines outline the cortical plate. V, ventricle; M, meninges. Scale bar, 100 µm. (E, F, H, I) Quantification of the number of EGFP-positive transfected cells in the VZ/SVZ (E, F) or in the cortical plate (H, I) that were also positive for Ki67, as shown in panels D and G. Numbers in B, C, E, F, H, I were obtained from analysis of 3-5 littermate pairs, four to five sections per embryo for each comparison between control and experimental groups. In total, an average of 110-192 cells/section was counted in each of 20-33 sections, for a total of 2641-6326 cells per experimental group. Error bars indicate SEM. **p<0.01, ***<0.001 relative to control-transfected sections.
embryos at varying timepoints. We first examined coronal sections through the cortex during the neurogenic period, 3 days post electroporation, performing double-label immunocytochemistry for EGFP to identify transfected cells, and the neuron-specific protein HuD, to identify the cortical plate and newly-born neurons (Fig. 21A, B). Analysis of the location of the EGFP-positive cells within the embryonic cortex revealed that while 40-50% of control plasmid-transfected cells were present within the cortical plate, presumably because they had become neurons and migrated there, only about half as many of the cells transfected with the CS H-Ras mutants were located in this region (Fig. 21C, E). Confocal analysis of cells coexpressing both EGFP and HuD (Fig. 21B) revealed a similar decrease in the numbers of neurons that were generated from transfected precursors; transfection with the CS H-Ras mutants decreased the percentage of EGFP-positive, HuD-positive neurons by two-or-more-fold (Fig. 21D, F). Moreover, of those transfected cells that had migrated to the cortical plate, fewer CS H-Ras-transfected cells expressed detectable levels of HuD (Fig. 21G).

These data support the conclusion that CS H-Ras mutants decrease neurogenesis. To verify this conclusion, we performed a number of additional experiments. First, we asked whether the decrease in neuronal numbers might be due to increased apoptosis of newly-born neurons that expressed activated H-Ras. To do this, we performed immunocytochemistry for cleaved caspase-3 on cortices that were electroporated with plasmids encoding nuclear EGFP and the empty vector or H-RasG12S for 3 days (Fig. 21H). This analysis demonstrated that only 1-3 cells per section were positive for cleaved caspase-3, and that this was similar in both the control and H-Ras-electroporated cortices. Second, we asked whether the decrease in neuronal numbers was persistent by performing electroporations at E13/14, and then analyzing the electroporated cortices at postnatal day 3. Quantification revealed that, even at this later
% of GFP+ cells in the cortical plate

C

D

E

F

G

H

I

% of HuD-GFP+ cells / total GFP+ cells

% of HuD-GFP+ cells / total GFP+ cells

% of HuD-GFP+ cells / total GFP+ cells

% of HuD-GFP+ cells in cortical plate

% of HuD-GFP+ cells in cortical plate

% of HuD-GFP+ cells in cortical plate

% of HuD-GFP+ cells / total GFP+ cells

% of HuD-GFP+ cells / total GFP+ cells

% of HuD-GFP+ cells / total GFP+ cells

142
Figure 21. CS H-Ras mutants decrease neurogenesis in vivo. Precursor cells of the embryonic cortex were transfected by in utero electroporation at E13/14 with plasmids encoding nuclear EGFP and an empty vector (control), H-RasG12V, or H-RasG12S, and then analyzed 3 days later at E16/17 (A-H) or at postnatal day 3 (I). (A) Confocal micrographs of electroporated cortical sections that were immunostained for EGFP (green) and the neuronal marker HuD (red). The red boxed area in the low magnification image on the left indicates the area that is shown in the higher magnification images. The right panels show the merged images. IZ, intermediate zone; CP, cortical plate; V, ventricle; M, meninges. Scale bar, 100µm. (B) Higher magnification confocal images of a section similar to that shown in (A). Arrows indicate transfected cells that are positive for both EGFP and HuD. Scale bar, 50µm. (C, E) Quantification of control and H-RasG12V (C) or H-RasG12S (E) transfected cells that were located within the cortical plate as a percentage of total EGFP transfected cells. (D,F,G) Quantification of the number of transfected, EGFP-positive, HuD-positive cells as a percentage of total transfected cells in the entire cortex (D,F), or specifically in the cortical plate (G). (H) Confocal micrographs of electroporated cortical sections that were immunostained for EGFP (green) and the apoptotic marker cleaved caspase-3 (CC3, red). The right panels show the merged images and arrowheads denote cells that were positive for CC3. (I) Quantification of the number of transfected, EGFP-positive, HuD-positive cells as a percentage of total transfected cells in the entire cortex at P3. Numbers in C-G, I were obtained from analysis of 3-5 littermate pairs, four to five sections per embryo for each comparison between control and experimental groups. For C,E, an average of 38-138 cells/section was counted in each of 22-38 sections, for a total of 1519-5241 cells per experimental group. For D,F, an average of 76-180 cells/section was counted in each of 16-48 sections for a total of 1215-7123 cells per experimental group. For G, an average of 16-23 cells/section was counted in each of 16-17 sections for a total of 276-394 cells per experimental group. For I, an average of 46-53 cells/section was counted in each of 16-24 sections for a total of 749-1329 cells per experimental group. Error bars indicate SEM. **p<0.01, ***<0.001 relative to control-transfected sections.
timepoint, expression of the CS H-Ras mutants caused a decrease in the total number of EGFP-positive, HuD-positive neurons (Fig. 21I). Finally, to ask whether this decrease reflected a deficit in neuronal migration, we looked specifically at the number of HuD-positive neurons present in the VZ/SVZ. This analysis showed that only 0.2%-0.3% of HuD-positive cells were in the VZ/SVZ and that this number was similar in cortices electroporated with the empty vector versus CS H-Ras mutants (P>0.05). Together, these data argue that CS H-Ras mutants directly decreased neurogenesis from cortical precursors in vivo.

5.3.4- Costello syndrome H-Ras mutants enhance proliferation of newly-generated astrocytes in vivo

These data indicate that CS H-Ras mutants caused increased precursor proliferation, decreased neuronal genesis, and the aberrant appearance of proliferating cells within the cortical plate. To ask whether these aberrantly-proliferating cells within the VZ/SVZ and cortical plate were or would become neurons or astrocytes, we performed in utero electroporations, and analyzed the electroporated cells 4.5 days later, immediately prior to birth. To ask if these proliferating cells were newly-born neurons that had not exited the cell cycle, we triple-labelled coronal sections through electroporated brains for EGFP, to monitor transfected cells, Ki67 to monitor proliferating cells, and one of two early neuronal markers, βIII-tubulin (Fig. 22A) or doublecortin (Fig. 22B). Confocal microscopy analysis revealed that only rare cells coexpressed βIII-tubulin or doublecortin and Ki67, regardless of the cortical region, and regardless of whether they were transfected with CS H-Ras mutants. We therefore performed similar analyses, triple-labelling for EGFP, Ki67, and GFAP to ask whether these proliferating cells were astrocytes (Fig. 22C, D). Confocal analysis demonstrated that, as seen at 3 days post-electroporation, at 4.5 days post-electroporation, transfection with CS H-Ras mutants caused an increase in the
% of GFAP-GFP+ cells / GFP+ cells

% of Ki67-GFP+ cells / GFP+ cells

% of Ki67-GFAP-GFP+ cells / GFAP-GFP+ cells

% of Ki67-GFAP-GFP+ cells / Ki67-GFP+ cells in CP

RasG12V-Ki67-GFP

RasG12S

RasG12V

RasG12S

Ctrl

RasG12V

**

*
Figure 22. **CS H-Ras mutants promote astrocyte proliferation in vivo.** Precursor cells of the embryonic cortex were transfected by in utero electroporation at E13/14 with plasmids encoding nuclear EGFP and an empty vector (control), H-RasG12V, or H-RasG12S, and then analyzed 4.5 days later at E17.5/18.5. **(A, B)** Confocal micrographs of cortical sections electroporated with H-RasG12S and triple-labeled for EGFP (green), the early neuronal marker βIII-tubulin or doublecortin (DCX) (both red) and Ki67 (blue). The right panel shows the merged images. Arrows indicate cells double-labelled for EGFP and Ki67 (A) or EGFP and doublecortin (B). Arrowheads in (B) indicated a cell that expresses EGFP only. Scale bar, 25µm (A), 10µm (B). **(C)** Confocal micrographs of a cortical section electroporated with H-RasG12V and triple-labeled for EGFP (green), Ki67 (red) and the astrocyte marker GFAP (blue). The right panels show the merged image. The bottom panels show higher magnification images of the section shown in the top panels. Arrows indicate a triple-labelled cell. The hatched lines in the top panels outline the VZ/SVZ. V; ventricle, CM; cortical mantle. Scale bar, 50µm (upper panels) and 25µm (bottom panels). **(D)** High magnification confocal images of a cortical section electroporated with H-RasG12S, and triple-labeled for EGFP (green), Ki67 (upper panels, red), and GFAP (bottom panels, red). The arrow denotes the same cell in all panels. The top right panel shows the merged image for EGFP and Ki67, and the bottom right the merged image for EGFP and GFAP. Scale bar, 10µm. **(E, F)** Quantification of the number of transfected cells that were positive for Ki67 (E), or GFAP (F) as a percentage of total transfected cells. **(G)** Quantification of the number of transfected cells that were positive for both Ki67 and GFAP, as a percentage of total GFAP-positive transfected cells in the entire cortex. **(H)** Quantification of the number of transfected cells that were positive for both Ki67 and GFAP, as a percentage of total transfected, Ki67-positive cells in the cortical plate (CP). **(I)** Quantification of the number of transfected cells that were positive for both Ki67 and GFAP in the VZ/SVZ versus cortical plate as a percentage of the total transfected, Ki67-positive, GFAP-positive cells. Numbers in E-I were obtained from analysis of 3-5 littermate pairs, four to five sections per embryo for each comparison between control and experimental groups. In total, an average of 105-276 cells/section was counted in each of 19-35 sections, for a total of 3674-5239 cells per experimental group. Error bars indicate SEM. *<0.05; **<0.01; ***<0.001 relative to control-transfected sections.
percentage of proliferating cells of approximately three-fold (Fig. 22E). Moreover, transfection with H-RasG12V caused an approximately four to five-fold increase in the percentage of astrocytes, while transfection with H-RasG12S caused a more modest, but still significant increase in astrogenesis (Fig. 22F). Interestingly, of those CS H-Ras mutants that had become astrocytes, many of them were still proliferating, something that was only occasionally seen in controls at this age (Fig. 22G). In addition, many of the Ki67-positive cells that were aberrantly observed in the cortical plate following CS H-Ras transfection also expressed GFAP (Fig. 22H), indicating that they were proliferating astrocytes. More specifically, in empty vector-electroporated cortices, all of the transfected proliferating astrocytes (Ki67-positive, GFAP-positive, GFP-positive cells) were localized to the VZ/SVZ, while in cortices electroporated with CS H-Ras mutants, approximately 60-80% were in the VZ/SVZ, with as many as 20-30% localized to the cortical plate (Fig. 22I). The identity of the remaining proliferating cells within the cortical plate that did not express either neuronal or astrocytic markers is still unclear, since they did not express the oligodendrocyte marker O4, and only very few expressed the cortical radial precursor marker Pax6 (data not shown). Thus, sustained H-Ras activation promotes the genesis and/or proliferation of astrocytes, and some of these proliferating astrocytes are aberrantly localized within the cortical plate.

**5.3.5- Sustained H-Ras activation promotes premature gliogenesis in the embryonic cortex**

During murine cortical development, gliogenesis starts at E18, peaks after birth, and continues postnatally. Our data with the CS H-Ras mutants indicate that they increase the number of astrocytes generated in the embryonic cortex either by promoting the genesis of astrocytes and/or by increasing proliferation of previously-generated astrocytes. To distinguish these two possibilities, we asked whether gliogenesis occurred prematurely in brains...
electroporated with either of the CS H-Ras mutants. Immunocytochemical analysis of brains electroporated at E13/14 and examined 3 days later revealed that, as predicted, no EGFP-positive, GFAP-positive cells were ever seen in control brains at this timepoint. In contrast, a subpopulation of H-RasG12V and H-RasG12S-transfected cells expressed GFAP (Fig. 23A-C), indicating that Ras activation promoted premature genesis and/or differentiation of astrocytes. However, none of these EGFP-positive, GFAP-positive cells were localized to the cortical plate, indicating that the aberrantly-proliferating cortical plate cells seen at this timepoint were likely precursor cells. Experiments were also performed 4.5 days after electroporation and revealed that as astrogenesis is starting in controls, brains transfected with H-RasG12V, or H-RasG12S, showed many GFAP-positive cells (Fig. 23D), consistent with the data 3 days post-electroporation. To determine whether H-Ras activity also ultimately increased the number of astrocytes, we examined electroporated brains at postnatal day 3 (P3) (Fig. 24A, B). Confocal microscopy showed that in the control brains, 25 to 30% of the total transfected cells were GFAP-positive, while in the H-RasG12V and H-RasG12S, approximately 40-55% of the total transfected cells were GFAP-positive (Fig. 24C). In addition, quantification demonstrated that astrocytes increased from approximately 20% in the controls to approximately 60 to 80% in the H-Ras mutants for the VZ/SVZ, and from approximately 18% in the controls to 40 to 50% in the H-Ras mutants for the cortical plate (Fig. 24D, E). Thus, not only do CS H-Ras mutants cause premature astrogenesis, but they also robustly increase the number of astrocytes present postnatally.

5.4- DISCUSSION
A

Control | GFAP | merge | V

RasG12S | GFAP | merge | V

VZ/SVZ

B

GFP-RasG12S

GFAP

merge

VZ/SVZ

C

% of GFAP-GFP+ cells / total GFP+ cells

Ctrl | RasG12V | RasG12S

***

10

5

15

20

**

D

Control | V

M

RasG12X | V

V

M

149
Figure 23. *CS H-Ras mutants cause premature astrogenesis embryonically.* Precursor cells of the embryonic cortex were transfected by in utero electroporation at E13/14 with plasmids encoding nuclear EGFP and an empty vector (control), H-RasG12V, or H-RasG12S, and then analyzed 3 (A-C), or 4.5 (D) days post-electroporation. (A, B, D) Confocal micrographs of cortical sections electroporated with empty vector (control, A,D), H-RasG12S (A,B) or H-RasG12V (D) and then immunostained for EGFP (green) and GFAP (red) 3 days (A,B), or 4.5 days (D) post-electroporation. In (A), the right panels show the merged images, and the hatched lines outline the VZ/SVZ. In (B) the images are higher magnification to show double-labelled cells with more resolution (arrows). In (D), the left panels show lower magnification images, and the hatched lines outline the tissue margins. The right panels show higher magnification images of the region adjacent to the ventricles (V; denoted by hatched lines). In all cases, arrows denote double-labelled cells. Scale bar, 50µm (A); 20µm (B); 100µm (D, left panels) and 50µm (D, right panels). V, ventricle; M, meninges. (C) Quantification of the number of transfected cells that were positive for GFAP as a percentage of total transfected cells at 3 days post-electroporation. Numbers are pooled from at least 6 different experiments, each involving the analysis of 3-5 littermate pairs, four to five sections per embryo for each comparison between control and experimental groups. For C, an average of 86-151 cells/section was counted in each of 37-56 sections, for a total of 4303-5600 cells per experimental group. Error bars indicate SEM. ***<0.001 relative to control-transfected sections.
Figure 24. CS H-Ras mutants increase astrocyte number postnatally. Precursor cells of the embryonic cortex were transfected by in utero electroporation at E13/14 with plasmids encoding nuclear EGFP and an empty vector (control), H-RasG12V, or H-RasG12S, and then analyzed at postnatal day 3 (P3). (A, B) Confocal micrographs of cortical sections electroporated with empty vector (control) or H-RasG12S and then immunostained for EGFP (green) and GFAP (red). The cortical plate (CP) is shown in (A), and the VZ/SVZ in (B) as denoted by the red boxes in the tissue images to the right. The hatched lines denote the boundaries of the cortical plate (A) or the ventricular margin (B). M, meninges. Scale bars, 50µm. (C, D, E) Quantification of the number of transfected cells that were positive for GFAP as a percentage of total transfected cells in the entire cortex (C), in the VZ/SVZ (D), or in the cortical plate (E). Numbers are pooled from at least 3 different experiments, each involving the analysis of 3-5 littermate pairs, four to five sections per embryo for each comparison between control and experimental groups. In total, an average of 28-55 cells/section was counted in each of 17-27 sections, for a total of 469-1258 cells per experimental group. Error bars indicate SEM. **<0.01; ***<0.001 relative to control-transfected sections.
The data presented here support three major conclusions. First, sustained activation of H-Ras promotes proliferation of cortical precursor cells in vivo. Second, this aberrant activation decreases the differentiation of cortical precursors into neurons. Finally, constitutive activation of H-Ras leads to premature formation of astrocytes, and ultimately increases the number of astrocytes postnatally. Thus, by perturbing the proliferation and differentiation of cortical precursors, sustained H-Ras activation disrupts the balance between the numbers of neurons and astrocytes, which can result in cortical dysgenesis and thus likely contribute to the cognitive impairments seen in Costello syndrome (CS).

CS patients all share multiple phenotypes. They have common facial traits, skin papillomata, short stature, and growth retardation. They present with heart defects, most often hypertrophic cardiomyopathies, and neurological conditions including ventricular dilatation that requires a ventriculo-peritoneal shunt, hydrocephaly, Chiari 1 malformation, and seizures (Delrue et al., 2003; Hennekam, 2003; Gripp et al., 2002, 2005; Zampino et al., 2007). In the majority of CS cases, mutations are found at codon 12 in H-Ras. The most common of these involve H-RasG12S, which accounts for approximately 80% of cases (Aoki et al., 2005), with H-RasG12V accounting for a further 5% of cases. This latter mutation is highly tumorigenic (Fasano et al., 1984; Seeburg et al., 1984), and its prevalence in CS is likely low as a consequence. In this regard, cancers, including neuroblastomas, rhabdomyosarcomas and bladder carcinomas, are more common in patients with CS than in any of the other NCFC syndromes likely because of the high relative tumorigenicity of H-Ras mutations in general.

Data presented here demonstrate that CS H-Ras mutants perturb neural precursor development in two ways; they induce these precursors to generate more astrocytes and fewer neurons, and they cause hyperproliferation of both precursors and newly-generated astrocytes.
How does H-Ras activation promote astrogenesis and decrease neurogenesis? We propose that they do so by maintaining cells in a proliferative state, preventing precursors from differentiating into neurons at the appropriate timepoint, and thereby ultimately enhancing astrogenesis. In the CNS, neuronal differentiation is tightly coupled to cell cycle exit. In this regard, pRb plays a key role in linking cell cycle exit and neuronal gene expression as cortical precursors become post-mitotic neurons (Toma et al., 2000), and Ras is known to regulate pRb to promote S-phase entry (Leone et al., 1997; Peeper et al., 1997). Thus, by directly regulating pRb and potentially other cell cycle regulators, activated Ras could prevent cell cycle exit and inhibit neurogenesis. In support of this idea, we have observed cycling precursors in the cortical plate region in this study, something that was also seen when the cell cycle inhibitors p19\textsuperscript{ink4d} and p27\textsuperscript{kip1} were genetically-ablated (Zindy et al., 1999). What happens then to these precursors that would normally make neurons, but have been inhibited from doing so by H-Ras activation? Our data indicate that they persist as cycling precursors throughout the neurogenic period. We suggest that they then find themselves in a neural environment that favors astrogenesis (Miller and Gauthier, 2007; Barnabé-Heider et al., 2005), and since there is no obligate link between cell cycle exit and adopting an astrocyte phenotype, they become astrocytes.

The second major phenotype we have documented here is hyperproliferation of cortical precursors and the astrocytes that they generate. These findings are relevant not only for the perturbed neural morphogenesis seen in CS, but also for the increased tumorigenesis seen in this syndrome. These findings may also have relevance for other NCFC syndromes, and in particular for neurofibromatosis-1 (NF1). NF1 is a tumor suppressor that functions as a negative regulator of Ras. Approximately 15–20% of children with NF1 develop low-grade glial cell neoplasms (Listernick et al., 1994, 1999) and in mice, inactivation of the NF1 gene leads to increased
proliferation of glial precursors and increased astrogenesis that ultimately results in formation of optic gliomas (Bajenaru et al., 2001, 2002, 2003; Zhu et al., 2005). These phenotypes are at least partially due to inactivation of NF1 in neural precursors since genetic ablation of NF1 in cultured neural stem cells or in BLBP-positive radial precursors in vivo enhanced gliogenesis (Dasgupta and Gutmann, 2005; Hegedus et al., 2007). Thus, activation of Ras either by inactivation of NF1 or by direct mutation, is sufficient to cause enhanced proliferation of glial precursors and astrocytes. However, while inactivation of NF1 had some effects on neuronal maturation (Hegedus et al., 2007), it did not decrease neurogenesis, arguing that while related, these two perturbations are distinct, consistent with the differences in cognitive impairments seen in these two disorders.

These findings provide a cellular mechanism to explain the severe cognitive deficits seen in CS patients. During development of the mammalian nervous system, cell genesis is a timed event, with neuronal genesis occurring first, followed by glial genesis. These neurogenic and gliogenic periods are growth-factor mediated and temporally distinct; within the murine cortex, for example, neurogenesis occurs from approximately E12-E17 while astrocyte formation is largely postnatal. This process is biologically-logical: neuronal circuitry is established first and then the numbers of glial cells are matched to that circuitry. Perturbation of these highly conserved events, in either timing or magnitude, could result in significant cortical abnormalities. In this regard, our finding that activated H-Ras actively promotes gliogenesis at the expense of neurogenesis is highly relevant. Previously published data from our group showed that sustained activation of SHP-2, as seen in Noonan syndrome, caused increased neurogenesis and decreased gliogenesis in vivo (Gauthier et al., 2007). How then can these different mutations, which cause opposite effects on neural cell genesis, still cause similar impairment of cognitive function? We
propose two potential explanations. First, it is not specifically the change in the number of neurons or astrocytes that causes mental dysfunction, but rather the overall balance between the two. Clearly the number of neurons would be critical to the establishment of appropriate neural circuitry, but emerging evidence indicates that the numbers of astrocytes are also important. When astrocyte numbers are decreased, this can lead to neuronal degeneration and dysfunction (Wagner et al., 2006), and astrocytes are now known to play a role in communication at the level of the synapse (Araque et al., 1999; Bezzi et al., 2004; Perra and Araque, 2007). Second, the timing of neurogenesis versus astrogenesis is key to the proper generation of neural architecture. For example, when neurons are born is a key determinant of their ultimate location within the cortex; when neurogenesis is acutely and transiently interrupted, the neurons that are inappropriately born later now localize to more superficial cortical layers (Mizutani and Saito, 2005; Paquin et al., 2005). Thus, perturbations at the level of the timing or extent of differentiation, as caused by CS H-Ras mutations, can affect cortical development, the efficiency and the functionality of the circuitry, and ultimately lead to mental retardation.

In summary, data presented here, together with previous work (Gauthier et al., 2007; Samuels et al., 2008), support the concept that the perturbations of the Ras-MAPK pathway that are seen in the NCFC disorders dysregulate neural precursor development, thereby causing the deficits in cognitive function that are seen in this family of genetic syndromes. Whether similar perturbations in neural precursor cell development play a role in other genetic syndromes that alter cognitive function is a key question for the future.
6.1- Summary of findings

The major goal of this study was to elucidate the role of different proteins implicated in cellular signalling during embryonic cortical development. This study was divided into three specific aims, examining different proteins involved in receptor tyrosine kinase (RTK) activated signalling cascades. First, I investigated the role of the C/EBP family of transcription factors during cortical development in vivo. It was previously shown that an upstream regulator of C/EBPs, MEK, as well as C/EBPs themselves, were able to promote neurogenesis from cultured cortical precursors (Ménard et al., 2002; Barnabé-Heider and Miller, 2003). However, their role during differentiation in vivo remained to be deciphered. Data presented in Chapter 3 demonstrated that C/EBP phosphorylation is necessary and sufficient to promote neurogenesis. Moreover, MEK activation was shown to be essential for cortical precursors to generate neurons in vivo through phosphorylation of C/EBPs. When MEK signalling was inhibited, many precursors remained in the VZ/SVZ in an undifferentiated state. Similarly, in the absence of C/EBP family activation, precursors initially remained in the VZ/SVZ as cycling precursors, but then had an enhanced propensity to generate astrocytes in the late embryonic and early neonatal environment, presumably when they were exposed to extrinsic gliogenic cues. In conclusion, growth factor-mediated activation of the MEK-C/EBP pathway is able to promote the genesis of neurons from cortical precursors, and at the same time, C/EBP activation inhibits the genesis of astrocytes when the same precursors are exposed to a gliogenic environment. Therefore, the
C/EBPs can act as growth factor-regulated switches that directly couple alterations in the neural environment to cell fate choices during embryogenesis.

The second aim of the study, covered in Chapter 4, was to examine the role of TrkB and TrkC receptors known for their role in regulating the biology of the CNS and of some precursor cells in culture (Huang and Reichardt, 2003; Barnabé-Heider and Miller, 2003), but whose role in cortical development in vivo remained unknown. I was able to show that neurotrophin-mediated activation of these two receptors is essential for the proliferation of embryonic cortical precursors in vivo. Moreover, the inhibition of TrkB and TrkC signaling lead to a delay in the generation of new neurons ultimately perturbing postnatal localization of principal cortical neurons. Conversely, BDNF overexpression enhanced the generation of neurons from precursors, indicating that Trk signaling regulates the timing and potentially the extent of cortical neurogenesis. It was also demonstrated that TrkB and TrkC receptor signaling was not required for cortical astrocyte formation, at least within the first few days postnatally. Finally, these studies show that inhibition of Trk signaling in embryonic cortical precursors leads to a decrease in the proportion of postnatal cortical precursors that are maintained within the SVZ, possibly as a direct consequence of the decrease in embryonic proliferation. Together, these results indicate that neurotrophin-mediated TrkB and TrkC activation regulates the proliferation, maintenance, and differentiation of embryonic cortical precursors, suggesting that this family of growth factors may play a more general role in the regulation of CNS neural precursor cells.

The final specific aim of this study, covered in Chapter 5, was to elucidate the role of Ras during cortical development. Many of the growth factors implicated in cellular differentiation during development converge onto the Ras signaling pathway. For example, Ras has been shown to play a central role in regulating neural precursor cell proliferation, survival, and cell fate
decisions through the ERK MAP kinase signaling cascade (Samuels et al., 2009). With specific regard to the developing cortex, aberrant H-Ras activation in postmitotic neurons causes neuronal cell bodies to hypertrophy and subsequent ventricular enlargement (Heumann et al., 2000; Hennekam, 2003). Moreover, components of the Ras signaling cascade, including SHP-2, MEK, ERK2 and C/EBP have been shown to be play important roles in cortical precursor cell neurogenesis (Gauthier et al., 2007; Ménard et al., 2002; Paquin et al., 2005; Samuels et al., 2008). In contrast, activation of the gp130-JAK-STAT pathway by cardiotrophin-1 has been shown to be required for astrocyte formation (Bonni et al., 1997; Barnabé-Heider et al., 2005). In Chapter 5, I specifically asked whether two particular H-Ras mutations, displayed by Costello syndrome patients and resulting in a hyperactivated form of Ras, could perturb cortical development. I found that sustained activation of H-Ras promotes proliferation of cortical precursor cells in vivo. Moreover, this aberrant activation decreases the differentiation of cortical precursors into neurons, and also leads to premature formation of astrocytes and ultimately increases the number of astrocytes postnatally. Thus, by perturbing the proliferation and differentiation of cortical precursors, sustained H-Ras activation disrupts the balance between the numbers of neurons and astrocytes, which can result in cortical dysgenesis and thus likely contribute to the cognitive impairments seen in Costello syndrome.

6.2- From extracellular cues to gene transcription: a model for cortical development

In light of the results obtained in this study, I propose a model where growth factors in the extrinsic environment play a key role in dictating cellular differentiation by activating specific proteins implicated in signalling cascades (Fig. 25). The inhibition of TrkB, TrkC, MEK,
Figure 25. From extracellular signals to gene transcription: a model for cortical development.
and C/EBPβ all resulted in a decrease in neurogenesis (Fig. 25A). In contrast, while TrkB and TrkC receptor activation was shown to be essential for cellular proliferation, the inhibition of MEK or C/EBPs seemed to have the opposite effect. This inconsistency could be explained by the fact that MEK and C/EBPs, which are part of the MAPK signalling cascade, are mostly responsible for cellular differentiation. I propose that the increase in proliferation associated with inhibition of MEK or C/EBPs is due in major part to decreased neurogenesis, where a proportion of precursor cells remain in an undifferentiated state during the neurogenic period. Finally, C/EBP knockdown also resulted in increased astrogenesis, possibly because these undifferentiated cells become astrocytes later during development. During development, the cortical environment changes from neurogenic to gliogenic (Morrow et al., 2001), in large part because of the accumulation of cytokines such as cardiotrophin-1 (Barnabe-Heider et al., 2005) as well as demethylation of astrocyte-specific genes (Takizawa et al., 2001). Therefore, once cortical precursor cells environment switches from neurogenic to gliogenic, the undifferentiated cells have a propensity to generate astrocytes.

In addition to knockdown experiments, the aberrant activation of the Trk-Ras-MEK-C/EBP pathway was also investigated (Fig. 25B). The overexpression of BDNF, or a constitutively active form of C/EBP results in an increase in the number of neurons, whereas the overexpression of an activated form of Ras caused a decrease in neurogenesis. In fact, the major effect of Ras overexpression was an important increase in cell proliferation and astrogenesis. How can the overexpression of Ras results in opposite effect from the overexpression of other proteins involved in the same signalling cascade? First, I suggest that by maintaining cells in a proliferative state and preventing neural differentiation of precursors during the neurogenic period, Ras overexpression leads to enhanced astrogenesis. In the CNS, neuronal differentiation
is tightly coupled to cell cycle exit. In this regard, pRb plays a key role in linking cell cycle exit and neuronal gene expression as cortical precursors become post-mitotic neurons (Toma et al., 2000), and Ras is known to regulate pRb to promote S-phase entry (Leone et al., 1997; Peeper et al., 1997). Thus, by directly regulating pRb and potentially other cell cycle regulators, activated Ras could prevent cell cycle exit and inhibit neurogenesis. In support of this idea, I have observed cycling precursors in the cortical plate region in this study, something that was also seen when the cell cycle inhibitors p19\textsuperscript{Ink4d} and p27\textsuperscript{Kip1} were genetically-ablated (Zindy et al., 1999). What happens then to these precursors that would normally make neurons, but have been inhibited from doing so by H-Ras activation? Data indicate that they persist as cycling precursors throughout the neurogenic period. They then find themselves in a neural environment that favors astrogenesis (Miller and Gauthier, 2007; Barnabé-Heider et al., 2005), and since there is no obligate link between cell cycle exit and adopting an astrocyte phenotype, they become astrocytes.

Results I have obtained in the course of this study also concur with other studies recently published. For example, our group has also investigated the role of SHP-2, a protein phosphatase, during cortical development (Gauthier et al., 2007). It was demonstrated that the genetic knockdown of SHP-2, both in culture or in vivo, could inhibit neurogenesis and enhanced astrogenesis. Conversely, the expression of a constitutively active form of SHP-2 was able to promote neurogenesis and inhibit astrogenesis. Interestingly, this specific form of constitutively active SHP-2 represent a common mutation found in patients diagnosed with Noonan syndrome, part of the neuro-cranio-facial cutaneous (NCFC) family of syndrome which also includes Costello syndrome. Another group has studied the effects of ERK2 knockdown during cortical development (Samuels et al., 2008). They have found that ERK2 conditional
knockouts display decreased neurogenesis and increased astrogenesis. These data support the hypotheses presented in this thesis, and a model for extrinsic cues dictating intrinsic mechanisms responsible for cortical differentiation is therefore presented which represents a significant contribution to the field.

What are the growth factors that can promote neurogenesis? I showed here that the TrkB and TrkC receptors play important roles since the inhibition of their function caused a decrease in neurogenesis and the activation of TrkB by the overexpression of its ligand, BDNF, caused an increase in the number of neurons. However, other tyrosine kinase receptors were shown to influence cellular differentiation during development. Previous work has identified a number of candidate receptor tyrosine kinase ligands, including FGF2, neurotrophins, HB-EGF, PDGF and IGF1. Specifically, FGF2 is mitogenic for cortical precursors (Lukaszewicz et al., 2002) and is essential for normal neurogenesis (Raballo et al., 2000). Cortical precursors express the neurotrophin receptors TrkB and TrkC, and ablation of either TrkB or the neurotrophins BDNF and/or NT-3 perturb the development of these precursors (Barnabé-Heider and Miller, 2003; Medina et al., 2004). Heparin-binding EGF (HB-EGF) is expressed in the embryonic VZ/SVZ (Nakagawa et al., 1998), and promotes neurogenesis in the adult CNS (Jin et al., 2003). Cultured cortical precursors express PDGF receptor and respond to PDGF with enhanced neurogenesis (Johe et al., 1996; Williams et al., 1997; Park et al., 1999). Finally, overexpression of IGF1 from the nestin promoter causes increased proliferation and neuron number in the embryonic cortex (Popken et al., 2004).

What are the sources of these growth factors during neurogenesis? Cortical precursors of the VZ/SVZ themselves express BDNF, NT-3 (Maisonpierre et al., 1990; Fukumitsu et al., 1998), FGF2 (Raballo et al., 2000), and HB-EGF (Nakagawa et al., 1998). Intriguingly,
endothelial cells also express BDNF (Kim et al., 2004), FGF2 (Albuquerque et al., 1998) PDGF and HB-EGF (Arkonac et al., 1998), and embryonic endothelial cells have recently been shown to promote self-renewal and neurogenesis in neural stem cells (Shen et al., 2004). Thus, it is likely that cortical precursors are exposed to diverse ligands from multiple sources, and signaling via these growth factors could converge on the MEK-C/EBP pathway to ultimately regulate the genesis of neurons. It is also likely that these same growth factors regulate survival and proliferation of cortical precursors via other pathways such as the PI3K-Akt pathway (Barnabé-Heider and Miller, 2003; Sinor and Lillien, 2004), and thus the precise repertoire of responses is likely defined by the relative levels and/or timecourse of activation of these different pathways.

What prevents astrocyte formation during the neurogenic period? One important factor would be DNA methylation, a major epigenetic silencing mechanism, of genes necessary for astrogenesis. Methylation of DNA within transcription factor-binding elements hinders the binding of these transcription factors to their target sequence. Moreover, other proteins can bind this methylated DNA and further suppress gene expression by acting as transcriptional repressors. A study conducted by McKay and colleagues, where the response of E12 versus E15 cortical precursor cell cultures to the cytokine LIF was compared. E15 cultures were found more responsive to gliogenic stimuli than E12 cultures, even if functional LIF receptors are expressed in both cultures, suggesting different intrinsic mechanisms in the two populations of cells (Molne et al., 2000). Further studies examining DNA methylation of similar populations of cells revealed that GFAP and S100β promoters were highly methylated at E11.5, and as development progresses become demethylated (Takizawa et al., 2001; Namihira et al., 2004). Thus, a gliogenic environment might not be sufficient to promote astrocyte formation when downstream effectors are silenced.
6.3- Biological implication of findings

During mammalian cortical development, neurons are generated first and glial cells second (Qian et al., 2000; Shen et al., 2006). This timing in differentiation makes biological sense, since the neurons first establish the backbone of the central nervous system, while glial cells are able to integrate and match this circuitry later. To date, many groups have worked intensively toward the elucidation of the key players regulating this sequential generation of neurons, astrocytes, and oligodendrocytes. In this thesis, I have presented important pieces of evidence that along with other published data, defined the complex interplay between environmental cues, signalling cascades, and transcription factors during cortical development.

Recently, many mutations in the MAPK pathway have been shown to result in similar human syndromes identified as the NCFC family of syndromes. Patients suffering from these syndromes all present common phenotypes such as short stature, coarse facial features, cardiac abnormalities, and mental retardation. Interestingly, the different mutations responsible for NCFC syndromes can either cause activation or inhibition of the MAPK pathway. How can these different mutations, which can also cause opposite effects on neural cell genesis, still produce similar impairments of cognitive function? I propose two potential explanations. First, it is not specifically the change in the number of neurons or astrocytes that causes mental dysfunction, but rather the overall balance between the two. Clearly, the number of neurons would be critical to the establishment of appropriate neural circuitry, but emerging evidence indicates that the number of astrocytes is also important. When astrocyte numbers are decreased, this can lead to neuronal degeneration and dysfunction (Wagner et al., 2006), and astrocytes are now known to
play a role in communication at the level of the synapse (Araque et al., 1999; Bezzi et al., 2004; Perra and Araque, 2007). Second, the timing of neurogenesis versus astrogensis is crucial to the proper generation of neural architecture. For example, the specific time at which neurons are born is a key determinant of their ultimate location within the cortex; when neurogenesis is acutely and transiently interrupted, the neurons that are inappropriately born later now localize to more superficial cortical layers (Mizutani and Saito, 2005; Chapter 3). Thus, perturbations at the level of the timing or extent of differentiation can affect cortical development, the efficiency and the functionality of the circuitry, and ultimately lead to mental retardation.

6.4- Future directions

In this thesis, the molecular mechanisms regulating cortical precursor cell fate determination were investigated. The results presented here suggest a complex interplay between growth factors that can activate receptor tyrosine kinase, such as the Trk receptors, and intrinsic components of signalling cascades that result in the activation of transcription factors, such as the C/EBPs. As previously mentioned, the disruption of the timing and/or extent of cellular differentiation can impact on the cortical architecture and ultimately affect the functionality of the central nervous system. The understanding of how the functionality is affected will be crucial for the determination of potential therapies. For example, the underlying cause of mental retardation seen in CS patient could be investigated in vivo using animal models. The generation of animal models will be informative in terms of the physiological and behavioural effects of H-Ras sustained activation. The cerebral cortex of these animals could be anatomically analyzed in detail, and studied using brain imaging. Alternatively or in addition to the animal model, a
combination of in utero electroporation and electrophysiological studies would be especially informative in terms of the consequences of developmental perturbations on synaptogenesis and synaptic activity.

Together, the data presented here significantly contribute to our basic knowledge of cortical development. Furthermore, combined with the suggested further experiments this will certainly unravel some of the complex interplay that determine embryonic cortical development, from cell fate determination to physiological functionality, and contribute to a better understanding of many developmental diseases.
REFERENCES


Haas NB, Cantwell CA, Johnson PF, Burch JB (1995) DNA-binding specificity of the PAR basic leucine zipper protein VBP partially overlaps those of the C/EBPs and CREB/ATF families and is influenced by domains that flank the core basic region. Mol Cell Biol 15:1923-1932.


Hendricks-Taylor LR, Darlington GJ (2005) Inhibition of cell proliferation by C/EBP alpha occurs in many cell types, does not require the presence of p53 or Rb, and is not affected by large T-antigen. Nucleic Acids Res 23:4726-4733.


