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# DIPLOMARBEIT

Titel der Diplomarbeit

## Involvement of Nf- $\kappa$ B in neural cortical development

angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag. rer.nat.)

|                                                      |                                       |
|------------------------------------------------------|---------------------------------------|
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Wien, im

September 2009



## Abstract

During mammalian neural development, a wide variety of neurons and glial cells differentiate from common precursor cells. Regulation of the transition from the initial growth phase to the subsequent differentiation of postmitotic cells is crucial for proper development of the nervous system. Premature differentiation and overproliferation both have severe impacts on neural development and the size, shape and integrity of brain structures.

Differentiation choices of multipotent neural progenitor cells are regulated by antagonistic activities of a number of positively and negatively acting transcription factors, including proteins containing the basic-helix-loop-helix (bHLH) DNA binding and dimerization motif. However, the precise mechanisms controlling neuronal and glial differentiation are not completely understood and additional transcription factors and signaling pathways controlling those events are being identified constantly.

The bHLH proteins of the Hairy and Enhancer of split (Hes) family are important regulators of brain development. Certain members, such as Hes 1 and Hes 5, are activated by the Notch signaling pathway and function to inhibit neuronal differentiation. In contrast, a different Hes family member, termed Hes 6, is not activated by Notch signaling and promotes, rather than inhibiting, neuronal differentiation. Hes 6 also acts to inhibit astrocyte differentiation, in contrast to Hes 1 and Hes 5. The molecular mechanisms underlying the opposite functions of Hes 6 and Notch-activated Hes proteins remain to be defined. In that regard, numerous previous studies have shown a functional cross-talk between Notch and NF- $\kappa$ B signaling pathways in a variety of tissues, suggesting that these pathways might also intersect in the nervous system. NF- $\kappa$ B signaling was shown to be important for neuronal survival, plasticity, learning, memory consolidation, neuroprotection and neurodegeneration. The NF- $\kappa$ B pathway was shown to be activated in the developing forebrain from at least embryonic day 13, but little is known about its involvement in brain development.

It is shown here for the first time that NF- $\kappa$ B signaling is activated in neural progenitor cells of the developing telencephalon. Moreover, the NF- $\kappa$ B complex protein, p65/RelA (RelA) physically and functionally interacts with the Hes 6, but not Hes 1. More importantly, RelA antagonizes Hes 6-mediated promotion of telencephalic neuronal differentiation, suggesting antagonistic roles for these factors during forebrain neurogenesis. Finally, it is shown that RelA also inhibits astrocyte differentiation, similar to Hes 6. Taken together, these results provide new evidence that NF- $\kappa$ B signaling is involved in the regulation of neuronal and glial differentiation during mammalian forebrain development and that at least some of its functions involve interactions with Hes 6.

## Zusammenfassung

Während der neuralen Entwicklung von Säugetieren differenziert eine Vielzahl an diversen Neuronen und Gliazellen aus gemeinsamen Vorläuferzellen. Die zeitliche Koordinierung von der Wachstumsphase auf die folgende Differenzierung der post-mitotischen Zellen ist für die normale Entwicklung des Nervensystems von entscheidender Bedeutung. Vorzeitige Differenzierung und übermäßiges Wachstum haben schwere Auswirkungen auf die neuronale Entwicklung sowie die Größe, Form und Integrität von Hirnstrukturen.

Differenzierungsprozesse von multipotenten neuralen Vorläuferzellen werden durch antagonistische Aktivitäten von Transkriptionsfaktoren, welche eine bestimmte DNA-bindungs- und Dimerisationsdomäne besitzen (die sogenannte basic-helix-loop-helix (bHLH) Domäne), reguliert. Die genauen Mechanismen sind noch nicht vollständig aufgeklärt und es werden laufend neue Faktoren und Signalwege identifiziert.

Die bHLH Proteine der Hairy and Enhancer of split (Hes) Familie sind wichtige Regulatoren der Gehirnentwicklung. Bestimmte Mitglieder der Familie, wie z.B. Hes1 und Hes5, werden über den Notch Signalweg aktiviert und inhibieren neuronale Differenzierung. Ein weiteres Mitglied, Hes6, hingegen wird nicht über den Notch Signalweg aktiviert und begünstigt die Differenzierung zu Neuronen. Weiters inhibiert Hes6 die Differenzierung zu Astrozyten, im Gegensatz zu Hes1 und Hes5. Die molekularen Mechanismen die den entgegensetzenden Funktionen von Hes6 und Notch-aktivierten Hes Proteinen unterliegen, sind noch zu definieren. In diesem Zusammenhang haben viele Studien einen funktionellen "cross-talk" zwischen Notch und NF- $\kappa$ B Signalwegen in verschiedenen Geweben gezeigt. Dies weist auf eine mögliche Interaktion der beiden Signalwege im Nervensystem hin. Es ist bekannt, dass NF- $\kappa$ B für viele Funktionen im Nervensystem wichtig ist, z.B. Lernen, Gedächtnis-Konsolidierung, Neuroprotektion und Neurodegeneration. Es wurde gezeigt, dass der NF- $\kappa$ B Signalweg im Vorderhirn ab dem 13. Tag der embryonalen Entwicklung aktiviert ist, bis jetzt aber nur sehr wenig über seine Rolle in der Gehirnentwicklung bekannt ist.

In dieser Arbeit wird erstmals gezeigt, dass NF- $\kappa$ B in neuronalen Vorläuferzellen im sich entwickelnden Telencephalon aktiviert ist. Weiters wird gezeigt, dass das NF- $\kappa$ B-Komplex-Protein p65/RelA (RelA) physisch und funktionell mit Hes6, aber nicht mit Hes1 interagieren kann. Wichtiger noch, RelA antagonisiert die Hes6-vermittelte Förderung neuronaler Differenzierung. Schlussendlich wird gezeigt, dass RelA – ähnlich wie Hes6 – die Differenzierung zu Astrozyten inhibiert. Zusammengekommen zeigen diese Ergebnisse eine Involvierung des NF- $\kappa$ B Signalweges in der Regulierung von neuronaler und glialer Differenzierung im Vorderhirn auf. Einige dieser Funktionen beinhalten dabei eine Interaktion mit Hes6.

## Acknowledgements

Foremost, I would like to express my sincere gratitude to my advisor Prof. Stefano Stifani for giving me the opportunity to pursue my thesis work in his lab at the Montreal Neurological Institute, his continuous support, mentoring and guidance.

Special thanks to Prof. Johannes Nimpf for supervision of my diploma thesis and his support throughout my studies.

I also want to thank my friends and colleagues at the MNI for all the fruitful discussions, inspirations and for making my stay in Montreal so wonderful.

I owe my deepest gratitude to my mother Marcela and my sister Anna-Marie for their unflagging love and support; this work would not have been possible without you.

Last but not least, I want to thank some very special friends: Martin Pfeffer, Murat Sahin, as well as Katharina and Stephan Wurzer, and all other members of the Molecular Biological Society.

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## Abbreviations

|              |                                                           |
|--------------|-----------------------------------------------------------|
| ACX          | archicortex                                               |
| AS-C         | Achaete-scute complex                                     |
| ATP          | Adenosine triphosphate                                    |
| $\beta$ -gal | $\beta$ -galactosidase                                    |
| bHLH         | basic-helix-loop-helix                                    |
| CBP          | CREB binding protein                                      |
| CGE          | caudal ganglionic eminences                               |
| CK2          | casein kinase 2                                           |
| CNTF         | ciliary neurotrophic factor                               |
| CP           | cortical plate                                            |
| CXH          | cortical hem                                              |
| DIV          | day in vitro                                              |
| Dll1         | Delta-like ligand 1                                       |
| DNA          | Deoxyribonucleic acid                                     |
| DTT          | Dithiothreitol                                            |
| EDTA         | 2,2',2'',2'''-(ethane-1,2-diyl dinitrilo)tetraacetic acid |
| FGF          | fibroblast growth factor                                  |
| GFAP         | glial fibrillary acidic protein                           |
| GFP          | green fluorescent protein                                 |
| HAT          | Histone acetyltransferase                                 |
| HBS          | HEPES buffered saline                                     |
| HCl          | Hydrochloric acid                                         |
| HDAC         | Histone deacetylase                                       |
| HEK          | human embryonic kidney                                    |
| Hes          | Hairy and Enhancer of split                               |
| HIV          | human immunodeficiency virus                              |
| HRP          | horseradish peroxidase                                    |
| IKK          | I $\kappa$ B kinase                                       |
| IL-1         | Interleukin-1                                             |
| IP           | immunoprecipitation                                       |
| LGE          | lateral ganglionic eminences                              |
| LPS          | Lipopolysaccharide                                        |

|                   |                                              |
|-------------------|----------------------------------------------|
| LTR               | long terminal repeat                         |
| MAP2              | Microtubule-associated protein 2             |
| MAPK              | mitogen activated protein kinase             |
| MgCl <sub>2</sub> | Magnesium chloride                           |
| MGE               | medial ganglionic eminences                  |
| MgSO <sub>4</sub> | Magnesium sulfate                            |
| NaCl              | Sodium chloride                              |
| NCX               | neocortex                                    |
| NEMO              | NFκB essential modifier                      |
| NeuN              | neuronal nuclei                              |
| Ngn2              | Neurogenin 2                                 |
| NICD              | Notch intracellular domain                   |
| NLS               | nuclear localization signal                  |
| ONPG              | Ortho-nitrophenyl-β-galactoside              |
| PBS               | phosphate buffered saline                    |
| PCR               | Polymerase chain reaction                    |
| PFA               | paraformaldehyde                             |
| ROS               | rat osteoblastic                             |
| SCF               | Skp1-Cullin-F-box                            |
| SDS               | Sodium dodecyl sulfate                       |
| SV40              | simian virus 40                              |
| TBS               | Tris buffered saline                         |
| TLE               | Transducin-like enhancer of split            |
| TNF               | Tumor necrosis factor                        |
| Tris              | 2-Amino-2-hydroxymethyl-propane-1,3-diol     |
| VZ                | ventricular zone                             |
| X-gal             | 5-bromo-4-chloro-3-indolyl-β-D-galactosidase |

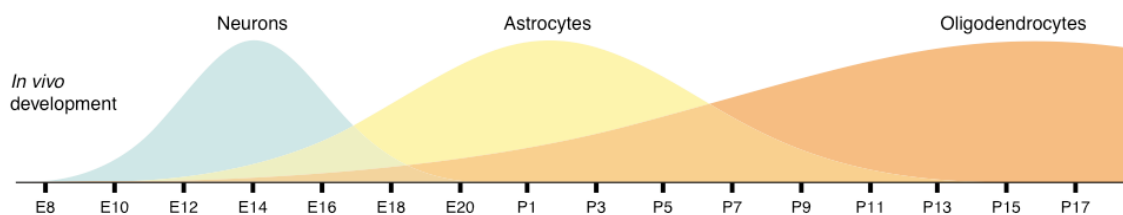


# 1 Introduction

## 1.1 Neural development in the mammalian forebrain

During mammalian neural development, a wide variety of neurons and glial cells differentiate from common precursor cells. Neuroepithelial cells are first generated from the ectoderm, forming the neural plate and then the neural tube.

These cells initially undergo symmetric divisions and then gradually become radial glia, the cells that acts as progenitors of the majority of brain neurons. During neurogenesis, radial glia divide asymmetrically into neuronal precursors and new radial glia. When gliogenesis starts, they rise to glial-restricted precursor cells that will generate astrocytes and oligodendrocytes [39]. Figure 1 shows a schematic representation of the temporary distinct, yet overlapping, phases of neuronal and glial differentiation in the mammalian forebrain. The transition from the initial proliferative phase to the subsequent differentiation of postmitotic cells needs to be tightly regulated for proper development of the nervous system. Premature differentiation and excessive proliferation both have severe impacts on neural development and the the size, shape and integrity of brain structures.



**Figure 1:** Temporal pattern of the generation of neurons, astrocytes and oligodendrocytes. In rats, neurogenesis peaks at E14, astrocytogenesis at P2, and oligodendrocytogenesis at P14. Taken from Sauvageot and Stiles [57].

## 1.2 Cortical development

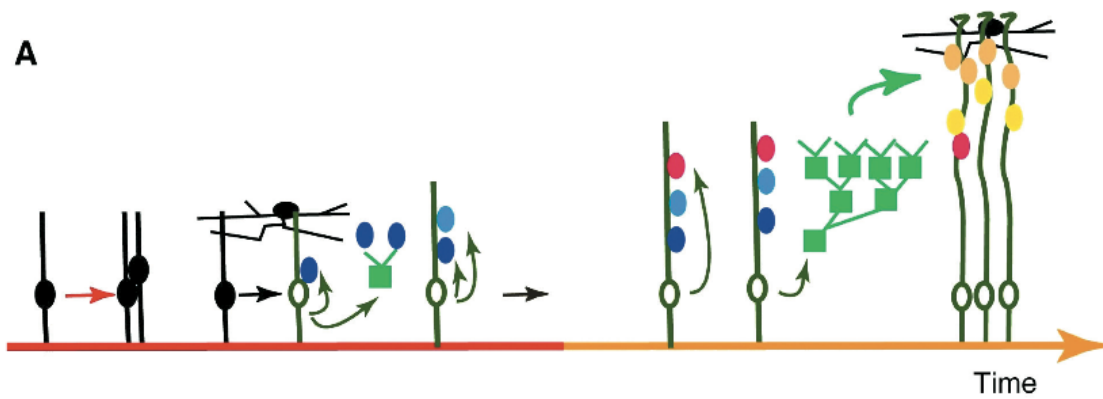
The most anterior part of the neural tube develops into the telencephalon. The dorsal part, or pallium, gives rise to the neocortex (isocortex) and hippocampus, while the ventral part of the telencephalon, or subpallium, forms the basal ganglia. The

neocortex is an evolutionary acquisition of mammals and represents the brain territory that has undergone a major increase in its relative size during the course of mammalian evolution (reviewed by Pierani and Wassef [54]). It is considered to be key of our cognitive, perceptual, and emotional abilities.

Development of the complex cortical architecture relies on precise and tightly controlled spatiotemporal generation of distinct cell types as well as control of their migration and cell death.

Progenitor cells that give rise to differentiated neural cells are located in the germinal zone (ventricular zone) lining the brain ventricles. Three types of neural progenitors are present during brain development: neuroepithelial cells, radial glia and basal progenitors (reviewed in [54]) (see Figure 2). First, neuroepithelial cells divide and expand before they give rise to radial glia. Radial glia then divide asymmetrically to form another radial glia cell and either a neuron, or a basal progenitor. Basal progenitors can perform a small number of symmetric divisions before they finally generate neurons. Later in development, radial glia cells give rise to astrocytes and oligodendrocytes.

In the dorsal telencephalon (neocortex) of rodents, glutamatergic neuronal subtypes are produced in the local pallium ventricular zone and reach their final destination through radial glia cell mediated migration. They form the neocortical layers II - VI through radial migration in an inside-out manner (neurons of deeper layers are generated earlier than those in more superficial positions). Interestingly, it was shown that timing of cortical neurogenesis is encoded within lineages of individual progenitor cells [60]. For instance, Cajal-Retzius cells (which together with subplate cells are among the first neuronal classes generated) reside in the most superficial layer (layer I) and play an important role in positioning of other layers via expression of the extracellular glycoprotein Reelin. All cortical layers also comprise GABAergic interneurons that are generated in extra-cortical regions (mainly the medial and caudal ganglionic eminences in the striatum) and reach their final position by tangential migration into the cortex. Oligodendrocytes in the neocortex are also generated from progenitors in the subpallium and reach their final destination by tangential migration.



**Figure 2:** Schematic representation of progenitor progression during neurogenesis. Neuroepithelial cells (black, left side) divide and then form radial glia (dark green circles, long extensions) which in turn give rise to basal progenitors (green squares) and neuronal precursors (dark blue, blue, red, yellow and orange filled circles). Taken from Pierani and Wassef [54].

### 1.3 Regulation of cortical development by basic helix-loop-helix factors

The differentiation of multipotent neural progenitor cells is regulated by antagonistic activities of a number of positively and negatively acting transcription factors containing the basic-helix-loop-helix (bHLH) DNA binding and dimerization motif.

Neurogenic bHLH-factors act as heterodimers with the ubiquitous bHLH protein E47 by binding to DNA sequences referred to as E-boxes (CANNTG) and activating genes that promote the neural fate. Examples of neurogenic proteins include members of the Mash, Math, Neurogenin and NeuroD families. These factors are related to the *Drosophila* pro-neuronal proteins Atonal and the achaete-scute complex (AS-C) belong to this group and are expressed at distinct stages of mammalian neural development. Evidence for the proneuronal function of those proteins is provided by targeted gene disruption – e.g. in Mash1-null mice differentiation of three types of neurons (autonomic, olfactory and retinal) is severely affected [37].

While bHLH-factors such as Mash1 promote neurogenesis, another set of bHLH-factors antagonizes the former and inhibits neuronal differentiation. This ‘repressor’ type bHLH-factors include proteins such as Hes 1 and Hes 5 from the *Hes* family (which are related to *Drosophila* Hairy and Enhancer of split proteins), as well as Id (Inhibitor of differentiation) family members [37]. Hes transcription factors mediate transcriptional repression and bind preferentially to DNA sequences referred to as

N boxes (CACNAG) [26]. Persistent expression of Hes 1 inhibits neuronal development, while disruption of its function leads to premature differentiation of neuronal cells and the up-regulation of proneuronal genes [33, 32, 65]. Inhibition of neuronal differentiation by Hes factors is based on multiple mechanisms, including negative regulation of proneuronal gene expression and inhibition of E47-proneuronal protein heterodimerization and transcriptional activity [3]. The WRPW sequence found in the carboxy-terminal regions of Hes factors is essential for transcriptional repression via recruitment of the Groucho corepressor (or its mammalian homologues Transducin-like enhancer of split; TLE).

Thus, by preventing premature neuronal differentiation, these 'repressor' type bHLH-factors play an important role in regulation of the timing of neuronal differentiation.

#### **1.4 Regulation of 'repressor-type' bHLH-genes by the Notch pathway**

Expression of bHLH genes of the Hes family is regulated by Notch, a transmembrane protein activated by ligands such as Delta and Jagged which are expressed by neighboring cells differentiating into neurons (reviewed by Kageyama and Nakanishi [37], Kageyama et al. [36], Kageyama and Ohtsuka [38]).

Upon activation of Notch, its intracellular domain ( $N^{ICD}$ ) is cleaved off and translocates into the nucleus where it forms a complex with the DNA binding protein RBP-J. When not in a complex with  $N^{ICD}$ , RBP-J acts as a transcriptional repressor and also represses Hes 1 and Hes 5 expression by binding to their promoters. In contrast, the  $N^{ICD}$ -RBP-J complex acts as a transcriptional activator and induces Hes 1 and Hes 5 expression. These factors then inhibit neuronal differentiation by transcriptional repression, and biochemical inhibition of proneuronal bHLH proteins.

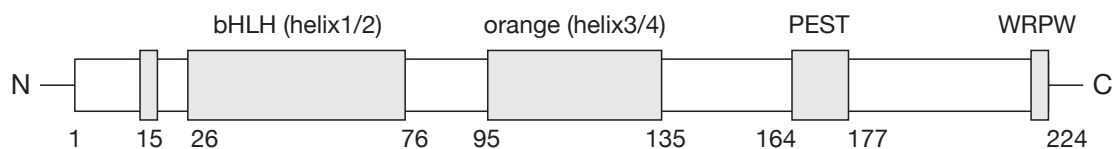
Lateral inhibition by Notch signaling is known to play a crucial role during neural development in *Drosophila*. In a classical view, the generation of a salt and pepper pattern of undifferentiated and differentiating cells depends on lateral inhibition by the Notch pathway. At first, all neural progenitor cells are equivalent, but some cells will eventually express higher levels of the Notch ligand, Delta due to stochastic variations and thus will more efficiently activate Notch in neighboring cells. Consequently, lateral inhibition amplifies stochastic variations and leads to

a salt and pepper pattern in gene expression. Recent findings in mammalian cells have challenged the stochastic notion of lateral inhibition by showing that Hes 1 expression oscillates in neural progenitor cells and, moreover, that Hes 1 drives the oscillation of Neurogenin 2 (Ngn2) and Delta-like ligand 1 (Dll1) [61]. Thus, a cell expressing high levels of Ngn2 and Dll1 at one point may not necessarily differentiate into a neuron. The expression pattern of these proteins is dynamic and subject to change, suggesting that while lateral inhibition is important for the maintenance of the progenitor pool, it is not involved in neuronal selection (see Kageyama et al. [40]).

### 1.5 The bHLH-factor Hes6

A unique member of the Hes family, termed Hes 6, was first identified in 2000 by several groups [3, 55, 45, 66]. In contrast to other Hes proteins, Hes 6 promotes neuronal differentiation and also inhibits astrocyte differentiation. Moreover, Hes 6 is not activated in response to Notch signaling; rather, it is positively regulated by neurogenin2, which itself is a target of Notch-mediated inhibition.

Hes 6 consists of 224 amino acid residues and is structurally closely related to other Hes family members. It comprises a basic-helix-loop-helix motif at its N-terminal end, followed by two helices forming the helix3/helix4 domain, a proline/glutamic acid/serine/threonine (PEST) region, and a WRPW tetrapeptide at its the C terminal end (see figure 3).



**Figure 3:** The bHLH transcription factor Hes 6. The mature murine Hes 6 protein consists of 224 amino acids. Functional domains are indicated and described in the main text.

The bHLH motif mediates DNA binding and homodimerisation, as well as heterodimerization with other bHLH factors. The loop region between the first and second helix of the bHLH domain of Hes6 is 4 or 5 residues shorter in compared to

other Hes factors. This unique feature of Hes6 is in part responsible for the different biological functions of Hes6 compared to other Hes family members [3].

The helix3/helix4 domain is important for functional specificity among Hes proteins [15]. In the case of Hes6, it contains a short sequence (LNHLL) which resemble the LXXLL motif found in other transcriptional regulators. LXXLL motifs were shown to mediate protein-protein interactions [34]. In many proteins, PEST regions are cis-acting sequences regulating protein turn-over and were suggested to be activated via phosphorylation. The PEST region of Hes6 contains SPXXSP and SDXE motifs, which were shown to be phosphorylated by mitogen activated protein kinase (MAPK) [4] or protein kinase CK2 (casein kinase 2) [26], respectively.

Phosphorylation of S183 in the SDXE motif has a positive effect on the ability of Hes6 to induce a proteolytic degradation of Hes1 and a S183A point mutation is correlated with a reduced pro-neuronal activity of Hes6 [26]. On the other hand, an intact SPXXSP motif is required for the anti-astrogenic activity of Hes 6, but is not correlated with suppression of Hes 1 transcription repression activity [4].

The WRPW motif is found in all Hes family members and is known to recruit co-repressors like Groucho/TLE [52] but also plays a role in the stability of proteins, as it was shown to mediate proteosomal degradation of Hes 6 [44]. The WRPW motif of Hes6 was reported to be important for its anti astrogenic, but not pro-neuronal activity.

The WRPW motif is found in all Hes family members where it recruits transcriptional co-repressors of the Groucho/TLE family[52]. The WRPW motif also plays a role in the stability of proteins, as it was shown to mediate proteosomal degradation of Hes 6 [44]. The WRPW motif of Hes6 is important for its anti astrogenic, but not pro-neuronal activity [34, 4].

### **1.5.1 Involvement of Hes 6 in neural development**

In mouse embryos, Hes6 expression first occurs first at E8.5 in the forebrain and optic vesicles, followed by a later expansion to other neuronal tissues. Hes6 is also expressed in non-neuronal tissues and it is suggested to be involved in the differentiation of a number of cell types. Hes6 is expressed in both undifferentiated and

differentiated cells, unlike other members of the Hes family which are preferentially, or exclusively expressed in the former [3]. Hes6 expression in *Xenopus* resembles that in the mouse, suggesting that aspects of its expression pattern are conserved in evolution [45].

Converse to the function of the related Hes family members Hes1 and Hes5, Hes6 does not inhibit, but promotes, neuronal differentiation, acting in a positive feedback loop with pro-neuronal bHLH factors [45].

Hes6 alone does not bind to N box or E box DNA sequences, like Hes1 or most other bHLH factors do [3, 45]. Mutants of Hes6 lacking the WRPW motif can still promote neuronal differentiation, suggesting that Hes6 does not need to bind DNA and/or the co-repressor Groucho/TLE for its pro-neuronal activity [45]. Although Hes6 was shown to interact with a specific type of the E box sequence, the ESE box (TGGCACGTGCCA) [14], the physiological significance of this observation is unclear since Hes6 does not mediate transcriptional repression from promoters containing ESE boxes in transfected cells Jhas et al. [34].

The loop region between the two helices of the bHLH domain was shown to be important for the specificity of Hes6 functions. Alterations in the loop region of Hes6 (insertion of five amino acids) or Hes1 (deletion of five amino acids) revealed that at least some of the specific features of those two proteins are interchangeable. While the insertion of five amino acids in the loop of Hes6 was not sufficient to fully confer activities typical of Hes1, deletion of just five amino acid residues from Hes1 was sufficient for functional conversion of Hes1 to Hes6 [3].

In addition to promoting neurogenesis, Hes6 inhibits astrocyte differentiation. These two activities involve different molecular mechanisms [34]. While dispensable for its neurogenic function, the WRPW motif and the LNHLL sequence in the helix3/helix4 domain of Hes6 are required for its anti-astrogenic activity [34]. Studies by Jhas et al. [34] suggested a correlation between the ability of Hes6 to suppress Hes1 mediated transcriptional repression and its anti-gliogenic, but not proneuronal, activity. However, more recent work has shown that Hes6 anti-astrogenic activity is not correlated with suppression of Hes1 -mediated transcriptional repression, but it requires amino- and carboxy-terminal motifs important for dimerization and phosphorylation [4]. These results suggest a model in which Hes6 homodimers form

a complex with other, yet unknown, proteins endowed with DNA-binding ability and recruit Grouch/TLE via the WRPW motif, resulting in transcriptional repression of the target gene. Separate studies showing the ability of Hes6 to mediate transcriptional repression in a WRPW motif-dependent manner when targeted to DNA by a heterologous DNA-binding domain [21], as well as the ability of the chicken homolog *hes6-2* to repress *Hes5* expression [18] support this model.

The precise mechanisms by which Hes 6 mediates pro-neuronal and anti-astrogenic effects are not well understood. Future identification of modes of Hes 6 recruitment to DNA and/or additional Hes 6 interacting proteins are expected to help to elucidate those mechanisms.

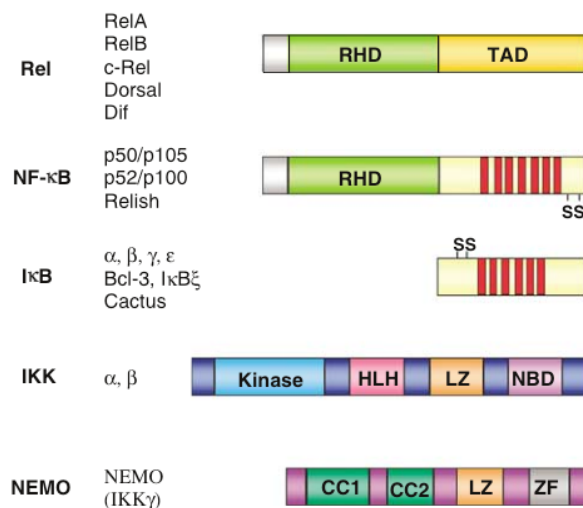
## **1.6 The NF- $\kappa$ B signaling pathway**

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) was first described in a paper by Sen and Baltimore [59] in 1986 as a nuclear factor binding to the  $\kappa$  light chain enhancer sequence in activated B cells. Soon, other proteins were found to be related to NF- $\kappa$ B, which together form the NF- $\kappa$ B superfamily. This superfamily comprises two subfamilies, namely 'Rel' proteins (RelA, RelB, c-Rel, *Drosophila* Dorsal and Dif) and 'NF- $\kappa$ B' proteins (p50/p105, p52/p100 and *Drosophila* Relish) [25]. All members contain a highly conserved Rel homology domain (RHD) at their N-terminal end, which is essential for dimerization and DNA-binding of these factors [24]. The C-terminus of Rel proteins contains a transactivation domain which is often not conserved across species (at the sequence level). 'NF- $\kappa$ B' proteins lack such transactivation domains; instead, their C-termini contain multiple ankyrin repeats, which act to negatively regulate these proteins. However, these ankyrin repeats are only present in the precursor proteins (p105, p100) which – upon stimulation – are autoproteolytically processed to form p50 and p52. Consequently, 'NF- $\kappa$ B' proteins do not act as activators of transcription themselves, except when they form dimers with members of the 'Rel' subfamily.

NF- $\kappa$ B factors act as hetero- or homodimers that bind 9-10 bp long  $\kappa$ B sites. These DNA sites have a high grade of variability, with the consensus sequence: 5' – GGGRNWYYCC–3' (where R is a purin, Y is a pyrimidine, W is A or T, and N is any nucleotide) [25]. All family members can form homo- and heterodimers, except for

RelB, which only forms heterodimers *in vivo*. p50/RelA form the most abundant dimer, which is also the one first identified by Sen and Baltimore [59] and therefore often referred to as simply 'NF- $\kappa$ B' or 'classical NF- $\kappa$ B'.

NF- $\kappa$ B dimers are permanently present in the cytosol in an inactive form and their activity is tightly regulated by proteins of the I $\kappa$ B family. Several I $\kappa$ B proteins are known, including, but not limited to, I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\epsilon$ , and *Drosophila* Cactus. I $\kappa$ B proteins directly bind NF- $\kappa$ B dimers and thereby generally cover NF- $\kappa$ B's nuclear localization signal and interfere with DNA binding. Each I $\kappa$ B protein has a different affinity for individual NF- $\kappa$ B dimers, is differently regulated by phosphorylation and proteolysis and displays distinct tissues specific expression patterns.



**Figure 4:** The NF- $\kappa$ B superfamily and regulatory proteins. The superfamily consists of the Rel and NF- $\kappa$ B subfamilies which form hetero- and homodimers in various combinations. They are negatively regulated by proteins from the I $\kappa$ B family. IKK proteins phosphorylate I $\kappa$ B proteins and thereby activate NF- $\kappa$ B. Schema taken from Gilmore [25].

### 1.6.1 NF- $\kappa$ B signaling

NF- $\kappa$ B signaling is ubiquitous and usually functions to rapidly reprogram gene expression in response to diverse stimuli [22]. There are two main NF- $\kappa$ B pathways, referred to as the 'canonical' and 'non-canonical' pathways. The common upstream regulatory step in both of these pathways is activation of an I $\kappa$ B kinase (IKK) complex [25] (see below).

In the canonical pathway, rapid activation and nuclear translocation of cytoplasmic NF- $\kappa$ B is achieved by degradation of its inhibitor I $\kappa$ B $\alpha$  as a result of phosphorylation of I $\kappa$ B $\alpha$  by an activated IKK complex. The IKK complex of the canonical pathway

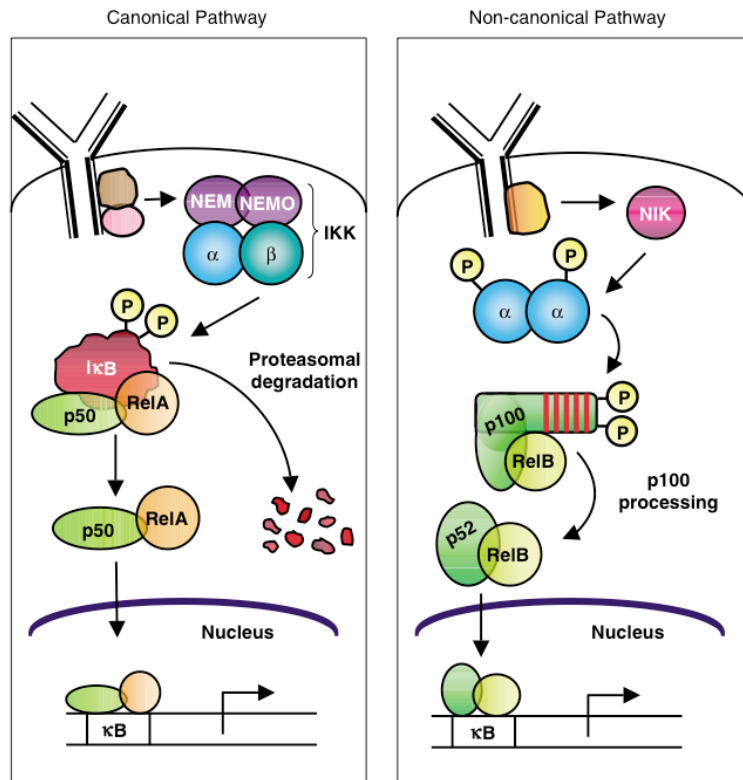
consists of the two catalytic kinase subunits IKK $\alpha$  and IKK $\beta$  and multiple copies of the regulatory subunit IKK $\gamma$  (aka 'NF- $\kappa$ B essential modifier' (NEMO)) [53]. Activation of the canonical IKK complex occurs in response to many external stimuli (e.g. TNF- $\alpha$ , IL-1, LPS) which signal through a variety of receptors. These stimuli lead to modifications of IKK complex components such as ubiquitination and phosphorylation of IKK $\gamma$  and phosphorylation of IKK $\beta$ . Activated IKK $\beta$  then phosphorylates I $\kappa$ B $\alpha$  at serine residues 32 and 36 [53]. Subsequently, I $\kappa$ B $\alpha$  is ubiquitinated by a SCF family ubiquitin ligase machinery and rapidly degraded via the proteasome [30] and NF- $\kappa$ B is free to relocate to the nucleus. Noteworthy, full activation of the canonical pathway may involve post-translational modifications of NF- $\kappa$ B subunits, including phosphorylation, acetylation, and prolyl isomerization of RelA [53, 6]. Termination of canonical NF- $\kappa$ B response can be achieved in several ways. Well characterized is the induction of I $\kappa$ B $\alpha$  gene transcription by activated NF- $\kappa$ B and subsequent binding of newly synthesized I $\kappa$ B $\alpha$  to NF- $\kappa$ B in the nucleus and relocation of the complex to the cytoplasm [30, 51].

In the non-canonical pathway the predominant NF- $\kappa$ B dimer being activated is p52/RelB, and signaling is independent of IKK $\beta$  and IKK $\gamma$ . Instead, NF- $\kappa$ B inducing kinase (NIK) phosphorylates and activates IKK $\alpha$  which in turn acts as homodimer to phosphorylate p100, inducing its proteolytic processing to p52. Cleavage of p100 C terminus, comprising the inhibitory ankyrin repeats, leads to activation of NF- $\kappa$ B complexes containing its processed form, p52, in the non-canonical pathway.

Beside the 'canonical' and 'non-canonical' pathways, which account for most of NF- $\kappa$ B activation, other atypical signaling pathways exist. This growing number of alternative mechanisms leading to NF- $\kappa$ B nuclear localization and DNA binding can be either IKK independent or utilize IKK activity in a manner distinct from that found with the two previously described pathways [53].

### **1.6.2 Modulation of NF- $\kappa$ B activity and the NF- $\kappa$ B subunit RelA**

NF- $\kappa$ B is activated in response to many different stimuli and in turn regulates hundreds of diverse target genes. Specificity of NF- $\kappa$ B function in all these processes needs to be assured and is achieved by a variety of strategies. While distinct NF- $\kappa$ B



**Figure 5:** NF-κB signaling. Shown are the canonical (left) and non-canonical (right) pathways. See main text for explanations. Taken from Gilmore [25].

dimer combinations allow for some specificity, mainly mechanisms such as modulation of NF- $\kappa$ B activity by post-translational modifications and cooperative interactions with other proteins are involved. In this regard, an important role has been ascribed to the NF- $\kappa$ B subunit RelA.

RelA is not only present in the predominant NF- $\kappa$ B dimer (p50/RelA) but can also form other complexes, including RelA homodimers [20]. Many post-translational modifications and interactions partners of RelA and their effects on NF- $\kappa$ B function have been described (reviewed by Campbell and Perkins [6]).

For example, of the seven putative RelA phosphorylation sites reported, five are thought to activate RelA transcriptional activity by either enhancing binding to co-activators (S276, S311), increasing RelA nuclear localization (T254) or increasing net negative charge of an acidic activation domain (S529, S536) [6]. In contrast, phosphorylation of T435 and T505 are thought to inhibit transcriptional activity of RelA [6]. Other phosphorylation sites have been proposed and reported, both in unstimulated cells and in response to some stimuli [2].

Reversible acetylation of RelA as well as its association with both histone acetyltransferases (HATs) and histone deacetylases (HDACs) has been described [9, 10, 11]. Furthermore, other post translational modifications such as ubiquitination and prolyl isomerization have been reported [6].

Interestingly, while RelA generally acts as a transcriptional activator, it has also been reported that it may act as repressor of transcription under certain circumstances [8, 7, 6].

### **1.6.3 Role of NF- $\kappa$ B in the nervous system**

NF- $\kappa$ B is best known for its biological functions in immune responses and inflammation. It is crucial for processes such as proliferation, cell survival and differentiation. In the brain, NF- $\kappa$ B was shown to be involved in numerous mechanisms, e.g. neuronal survival, learning, memory consolidation, plasticity and neurodegeneration [42, 47]. Furthermore, NF- $\kappa$ B activated by the non-canonical pathway was shown to be involved in modulation of brain inflammation by astrocytes [69]. More recent

data also suggest a role of NF- $\kappa$ B in proliferation, migration and differentiation of adult brain neural stem cells [41].

The precise mechanisms of NF- $\kappa$ B signaling in many of the aforementioned processes are not characterized. Activation of NF- $\kappa$ B in the brain via both the canonical and non-canonical pathway as well as through IKK independent mechanisms has been described [69, 29]. Furthermore, Kaltschmidt et al. [43] first showed that NF- $\kappa$ B signaling is activated in neurons of the hippocampus and cerebral cortex *in vivo*, although this is true only for a subset of neurons. Activation of NF- $\kappa$ B signaling was shown to be required for neuron survival [5] or neurite growth regulation [29].

NF- $\kappa$ B and Notch signaling pathways are known to functionally interact in various cell types (reviewed by Osipo et al. [50]). Regulation of NF- $\kappa$ B by Notch and vice versa occurs through many different, context-dependent mechanisms which in many cases are not well understood. For instance, it was shown that NF- $\kappa$ B can positively modulate Hes1 and Hes5 expression [16, 49] and that Notch can up-regulate the expression of p50, RelA, RelB and c-Rel [12]. Moreover, physical interaction between Notch1 and p50 has been described [28, 67]. The functional implication of this interaction, however, remains unclear [50]. Cross talk between Notch and NF- $\kappa$ B is also evident in the nervous system. Long term potentiation in hippocampal neurons is impaired in Notch1 knock-down mice, and NF- $\kappa$ B DNA binding activity was found to be defective in Notch1-deficient neurons [68]. However, the mechanism through which Notch stimulated NF- $\kappa$ B activity in this system remains undetermined [50]. Moreover, parallel signaling of both pathways in the nervous system is evident [1, 47], suggesting that many brain functions might involve functional cross talk of NF- $\kappa$ B and Notch pathways.

NF- $\kappa$ B is known to be essential during development, since NF- $\kappa$ B knockout models are embryonic lethal [22]. More specifically, mice lacking RelA (*rela*<sup>-/-</sup>, *p50*<sup>-/-</sup> *rela*<sup>-/-</sup>, *rela*<sup>-/-</sup> *c-rel*<sup>-/-</sup>) die during embryonic development (E 15 (*rela*<sup>-/-</sup>) and E 13 (the other two)) due to liver degeneration. Interestingly, while the *Drosophila* homolog of the mammalian Rel family proteins, Dorsal, is involved very early in development, mammalian NF- $\kappa$ B proteins seem to be required only later on, e.g. around E 12 in mouse embryos [58].

Little is currently known about the involvement of NF- $\kappa$ B in neural development.

Previous evidence suggests that the pathway is activated in the telencephalon of E13 mouse embryos [5]. Moreover, work by Youssef and Steinman [69] suggested that neural progenitor cell number is regulated by NF- $\kappa$ B-dependent control of proliferation *in vitro*. Based on these observations, the main objective of this thesis was to determine whether or not NF- $\kappa$ B signaling is important for the proliferation and/or differentiation of proliferating neural progenitor cells in the developing telencephalon of mouse embryos.

## 2 Materials and Methods

### 2.1 DNA Plasmids

DNA plasmids used in this work were previously described and are listed in table 2.

**Table 2:** DNA Plasmids

| plasmid                             | comment                                              | reference  |
|-------------------------------------|------------------------------------------------------|------------|
| pCMV2-Flag-Hes6 <sup>WT</sup>       | wild type mouse Hes6 cDNA                            | [34]       |
| pCMV2-Flag-Hes6 <sup>AMD</sup>      | acidic motif defective; <sup>15</sup> EDED to EAKA   | [4]        |
| pCMV2-Flag-Hes6 <sup>BAD</sup>      | basic arm defective; <sup>35</sup> KKRR to EKER      | [34]       |
| pCMV2-Flag-Hes6 <sup>AQ</sup>       | AQ insertion at position 39                          | [34]       |
| pCMV2-Flag-Hes6 <sup>Δ55-95</sup>   | aka Δhelix 2; delition of aa. 55 through 95          | [4]        |
| pCMV2-Flag-Hes6 <sup>LMD</sup>      | LXXLL motif defective; <sup>126</sup> LNHLL to ANHAL | [34]       |
| pCMV2-Flag-Hes6 <sup>Δ164-177</sup> | aka ΔPEST; delition of aa. 164 through 177           | [4]        |
| pCMV2-Flag-Hes6 <sup>ΔWRPW</sup>    | delition of the four C term. residues WRPW           | [34]       |
| pCMV2-Flag-Hes1                     | rat Hes1 cDNA                                        | [48]       |
| pCMV-p65                            | human p65/RelA wild type cDNA                        | [64]       |
| pRSV-βgal                           | constitutive β-galactosidase                         | [48]       |
| pNF-κB-Luc                          | luciferase gene contr. by 5 κB binding sites         | Stratagene |

### 2.2 Mouse lines and genotyping

Male NF-κB reporter mice (kindly provided by Dr. Phil Barker, McGill University) were crossed to C57/Bl6 females (Charles River, Montreal, Quebec, Canada) to obtain heterozygous litters. In all studies, NF-κB reporter mice containing a single transgenic allele were used. For embryonic staging, the day of appearance of the vaginal plug was considered as E0.5. Genotyping of transgenic mice was performed by PCR analysis from tail biopsies using primers specific for β-galactosidase (5' -CTGCAGATAACTGCCGTCCTCC-3' and 5' -CTTAATCGCCTTGACGACAT-3'). PCR was performed with Taq polymerase (Promega) with the following parameters: 94°C for five minute followed by 30 cycles of 94°C for 15 seconds, 60°C for 30 seconds, 72°C for 90 seconds and a final 5 minute extension at 72°C. All animal proce-

dures were conducted in accordance with the guidelines of the Canadian Council for Animal Care.

Primary cultures were prepared from either NF- $\kappa$ B reporter mice or CD1 mice which were obtained from Charles River.

## **2.3 Cell cultures**

### **2.3.1 Established cell lines**

Human embryonic kidney (HEK) 293 and ROS (rat osteoblastic cell line) cells were grown in Dulbecco's Modified Eagle's Medium (high glucose) (HyClone), supplemented with 10% fetal bovine serum, 2 mM Glutamine and antibiotics (Penicillin and Streptomycin, 10,000 U/ml and 10 mg/ml, resp.) at 37 °C and 5% CO<sub>2</sub>. Cells were routinely passaged by washing with phosphate-buffered saline (PBS), treatment with 0.05 % Trypsin-EDTA solution for one to two minutes at 37°C and following resuspension in culture medium.

Cells were transiently transfected using Superfect reagent (Qiagen), following the manufacturer's instructions and according to previously published protocols [26, 4].

### **2.3.2 Primary cultures of cortical neural progenitor cells**

Primary neural progenitor cell cultures were established from dorsal telencephalic cortices obtained from mouse embryos collected at stage E 12.5–13.5. Tissues from littermates were collected in ice-cold Neurobasal medium (Invitrogen) and homogenized by carefully pipetting up and down ten times. The cell suspension was then diluted into the desired amount of Neurobasal medium containing 40 ng/ml FGF2 (Collaborative Research, Bedford, MA), 2% B27, 1% N2, Penicillin/Streptomycin (10,000 U/ml and 10 mg/ml, resp.) and 0.5 mM glutamine (Invitrogen). 200000 cells/ml (total volume 800µl) were seeded into four-well chamber slides (Nalgene Nunc) coated with 0.1 % poly-D-lysine and 0.2 % laminin (BD Biosciences). Cells were cultured at 37 °C and 5 % CO<sub>2</sub>.

After 48 h *in vitro*, cells were transfected with plasmids encoding either enhanced green fluorescent protein (GFP) alone (0.2 µg/well), GFP (0.2 µg/well) and Hes6

(0.5 µg/well), GFP (0.2 µg/well) and RelA (0.8 µg/well), or all three together. When needed, the total amount of DNA was adjusted to 1.2 µg using pcDNA3 plasmid. DNA was mixed with 50 µl of OptiMEM medium and incubated for five minutes. 50 µl OptiMEM medium were mixed separately with Lipofectamine 2000 reagent (Invitrogen; 2 µl/µg DNA), then mixed with the DNA mixture and incubated for 20 minutes at room temperature. The DNA-Lipofectamine 2000 mixture was then added drop-wise to each well (after 400 µl culture medium of each well were removed, collected in a 50 ml tube and stored at 37 °C ('conditioned' medium)). After five hours, the transfection medium was removed and replaced with a 1:1 mixture of fresh Neurobasal medium and 'conditioned medium'. On day in vitro (DIV) five, cells were fixed with 4 % paraformaldehyde (PFA) for 20 minutes, washed four times with HBS (HEPES buffered saline) and permeabilized with blocking/permeabilization solution (5 % normal goat serum, 0.1 % bovine serum albumin, and 0.2 % IGEPAL in HBS) for 45 minutes, followed by immunocytochemistry.

## **2.4 Immunocytochemistry**

Fixed cells were incubated with appropriate primary antibodies (diluted in blocking solution) in a wet chamber for two hours at room temperature or overnight at 4°C. Primary and secondary antibodies are listed in Tables 3 and 4. Cells were then washed five times with blocking solution, followed by incubation with appropriate secondary antibodies in a dark chamber for 60 minutes at room temperature. Cells were then washed three times with blocking solution and three times with HBS. If desired, cell nuclei were counterstained with H $\ddot{o}$ chst reagent (one minute at room temperature), followed by washing with HBS (three times) and mounting with GelTol Aqueous Mounting Medium (Thermo Scientific).

## **2.5 Transcription assays**

HEK-293 or ROS cells were seeded in six well dishes and transiently transfected with the desired combinations of plasmids using the Superfect reagent. In each case, a  $\beta$ -galactosidase expression plasmid was co-transfected to provide a means of normalizing the assays. Forty-eight hours after transfection, cells were harvested and

**Table 3: Primary antibodies**

| product name                       | host species | company                     | serial number |
|------------------------------------|--------------|-----------------------------|---------------|
| anti-Flag M2                       | mouse        | Sigma                       | F 3165        |
| anti-mouse GFAP                    | mouse        | Sigma                       | AB5804        |
| anti-mouse S100 $\beta$            | mouse        | Sigma                       | S2532         |
| anti-human NF- $\kappa$ B p65 C-20 | rabbit       | Santa Cruz Biotechnology    | sc-372        |
| anti- $\beta$ III Tubulin          | mouse        | Promega                     | G7121         |
| anti-rat Nestin                    | rabbit       | Chemicon International      | MAB353        |
| anti-mouse NeuN                    | mouse        | Chemicon International      | MAB337        |
| anti- $\beta$ -Galactosidase       | mouse        | Dev. Studies Hybridoma Bank | JIE7          |
| anti- $\beta$ -Galactosidase       | rabbit       | Cappel                      | 55978         |
| anti-human Ki67                    | rabbit       | BD Biosciences              | 556003        |

**Table 4: Secondary antibodies**

| product name                                   | company                             | serial number |
|------------------------------------------------|-------------------------------------|---------------|
| HRP-conjugated Goat Anti-Mouse                 | Jackson ImmunoResearch Laboratories | 115-035-166   |
| HRP-conjugated Goat Anti-Rabbit                |                                     | 111-035-003   |
| HRP-conjugated Donkey Anti-Goat                |                                     | 705-035-003   |
| Cy <sup>TM</sup> 3-conjugated Goat Anti-Mouse  |                                     | 115-165-166   |
| Cy <sup>TM</sup> 3-conjugated Goat Anti-Rabbit |                                     | 115-165-166   |

lysates prepared as follows: cells were washed two times with PBS and then resuspended in lysis buffer (0.2 ml/well; 40 mM Tris·HCl pH 7.8, 2 mM EDTA, 1% Triton X-100, 50 mM NaCl, 1 mM MgSO<sub>4</sub>, 5 mM DTT) by pipetting up and down with a syringe and a 27G needle several times. Cell lysates were incubated on a rotating wheel at 4 °C for 10 to 20 minutes and then centrifuged at 12000 rpm, 4 °C for 5 minute. Supernatants were collected and used to determine luciferase activities as follows. Fifty µl of each lysate was mixed with freshly prepared luciferase assay reagent (40 mM Tris·HCl pH 7.8, 0.5 mM EDTA, 0.5 mM ATP, 0.5 mM Coenzyme A, 0.5 mM Luciferin, 10 mM MgSO<sub>4</sub> and 10 mM DTT), followed by measurement of luciferase activity with a luminometer. For β-galactosidase assays, 10 µl of cell lysate were mixed with 200 µl assay buffer (100 mM phosphate buffer, 2 mM MgCl<sub>2</sub>, 0.2 % IPEGAL, 0.1 % sodium deoxycholate, 5 mM DTT and 0.01 % ONPG). After incubation at 37 °C β-galactosidase activity was determined by photometric measurement at 420 nm.

## 2.6 Coimmunoprecipitation assays

Cells were rinsed with PBS and resuspended in lysis buffer (25 mM Tris·HCl pH 8.0, 0.5 mM EDTA, 0.5% Triton X-100, 150 mM NaCl, 1 mM DTT and 1×*Complete Protease Inhibitor Cocktail* (Roche). Suspensions were incubated on a rotating wheel at 4 °C for 10 to 20 minutes and then centrifuged at 12000 rpm, 4 °C for 5 minutes to remove cell debris. Supernatants were collected (lysates) and used immediately for immunoprecipitation. One tenth to one-twentieth of each supernatant was saved ('input') while the remaining sample was brought to a volume of 600 µl by adding lysis buffer (without DTT and protease inhibitor). Then 3 µl of an 80 mg/ml bovine serum albumin solution and 30 µl Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) were added together with the appropriate amount of desired antibody, followed by incubation on a rotating wheel at 4 °C overnight. The next day, samples were washed 5 times with wash buffer (25 mM TRIS pH 7.8, 200 mM NaCl, 0.5 % Triton X-100), beads were resuspended in 30 µl reducing gel loading buffer, incubated at 95 °C for 5 minutes and either loaded on SDS-polyacrylamide gels immediately or stored at -80 °C.

## 2.7 Western blot analysis

Protein samples were fractionated on 10 % SDS-polyacrylamide gels (10 % acryl amide, 1 % SDS, 25 mM TRIS·HCl pH 8.8; stacking gel: 4 % acryl amide, 1 % SDS, 25 mM TRIS·HCl pH 6.8) at 180 V for approximately 45 minutes (running buffer: 2.5 mM Tris, 19.2 mM Glycine, 0.01 % SDS). Proteins were transferred to nitrocellulose membranes (Biorad Trans-Blot®, 0.45 µm) at 100 V for 80 minutes in wet blot transfer buffer (25 mM Tris·HCl, 0.2 M glycine, 20% methanol). Transfer membranes were stained with Ponceau red. For immunodetection nitrocellulose membranes were blocked in blocking solution (Tris-buffered saline (TBS) containing 5 % milk-powder) for one hour, then incubated with the appropriate primary antibody for two hours or overnight. After several short washes with TBS, membranes were incubated with the appropriate secondary antibody for one hour and then again washed several times with TBS for appr. 20 minutes before treatment with *Amersham ECL™ Western Blotting detection kit*, according to the manufacturer's instructions, followed by autoradiography.

## 2.8 Preparation of cryostat sections

Mouse embryos were harvested at the desired gestational stage and the tails removed for preparation of genomic DNA and genotyping (see 2.2). Embryos were rinsed in PBS followed by fixation in 2 % PLP solution (2 % PFA, 74 mM L-lysine monochloride, 10 mM sodium periodate, 0.1 M phosphate buffer, pH 7.4) for 15 to 120 minutes. After washing four times with PBS, embryos were placed in tubes filled with 12 % sucrose and left at 4 °C until they sank to the bottom. The solution was then changed to 16 % sucrose, and after embryos sank again, to 18 % sucrose. Finally the procedure was repeated one more time with 30 % sucrose. Embryos were left in 30 % sucrose until they sank, before they were embedded in O.C.T. compound and stored at -80 °C. Frozen sections were obtained on a cryostat and were stored at -20 °C.

## 2.9 X-Gal staining

To analyze  $\beta$ -galactosidase activity on tissue sections, slides were placed in coplin jars, washed three times (10 minutes each) with rinse solution (100 mM phosphate buffer, 2 mM  $\text{MgCl}_2$ , 0.2 % IPEGAL, 0.1 % sodium deoxycholate) and then incubated with stain solution (rinse solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactosidase)) at 37 °C overnight. The next day, slides were rinsed with PBS and counterstained with eosin before mounting with GelTol Aqueous Mounting Medium.



### 3 Results

#### 3.1 Activation of NF- $\kappa$ B signaling in the developing mouse forebrain

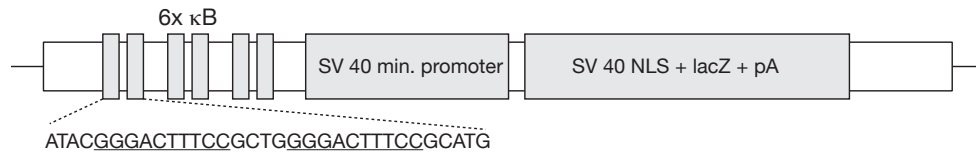
The  $\beta$ -galactosidase-NF- $\kappa$ B reporter mouse model generated by Bhakar et al. [5] was used to analyze NF- $\kappa$ B signaling in the developing brain. A schema of the reporter construct is shown in figure 6. In order to examine the activation of the NF- $\kappa$ B pathway during mouse neural development, coronal sections of transgenic litters at various stages (E 10.5, E 13.5, E 17.5) were prepared and examined for  $\beta$ -galactosidase activity by histochemical staining with the substrate, X-gal.

At E 10.5, a phase when the telencephalon contains mostly proliferating neural progenitor cells, no  $\beta$ -gal+ cells were generally observed in the brain or spinal cord (not shown). However, a few  $\beta$ -gal+ cells were visible in the ventral part of the telencephalon (data not shown). These cells might represent the first examples of brain cells in which the NF- $\kappa$ B pathway is activated.

At E13.5, when active neurogenesis is in full progress, I observed strong NF- $\kappa$ B activation in most of the telencephalon, but not in other brain regions (Fig. 7 and data not shown). Strong staining was observed in the ventricular zone of the neocortex in the pallium, as well as in the archicortex, albeit at somewhat lower levels (figure 7).  $\beta$ -gal expression was also detected in the subpallium, including the lateral, medial and caudal ganglionic eminences.

At E17.5, when neurogenesis has almost ended and astrogenesis has begun, cells exhibiting activated NF- $\kappa$ B signaling were still observed in the ventricular zone of the neocortex, as well as in regions of the ventral telencephalon. In addition,  $\beta$ -gal expression was robust in the cortical plate, where differentiated neurons are found (Fig. 7).

In summary, these results provide evidence that NF- $\kappa$ B signaling is activated in specific regions of the forebrain that contain undifferentiated neural progenitor cells or differentiated neurons. Thus, these findings raise the novel possibility that NF- $\kappa$ B signaling might be involved in mechanisms underlying the progenitor-to-neuron transition in the developing telencephalon.



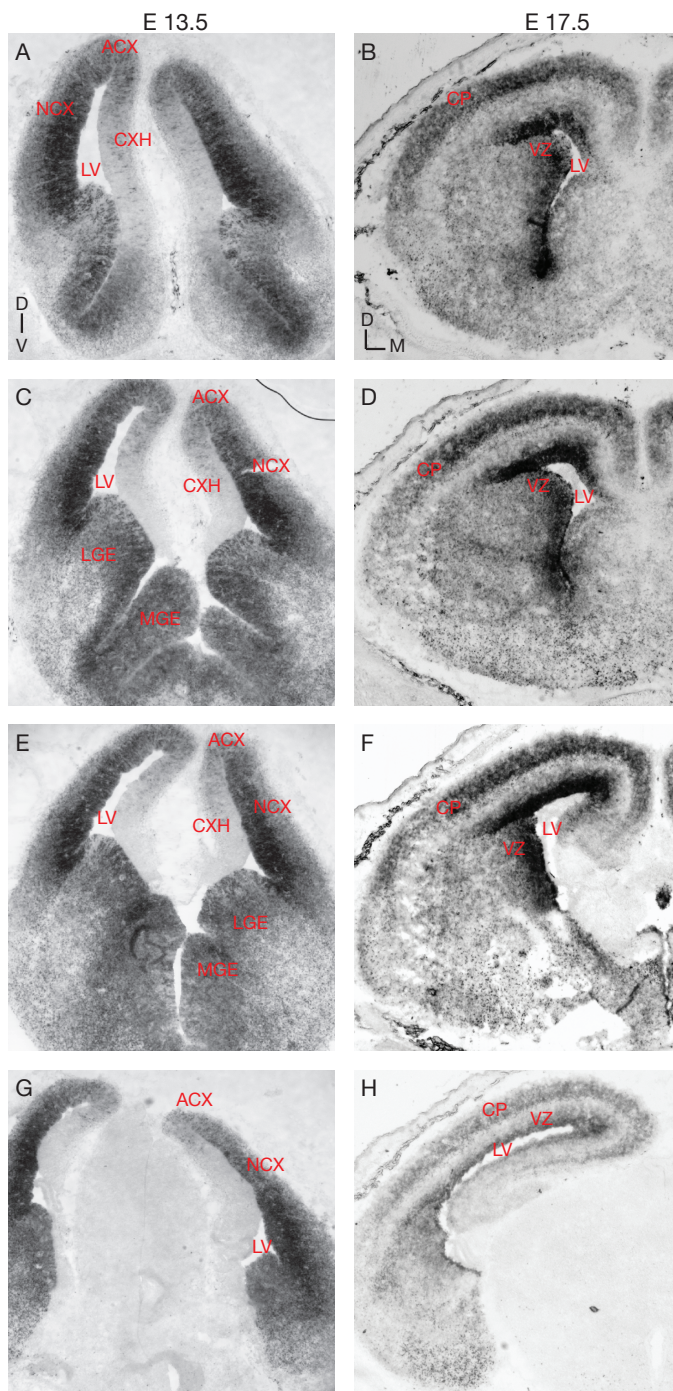
**Figure 6: Schema of the NF- $\kappa$ B reporter gene construct in transgenic mice.** The E.coli  $\beta$  galactosidase cDNA was modified to contain a mammalian Kozak consensus, an SV40 T-antigen-derived nuclear localization signal, and a polyA tract derived from the protamine I gene. Three tandem HIV-LTR repeats were inserted upstream of the SV40 minimal promoter. Modified from Bhakar et al. [5].

### 3.2 Activation of the NF- $\kappa$ B pathway in neural progenitors cells

To determine in which telencephalic cell types the NF- $\kappa$ B pathway is activated, primary cultures of dorsal telencephalic neural progenitor cells were established from E12.5 transgenic NF- $\kappa$ B reporter mice, followed by immunocytochemical analysis after two days in vitro. Figure 8 depicts the results of double labeling analysis of cultured cells with antibodies against  $\beta$ -galactosidase, the neural progenitor cell markers, nestin, Groucho/TLE, and Ki67 (a marker of cell proliferation), and the neuronal markers  $\beta$ III tubulin and NeuN).

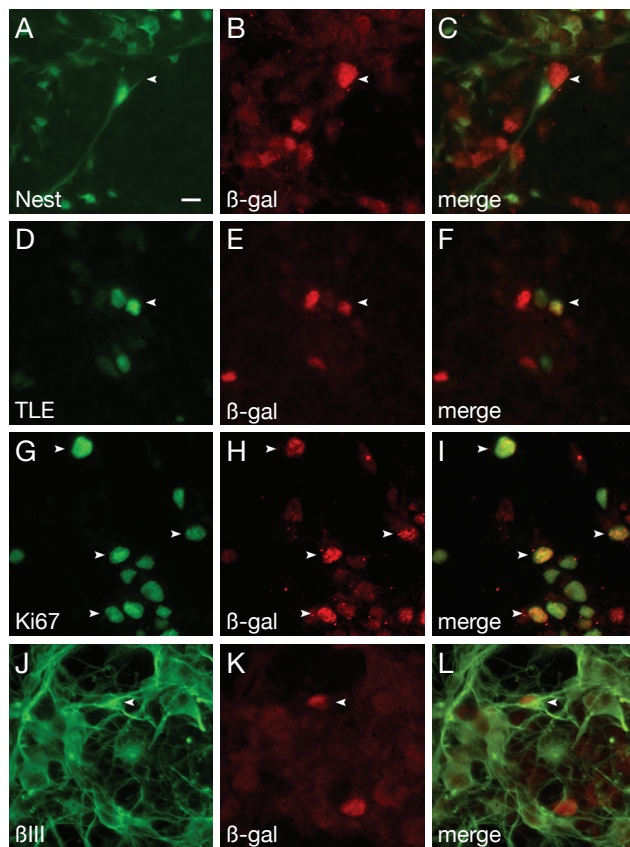
These studies revealed the presence of cells expressing both  $\beta$ -galactosidase and markers of neural progenitors (figure 8, panels A-F), suggesting the NF- $\kappa$ B pathway is activated in at least certain telencephalic neural progenitor cells. The co-expression of  $\beta$ -galactosidase with Groucho/TLE was particularly interesting because the latter was shown to functionally interact with the NF- $\kappa$ B complex [64] and also forms transcription complexes with Hes family members [19, 27]. I also detected cultured cells positive for  $\beta$ -galactosidase which also expressed markers of postmitotic neurons (Fig. 8, panels J-L). This finding is in agreement with the previous demonstration that NF- $\kappa$ B signaling is activated in differentiated cortical neurons [5].

In summary, these results provide evidence that NF- $\kappa$ B signaling is activated in neural progenitor cells. This new finding suggests an involvement of the NF- $\kappa$ B signaling pathway in cortical development.



**Figure 7: NF- $\kappa$ B activity analysis in the developing telencephalon.**

Coronal sections through the brains of E13.5 (left column) or E17.5 (right column) transgenic littermates were prepared and stained for  $\beta$ -galactosidase activity. Four representative pictures for each stage from rostral (top) to caudal (bottom) telencephalon are shown. NCX: neocortex, ACX: archycortex, CXH: cortical hem, LGE: lateral ganglionic eminences, MGE: medial ganglionic eminences, LV: lateral ventricle, VZ: ventricular zone, CP: cortical plate.

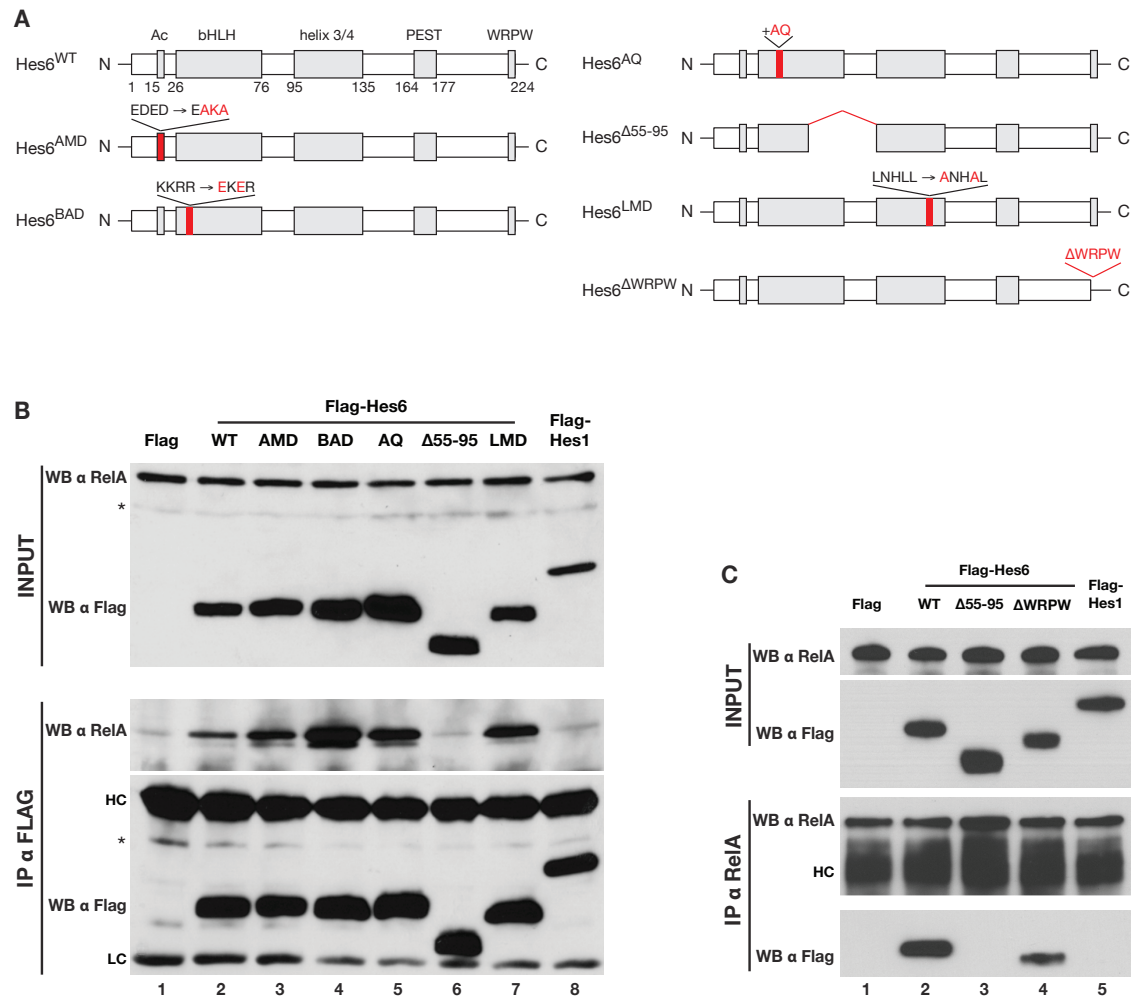


**Figure 8: Analysis of NF- $\kappa$ B pathway activation in cortical progenitor cell cultures.** Primary cultures of cortical progenitor cells were established from E13.5 NF- $\kappa$ B reporter embryos and subjected to double label immunocytochemistry with antibodies against  $\beta$ -galactosidase (2nd column) and the indicated neural progenitors (nestin, Gro/TLE, Ki67) and neuronal ( $\beta$ III tubulin) marks. Arrowheads point to double labeled cells. Scale bar: 10  $\mu$ m.

### 3.3 Molecular interaction of RelA with Hes6

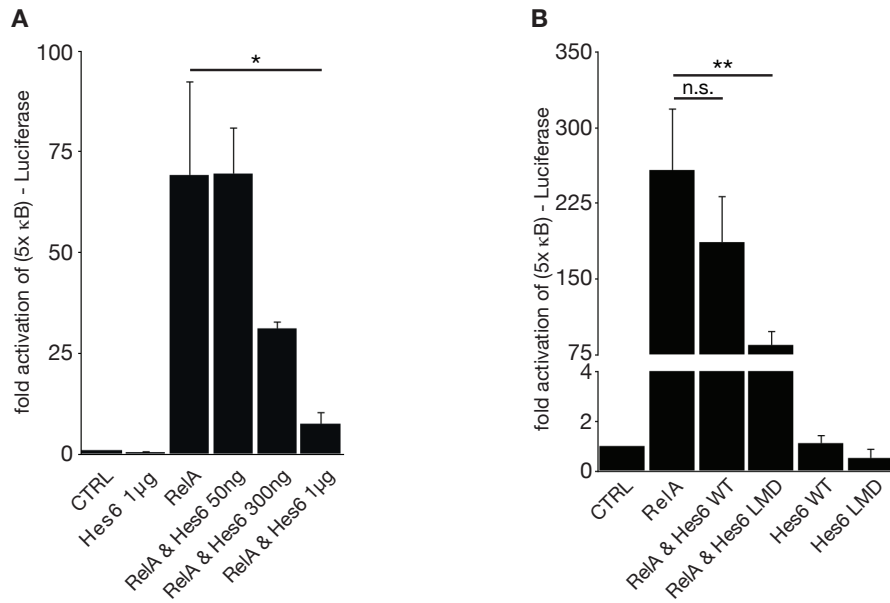
The new observation that the NF- $\kappa$ B pathway is activated in neocortical neural progenitor cells undergoing proliferation and differentiation raised the possibility that NF- $\kappa$ B signaling might play a role in cortical neurogenesis. Previous studies have shown functional interactions between the NF- $\kappa$ B and Notch signaling pathways. In particular, the NF- $\kappa$ B subunit RelA physically interacts with Gro/TLE [64], an important regulator of cortical neurogenesis (reviewed by Louvi and Artavanis-Tsakonas [46]). Based on my demonstration that NF- $\kappa$ B signaling is active in cells expressing Gro/TLE, I tested the possibility that RelA might associate with Gro/TLE and/or Gro/TLE-binding proteins important for the regulation of neuronal differentiation, such as Hes1 and Hes6. Although I was unable to observe an interaction between RelA and either Gro/TLE (data not shown) or Hes1 (Fig. 9 B and C), I detected a specific interaction of RelA with Hes6 in transfected cells (Fig. 9 B and C). More specifically, HEK-293 cells were transiently transfected with either Flag-Hes1 or Flag-Hes6, followed by co-immunoprecipitation experiments using anti-FLAG or anti-RelA antibodies. Endogenous RelA coimmunoprecipitated with Hes6, but not Hes1 (Fig. 9 B, cf. lanes 2 and 8). Similarly, Hes6, but not Hes1, coimmunoprecipitated with RelA (Fig. 9 C, cf. lanes 2 and 5). I next mapped the domain important for binding of Hes6 to RelA. Several previously described [34, 4] mutated forms of Hes6 were tested for their ability to interact with RelA by co-immunoprecipitation studies. I found that only one mutant, Hes6 <sup>$\Delta$ 55-95</sup> failed to interact with RelA (Fig. 9 B and C). Hes6 <sup>$\Delta$ 55-95</sup> lacks the second helix of the bHLH domain, which is required for protein homo- and heterodimerization [4]. Taken together, these results show that RelA forms complexes with Hes6 and that the bHLH domain of Hes6 mediated this association.

Transient transfection / transcription assays were performed next to analyze the effect of Hes6 on RelA transcriptional activity. HEK-293 (Fig. 10 A) or ROS (Fig. 10 B) cells were transiently transfected with a reporter construct containing the luciferase gene under the control of five  $\kappa$ B binding sites, in the absence or presence of RelA and Hes6 (or mutated forms thereof). Expression of RelA alone led to a significant activation of reporter gene expression and this activation was decreased in a dose-dependent manner by the presence of Hes6 (Fig. 10 A, experiment performed



**Figure 9: Interaction of RelA with Hes6. A |** Schematic representation of Hes6 and mutated forms thereof. Shown are the N-terminal acidic patch (Ac) and bHLH domain [3], followed by the helix3/helix4 domain containing the LNHLL motif, the SPXXSP motif in the PEST region, and the C-terminal WRPW motif. Hes6<sup>AMD</sup> contains a <sup>15</sup>EDED-to-<sup>15</sup>EAKA mutation in the acidic patch, Hes6<sup>BAD</sup> contains a <sup>35</sup>KKRR-to-<sup>35</sup>EKER mutation within the basic arm of the bHLH domain. Hes6<sup>AQ</sup> harbors a two amino acid insertion (AQ) at position 39 at the end of the basic arm. Hes6<sup>Δ55-95</sup> contains a deletion of residues 55 through 95 removing the second helix of the bHLH domain, Hes6<sup>LMD</sup> contains a <sup>126</sup>LNHLL-to-<sup>126</sup>ANHAL mutation near the end of the helix3/helix4 domain. Hes6<sup>ΔWRPW</sup> lacks the last four amino acids, required for Groucho/TLE binding. **B & C |** Co-immunoprecipitation of endogenous RelA and Hes6. HEK-293 cells were transiently transfected with the indicated FLAG epitope-tagged proteins, followed by cell lysis and immunoprecipitation (IP) with either Flag (B) or RelA (C) antibodies. IPs and input lysates were subjected to westernblot (WB) analysis with the indicated antibodies. HC: immunoglobulin heavy chain, LC: immunoglobulin light chain, \* non-specific signal with RelA antibody.

by Sumit Jhas). This finding suggests that Hes6 can negatively modulate the trans-activation ability of RelA. These studies also revealed that one particular mutated form of Hes6 that carries a mutated LXXLL motif (Hes6<sup>LMD</sup> [34]), had a stronger inhibitory effect of RelA transcriptional activity than wild type Hes6 (Fig. 10 B). Together, these results show a functional interaction between RelA and Hes6 and suggest that protein-protein interactions mediated by the LXXLL motif of Hes6 might interfere with the RelA:Hes6 association.



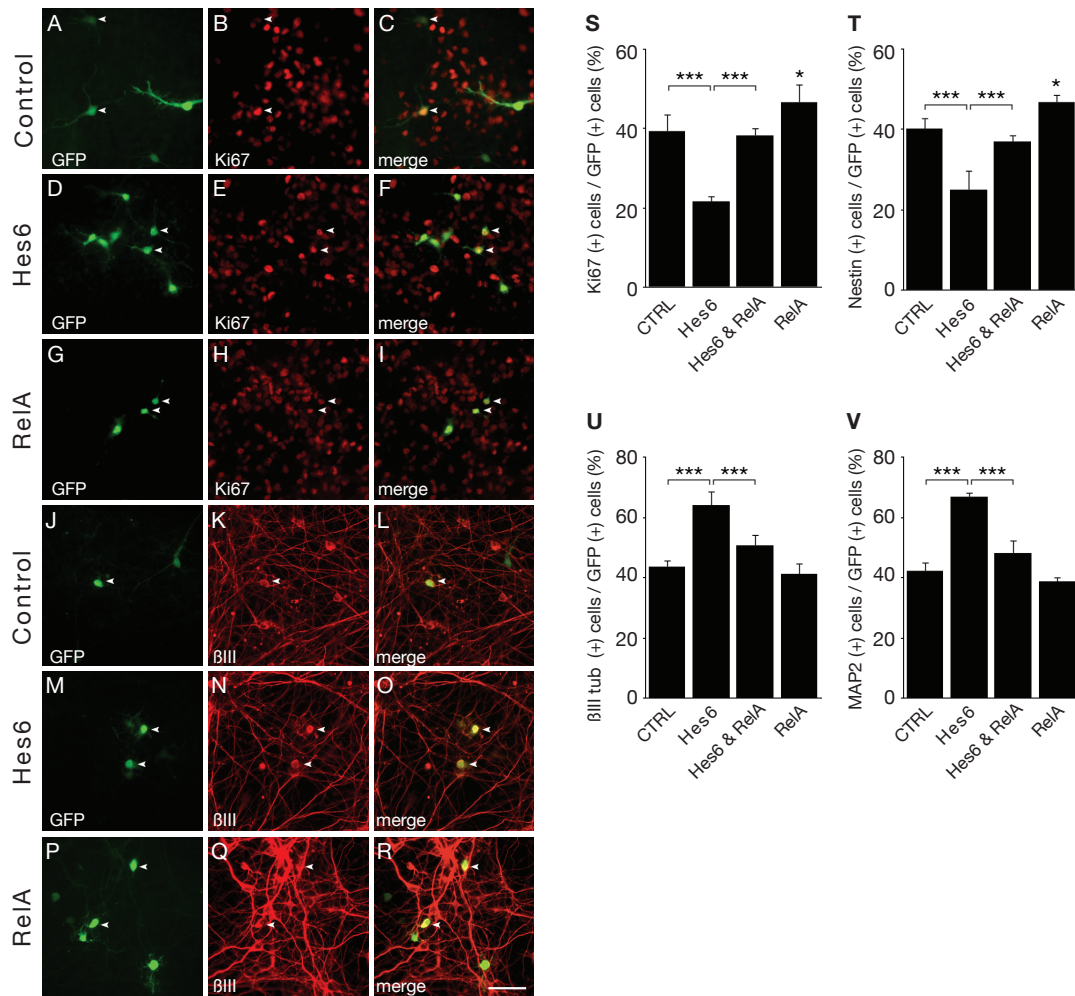
**Figure 10: Transient transfection / transcription assays. A |** HEK-293 cells were transfected with a 5 ×κB-luciferase reporter construct (1.0 μg/transfection) in the absence (bar 1) or presence of RelA (bars 3–6; 0.5 μg/transfection) and Hes6 (bars 2 and 4–6; 0.05, 0.3 or 1 μg/transfection). Basal luciferase activity in the absence of effector plasmids was considered 1 and values in the presence of effector plasmids are shown as the mean ± S.D. of three separate experiments performed in duplicate; \*p < 0.05, \*\*p < 0.01, analyzed by one-way ANOVA followed by Tuckeys post hoc test. **B |** ROS cells were transfected with a 5 ×κB-luciferase reporter construct (1.0 μg/transfection) in the absence (bars 1,5 and 6) or presence of RelA (bars 2–4; 0.5 μg/transfection), wild type Hes6 (bars 3 and 5; 0.5 μg/transfection) and Hes6<sup>LMD</sup> (bars 4 and 6; 0.5 μg/transfection). Basal luciferase activity in the absence of effector plasmids was considered 1 and values in the presence of effector plasmids are shown as the mean ± S.D of four separate experiments performed in duplicate; \*p < 0.05, \*\*p < 0.01, analyzed by one-way ANOVA followed by Tuckeys post hoc test.

### **3.4 Inhibition of Hes6-induced neuronal differentiation by RelA**

Based on the observation that NF- $\kappa$ B signaling is active in neural progenitor cells during the peak of the neurogenic phase and the physical and functional interaction between Hes6, a positive regulator of neurogenesis, and RelA, I examined the possibility that RelA might have a role in neuronal differentiation. Primary cultures of undifferentiated, pluripotent neural progenitor cells established from the dorsal telencephalon of E12.5 embryos were transiently transfected with Hes6, RelA, or a combination of both (in each case, enhanced GFP was coexpressed to mark transfected cells). Three days following transfection, cells were subjected to double-labeling analysis of the expression of GFP and markers of either undifferentiated neural progenitor cells or differentiated neurons (Fig. 11 A-R) to determine the numbers of transfected cells that displayed features of progenitor or neuronal cells in each case. These studies showed that exogenous expression of Hes6 alone caused a decrease in the number of progenitor cells and a parallel increase in the number of neurons, compared to expression of GFP alone (Fig. 11 S-V, cf. bars 1 and 2), as described previously [34, 4]. Co-expression of RelA with Hes6 abrogated the pro-neuronal effect of Hes6 (Fig. 11 S-V, cf. bars 2 and 3). Exogenous expression of RelA alone led to a slight, but statistically significant, increase in the number of progenitor cells, whereas no significant change in the number of neurons was observed (Fig. 11 S-V, bar 4). Together, these results suggest that RelA can counteract Hes6 neurogenic function and promote the undifferentiated state.

### **3.5 Inhibition of astrocyte differentiation by RelA**

Because the NF- $\kappa$ B pathway is active in the neocortex during phases of astrogenesis, and because Hes6 is a known regulator of astrocyte differentiation, I next examined whether or not RelA might regulate the latter process. Primary cultures of E12.5 mouse cortical progenitor cells were transiently transfected with RelA, Hes6 or a combination of both (together with enhanced GFP to mark transfected cells) and grown in the presence of CNTF, a potent inducer of astrocyte differentiation [31, 35]. Three days later, I performed immunocytochemical analysis of transfected cells using a panel of antibodies against markers of undifferentiated progenitors,

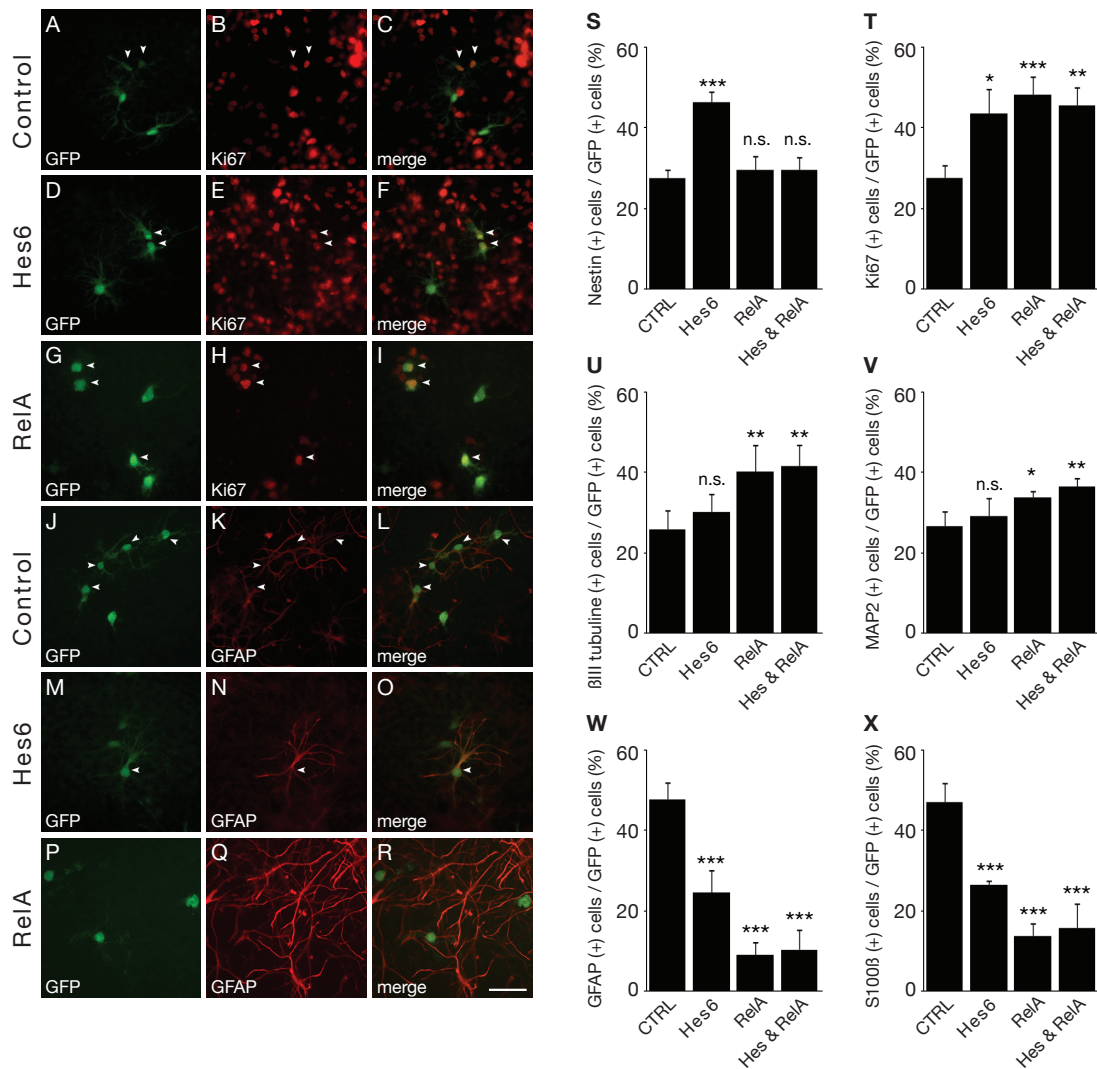


**Figure 11: Inhibition of Hes6-induced neuronal differentiation by RelA. A-R |** E12.5 mouse embryo cortical progenitor cells were transfected with either enhanced GFP alone (A-C and J-L) or GFP in combination with either Hes6 alone (D-F and M-O), RelA alone (G-I and P-R), or Hes6 and RelA (not shown) followed by double-labeling analysis of the expression of GFP (left column) and the indicated markers (middle column, B, E, H = Ki67; K, N, Q =  $\beta$ III tubulin). Combined stainings are shown in the right column. Arrowheads point to examples of double-labeled cells. Scale bar: 50  $\mu$ m. **S-V |** Quantitation of double-labeling studies was performed to calculate the percentage of GFP-positive cells that were also positive for the expression of either Ki67 (S), Nestin (T),  $\beta$ III-tubulin (U) or MAP2 (V). The results are shown as mean  $\pm$  SD (at least 500 cells were counted for each condition; n = 5; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001, analyzed by one-way ANOVA followed by Tuckeys post hoc test.)

differentiated neurons, or astrocytes (Fig. 12 A-R). As described previously [34], exogenous expression of Hes6 inhibited astrocyte differentiation (Fig. 12 W and X, cf. bars 1 and 2). This effect was accompanied by an increase in the number of undifferentiated cells (Fig. 12 S and T, cf. bars 1 and 2). Coexpression of RelA did not counteract Hes6 anti-astrogenic, rather it enhanced it (Fig. 12 W and X, cf. bars 2 and 4). This is in contrast to the antagonistic effect of RelA on Hes6 during neuronal differentiation. Importantly, under these conditions, RelA was able to inhibit astrocyte differentiation when transfected alone (Fig. 12 W and X, bar 3). Together, these findings suggest a previously uncharacterized function for RelA during cortical astrogenesis.

My studies also showed that exogenous expression of RelA, alone or together with Hes6, resulted in increased numbers of neuronal cells expressing the early neuron-specific marker,  $\beta$ III tubulin (Fig. 12 U), as well as an increase in Ki67-positive mitotic cells (Fig. 12 T). These apparently contradictory finding could be caused by the presence of 'mixed' cells, ie, cells that misexpress early neuronal differentiation programs when they are still proliferating. Interestingly, nestin-positive cells did not increase in the presence of RelA (Fig. 12 S), suggesting that the latter might induce a down-regulation of nestin expression.

Together, these data suggest that RelA inhibits astrocyte differentiation, but does not antagonize Hes6 during this process – in contrast to the situation observed during neuronal differentiation.



**Figure 12: Inhibition of astrocyte differentiation by RelA. A-R** | E12.5 mouse embryo cortical progenitor cells were transfected with either enhanced GFP alone (A-C and J-L) or GFP in combination with either Hes6 alone (D-F and M-O), RelA alone (G-I and P-R), or Hes6 and RelA (not shown) followed by double-labeling analysis of the expression of GFP (left column) and the indicated markers (middle column, B, E, H = Ki67; K, N, Q = GFAP). Combined stainings are shown in the right column. Arrowheads point to examples of double-labeled cells. Scale bar: 50  $\mu$ m. **S-X** | Quantitation of double-labeling studies was performed to calculate the percentage of GFP-positive cells that were also positive for the expression of either nestin (S), Ki67 (T),  $\beta$ III-tubulin (U), MAP2 (V), GFAP (W), or S100 $\beta$  (X). The results are shown as mean  $\pm$  SD (at least 500 cells were counted for each condition; n = 5; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001, analyzed by one-way ANOVA followed by Tuckeys post hoc test.)



## 4 Discussion

Several NF- $\kappa$ B reporter mice have been generated in the last two decades, allowing the investigation of NF- $\kappa$ B activity patterns in response to various stimuli and revealing cell types where this ubiquitous expressed transcription factor is constitutively activated. The various reporter mice do not show the same activity patterns because in each case, different  $\kappa$ B binding sites were used. The reporter mouse generated by Bhakar et al. [5] utilized  $\kappa$ B binding sites from the HIV LTR promoter region, which were shown to be sensitive to neuronal NF- $\kappa$ B activity [56, 13]. Indeed, this reporter mouse revealed regions of constitutive active NF- $\kappa$ B in the developing brain which were not described before. In this work, the role of NF- $\kappa$ B in proliferation and differentiation of neural progenitor cells in the developing telencephalon was investigated.

### 4.1 Activation of the NF- $\kappa$ B pathway in neural progenitors cells

Previous work has shown that NF- $\kappa$ B is constitutively activated in the developing telencephalon of E 13 mouse embryos [5]. However, neither the cell types comprising this activity, nor its function at this stage of development have been investigated. Here it is shown for the first time, that cortical neural progenitor cells comprise constitutive active NF- $\kappa$ B. First indications for this finding became evident after coronal sections of E 13.5 transgenic embryos were stained for  $\beta$ -galactosidase activity. The strong staining in the ventricular zone of the neocortex suggested that NF- $\kappa$ B might be constitutively active in neural progenitors in addition to neurons. To proof this hypothesis, primary cultures of neural cortical progenitors were established and tested for co-expression of  $\beta$ -galactosidase and either markers for neural progenitors or neuronal cells. These experiments confirmed the existence of neural progenitor cells comprising constitutive NF- $\kappa$ B activity. Moreover, results from similar stainings performed on coronal sections of E 13.5 transgenic embryos support these findings (Hosam Al-Jehani, personal communication). Together, these findings provide evidence that NF- $\kappa$ B signaling is activated in neural progenitor cells.

## 4.2 Molecular interaction of RelA with Hes6

Previous studies have shown functional interactions between the NF- $\kappa$ B and Notch signaling pathways. RelA was reported to directly interact with Groucho/TLE [64], an important regulator of cortical neurogenesis. Based on my demonstration that NF- $\kappa$ B signaling is active in cells expressing Gro/TLE, I tested the possibility that RelA might associate with Gro/TLE and/or Gro/TLE-binding proteins important for the regulation of neuronal differentiation, such as Hes1 and Hes6. Co-immunoprecipitation experiments with transiently transfected HEK-293 cells were performed and revealed that RelA is able to interact with Hes6, but not Hes1 *in vitro*. Co-immunoprecipitation of endogenous Hes6 and RelA was technically not feasible up until to date. Better antibodies for Hes6 might resolve this problem and deliver evidence for their interaction *in vivo*.

RelA is known to be able to bind to other proteins via their LxxLL motif, and Hes6 comprises such a motif (LHHLL) in its sequence. A form of Hes6 in which this domain is mutated, Hes6<sup>LMD</sup>, as well as other mutated forms of Hes6 previously generated and available in our laboratory were tested for their potential to interact with RelA by co-immunoprecipitation experiments. Interestingly, the LxxLL motif seems not to be required for binding, as Hes6<sup>LMD</sup> still could bind to RelA. However, another mutated form of Hes6, Hes6 <sup>$\Delta$ 55-95</sup>, was found to not be able to interact with RelA. This mutant is lacking the second helix of the bHLH domain, which was shown to be important for dimerization of Hes6 [4].

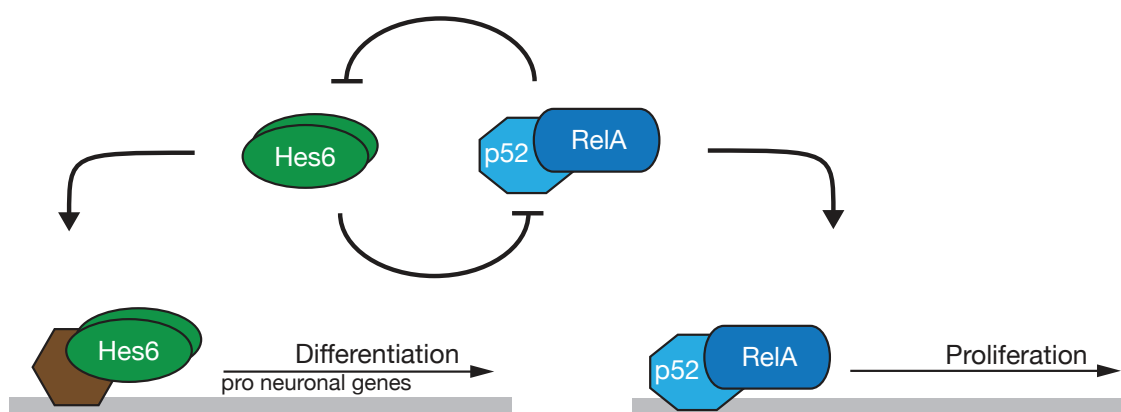
Summarizing, I provide evidence that RelA is able to bind Hes6 *in vitro* and that the helix 2 of the bHLH domain is essential for this interaction to occur (whether it is sufficient or not, needs to be proven in future experiments).

## 4.3 Inhibition of Hes6-induced neuronal differentiation by RelA

Hes6 was shown to promote neurogenesis as well as inhibit astrocyte differentiation through different mechanisms [34]. Given the ability of RelA to bind to Hes6 and its constitutively active state in neural progenitor cells, its involvement in neuronal and astrocyte differentiation was examined. Primary neural cortical progenitor cells

were transiently transfected with RelA, Hes6, or a combination of both and individual cells examined by immunocytochemistry. Exogenous Hes6 led to an increase of cells expressing neuronal markers and a decrease in cells expressing progenitor markers compared to control conditions (see figure 12). However, this effect was reverted when RelA was co-transfected with Hes6. RelA alone increased the number of undifferentiated cells but did not significantly reduce the number of neurons. These results suggest that RelA is able to antagonize Hes6 during neuronal differentiation. However, the mechanisms through which this effect is accomplished are not clear. Proposed models for RelA action during neurogenesis are shown in figure 13. RelA and Hes6 might inhibit each other during neuronal differentiation by forming an inactive complex or preventing interaction with other proteins. Binding of RelA to Hes6 likely already occurs in the cytosol, since a mutant of Hes6, Hes6<sup>BAD</sup>, which is not able to locate to the nucleus [34] is still able to bind RelA *in vitro*. Interaction of Hes6 with RelA might prevent them from translocating to the nucleus and thereby inhibit their normal functions. In fact, RelA-Hes6 complexes might be less stable and subjected to degradation (unpublished observations), which could contribute to their antagonistic effects. Hes6 might also bind to RelA in the nucleus to modulate NF- $\kappa$ B transcriptional activity. The strength of Hes6 repression of NF- $\kappa$ B likely depends on other factors binding Hes6, since Hes6<sup>LMD</sup>, which is not able to interact with other proteins via its LXXLL motif, was able to repress NF- $\kappa$ B dependent transcription much stronger than the wild type *in vitro* (see figure 10 B). A model for mutual repression between RelA and myocardin, a transcription factor in cardiac and smooth muscle cells, has been proposed by Tang et al. [63]. They showed that myocardin inhibits cellular proliferation by inhibiting NF- $\kappa$ B dependent cell cycle progression and that RelA is able to inhibit differentiation by inhibiting myocardin transcriptional activity. A similar scenario involving Hes6 and RelA is possible in the case of neuronal differentiation versus ongoing proliferation during neural development. Hes6 was reported to inhibit cell proliferation via mechanisms involving binding to CBP and induction of p21 [17]. CBP is a general co-activator and was also shown to interact with RelA [23]. However, the paper by Eun et al. [17] did not investigate an involvement of RelA in this matter and I was not able to reproduce co-immunoprecipitation of Hes6 and CBP under conditions that allow co-immunoprecipitation of Hes6 with RelA.

When neural cortical progenitor cells were transfected with RelA alone, a slight increase in cells expressing Nestin and Ki67 could be observed, without a notable change in the number of cells expressing the neuronal markers  $\beta$ III-tubulin and MAP2 (see figure 11, column four in graphs S-V). If RelA antagonizes Hes6, one would expect a decrease in the neuronal cell population. However, positive effects of NF- $\kappa$ B on neuronal survival have been published [5] and might explain the outcome of the experiment. Because the total percentage of counted cell populations did not change in a notable extent (progenitors and neurons accounted for approximately 85 % of all GFP positive cells counted), an effect on survival of either neurons or progenitors is not evident from obtained results.



**Figure 13: Working model for RelA action during the neurogenic phase.**

#### 4.4 Inhibition of astrocyte differentiation by RelA

After finding that RelA seems to be involved in the regulation of neuronal differentiation, I next investigated its role during astrocyte differentiation. Neural cortical progenitor cells from E 12.5 mouse embryos were transiently transfected and, thereafter, grown under presence of ciliary neurotrophic factor (CNTF) to promote differentiation into astrocytes. In accordance with previously published results [34], exogenous Hes6 in the presence of CNTF did not increase neuronal differentiation but instead inhibited differentiation into astrocytes. When RelA was co-transfected, it failed to antagonize Hes6 function under these conditions. Strikingly, exogenous expression of RelA alone had a strong inhibitory effect on astrocyte differentiation.

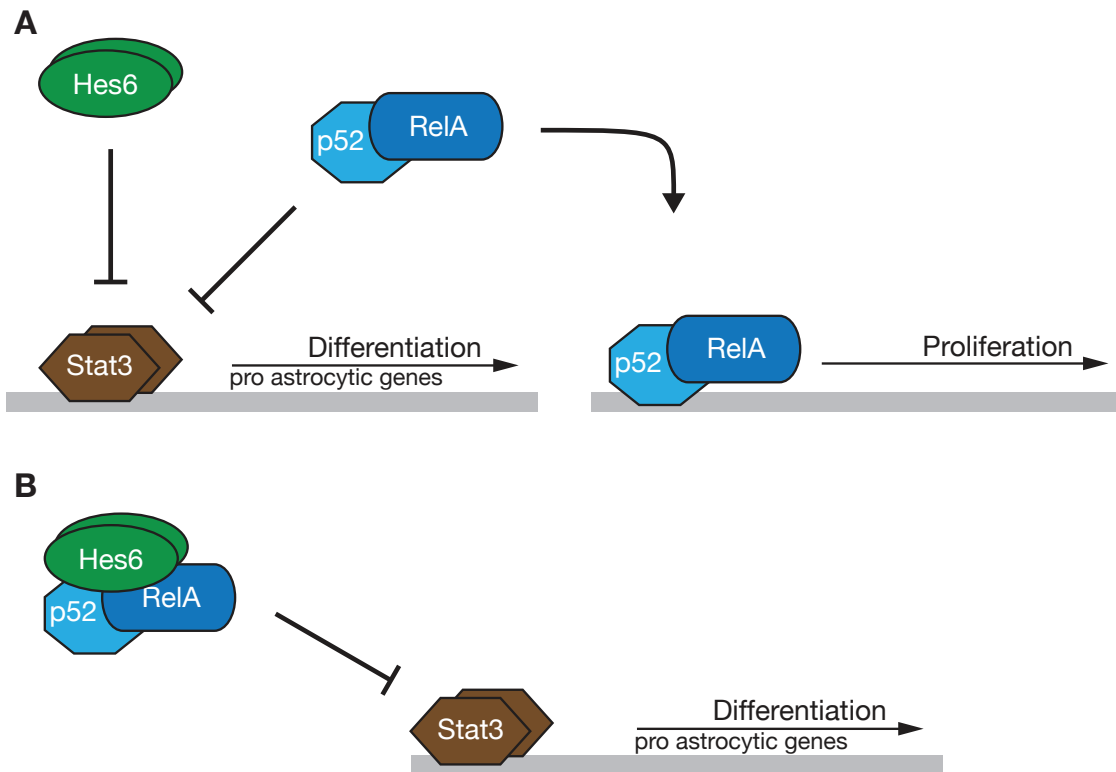
In fact, no significant differences between the two conditions – RelA alone and co-transfected with Hes6 – could be observed. Neither an antagonistic nor synergistic effect of RelA on Hes6, or vice versa, is evident from the available data. Thus, it is possible that both act independently of each other during astrocyte differentiation.

The strong anti-astrogenic effect of RelA alone is very interesting and possible mechanisms and modes of RelA and/or NF- $\kappa$ B action are shown in figure 14. CNTF induces astrocyte differentiation via the Jak-Stat pathway to directly activate expression of pro astrocytic genes such as glial fibrillary acidic protein (GFAP). RelA is known to directly interact with Stat 3 [70] and might inhibit astrocyte differentiation by repressing Stat 3 transcriptional activity. Noteworthy, Stat 3 recruits the co-activator CBP to induce expression of GFAP [62]. Hes6 could possibly prevent this recruitment by binding to CBP and thereby inhibit astrocyte differentiation. In such a model, RelA and Hes6 would act independently to prevent premature astrocyte differentiation.

Noteworthy, the number of neurons was slightly, but significantly increased in cultures transfected with RelA alone or in combination with Hes6 under presence of CNTF. This was rather unexpected, since RelA did not promote neuronal differentiation in previous experiments where its effect on neuronal differentiation was tested.

Interestingly, in cultures transfected with RelA alone or together with Hes6, the number of Ki67 positive cells increased significantly, while no change could be observed when cells were examined for presence of the Nestin marker. While Nestin is a specific marker for neural progenitors, Ki67 is a general proliferation marker. However, in the present primary culture system the only proliferating cell population are neural progenitor cells, thus Ki67 is considered as suitable marker for this cell type under these conditions. The discrepancy between the number of Nestin and Ki67 positive cells was only ever observed in cells transfected with RelA (either alone or together with Hes6), thus RelA seems to be responsible for this phenomenon. Potentially RelA strongly promotes cell proliferation under these conditions but fails to prevent down-regulation of progenitor markers when exogenously expressed in these cells. Those cells would then stay in a proliferative state, without expressing progenitor markers, and also being unable to differentiate into astrocytes due to the anti-astrogenic effect of RelA. It is possible that some of those cells then start expressing neuronal markers and eventually differentiate into neurons. Double labeling experiments (e.g. Ki67/Nestin, Ki67/ $\beta$ III-tubulin, Ki67/GFAP) and examination

of more markers in RelA transfected cells will shed some light on this phenomena.



**Figure 14: Working model for RelA action during the astrocyte differentiation.**

#### 4.5 Concluding remarks

The provided results in this work suggest an important role of NF- $\kappa$ B signalling in proliferation and differentiation processes during mammalian neural development. NF- $\kappa$ B is constitutively active in neural progenitors in addition to neurons *in vivo* and is able to interact with the bHLH transcription factor Hes6 *in vitro*, which was shown to play an important role in controlling neuronal and astrocyte differentiation. Moreover, presented results suggest an antagonistic effect of RelA on Hes6 during neuronal differentiation. Furthermore, RelA was shown to inhibit astrocyte differentiation, likely independent from Hes6.

This work provides first important insights into NF- $\kappa$ B signaling in neural progenitors during development and raises numerous interesting questions to be answered by future research.

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# curriculum vitæ

## Profile

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## Education

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