Ph. D. Thesis

Title: **Regulation of mammalian cerebral cortex development by Nuclear Factor-kappaB**

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## I. Table of contents

## II. List of figures and tables

Figures

Tables

## III. Abstract

The cerebral cortex is a stratified structure on the surface of the cerebral hemispheres that controls important functions such as consciousness, memory, attention, language, abstraction, and production of voluntary movements. A comprehensive description of human cortical development is still incomplete and is essential for understanding the etiology of neurodevelopmental disorders with cognitive impairments. This thesis describes studies aimed at identifying some of the molecular mechanisms regulating the generation of key neural components of cortical networks, namely neurons, astrocytes and oligodendrocytes. In this regard, although the involvement of nuclear factor-kappaB (NF-κB) in a number of processes in the postnatal and adult brain, ranging from neuronal survival to synaptogenesis and plasticity has been documented, little is known about the functions of this pleiotropic transcription factor during early phases of brain development. It is shown here that the NF-κB signaling pathway is activated in both neurogenic and gliogenic cortical neural progenitor cells (NPCs) in the developing embryonic and postnatal cerebral cortex. During cortical neurogenesis, blockade of NF-κB activity leads to premature neuronal differentiation and depletion of the NPC pool. Conversely, NF-κB activation causes an arrest/delay of cortical neuronal differentiation and expansion of the NPC compartment. This effect is antagonized by the pro-neuronal transcription factor, Hes6, which is expressed in cortical NPCs in which NF-κB signaling is activated and physically and functionally interacts with RelA-containing NF-κB complexes. These results reveal previously uncharacterized functions, and modes of regulation, for NF-κB and Hes6 transcription factors during cortical neuronal development. During cortical gliogenesis, the NF-κB signaling pathway is downregulated in the transition of NPCs to the oligodendrocyte precursor cell (OPC) fate, the earliest stage of oligodendrocyte differentiation. Blockade of endogenous NF-κB activity results in an increase in the number of cells expressing OPC marker Olig2**,** together with an increase in the number of cells populating the white matter. Blockade of NF-κB also results in a reduction in the number of cells expressing the astrocytic lineage marker GFAP. These results suggest that NF-κB inhibits progression to the oligodendrocytic lineage and promotes acquisition of the astrocytic lineage. Together, this work describes different role for the NF-κB pathway in the regulation of NPC biology during cortical neurogenesis and gliogenesis.

## IV. Résumé

Le cortex cérébral est une structure stratifiée située à la surface des hémisphères cérébraux qui contrôle des fonctions importantes telles que la conscience, la mémoire, l'attention, le langage, l'abstraction et la production de mouvements volontaires. Aujourd'hui encore, il est impossible de donner une description complète du développement cortical humain. Malgré tout, celle-ci reste essentielle à la compréhension de l'étiologie des troubles du développement neurologique avec déficits cognitifs. Cette thèse démontre des études visant à identifier certains des mécanismes moléculaires qui régulent la production de composants neuraux des réseaux corticaux, à savoir les neurones, les astrocytes et les oligodendrocytes. À cet égard, bien que l'implication du Nuclear Factor-kappaB (NF-κB) dans certains processus du cerveau adulte et postnatal, allant de la survie neuronale à la plasticité de la synaptogénèse, ait été documentée, on en connait peu sur les fonctions de ce facteur de transcription pléiotropique au cours des premières phases du développement du cerveau. Il est démontré ici que la voie de signalisation NF-κB est activée dans les cellules progénitrices neurales (NPC), neurogéniques et gliogéniques dans le cortex cérébral en développements embryonnaire et postnatal. Au cours de la neurogenèse corticale, le blocage de l'activité de NF-κB conduit à la différenciation neuronale précoce et à l'appauvrissement de la population de NPC. En revanche, l'activation de NF-κB provoque un arrêt et/ou un retard de la différenciation neuronale corticale et une expansion du bassin de NPC. Cet effet est inhibé par le facteur de transcription pro-neuronal Hes6 qui est exprimé dans les NPC corticales, dans lesquelles la signalisation de NF-κB est activée, et qui interagit physiquement et fonctionnellement avec les complexes NF-κB contenant la protéine RelA. Ces résultats révèlent des fonctions et des modes de régulation auparavant non définis des facteurs de transcription NF-κB et Hes6 au cours du développement neuronal cortical. Pendant la gliogenèse corticale, la voie de signalisation NF-κB est régulée à la baisse dans la transition des NPC à des cellules précurseurs d'oligodendrocytes (OPC), la première étape de la différenciation de la lignée oligodendrocytaire. Le blocage de l'activité endogène de NF-κB mène à une augmentation du nombre de cellules exprimant le marqueur d'OPC Olig2 et à une augmentation du nombre de cellules qui peuplent la matière blanche. Le blocage de NF-κB se traduit également par une réduction du nombre de cellules exprimant le marqueur de la lignée

astrocytaire GFAP. Ces résultats suggèrent que NF-κB inhibe la transition vers la lignée oligodendrocytaire et favorise le passage à la lignée astrocytaire. Dans son ensemble, cet ouvrage décrit les différents rôles de la voie de signalisation NF-κB dans la régulation de la biologie des NPC pendant la neurogenèse et la gliogenèse corticale.

## V. Acknowledgements

First, I would particularly like to thank my thesis supervisor, Dr. Stefano Stifani, for his constant and invaluable mentoring and support over the years. This thesis would not have been possible without his help and guidance.

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These acknowledgments would not be complete without thanking my family for their constant support and care. I thank my uncle, Federico, for advice. I thank my father, Francis, and my mother, Joanne, for their love and encouragements. Above all, I owe my deepest gratitude to my wife, Maryse, and my son, Léon, for their unconditional love.

## VI. Preface & contribution of authors

An important portion of the studies presented in this thesis were published in an article on which I am the first author, entitled "Interaction and antagonistic roles of NF-κB and Hes6 in the regulation of cortical neurogenesis" published in the journal Molecular and Cellular Biology (July 2013, volume 33, number 14, pages 2797-2808). Specifically, the studies presented in Figures 7, 8, 9, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, and 32, were adapted from the published article.  doi:10.1128/MCB.01610-12.

The studies presented in Figures 10, 11, 12, 25, 26, 27, 29, 30, 31, 33, 34, and 35, are original contributions to knowledge that were not published at the time of submission of this thesis.

A number of studies presented in this thesis are the result of collaborative work conducted together with a) other previous and current students in the Stifani laboratory at the Montreal Neurological Institute, Robert Hermann, Hosam Al-Jehani, Sally Li, and Erin Campos, b) a student in Dr. Ruth Slack's laboratory at University of Ottawa, Devon Svoboda, and c) Laboratory Technicians in the Stifani lab, Rita Lo and Yeman Tang. The acknowledgment of contributions to the specific studies in which each of these people were involved is included in this thesis where appropriate.

The staff of the Animal Care Facility at the Montreal Neurological Institute provided invaluable assistance. Specifically, for management of the mice colony, Mireille Bouchard-Levasseur, Ivannia Murillo, and Stephanie Perrino; for assistance with surgeries, Dr. Fernando Chaurand, Cathy Hunt, and Benoit Chambaron; and for their very efficient advisory and administrative work, Janet Green, Lisa Volume, and Madeleine Arkilanian.

Dr. Stefano Stifani significantly contributed to the design of the experiments and interpretation of the results presented in this thesis. Dr. Phil Barker also contributed to the interpretation of the

results presented in this thesis and generously provided transgenic mice, material and reagents to conduct these studies. Dr. Jean-Francois Cloutier has kindly provided equipment. Dr. Ryoichiro Kageyama, Dr. Ruth Slack, and Dr. Charles D. Stiles generously provided reagents.

## VII. Abbreviations

| | |
|---|---|
| A | Astrocyte |
| Acx | Archicortex |
| β-Gal | β-Galactosidase |
| C | Caudate nucleus |
| cc | Corpus callosum |
| CP | Cortical plate |
| CH | Cortical hem |
| Chp | Choroid plexus |
| ctx | Cerebral cortex |
| DIV | Days *in vitro* |
| E | Embryonic day |
| GE | Ganglionic eminence |
| GFP | Green fluorescent protein |
| gw | Gestational week |
| GZ | Germinative zone |
| H | Hippocampus |
| HC | Heavy chain |
| Hcx | Hippocampal cortex |
| Hth | Hypothalamus |
| Hoe/Hst | Hoechst |
| IP | Intermediate progenitor |
| IKK | Inhibitor of κB Kinase |
| LC | Light chain |
| IZ | Intermediate zone |
| IκB | Inhibitor of κB |
| LGE | Lateral ganglionic eminence |
| LV | Lateral ventricle |
| Mbr | Midbrain |
| MGE | Medial ganglionic eminence |

| | |
|---|---|
| MgZ | Marginal zone |
| MZ | Mantle zone |
| Ncx | Neocortex |
| NE | Neuroepithelial cell |
| NF-κB | Nuclear Factor-kappaB |
| NPC | Neural progenitor cell |
| OL | Oligodendrocyte |
| OPC | Oligodendrocyte precursor cell |
| oRG | Outer radial glial cell |
| P | Putamen |
| POA | Preoptic area |
| siRNA | Small interfering RNA |
| shRNA | Short hairpin RNA |
| spt | Septum |
| str | Striatum |
| SVZ | Subventricular zone |
| SVZdl | Dorsolateral subventricular zone |
| SVZspt | Septal subventricular zone |
| SVZstr | Striatal subventricular zone |
| Th | Thalamus |
| vRG | Ventricular radial glial cell |
| VZ | Ventricular zone |
| WB | Western blot |
| WM | White matter |

## 1. Introduction

The cerebral cortex is a stratified (layered) structure on the surface of the cerebral hemispheres. It is composed of complex networks of neuronal cells, nerve fibers, glial cells, and blood vessels, which together control important functions such as consciousness, memory, attention, language, abstraction, and production of voluntary movements. Assembly of the fundamental components of the cerebral cortex occurs during prenatal development, with the establishment of a primordial cortical organization taking place during embryonic development, the generation of various neural cell types and their migration and organization into stratified structures and white matter tracts during fetal development, and maturation into functional cortical networks in late fetal and postnatal development (Paxinos and Mai, 2004).

A comprehensive description of human cerebral cortex development is still incomplete and is essential for understanding the etiology of several neurodevelopmental disorders with cognitive impairments, the pathophysiology of perturbed developmental processes involved in at least certain forms of brain cancer, and, importantly, human nature. Because of the inherent difficulty of studying the development of the human brain, considerable efforts have been directed toward the characterization of the events underlying the development of the murine cerebral cortex. In this regard, this thesis will describe studies aimed at understanding the molecular mechanisms regulating the biology of cortical neural progenitor cells (NPCs) endowed with the potential to give rise to neuronal and glial cells that will populate the mature cerebral cortex. In doing so, this thesis will first describe the developmental contributions of NPCs to human corticogenesis, followed by an introduction to the developing mouse cerebral cortex as an attractive experimental model system to investigate cortical development. Lastly, this thesis will describe studies that have revealed a previously uncharacterized role for the Nuclear Factor-kappaB (NF-κB) signaling pathway in the regulation of both cortical neurogenesis and gliogenesis in mice.

## 1.1. Developmental roles of neocortical neural progenitor cells in human corticogenesis

### *1.1.1 Embryonic forebrain structures*

The prosencephalon, the embryonic structure that gives rise to the forebrain, appears at the rostral end of the neural tube by the end of gestational week 4 in humans (Nieuwenhuys *et al.*, 2008). The prosencephalon then separates into the telencephalon (rostral part) and diencephalon (medial part), and also gives rise to the eyes, the optic nerves and the olfactory bulbs. The telencephalon is the embryonic structure that develops into the cerebrum, where the dorsal part (pallium) develops into the cerebral cortex and the ventral part (subpallium) develops into the basal ganglia. The developing cerebral cortex comprises three cortical formations: the dorsolateral neocortex, the medial hippocampal cortex and the ventrolateral piriform cortex (Figure 1). Neocortex, a term that refers to its late phylogenetic appearance, comprises six cortical layers and composes the vast majority of the cerebral cortex. The other cortical areas, termed allocortex, are phylogenetically older areas, and comprise two components: palaeocortex, which includes the piriform cortex, and archicortex, which includes the hippocampal cortex. The diencephalon is the embryonic structure that gives rise to posterior forebrain structures such as the thalamus. This thesis focuses on the development of the six-layered neocortex.

### *1.1.2. Telencephalic germinative zones in prenatal development*

Corticogenesis is initiated with the establishment of a primordial cortical organization and the specification of different NPC populations in distinct cortical regions. Together, these NPC populations are responsible for generating the various neural cell types of the mature cerebral cortex (Franco and Müller, 2013). The different populations of NPCs are located in germinative zones (GZs) along the walls of the lateral ventricle (LV) (Figure 1). Different cortical regions are generated from distinct pools of NPCs occupying anatomically-defined GZs around the LV. For example, dorsolateral pallial (neocortical) NPCs generate projection neurons that migrate radially into the neocortex, while medial pallial NPCs generate neurons that form the hippocampal cortex. At the same time, different components of a single cortical structure can be generated by distinct pools of NPCs. This is best exemplified by the different origins of

excitatory glutamatergic projection neurons and inhibitory GABAergic interneurons that populate the neocortex: while projections neurons are generated by dorsolateral pallial NPCs, interneurons are generated by subpallial NPCs located in the ganglionic eminences (GEs) and migrate tangentially to their final position in the neocortex (Figure 1) (Zecevic *et al.*, 2011; Greig *et al.*, 2013).

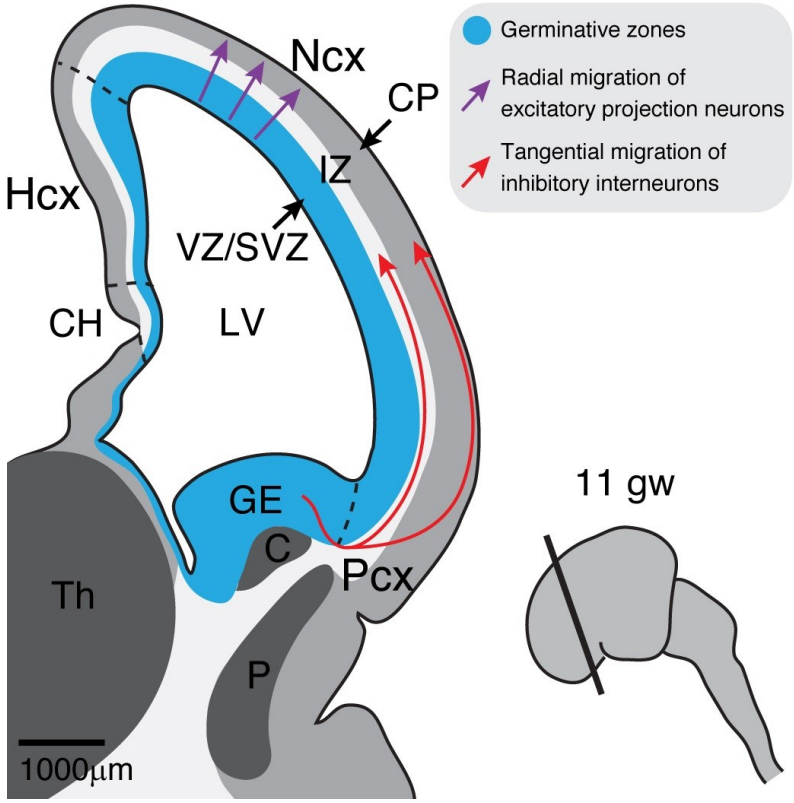


**Figure 1.** Anatomical regions and distinct germinative zones in the early fetal human telencephalon. Schematic cross-section through a human dorsal forebrain at gestational week 11 depicting the main thelencephalic structures: the medial pallium (hippocampal cortex [Hcx] and cortical hem [CH]), the lateral pallium (neocortex [Ncx] and piriform cortex [Pcx]) and subpallium (ganglionic eminence [GE] and striatum, which comprises the caudate nucleus [C] and putamen [P]). The neocortex can be divided in three principal regions: the apical germinative zone (comprising the ventricular zone [VZ] and subventricular zone [SVZ]), the intermediate zone [IZ], and the basal cortical plate [CP], which develops into the 6-layered neocortex. The different cortical germinative zones lining in the lateral ventricle (LV), depicted in blue, are composed of distinct populations of NPCs that collectively generate the various differentiated neural cells of the cortex. Th: thalamus.

### *1.1.3. Establishment of a radial glial cell system*

During embryonic development, prior to the generation of the first-born neurons, the neocortex is a neuroepithelium composed of NPCs that have a neuroepithelial (NE) cell identity. NE cells are actively mitotic and the majority undergo symmetrical amplifying cell divisions that give rise to daughter cells of equivalent fates. This mode of cell division takes place for several weeks in order to increase the number of NE cells until a sufficiently large pool of NE cells is generated. In the neocortex, the onset of cortical neurogenesis occurs at gestational weeks 5-6 (Lui *et al.,* 2011; Paxinos and Mai, 2004). At the onset of cortical neurogenesis, a radial glial cell system is established: neocortical NE cells gradually adopt a ventricular radial glial (vRG) cell identity (Kriegstein and Alvarez-Buylla, 2009), start expressing glial fibrillary acid protein (GFAP), and form a ventricular zone (VZ) (Figure 2). vRG cells have long basal radial fibres that extend up to the pial surface and apically-attached (ependymal) end feet that contact the ventricle. vRG cells serve several key functions in corticogenesis: (1) mainly through asymmetrical divisions, they generate cortical projection neurons and maintain a pool of undifferentiated NPCs; moreover, through both asymmetrical and symmetrical divisions, they generate macroglial cells (eg, astrocytes and oligodendrocytes); (2) through their long radial processes that extend to the pial surface, they provide structural support for radially-migrating neurons, precursor cells, and glial cells; and (3) through their multiple endfeet reaching the pial surface, they construct and transiently maintain the external glial limiting membrane (EGLM) (discussed further below). The thickness of the neocortex increases with the addition of newly-born neurons, which is paralleled by a lengthening of radial glial processes that retain their ependymal and pial attachments.

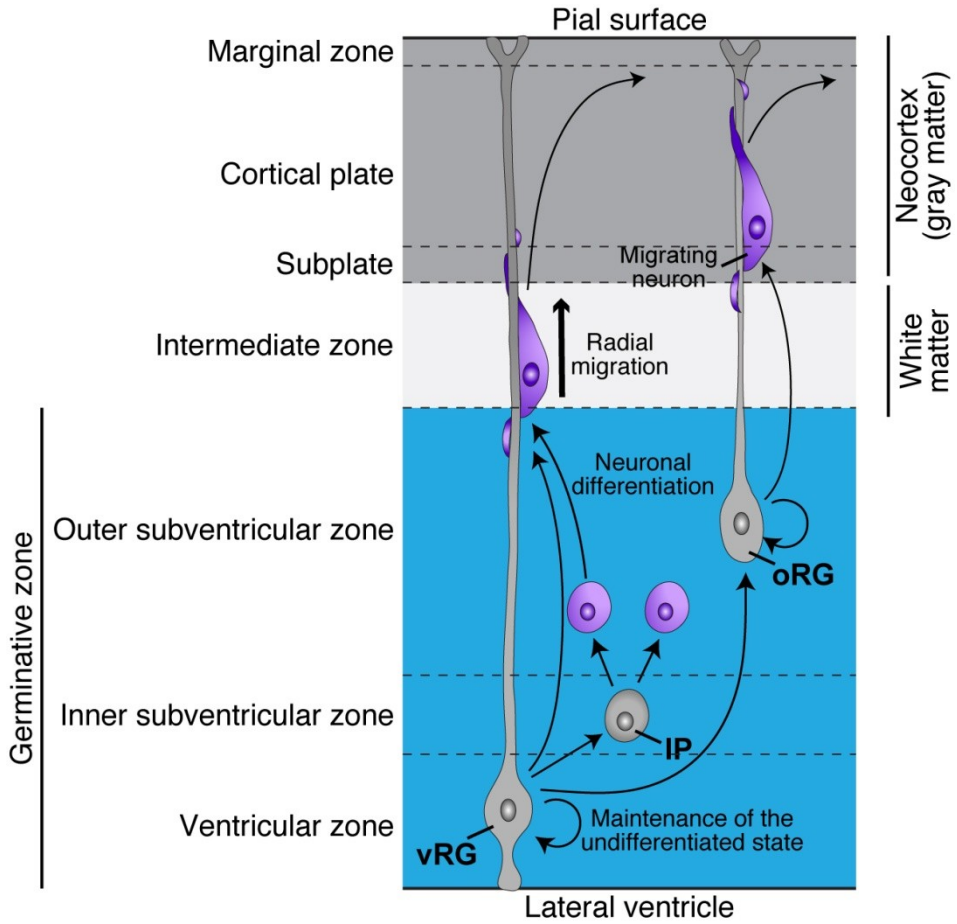


**Figure 2.** The radial glial cell system and modes of neural progenitor cell division and neuronal differentiation during cortical neurogenesis. The neocortical germinative zone during cortical neurogenesis is composed of three populations of cortical NPCs: ventricular radial-glial (vRG) cells in the ventricular zone, intermediate progenitor (IP) cells in the inner subventricular zone, and outer radial-glial (oRG) cells in the outer subventricular zone. vRG and oRG cells mainly undergo asymmetrical self-renewing divisions that give rise to a daughter cells that maintains an undifferentiated fate and a daughter cell that undergoes neuronal differentiation. IP cells undergo a limited number of symmetrical divisions (either transit-amplifying or differentiative) to generate neurons. Newly-born neurons migrate radially along the basal radial fibers of vRG and oRG cells toward the most superficial part of the cortical plate to populate the cortex in an inside-out fashion.

### *1.1.4. Modes of neural progenitor cell division and neuronal differentiation during cortical neurogenesis*

During human cortical neurogenesis, most vRG cells undergo asymmetrical self-renewing divisions to generate a daughter cell that remains undifferentiated and retains a vRG cell identity, and a daughter cell that either: (1) differentiates into a neuron that migrates radially to the cerebral cortex; (2) differentiates into a neurogenic intermediate progenitor (IP) cell that migrates away from the VZ to establish an inner subventricular zone (SVZ) and undergoes many transit-amplifying rounds of symmetrical cell division before producing projection neurons; or (3) transforms into an outer radial glial (oRG) cell that migrates away from the VZ to establish an outer SVZ (Figure 2). oRG cells have basal fibres that extend to the pial surface to provide a structure for radial neuronal migration, but lack ependymal (apical) processes that contact the ventricle (Hansen *et al.,* 2010; LaMonica *et al.,* 2012). Like vRG cells, oRG cells undergo self-renewing asymmetrical divisions to generate projection neurons and maintain an undifferentiated pool of oRG cells. oRG cells are heterogeneous and display different features and behaviours, such as diverse apical and basal fiber morphologies and dynamics, or the presence and distance of mitotic somal translocation (MST, also referred to as mitotic translocating movement) prior to mitosis (Gertz *et al.,* 2014; Betizeau *et al.,* 2013). Interestingly, oRG cells are most abundant in primates. This underlies in part the massive increase in primate neocortical size (Lui *et al.,* 2011). It is important to note that the symmetry of division described here does not refer to the physical orientation of the plane of division, but rather to the fate of the daughter cells. Together, vRG and oRG cells serve as the neocortical NPCs and their localization in the VZ and SVZ represents the neocortical GZ.

### *1.1.5. Cortical neurogenesis and formation of a six-layered neocortex*

The first cortical neurons to be generated are called Cajal–Retzius cells, a heterogeneous cell population that migrates radially to form a preplate immediately below the pial surface (Marín-Padilla, 2010). The onset of human fetal development at gestational week 8 coincides with the appearance of the first projection neurons. Projection neurons migrate radially from the GZ to form the cortical plate (CP) within the subplate. Development of the CP gradually splits the

subplate in two: a more superficial marginal zone and a transient deeply-located subplate (Figure 2). Together, the marginal zone, the CP and the subplate form the developmental anlage of the human cerebral cortex.

The CP is populated by sequential waves of birth and migration of distinct subtypes of projection neurons. The neocortex develops into a stratified structure in an "inside-out" fashion, where later-born neurons migrate radially into the CP past earlier-born neurons and settle more superficially. For example, layer VI corticothalamic projection neurons are born before layer V subcerebral projection neurons (Greig *et al.,* 2013). The bulk of cortical neurogenesis is almost completed by gestational week 15, although few newly-born neurons are still generated at least up to mid-gestation (Bystron *et al.,* 2008; Jakovcevski *et al.,* 2009). Marginal zone cells become part of layer I, while subplate cells disappear gradually postnatally. Concomitant with the development of the CP and during the remainder of fetal development, other cell populations, such as interneurons, astrocytes and oligodendrocytes, endothelial cells, and microglial cells (Wierzba-Bobrowicz *et al.,* 1998), integrate into the cerebral cortex to form functional networks. The six layers of the cerebral cortex become clearly visible at the 7th gestational month (Bystron *et al.,* 2008).

### *1.1.6. Cortical gliogenesis*

Once neurogenesis is mostly completed, the neocortex undergoes neuronal maturation and development of its intrinsic vascular system. These two processes rely on the uninterrupted incorporation of new glial cells in the cerebral cortex through active cortical gliogenesis. Human cortical gliogenesis can be divided into three phases: (1) early gliogenesis, with the establishment, during early neurogenesis, of a radial glial cell system that remains present at least until gestational week 30, together with the formation of glia limitans; (2) intermediate gliogenesis, with the generation of white matter fibrous astrocytes and oligodendrocytes, and their integration predominantly into the expanding white matter; and (3) late gliogenesis, with the generation of layer I astrocytes and gray matter protoplasmic astrocytes (Marín-Padilla, 2010). While some differentiated glial cells appear in different cortical regions during the neurogenic phase, the bulk of gliogenesis temporally follows neurogenesis.

Towards the end of cortical neurogenesis, vRG cells gradually acquire a gliogenic competence and undergo a transition from a neurogenic to a gliogenic mode (Rowitch and Kriegstein, 2010). During this gliogenic competency acquisition, neurogenic genes are repressed and gliogenic genes are de-repressed through epigenetic modifications (discussed in greater details below). This allows gliogenic-competent NPCs to respond to intrinsic and extrinsic pro-gliogenic signals and undergo a transition to specific glial cell lineages.

### *1.1.7 Cortical oligodendrogenesis*

The description of the developmental history of oligodendrocytes, the myelin-forming cells of the CNS, is more comprehensive than that of astrocytes thanks to a broader knowledge of the marker proteins expressed by cells at different maturation stages along the oligodendrocytic lineage, from the early oligodendrocyte precursor cells (OPCs), to the pre-myelinating mature oligodendrocytes (Rowitch and Kriegstein, 2010). Oligodendrocytes have multiple distinct origins in the forebrain, including the GEs and the cortical VZ (Rakic and Zecevik, 2003). Oligodendrocytes originating in the cortical VZ are derived from vRG cells (Jakovcevski *et al.,* 2009). Cortical oligodendrogenesis starts as early as gestational week 10, with the first appearance in the pallium of platelet-derived growth factor α receptor (PDGFRα)-positive OPCs, and then continues into adulthood (Jakovcevski *et al.,* 2009). At midgestation, OPCs are present throughout the cortical SVZ and can be found dispersed in all cortical layers. At the same time the first myelin basic protein (MBP)-positive premyelinating oligodendrocytes start to appear (Jakovcevski and Zecevik, 2005). Complete axonal myelination by oligodendrocytes takes decades in humans (Miller *et al.,* 2012).

### *1.1.8 Cortical astrogenesis*

The developmental history of astrocytes in the cerebral cortex is not yet fully understood. The investigation of astrocytic development is hampered by a lack of knowledge about the expression of marker proteins that could allow identifying and discriminating between the different developmental stages along the astrocytic lineages, such as astrocyte precursors, immature astrocytes, and differentiated astrocytes (Molofsky *et al.,* 2012). In addition, the complex

astrocytic heterogeneity in the cerebral cortex, which is tightly linked to their developmental heterogeneity, is incompletely characterized, again in part due to a lack of marker proteins to study this question. It is proposed that astrocytes are generated through a number of different developmental routes (Figure 3). Glial-competent (gliogenic) cortical vRG cells give rise to different glial cell lineages (astrocytes, oligodendrocytes and ependymal cells) (Rowitch and Kriegstein, 2010). With the gradual disappearance of the radial glial cell system (discussed below), gliogenic vRG cells are thought to differentiate into astrocytes or develop into SVZ glial progenitor cells that can give rise to different glial cell lineages. It has also been proposed that gliogenic vRG cells can give rise to intermediate astrocyte precursors or glial precursors that undergo expansion before differentiating into immature astrocytes (Molofsky *et al.,* 2012; Marín-Padilla, 2010).

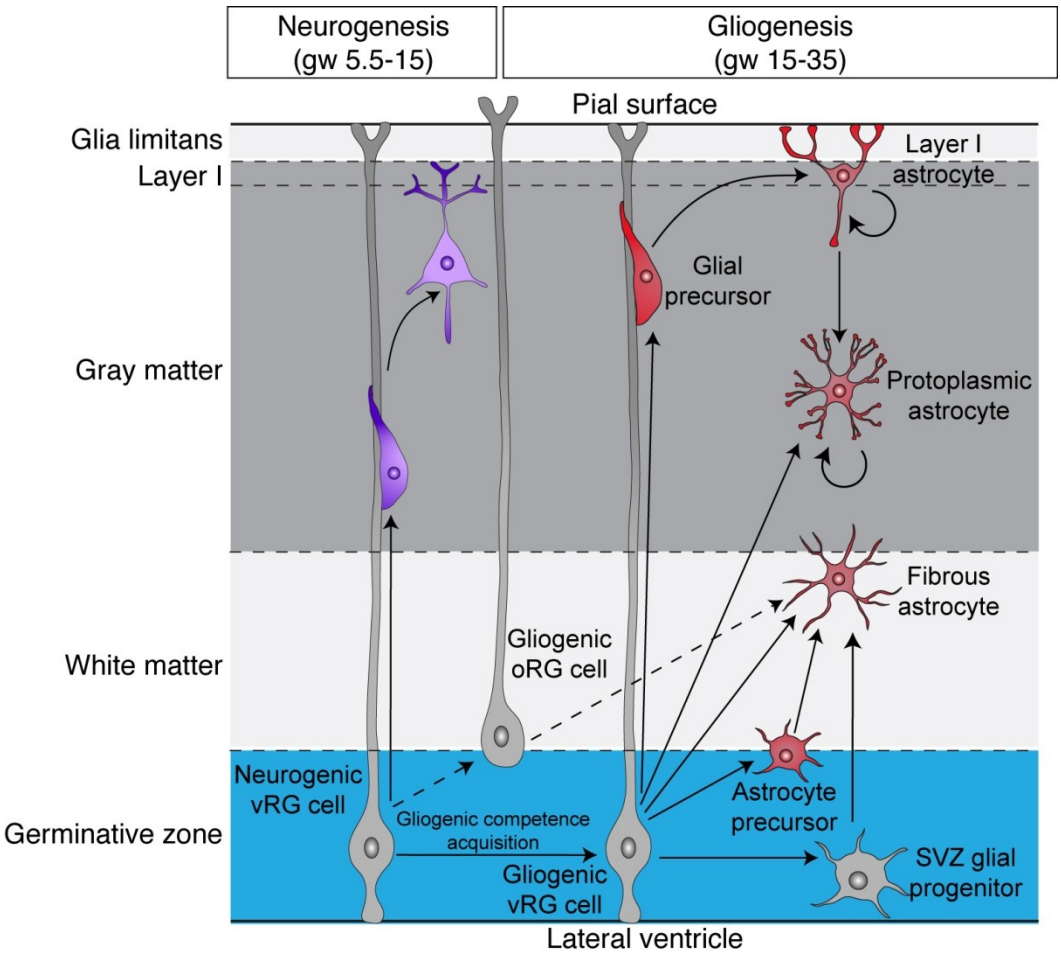


**Figure 3.** Proposed model of astrogenesis in the developing human cerebral cortex. Gliogenic cortical vRG cells are suggested to give rise to astrocytes through a number of routes: by differentiating directly into astrocytes (fibrous or protoplasmic) or by differentiating first into glial and astrocyte precursors that proliferate and differentiate into astrocytes. Protoplasmic astrocytes are also thought to be generated through the differentiation of layer I astrocytes and through symmetrical divisions of protoplasmic astrocytes. vRG cells gradually develop into SVZ glial progenitors that can differentiate in turn into astrocytes. Additionally, I propose that oRG cells can acquire a gliogenic potential and differentiate into astrocytes.

The behaviour of oRG cells during gliogenesis and their involvement in the generation of astrocytes and oligodendrocytes are still poorly characterized (Rubenstein and Rakic, 2013). Based on available evidence, I propose that a specific glial cell population in the developing human cerebral cortex described by Dr. Marín-Padilla (Marín-Padilla, 1995; Marín-Padilla, 2010) could correspond to oRG cells that have acquired a gliogenic competence and that generate white matter fibrous astrocytes. Histological investigation of prenatal human cortical development, primarily based on the rapid Golgi procedure, has led to the hypothesis that white

matter fibrous astrocytes originate from a glial cell population described as being located in paraventricular zones (in the outer SVZ and in the adjacent white matter), having long ascending processes toward the pial surface but lacking ependymal attachments, and ascending toward the white matter independently of RG cells (Marín-Padilla, 1995). These features correspond to those described for oRG cells during cortical neurogenesis (Hansen *et al.,* 2010).

Fibrous astrocytes are predominantly distributed along the white matter tract. They have a ‘‘star-like’’ morphology, and abundant and dense gliofilaments that stain with the intermediate filament marker glial fibrillary acidic protein (GFAP) (Dąmbska and Wisniewski, 1999). White matter fibrous astrocytes are generated during intermediate cortical gliogenesis: the first recognizable GFAP-positive white matter astrocytes appear in the upper white matter and subplate at gestational week 15 (Wilkinson *et al.,* 1990), a time-point corresponding to the end of the neurogenic phase. By gestational week 25, mature fibrous astrocytes with vascular endfeet are found throughout the white matter. As described above, the current literature suggests that white matter fibrous astrocytes are generated by cortical vRG cells, by SVZ glial progenitor cells, and by astrocyte precursors (Figure 3). Additionally, I propose that fibrous astrocytes could also derive from oRG cells.

Layer I astrocytes (or first lamina special astrocytes) and gray matter protoplasmic astrocytes are generated during late gliogenesis. Layer I astrocytes occupy the upper region of layer I and the subpial zone. They increase in number during prenatal development starting at around gestational week 15 (Marín-Padilla, 1995). Like radial glial cells and fibrous astrocytes, layer I astrocytes have gliofilaments that abundantly express GFAP. Layer I astrocytes incorporate the enlarged endings of their astrocytic processes (astrocytic endfeet) into the adjacent EGLM to allow for its maintenance and expansion (Marín-Padilla, 2010). The EGLM is a type of glia limitans that envelops the cortex between the marginal zone and the pial surface to serve as a laminar boundary between neuroectodermal tissue and adjacent structures. During neurogenesis, the EGLM is constructed and maintained by astrocytic endfeet of radial glial cells. During gliogenesis, astrocytic endfeet of layer I astrocytes progressively replace the radial glial endfeet in the EGLM. Layer I astrocytes also develop processes that penetrate the underlying gray matter to establish contact with developing capillaries. Layer I astrocytes derive from vRG cells in the cortical GZ. It has been proposed that these astrocytes are not born in the cortical GZ; instead vRG cells are thought to give rise, between gestational weeks 15 to 30, to glial precursors

that migrate radially up to the subpial zone, where they undergo astrocytic differentiation into layer I astrocytes (Marín-Padilla, 2010) (Figure 3). After birth, layer I astrocytes can undergo mitosis and replenish themselves.

Protoplasmic astrocytes are distributed in the gray matter. They have few gliofilaments, and express low levels of GFAP under normal conditions. Instead, they have numerous complex fine processes that contact and ensheath synapses and make few contacts with capillaries (Oberheim *et al.,* 2012; Molofsky *et al.,* 2012). Based on the current literature, it appears that protoplasmic astrocytes can be generated through three distinct developmental processes: (1) through the astrocytic differentiation of RG cells and progenitor cells located in the cortical GZ, which would account for a minority of protoplasmic astrocytes; (2) through the differentiation of layer I astrocytes into protoplasmic astrocytes and downward migration into the gray matter; and (3) through mitosis of local gray matter protoplasmic astrocytes (Figure 3) (Marín-Padilla, 2010).

### *1.1.9 Disappearance of the radial glial cell system*

Towards the end of prenatal development, the radial migration of various neural cell types into the maturing cerebral cortex is almost complete. There is therefore no more need for a system of radial fibers. RG (vRG and oRG) cells gradually disappear, removing radial fibers through autophagy, and transform into astrocytes or SVZ glial progenitor cells. A subpopulation maintains an undifferentiated state and become astrocyte-like SVZ adult neural stem cells which persist into the adult mammalian brain (Kadhin *et al.,* 1998; Rakic, 2003). SVZ neural stem cells can self-renew and give rise to neurons that migrate tangentially to the olfactory bulb and to oligodendrocytes (Alvarez-Buylla *et al.,* 2008).

## 1.2. The developing mouse neocortex as an experimental model system to investigate the molecular mechanisms regulating neocortical neural progenitor cell biology and neural differentiation

Different experimental model systems have been used to study the molecular mechanisms regulating NPC biology during corticogenesis, including cultured human fetal cerebral cortex tissue and cortical NPCs derived from human pluripotent stem cells (hPSCs) (Hansen *et al.,* 2010; Shi *et al.,* 2012). Due to the ethical and practical issues associated with the study of human

tissues, the majority of studies of cortical development have been conducted in experimental animals, among which the most commonly used are murine species. Development of the mouse nervous system is relatively short and can be manipulated *in vivo* through a number of different approaches such as genetic modifications or *in utero* electroporation techniques. Most importantly, the developing mouse cerebral cortex shares numerous similarities with the developing human cerebral cortex. Humans and mice have many common cortical structures and a similar cortical organization, and their development follows predominantly homologous routes where comparable populations of NPCs undergo similar modes of division and neural cell differentiation. This situation makes the developing mouse brain an attractive experimental system to study corticogenesis.

There are, however, some differences between the human and mouse developing cerebral cortex: (1) the human cerebral cortex develops into a gyrencephalic structure (folded appearance) while the mouse cerebral cortex develops into a lissencephalic structure (smooth appearance) (Sun and Hevner, 2014); (2) the vast majority of neuronal and glial cells forming the human cerebral cortex are generated during prenatal development, while in mice, neuronal cells are generated prenatally while most cortical glial cells are generated postnatally (Kriegstein and Alvarez-Buylla, 2009); (3) oRG cells are abundant in humans, where they play a central role in corticogenesis and form an outer SVZ, while oRG cells are scarce in mice and do not form a separate SVZ (Wang *et al.,* 2011); (4) human IP cells undergo several rounds of transit-amplification before undergoing neuronal differentiation, while mice IP cells undergo limited proliferative divisions before undergoing neuronal differentiation (LaMonica *et al.,* 2013); (5) GFAP is expressed by human neurogenic RG cells but not by mouse neurogenic RG cells, which could entail, in addition to mechanical/structural differences, functional differences based on proposed functions of GFAP (Rutka *et al.,* 1997; Middeldorp and Hol, 2011) (6) astrocyte biology is different between humans and mice, which could underlie differences in astrocytic populations and astrocytic development (Oberheim *et al.,* 2012; Molofsky *et al.,* 2012).

The complex developmental programs orchestrated by NPCs are regulated by the expression of specific combinations of genes that promote or inhibit biological functions such as cell survival, proliferation, self-renewal or cell differentiation. The expression of such genes is controlled by a number of mechanisms: (1) the chromatin structure around the genes, where open chromatin allows for DNA-binding of protein complexes while closed chromatin inhibits it; (2)

the presence or absence and post-translational modifications of transcriptional activators/coactivators and repressors/corepressors that activate or repress gene expression through a number of different molecular mechanisms; (3) post-transcriptional mechanisms such as non-coding miRNAs that inhibit the translation of target mRNAs through promotion of mRNA degradation or suppression of protein translation; and (4) extrinsic signaling factors such as growth factors, cytokines, or cell-cell interactions, that activate intracellular signaling cascades (Kriegstein and Alvarez-Buylla, 2009; Rowitch and Kriegstein, 2010; Franco and Müller, 2013; Paridaen and Huttner, 2014; Imayoshi and Kageyama, 2014; Sun and Hevner, 2014; Greig *et al.,* 2013). Although the complex molecular mechanisms regulating the developmental programs of NPCs have been extensively investigated, a complete description of these mechanisms is not yet available.

### *1.2.1. Neocortex specification and progenitor expansion*

A neocortical identity is specified toward the rostral end of the mouse neuroepithelium (developing telencephalon) as early as embryonic day (E) 8.0 through the cooperation of transcription factors Foxg1 and Lhx2 (Mangale *et al.,* 2008; Kumamoto *et al.,* 2013). Closure of the neural tube starts at E8.5 and is completed by E10.0 (Copp *et al.,* 2013). From E8 to E10.5, the neuroepithelium is composed of NE cells that undergo an expansion phase during which they divide symmetrically to increase in number, where one NE cell divides into two NE cells (Figure 4) (Paridaen and Huttner, 2014). During the expansion phase, it is essential that NE cells maintain an undifferentiated neural progenitor fate and expand sufficiently before undergoing neuronal differentiation. Precocious neuronal differentiation of NE cells during the expansion phase is inhibited by oscillations in the expression of proneuronal genes such as *Neurogenin2* (*Ngn2)* caused by oscillation of the hairy /enhancer of split (Hes) transcription factor Hes1, a basic helix-loop-helix (bHLH) transcriptional repressor of proneuronal genes (Shimojo *et al.,* 2008; Shimojo *et al.,* 2011). bHLH factors usually bind a consensus DNA sequence present in promoter regions of specific genes, termed E-box. In contrast to bHLH factors that bind only to E-box DNA elements, Hes1 has a particular basic domain that allows it to bind slightly different, N-box, DNA elements with high affinity and E-box DNA elements with low affinity (Sasai *et*

*al.,* 1992). As a result of Hes1 oscillations, *Ngn2* expression is not sustained long enough to trigger the neuronal differentiation program (Imayoshi and Kageyama, 2014).

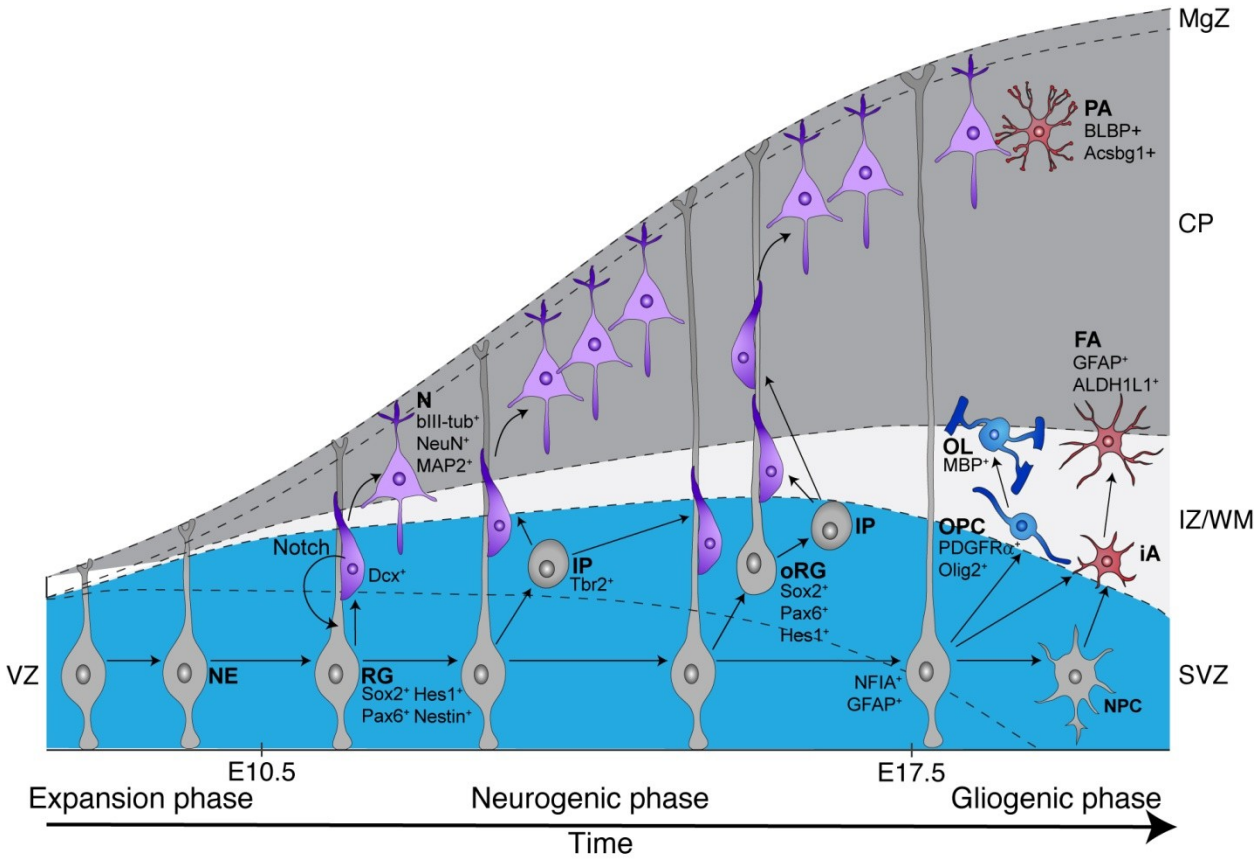


**Figure 4.** Neural cell differentiation in the developing murine cerebral cortex. See text for details. CP, cortical plate; FA, fibrous astrocyte; iA, immature astrocyte; IP, intermediate progenitor cell; IZ, intermediate zone; MgZ, marginal zone; N, neuron; NE, neuroepithelial cell; NPC, neural progenitor cell; OL, oligodendrocyte; OPC, oligodendrocyte precursor cell; oRG, outer radial-glial cell; PA, protoplasmic astrocyte; RG, radial-glial cell; SVZ, subventricular zone; VZ, ventricular zone; WM, white matter.

### *1.2.2. Neuronal differentiation of neural progenitor cells*

With the onset of mouse cortical neurogenesis between E10.5 and E11.0, NE cells gradually adopt a RG cell identity (Figure 4). Similar to human cortical RG cells, mouse RG cells undergo asymmetric self-renewing divisions to generate projection neurons and maintain a pool of undifferentiated progenitor cells throughout corticogenesis (Kriegstein and Alvarez-Buylla, 2009). RG cells also differentiate into neurogenic IP cells, which form a SVZ and undergo

limited rounds of symmetrical divisions before differentiating into projection neurons. In addition, few RG cells also differentiate into ependymally-detached oRG cells that migrate to the SVZ. The asymmetric, self-renewing cell divisions of RG cells (and to a lower extent, oRG cells) allow maintenance of a pool of NPCs throughout cortical development. Importantly, deregulation of these divisions can lead to perturbations in identity and number of the neural cells generated. Although extensive work has been done to understand how this mode of asymmetric division is regulated, a complete description of the underlying molecular mechanisms is still lacking.

### *1.2.3. Lateral inhibition of neuronal differentiation by Notch signaling*

Asymmetrical, self-renewing NPC cell divisions, both in RG and oRG cells, are regulated in part through lateral inhibition by Notch signaling (Figure 5). In this cell-cell communication mechanism, postmitotic immature neuronal cells and IP cells activate Notch signaling in NPCs to inhibit their neuronal differentiation and maintain their undifferentiated fate (Shimojo *et al.,* 2011, Neslon *et al.,* 2013). The expression of the proneuronal genes *Ngn2* and *Mash1 (Ascl1)* is upregulated during neuronal differentiation. Ngn2 and Mash1 are bHLH factors that act as activator of neuronal differentiation genes and induce the expression of the Notch ligands Delta-like1 (Dll1) and Jagged1. Dll1 and Jagged1 bind to the transmembrane receptor protein Notch in neighbouring cells, which causes the cleavage of Notch and the release of the Notch intracellular domain (NICD). NICD translocates to the nucleus and forms a complex with the DNA-binding protein Rbpj to induce the expression of antineurogenic bHLH transcription repressors such as members of the Hes family *Hes1* and *Hes5*. Hes1 and Hes5 in turn repress the expression of proneuronal genes and in this way promote the maintenance of the undifferentiated state.

A related Hes family member, Hes6, is expressed in both undifferentiated NPCs and postmitotic neurons throughout corticogenesis (Bae *et al.,* 2000; Koyano-Nakagawa *et al.,* 2000). Hes6, unlike Hes1, does not bind directly to N- or E-box DNA elements (Bae *et al.,* 2000). Hes6 functions in NPCs downstream of proneuronal factors and acts as a positive regulator of neuronal differentiation. Hes6 can promote neuronal differentiation, at least in part, by antagonizing Hes1 functions through inhibition of the interaction between Hes1 and corepressors of the Groucho (Gro)/Transducin-like Enhancer of split (TLE) family, and through promotion of Hes1

degradation (Gratton *et al.,* 2003). Interestingly, Hes6 expression in the embryonic mouse forebrain is first detectable at E8.5, 2 days prior the onset of cortical neurogenesis. The proneuronal functions of Hes6 during the expansion phase are antagonized in part by oscillations in the expression of proneuronal factors. The modes of regulation of Hes6 activity during cortical neurogenesis remain to be fully elucidated. A novel molecular mechanism by which the proneuronal functions of Hes6 are antagonized in NPCs to allow maintenance of the undifferentiated state will be addressed below.

### *1.2.4. Acquisition of gliogenic competence by neural progenitor cells*

During late mouse embryogenesis (E17.5), the cortical neurogenic phase is almost complete and the cortical gliogenic phase starts progressively. Towards the end of the neurogenic phase, RG cells acquire a gliogenic competence and undergo a transition from a neurogenic to a gliogenic mode (Li *et al.,* 2012; Rowitch and Kriegstein, 2010). This transition is required for the generation, and massive expansion, of astrocytes and oligodendrocytes in the postnatal mouse cortex during the first three postnatal weeks (Li *et al.,* 2012).

The neurogenic and gliogenic potential of RG cells is regulated by a combination of extrinsic and intrinsic signals. During the neurogenic phase, RG cells are already exposed to progliogenic signals, such as JAK-STAT signaling which promotes the expression of glial differentiation genes (Bonni *et al.,* 1997; He *et al.,* 2005). A number of molecular mechanisms prevent precocious glial cell differentiation of RG cells during the neurogenic phase. First, through epigenetic modifications, the promoters of proneuronal bHLH factor genes are de-methylated while the promoters of genes required for gliogenic differentiation, such as *GFAP*, *S100B* and *nuclear factor I A* (*Nfia*), are methylated (Rowitch and Kriegstein, 2010; Piper *et al.,* 2010). DNA methylation of specific cytosine residues present in gene promoter regions is a common epigenetic silencing mechanism involved in numerous biological processes which usually results in repression of gene transcription. DNA can be actively demethylated by a number of enzymes, which allows for transcriptional activation (Wu and Zang, 2010; Gaspar−Maia *et al.,* 2011). Second, gliogenic STAT proteins are inhibited by proneuronal bHLH factors expressed during the neurogenic phase (Hirabayashi *et al.,* 2009). Third, in response to external

signals like fibroblast growth factor 2 (Fgf2), nuclear receptor co-repressor (NCoR) signaling acts on the promoter of astrocytic genes to inhibit their expression (Hermanson *et al.,* 2002).

At the onset of the gliogenic phase, RG cells become competent to respond to progliogenic signals through several molecular mechanisms. First, epigenetic modifications toward the end of neurogenesis lead to the progressive repression of proneuronal genes and de-repression of progliogenic genes. For example, Polycomb group proteins epigenetically suppress proneuronal gene loci, such as the *Neurogenin1* (*ngn1*) gene locus (Hirabayashi *et al.,* 2009), and in parallel NFIA participates in promoter demethylation of *Gfap*, thereby allowing it to become responsive to JAK-STAT signaling (Namihira *et al.,* 2009). Second, activation of Notch signaling in RG cells by Dll1 and Jagged1 expressed by IP cells and newly-born neurons leads to the repression of proneuronal bHLH factors and promotion of JAK-STAT signaling to upregulate glial gene expression (Namihira *et al.,* 2009; Kamakura *et al.,* 2003). Third, embryonic cortical neurons secrete factors, such as the neurotrophic cytokine cardiotrophin-1 (CT1), that activate JAK-STAT signaling in RG cells and promote gliogenesis (Barnabe-Heider *et al.,* 2005).

Glial-committed (gliogenic) RG cells undergo glial cell differentiation along different glial cell lineages: (1) RG cells can transition to the oligodendrocytic lineage by generating OPCs (Yue *et al.,* 2006); (2) RG cells can transition to the astrocytic lineage by differentiating into astrocytes or by generating astrocyte progenitors that proliferate and diversify into fibrous and protoplasmic astrocytes (Molofsky *et al.,* 2012; Rowitch and Kriegstein, 2010); (3) RG cells can differentiation into ependymal cells that form an ependymal layer lining most of the ventricular surface; (4) RG cells can convert into in adult SVZ neural stem cells (type B cells) (Kriegstein and Alvarez-Buylla, 2009). Because RG cells progressively disappear shortly after birth, their behaviour in the short period that starts around the time of birth is central for the establishment of the different cortical glial cell lineages. The molecular mechanisms regulating the choice of glial cell specification by gliogenic RG cells during cortical gliogenesis are still poorly understood. Results of studies that have addressed these questions will be described in this thesis.

*1.2.5. Oligodendrogenesis in the developing mouse cerebral cortex*

Oligodendrocyte development starts with the oligodendrocytic specification of multipotent NPCs and their transition to the OPC fate. This transition is regulated by a combination of transcriptions factors (Nakatani *et al.,* 2013; Yu *et al.,* 2013), such as the bHLH transcription factors Olig2 and Mash1, which are critical for OPC generation (Yue *et al.,* 2006; Nakatani *et al.,* 2013). For example, Olig2 knockout mice are devoid of oligodendrocytic lineage cells (Takebayashi *et al.,* 2002) while forced expression of Olig2 in NPCs is sufficient to promote the transition to the OPC fate (Zhou *et al.,* 2001). Olig2 acts both as a transcriptional repressor and an activator (Imayoshi and Kageyama, 2014). For example, it promotes the expression of the oligodendrocyte-specific gene *Sox10* (Küspert *et al*., 2011). Once OPCs are specified, they migrate, proliferate, and differentiate along the oligodendrocytic lineage by becoming first immature oligodendrocytes, which express the marker proteins Oligodendrocyte Marker O4 (O4), galactosylceramidase (GalC), and 2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase). Immature oligodendrocytes develop intro myelinating mature oligodendrocytes that express the marker protein Myelin basic protein (MBP) (Zhang, 2001; Baumann and Pham-Dinh, 2001).

Oligodendrogenesis in the developing mouse forebrain occurs in three spatiotemporally distinct sequential waves that follow a ventral to dorsal progression (Rowitch and Kriegstein, 2010). The first wave of oligodendrogenesis starts as early as E12.5 with OPCs being generated by the medial ganglionic eminence and migrating to the developing cerebral cortex by E16. Oligodendrocytic cells deriving from this first wave are replaced postnatally by oligodendrocytic cells from subsequent waves (Kessaris *et al.,* 2006). The second wave of oligodendrogenesis starts at E15.5 in the lateral and/or caudal ganglionic eminences and generates oligodendrocytes that migrate to the cortex and compete with oligodendrocytes generated in the third wave. The third wave of oligodendrogenesis starts at birth in the neocortex, where gliogenic cortical $GFAP^+$ RG cells generate $PDGFR\alpha^+$ OPCs that rapidly increase in number in the early postnatal cortex (Kessaris *et al.,* 2006). Even though OPCs of ventral and dorsal origin compete to occupy the cerebral cortex and can replace one another upon removal of one population, dorsal oligodendrogenesis is critical for proper cortical myelination: conditional ablation of Olig2 in GFAP-expressing cells results in a defect in dorsal oligodendrogenesis (third wave) and leads to

increased dorsal migration of ventrally-generated oligodendrocytes (second wave) that can populate the neocortex but cannot properly myelinate (Yue *et al.,* 2006).

### *1.2.6. Astrogenesis in the developing mouse cerebral cortex*

The developmental history of astrocytes in mice is not yet fully understood (Molofsky *et al.,* 2012), however recent evidence has shed light into the distinct developmental routes of white matter and gray matter astrocytes (Ge *et al.,* 2012). The evidence provided by Ge and colleagues suggests that astrogenesis in the developing postnatal mouse cerebral cortex happens through two developmental processes. First, there is an early phase of astrogenesis where SVZ RG cells and progenitor cells generate astrocytes, during which the vast majority of astrocytes generated localize in the SVZ and white matter while a minority of astrocytes localize in the gray matter, with very few astrocytes migrating to cortical layers I-IV. This observation suggests that most RG cells undergoing a transition to the astrocytic lineage differentiate into white matter fibrous astrocytes, while the vast majority of layer I and protoplasmic astrocytes are not generated through the astrocytic differentiation of SVZ RG cells and progenitor cells. Second, there is a later phase of astrogenesis during which layer I astrocytes ($GFAP^+$) and gray matter protoplasmic astrocytes (GFAP-negative, $BLBP^+$) are generated locally through symmetrical cell divisions of a small pool of local astrocytes derived from RG cells. This is proposed to constitute the main source of layer I-IV astrocytes during postnatal development. Whether protoplasmic astrocytes in the postnatal mouse cortex are generated first from layer I astrocytes, like it is proposed to be the case in the developing human cerebral cortex, has not been addressed. The molecular mechanisms regulating the establishment of heterogeneous populations of astrocytic cells are poorly understood.

## 1.3. The NF-κB pathway and its functions in the nervous system

### *1.3.1. NF-κB proteins*

The NF-κB pathway regulates the expression of a wide array of genes involved in developmental processes, cellular growth, immune and inflammatory responses, and apoptosis. This pathway comprises the NF-κB family of transcription factors composed of 5 members: RelA/p65 (RelA hereafter), RelB, c-Rel, p50, and p52. NF-κB proteins act as dimeric transcription factors existing in the form of homo- or heterodimers containing specific combinations of these 5 members (Hayden and Ghosh, 2008). NF-κB proteins activate target genes through a highly-conserved Rel homology domain/region (RHD) important for DNA-binding and dimerization. NF-κB dimers bind to specific sequence motifs known as κB sites within the promoters/enhancer regions of target genes and regulate transcription through the recruitment of coactivators or corepressors. κB sites display high levels of variability (Zheng *et al.,* 2011). NF-κB proteins RelA, RelB and c-Rel, but not p50 and p52, contain a transactivation domain (TAD) required for transcriptional activation of target genes. As a result, NF-κB dimers containing one or two members with a TAD (e.g., RelA/p50) act as transcriptional activators, while NF-κB dimers lacking a TAD (e.g. p50-p50) passively repress transcription.

### *1.3.2. NF-κB pathway*

Under basal/unstimulated conditions, the NF-κB pathway is kept inactive by mechanisms that maintain NF-κB dimers in the cytosol and prevent DNA-binding. NF-κB proteins contain a nuclear localization signals (NLS) that can be masked to prevent nuclear translocation. NF-κB proteins RelA, RelB and c-Rel are tightly regulated through interaction with inhibitors of κB (IκB), such as IκBα. The interaction with IκB masks the NLS of NF-κB and promotes cytoplasmic retention. In contrast, nuclear translocation of NF-κB proteins p50 and p52 is regulated by their precursor proteins p105 and p100, respectively. p105 and p100 have an IκB-like domain at their C-terminal region that inhibits *in cis* the functions of the RHD (Zheng *et al.,* 2011).

In canonical (classical) NF-κB pathway, activation of an IκB kinase (IKK) complex (composed of IKKα, IKKβ, and NEMO/IKKγ) leads to phosphorylation of IκB by IKKβ and subsequent polyubiquitination and proteosome-dependent degradation of IκB and/or dissociation of NF-κB dimers from IκB. This releases the NF-κB dimers and unmasks their NLS. NF-κB dimers translocate to the nucleus and trigger specific transcriptional responses (Figure 5). In non-canonical (alternative) NF-κB pathway, proteasomal processing of p105 to p50 and p100 to p52 promotes a transcriptional response that is distinct from that of the canonical pathway. Although canonical and non-canonical NF-κB pathways were originally thought to be distinct and independent signaling pathways, recent studies have shown great levels of cross-talk between the two signaling pathways (Shih *et al.,* 2011).

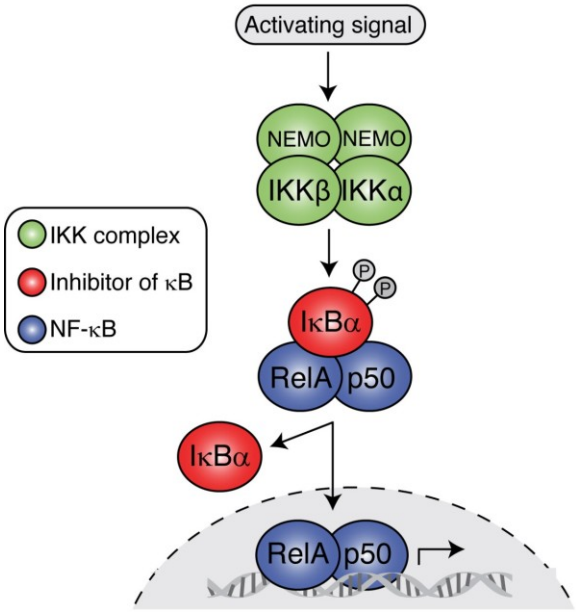


**Figure 5.** The canonical NF-κB pathway. Activation of the Inhibitor of κB kinase (IKK) complex (usually composed of IKKβ, IKKα, and NEMO/IKKγ) leads to the phosphorylation of Inhibitor of kB (IκB), such as IκBα, by IKKβ. Phosphorylated IκB dissociates from NF-κB dimers (such as RelA/p50, the most common DNA-bound NF-κB dimers in the embryonic brain) and undergoes proteosome-dependent degradation. Dissociation of NF-κB dimers from IκB unmasks their nuclear localization signal which leads to the nuclear translocation and ultimately binding to κB sites in specific gene promoters. DNA-bound NF-κB dimers containing at least one transactivation domain (TAD) can transactivate transcription of target genes, while DNA-bound NF-κB dimers lacking a TAD can passively repress transcription of target genes.

*1.3.3. NF-κB pathway functions in the nervous system*

NF-κB family transcription factors are ubiquitously expressed but their DNA binding ability and transcriptional activity is tissue-specific and developmentally regulated. In the embryonic rodent brain, dimers composed of RelA/p50, RelA/c-Rel and p50/c-Rel are the most abundant DNA-bound dimers (Bakalkin *et al.,* 1993). Activation and functions of the NF-κB pathway in neuronal and glia cells were described by several groups showing that NF-κB plays roles in neuronal survival, neural cell proliferation, synaptic plasticity, neurite outgrowth, neural differentiation, regulation of neural inflammatory responses, and pathological events associated with neurodegeneration (Bhakar *et al.,* 2002; Young *et al.,* 2006; O'Mahony *et al.,* 2006; Boersma *et al.,* 2011; Gavaldà *et al.,* 2009; Limpert *et al.,* 2013; Sabolek *et al.,* 2009; Maqbool *et al* 2003).

NF-κB was shown to play diverse roles in distinct populations of NPCs in the developing and adult brain, where it can regulate biological functions such as maintenance of the undifferentiated state, cell proliferation, and neuronal differentiation (Young *et al.,* 2006; Sabolek *et al.,* 2009; Zhang *et al.,* 2012; Andreu-Agulló *et al.,* 2009; Denis-Donini *et al.,* 2008; Koo *et al.,* 2010). For example, the activation and function of the NF-κB pathway in embryonic subpallial NPCs was investigated *in vitro* (Young *et al.,* 2006). Primary cultures of embryonic subpallial NPCs displayed immunostaining for RelA both in the cytoplasm and nucleus, suggesting activated NF-κB pathway. The genetic loss of either RelA alone or both RelA and p50 caused a reduction in the proliferation of subpallial NPCs (cultured as floating "neurospheres") together with increased neuronal differentiation. In adult neural stem cells of the subebendymal zone (cortical SVZ), the NF-κB pathway synergizes with Notch signaling to promote neural stem cell self-renewal through activation of *Hes1* and *epidermal growth factor receptor* (*Egfr*) genes (Andreu-Agulló *et al.,* 2009).

Endogenous activation of the NF-κB pathway *in vivo* in the embryonic dorsolateral telencephalon, the structure that gives rise to the neocortex, was first reported using NF-κB reporter transgenic mice (termed 'NF-κB$^{LacZ}$' hereafter), which are heterozygous for a reporter cassette harbouring the bacterial *LacZ* gene under the control of a promoter containing tandem NF-κB binding sites (Bhakar *et al.,* 2002) (Fig. 6). This construct encodes a nuclearly localized

form of β-Galactosidase (β-Gal), thereby enabling monitoring of the NF-κB pathway activation in NF-κB$^{LacZ}$ mice through analysis of the expression of nuclear β-Gal (Bhakar *et al.,* 2002; Lebrun-Julien *et al.,* 2009). Whole-mount staining of NF-κB$^{LacZ}$ mice embryos for β-Gal activity first revealed robust activation of the NF-κB pathway in the telencephalon during the neurogenic phase (Bhakar *et al.,* 2002). Bhakar and colleagues reported robust activation in post-mitotic cortical neurons, where they described a role for NF-κB in the promotion of cell survival. The activation of the NF-κB pathway in other cell types of the developing cerebral cortex, including cortical NPCs, was not investigated.

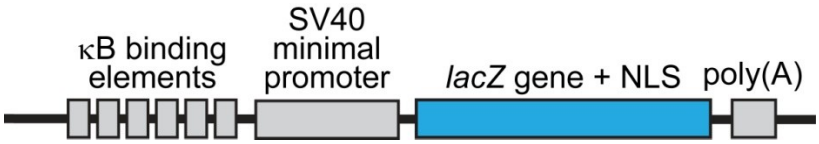


**Figure 6.** NF-κB reporter gene. The reporter gene harbours a bacterial *LacZ* gene under the control of an SV40 minimal promoter containing 6 tandem κB binding sites. The *LacZ* gene encodes a modified form of the β-Galactosidase enzyme containing a nuclear localization signal (NLS) that leads to its nuclear localization. poly(A): polyadenylation tail.

## 1.4. Rationale and specific aims

### *1.4.1. Rationale*

The reported activation and involvement of the NF-κB pathway in subpallial NPC behaviour *in vitro* (Young *et al.,* 2006) together with the activation of the NF-κB pathway in the embryonic cortex *in vivo* (Bhakar *et al.,* 2002) provided a rationale to investigate the activation and potential regulatory functions of the NF-κB pathway in NPCs during cerebral cortex development. Together with two former students in the Stifani laboratory, Robert Hermann and Hosam Al-Jehani, I conducted studies to characterize the spatiotemporal pattern of endogenous activation of the NF-κB pathway in GZs of the developing embryonic forebrain utilizing NF-κB$^{LacZ}$ mice. In this thesis, I will describe studies that I conducted on my own or together with Robert Hermann and Hosam Al-Jehani (with acknowledgment of contributions where needed). Studies performed

solely by the latter will not be shown but they will be described where needed (with acknowledgment of contributions) with reference to our published work (Methot *et al*., 2013).

Robert Hermann, Hosam Al-Jehani and I obtained evidence that activation of the NF-κB pathway in the embryonic forebrain of NF-κB$^{LacZ}$ mice is first detected between E10.5 and E11.0 (Figure 7A), a time point that approximately corresponds to the onset of cortical neurogenesis (Kriegstein and Alvarez-Buylla, 2009). At this stage, NF-κB activation was observed in both the dorsolateral telencephalon (lateral pallium), a region that will give rise to the developing neocortex, and the ventral telencephalon (subpallium), a region that will give rise the GEs (Flandin *et al.,* 2010).

At E13.5, a period of active cortical neurogenesis, activation of the NF-κB pathway was observed preferentially in the telencephalon (not shown but see Methot *et al*., 2013), while little or no activation could be detected in other regions of the brain of NF-κB$^{LacZ}$ mice. In the E13.5 telencephalon, NF-κB activation was robust in the germinative zone (VZ and SVZ) of the neocortex and ganglionic eminences. In contrast, NF-κB activation was undetectable in the medial pallium, a structure that contains the hippocampal anlage and cortical hem (Subramanian *et al.,* 2009). This activation pattern was observed at different rostrocaudal levels (Methot *et al*., 2013). Activation of the NF-κB pathway was also detected in the mantle zone, an embryonic neocortical region containing postmitotic neurons and comprising the preplate, cortical plate, and marginal zone at E13.5 (Methot *et al*., 2013). This situation is in agreement with previous studies showing activation of the NF-κB pathway in postmitotic neurons (Mattson *et al.,* 1997; Bhakar *et al.,* 2002; Aleyasin *et al.,* 2004). These results provide evidence that NF-κB signaling is activated in the developing neocortex during the phase of active cortical neurogenesis. Consistent with these findings, the two NF-κB subunits that were shown to be most abundantly bound to DNA as a complex in the embryonic forebrain, RelA and p50 (Bakalkin *et al.,* 1993), were endogenously expressed in the dorsolateral embryonic telencephalon as detected by Western blotting analysis of dissected tissues (Figure 8). These results suggest that a RelA-p50 complex is present in the developing neocortex during cortical neurogenesis.

At E17.5, a period when neurogenesis is coming to an end and gliogenesis is starting in the developing neocortex, activation of the NF-κB pathway was still detectable in the GZ of the neocortex, as well as in the ganglionic eminences of NF-κB$^{LacZ}$ mice (Methot *et al*., 2013).

Within the neocortex, the robust activation in the GZ contrasted with little or no activation in the intermediate zone (IZ), a region containing a number of cell populations including radially-migrating newly born neurons, suggesting that NF-κB signaling is down-regulated during early stages of neuronal differentiation. However, robust NF-κB activation was observed in the cortical plate, a region that contains the vast majority of developmentally mature cortical neurons, providing evidence that NF-κB signaling is only transiently down-regulated during neuronal development. Similar to the activation pattern observed at E13.5, at E17.5 there was little to non-detectable NF-κB activation in the medial pallium (hippocampus) nor in septum, a subpallial (Visel *et al.,* 2013) structure lining the medial-lateral ventricle in the rostral forebrain (Methot *et al*., 2013). Taken together, these results show that the NF-κB pathway is selectively activated in the neocortical GZ, an anatomical region containing the NPCs that generate excitatory cortical neurons. Importantly, they suggest that the NF-κB pathway could be activated in cortical NPCs and potentially involved in regulatory mechanisms in these cells.

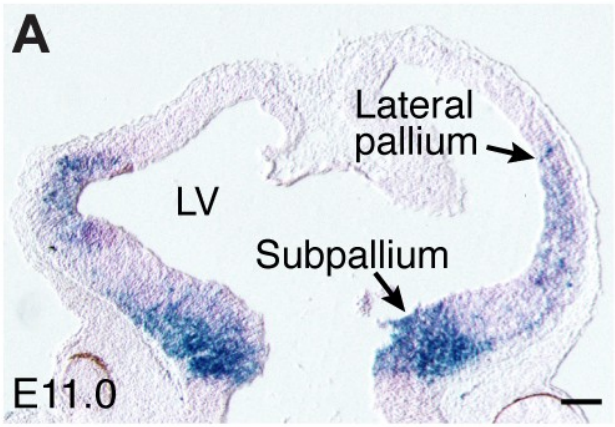


**Figure 7.** Activation of the NF-κB pathway in lateral pallial and subpallial GZs during neurogenesis. Coronal view of β-Gal activity (blue staining) in the telencephalon of E11.0 NF-$\kappa B^{LacZ}$ embryos. Dorsal is at the top and ventral is at the bottom. LV: lateral ventricle.

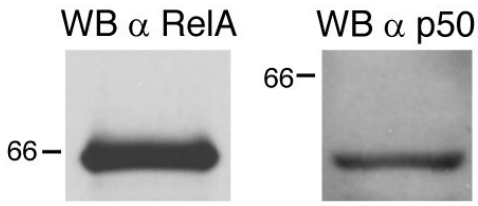


**Figure 8.** Expression of NF-κB subunits RelA and p50 in the E13.5 telencephalon, as detected by Western blotting.

*1.4.2. Specific aims*

Prior to the start of my doctoral studies, a detailed description of the spatiotemporal activation and biological functions of the NF-κB pathway in the developing cerebral cortex was not available. The experiments described in this thesis were aimed at characterizing the endogenous activation of the NF-κB pathway *in vivo* in embryonic and postnatal cortical NPCs during corticogenesis, and investigating the biological relevance of the endogenous activation of the NF-κB pathway on the biology of cortical NPCs. More specifically, this thesis describes experiments aimed at characterizing, during cortical neurogenesis: (1) the cell population(s) that display robust activation of the NF-κB pathway in cortical GZs; (2) the function(s) of the NF-κB pathway in cells of the cortical GZs; (3) the molecular mechanism(s) modulating activation of the NF-κB pathway; and (4) the signaling event(s) that lead to endogenous NF-κB activation in the cortex.

Furthermore, the pattern of activation of the NF-κB pathway in the neocortical GZ at the end of cortical neurogenesis and onset of cortical gliogenesis suggested that the NF-κB pathway could remain activated in GZ of the cerebral cortex during cortical gliogenesis. This provided a rationale for investigating the activation and functions of the NF-κB pathway during cortical gliogenesis. More specifically, this thesis describes experiments aimed at characterizing, during cortical gliogenesis: (5) the spatiotemporal activation pattern of the NF-κB pathway; (6) the identity of cells with activated NF-κB pathway in the cerebral cortex; (7) the functions of the NF-κB pathway; and (8) the target genes regulated by the NF-κB pathway.

## 2. Material and methods

### 2.1. Animals

Animal procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Montreal Neurological Institute Animal Care Committee. Timed pregnant CD1 mice were purchased from Charles River (Saint-Constant, Quebec). NF-κB$^{LacZ}$ reporter mice were generated and genotyped as described previously (Bhakar *et al.*, 2002). Briefly, an enhancer/promoter fragment composed of three tandem NF-κB binding sites upstream of a minimal SV40 promoter was cloned upstream of a modified *E. coli lacZ* gene, which encodes a nuclearly-localized form β-*Galactosidase (β-Gal*), to generate an NF-κB reporter gene cassette. For analysis of staged embryos, NF-κB$^{LacZ}$ male mice were crossed to C57BL/6 females to obtain heterozygous embryos containing a single transgenic allele. For staging of mouse embryos, the day of the appearance of the vaginal plug was considered embryonic day 0.5 (E0.5).

### 2.2. DNA Plasmids

The following expression and reporter plasmids have been described previously: pCAGGS-EGFP (where EGFP is enhanced green fluorescent protein) (Maira *et al.,* 2010); pCig2-IRES-EGFP (which contains a cDNA-internal ribosome entry site [IRES]-EGFP expression cassette under the control of a CMV enhancer and chicken β-actin promoter) (Hand *et al.,* 2005); pcDNA3-IκBαM (where IκBaM is a dominant-negative form of IκBα) (Bhakar *et al.,* 2002); pAd-Track-IKKβ-DN (where IKKβ-DN is a dominant-negative form of IKKβ) (Ho *et al.,* 2005); pCMV2-FLAG-Hes6WT (Belanger-Jasmin *et al.,* 2007; Jhas *et al.,* 2006); and pCMV-RelA (Tetsuka *et al.,* 2000). For the generation of pCig2-IκBαM-IRES-EGFP, the IκBαM coding sequence was subcloned as an EcoRI fragment into the EcoRI site of the pCig2-IRES-EGFP vector. For the generation of pCig2-IKKβ-DN-IRES-EGFP, the IKKβ-DN coding sequence was subcloned into a blunt-ended NotI/HindIII fragment intro the SmaI site of the pCig2-IRES-EGFP vector.

### 2.3. Embryonic and postnatal brain preparation

Embryonic and postnatal brains were dissected and rinsed in ice-cold phosphate-buffered saline (PBS) followed by fixation in 4% paraformaldehyde (PFA) for 25-90 min at 4°C. Fixed brains were rinsed in PBS, cryoprotected in 30% Sucrose in PBS, and embedded in O.C.T. (optimum cutting temperature) compound (Tissue-Tek) for sectioning on a cryostat (14 μm sections).

### 2.4. Analysis of NF-κB reporter mice

Heterozygous NF-$\kappa B^{LacZ}$ embryonic and postnatal brains were collected and genotyped based on β-Gal activity in the nose bud (for embryos between E10.5 and E15.5) or in the olfactory bulb (for embryos E16.5 and older, and pups). β-Gal activity was detected by incubation of tissue at 37°C for 1 hour in X-Gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) (Invitrogen) staining solution composed of solution A (80 mM $Na_2HPO_4$, 20 mM $NaH_2PO_4$ [pH 7.4], 2 mM $MgCl_2$, 0.2% IGEPAL [octylphenoxypolyethoxyethanol], 0.1% sodium deoxycholate) containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-Gal. Heterozygous NF-$\kappa B^{LacZ}$ embryonic and postnatal brains were prepared as described above. For detection of β-Gal activity histochemically on cryosections, sections were rinsed three times in solution A, followed by incubation at 37°C for 1-16 hours in X-Gal solution. After this, sections were rinsed in PBS and counterstained with eosin before being mounted with Fluoromount-G (SoutherBiotech). For immunohistochemical analysis of cryosections, sections were processed as described below.

### 2.5. *In utero* electroporation

Pregnant CD1 mice at gestational stage E13.5 (for analysis of cortical neurogenesis) or E17.5 (for analysis of cortical gliogenesis) were anesthetized with isoflurane, and uterine horns were exposed as described previously (Walantus *et al.,* 2007). Embryos were injected into the lateral ventricles with an empty pCIG2-IRES-EGFP plasmid, a pCIG2-IκBαM-IRES-EGFP construct, or a pCIG2-IKKβ-DN-IRES-EGFP construct (2 μg/μl in each case) using an Eppendorf Femtojet

microinjector (Ghanem *et al.,* 2012) or a WPI PV830 Pneumatic PicoPump. The head of each embryo was held between tweezer-type circular electrodes (Harvard Apparatus) across the uterine wall, and five electrical pulses (amplitude, 50 V; duration, 50 ms; intervals, 950 ms) were delivered using an ECM830 square wave electroporator (BTX Harvard Apparatus). Gestation was then allowed to continue for 2 or 4 days, after which embryos or pups were collected and prepared as described above. Double-labeling immunofluorescence analysis was performed as described below.

**2.6. Organotypic forebrain slice culture and electroporation**

E13.5 embryos from staged CD1 pregnant mice were collected, and their brains were recovered in ice-cold Hanks' balanced salt solution (HBSS) supplemented with 0.7% glucose and 2.5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (pH 7.3). Brains were embedded in 4% low-melting-point agarose (Invitrogen) in HBSS, followedby preparation of 250-μm coronal slices of the forebrain by using a Leica VT1000S vibratome. Slices were collected on Nucleopore Track-Etched polyvinylpyrrolidone-free polycarbonate membranes (8.0-μm pores; Whatman Schleicher & Schuell) floating in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% glucose, and 1% penicillin-streptomycin. Slices were incubated at 37°C for 2 h prior to nucleic acid injection and electroporation. DNA plasmids (1 μg/μl) or small interferingRNA (siRNA) reagents (10 μM; Ambion) were mixed with Fast green dye and injected into the neocortical VZ. The ratio of effector nucleic acids (i.e., plasmids encoding IκBαM, IKKβ-DN, RelA, or FLAG-Hes6 or RelA or scrambled siRNA reagents) to GFP expression plasmid was kept at 4:1 to ensure that GFP-expressing cells also contained the effector plasmids. This approach normally results in overlapping delivery of the two plasmids in greater than 90% of the electroporated cells (Maira *et al.,* 2010). Electroporations were performed using a TSS20 Ovodyne electroporator (Intracel) as described previously (Maira *et al.,* 2010), with the following settings: three pulses (25 V, 5-ms width), 500-ms interval between pulses, zero resistance, boost option selected. Electroporated slices on polycarbonate membranes were transferred to neurobasal medium supplemented with 1% B27, 1% glucose, 1% penicillin-streptomycin, and 1% glutamine. Electroporated slices were incubated at 37°C for either 48 h (loss-of-function experiments) or 96 h (RelA overexpression experiments), followed by fixation

in 4% PFA for 20 min, cryoprotection, and embedding in OCT compound (Tissue-Tek) for sectioning on a cryostat (16 μm). Double-labeling immunofluorescence analysis was performed as described below. The percentage of GFP-positive cells that were also positive for the expression of Ki-67, nestin, MAP2, or NeuN, was quantitated. In each experiment, an average of 5 or 6 slices were electroporated and analyzed for expression of the different markers mentioned above.

### 2.7. Primary culture of neurogenic cortical neural progenitor cells

Primary neural progenitor cells were established from dissociated dorsal telencephalic cortices obtained from CD1 or NF-κB$^{LacZ}$ mouse embryos collected between E12.5 and E13.5 and cultured in four-well chamber slides (Thermo Scientific) for immunocytochemical analysis or six-well dishes (Thermo Scientific) for transcription assays, as described above (Gratton *et al.,* 2003). Briefly, cortices were dissected in ice-cold HBSS and transferred in culture medium (Neurobasal medium [Gibco] supplemented with 1% N2 [Gibco], 2% B27 [Gibco], 0.5 mM glutamine [Gibco], 1% penicillin-streptomycin [Gibco], and 40 ng/ml of fibroblast growth factor 2 [PeproTech]). Cortical tissue was triturated to yield single-cell suspensions and plated on chambers or culture dishes coated with 0.1% poly-d-lysine and 0.2% laminin (BD Biosciences) at a cell density of 2.5 x $10^5$ cells/ml. For Western blotting analysis or immunoprecipitation assays, cells were washed in ice-cold PBS, collected and lysed in lysis buffer. For immunofluorescence staining, cells were washed in ice-cold PBS and fixed in 4% PFA for 20 min.

### 2.8. Pharmacological treatment of primary cultures of neurogenic cortical neural progenitor cells

For treatment with the NF-κB inhibitor SN50, half of the culture medium was removed on day 1 *in vitro*, kept as conditioned medium, and replaced with fresh medium together with 2.5 μM SN50 peptide (Enzo Life Sciences, cat#BML-P600-0005), 2.5 μM SN50M mutated peptide (Enzo Life Sciences, cat#BML-P601-0500), or no peptide. Six hours later, the culture medium was replaced with a 1:1 mix of conditioned medium and fresh medium and cells were cultured

for 18 h, followed by a second round of pharmacological treatment as described above. Cells were fixed on day 3 and subjected to immunocytochemistry for Ki-67, nestin, MAP2, or βIII-tubulin, as described below. For each condition and each antibody, 5 different fields were photographed using a 20X objective, with an average of about 300 cells per field. Cells were identified manually and quantitated in ImageJ (National Institutes of Health). For treatment with the PI3K inhibitor Wortmannin, primary cortical progenitor cells were established from E13.5 NF-κB$^{LacZ}$ embryos. In the morning of day 2 *in vitro*, half of the culture medium was removed and replaced with fresh medium together with a first dose of Wortmannin (Enzo Life Sciences). A second dose of Wortmannin was added directly to the culture medium on the evening of day 2 *in vitro*. For the cells that received 4 treatments of Wortmannin, two additional doses of Wortmannin were added directly to the culture medium in the morning and in the evening of day 3 *in vitro*. On day 4 *in vitro*, cells were collected, lysed, and subjected to determination of β-Gal activity as previously described (Gratton *et al.,* 2003).

### 2.9. Adenoviral transduction of primary cultures of neurogenic cortical neural progenitor cells

In transduction experiments, primary cortical progenitor cells were established from E13.5 NF-κB$^{LacZ}$ embryos. After 2 days in vitro, half of the culture medium was removed and replaced with fresh medium containing no adenovirus or adenovirus expressing RelA and GFP (Bhakar *et al.,* 2002), IKKβ-DN and GFP (Ho *et al.,* 2005), or GFP alone (Theriault *et al.,* 2005). Cells were collected and subjected to determination of β-Gal activity as described previously (Gratton *et al.,* 2003). Western blotting analysis was performed with the antibodies described in Table 1.

**Table 1.** Antibodies for Western blotting.

| Antibody | Company | Catalog # | Host species | Dilution |
|---|---|---|---|---|
| **Acsbg1** | Abcam | ab65154 | Rabbit | 1:1,000 |
| **Akt** | Cell Signaling | 9272 | Rabbit | 1:1,000 |
| **ALDH1L1** | Abcam | ab87117 | Rabbit | 1:1,000 |
| **βIII-tubulin** | Promega | G7121 | Mouse | 1:10,000 |
| **β-actin** | Abcam | ab6276 | Mouse | 1:10,000 |
| **CNPase** | Millipore | MAB326R | Mouse | 1:1,000 |
| **FLAG** | Sigma | F3165 | Mouse | 1:5,000 |
| **GFAP** | Sigma | G3893 | Mouse | 1:5,000 |
| **Hes6** | Novus Biologicals | NB100-81022 | Rabbit | 1:1,000 |
| **IKKβ** | Millipore | 05-535 | Mouse | 1:1,000 |
| **Olig2** | Millipore | AB9610 | Rabbit | 1:1,000 |
| **p50** | Santa Cruz Biotechnology | sc-8414 | Mouse | 1:500 |
| **PDGFRα** | Santa Cruz Biotechnology | sc-338 | Rabbit | 1:500 |
| **Phospho-Akt (Ser473)** | Cell Signaling | 9271 | Rabbit | 1:1,000 |
| **RelA/p65** | Santa Cruz Biotechnology | sc-372 | Rabbit | 1:5,000 |
| **Sox2** | R&D Systems | AF2018 | Goat | 1:500 |

### 2.10. Primary culture of gliogenic cortical neural progenitor cells

Mixed primary cortical cell cultures were established from dissociated dorsal telencephalic cortices obtained from CD1 embryos collected at E17.5, triturated and cultured in suspension in 'proliferation medium' (Neurobasal medium [Gibco] supplemented with 1% N2 [Gibco], 2% B27 [Gibco], 0.5 mM glutamine [Gibco], 1% penicillin-streptomycin [Gibco], and 40 ng/ml of fibroblast growth factor 2 [PeproTech]) on uncoated petri dishes. After 3 days in culture, floating neurospheres were collected and plated on culture dishes coated with 0.1% poly-d-lysine and 0.2% laminin (BD Biosciences). After 24 hours, culture medium was replaced with 'glial differentiation medium' (1:1 mix of Neurobasal medium and DMEM [Gibco] supplemented with 0.5% N2 [Gibco], 1% B27 [Gibco], 0.5 mM glutamine [Gibco], 1% penicillin-streptomycin [Gibco], 1% fetal bovine serum).

## 2.11. Pharmacological treatment of primary cultures of gliogenic cortical neural progenitor cells

Primary cultures of gliogenic cortical neural progenitor cells were subjected to pharmacological inhibitor IKK-2 Inhibitor IV (Calbiochem, cat#401481) after 24 hours in culture in glial differentiation medium. Primary cultures were kept untreated, treated with DMSO alone, or treated with different concentrations of IKK-2 Inhibitor IV suspended in DMSO. After 24 hours, half of the culture medium was replaced with fresh culture medium. 3 days after pharmacological inhibition, cells were washed in ice-cold PBS and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris·HCl pH 7.8, 0.5% Triton-X100, 0.1% SDS, Complete protease inhibitor cocktail [Roche Applied Science], 50 mM NaF, 0.4 mM $NaVO_4$, 10 mM Na-pyrophosphate). Samples were analyzed with Western blotting with the antibodies described in Table 1.

## 2.12. Immunofluorescence

Cryosections from staged embryos or fixed primary cultures were rinsed twice in PBS or HEPES-buffered saline (HBS) and then preincubated for 1 h in blocking solution. With primary antibodies raised in mice, the blocking solution was provided by the "mouse-on-mouse" (MOM) kit (Vector Laboratories Inc). With primary antibodies raised in other species, the blocking solution consisted of 5% normal donkey serum (Jackson ImmunoResearch Laboratories), 0.1% Triton X-100, and 0.5 mg/ml bovine serum albumin (Sigma) in PBS or HBS. Samples were then incubated for 2 h at room temperature in blocking solution containing primary antibodies (Table 2). Samples were then extensively rinsed in blocking solution, followed by incubation with the appropriate secondary antibodies for 1 h at room temperature. Secondary antibodies against primary reagents raised in various species were conjugated to Alexa Fluor 555, Alexa Fluor 488, or Alexa Fluor 405 (1/1,000; Invitrogen). Samples were then rinsed twice with blocking solution and several times with PBS, counterstained with Hoechst 33258 (1/5,000; Sigma) for 2 min, rinsed twice with PBS, mounted with Fluoromount-G, and examined by fluorescence microscopy. Images were acquired using either a digital video camera mounted on a Zeiss Axioskop 2 microscope, a Retiga EXi camera (Qimaging) on a Zeiss Axio Imager.M1

microscope, or an AxioCam MRm Rev.3 camera on a fluorescent microscope Zeiss Axio observer z1 equipped with Xenon illumination. Images were digitally assigned to the appropriate red, green, or blue channels using Northern Eclipse image acquisition software (Empix) or ZEN imaging software (Zeiss).

**Table 2.** Antibodies for immunofluorescence

| Antibody | Company | Catalog # | Host species | Dilution |
|---|---|---|---|---|
| **Acsbg1** | Abcam | ab65154 | Rabbit | 1:500 |
| **Active Caspase-3** | BD Pharmingen | 557035 | Rabbit | 1:200 |
| **ALDH1L1** | Abcam | ab87117 | Rabbit | 1:500 |
| **βIII-tubulin** | Promega | G7121 | Mouse | 1:1,000 |
| **β-Gal** | Cappel | 559761 | Rabbit | 1:2,000 |
| **β-Gal** | Biogenesis | 4600-1409 | Goat | 1:1,000 |
| **β-Gal** | Developmental Studies Hybridoma Bank | JIE7 | Mouse | 1:50 |
| **β-Gal** | Abcam | ab9361 | Chicken | 1:2,000 |
| **BLBP** | Abcam | ab32423 | Rabbit | 1:1,000 |
| **CNPase** | Millipore | MAB326R | Mouse | 1:250 |
| **FoxG1** | Abcam | ab18259 | Rabbit | 1:500 |
| **GFAP** | Sigma | G3893 | Mouse | 1:1,000 |
| **GFP** | Invitrogen | A6455 | Rabbit | 1:1,000 |
| **GFP** | Abcam | ab6673 | Goat | 1:1,000 |
| **Hes1** | Gift from Dr. Ryoichiro Kageyama (Kyoto University) | | Guinea pig | 1:500 |
| **Hes6** | Abcam | ab66461 | Rabbit | 1:1,000 |
| **Iba1** | Wako | 019-19741 | Rabbit | 1:1,000 |
| **Ki-67** | BD Pharmingen | 556003 | Mouse | 1:200 |
| **MAP2** | Sigma | M 1406 | Mouse | 1:500 |
| **Nestin** | Developmental Studies Hybridoma Bank | Rat401 | Mouse | 1:10 |
| **NeuN** | Millipore | MAB377 | Mouse | 1:400 |
| **NFIA** | Sigma | HPA008884 | Rabbit | 1:500 |
| **Olig2 (DF308)** | Gift from Dr. Charles D. Stiles (Harvard University) | | Rabbit | 1:5,000 |
| **Pax6** | Covance | PRB-278P | Rabbit | 1:500 |
| **PDGFRα** | BD Pharmingen | 558774 | Rat | 1:500 |
| **Phospho-Histone H3 (Ser10)** | Millipore | 06-570 | Rabbit | 1:500 |
| **RC2** | Developmental Studies Hybridoma Bank | RC2 | Mouse | 1:15 |
| **Sox2** | R&D Systems | AF2018 | Goat | 1:500 |
| **Tbr2** | Abcam | Ab23345 | Rabbit | 1:500 |

## 2.13. Immunoprecipitation

Dorsal telencephalon from E13.5 or E14.5 CD1 mouse embryos were dissected and quickly rinsed in ice-cold HBSS and then incubated in 10 packed-tissue volumes of hypotonic buffer (20 mM Tris-HCl [pH 7.8], 10 mM KCl, 1.5 mM $MgCl_2$) supplemented with 20 mM iodoacetamide, 10 μM MG132 (Boston Biochem), Complete protease inhibitor cocktail (Roche Applied Science), and 1mM phenylmethylsulfonyl fluoride (PMSF) (Husain *et al.,* 1996). Tissue was mechanically triturated and incubated for 15 min on ice, followed by the addition of 0.5 packed-tissue volume of hypertonic buffer (20 mM Tris-HCl [pH 7.8], 1 M KCl, 30 mM $MgCl_2$, 20 mM iodoacetamide) supplemented with protease inhibitors. Tissue suspension was thoroughly mixed and centrifuged at 1,500 *g* for 10 min. The resulting supernatant (postnuclear supernatant) and pellet (crude nuclear fraction) were collected. The crude nuclear pellet was rinsed and then resuspended in 2 packed-tissue volumes of lysis buffer (30 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 mM iodoacetamide) supplemented with protease inhibitors, followed by mechanical trituration and a brief sonication. The ensuing suspension was centrifuged at 14,000 *g* for 15 min, and the supernatant was recovered (nuclear extract). This fraction was subjected to immunoprecipitation of endogenous RelA using rabbit anti-RelA or control rabbit antibodies using protein A/G-Plus agarose beads (Santa Cruz Biotechnology). Collected and rinsed immunoprecipitates were resuspended in 60 mM Tris-HCl (pH 6.8), 10% glycerol, and 2% SDS, followed by incubation at room temperature for 10 min and recovery of the eluted material by quick centrifugation. Eluates, together with 1/10 of input lysate, were incubated in the presence of 5% β-mercaptoethanol, followed by SDS polyacrylamide gel electrophoresis on 12% gels, transfer to nitrocellulose, and Western blotting with anti-Hes6 or anti-RelA antibodies (Table 2).

## 2.14. Statistical analysis

All quantitative data are expressed as means ± standard errors of the means (SEM). Statistical analyses were done with Student's *t* test or one-way analysis of variance (ANOVA), as indicated. A $p$ value of $<0.05$ was considered statistically significant.

## 3. Results

### 3.1. Characterization of the spatiotemporal activation of the NF-κB pathway in the developing mouse forebrain

*3.1.1 Activation of the NF-κB pathway in neocortical neural progenitor cells during cortical neurogenesis*

In order to determine whether the NF-κB pathway was activated in NPCs during cortical neurogenesis, we first characterized the neocortical GZ cells of NF-κB$^{LacZ}$ embryos displaying activated NF-κB (β-Gal$^{+}$ cells) by double-labelling immunohistochemical analysis of E13.5 NF-κB$^{LacZ}$ forebrain. These studies showed that β-Gal$^{+}$ cells in the neocortical VZ expressed the specific cortical NPC marker protein, Pax6 (Figure 9A). β-Gal$^{+}$ cells in the neocortical VZ also expressed the NPC markers FoxG1, Hes1, and nestin (Figures 9B-D). Moreover, primary cultures of cortical NPCs established from NF-κB$^{LacZ}$ embryos exhibited overlapping β-Gal and nestin immunoreactivity (not shown; but see Methot *et al*., 2013). In agreement with these results, β-Gal$^{+}$ cells located in the neocortical VZ did not express the early neuronal marker protein, βIII-tubulin (not shown; but see Methot *et al*., 2013). An overlap of β-Gal and βIII-tubulin expression was only observed in cells located in the neocortical mantle zone at this and later stages of corticogenesis (not shown; but see Methot *et al*., 2013). Together, these results show that the NF-κB pathway is activated in NPCs during cortical neurogenesis.

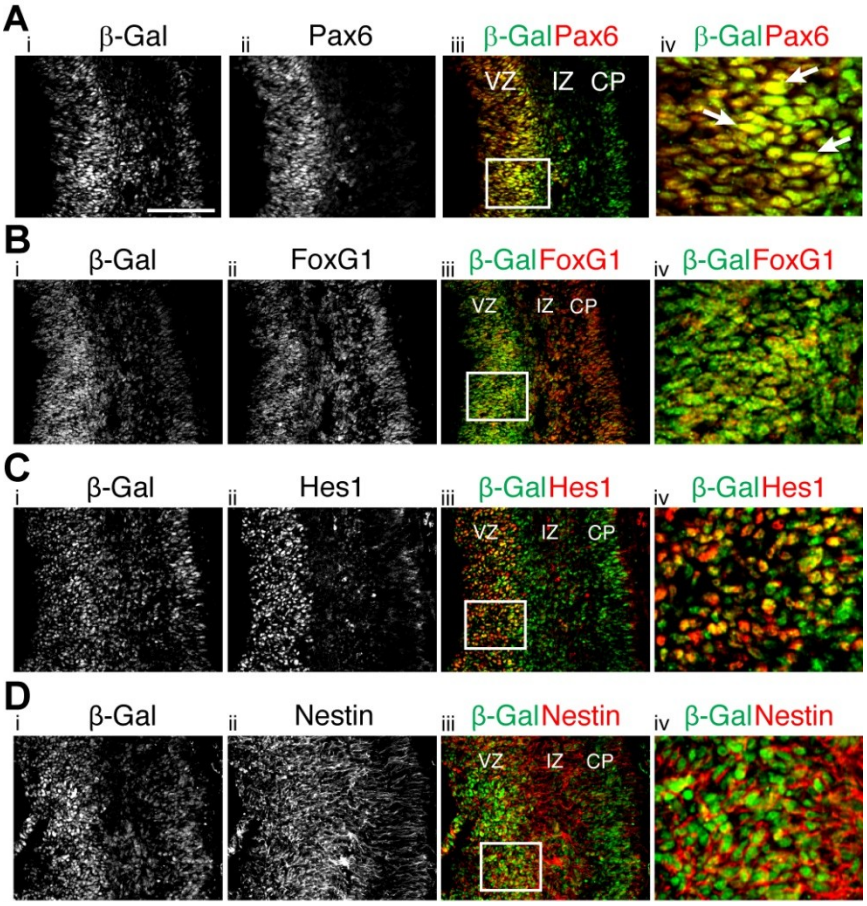


**Figure 9.** Activation of the NF-κB pathway in neocortical NPCs during cortical neurogenesis. Double-label immunofluorescence analysis of β-Gal and Pax6 (A), FoxG1 (B), Hes1 (C), or Nestin (D) expression in the pallium of E13.5 NF-κB$^{LacZ}$ embryos showing β-Gal expression in neural progenitor cells in the neocortical VZ. The iv panels represent high-magnification views of the boxed areas in the iii panels. Arrows point to examples of double-labeled cells. Dorsal is at the top and lateral is to the right in all panels. CP, cortical plate; IZ, intermediate zone. Scale bar, 100μm.

*3.1.2. Activation of the NF-κB pathway in cortical and striatal neural progenitor cells during cortical gliogenesis*

Based on the robust activation of the NF-κB pathway in neocortical GZs at E17.5, roughly corresponding to the onset of cortical gliogenesis, we next examined whether the NF-κB pathway remains activated in the GZ of the cerebral cortex during cortical gliogenesis. To this end, we analyzed β-Gal expression in late embryonic (E18.5) and postnatal (P1 to P15) forebrain of NF-κB$^{LacZ}$ mice. Throughout cortical gliogenesis, robust expression of β-Gal was observed in the dorsolateral SVZ (SVZdl) but not in the septal SVZ (SVZspt), similar to the selective activation in the dorsolateral, but not medial, pallium during the neurogenic phase (Figure 10). Robust activation was also observed in the striatal SVZ (SVZstr), another anatomically-defined GZ lining the lateral ventricles (Brazel *et al.*, 2003). Interestingly, a small group of cells with undetectable β-Gal expression was consistently observed in a region that appeared to correspond to the boundary between the SVZdl and SVZstr ('cortico-striatal boundary') (arrowheads in Figure 10). β-Gal expression was also robust in post-mitotic neurons of the cortex, striatum and septum, in accordance with previously described functions of NF-κB in the regulation of a number of biological processes in forebrain neurons.

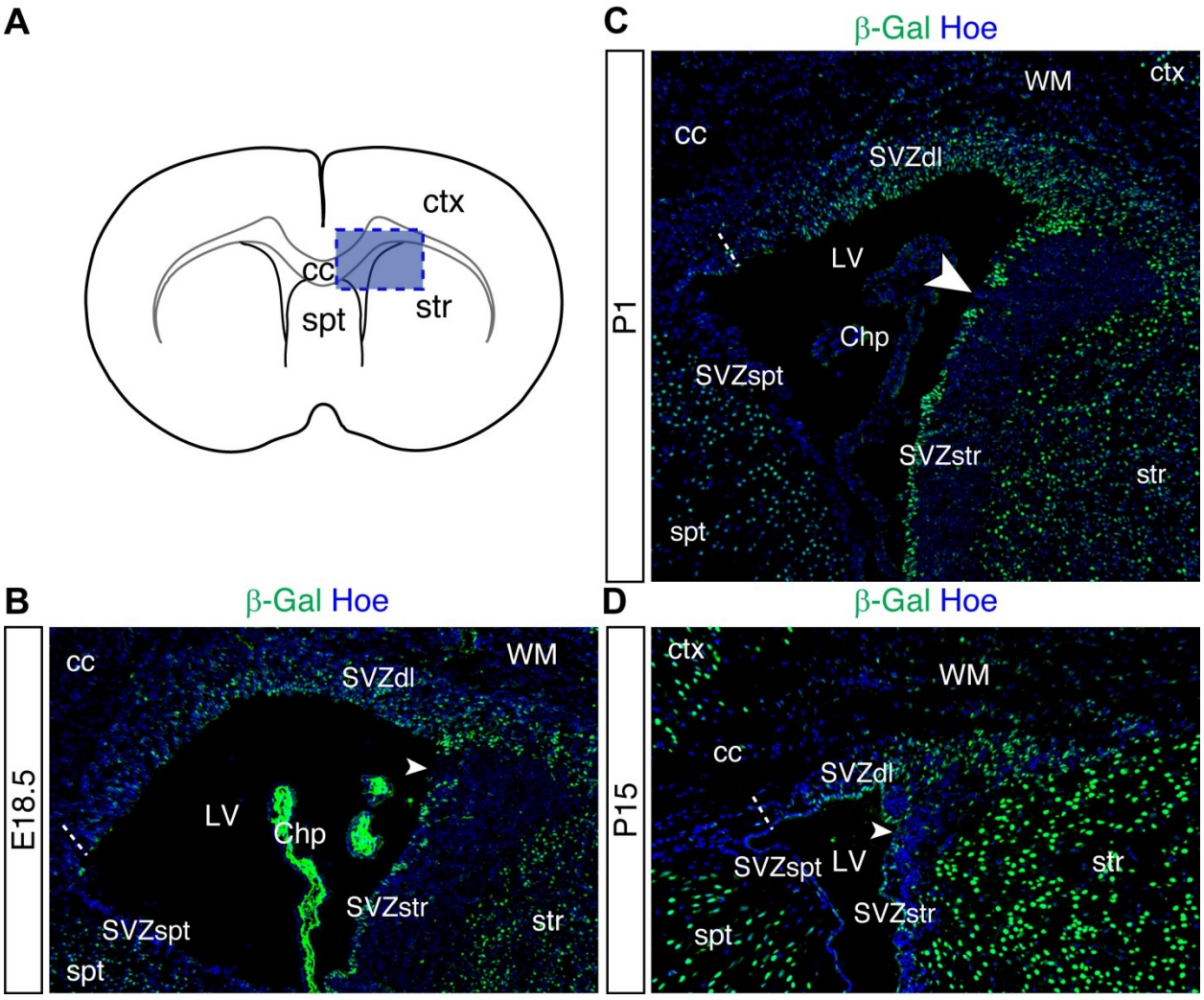


**Figure 10.** Spatiotemporal activation of the NF-κB pathway in the embryonic and postnatal mouse forebrain during cortical gliogenesis. (A) Schematic illustration of a coronal section through the rostral postnatal mice forebrain, showing in the blue boxed area the region surrounding the lateral ventricle (LV) analyzed in panels (B-D). (B-D) Immunofluorescence analysis of β-Gal expression in the telencephalon of E18.5 (B), P1 (C), or P15 (D) NF-κB$^{LacZ}$ mice showing β-Gal expression in the dorsolateral subventricular zone (SVZdl) and striatal subventricular zone (SVZstr), and lack of detectable expression in the septal subventricular zone (SVZspt). The dashed line demarcates the anatomical boundary between the SVZdl and SVZspt. The arrowheads point to a region with no detectable β-Gal expression between the SVZdl and the SVZstr. Dorsal is at the top and lateral is to the right in all panels. cc, corpus callosum; Chp, choroid plexus; ctx, cortex; spt, septum; str, striatum; WM, white matter.

To characterize the identity of the cells exhibiting activated NF-κB pathway in the SVZdl and SVZstr, double-labelling immnohistochemical analysis of NF-κB$^{LacZ}$ embryonic and postnatal forebrains was performed. At all stages examined (E18.5, P1 and P10), the vast majority of β-Gal$^{+}$ cells in the SVZdl and SVZstr expressed the NPC marker protein, Sox2 (Figure 11A), suggesting that they were NPCs. In agreement with this possibility, SVZdl β-Gal$^{+}$ cells also expressed the neocortical NPC marker protein, Pax6 (Figure 11B), as well as the general NPC marker, nestin (Figure 11C). Additionally, in the early postnatal brain, a subpopulation of β-Gal$^{+}$ cells in the outer region of the SVZdl expressed the intermediate progenitor marker Tbr2 (Figure 11D). Tbr2$^{+}$ progenitor cells are thought to still be neurogenic at this stage as they generate no glial cells, and they deplete themselves during the first few days of post-natal life (Kowalczyk *et al.,* 2009), suggesting a role for NF-κB in late cortical neurogenesis. NF-κB signaling can be activated in microglia, the resident immune cells of the nervous system, usually under pathophysiological conditions (Kaltschmidt *et al.,* 1994; Frakes *et al.,* 2014). We also examined whether at least some of the β-Gal$^{+}$ cells in the postnatal SVZdl could correspond to microglial cells, which are abundant in the early postnatal SVZ and IZ (Zusso *et al.,* 2012). Virtually no cell expressing the microglia/macrophage marker Iba1 displayed detectable expression of β-Gal in NF-κB$^{LacZ}$ mice (Figure 11E), suggesting that activation of the NF-κB pathway does not occur, or is below detection levels, in postnatal forebrain amoeboid microglia. Taken together, these results provide evidence that the NF-κB pathway is selectively activated in NPCs in the SVZdl and SVZstr during cortical gliogenesis.

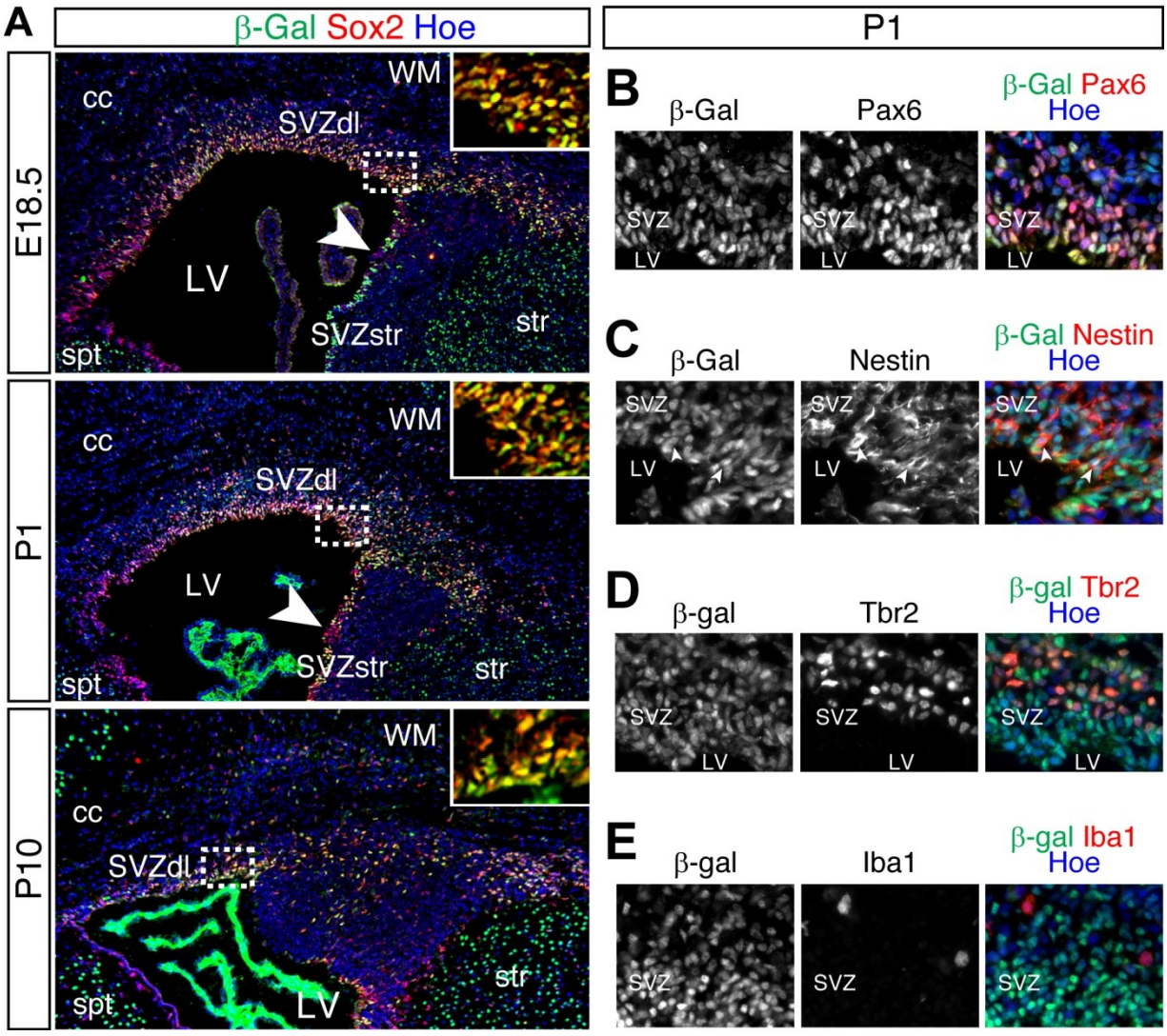


**Figure 11.** Activation of the NF-κB pathway in cortical NPCs of the SVZdl and SVZstr during cortical gliogenesis. (A) Double-label immunofluorescence analysis of β-Gal and Sox2 expression in the telencephalon of E18.5 (top panel), P1 (middle panel), or P10 (bottom panel) NF-κB$^{LacZ}$ mice showing β-Gal expression in neural progenitor cells of the dorsolateral subventricular zone (SVZdl) and striatal subventricular zone (SVZstr). The insets represent high-magnification views of the boxed areas in main panels. Arrowheads point to a region with no detectable β-Gal expression between the SVZdl and the SVZstr. cc, corpus callosum; LV, lateral ventricle, spt, septum; str, striatum; WM, white matter. (B-E) Double-label immunofluorescence analysis of β-Gal and Pax6 (B) Nestin (C), Tbr2 (D), or Iba1 (E) expression in the SVZdl of P1 NF-κB$^{LacZ}$ pups showing β-Gal expression in cortical neural progenitor cells (Pax6$^{+}$, Nestin$^{+}$) and intermediate progenitor cells (Tbr2$^{+}$), and lack of detectable β-Gal expression in microglia (Iba1$^{+}$). Arrowheads in panel C point to examples of double-labeled cells. LV, lateral ventricle; SVZ, subventricular zone.

*3.1.3. Activation of the NF-κB pathway in gliogenic cortical and striatal NPCs during cortical gliogenesis*

To characterize the NPCs that display activation of the NF-κB pathway during cortical gliogenesis, we examined the expression of proteins involved in glial cell differentiation in the postnatal SVZdl of NF-κB$^{LacZ}$ pups. Repression of pro-neurogenic genes and de-repression of pro-gliogenic genes, such as *nuclear factor I A* (*Nfia*) through promoter demethylation, normally occur during the neurogenic-to-gliogenic switch (Piper *et al.,* 2010). In the SVZdl, β-Gal was robustly expressed, together with NFIA, in Sox2$^{+}$ cortical NPCs (Figure 12A). NFIA is involved in promoter demethylation of the astrocyte-specific gene glial fibrillary acidic protein (*Gfap*), thereby allowing it to become responsive to JAK-STAT signaling (Namihira *et al.,* 2009). While NFIA expression in NPCs is upregulated during mid-neurogenesis, the expression of GFAP by cortical NPCs is low in late embryogenesis and becomes robust in the SVZdl during the first days of postnatal life. Many β-Gal$^{+}$ cells in the postnatal SVZdl expressed GFAP at P1 (Figure 12B), suggesting that these cells are responsive to pro-gliogenic signals and are gliogenic in potential. Because GFAP expression in the postnatal SVZdl marks not only gliogenic cortical NPCs but also newly-born astrocytes, we examined additional NPC markers. Double-label immohistochemical analysis showed that most Sox2$^{+}$ cells in the SVZdl expressed GFAP (Figure 12C). In addition, SVZdl β-Gal$^{+}$ cells expressed the transcription factor FoxG1 (Figure 12D). FoxG1, which is expressed in the SVZ at the time of gliogenesis (Miyoshi *et al.,* 2012), promotes NPC self-renewal and inhibits astrocyte differentiation (Brancaccio *et al.,* 2010). Together, these results provide evidence that the NF-κB pathway is activated in cortical NPCs with gliogenic potential during cortical gliogenesis.

Similar results were obtained when the postnatal SVZstr of NF-κB$^{LacZ}$ pups was analyzed. Specifically, β-Gal was robustly expressed together with NFIA in Sox2$^{+}$ cells in this anatomical region (Figure 12E). In the postnatal SVZstr of newborn mice, striatal NPCs express the radial glial cell marker RC2 and display low or no GFAP immunoreactivity. GFAP expression appears in striatal NPCs and astrocytes in the SVZstr only by the end of the first postnatal week and correlates with a parallel loss of RC2 expression (Tramontin *et al.,* 2003; Merkle *et al.,* 2004). In agreement with this, we observed that in the early postnatal brain (P1), SVZstr β-Gal$^{+}$ cells expressed RC2 (Figure 12F), with little or no expression of GFAP (Figure

12G). Robust expression of GFAP by β-Gal$^{+}$ cells was observed at P10 (Figure 12H). Taken together, these observations suggest that the NF-κB pathway is activated in striatal NPCs with gliogenic potential during cortical gliogenesis.

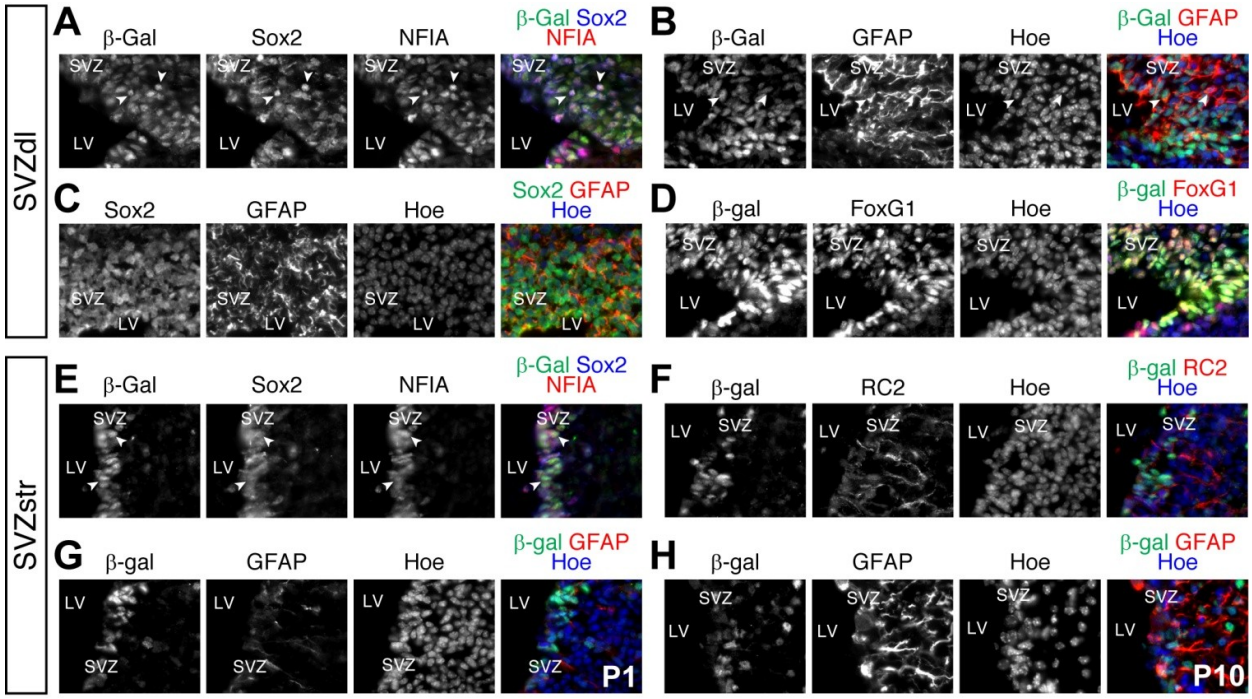


**Figure 12.** Activation of the NF-κB pathway in gliogenic cortical and striatal neural stem/progenitor cells during cortical gliogenesis. (A) Triple-label immunofluorescence analysis of β-Gal, Sox2, and NFIA expression in the SVZdl of P1 NF-κB$^{LacZ}$ pups showing β-Gal expression in gliogenic cortical neural progenitor cells (Sox2$^{+}$, NFIA$^{+}$). Arrowheads point to examples of triple-labeled cells. (B and D) Double-label immunofluorescence analysis of β-Gal and either GFAP (B) or FoxG1 (D) expression in the SVZdl of P1 NF-κB$^{LacZ}$ pups showing β-Gal expression in gliogenic neural progenitor cells. Arrowheads in panel B point to examples of double-labeled cells. (C) Double-label immunofluorescence analysis of GFAP and Sox2 expression in the SVZdl of P1 NF-κB$^{LacZ}$ pups showing that the vast majority of Sox2$^{+}$ cells express GFAP. (E) Triple-label immunofluorescence analysis of β-Gal, Sox2, and NFIA expression in the SVZstr of P1 NF-κB$^{LacZ}$ pups showing β-Gal expression in gliogenic striatal neural progenitor cells (Sox2$^{+}$, NFIA$^{+}$). Arrowheads point to examples of triple-labeled cells. (F-H) Double-label immunofluorescence analysis of β-Gal and RC2 (F) or GFAP (G and H) expression in the SVZstr of P1 (E-G) or P10 (H) NF-κB$^{LacZ}$ pups. LV, lateral ventricle; SVZ, subventricular zone.

### 3.2. Regulation of cortical neurogenesis by the NF-κB pathway

*3.2.1. Activation of the NF-κB pathway in neocortical neural progenitor cells is required to maintain the undifferentiated neocortical progenitor state and prevent premature neuronal differentiation*

To characterize the biological functions of the NF-κB pathway in neocortical NPCs during cortical neurogenesis, we first examined the phenotype resulting from suppression of endogenous NF-κB in neocortical NPCs *in vivo* using two previously characterized dominant-negative inhibitors of NF-κB activation (Figure 13). One dominant-negative inhibitor corresponded to a mutated form of IκBα, termed IκBαM, which contains mutations on phosphorylation sites Ser32 and Ser36 preventing phosphorylation by the IKK complex, as well as mutations in the PEST sequence rendering the protein more resistant to proteosome-dependent degradation (Van Antwerp *et al.,* 1996). As a result, IκBαM interferes with pathways that lead to NF-κB through phosphorylation and proteosomal degradation of IκBα, causing cytosolic retention of NF-κB dimers such as p50:RelA. The second reagents utilized corresponded to a catalytically inactive form of IKKβ that exerts a dominant-negative effect on the IKK complex (termed IKKβ-DN) (Manning *et al.,* 1997). Both IκBαM and IKKβ-DN were shown to block principally the canonical NF-κB pathway, although IκBαM is expected to also be competent to interfere with atypical pathways converging on IκBα (Van Antwerp *et al.,* 1996, Li *et al.,* 1999).

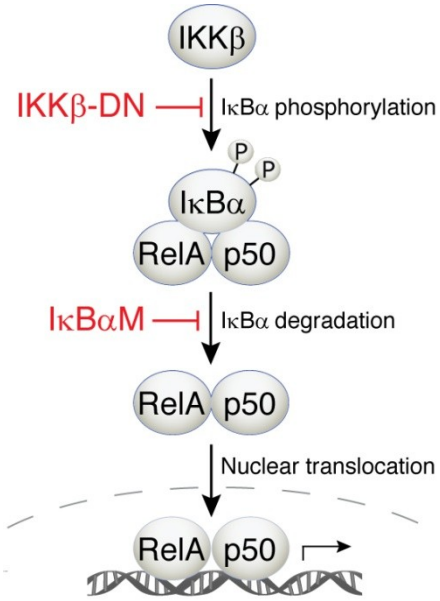


**Figure 13**. Dominant-negative inhibition of canonical NF-κB pathway. IKKβ-DN is a catalytically inactive form of IKKβ that exerts a dominant-negative effect on the IKK complex. IκBαM is a mutated form of IκBα which contains mutations preventing phosphorylation by the IKK complex, as well as mutations rendering the protein more resistant to proteosome-dependent degradation. IκBαM sequesters NF-κB dimers in the cytoplasm and prevents DNA-binding.

In a first set of experiments conducted together with a student in Dr. Ruth Slack's laboratory at University of Ottawa, Devon Svoboda, either a bicistronic DNA plasmid driving the coexpression of IκBαM and EGFP (IκBαM-IRES-EGFP) or a control DNA plasmid expressing EGFP alone (IRES-EGFP), both under the control of the chicken β-actin promoter, was injected *in utero* into the lateral ventricle of E13.5 mouse embryos, followed by electroporation into the neocortical VZ, which containing mostly undifferentiated NPCs. The phenotype resulting from IκBαM-mediated blockade of the NF-κB pathway was examined 48 hours post-electroporation (E15.5). During normal neocortical neurogenesis, NPCs can either maintain an undifferentiated state and remain in the VZ or undergo neuronal differentiation giving rise to cells that move radially towards the pial surface. Compared to expression of EGFP alone, forced expression of IκBαM resulted in a significant decrease in the number of electroporated cells ($EGFP^+$) expressing the NPC marker proteins, nestin and Sox2 (Figures 14A, B, and D). In parallel, IκBαM expression caused a significant increase in the number of electroporated cells expressing the early neuronal marker, βIII-tubulin (Figure 14C and D). A

significant number of electroporated cells expressing IκBαM already expressed βIII-tubulin while still located in the neocortical VZ, where βIII-tubulin is normally expressed at low or no detectable levels (Figure 14C).

Similar results were observed in a second set of experiments in which forced expression of IKKβ-DN, a dominant-negative form of IKKβ harbouring a K44M mutation in the kinase domain (Mercurio *et al.,* 1997), was used to block endogenous NF-κB in the developing neocortex. We first showed that expression of IKKβ-DN via adenoviral transduction caused decreased β-Gal activity in primary cultures of cortical NPCs established from NF-κB$^{LacZ}$ embryos (Figures 14E and F), in agreement with previous studies showing that exogenous IKKβ-DN expression efficiently inhibits NF-κB (Russo *et al.,* 2009). Using *in utero* electroporation approaches, a bicistronic DNA plasmid driving the coexpression of IKKβ-DN and EGFP (IKKβ-DN-IRES-EGFP) or control IRES-EGFP plasmid was transfected *in vivo* into the developing neocortex. Blockade of NF-κB by expression of IKKβ-DN resulted in a significant reduction in the number of electroporated cells expressing the undifferentiated NPC marker proteins, nestin and Sox2. Concomitantly, the number of electroporated cells expressing βIII-tubulin was significantly increased (Figure 14G). Together, these results show that inhibition of endogenous NF-κB pathway leads to precocious cortical neurogenesis, suggesting that the NF-κB pathway is required to maintain the undifferentiated neocortical NPC fate and prevent premature neuronal differentiation during cortical neurogenesis.

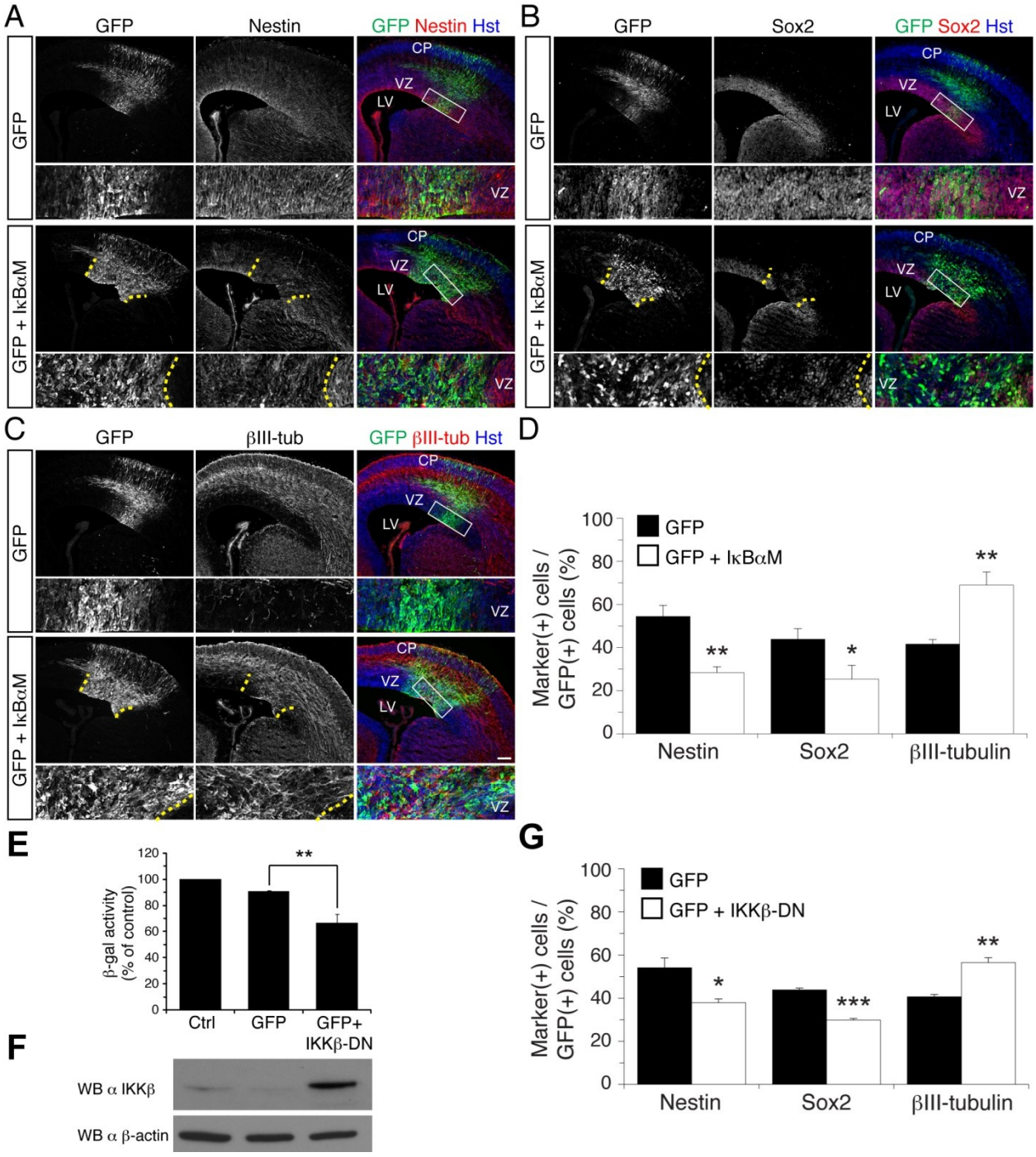


**Figure 14.** Precocious cortical neurogenesis by inhibition of endogenous NF-κB pathway in neocortical NPCs *in vivo*. (A to C) Double-label immunofluorescence analysis of GFP and either nestin (A), Sox2 (B), or βIII-tubulin (βIII-tub) (C) expression 48 h after *in utero* electroporation of E13.5 mouse embryos with either a bicistronic plasmid expressing both IκBαM and EGFP or the empty vector expressing EGFP alone. Bottom rows, high-magnification views of the areas indicated by rectangles in the right-hand panels. Where shown (when IκBαM was used), dotted lines roughly demarcate the $GFP^+$ electroporated area in the VZ. Hst, Hoechst. Scale bar, 100

μm. (D) Quantification of the fraction of $GFP^+$ cells coexpressing nestin, Sox2, or βIII-tubulin. Results are shown as the means ± standard errors of the means (SEM) (*, $p < 0.05$; **, $p < 0.01$; n = 5 electroporated embryos per condition; *t* test). (E and F) IKKβ-DN-mediated inhibition of NF-κB signaling in cortical progenitor cells. (E) Quantification of β-gal activity in primary cultures of cortical progenitor cells from E13.5 NF-κBLacZ embryos. Cells were either not infected ("Ctrl"; considered as 100%) or infected with adenovirus expressing EGFP alone or EGFP+IKKβ-DN, as indicated. Determination of β-gal activity was performed 48 h after transduction (mean ± SEM; **, $p < 0.01$, n = 3 separate experiments performed in duplicates, *t* test). (F) Expression of IKKβ-DN in the transduced cells was detected by Western blotting with an anti-IKKβ antibody. (G) Quantification of the fraction of $GFP^+$ cells coexpressing nestin, Sox2, or βIII-tubulin 48 h after in utero electroporation of E13.5 embryos with either a bicistronic plasmid expressing both IKKβ-DN and GFP or the empty vector expressing GFP alone. Results are shown as the means ± SEM (***, $p < 0.001$; n = 3 electroporated embryos per condition; *t* test).

To examine this possibility further, we next conducted *ex vivo* studies in which specific DNA plasmids were focally injected into the neocortical VZ, followed by electroporation, using organotypic forebrain slices prepared from E13.5 mouse embryos. In *ex vivo* organotypic cultures, a plasmid co-electroporation strategy was used instead of a bicistronic plasmid electroporation strategy to allow for the concomitant and robust expression of multiple exogenous protein combinations in order to investigate molecular mechanisms underlying the roles of the NF-κB pathway in the regulation of neocortical NPC biology. In one set of studies mimicking the *in vivo* experiments described above, an EGFP-expressing plasmid was electroporated alone (as control) or together with an IκBαM-expressing plasmid (in a 1:4 ratio). This plasmid ratio ensures that most, if not all, $EGFP^+$ cells coexpress IκBαM when the two plasmids are co-electroporated (Maira *et al.,* 2010). Conversely, a number of IκBαM-expressing cells are expected not to express EGFP: this latter type of cells (IκBαM+;EGFP-) was not included in our analysis. Exogenous expression of IκBαM resulted in a significant reduction in the number of cells expressing the mitotic marker, Ki-67, which is expressed in NPCs but not post-mitotic neurons (Figures 15A and C), and the NPC marker, nestin (Figure 15D), further indicating that blockade of endogenous NF-κB pathway in neocortical NPCs results in a perturbed ability to maintain an undifferentiated, mitotic state. This effect was paralleled by a significant increase in the number of electroporated cells expressing the neuronal marker proteins, MAP2 (Figures 15B and C), NeuN (Figure 15E), and βIII-tubulin (Figure 15F). We did

not observe any detectable changes in the number of $EGFP^+$ cells expressing the apoptotic marker protein, active caspase-3, in CP cells expressing EGFP alone or together with IκBαM (EGFP, 4.7% ± 2.8%; EGFP+IκBαM, 9.6% ± 7.1%; *n*=5 electroporated slices per condition from each of 5 separate electroporations). In addition, no changes in the expression of the astrocytic marker protein, GFAP, were detected following ectopic expression of IκBαM, suggesting no difference in astrocytic differentiation (Figure 15G). A similar phenotype was observed when endogenous NF-κB pathway was blocked by exogenous expression of IKKβ-DN. This approach also caused a significant reduction in the number of $EGFP^+$ cells expressing Ki-67 (Figures 16A and C), together with a significant increase in the number of $EGFP^+$ cells expressing the neuronal marker protein, MAP2 (Figures 16B and C).

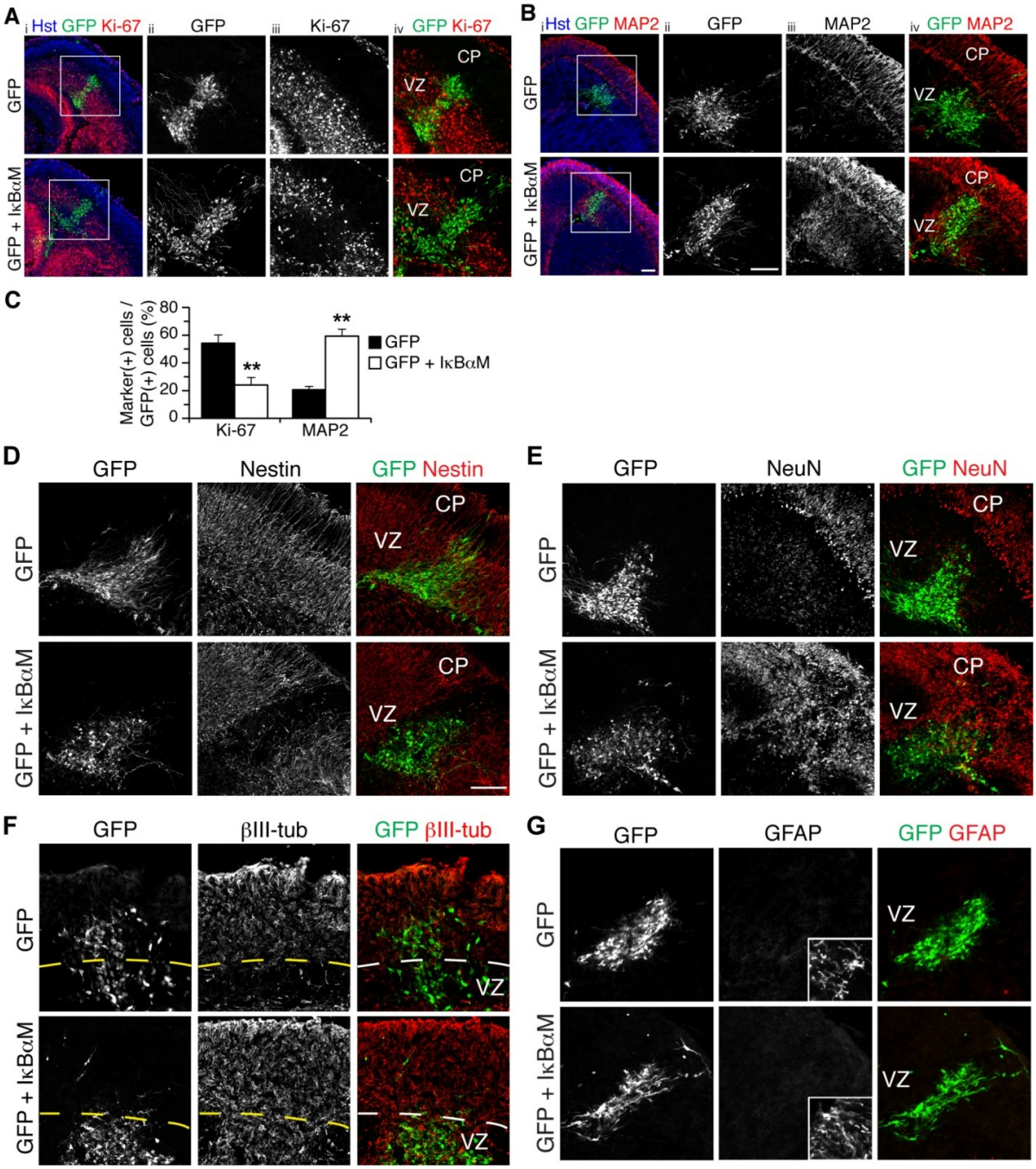


**Figure 15.** Precocious cortical neurogenesis by inhibition of endogenous NF-κB pathway in neocortical NPCs *ex vivo* (Part 1: IκBαM dominant-negative inhibition). (A,B, and D-G) Double-label immunofluorescence analysis of GFP and either Ki-67 (A), MAP2 (B), Nestin (D), NeuN (E), βIII-tubulin (F), or GFAP (G) expression in organotypic slice cultures from E13.5 mouse forebrain, 48 h after electroporation of plasmids encoding the indicated proteins. (A and B) Columns ii to iv, high-magnifications from boxed areas in column i. Scale bars, 100 μm. (C) Quantification of the fraction of GFP+ cells coexpressing either Ki-67 or MAP2 (means ± SEM; **, $p < 0.01$; $n = 16$ electroporated slices per condition; *t* test).

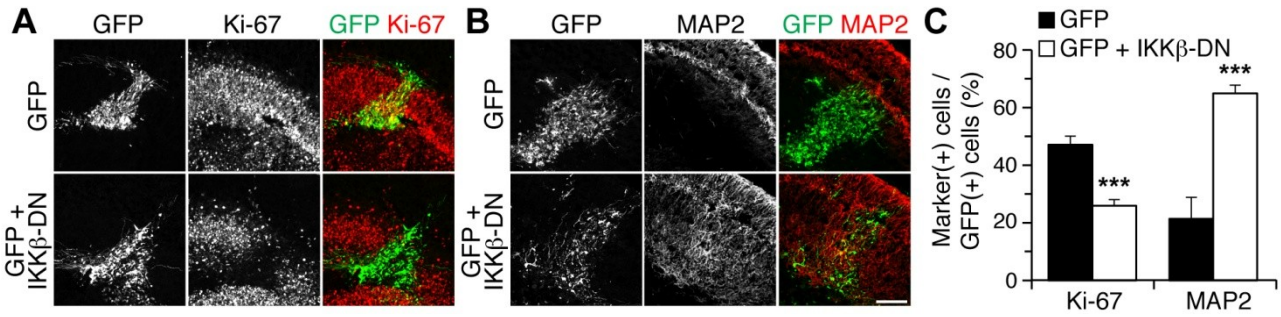


**Figure 16.** Precocious cortical neurogenesis by inhibition of endogenous NF-κB pathway in neocortical NPCs *ex vivo* (Part 2: IKKβ-DN dominant-negative inhibition). (A and B) Double-label immunofluorescence analysis of GFP and either Ki-67 (A) or MAP2 (B) expression in organotypic slice cultures from E13.5 mouse forebrain, 48 h after electroporation of plasmids encoding the indicated proteins. Scale bar, 100 μm. (C) Quantification of the fraction of $GFP^+$ cells coexpressing Ki-67 or MAP2 (means ± SEM; ***, $p < 0.001$; $n = 21$ electroporated slices per condition; *t* test).

To complement the *in vivo* and *ex vivo* studies using dominant-negative inhibitors of NF-κB, experiments were next performed in which the NF-κB pathway was blocked by targeting the NF-κB subunits RelA and p50. In one approach, RelA, which contains a transactivation domain, was knocked down by RNA interference through *ex vivo* electroporation of EGFP together with a *RelA* siRNA, or a scrambled siRNA, in neocortical VZ cells of organotypic slices established from E13.5 mouse embryos. RelA knock down resulted in a significant reduction in the number of $EGFP^+$ cells expressing the mitotic marker, Ki-67 (Figures 17A and C), as well as a significant increase in the number of $EGFP^+$ cells expressing the neuronal marker, MAP2 (Figures 16B and C), consistent with the results described above. In a second approach targeting NF-κB subunits, primary cultures of cortical NPCs established from E13.5 embryos were treated with the SN50 peptide, which robustly inhibits nuclear translocation of p50:RelA dimers (Lin *et al.,* 1995), or the SN50M control, a mutated form of SN50 that does not interfere with p50:RelA nuclear translocation. As was the case with the other conditions interfering with NF-κB activity, treatment of primary cultures of cortical NPCs with SN50 led to a significant reduction in the number of cells expressing NPC markers, like nestin and Ki-67, and a significant increase in the number of cells expressing neuronal markers, such as βIII-tubulin and MAP2 (Figure 18).

Together, these combined results demonstrate that NF-κB is required to prevent premature neuronal differentiation of neocortical NPCs during cortical neurogenesis.

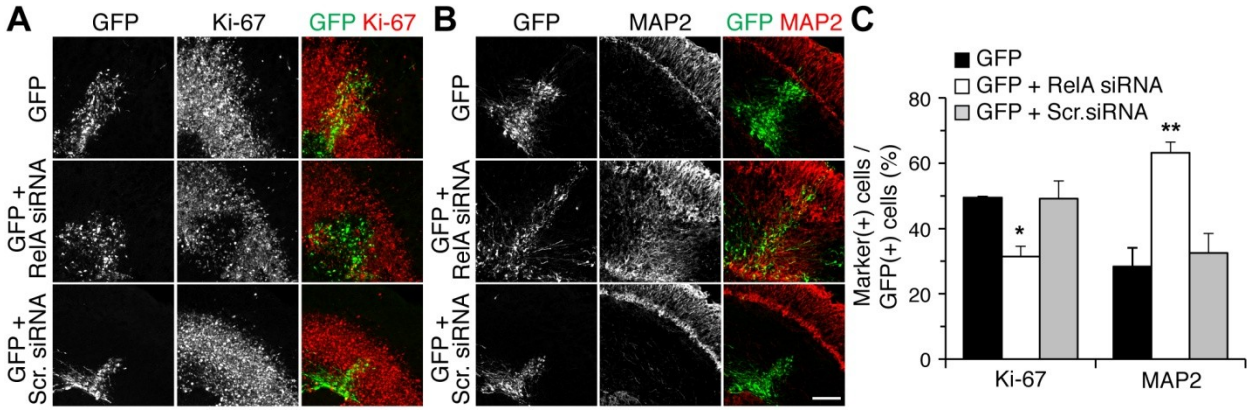


**Figure 17.** Precocious cortical neurogenesis by inhibition of endogenous NF-κB pathway in neocortical NPCs *ex vivo* (Part 3: RelA knockdown). (A and B) Double-label immunofluorescence analysis of GFP and either Ki-67 (A) or MAP2 (B) expression in organotypic slice cultures from E13.5 mouse forebrain, 48 h after electroporation of the indicated siRNA reagents together with GFP. Scr., control scrambled. Scale bar, 100 μm. (C) Quantification of the fraction of $GFP^+$ cells coexpressing Ki-67 or MAP2 (means ± SEM; *, $p < 0.05$; $n = 23$ electroporated slices per condition; one-way analysis of variance [ANOVA]).

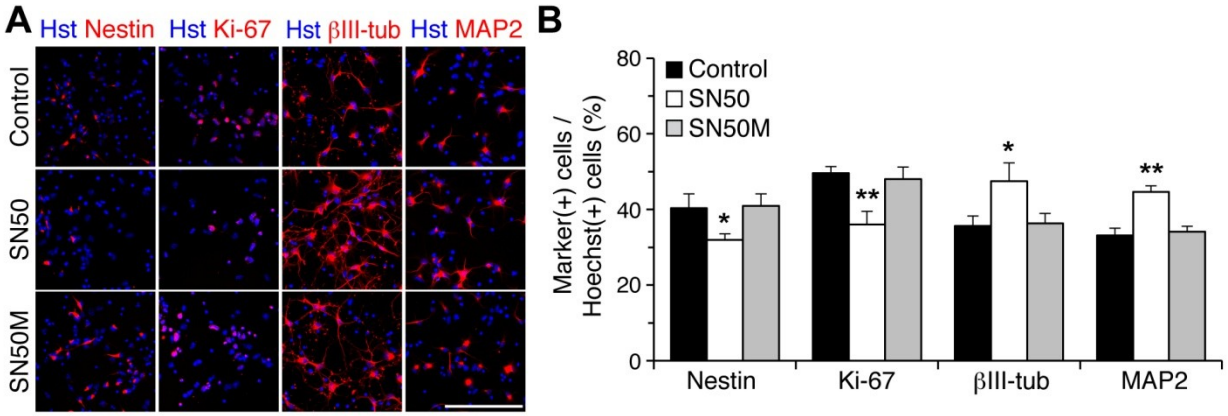


**Figure 18.** The NF-κB pathway is required to prevent premature neuronal differentiation of neocortical neural progenitor cells during cortical neurogenesis *in vitro*. (A) Double-label immunofluorescence analysis of cortical progenitor cells incubated in the absence (control) or presence of either SN50 or SN50M peptides, as indicated. Nestin and Ki-67 were used as progenitor cell markers, while βIII-tubulin (βIII-tub) and MAP2 were used as neuronal markers. Scale bar, 100 μm. (B) Quantification of the percentage of Hoechst-positive cells expressing either Ki-67, nestin, βIII-tubulin, or MAP2 under the three experimental conditions (means ± SEM; 500 cells were counted in each case; *, $p < 0.05$; **, $p < 0.01$; $n = 4$ separate experiments; one-way ANOVA).

*3.2.2. Activation of the NF-κB pathway in neocortical progenitor cells is sufficient to maintain the undifferentiated progenitor state and prevent premature neuronal differentiation during cortical neurogenesis*

To determine whether the activation of the NF-κB pathway is sufficient to inhibit/delay neuronal differentiation in the developing neocortex, we next conducted gain-of-function studies in which the NF-κB subunit RelA was overexpressed in neocortical NPCs. This approach has been previously shown to cause NF-κB pathway activation in other cells types (Bhakar *et al.,* 2002; Schmitz *et al.,* 1991). In agreement with those studies, we showed that adenoviral vector-mediated expression of exogenous RelA in primary cultures of cortical NPCs derived from NF-$\kappa B^{LacZ}$ embryos resulted in increased β-Gal activity, indicating that RelA overexpression is sufficient to cause NF-κB pathway activation in these cells (Figure 19). Based on these findings, EGFP alone or together with RelA was electroporated in the neocortical VZ of organotypic forebrain slices *ex vivo*, and the resulting phenotype analyzed 4 days post-electroporation. The location of $EGFP^+$ cells was assessed first, on the premise that $EGFP^+$ cells that have maintained

an undifferentiated NPC fate are expected to remain in the GZ, while $EGFP^+$ cells that have undergone neuronal differentiation are expected to migrate towards the CP. To quantitate the radial migration of $EGFP^+$ cells, the cerebral cortex was operationally divided into 5 radial sectors emanating from the VZ (sector 1) toward the CP (sector 5 being the outermost sector) (Figure 20A), and the number of $EGFP^+$ cells was quantified in each sector. RelA electroporation resulted in a significant increase in the number of $EGFP^+$ cells in sectors 1 and 2 compared to EGFP alone, with a parallel significant reduction in the number of $EGFP^+$ cells in sector 5 (Figure 20B). Expression of RelA caused neither a detectable alteration of the thickness of the neocortex (EGFP, 484.1 ± 30.5 μm; EGFP+RelA, 471.9 ± 10.2 μm; $n$=20 slices from 4 separate electroporation experiments) nor a significant change in the total number of $EGFP^+$ cells present 4 days after electroporation (EGFP, 119.4 ± 27.2; EGFP and RelA, 105.3 ± 9.5; $n$=25 slices from 5 separate electroporation experiments), suggesting that the observed changes were not due to morphological alterations of the tissue or differential survival of the electroporated cells. These results suggest that NF-κB activation caused by exogenous RelA expression inhibits cortical neuronal differentiation, leading to a reduction in the number of differentiating/ed cells located in outer sectors.

To determine whether the accumulation of $EGFP^+$ cells exogenously expressing RelA in the neocortical GZ (sectors 1 and 2) was caused by either an inhibition of the progenitor-to-neuron transition or a defect in radial neuronal migration, we analyzed the expression of NPC and neuronal markers in the electroporated cells. Compared to EGFP alone, forced RelA overexpression resulted in a significant increase in the number of $EGFP^+$ cells expressing Ki-67, Pax6, and Sox2, indicating the presence of supernumerary undifferentiated progenitors (Figures 20C-G). Parallel analysis of the expression of the neuronal marker, MAP2, revealed a reduction in the number of $EGFP^+$ cells expressing this protein in or near the GZ when RelA was exogenously expressed, suggesting that postmitotic neurons are not blocked or delayed in their radial migration towards the neocortical CP (EGFP, 16.4% ± 1.7%; EGFP+RelA, 9.1% ± 0.3%; $p$=0.014; $n$=15 electroporated slices per condition; $t$ test) (Figure 20H). Together, these results show that NF-κB pathway activation in neocortical NPCs is sufficient to maintain the undifferentiated neocortical NPC state and prevent premature neuronal differentiation during cortical neurogenesis.

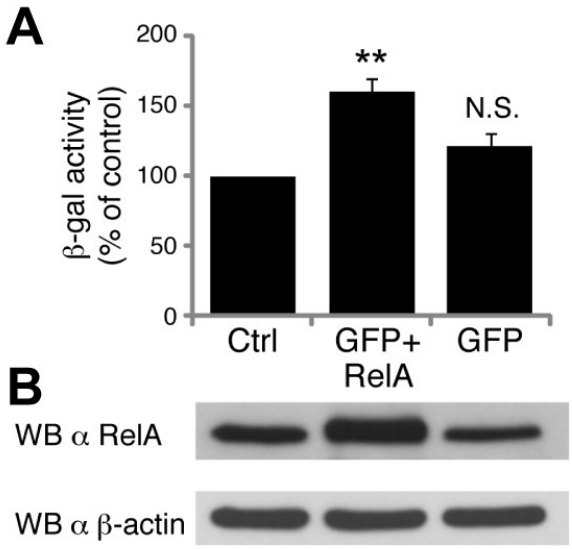


**Figure 19.** Ectopic activation of the NF-κB pathway in cortical NPCs by exogenous RelA expression. RelA-mediated activation of NF-κB signaling in cortical progenitor cells. (A) Quantification of β-gal activity in primary cultures of cortical progenitor cells from E13.5 NF-κBLacZ embryos. Cells were either not infected ("Ctrl"; considered as 100%) or infected with adenovirus expressing GFP+RelA or GFP alone, as indicated. Determination of β-gal activity was performed 24 h after transduction. Data are shown as the mean ± SEM (**, $p < 0.01$, $n = 4$ separate experiments performed in duplicates, *t* test). (B) Increased expression of RelA in the transduced cells was detected by Western blotting with an anti-RelA antibody.

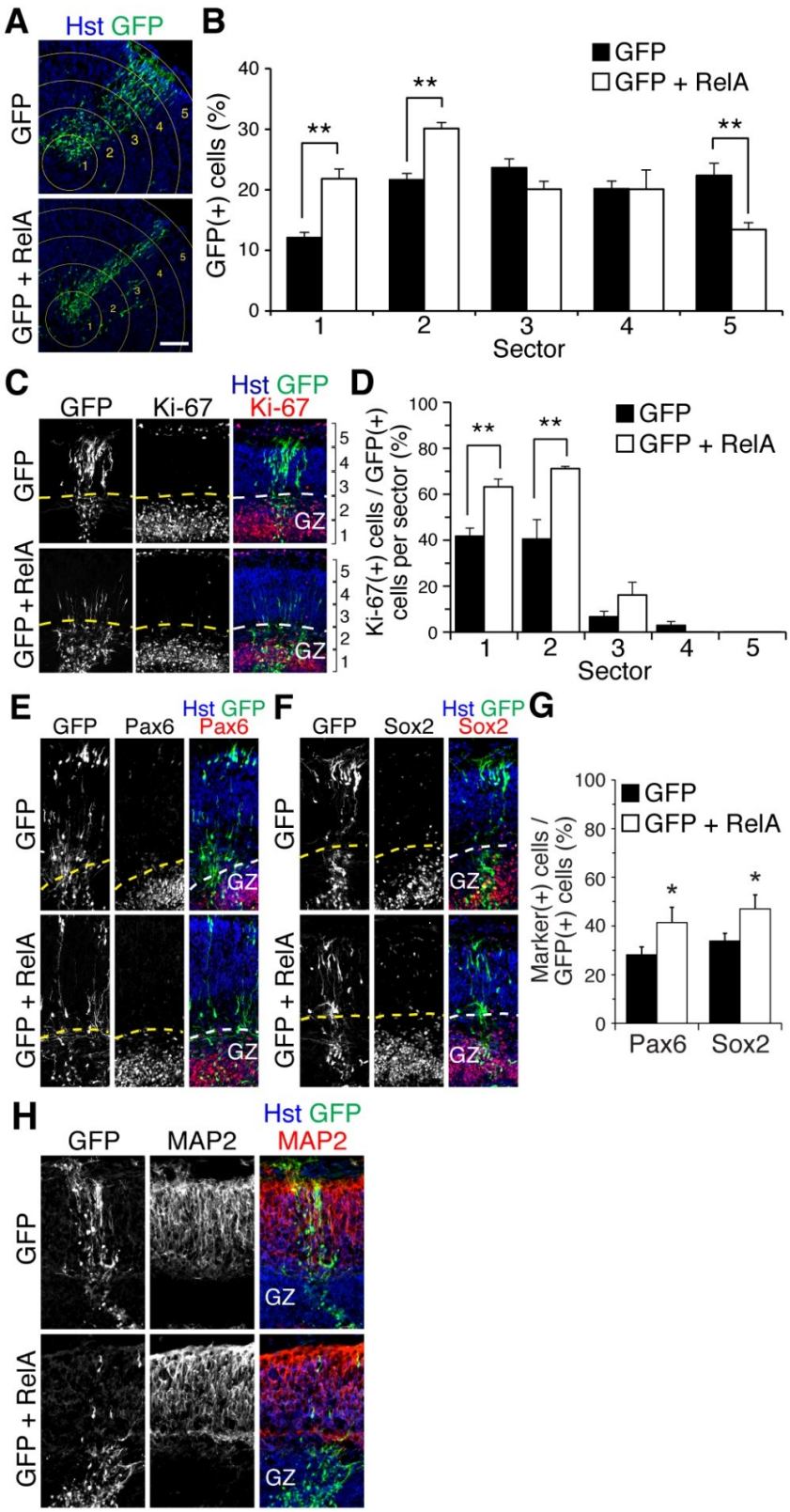


**Figure 20.** Inhibition of cortical neurogenesis in neocortical NPCs by ectopic activation of the NF-κB pathway. (A) Depiction of GFP$^{+}$ cell distribution in slice cultures from E13.5 mouse

forebrains, 96 h after electroporation of plasmids encoding GFP or GFP-RelA. Neocortex was operationally divided into 5 sectors; sector 1 is in the GZ, sector 5 is close to the pial surface. Scale bar, 100 μm. (B) Quantification of the fraction of $GFP^+$ cells (percentage of total) in each of the 5 different sectors (means ± SEM; **, $p < 0.01$; $n = 25$ electroporated slices per condition; two-way ANOVA). (C) Double-label immunofluorescence analysis of GFP and Ki-67 expression in organotypic slice cultures from E13.5 mouse forebrain, 96 h after electroporation of plasmids encoding GFP or GFP plus RelA. (D) Quantification of the fraction of $GFP^+$ cells coexpressing Ki-67 in each of the 5 different sectors (means ± SEM; **, $p < 0.01$; $n = 13$ electroporated slices per condition; two-way ANOVA). (E and F) Double-label immunofluorescence analysis of GFP and either Pax6 (E) or Sox2 (F) expression in organotypic slice cultures from E13.5 mouse forebrain, 96 h after electroporation of plasmids encoding GFP or GFP plus RelA. (G) Quantification of the fraction of $GFP^+$ cells coexpressing either Pax6 or Sox2 (means ± SEM; *, $p < 0.05$; $n = 12$ electroporated slices per condition; *t* test). (H) Double-label immunofluorescence analysis of GFP and MAP2 expression in organotypic slice cultures from E13.5 mouse forebrain, 96 h after electroporation of plasmids encoding GFP or GFP+RelA. Abbreviations: GZ, germinative zone; Hst, Hoechst.

### *3.2.3. Suppression of NF-κB pathway-mediated inhibition of cortical neurogenesis by Hes6*

It is likely that mechanisms that can modulate the activation of NF-κB in neocortical NPCs must be in place for these NPCs to undergo neuronal differentiation at the right time during cortical neurogenesis. We observed that the pro-neuronal bHLH protein Hes6 is co-expressed by most β-$Gal^+$ neocortical NPCs in E13.5 NF-$κB^{LacZ}$ embryos (Figure 21A). Overlapping expression of Hes6 and β-Gal was also observed in primary cultures of cortical NPCs derived from NF-$κB^{LacZ}$ embryos (Figure 21B). To investigate this situation further, we validated a commercial anti-Hes6 antibody by showing that a ~28-kDa band, the predicted size for mouse Hes6, recognized by this antibody could be abolished after transduction of primary cultures of cortical NPCs with a lentivirus expressing a Hes6 shRNA, strongly suggesting that the ~28-kDa band corresponds to endogenous Hes6 (Figure 22). With this antibody, together with Rita Lo from the Stifani lab, I performed coimmunoprecipitation studies using dissected cortices from E13.5 mouse embryos to examine whether endogenous Hes6 and the NF-κB subunit RelA might form a complex *in vivo*. These experiments showed that immunoprecipitation of endogenous RelA resulted in the coimmunoprecipitation of the ~28-kDa band recognized by the anti-Hes6 antibody (Figure 23). To understand how this physical interaction between RelA and Hes6 modulates the

transactivating function of RelA, Dr Yeman Tang from the Stifani lab performed transient-transfection/transcription assays that provided evidence that Hes6 can downregulate the transactivating ability of RelA (Methot *et al*., 2013). Taken together, these results provide evidence that activation of the NF-κB pathway and Hes6 expression are concurring events in neocortical NPCs during cortical neurogenesis, and that the NF-κB subunit RelA and Hes6 physically and functionally interact.

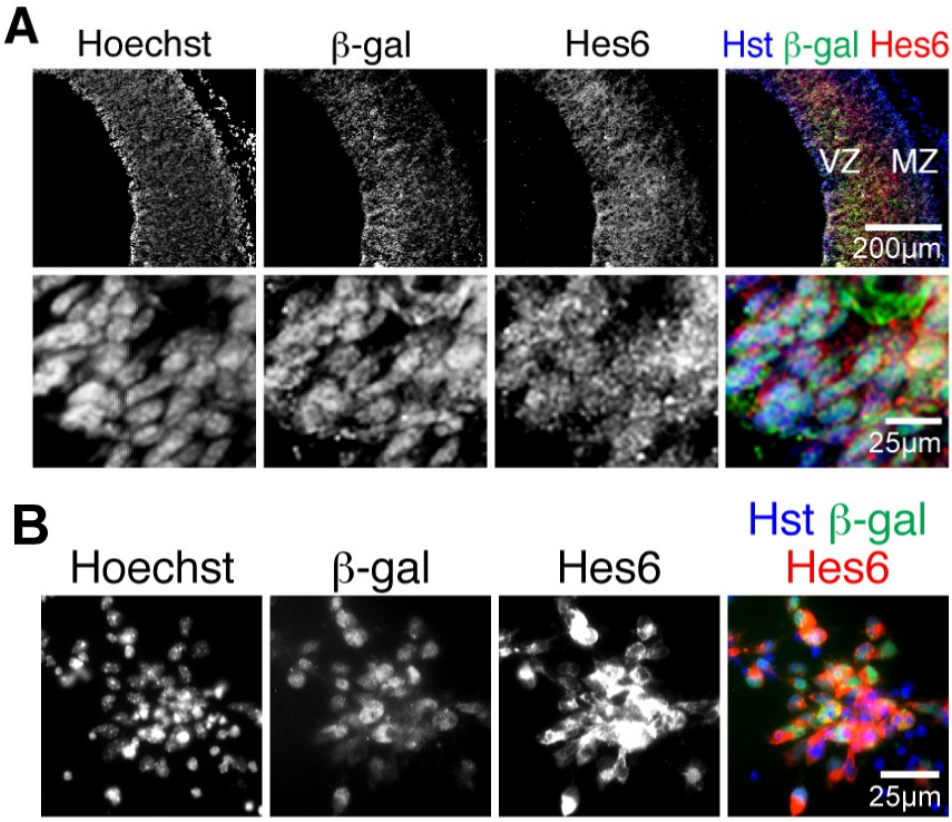


**Figure 21.** Hes6 is expressed in neocortical neural progenitor cells in which the NF-κB pathway is activated *in vivo* and *in vitro*. (A) Double-label immunofluorescence analysis of β-Gal and Hes6 expression in the neocortex of E13.5 NF-κB$^{LacZ}$ embryos. The bottom row depicts high-magnification views of β-Gal and Hes6 coexpression in VZ cells. MZ, mantle zone. (B) Double-label immunofluorescence analysis of β-gal and Hes6 expression in primary cortical neural progenitor cells from E13.5 NF-κB$^{LacZ}$ embryos. Hst, Hoechst. Scale bars, 200 μm or 25 μm, as indicated.

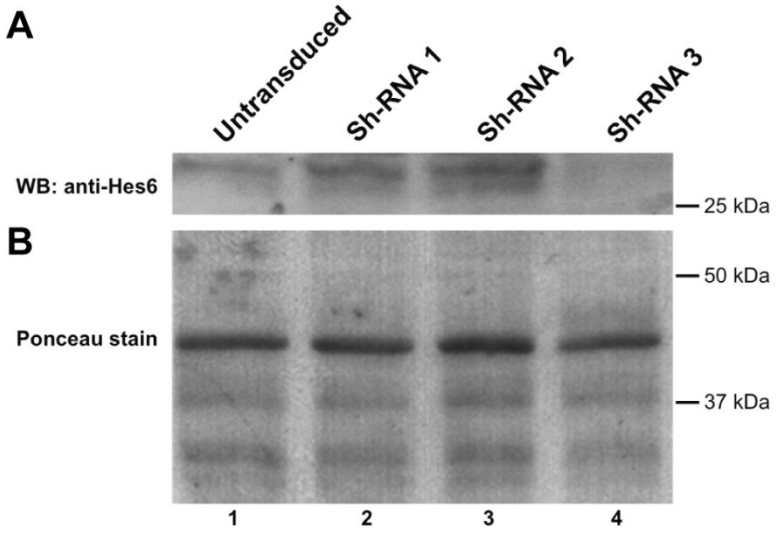


**Figure 22.** Validation of the anti-Hes6 antibody for Western blotting. Primary cultures of cortical neural progenitor cells were established from E13.5 CD1 mouse embryos. After 3 days in vitro, cells were transduced with lentiviral particles expressing three different shRNA sequences under the control of the human H1 promoter (GenTarget Inc.), followed by culture for an additional period of 48 hours and preparation of cell lysates. (A) Lysates were subjected to Western blotting using rabbit anti-Hes6 antibody (1/1,000, Novus Biologicals). shRNA directed against mouse Hes6 ("shRNA 3"; GenTarget Inc.; sequence, TGCAGGTCCCTAGCACTATTT) caused a significant decrease in the ~28-kDa band recognized by the anti-Hes6 antibody (lane 4). No changes were observed when shRNA reagents 1 and 2 were used (lanes 2 and 3). (B) Ponceau S staining prior to Western blotting was used to compare protein loading in the different lanes. Molecular size markers are indicated.

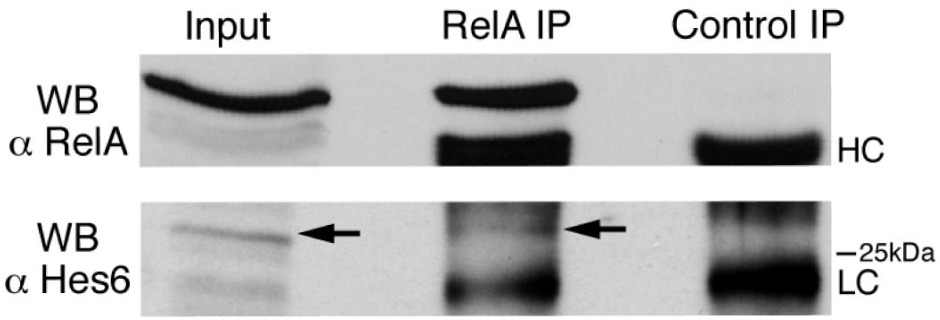


**Figure 23.** Hes6 forms a complex with the NF-κB subunit RelA *in vivo*. Nuclear extracts from dissected cortices from E14.5 CD1 mouse embryos were subjected to immunoprecipitation (IP) with anti-RelA or control antibody. Immunoprecipitates were analyzed with each input lysate by Western blotting (WB) with anti-RelA (top) or anti-Hes6 (bottom) antibody. The arrow points to the position of the Hes6 immunoreactive band. HC, immunoglobulin heavy chain; LC, immunoglobulin light chain.

Based on studies showing that Hes6 has the ability to inhibit RelA-mediated transactivation (Methot *et al.,* 2013), we examined whether Hes6 would antagonize NF-κB-mediated maintenance of the undifferentiated neocortical NPC state. To this end, we took advantage of the gain-of-function experimental design described above in Figure 20, in which exogenous RelA expression in neocortical NPCs *ex vivo* causes an inhibition/delay of the progenitor-to-neuron transition leading to an accumulation of undifferentiated cells in inner sectors of the neocortex. In agreement with the previously characterized pro-neurogenic function of Hes6 (Bae *et al.,* 2000; Gratton *et al.,* 2003), we observed that co-electroporation of EGFP and a Hes6-expressing vector in the neocortical VZ of organotypic forebrain slices resulted in a detectable decrease in the number of $EGFP^+$ electroporated cells located in the inner sectors (Figures 24A and B, sector 2, first and fourth bars), with a parallel increase in the number of $GFP^+$ cells in the most outer sector (Figures 24A and B, sector 5, first and fourth bars), compared to GFP expression alone. More importantly, the coexpression of Hes6 together with RelA abolished both the RelA-mediated accumulation of electroporated cells in sectors 1 and 2 and the concomitant reduction in the number of electroporated cells in sector 5 (Figures 24A and B, sectors 1 and 5, second and third bars). In agreement with these results, exogenous Hes6 expression antagonized RelA-mediated increase in the number of Ki-$67^+$ cells and decrease in the number of $MAP2^+$ cells (Figures 24C and D).

Although exogenous coexpression of EGFP and Hes6 *ex vivo*, compared to EGFP alone, resulted in a reduction in the proportion of manipulated cells located in the neocortical GZ, it did not result in a significant reduction in the number of manipulated cells expressing Ki-67 (corresponding to mitotic progenitor cells), a situation that was observed *in vitro* in transfected cultured cortical progenitor cells (Gratton *et al*., 2003; Methot *et al*., 2013) and would have been expected based on the described role of Hes6 in promoting neuronal differentiation of cortical NPCs at the expense of maintenance of the undifferentiated progenitor identity. This discrepancy could be explained by differences in the levels of forced Hes6 expression in *ex vivo* organotypic cultures compared to in vitro primary cultures: exogenous Hes6 proteins in electroporated GZ cells *ex vivo* were abundant enough to antagonize the function of NF-κB when coexpressed together with RelA (Figure 24), but might not have been sufficient to lead to a significant change

on the ability of progenitor cells to maintain an undifferentiated fate when exogenously expressed only together with EGFP in this specific forced expression system.

Together, these results identify Hes6 as a previously uncharacterized modulator of NF-κB transactivating activity, possibly through formation of a physical complex between Hes6 and the NF-κB subunit RelA. Furthermore, they suggest a function for Hes6 in the modulation of RelA-mediated maintenance of the undifferentiated NPC fate during cortical neurogenesis.

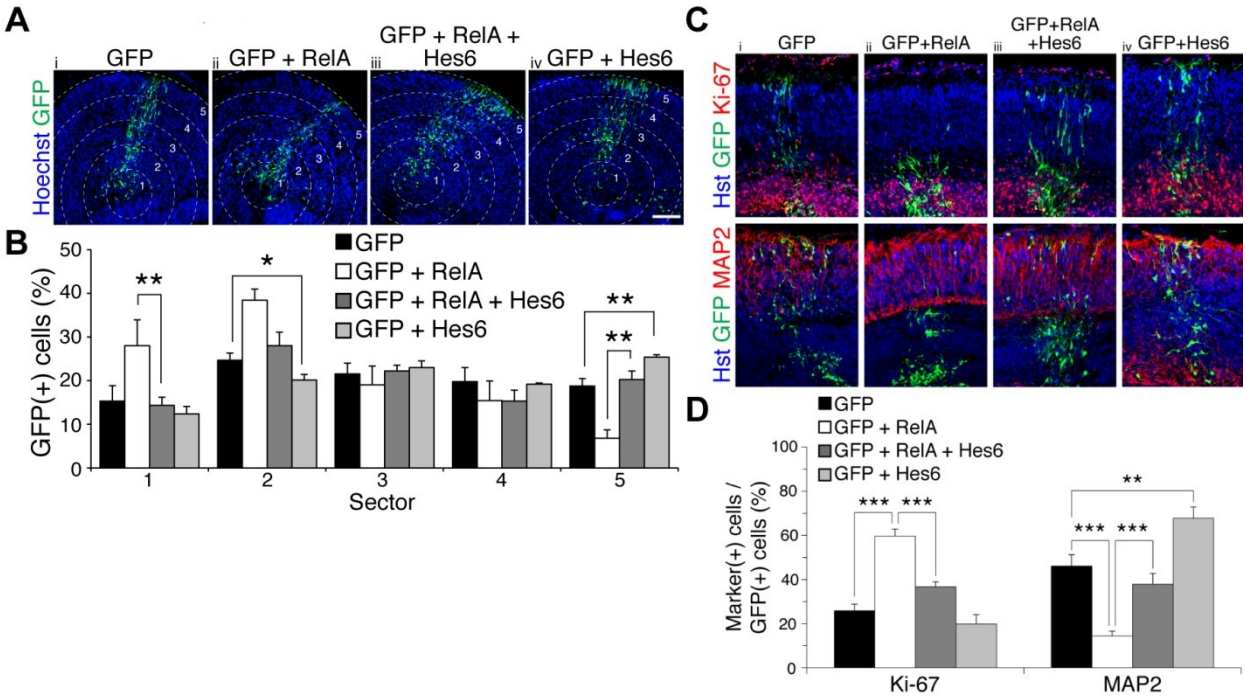


**Figure 24.** Hes6 antagonizes RelA-mediated inhibition or delay of neocortical neuronal differentiation. (A) Distribution of $GFP^+$ cells in slice cultures from E13.5 mouse forebrain, 96 h after electroporation of plasmids encoding the indicated combinations of proteins. Neocortex was operationally divided into 5 sectors. Scale bar, 100 μm. (B) Quantification of the fraction of $GFP^+$ cells present in each of the 5 different sectors (means ± SEM; *, $p < 0.05$; **, $p < 0.01$; $n$ = 19 electroporated slices per condition; $t$ test). (C) Double-label immunofluorescence analysis of GFP and either Ki-67 or MAP2 expression in organotypic slice cultures from E13.5 mouse forebrain, 96 h after electroporation of plasmids encoding the indicated combinations of proteins. (D) Quantification of the fraction of $GFP^+$ cells coexpressing either Ki-67 or MAP2 (means ± SEM; **, $p < 0.01$; ***, $p < 0.001$; $n$ = 14 electroporated slices per condition; $t$ test).

*3.2.4. PI3-Kinase/Akt pathway-mediated activation of the NF-κB pathway in neocortical neural progenitor cells*

To begin to characterize the molecular events underlying the activation of the NF-κB pathway in neocortical NPCs, we examined whether the phosphatidylinositol (PI) 3-Kinase/Akt pathway might be important for NF-κB activation in these cells. The PI3-Kinase/Akt pathway is activated by growth factors and certain cytokines and in turn leads to activation of the IKK complex and canonical NF-κB pathway (Sizemore *et al.,* 1999; Richmond, 2002). Akt signaling has been shown to be activated in cortical NPCs during embryonic neurogenesis (Poduri *et al.* 2012), to promote maintenance of the undifferentiated state of cortical NPCs *in vivo* (Zhang *et al*., 2013), and to be involved in the regulation of neural stem/progenitor cell self-renewal (Sato *et al.,* 2010; Le Belle *et al*., 2011). The PI3-Kinase/Akt pathway can be inhibited pharmacologically with Wortmannin, a potent inhibitor of PI3-Kinases activity (Arcaro *et al.,* 1993). To determine whether treatment of primary cultures of cortical NPCs with Wortmannin would lead to an inhibition of PI3-Kinases, we examined the phosphorylation status of serine residue 473 of the PI3-Kinase target protein, Akt. Treatment with Wortmannin caused a robust reduction in phosphorylated Akt, but no change in total levels of Akt (Figure 25A), indicating that Wortmannin efficiently inhibits the PI3-Kinase/Akt pathway in neocortical NPCs. We then treated primary cultures of cortical NPCs established from E13.5 NF-κB$^{LacZ}$ embryos with Wortmannin and measured β-Gal activity as an indicator of NF-κB pathway activation. Compared to untreated cells, Wortmannin treatments led to a significant reduction in β-Gal activity (Figure 25B). These results suggest that activation of the PI3-Kinase/Akt pathway occurs in cortical NPCs *in vitro* and is important for NF-κB activation in these cells. Furthermore, they suggest that growth factors or specific cytokines might be important in the activation of the NF-κB pathway during corticogenesis.

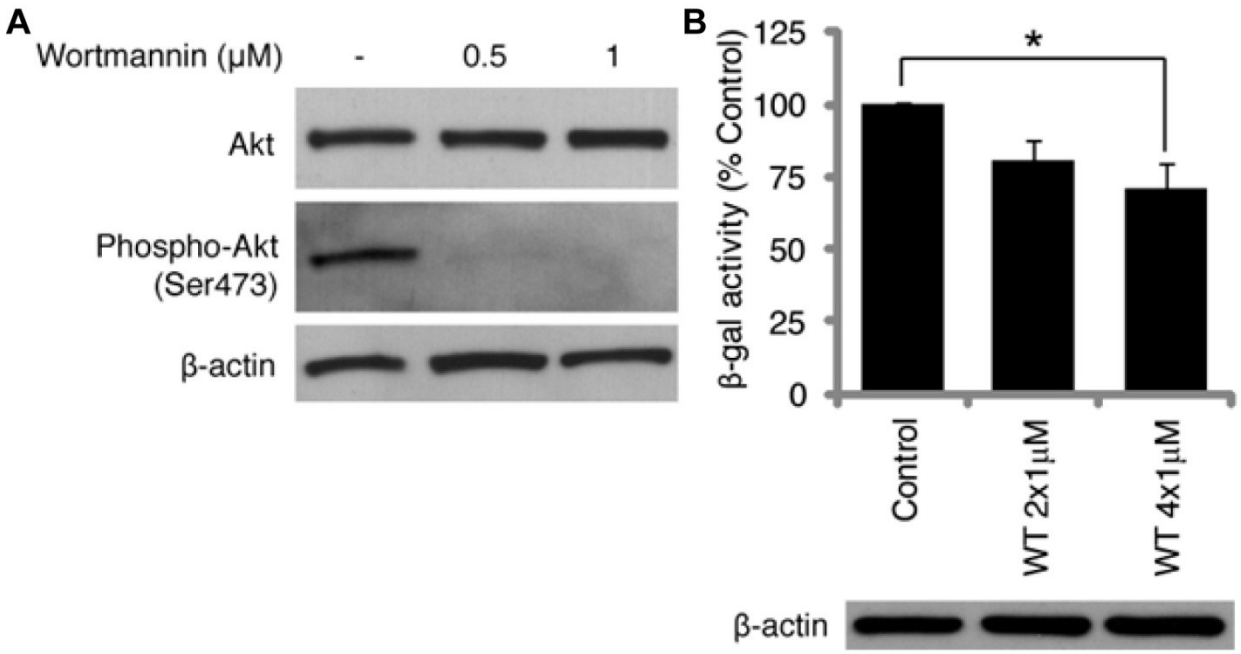


**Figure 25.** PI3K-mediated activation of the NF-κB pathway in neocortical neural progenitor cells *in vitro*. (A) Unchanged levels of Akt expression and reduced levels of Phospho-Akt (Ser473) expression in primary cultures of cortical progenitor cells from E13.5 embryos treated with DMSO alone or with 0.5 or 1 μM Wortmannin, detected by Western blotting with anti-Akt and anti-Phospho-Akt (Ser473) antibodies. (B) Quantification of β-gal activity in primary cultures of cortical progenitor cells from E13.5 NF-κB$^{LacZ}$ embryos. Cells were either treated with DMSO alone ("Control"; considered as 100%) or with 2 or 4 doses of 1 μM Wortmannin, as indicated. Determination of β-gal activity was performed 48 h after transduction. Data are shown as the mean ± SEM (*, $p$ <0.05, $n$ = 3 separate experiments performed in duplicates, $t$ test). (B) Unchanged levels of β-actin expression as detected by Western blotting with an anti-β-actin antibody.

### 3.3. Regulation of cortical gliogenesis by the NF-κB pathway

#### *3.3.1 Pattern of NF-κB pathway activation during astrocytic differentiation*

To characterize the pattern of NF-κB activation during astrocytic differentiation in the developing cortex of NF-κB$^{LacZ}$ mice, we monitored the expression of β-Gal in the two main populations of cortical astrocytes, namely white matter and gray matter astrocytes (Figure 26A). White matter fibrous astrocytes can be identified by their localization in the white matter and the expression of the proteins, GFAP and ALDH1L1 (Cahoy *et al.,* 2008). In the postnatal forebrain of NF-κB$^{LacZ}$ pups, β-Gal expression was detected in roughly half of all GFAP$^{+}$ cells localized in the white matter (Figures 26B and C). Fewer white matter ALDH1L1$^{+}$ cells, which correspond to more mature astrocytes, showed detectable levels of β-Gal expression (Figures 26D and E). These observations suggest that the NF-κB pathway continues to be activated in maturing white matter astrocytes and that its activity may be downregulated during the developmental maturation of these cells.

Most gray matter astrocytes can be identified by the expression of the proteins, Acsbg1 and BLBP, and by their anatomical localization in the gray matter (Cahoy *et al.,* 2008; Ge *et al.,* 2012). We observed that about half of BLBP$^{+}$ cells or Acsbg1$^{+}$ cells in the gray matter displayed expression of β-Gal in the cortex of NF-κB$^{LacZ}$ pups (Figures 26F). NF-κB activation in these cells was modest when compared to the robust activation observed in cortical neurons. Taken together, these results suggest that the NF-κB pathway is activated in a subset of white and gray matter astrocytes that could correspond to cells that are not yet developmentally mature. They suggest further that activation of the NF-κB pathway is maintained during the transition from cortical NPCs to the astrocytic lineage but later downregulated in more mature astrocytes. Due to the lack of appropriate markers, it is not possible to determine whether NF-κB is initially downregulated during the transition from cortical NPCs to the newly-born astrocytes and then re-activated at early stages of astrocytic differentiation.

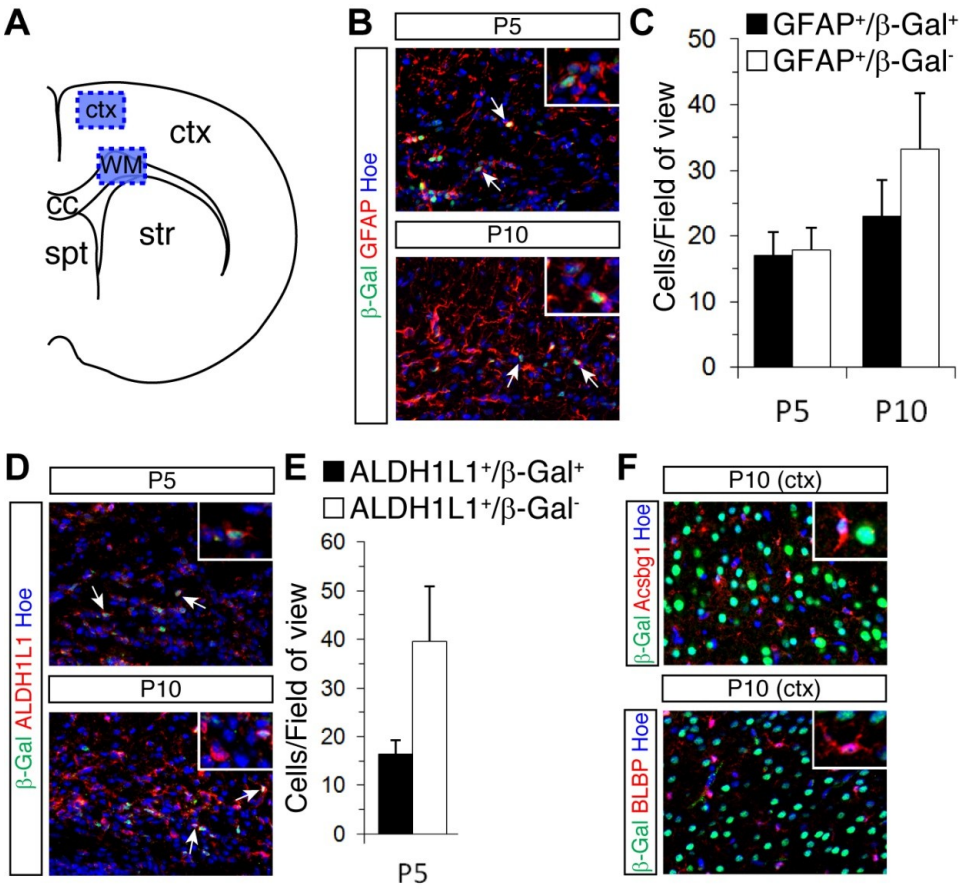


**Figure 26.** Activation of the NF-κB pathway in certain white matter and gray matter astrocytes. (A) Schematic illustration of a coronal section through the rostral postnatal mouse forebrain, showing in the blue boxed areas the regions analyzed in panels (B-F) (WM, white matter; ctx, cortex). cc, corpus callosum;, spt, septum; str, striatum. (B) Double-label immunofluorescence analysis of β-Gal and GFAP expression in the white matter of P5 (top panel) or P10 (bottom panel) NF-κB$^{LacZ}$ pups showing β-Gal expression in a fraction of white matter astrocytes. The insets represent high-magnification views of an area in main panels. Arrows point to examples of double-labeled cells. (C) Quantification of the fraction of β-Gal$^{+}$ cells coexpressing GFAP in the white matter of P5 or P10 NF-κB$^{LacZ}$ pups, as indicated (means ± SEM; *n* = 3 pups per age). (D) Double-label immunofluorescence analysis of β-Gal and ALDH1L1 expression in the white matter of P5 (top panel) or P10 (bottom panel) NF-κB$^{LacZ}$ pups showing β-Gal expression in a portion of white matter astrocytes. The insets represent high-magnification views of an area in main panels. Arrows point to examples of double-labeled cells. (C) Quantification of the fraction of β-Gal$^{+}$ cells coexpressing ALDH1L1 in the white matter of P5 NF-κB$^{LacZ}$ pups (means ± SEM; *n* = 3 pups). (F) (D) Double-label immunofluorescence analysis of β-Gal and Acsbg1 (top panel) or BLBP (bottom panel) expression in the cerebral cortex of or P10 NF-κB$^{LacZ}$ pups showing β-Gal expression in a portion of gray matter astrocytes. The insets represent high-magnification views of an area in main panels.

*3.3.2. Downregulation of NF-κB activation during transition of gliogenic neural progenitor cells to the oligodendrocytic lineage*

We next sought to characterize the pattern of NF-κB activation during the transition from undifferentiated cortical NPCs to differentiating glial cells of the oligodendrocytic lineage. To examine the oligodendrocytic lineage in the postnatal cerebral cortex of NF-κB$^{LacZ}$ pups, we monitored the expression of β-Gal in OPCs, the most immature cell population expressing the proteins, Olig2 and PDGFRα. Approximately half of Olig2$^+$ cells in the postnatal SVZdl showed detectable expression of β-Gal (Figures 27A and C). Olig2 is expressed not only in OPCs, but also in at least some gliogenic NPCs giving rise to astrocytes (Marshall *et al.,* 2005; Cai *et al.,* 2007). In the transition towards the astrocytic lineage, Olig2 expression is downregulated in maturing astrocytes (Cai *et al.,* 2007) while maintained in OPCs, where it orchestrates a transcriptional program required for oligodendrocyte differentiation (Yu *et al.,* 2013). Olig2$^+$ SVZ cells are therefore not yet committed to a specific glial lineage. Olig2$^+$ SVZ cells that undergo a transition to the oligodendrocyte lineage migrate to the adjacent white matter and upregulate PDGFRα expression. We observed that the majority of Olig2$^+$ cells in the adjacent white mater did not express detectable levels of β-Gal (Figures 27A and C), suggesting that the NF-κB pathway is downregulated at early stages in the transition to the oligodendrocytic lineage. In agreement with this hypothesis, we observed virtually no overlap between PDGFRα and β-Gal expression in the white matter, from P1 to P10 (Figures 27B and C). Almost all PDGFRα$^+$ cells were β-Gal-negative and almost all β-Gal$^+$ cells were PDGFRα-negative (not shown). We also examined whether the NF-κB pathway could be activated in more developmentally mature oligodendrocyte lineage cells expressing the protein, CNPase (immature oligodendrocytes). The vast majority of CNPase$^+$ cells did not show detectable levels of β-Gal expression (Figures 27D and E). Taken together, these results provide evidence that the NF-κB pathway is downregulated during the transition from cortical NPCs to OPCs and immature oligodendrocytes.

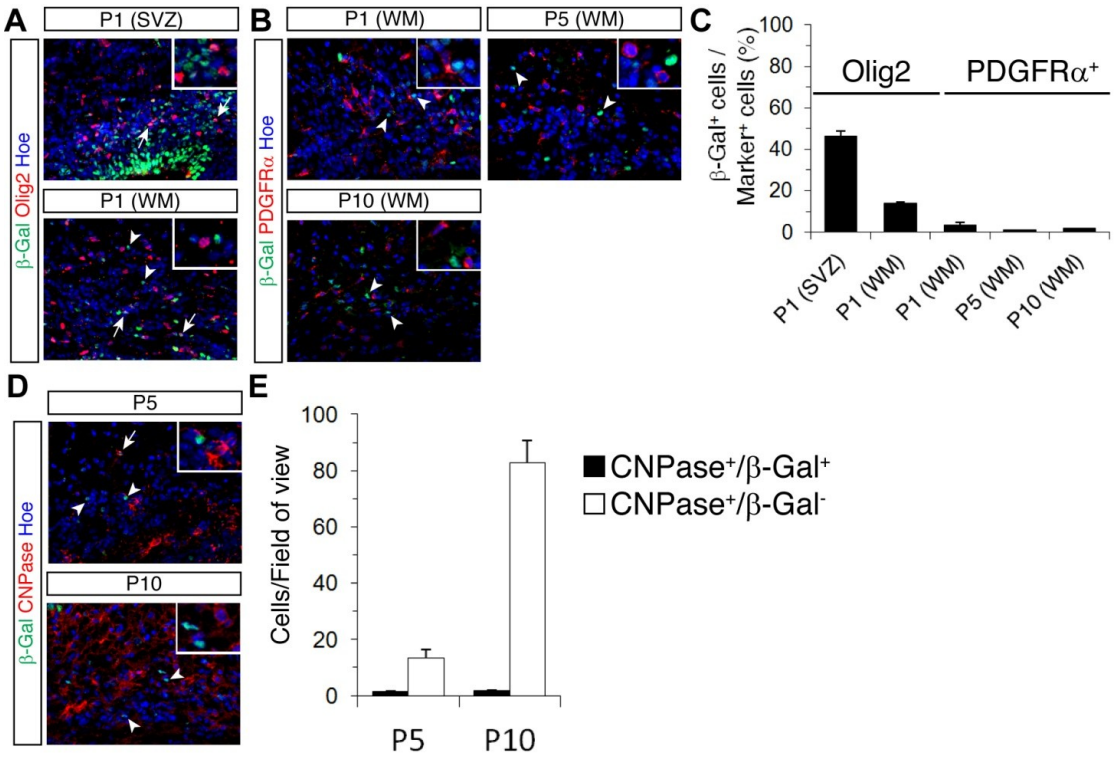


**Figure 27.** Downregulation of the NF-κB pathway in immature cells of the oligodendrocytic lineage. (A) Double-label immunofluorescence analysis of β-Gal and Olig2 expression in the subventricular zone (SVZ, top panel) or in the white matter (WM, bottom panel) of P1 NF-κB$^{LacZ}$ pups showing a lack of detectable activation of β-Gal expression in most Olig2$^+$ cells in the white matter. The insets represent high-magnification views of an area in main panels. Arrows point to examples of double-labeled cells and arrowheads point to examples of β-Gal$^+$, Olig2-negative cells. (B) Double-label immunofluorescence analysis of β-Gal and PDGFRα expression in the white matter (WM) of P1 (top left panel), P5 (top right panel), or P10 (bottom panel) NF-κB$^{LacZ}$ pups showing a lack of detectable activation of β-Gal expression in almost all PDGFRα$^+$ cells in the white matter. The insets represent high-magnification views of an area in main panels. Arrowheads point to examples of β-Gal$^+$, PDGFRα-negative cells. (C) Quantification of the fraction of β-Gal$^+$ cells coexpressing GFAP or PDGFRα in the subventricular zone (SVZ) or white matter (WM) of P1, P5 or P10 NF-κB$^{LacZ}$ pups, as indicated (means ± SEM; $n$ = 3 pups per condition). (D) Double-label immunofluorescence analysis of β-Gal and CNPase expression in the white matter of P5 (top panel), or P10 (bottom panel) NF-κB$^{LacZ}$ pups showing a lack of detectable activation of β-Gal expression in almost all CNPase$^+$ cells in the white matter. The insets represent high-magnification views of an area in main panels. Arrows point to examples of double-labeled cells and arrowheads point to examples of β-Gal$^+$, CNPase-negative cells. (E) Quantification of the fraction of β-Gal$^+$ cells coexpressing CNPase in the white matter of P5 or P10 NF-κB$^{LacZ}$ pups, as indicated (means ± SEM; $n$ = 3 pups per age).

*3.3.3. NF-κB pathway in the maintenance of the undifferentiated gliogenic identity of neural progenitor cells during cortical gliogenesis.*

Based on the function of the NF-κB pathway in promoting the maintenance of the undifferentiated NPC state during cortical neurogenesis, we first sought to determine whether NF-κB might play a similar role in NPCs during cortical gliogenesis. To this end, we examined the *in vivo* phenotype resulting from inhibition of endogenous NF-κB in cortical NPCs at the onset of gliogenesis (E17.5) using the two dominant inhibitors of NF-κB activation, IκBαM and IKKβ-DN, described above (Figure 28). Plasmids expressing IκBaM and EGFP (IκBαM-IRES-EGFP), IKKβ-DN and EGFP (IKKβ-DN-IRES-EGFP), or EGFP alone (IRES-EGFP) were electroporated in the SVZdl *in utero* at E17.5 to target gliogenic cortical NPCs. Electroporated pups were then analyzed 4 days post-electroporation (P2).

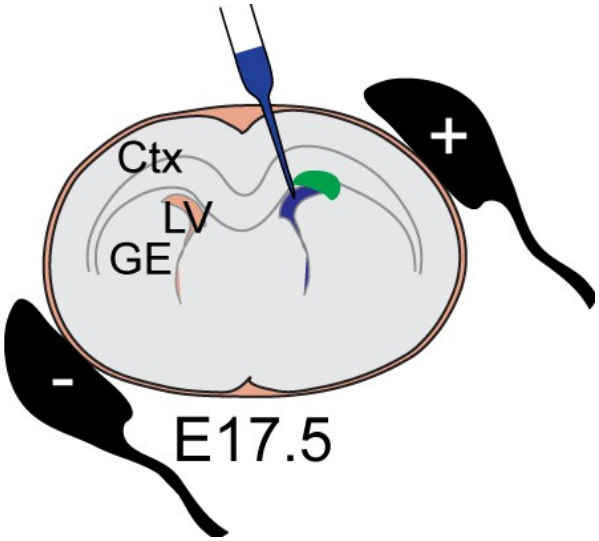


**Figure 28**. Schematic representation of *in utero* electroporation strategy at the onset of cortical gliogenesis. The position of the electrodes relative to the brain is indicated. DNA mixture is injected in the lateral ventricle (LV). Ctx, cortex; GE, ganglionic eminence.

To determine whether the NF-κB pathway is required for the maintenance of the undifferentiated identity of NPCs during cortical gliogenesis, we quantitated the number of $EGFP^+$ cells expressing the NPC marker, Sox2 in the SVZdl in each of three experimental conditions. Forced expression of neither IκBαM nor IKKβ-DN caused a significant change in

the number of EGFP$^+$ cells expressing Sox2 (Figure 29). These results suggest that the NF-κB pathway is not required for the maintenance of the undifferentiated NPC state cortical gliogenesis.

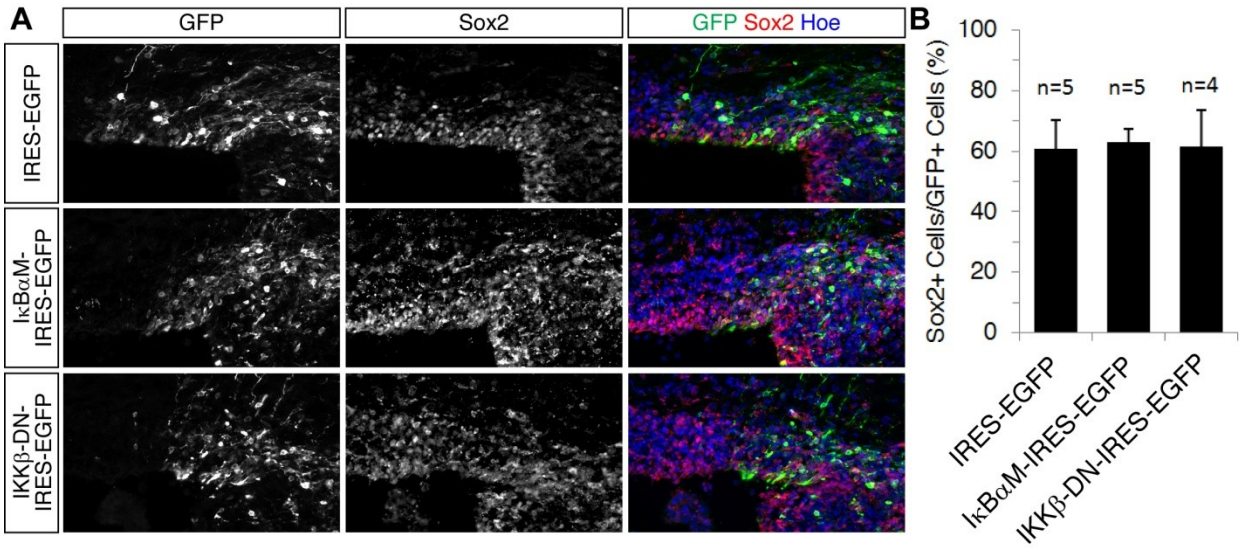


**Figure 29.** Unperturbed number of cortical NPCs after inhibition of endogenous NF-κB. (A) Double-label immunofluorescence analysis of GFP and Sox2 expression 96 h after *in utero* electroporation of E17.5 mouse embryos with either a bicistronic plasmid expressing both IκBαM and EGFP, both IKKβ-DN and EGFP, or the empty vector expressing EGFP alone, as indicated. Hoe, Hoechst. (B) Quantification of the fraction of GFP$^+$ cells coexpressing Sox2 (means ± SEM; $n$ = 4-5 pups per condition, $t$ test).

To determine the function of NF-κB pathway activation in postnatal cortical NPCs in the maintenance of the radial-glial and/or gliogenic identity, we quantitated the number of GFP$^+$ cells in the SVZdl expressing GFAP 4 days post-electroporation (Figure 30). Electroporation of EGFP alone resulted in a proportion of GFAP$^+$/GFP$^+$ cells that was similar to the proportion of Sox2$^+$/GFP$^+$ cells (Figure 29). These results are consistent with the observation that, under normal conditions, the vast majority of SVZdl cortical NPCs in the early postnatal brain have a radial-glial/gliogenic identity (Sox2$^+$GFAP$^+$) (see Figure 12C). Blockade of the NF-κB pathway in cortical NPCs resulted in a significant reduction in the number of GFAP$^+$ electroporated cells in the SVZdl (Figure 30). Importantly, these results are not consistent with the observation that the proportion of Sox2$^+$/GFP$^+$ cells does not change when the NF-κB pathway is blocked. These results suggest a number of possibilities: (1) the NF-κB pathway may be important for

maintenance of the radial-glial/gliogenic identity of NPCs during cortical gliogenesis; and/or (2) the NF-κB pathway may be important  may be important for the induced/sustained expression of the *Gfap* gene in cortical NPCs (see Discussion).

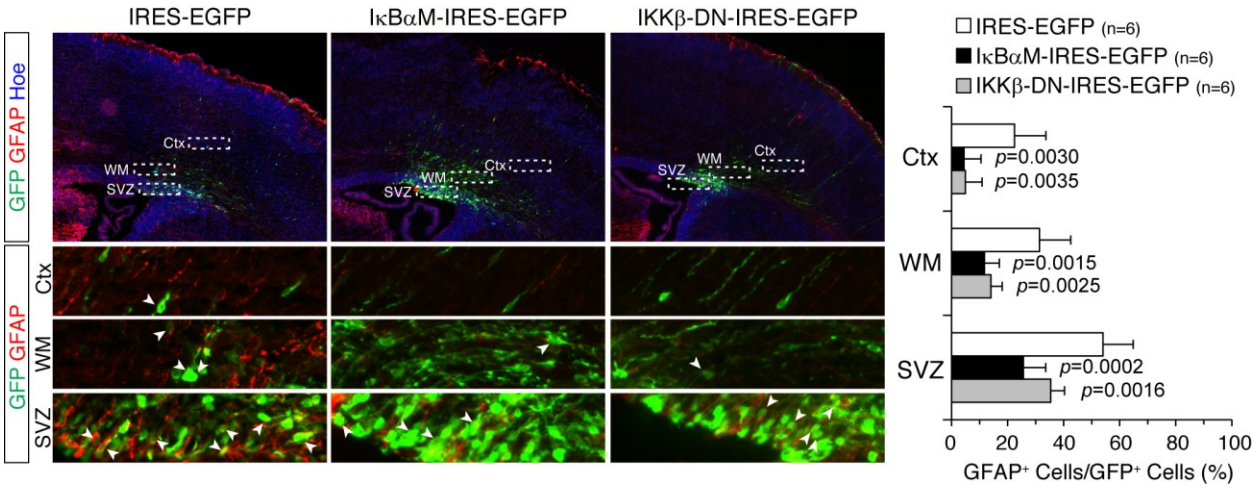


**Figure 30.** Suggested loss of undifferentiated gliogenic NPCs and reduced/delayed transition of cortical NPCs to the astrocytic lineage after inhibition of endogenous NF-κB. (A) Double-label immunofluorescence analysis of GFP and GFAP expression 96 h after *in utero* electroporation of E17.5 mouse embryos with either a bicistronic plasmid expressing both IκBαM and EGFP, both IKKβ-DN and EGFP, or the empty vector expressing EGFP alone, as indicated. Hoe, Hoechst. Panels in bottom rows represent high magnification views of boxed areas in different anatomical regions in top row panels, as indicated. (B) Quantification of the fraction of $GFP^+$ cells coexpressing GFAP in the cortex (Ctx), white matter (WM), or subventricular zone (SVZ), as indicated (means ± SEM; $n = 6$ pups per condition, *t* test).

*3.3.4. Activation of the NF-κB pathway in gliogenic cortical neural progenitor cells is required to promote acquisition of the astrocytic lineage and/or to promote expression of GFAP gene*

To determine whether activation of NF-κB pathway in cortical NPCs was also involved in the regulation of the acquisition of an astrocyte identity, we analyzed the number of electroporated cells expressing the astrocytic marker GFAP outside of the SVZ (in the white matter and cerebral cortex), which are cells that have acquired an astrocytic identity, under control condition (electroporated with EGFP alone) are upon blockade of the NF-κB pathway (electroporated with EGFP together with IκBαM or IKKβ-DN) (Figure 31). Both IκBαM and IKKβ-DN caused a significant reduction in the number of $EGFP^+$ cells expressing GFAP in the white matter and

cerebral cortex (Figure 31). These results suggest that the NF-κB pathway may be important to promote the acquisition of the astrocytic lineage identity and/or to sustain *Gfap* expression in developing astrocytes.

### *3.3.5. Activation of the NF-κB pathway in gliogenic cortical neural progenitor cells is required to inhibit/delay the acquisition of the oligodendrocytic lineage*

To determine whether inhibition of endogenous NF-κB signaling in NPCs at the onset of cortical gliogenesis might modulate the transition of cortical NPCs to the oligodendrocytic lineage, with the assistance of an undergraduate student in the Stifani lab, Sally Li, I compared the number of $EGFP^+$ cells expressing the early oligodendrocytic lineage marker protein, PDGFRα, in the three different anatomically-defined regions described above. Both IκBαM and IKKβ-DN caused a significant increase in the number of $EGFP^+$ cells expressing PDGFRα in the SVZ and white matter (Figures 32A and B). A significant increase in the number of electroporated cells expressing PDGFRα was observed in the cortex with IκBαM, but not IKKβ-DN. The presence of increased numbers of PDGFRα+ cells in the white matter was consistent with the observation that inhibition of endogenous NF-κB led to a significant accumulation of electroporated cells in the white matter, compared to control condition (Figure 32C and D). Because cortical NPCs are normally located in the SVZ while differentiating/ed oligodendrocytes move into the white matter, these combined results suggest that activation of the NF-κB pathway in cortical NPCs is required to inhibit/delay the NPC-to-oligodendrocytic lineage transition during cortical development.

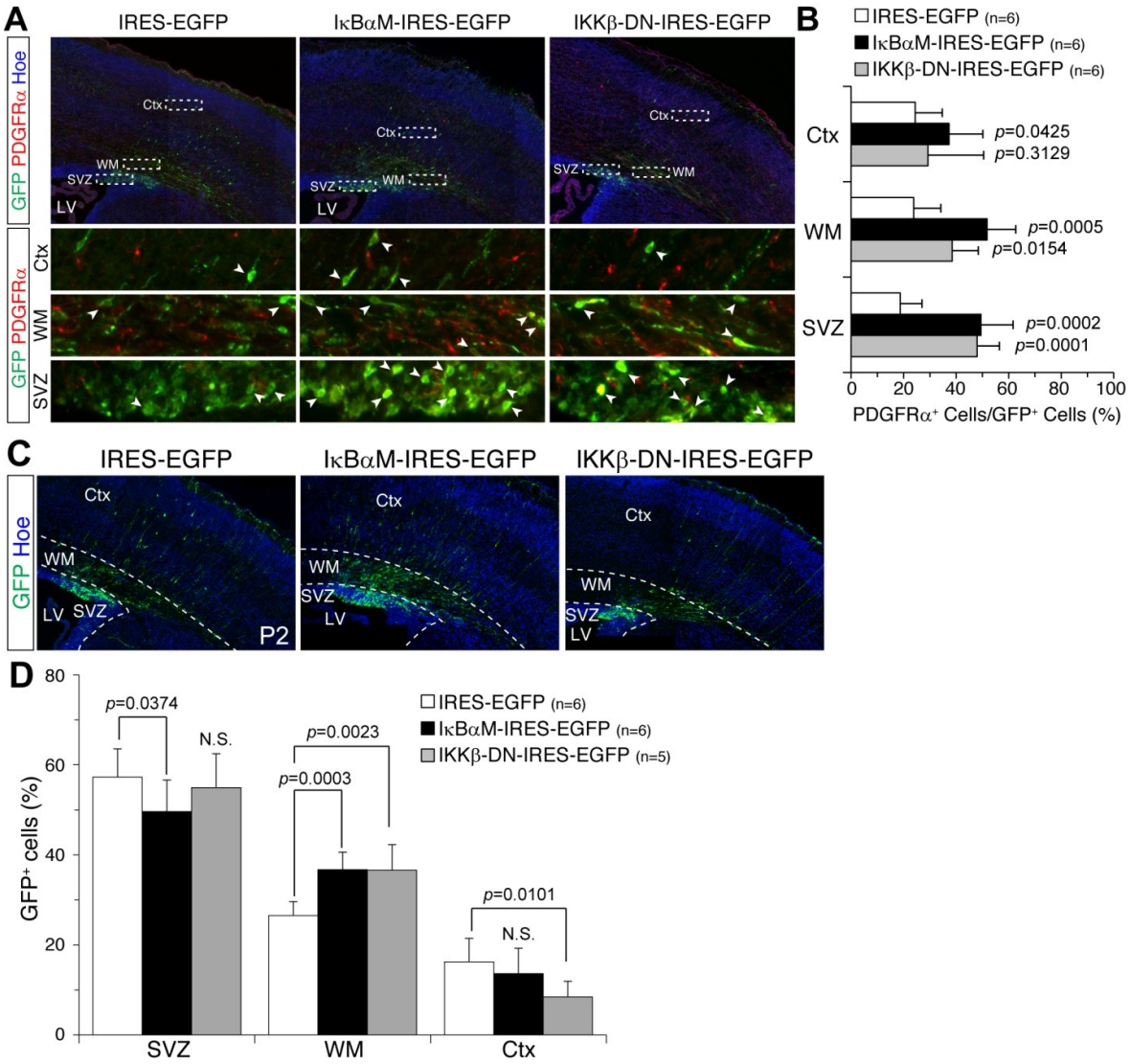


**Figure 31.** Increased progression of cortical NPCs to the oligodendrocytic lineage after inhibition of endogenous NF-κB. (A) Double-label immunofluorescence analysis of GFP and PDGFRα expression 96 h after *in utero* electroporation of E17.5 mouse embryos with either a bicistronic plasmid expressing both IκBαM and EGFP, both IKKβ-DN and EGFP, or the empty vector expressing EGFP alone, as indicated. Hoe, Hoechst. Panels in bottom rows represent high magnification views of boxed areas in different anatomical regions in top row panels, as indicated. (B) Quantification of the fraction of $GFP^+$ cells coexpressing PDGFRα in the cortex (Ctx), white matter (WM), or subventricular zone (SVZ), as indicated (means ± SEM; $n = 6$ pups per condition, *t* test). (C) Depiction of $GFP^+$ cell distribution 96 h after *in utero* electroporation of E17.5 mouse embryos with either a bicistronic plasmid expressing both IκBαM and EGFP, both IKKβ-DN and EGFP, or the empty vector expressing EGFP alone, as indicated. (D) Quantification of the fraction of $GFP^+$ cells (percentage of total) in each of the three different anatomical regions (subventricular zone, white matter, or cortex), as indicated (means ± SEM; $n = 6$ pups per condition, *t* test).

# 4. Discussion

## 4.1. Biological implications of NF-κB pathway activity during cortical neurogenesis

### *4.1.1. Biological model of NF-κB-mediated maintenance of neocortical neural progenitor cell fate during cortical neurogenesis*

Our understanding of the molecular mechanisms regulating the biology of cortical NPCs endowed with the potential to give rise to neuronal and glial cells that populate the cerebral cortex is still incomplete. Although roles for the NF-κB pathway in regulating a number of biological functions of distinct populations of NPCs in the developing and adult brain, such as maintenance of the undifferentiated state, cell proliferation, and neuronal differentiation, have been described (Young *et al.,* 2006; Sabolek *et al.,* 2009; Zhang *et al.,* 2012; Andreu-Agulló *et al.,* 2009; Denis-Donini *et al.,* 2008; Koo *et al.,* 2010), its precise activation pattern during corticogenesis and involvement in the regulation of cortical neurogenesis and gliogenesis were not known.

In this regard, together with two former students in the Stifani laboratory, Robert Hermann and Hosam Al-Jehani, I conducted studies to characterize the spatiotemporal pattern of endogenous NF-κB pathway activation in GZs of the developing embryonic forebrain utilizing NF-κB$^{LacZ}$ reporter mice. We demonstrated that the NF-κB pathway becomes activated in the lateral pallium (precursor of the neocortex) at developmental stages coinciding with the onset of neurogenesis in this region of the brain (Kriegstein and Alvarez-Buylla, 2009). Throughout the period of cortical neurogenesis, NF-κB activation persists in the lateral pallial GZ, a region containing neocortical NPCs responsible for generating excitatory neurons. These observations provided a rationale to perform studies aimed at characterizing the cell types in the GZ in which the NF-κB pathway is endogenously activated *in vivo* during embryonic and postnatal corticogenesis, and investigating the biological relevance of the endogenous activation of the NF-κB pathway in these cells.

In this thesis, I provided evidence that the NF-κB pathway is activated in neocortical NPCs during cortical neurogenesis. Additionally, I described functional studies aimed at investigating the biological significance of NF-κB activation in neocortical NPCs. Studies that

either blocked or ectopically activated the NF-κB pathway in neocortical NPCs showed a role for this transcription factor in maintaining the undifferentiated neocortical NPC fate and inhibiting and/or delaying neuronal differentiation during corticogenesis. *In vivo* and *in vitro* inhibition of canonical NF-κB pathway activity in cortical NPCs resulted in an increase in the number of cells expressing neuronal markers, together with a reduction in the number of cells expressing markers typical of undifferentiated NPCs. A reverse phenomenon was observed when the NF-κB pathway was ectopically activated through overexpression of the TAD-containing NF-κB subunit RelA: an expansion of the NPC compartment and a delay in neuronal differentiation.

These functional studies did not suggest that the migration of postmitotic neurons was perturbed. Indeed, we did not observe any change in the distribution of cells electroporated *in utero* with a control vector or vectors encoding dominant-negative protein to block endogenous NF-κB activity in the different anatomical zones of the developing neocortex (Methot *et al*., 2013). In addition, functional studies in which RelA was overexpressed did not result in any accumulation of postmitotic neurons in or near the neocortical GZ that could have resulted from perturbed neuronal migration.

The studies presented in this thesis strongly suggest that factors of canonical NF-κB pathway (IKKβ, IκBα, RelA) are involved in NF-κB-mediated promotion of the undifferentiated NPC fate. These studies cannot rule out however the involvement of non-canonical and atypical NF-κB pathways in the regulation of neocortical NPCs since redundancy and cross-talk between the different NF-κB signaling pathways has been described in a variety of contexts (Shih et al., 2011). For example, the canonical signaling pathway subunit RelA can promote the expression of the non-canonical subunit RelB in murine embryonic fibroblasts (MEFs) (Basak et al., 2008). As well, some stimuli have been shown to activate both canonical and non-canonical NF-κB signaling. For example, bacterial lipopolysaccharide (LPS) is a classical activator of canonical NF-κB signaling through Toll-like receptor 4 (TLR4) (Sen et al., 2010). LPS can also activate non-canonical NF-κB signaling through TLR4, which leads to phosphorylation of NF-κB inducing kinase (NIK) (the kinase that activates the IKK complex in the non-canonical pathway), and the subsequent proteosomal processing of p100 into p52 (Mordmüller et al., 2003; Bhattacharyya et al., 2010). Whether cross-talk between different NF-κB pathways is occurring in neocortical progenitor cells during neurogenesis is still unclear.

Together, the studies described in this thesis led us to propose a biological model where NF-κB activity promotes the maintenance of the undifferentiated NPC fate at the expense of neuronal differentiation (Figure 32). This regulatory function for the NF-κB pathway in asymmetrically-dividing cortical NPC may ensure that a pool of undifferentiated neocortical NPCs is maintained throughout corticogenesis and not depleted prematurely through precocious neuronal differentiation.

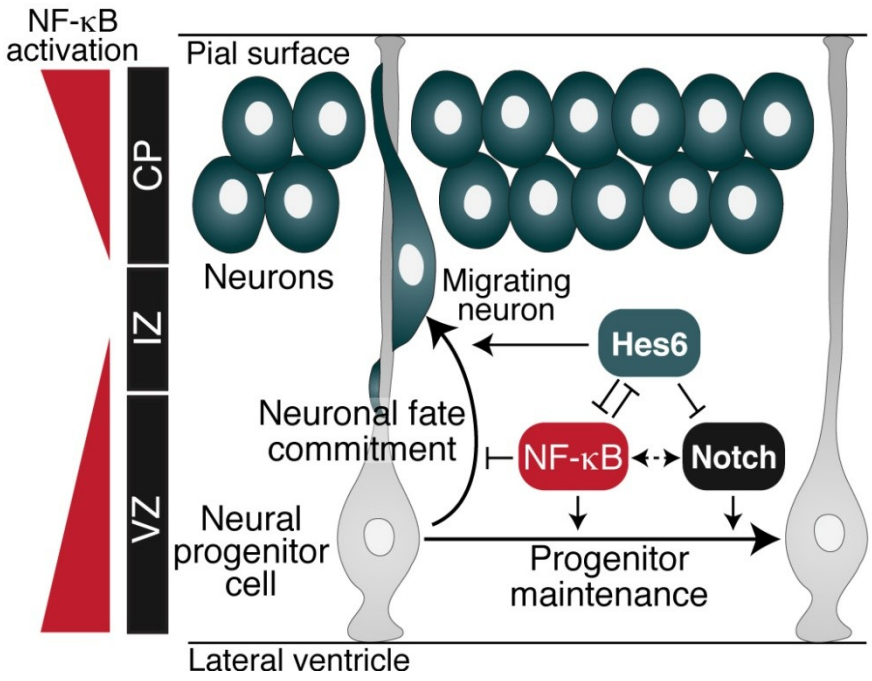


**Figure 32.** Biological and molecular model of NF-κB-mediated maintenance of neocortical neural progenitor cell fate during cortical neurogenesis. NF-κB signaling is robustly activated in neocortical VZ progenitors, transiently downregulated during early stages of neuronal differentiation, and then strongly activated in more developmentally mature neurons in the CP. NF-κB acts in undifferentiated progenitors to prevent/delay neuronal differentiation, thereby supporting progenitor cell maintenance. This role is similar to the function of the Notch signaling pathway (dotted double-pointed arrow). Hes6 antagonizes NF-κB in progenitor cells that are committed to undergo neuronal cell lineage progression. Conversely, NF-κB antagonizes the proneuronal activity of Hes6 to promote preservation of the neural progenitor pool. Hes6 also opposes Notch signaling-mediated inhibition of neuronal differentiation.

*4.1.2. Molecular mechanisms of NF-κB-mediated maintenance of neocortical neural progenitor cell fate during cortical neurogenesis*

The biological functions identified for the NF-κB pathway in promoting the undifferentiated neocortical neurogenic NPC fate raised an intriguing question: how is NF-κB activity modulated in neocortical NPCs to allow for neuronal differentiation to take place? To answer this question, we sought to investigate the molecular mechanisms modulating NF-κB activity in neocortical NPCs. In this regard, we provided evidence that activation of the NF-κB pathway and expression of the pro-neuronal factor Hes6 are concurring events in neocortical NPCs *in vivo* at the time of cortical neurogenesis, and that the NF-κB subunit RelA and Hes6 physically interact. Additionally, studies performed by Robert Hermann in transfected HEK 293 cells showed that the association of Hes6 with RelA in a complex is specific because RelA did not coprecipitate with the related Hes family member Hes1, which performs an anti-neurogenic function that is antagonistic to Hes6 activity (Methot *et al.*, 2013). Furthermore, even though both Hes6 and RelA can interact with the transcriptional corepressor Groucho/TLE (Gratton *et al.,* 2003; Tetsuka *et al.,* 2000), co-immunopercipitation studies performed by Robert Hermann showed that a mutated form of Hes6 lacking Groucho/TLE-binding ability was competent to interact with RelA, indicating that the ability of Hes6 to form a complex with the latter did not require the Groucho/TLE association (Methot *et al.*, 2013). To understand how this physical interaction between RelA and Hes6 modulates the transactivating function of RelA, Dr Yeman Tang from the Stifani lab performed transient-transfection/transcription assays that provided evidence that Hes6 can downregulate the transactivating ability of RelA (Methot *et al*., 2013). More importantly, this thesis provided evidence that this physical and functional interaction between RelA and Hes6 had biological significance, as Hes6 could antagonize NF-κB-mediated promotion of the maintenance of the undifferentiated fate by neocortical NPCs. In addition, this physical and function interaction between RelA and Hes6 is also suggested to be mutually inhibitory because transient-transfection/transcription assays performed by Dr. Yeman Tang showed that RelA antagonized the inhibitory effect of Hes6 on Hes1 transcription repression activity (Methot *et al*., 2013). Studies conducted by Robert Hermann in primary cultures of cortical NPCs provided evidence that overexpression of RelA antagonizes Hes6-mediated promotion of neocortical neuronal differentiation (Methot *et al*., 2013). Taken together, these

studies showed that the NF-κB subunit RelA and Hes6 physically and functionally interact and exert cross-inhibitory actions on each other.

We propose a molecular mechanism where the NF-κB pathway synergizes with the Notch effector protein Hes1 by antagonizing Hes6 to promote maintenance of the undifferentiated neocortical NPC fate. This mechanism could have important biological significance in the context of cortical neurogenesis. The ubiquitous expression of the pro-neuronal protein Hes6 during corticogenesis and its inhibitory effect on Hes1-mediated repression of transcription in cortical NPCs (Gratton et al., 2003) implied that some molecular mechanism is in place to modulate Hes6 activity, and in this way de-repress Hes1 activity to maintain the undifferentiated NPC fate. We propose that the presence of high levels of activated RelA that are sufficiently elevated to antagonize Hes6 activity in one of the daughter cells born from an asymmetrical NPC division is correlated with maintenance of the undifferentiated state prior to neuronal differentiation. In contrast, conditions where Hes6 levels are increased as a result of strong neurogenic signals caused by persistent neurogenic gene expression may result in an inhibition of NF-κB signaling by Hes6 during the neurogenic phase. Based on the studies presented in this thesis, we suggest that Hes6 might inhibit the ability of RelA to transactivate specific target genes in neocortical NPCs. These as yet unidentified target genes would most probably be important for the maintenance of the undifferentiated NPC fate and/or for the inhibition of neuronal differentiation. This inhibitory cross-talk would be in part achieved through formation of a physical complex between RelA and Hes6. We propose that the presence of levels of Hes6 that are sufficiently abundant to antagonize RelA-mediated transactivation would promote neuronal differentiation of a daughter cell resulting from an asymmetrical NPC cell division. This proposed model of cross-inhibitory actions of RelA and Hes6 on each other would imply that the relative abundance and/or availability of each protein compared the other is an important mediator of NPC fate.

Based on the identified biological function of the NF-κB pathway in promoting the maintenance of the undifferentiated state of neocortical NPCs, it might have been expected to observe activation of this pathway in cortical GZs prior to the onset of the cortical neurogenic phase (E10.5), during the expansion phase, when NPCs are actively dividing and maintaining an undifferentiated state. The presence of NF-κB activity in this population of cells would have been even more relevant considering the early onset of *Hes6* expression during the expansion

phase (Bae et al, 2000). We defined however that the first detectable activation of the NF-κB pathway in the cortical GZ coincides with the appearance of the first postmitotic cortical neurons. We hypothesize that NF-κB activity in cortical NPCs during the expansion phase is not required due to the oscillatory pattern of Notch signaling and Hes1 expression that results in a transient expression of pro-neuronal genes that does not permit neuronal differentiation. With the onset of cortical neurogenesis and the start of asymmetrical self-renewing NPC divisions, the expression of Hes1 is maintained low in the daughter cell that undergoes neuronal differentiation (Imayoshi and Kageyama, 2014). In turn, low expression of Hes1 allows for the expression of pro-neuronal bHLH factors that promote neuronal differentiation and the expression of Notch ligands that lead to stable activation of the Notch signaling pathway in the daughter cell that needs to remain undifferentiated. In this daughter cell, the inhibitory function of Hes6 on Hes1-mediated repression of transcription has to be downregulated in order to allow maintenance of the undifferentiated state. Studies described in this thesis provide a molecular mechanism that may explain in part how Hes6 activity is modulated to allow for Hes1-mediated repression of pro-neuronal genes to take place during cortical neurogenesis.

We provided evidence that the interaction between Hes6 and NF-κB signaling pathways is occurring in the developing lateral pallium during the cortical neurogenic phase. Whether this interaction also occurs in other developmental processes where activation of both NF-κB and Notch signaling pathways co-occurs with the expression of Hes6 protein and/or gene, for example in the developing GEs (Bae *et* al., 2000; Methot *et al*., 2013; Wang *et al.*, 2009), the postnatal cortex during gliogenesis (Allen Developing Mouse Brain Atlas; Sauvageot and Stilles, 2002), or in pathological events, such as glioblastoma (Bredel *et* al., 2011; Haapa-Paananen *et al*., 2012), is an open question. It should be emphasized that the proposed mode of interaction between NF-κB and Hes6 does not seem to be occurring in medial pallial NPCs, where Hes6 is expressed (Bae et al., 2000) but NF-κB is not detectably activated.

A complex cross-talk and interaction between the NF-κB and Notch signaling pathways at multiple levels have been documented in a number of different contexts, ranging from vertebrate development to cancer (Osipo *et al.*, 2008; Ang and Tergaonkar, 2007). These interactions can be either synergistic or cross-inhibitory depending on the context. Notch can promote NF-κB activity through transcriptional mechanisms, for example through the promotion of transcription of all 5 NF-κB proteins (Osipo *et al.*, 2008), or through non-transcriptional

mechanisms, for example through the binding of NICD to the IKK complex and subsequent activation of canonical NF-κB pathway (Bellavia *et al*., 2010), or through the sustained activation of the IKK complex by Hes1 (Espinosa *et al.*, 2010). The Notch pathway can also exert inhibitory interaction with the NF-κB pathway through interactions between NICD and p50 that inhibit RelA/p50-mediated transactivation (Wang *et al*., 2001). Conversely, the NF-κB pathway can promote Notch activity, for example in adult neural stem cells of the SVZ where activated RelA synergizes with Notch to promote *Hes1* expression (Andreu-Agullo et al., 2009), or through NF-κB-mediated expression of the Notch ligand *Jagged1* (Bash *et al.,* 1999). The studies presented in this thesis suggest a novel way by which the NF-κB and Notch pathways cooperate at the level of a physical and functional complex between RelA and Hes6.

### *4.1.3. Differences in NF-κB pathway activity between the lateral pallium and medial pallium*

Studies conducted by Robert Hermann, Hosam Al-Jehani, and myself have shown that, in contrast to the robust activation of the NF-κB pathway observed in the lateral pallial GZ, no detectable activation could be observed in the medial pallial GZ, a structure comprising the developing hippocampal cortex and cortical hem. Although these studies cannot exclude the possibility that the NF-κB pathway is activated below detection levels in this region, there is a significant difference in the activation levels between the medial and lateral pallium. This suggests that the NF-κB pathway is important for the regulation of cortical neurogenesis but not hippocampal neurogenesis during embryonic development. The molecular mechanisms involved in this significant difference in activation between the lateral and medial pallium are still unknown. It is possible that the signal(s) activating the NF-κB pathway in the GZ during pallial development is specific to the developing neocortex. Alternatively, uncharacterized processes may inhibit NF-κB pathway activation specifically in the medial pallial GZ. Regardless of the mechanisms, this restricted activation pattern suggests that NF-κB is a selective regulator of cortical, but not hippocampal, neural progenitor behavior. In this regard, recent studies have shown that stress-induced activation of the NF-κB pathway in the adult hippocampus causes impaired adult hippocampal neurogenesis (Koo et al., 2010), raising the possibility that

activation of the NF-κB pathway might be incompatible with neuronal differentiation in the developing ventromedial pallium.

## 4.2. Biological implications of NF-κB pathway activity in cortical gliogenesis

### *4.2.1. Proposed biological model of NF-κB-mediated promotion of astrocytic fate acquisition and/or inhibition of oligodendrocytic fate acquisition during cortical gliogenesis*

In the developing mouse brain, the end of cortical neurogenesis coincides with the onset of the gliogenic phase (Kriegstein and Alvarez-Buylla, 2009). During this transition, cortical NPCs become competent to respond to progliogenic signals. Gliogenic cortical NPCs can undergo glial cell differentiation along different glial cell lineages, mainly along the oligodendrocytic lineage (by differentiating first into OPCs) and the astrocytic lineage (by differentiating into astrocytes directly or by generating astrocyte progenitors that proliferate and diversify into fibrous and protoplasmic astrocytes) (Yue *et al.,* 2006; Molofsky *et al.,* 2012; Rowitch and Kriegstein, 2010). The studies presented in this thesis were aimed at identifying some of the molecular mechanisms that regulate the differentiation of cortical NPCs along the different glial lineages.

More specifically, the described activation of the NF-κB pathway in neocortical GZ at the end of cortical neurogenesis led us to hypothesize that the pathway could remain activated in the cortical GZ during cortical gliogenesis. To this end, I analyzed the spatiotemporal activation pattern of the NF-κB pathway and I obtained evidence that throughout cortical gliogenesis, the pathway is robustly activated specifically in both SVZdl (the cortical GZ during cortical gliogenesis) and SVZstr, but not in SVZspt. This activation pattern was reminiscent of the selective activation in the dorsolateral, but not medial, pallium during the neurogenic phase. Studies aimed at characterizing the identity of the cells in which we observed activation of the NF-κB pathway provided evidence that it is selectively activated in NPCs in the SVZdl and SVZstr during cortical gliogenesis. Importantly, these studies suggested that the NF-κB pathway is activated in cortical and striatal NPCs with gliogenic potential.

To investigate the biological significance of NF-κB pathway activation in cortical NPCs, the cell populations that give rise to the vast majority of oligodendrocytes and astrocytes that

population the cortex, I first sought to characterize the activation pattern of the NF-κB pathway during the transition of cortical NPCs along the astrocytic and oligodendrocytic lineages. These studies suggested that the NF-κB pathway is activated in a subset of white and gray matter astrocytes that could correspond either to (1) cells that are not yet developmentally mature, or (2) specific subpopulations of astrocytes. Due to the lack of appropriate markers, it was not possible to determine whether NF-κB is initially downregulated during the transition from cortical NPCs to newly-born astrocytes and then re-activated at subsequent stages of astrocytic differentiation. Importantly, studies presented in this thesis provided evidence that the NF-κB pathway is downregulated during the transition from cortical NPCs to the oligodendrocytic lineage. This observation is in agreement with studies that provided evidence that NF-κB activation is not required for oligodendrocyte maturation and subsequent myelination of axons in the CNS (Raasch et al., 2011). In the context of NPC biology, this observation suggests that downregulation of NF-κB activity is an important event during the transition of cortical NPCs to the oligodendrocytic lineage.

I described functional studies aimed at investigating the biological significance of NF-κB activation in gliogenic cortical NPCs, in which the NF-κB pathway was blocked at the onset of cortical gliogenesis. These studies provided evidence that the pathway is not required for the maintenance of the undifferentiated NPC state cortical gliogenesis, unlike its role in maintaining the undifferentiated state during the neurogenic phase. During the cortical neurogenic phase, it is crucial that a pool of undifferentiated NPCs is maintained in order to generate late-born neurons and subsequently glial cells. This situation is very different from the gliogenic phase, where the pool of undifferentiated NPCs is progressively depleted until it is completely exhausted by the end of the gliogenic phase (personal observation, not shown here). For this reason, I suggest that there is no requirement for a combination of synergistic and/or parallel molecular mechanisms involved in maintaining the undifferentiated NPC fate. As a result, I suggest that NF-κB activation in cortical gliogenic NPCs is involved in regulatory mechanisms that are not involved in the maintenance of the undifferentiated state.

Instead, my studies suggested that the NF-κB pathway could be important for maintenance of the radial-glial/gliogenic identity of NPCs during cortical gliogenesis and/or for the induced/sustained expression of the *Gfap* gene in cortical NPCs (see section 4.1.2 below). As well, these studies provided evidence that the NF-κB pathway might be important to promote the

acquisition of the astrocytic lineage identity and/or to sustain *Gfap* expression in developing astrocytes. Importantly, I also presented studies suggesting that NF-κB activity in cortical NPCs is required to inhibit/delay the NPC-to-oligodendrocytic lineage transition during cortical development. Together, these studies suggest a possible novel role for the NF-κB pathway in promoting the progression of gliogenic cortical NPCs along the astrocytic lineage while at the same time inhibiting/delaying progression along the oligodendrocytic lineage.

The biological significance of NF-κB-mediated inhibition/delay of oligodendrocytic fate acquisition by NPCs specifically in the developing cortex could be explained in part by the unique ability of cortical NPCs to generate both oligodendrocytes and astrocytes during late embryonic and early postnatal development. In the developing mouse cortex during the gliogenic phase, cells that have acquired an oligodendrocytic fate appear a few days prior to cells that have acquired an astrocytic fate (not shown), suggesting the presence of uncharacterized molecular mechanisms that initially delay/inhibit transition along the astrocytic lineage and promote differentiation along the oligodendrocytic lineage. In this context, the proposed biological role of the NF-κB pathway could be important to antagonize these mechanisms and in this way allow for the maintenance of a population of cortical NPCs that does not undergo oligodendrocytic differentiation, but instead transitions to the astrocytic lineage.

This proposed biological role could also explain the differences in NF-κB pathway activity between SVZdl (robust activation) and SVZspt (no detectable activation). Unlike the SVZdl, where the generation of astrocytes is postponed to postnatal development when the bulk of oligodendrocyte lineage commitment has taken place, astrocytic differentiation in the SVZspt takes place in late embryonic development (personal observation, not shown here). In addition, while the SVZdl generates a large amount of OPCs, it is not clear whether the SVZspt generates any OPCs at all. These differences could suggest that molecular mechanism(s) in place in the SVZdl to delay/inhibit astrocytic differentiation are not present/activated in the SVZspt. This could therefore explain in part why NF-κB activity and its role in inhibiting/delaying oligodendrocyte fate acquisition are not required in the SVZspt.

Finally, the 'gap' in detectable NF-κB activation between the SVZdl and SVZstr, which we hypothesize to correspond to the cortico-striatal boundary, contains $Sox2^{+}$ cells (Figure 33), suggesting that this putative boundary contains a population of neural stem/progenitor cells in which the NF-κB pathway is selectively not activated or selectively downregulated. The

selective expression of *Dbx1*, *Sfrp2*, and *Tgfα* (Mangale *et al.,* 2008; Carney *et al.,* 2009; Caronia-Brown and Grove, 2011) should reveal whether or not this boundary region corresponds to the cortico-striatal boundary.

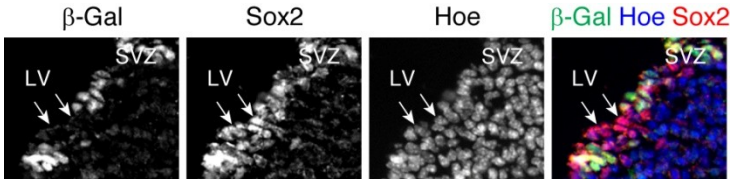


**Figure 33.** No detectable activation of the NF-κB pathway in hypothesized cortico-striatal boundary during cortical gliogenesis. Double-label immunofluorescence analysis of β-Gal and either Sox2 expression in the boundary region between the SVZdl and SVZstr of P1 NF-κB$^{LacZ}$ Arrows point to examples of Sox2$^{+}$ with no detectable β-Gal expression.

*4.2.2. Investigation of the molecular mechanisms underlying NF-κB-mediated promotion of astrocytic fate acquisition and/or inhibition of oligodendrocytic fate acquisition during cortical gliogenesis*

Similar to its functions in other systems, activated NF-κB in gliogenic cortical NPCs could regulate NPC biology and glial cell differentiation through a number of different molecular mechanism(s), including protein-protein interactions and regulation of transcription.

A number of proteins that were shown to physically interact with proteins of the NF-κB pathway in different contexts were also shown to be expressed in the developing cortex at the time of cortical gliogenesis and to regulate various aspects of glial cell differentiation. These proteins could represent potential candidates involved in NF-κB-mediated regulation of gliogenic NPC biology. One of these proteins, Hes6, which forms a complex with the NF-κB subunit RelA in the embryonic cortex (shown here and in Methot *et al*., 2013), is expressed in the postnatal cortical GZ (Allen Developing Mouse Brain Atlas), and was shown to inhibit astrocytic differentiation in the developing cerebral cortex (Jhas *et al.,* 2006). Thus, cortical astrogenesis appears to be another example of a developmental process where NF-κB (promotion) and Hes6 (inhibition) play opposing roles. Another example is the Gro/TLE family of proteins. The function of Hes6 in inhibiting astrocyte differentiation is correlated with its ability to interact with Gro/TLE (Jhas *et al*., 2006). Gro/TLE proteins act as transcriptional

corepressors that participate in mechanisms that can inhibit differentiation in various tissues (Buscarlet and Stifani, 2007). Interestingly, Gro/TLE can also form a physical complex with RelA in other contexts (Tetsuka *et al.*, 2000), and its expression in the SVZdl during cortical gliogenesis co-occurs with activation of the NF-κB pathway (Figure 34). In the future, it will be interesting to investigate the presence and functional significance of interactions between NF-κB, Hes6, and/or Gro/TLE in cortical gliogenesis.

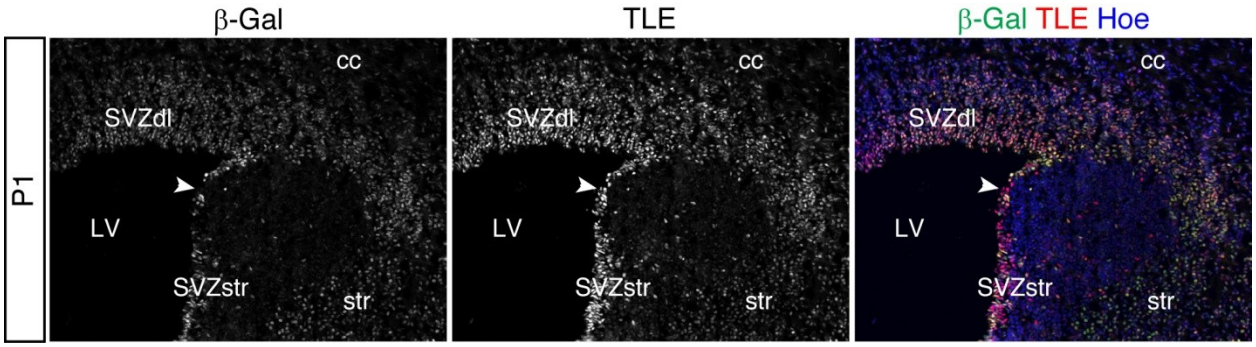


**Figure 34**. Expression of Gro/TLE family of proteins in cells of the SVZdl in which NF-κB pathway is activated. Double-label immunofluorescence analysis of β-Gal and Gro/TLE (TLE) expression in the telencephalon of P1 NF-κB$^{LacZ}$ mice showing almost complete co-expression in dorsolateral subventricular zone (SVZdl) and striatal subventricular zone (SVZstr). Arrowheads point to a region with Gro/TLE expression but no detectable β-Gal expression between the SVZdl and the SVZstr. cc, corpus callosum; LV, lateral ventricle; str, striatum.

Additionally, NF-κB could also regulate cortical gliogenesis through transactivation of target genes involved in glial cell differentiation. Because hundreds of NF-κB target genes have been described and validated in different contexts (Pahl, 1999), I propose that a candidate gene approach may represent an efficient strategy to identify NF-κB target genes in gliogenic NPCs. Specifically, possible NF-κB candidate target genes could be proposed on the basis of three criteria: (1) validated binding of NF-κB to their promoter; (2) their expression in the SVZdl during cortical gliogenesis; and (3) proteins encoded by candidate genes are known to be involved in astrocytic development and maturation. A number of genes match these criteria, including the cytokines *Interleukin-6* (*Il-6*) (Nakanishi *et al.*, 2007) and *Ccl2* (Lawrence *et al.,* 2006), the astroglial glutamate transporter *Slc1a2* (Gosh *et al.,* 2011), and the phosphatase *Pten* (Banerjee *et al.,* 2011), to name only a few.

In order to perform studies aimed at identifying target genes of NF-κB in cortical gliogenesis, together with a former undergraduate student in the Stifani lab, Erin Campos, I established an *in vitro* experimental system of cortical glial cell differentiation in which primary cultures of cortical NPCs are allowed to undergo glial cell differentiation along the astrocytic and oligodendrocytic lineages. This *in vitro* experimental system can allow testing for the regulation of candidate gene and protein expression by the NF-κB pathway through qRT-PCR analysis and Western blotting following NF-κB inhibition or knockdown, and testing for binding of endogenous NF-κB to promoter of candidate gene through chromatin immunoprecipitation (ChIP). Candidate genes validated *in vitro* will have to be validated *in vivo* to confirm that target gene expression is regulated by the NF-κB pathway and that these genes perform biological functions in the regulation of cortical glial cell differentiation through rescue experiments.

We have performed initial studies with this *in vitro* experimental system of glial cell differentiation by blocking endogenous NF-κB pathway through pharmacological inhibition of IKKβ with the inhibitor IKK-2 Inhibitor IV. Remarkably, this approach led to a significant reduction in the expression of the astrocytic protein GFAP (Figure 35), while the levels of several other proteins analyzed were not significantly modulated under these specific *in vitro* conditions. These initial studies suggest that *Gfap* expression is specifically regulated by NF-κB in cortical NPCs during cortical gliogenesis. This possibility is supported by the presence of conserved NF-κB binding sites in the mouse *Gfap* promoter (Bae *et al.,* 2006). Determining whether *Gfap* is a direct target of NF-κB in gliogenic cortical NPCs will be key for interpreting the phenotype observed *in vivo* where blockade of the NF-κB pathway resulted in the reduction in the number of manipulated cells expressing GFAP.

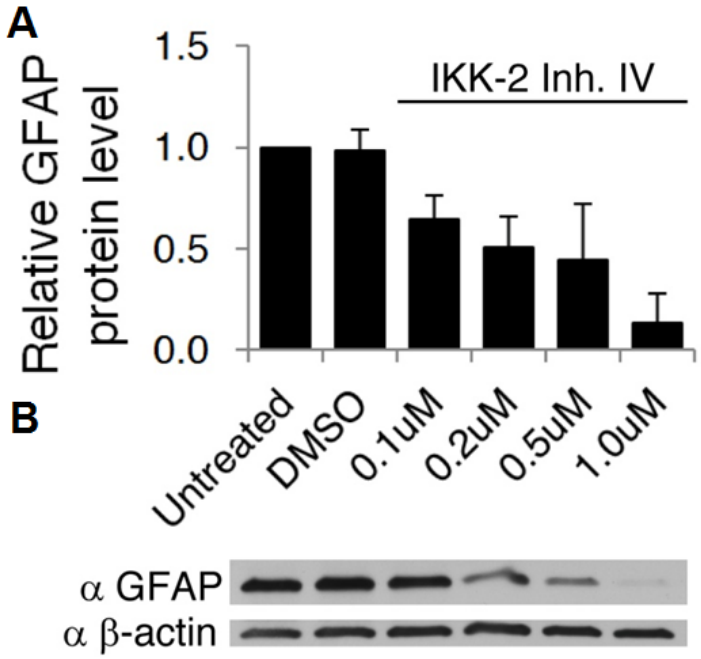


**Figure 35.** Regulation of GFAP expression by the NF-κB pathway during cortical gliogenesis. (A) Quantification of GFAP expression detected by Western blotting analysis in primary cultures of cortical progenitor cells untreated, treated with DMSO alone, or treated with increasing concentrations of IKK-2 Inhibitor IV, as indicated ($n$ = 2). (B) One representative Western blotting analysis ($n \geq 3$) of GFAP expression quantitated in (A). β-actin is shown as loading control.

Finally, it is important to note that target genes of NF-κB in cortical neurogenesis and in cortical gliogenesis are not expected to be the same. Chromatin modifications during the neurogenic-to-gliogenic transition will allow for the binding of NF-κB to a distinct set of genes in neurogenesis compared to gliogenesis. For example, if NF-κB does indeed bind to the *Gfap* promoter and activate transcription during cortical gliogenesis, the same event could not occur during cortical neurogenesis as the *Gfap* promoter is not accessible to DNA-binding proteins in neurogenic cortical NPCs. This possible discrepancy between NF-κB target genes during cortical neurogenesis and gliogenesis could potentially underlie some of the differences between the biological functions described for NF-κB at different stages of corticogenesis.

## 4.3 Perturbations in neural progenitor cell biology in neurodevelopmental disorders

### *4.3.1. The NF-κB pathway and inflammatory and immune system response in cortical neurogenesis*

The NF-κB pathway mediates inflammation and immune response in numerous diseases, including neuropathologies of the central nervous system such as neurodegenerative diseases, brain cancer, cerebral ischemia, traumatic brain injury, and spinal cord injury. In neurons, astrocytes and microglia, the NF-κB pathway is modulated by inflammatory signals and has been shown to regulate pro- and anti-inflammatory responses that either contribute to pathogenesis or promote neuroprotection (Maqbool *et al*., 2013; Bracchi-Ricard *et al*., 2013). The functions of the NF-κB pathway in cerebral cortex development in the context of prenatal neuroinflammation, however, are still uncharacterized.

Prenatal inflammation has been linked to perturbations of cerebral cortex development underlying pathogenesis of neurodevelopmental defects such as Schizophrenia and Autism spectrum disorders (Patterson, 2007; Patterson, 2011). This type of inflammation can be caused by a maternal immune response to pathogens or viral infection which releases pro-inflammatory cytokines that are capable of entering fetal circulation and crossing the blood-brain-barrier (Watanabe *et al.,* 2010). In addition to maternally secreted cytokines, the placenta and fetal microglia can also secrete pro-inflammatory molecules as a response to the maternal immune response, and in this way constitute a second source of cytokine that amplify the pro-inflammatory response (Meyer el al, 2009; Hagberg *et al.,* 2012). These events trigger a cytokine surge that perturb cytokine levels in the prenatal brain and disrupt the balance between pro- and anti-inflammatory cytokines required for normal nervous system development (Deverman and Patterson, 2009;Meyer *et al.,* 2009). In addition, a cytokine surge can cause immune cells priming which renders the nervous system more vulnerable to secondary insults (Madhusudan *et al.,* 2012). Such events have been suggested to alter fetal and neonatal brain development in a number of ways, for example as through perturbations of neuronal number, migration, apoptosis, dendritic morphology, neurotransmission, myelination, and embryonic NPC biology (Hagberg *et al.,* 2012; Gallagher *et al.,* 2013).

Several groups have described functions for various cytokines and cytokine receptors in the regulation of embryonic forebrain NPCs in normal and pathological prenatal brain development (Devermann and Patterson, 2009). Combinations of *in vivo* and *in vitro* studies have shown that cytokine signaling is important for the maintenance of embryonic NPC pool through regulation of NPC self-renewal and neuronal differentiation. Importantly, rodent studies have provided evidence that a single maternal cytokine surge during prenatal development can alter embryonic NPC biology and lead to perturbations in neural stem cell biology sustained in neonatal and adult life (Gallagher *et al.,* 2013). More specifically, embryonic NPC self-renewal and pool number is regulated by an endogenous interleukin-6-dependent pathway, which can be aberrantly activated in the context of maternal inflammatory response, leading to deregulated NPC self-renewal and an increased NPC number. Based on the ability of cytokine signaling to activate the NF-κB pathway in neural cells, I hypothesize that endogenous and pathological cytokines and cytokine receptor activation could regulate embryonic NPC self-renewal and neuronal differentiation in part through regulation of NF-κB pathway activation. Investigating this hypothesis would allow identifying novel factors involved in the response of NPCs to cytokine signaling. A comprehensive description of the functions of the NF-κB pathway in mediating the response of NPCs to neuroinflammation could also provide important information about the etiology of neurodevelopmental disorders that involve dysregulated inflammatory signaling in the central nervous system. For example, patients with Autism spectrum disorders display increased NF-κB expression and pathway activation in the orbitofrontal cortex (Young *et al.,* 2011) raising the possibility that aberrant NF-κB pathway activation could have also been present in the prenatal brain and perturb cortical neurogenesis.

### *4.3.2. Perturbations in NF-κB pathway and intellectual disability*

A number of studies have identified mutations associated with intellectual disability within two genes involved in NF-κB signaling. Mutations in the *TRAPPC9* gene, which encodes a NIK and IKKβ Binding Protein (NIBP), were detected in four separate families with individuals affected by autosomal recessive non-syndromic mental retardation (Mir *et al.*, 2009; Mochida *et al.*, 2009; Philippe *et al.*, 2009). Cultured skin fibroblasts from individuals with homozygous mutations in *TRAPPC9* show impaired NF-κB signaling (Philippe *et al.*, 2009). Importantly,

NIBP is expressed throughout the embryonic cerebral cortex during neurogenesis (Mochida *et al.*, 2009) and enhances canonical NF-κB signaling in neuronal cells (Hu et al., 2005). Mutations in the *CC2D1A* gene have also been linked to autosomal-recessive non-syndromic mental retardation (Basel-Venagaite *et al.*, 2006; Rogaeva *et al.*, 2007). *CC2D1A* is expressed in the VZ of the developing brain during neurogenesis (Basel-Venagaite *et al.*, 2006) and the CC2D1A protein promotes activation of canonical NF-κB signaling (Zhao *et al.*, 2010). Taken together with the present findings, these results suggest that NF-κB signaling might be defective in these patients during brain development, resulting in an impaired generation and/or maturation of cortical neurons. This possibility is consistent with the microcephaly observed in some affected individuals carrying *TRAPPC9* mutations. In the future, it will be important to determine whether disruption of the functions of the mouse orthologs of NIBP or CC2D1A might cause perturbations of cortical neurogenesis.

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