Doctoral Thesis

Conditional ablation of Numb from the vertebrate central nervous system

Author(s):
Klein, Anne Laurence

Publication Date:
2004

Permanent Link:
https://doi.org/10.3929/ethz-a-004713115

Rights / License:
In Copyright - Non-Commercial Use Permitted

This page was generated automatically upon download from the ETH Zurich Research Collection. For more information please consult the Terms of use.
Conditional Ablation Of Numb From The Vertebrate Central Nervous System

A dissertation submitted to the
EIDGENOESSISCHE TECHNISCHE HOCHSCHULE (ETH) ZUERICH

for the degree of
Doctor of Natural Science

presented by
Anne Laurence KLEIN
dipl.sc.nat.
Swiss Federal Institute of Technology (ETH), Zürich, Switzerland
Born October 6, 1976 in Luxembourg
Citizen of Luxembourg

Prof. Dr Ueli Suter, examiner
Prof. Dr Isabelle Mansuy, co-examiner
Dr Verdon Taylor, co-examiner

2004
NUMB function in CNS

TABLE OF CONTENTS 1

SUMMARY 3

ZUSAMMENFASSUNG 5

1 INTRODUCTION 7

1.1 DEVELOPMENT OF THE CNS 7
  1.1.1 THE CEREBELLUM AS A MODEL SYSTEM TO STUDY VERTEBRATE CNS DEVELOPMENT 7
  1.1.2 REBUILDING THE DAMAGED CNS 9

1.2 NOTCH SIGNALLING IN INVERTEBRATES 11
  1.2.1 NOTCH SIGNALLING IN DROSOPHILA SENSILLIUM FORMATION 11
  1.2.2 NUMB MODULATES NOTCH SIGNALING IN DROSOPHILA SENSILLIUM FORMATION 12

1.3 NOTCH FUNCTION IN VERTEBRATE CNS DEVELOPMENT 13
  1.3.1 THE NOTCH SIGNALING PATHWAY IS CONSERVED BETWEEN INVERTEBRATES AND VERTEBRATES 13
  1.3.2 NOTCH1 EXPRESSION IN THE MAMMALIAN CNS 14
  1.3.3 NOTCH1 FUNCTION IN THE MAMMALIAN CNS 14

1.4 NUMB FUNCTION IN VERTEBRATE CNS DEVELOPMENT 15
  1.4.1 NUMB IS A CONSERVED CYTOPLASMIC ADAPTER PROTEIN 15
  1.4.2 NUMB EXPRESSION IN MAMMALIAN CNS 17
  1.4.3 NUMB FUNCTION IN THE MAMMALIAN CNS 17
    1.4.3.1 NUMB REGULATES NEUROGENESIS IN MAMMALIAN CELL LINES 17
    1.4.3.2 NUMB IS INVOLVED IN CELL FATE CHOICES IN THE DEVELOPMENT OF THE MAMMALIAN CNS 18
    1.4.3.3 BIOCHEMICAL MECHANISMS UNDERLYING THE FUNCTION OF NUMB 19
    1.4.3.4 NUMB-DEFICIENT MICE 20

2 NOTCH1 AND ITS LIGANDS DELTA-LIKE 1 AND JAGGED ARE EXPRESSED AND ACTIVE IN DISTINCT CELL POPULATIONS IN THE POSTNATAL BRAIN 21

2.1 ABSTRACT AND INTRODUCTION 21

2.2 MATERIALS AND METHODS 22
  2.2.1 IN SITU HYBRIDIZATION AND PROBES 22

2.3 RESULTS AND DISCUSSION 22

2.4 OUTLOOK 34

3 MURINE NUMB REGULATES GRANULE CELL MATURATION IN THE CEREBELLUM 36

3.1 ABSTRACT 36

3.2 INTRODUCTION 36

3.3 MATERIALS AND METHODS 39
  3.3.1 GENERATION OF MICE AND BREEDING 39
  3.3.2 IN SITU HYBRIDIZATION ANALYSIS OF GENE EXPRESSION 39
  3.3.3 IMMUNOHISTOCHEMISTRY AND CELL FATE ANALYSIS 39
  3.3.4 ISOLATION OF CEREBELLAR NEUROEPITHELIAL CELLS AND IMMUNOCYTOCHEMISTRY 40
  3.3.5 WESTERN BLOT ANALYSIS 41

3.4 RESULTS 41
  3.4.1 CONDITIONAL DISRUPTION OF mNUMB DOES NOT AFFECT MIDBRAIN/HINDBRAIN FORMATION BETWEEN E10.5 AND E15 42
  3.4.2 CONDITIONAL ABLATION OF THE mNUMB GENE LEADS TO A DELAY IN GRANULE CELL PRECURSOR MATURATION 44
SUMMARY

Understanding the generation of the enormous diversity of cells in the mammalian CNS represents a major challenge in developmental neurobiology. Neuroepithelial stem cells can use different mechanisms to create diversity among their progeny. They can produce two identical daughter cells, which then become different through interaction with different environmental cues. Alternatively, they can produce different daughter cells through asymmetric segregation of intrinsic cell fate determinants before mitosis (reviewed in Horvitz et al., 1992). The developing Drosophila CNS combines both mechanisms. Signaling through the Notch receptor as well as the asymmetric distribution of Numb were shown to be crucial for the proper development of the Drosophila nervous system (Hartenstein and Posakony, 1990; Rhyu et al., 1994; Spana and Doe, 1998).

The importance of understanding the basic mechanisms in early neurogenesis becomes obvious in view of possible treatments of neurological diseases, which damage CNS neurons. We therefore analyzed the function of mouse Numb in the development of the CNS. The lethality of the Numb knockout only provided limited information on Numb function in neurogenesis (Zhong et al., 2000; Zilian et al., 2001) and prompted us to analyze the function of Numb in the development of the cerebellum by conditional gene ablation. We chose the cerebellum to study CNS neurogenesis since it represents a non-vital CNS structure that is anatomically well-defined. Numb was shown to be expressed in the neuroepithelium from E8.5 onwards (Zhong et al., 1996). Furthermore, Numb expression was detected postnatally in the cerebellum, the dentate gyrus and in some defined cortical regions (Stump et al., 2002). Our analysis revealed that Numb ablation does not affect early cell fate decisions in the midbrain/hindbrain region of the neural tube and that cerebellar development occurs normally until birth. However, it became obvious that Numb-deficient granule cell precursors in the external germinal layer (EGL) are delayed in their maturation progression: they are retained in the EGL which leads to an increase in EGL thickness and in consequence, the establishment of the internal granule cell layer (IGL) is retarded. These findings were confirmed in vitro. The proportion of ‘intermediate’ granule cell precursors, expressing Math1, generated from Numb-deficient neuroepithelial cells was increased after 6d in culture and the generation of mature β-tubulin expressing granule cells was delayed. These results suggested that Numb regulates the transition of a mitotic progenitor to a fully differentiated granule cell in the cerebellum. In addition, the maturation of Purkinje cells is also delayed in
NUMB function in CNS

Numb-deficient mice in vivo. Taken together, these result suggest that, in cerebellar development, Numb is not an instructive factor of cell fate determination, but that its function only gets crucial in late neuronal maturation.

In future, several remaining questions need to be addressed. First, we must address whether the relatively weak phenotype observed upon Numb ablation in the cerebellum is due to a compensatory function of Numblike. Second, the biochemical mechanisms of Numb underlying the function of Numb must be addressed. The identification of additional interaction partners of Numb may enlarge the view on Numb function and clarify the network of signalling cascades involving Numb, which possibly regulates endocytic processes.
ZUSAMMENFASSUNG


zweite wichtige Nervenzellpopulation im Kleinhirn darstellt, im letzten Differenzierungsschritt verspätet sind. Unsere Ergebnisse zeigen, dass Numb nicht ausschlaggebend ist um einen bestimmten Zelltyp zu bilden, jedoch eine wichtige Rolle in der späteren neuronalen Entwicklung spielt, indem es die Vorgänge zur endgültigen 'Reifung' der Nervenzellen im Kleinhirn kontrolliert.

1 INTRODUCTION

1.1 DEVELOPMENT OF THE CNS

The vertebrate central nervous system (CNS) derives from the neural plate, an epithelial sheet arising from the dorsal ectoderm of a gastrula-stage embryo. Folding of the neural plate in its anterio-posterior (AP) axis results in the formation of a tube. The most anterior part of this tube becomes subdivided into a series of vesicles that represent the forming fore-, mid-, and hindbrain. The posterior part will form the future spinal cord (Lumsden and Krumlauf, 1997). Regionalization along the AP axis of the early embryo is controlled by the action of spatially restricted signals like retinoic acid (RA) and fibroblast like growth factors (FGFs) (Marshall et al., 1992). The emergence of restricted expression patterns of transcription factors, e.g. Hox genes in the hindbrain (Studer et al., 1994), confers additional positional information along the AP axis. Neural patterning also requires positional cues along the dorso-ventral (DV) axis of the neural tube that together with the AP values elicit regionally appropriate neuronal differentiation (Tanabe and Jessel, 1996). Members of the bone morphogenetic (BMP) family have been implicated in the induction of dorsal cell types (Liem et al., 1995), whereas Sonic Hedgehog (SHH) induces ventral floor plate and motoneuron identity (Ericson et al., 1997; Roelink et al., 1994; Wijgerde et al., 2002). Since the development of the CNS is strongly dependent on the controlled specification, generation and migration of distinct classes of neurons in different regions within the nervous system, it is critical to elucidate molecular mechanisms underlying these events.

1.1.1 THE CEREBELLUM AS A MODEL SYSTEM TO STUDY VERTEBRATE CNS DEVELOPMENT

The cerebellum is part of the CNS and shows a well-defined anatomy and physiology (see fig1-1). Limited number of neurons undergo typical patterns of CNS development including morphogenetic movements and the establishment of different neuronal layers (Chizhikov and Millen, 2003; Hatten and Heintz, 1995; Hatten et al., 1997). Although the cerebellum is required for controlling balance and eye movements, adjusts and coordinates ongoing movements and might participate in learning and memory (Ito et al., 1998; Okano H, 2000; Linden et al., 2003), it is not a vital structure. These characteristics of the cerebellum make it an ideal system to address molecular mechanisms of early and late neurogenesis by using conditional gene ablation approaches.
The cerebellum arises from the alar plate of the most anterior hindbrain rhombomere of the developing neural tube (Wingate, 2001). The formation of the murine cerebellar anlage starts at approximately embryonic day 9-11 rostro-caudal to the mid-hindbrain region of the neural tube (reviewed in Hatten et al., 1997). At E10-11, deep nuclei neurons, which are responsible for transmitting information out of the cerebellum, are the first neurons to arise from the ventricular zone (VZ) (reviewed in Goldowitz and Hamre, 1998; Altman and Bayer, 1985a). At E13, Purkinje cell precursors arise from the VZ of the neuroepithelium (Altman and Bayer, 1985b). At this time Purkinje cell precursors cease proliferation and migrate radially along the glial fibre system towards the surface of the cerebellum. At E16-E18, postmigratory, immature Purkinje cell precursors settle in a dense zone, the future Purkinje cell layer of the adult cerebellum. Terminal differentiation of Purkinje cells occurs between P10 and P20 when they form a single cell layer and make contact to the parallel fibres of the granule cells and afferent axons of the climbing fibres (Chizhikov and Millen, 2003; Hatten and Heintz, 1995). Purkinje cells are the only efferent neurons of the cerebellar cortex, innervating mainly the deep cerebellar neurons. (Herrup and Kuemerle, 1997).

Granule cells constitute the dominant population of cerebellar neurons in the adult. In contrast to Purkinje cell precursors that arise from the VZ, granule cells are derived from the rhombic lip, a structure formed by the neuroepithelium at the posterior edge of the cerebellar anlage. At E13-E14, granule cell precursors migrate from the rhombic lip over the surface of the cerebellar anlage to form the external germinal layer (EGL). Granule cell proliferation within the EGL is maintained peri- and postnatally until the EGL becomes an 8 cell-layer structure. The terminal differentiation occurs during the second and third week postnatally when cell bodies of the granule cells migrate radially into the cerebellar anlage and establish the internal granule layer (IGL) of the adult cerebellum. Here they form synapses with ingrowing afferent mossy fibres (Alder, 1996; Hatten et al., 1997). The stellate and basket cells that form the interneurons of the ML arise from both the mes-and metencephalon and are generated at an early postnatal stage. Cell proliferation continues until the interneuron precursors migrate into the molecular layer (ML) of the adult cerebellum (Goldowitz and Hamre, 1998).
The cerebellum is a highly organized structure containing three layers. The PCL is a single cell layer composed of Purkinje cells, which represent the only efferent cerebellar neurons and which synapse on the deep cerebellar neurons. The dendritic tree of Purkinje cells reach into the molecular layer (ML) where they make contact with the parallel fibres of granule cells the cell bodies of which are located in the inner granular layer (IGL). Sensory information from outside the cerebellum is brought via mossy fibres to granule cells and via climbing fibres to Purkinje cells.

1.1.2 REBUILDING THE DAMAGED CNS

Until recently it was believed that CNS neurons, once lost, could not be regenerated in the postnatal brain. Currently, it is known that neuronal degeneration in the CNS leads to the formation of glial scars by astrocytes (Hatten et al., 1991; Rhodes et al., 2003). Research has focused almost entirely on therapeutic approaches limiting further damage. However, the
recent discovery of both embryonic and adult stem cells, able to generate neural tissue, represents a challenging tool for repairing the nervous system.

Neural stem cells are characterized as a subtype of progenitor cells in the nervous system that are able to self-renew and to produce neurons, glia and oligodendrocytes (Reynolds and Weiss, 1992 and 1996; McKay, 1997; Gage et al., 1995b; Gritti et al., 1996; Laywell et al., 2000). Neural stem cells have been isolated from diverse regions of the developing and adult rodent brain and cultured using different growth factors, such as EGF and FGF (Gage, 2000).

Adult neural stem cells are located in two main neurogenic regions of the vertebrate brain, the subventricular zone (SVZ) of the lateral ventricle (Doetsch et al., 1999) and the subgranular zone (SGZ) of the hippocampal formation (Gage, 1998), but may also be found in some non-neurogenic regions of the brain, including the spinal cord (McKay, 1997; Rao, 1999).

For brain repair in regions outside of these germinal centers, such as the cerebral cortex, stem cells may contribute to repair through replacement or recruitment (reviewed in Hallbergson et al., 2003). In the case of replacement, neural stem cells derived from embryonic, fetal, or adult sources could be expanded in vitro and fated to specific neuronal lineages. Thus when committed to the required phenotype, these cells could then be grafted to the target region. Studies using fetal tissue grafts to replace damaged cells in the CNS have proven to be successful (Studer et al., 1998; Sanchez-Pernaute et al., 2001) and have been used in clinical trials (Piccini et al., 2000; Wenning et al., 1997). However, the limited accessibility of stem cells on one hand and the ethical hurdles on the other, set constraints on 'replacement' therapy. An alternative solution could be to recruit endogenous neural stem cells for repairing CNS lesions. The environment of damaged non-neurogenic regions of the brain must be stimulated with appropriate signals to attract endogenous neural stem cells from the germinal centers and to instruct their proliferation, their migration and their differentiation into the appropriate neurons. Indeed, induction of de novo cortical neurogenesis could be observed in the cortex of adult mice (Fallon et al., 2000, Magavi et al., 2000; Arlotta et al., 2003). A persisting idea to explain the mechanisms of de novo neurogenesis in the cortex is the reactivation of a developmental program. Hence, it is of great interest to increase our understanding of mechanisms controlling cell specification and differentiation during early CNS development.

The receptor molecule Notch has been shown to play a role in cell fate decisions and differentiation in both invertebrate and vertebrate neurogenesis. However, mechanisms regulating the Notch signaling pathway itself still remain to be elucidated. Work in
Drosophila and vertebrates have indicated Numb to be a good candidate to modulate Notch signaling and to be involved in CNS development. Therefore, we have addressed the role of Numb in the formation of the mammalian brain (see part 3).

1.2 NOTCH SIGNALLING IN INVERTEBRATES

Drosophila has enabled an insight into invertebrate neurogenesis in the context of the Delta-Notch signaling pathway and the neural bHLH transcription factors that either promote or antagonize neural differentiation. The neurogenic genes encoded by Delta and Notch mediate lateral inhibition through cell-cell interactions. Notch is a transmembrane receptor that regulates cellular differentiation upon activation by one of its ligands, for example Delta, by inhibiting neural differentiation. The ligand Delta is expressed by the cells that deliver the inhibition signal. In the developing Drosophila nervous system, the Delta-Notch pathway is used to single out a cell from a group of ectodermal cells, the proneural cluster, to become a neural precursor (Lewis, 1996). The equal expression of the proneural genes ‘achaete-scute’ and ‘atonal’ enables all cells within the cluster to adopt a neural fate. However, the binding of Delta to Notch in a given cell triggers a signal involving the activity of anti-neural genes such as Suppressor of Hairless (Su(H)) and Enhancer of split (E(spl)), which result in the downregulation of proneural genes and the inhibition of this cell from adopting a neural fate (Beatus and Lendahl, 1998) (see also Fig 1-2).

1.2.1 NOTCH SIGNALING IN DROSOPHILA SENSILLIUM FORMATION

The development of sensory bristles, which cover the surface ectoderm of Drosophila, is regulated by Notch. Notch is involved in singling out a cell, the sensory organ precursor (SOP) cell, from an equipotent proneural cluster (Heitzler and Simpson, 1991; Muskavitch, 1994). The SOP cell then gives rise to the different cell types of the sensory bristle (Artsavanis-Tsakonas and Simpson, 1991). By delivering inhibitory signals to surrounding cells in the proneural cluster, the Delta-expressing SOP cell diverts its Notch-expressing neighbors from a neural fate and directs them to a secondary fate.

Notch signaling is not only involved in the generation of the SOP cell, but also later during the asymmetric divisions of the SOP cell which leads to the generation of distinct daughter cells. In the first division, the SOP cell divides asymmetrically to produce an SpIIA and an SpIIB cell. The SpII cells then undergo a second asymmetric division to generate a hair and a socket cell, the outer support cells as well as a sheath cell and a neuron, the inner
cells. Loss of *notch* function ablates asymmetric divisions and leads to the generation of two SpIIb cells and furthermore to the generation of multiple neurons (Hartenstein and Posakony, 1990). Conversely, overexpression of Notch similarly ablates asymmetric division and leads to the production of two SpIIa cells and socket cells at the expense of SpIIb progeny and bristles (Artavanis, 1995).

### 1.2.2 NUMB MODULATES NOTCH SIGNALING IN DROSOPHILA SENSILLIUM FORMATION

Beside cell-extrinsic mechanisms, cell-intrinsic cues influence asymmetric SOP divisions. These intrinsic mechanisms include the differential segregation of cell fate determinants to one of the two daughter cells and thus allow daughter cells to adopt distinct fates (Hawkins and Garriga, 1998). In contrast to Notch, the membrane-associated cytoplasmatic protein Numb is not involved in the generation of the SOP cell from the proneural cluster, but regulates asymmetric cell division by acting as a cell-intrinsic factor (Rhyu et al., 1994; Knoblich et al., 1995). Hence, Numb is essential for cell-fate determination of SOP progeny. Genetic manipulations have shown that loss of *numb* function, similarly to Notch overexpression, transforms the SpIIb cell into the SpIIa lineage (Uemura et al., 1989). In contrast, overexpression of Numb, similar to Notch ablation, transforms the SpIIa cell into the SpIIb lineage (Rhyu et al., 1994; Wang et al., 1997). These data indicate that Notch and Numb may have reciprocal functions in *Drosophila* sensillium formation. In addition, genetic epistasis experiments suggest that Notch acts downstream of Numb in the SOP lineage and that Numb might regulate Notch function through direct protein-protein interaction (Guo et al., 1996).

The interaction between Numb and Notch in *Drosophila* provides a way of integrating intrinsic and extrinsic cues. The task remains to elucidate whether similar mechanisms may be active during vertebrate neurogenesis.
1.3 NOTCH FUNCTION IN VERTEBRATE CNS DEVELOPMENT

1.3.1 THE NOTCH SIGNALING PATHWAY IS CONSERVED BETWEEN INVERTEBRATES AND VERTEBRATES

Notch belongs to a conserved family of transmembrane receptors that are activated upon binding to ligands of the Delta-Serrate-Lag2 family. To date, four members of the Notch family have been identified in mammals (Notch 1-4) that may interact with six ligands, Delta-like 1-4 and Jagged 1-2 (reviewed in Lendahl, 1998). The binding of Notch ligand induces a proteolytic cleavage of the Notch protein (Brou et al., 2000; Berezovska et al., 2000). Upon proteolysis, the intracellular domain (ICD) of Notch translocates, in complex with RBP-Jk (mammalian homologue to Drosophila Suppressor of Hairless (Su(H)) (reviewed in Honjo, 1996; Weinmaster; 1997), a member of the CSL Family, to the nucleus where it activates the transcription of the anti-neural Hes bHLH factors, homologs of the Enhancer of split complex (E(spl)) in Drosophila (Jarriault et al., 1998; reviewed in Kopan, 2002). Hes family members in turn repress the activation of the proneural bHLH factors like Mashl, a mammalian homologue to the Drosophila achaete-scute complex (reviewed in Lee, 1997). Mashl has been shown to be a determination factor in early neural precursor cells (Guillemot and Joyner, 1993). Thus Notch activity results in suppressing neural fate in the signal-receiving cell (Fig.1-2).

Fig 1-2
NUMB function in CNS

Fig 1-2:
Ligand induced cleavage of Notch leads to the release of Notch ICD that migrates to the nucleus; in collaboration with a CSL family member it activates transcription of Hes genes that in turn repress the expression of neural-specific genes. Interaction between Numb and Notch leads to proteasomal degradation of Notch ICD.

1.3.2 NOTCH1 EXPRESSION IN THE MAMMALIAN CNS

During early development, Notch1 transcripts can be found in a variety of tissues. In the postimplantation mouse embryo Notch1 expression is first detectable in the mesoderm at E7.5. By E8.5, transcript levels are highest in presomitic mesoderm, mesenchyme and endothelial cells, while much lower levels are detected in the nervous system (Reaume et al., 1992; del Amo et al., 1992). At E9.5, Notch1 expression in the nervous system becomes prominent and is restricted to the ventricular proliferative zone of the embryonic neuroepithelium (Lindsell, 1996; Weinmaster et al., 1991). In the cerebellum, Notch1 transcripts can be first detected at E9.5. Notch1 expression is downregulated as Purkinje and granule cell precursors leave the germinal zones by E16. A second wave of Notch1 expression is observed postnatally in Purkinje cell precursors.

Expression of Notch family members and their ligands is described in more detail in section 2: Notch signaling in postnatal brain.

1.3.3 NOTCH1 FUNCTION IN THE MAMMALIAN CNS

Targeted deletion of Notch1 in the mouse has primarily demonstrated a role for Notch1 in somitogenesis (Conlon et al., 1995; Swiatek et al., 1995). Unlike studies in Drosophila or Xenopus, where Notch was shown to influence neuronal cell fate determination and differentiation in the CNS (Spana and Doe, 1996; Chitnis et al., 1995), no such function could be detected in Notch1-/- deficient mice likely due to the early embryonic lethality of the mutation. Nevertheless, a slight increase in the expression of the proneural gene Mash1 in the neural tube of Notch1-/- mice has been reported (de La Pompa et al., 1997). However, it remained difficult to ascertain whether Notch1-deficiency leads to increased neurogenesis, since Notch1-deficient mice die shortly after E9.5, a time point preceding the major onset of neurogenesis. In order to study the role of Notch1 in development of the CNS and to circumvent the early lethality, Lütolf et al. conditionally ablated Notch1 from the neuroepithelium in the midbrain/hindbrain region of the neural tube at E9.0 (Lütolf et al., 1998).
They demonstrated a premature onset of neurogenesis by Notch1-deficient neuroepithelial cells in the midbrain/hindbrain neural tube, revealed by the premature onset of proneural and early neuronal marker expression. The precociously differentiating neurons are later eliminated by apoptosis, probably due to a lack of trophic support. Furthermore, the *in vivo* requirement of Notch1 in the generation of cerebellar Bergman glial cells was described (Lütolf et al., 2002).

Evidence for Notch1 being involved in vertebrate gliogenesis has been substantiated by other studies. *In vitro*, it could be shown that Jagged1 strongly inhibits oligodendrocyte differentiation through activation of the Notch1 receptor that is expressed in oligodendrocyte precursor cells (Wang et al., 1998). Furthermore, *in vivo* heterozygote Notch1 receptor mutant mice show increased oligodendrocyte differentiation suggesting that the expression of Notch ligands prevents myelination in certain areas of the brain (Givogri et al., 2002). The conditional Notch1 ablation in oligodendrocyte precursor cells in the spinal cord revealed premature oligodendrocyte differentiation and ectopic appearance of immature oligodendrocytes in the gray matter (Genoud et al., 2002). Very recently, it was shown that activation of Notch1 via F3 contactin controls later steps of oligodendrocytes maturation by activating the expression of the myelin associated glycoprotein MAG (Hu et al., 2003). In addition, other groups claim yet another role for Notch1 in gliogenesis (reviewed in Wang and Barres, 2000). Activated Notch is reported to promote the formation of radial glia in the fetal forebrain (Gaiano et al., 2000; Chambers et al., 2001) and of Müller glia in the retina (Furukawa et al., 2000). *In vitro*, it was found that transient activation of Notch is sufficient to induce differentiation of adult hippocampus-derived progenitors into astroglia (Tanigaki et al., 2001).

1.4 NUMB FUNCTION IN VERTEBRATE CNS DEVELOPMENT

1.4.1 NUMB IS A CONSERVED CYTOPLASMIC ADAPTER PROTEIN

Numb was initially identified in *Drosophila*, but species homologues in chicken, rat, mouse and human were shown to be quite conserved. mNumb is 593 amino acid (aa), cytoplasmic, membrane associated protein which displays many features of an adapter or scaffold protein. Numb is reported to be asymmetrically distributed during cell division (Zongh et al. 1996). The Numb protein is composed of three characteristic structural domains. The N–terminus all Numb homologues contains a conserved phosphotyrosine binding (PTB)
NUMB function in CNS
domain. PTB domains have been identified in several other proteins, e.g. Shc and have been shown to bind to phosphotyrosine-binding proteins such as EGF or NGF receptors (reviewed by van der Geer and Pawson, 1995). Moreover, the PTB domain of Numb was shown to bind in vitro to the ICD of Notch1 in a phosphotyrosine-independent manner (Zhong et al., 1996; Wakamatsu et al., 1999) and is thus hypothesized to modulate Notch signaling. The Numb protein contains a second structural domain, a proline rich region (PRR), which contains SH3 binding sites (Verdi et al., 1996). Finally, a third potential protein interaction domain at the N-terminus, an Eps15 homology (EH) domain-binding motif has been identified (Salcini et al., 1997) (see fig 1-3).

A second Numb-related protein, Numblike, was cloned in the mouse. Numblike encodes a 603 aa protein showing 76% identity and 46.7% identity to the Numb PTB domain and C-terminus, respectively (Zong et al., 1997). Similarly to Numb, Numblike is able to bind to the Notch1 ICD and localizes to the cytoplasm. However, Numblike has not been shown to distribute asymmetrically during cell division as shown for Numb (Zongh et al. 1996).

Recently, it was shown that mouse and human Numb proteins may exist in four alternatively spliced transcript forms that encode variants of the PTB and PRR domains. One splice variant shows an 11 aa insertion into the PTB domain, while a second splice variant of the N-terminus contains an 49 aa insertion into the PRR domain (Dho et al., 1999; Verdi et al., 1999) (see fig 1-3). The different isoforms confer distinct subcellular localization and moreover allow alternative protein interactions and functions for the different Numb isoforms (see 1.4.3.1).

Fig.1-3: Schematic representation of mNumb protein. The N-terminus of Numb consists of an alternatively spliced PTB domain followed by an alternatively spliced proline rich region (PRR), containing SH3 binding sites. The C-terminus contains an EH-domain binding motif.
1.4.2 NUMB EXPRESSION IN MAMMALIAN CNS

Numb RNA can be detected by Northern Blot analysis in most adult tissues, including brain, heart, liver, lung, spleen and intestine with the exception of skeletal muscle (Verdi et al., 1996; Zhong et al., 1996). During embryogenesis, Numb is expressed from E9.5 onwards in the VZ neuroepithelial cells of the developing CNS and PNS in a pattern overlapping the Notch1 expression. Recently, the expression of Numb in myelinating oligodendrocytes in the white matter of P30 cerebellum was reported (Givogri et al., 2003). Numblike RNA is detected first at E9.5 in trigeminal and dorsal root ganglia and from E12.5 onwards throughout the neural tube (Zhong et al., 1997). Expression patterns of Numb and Numblike are described in greater detail in section 2: ‘Notch signaling in postnatal brain’.

The specific Numb isoforms generated through alternative splicing reveal a dynamic developmental expression pattern: in the developing CNS, the expression of PRR_L (long PRR) isoforms peaks at E10 when neural precursor proliferation is occurring and declines after E14 while the expression of PRR_S (short PRR) remains constant (Verdi et al., 1999). Additionally, Numb distributes asymmetrically to the apical pole of cortical progenitor cells in the mouse (Zhong et al., 1996), whereas Numblike localizes to the cytoplasm and does not show asymmetric distribution (Zhong et al., 1997).

1.4.3 NUMB FUNCTION IN THE MAMMALIAN CNS

1.4.3.1. NUMB REGULATES NEUROGENESIS IN MAMMALIAN CELL LINES

In vitro studies using the overexpression of Numb in mammalian cell lines, provided the first evidence for Numb being involved in neurogenesis. Overexpression of Numb in the mouse embryonic carcinoma line P19 induces differentiation into neurons, whereas a truncated dominant-negative form of Numb, containing only the PTB domain, induces differentiation away from the neuronal lineage (Verdi et al., 1996). In contrast, Numb overexpression in the neural crest stem cell line MONC-1 leads to an increased neuronal differentiation as opposed to glia or smooth muscle generation (Verdi et al., 1996). Distinct human Numb isoforms were shown to have distinct functions in the neuronal lineage. Isoforms of Numb containing a short PRR promote neuronal differentiation while Numb protein isoforms containing the long PRR promote proliferation upon overexpression in the P19 cell line (Verdi et al., 1999). In the rat PC12 cell system, NGF treatment leads to the
NUMB function in CNS

generation of sympathetic neurons which then require NGF for their survival. Numb isoforms comprising a short PTB domain induce differentiation into neurons upon NGF administration while they enhance apoptosis upon NGF withdrawal. In addition, PC12 cells stably expressing a Numb isoform comprising a long PTB domain do not react in response to NGF (Pedersen et al., 2002).

1.4.3.2 NUMB IS INVOLVED IN CELL FATE CHOICES IN THE DEVELOPMENT OF THE MAMMALIAN CNS

During mammalian CNS development, neurons and glia arise from the subventricular zone neuroepithelium. In order to produce large number of different cell types, neuroepithelial cells display stem cell like properties thereby maintaining the neuroepithelial population and giving rise to neural precursors. Thereafter, precursors delaminate from the ventricular surface and start to differentiate. To achieve this, neuroepithelial cells undergo symmetric and asymmetric divisions (Chenn and McConnell, 1995). Asymmetric divisions are defined as divisions in which the two daughter cells inherit different cell-fate determinants from the mother cell and are thus able to adopt distinct fates. Similar to dNumb and to avian cNumb (see part 3.1), mammalian Numb is asymmetrically localized in dividing rat retinal neuroepithelial cells (Cayouette et al., 2001) and in progenitor cells during mouse cortical neurogenesis (Zhong et al., 1996; Shen et al., 2002).

The different cell types of the retina are produced in a well-defined chronological order and thus represent a good system to study cell fate determination in the vertebrate CNS. The proportion of retinal neuroepithelial cells undergoing asymmetric division increases around birth, a time point where Müller glial and bipolar cells develop. At P0, immunostaining of rat retinal sections and on isolated retinal cells revealed a diffuse localization of Notch1 at the cell surface and the asymmetric localization of Numb to the apical pole in both interphase and mitotic neuroepithelial cells (Cayouette, 2001; Silva et al., 2002). Asymmetric division in the retina results in the apical daughter cell inheriting Numb. Because Numb inhibits Notch signaling and Notch is inherited equally by the two daughter cells, the Numb expressing apical daughter cell might be inhibited from differentiating into a Müller glial cell. In contrast, the basal daughter cell is able to adopt a Müller glial fate. Here Notch signaling is not hampered through the action of Numb. Indeed, active Notch signaling has been shown to promote Müller glia formation (Furukawa et al., 2000). This hypothesis was further substantiated by a study that followed the fate of GFP-labelled daughter cells in retinal
explants. Daughter cells inheriting the same amount of Numb adopted a similar cell fate, while daughter cells inheriting unequal amounts of Numb adopted different fates. Furthermore, overexpression of Numb in mitotic cells promotes photoreceptor cell formation at the expense of Müller glia and interneurons (Cayouette and Raff, 2003).

Asymmetric distribution of Numb could further be associated with asymmetric divisions in mouse cortical progenitor cells (Shen et al., 2002). In cortical progenitor cells isolated at E10, Numb acquisition does not correlate with the acquisition of a certain cell fate. However, at E13-14, which represents the peak of cortical neurogenesis, Numb is preferentially localized to the neuronal daughter. Upon differentiation in vitro, two neurons generated from a common mother cell show different morphology upon unequal Numb distribution. Again, it was suggested that the adoption of different cell fates arising through unequal Numb distribution might be based on the inhibition of Notch signaling via Numb (Shen et al., 2002).

1.4.3.3 BIOCHEMICAL MECHANISMS UNDERLYING THE FUNCTION OF NUMB

Whereas the importance of Notch and Numb function during development has been recognized in both invertebrates and vertebrates (see sections above), the molecular and biochemical mechanisms underlying the regulatory effect of Numb on Notch signaling, remain largely obscure. Although studies in Drosophila and chicken suggested that Numb interaction with the ICD of Notch prevents its nuclear localization and transmission of the Notch signal (Frise et al., 1996; Wakamatsu et al., 1999), two recent studies present molecular explanations for these observations.

The finding that Numb interacts with the endocytic molecule Eps15 (Salcini et al., 1997) was the first hint that linked Numb to endocytosis. EH-containing and -binding proteins are involved in trafficking or internalization processes within a cell (Santolini et al., 1999). Further in vitro studies revealed that Numb is located in endocytic organelles, including endosomes, clathrin-coated pits and vesicles (Santolini et al., 2000). Surprisingly, Numb did not colocalize with Eps15 on the same coated pit. Numb was also shown to interact with the appendage domain (ear-domain) of α-adaptin, a subunit of AP2, a major component of clathrin-coated pits. Moreover, Numb cotrafficks with internalized EGF receptors and C-terminal fragments of Numb act as dominant/negative fragments inhibiting internalization of EGF receptors. Although established in cell lines, these results link Numb to the endocytic machinery, but it still remains to be shown whether Numb is also involved in receptor-mediated endocytosis of Notch.
Several studies suggested that the ubiquitin/proteasome degradation pathway is involved in controlling Notch signaling (reviewed Lai et al., 2002). Membrane bound Notch1 is ubiquitinated by the ubiquitin E3 ligase Itch (Qiu et al., 2000). Given that Numb was shown to interact with several ubiquitin E3 ligases (see below), but does not possess intrinsic E3 ligase activity, McGill and McGlade demonstrated that Numb interacts with Itch to enhance cooperatively ubiquitination of membrane tethered Notch, to target Notch1 ICD for proteasomal degradation and to downregulate Notch1-dependent signal transduction (McGill and McGlade, 2003) (see fig. 1.2).

Numb itself is regulated by the ubiquitin/proteasome pathway through the action of several molecules showing E3 ubiquitin ligase activity. The scaffold protein Lnx, the oncoprotein Mdm2 and the tumor suppressor protein Siah1 possess E3 ubiquitin ligase activity and selectively bind to Numb, promote its ubiquitination and proteasomal degradation (Nie et al., 2002; Yogosawa et al., 2003; Susini et al., 2001). Reduced Numb protein levels resulted in an increase of Notch nuclear activity (Nie et al., 2002).

### 1.4.3.4 NUMB-DEFICIENT MICE

Two independent lines of Numb-deficient mice have been generated (Zhong et al., 2000; Zilian et al., 2001). Both groups describe that Numb-deficient embryos die before E11.5, very likely due to vascular defects. These mice show also a failure of neural tube closure. However, the reported effects of Numb ablation on nervous system development differ. Zhong and colleagues report depletion of the progenitor pool and precocious neuron production in the forebrain of Numb-deficient mice. Hence, these authors claim that Numb as well as Numblike (Petersen et al., 2002) are required for progenitor cell maintenance. In contrast, Zilian and colleagues report that neurogenesis in the spinal cord was reduced. In the developing PNS, neural-crest derived sympathetic neurons differentiated normally whereas sensory neurogenesis seemed to be blocked. The authors claim Numb to be required only in a subset of neuronal lineages and to promote neurogenesis in these same neuronal lineages. An inherent problem of both studies is the early lethality of Numb-deficient embryos, which makes it impossible to study the effects of Numb ablation in later neurogenesis and may explain the seemingly conflicting reports on Numb ablation. In our study, we temporally and spatially restricted the ablation of Numb to the neuroepithelial cells of the midbrain/hindbrain region that gives rise to the cerebellum, a non vital CNS structure during development (see part 3).
2 NOTCH1 AND ITS LIGANDS DELTA-LIKE 1 AND JAGGED ARE EXPRESSED AND ACTIVE IN DISTINCT CELL POPULATIONS IN THE POSTNATAL BRAIN

Published: Gila Stump, André Durrer, Anne-Laurence Klein, Simone Lütolf, Ueli Suter and Verdon Taylor (2002). Notch1 and its ligands Delta-like and Jagged are expressed and active in distinct cell populations in the postnatal mouse brain, Mech Dev. 114 (1-2):153-159.

Contribution Anne-Laurence Klein: generation of Numb and Numblike RNA probes, in situ hybridization analysis of Numb and Numblike in postnatal and adult brain

2.1 ABSTRACT AND INTRODUCTION

Notch signaling plays a pivotal role in the regulation of vertebrate neurogenesis. However, in vitro experiments suggest that Notch1 may also be involved in the regulation of later stages of brain development. We have addressed putative roles in the central nervous system by examining the expression of Notch signaling cascade components in the postnatal mouse brain. In situ mRNA hybridization revealed that Notch1 is associated with cells in the subventricular zone, the dentate gyrus and the rostromigratory stream, all regions of continued neurogenesis in the postnatal brain. In addition, Notch1 is expressed at low levels throughout the cortex and olfactory bulb and shows striking expression in the cerebellar Purkinje cell layer. The Notch ligands, including Delta-like1 and 3 and Jagged1 and Jagged2, show distinct expression patterns in the developing and adult brain overlapping that of Notch1. In addition, the downstream targets of the Notch signaling cascade Hes1, Hes3, Hes5 and the intrinsic Notch regulatory proteins Numb and Numblike also show active signaling in distinct brain regions. Hes5 coincides with the majority of Notch1 expression and can be detected in the cerebral cortex, cerebellum and putative germinal zones. Hes3 on the other hand shows a restricted expression in cerebellar Purkinje cells. The distribution of Notch1 and its putative ligands suggest distinct roles in specific subsets of cells in the postnatal brain including putative stem cells and differentiated neurons.
2.2 MATERIALS AND METHODS

2.2.1 IN SITU HYBRIDIZATION AND PROBES

Brains were isolated and frozen in OCT (TissueTech) on dry ice. Twenty μm frozen sections were thaw mounted onto Superfrost slides (Mettler), air-dried, and fixed in 4% paraformaldehyde. The midline parasagittal sections of the postnatal brains were orientated between the cerebellar peduncles. In situ RNA hybridization was performed with digoxigenin-labeled RNA probes for Notch1 (867 bp encoding amino acid 1233-1520), Notch3 (Lindsell et al., 1995), Dll1 (dbEST Id:685391), Dll3 (mRNA 1-2100 bp (Dunwoodie et al., 1997), Jagged1 (dbEST Id:544372), Jagged2 (dbEST Id:1183493), Hes1 (mRNA 1-708 bp), Hes3 (mRNA 1-661 bp), Hes5 (mRNA 1-992 bp), Numb and Numblike overnight at 72°C in buffer containing 50% formamide, and detected using an anti-DIG-AP antibody according to manufacturer’s instructions (Roche Diagnostics). Expression was detected by colorimetric reaction using NBT and BCIP as reaction substrates. Coverslips were mounted in glycerol and images taken using 2.5-10X objectives and an Axioplan microscope in conjunction with an Axiocam CCD camera. Images were processed with PhotoShop 5.0 software.

2.3 RESULTS AND DISCUSSION

The role of Notch signaling in the regulation of embryonic progenitor cell differentiation has been extensively studied (Chitnis et al., 1995; Dorsky et al., 1997; Furukawa et al., 2000; Gaiano et al., 2000; Henrique et al., 1997; Lanford et al., 1999; Lütolf et al., 2002; Morrison et al., 2000; Muskavitch, 1994; Nieto et al., 2001; Scheer et al., 2001; Tanigaki et al., 2001; Wakamatsu et al., 2000). However, currently little is known about the involvement of Notch signaling in other phases of neural development. In vitro experiments indicate that Notch1 may play a role in postmitotic neurons by regulating neurite formation (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). Therefore, we examined the expression patterns of Notch1 and its putative ligands in the postnatal mouse brain. At P4, high levels of Notch1 transcripts were detected in the subventricular zone (SVZ), the region that gives rise to the olfactory neurons and contains putative stem cells of the postnatal brain (a summary of the expression data at P4 is shown in Table 2-1). Notch1 expression is also associated with cells lining the ventricular zone overlying the SVZ and the lateral ventricles over the hippocampal structure (Table 2-1). Punctate expression of Notch1 mRNA is evident in the dentate gyrus but is not associated with the majority of granule cells (Table 2-1). Notch1 transcripts are also evident in cells along the putative rostromigratory stream (RMS) towards the olfactory bulb (data not shown). Expression of the ligand Jagged1 can be found in the
SVZ and dentate gyrus where transcripts are mainly localized to granule neurons and neurons of the CA3 region (Table 2-1). By contrast, at P4 Jagged2 mRNA is detectable in the pyramidal neurons of the hippocampus (Table 2-1). The Notch ligands Dll1, Dll3 and Jagged2 are not detectable in the SVZ at P4.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Notch1</th>
<th>Dll1</th>
<th>Dll3</th>
<th>Jagged1</th>
<th>Jagged2</th>
<th>HES1</th>
<th>HES3</th>
<th>HES5</th>
<th>Numb</th>
<th>Numblike</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SVZ</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DG (sgl)</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Forebrain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
</tr>
<tr>
<td>Hip</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>DG (gel)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGL</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>PC</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2-1:
Summary of expression patterns of Notch signaling components in the P4 mouse brain. Expression in the subventricular zone (SVZ) and subgranule cell layer of the dentate gyrus (DG (sgl)) likely represent expression by or adjacent to putative postnatal neural stem cells. Forebrain region: cerebral cortex (Co), Hippocampus (Hip), dentate gyrus granule cell layer (DG (gel)). Cerebellum: external germinal layer (EGL), internal granule cell layer (IGL), Purkinje cells (PC). +++-prominent expression, ++-intermediate expression, +-low expression, +*-Numblike expression is elevated in the inner cells of the EGL. (+)-low level expression in a subpopulation of cells, ++(+) -intermediate HES5 expression in most cells but prominent expression in a subpopulation of cells.
In the adult, Notch1 transcripts remain prominently expressed in the germinal zones as well as in the RMS (Figure 2-1A). Within the SVZ clusters of cells lying adjacent to the ventricle lining express Notch1 mRNA (Figure 2-1B). A few cells lining the lateral ventricles also retain Notch1 expression (Figure 2-1A). Within the hippocampus, small clusters of Notch1 expressing cells can be detected in the subgranule cell layer, another region of continued neurogenesis in the adult brain (Figure 2-1C). In addition, scattered Notch1 expressing cells can also be found in the brain outside the SVZ (arrows in Figure 2-1B) as well as in the stratum radialis (arrows in Figure 2-1C) and stratum oriens (arrowheads in Figure 2-1C) of the hippocampus. As in the early postnatal brain, Jagged1 expression can be detected in cells within and around the SVZ (Figure 2-1D) and in the granule cells of the dentate gyrus (Figure 2-1E). Expression of the Dll ligands could not be detected in the SVZ or dentate gyrus and Jagged2 transcripts were also not detected in the SVZ (Figure 2-1F) but were found to be restricted to the granule cells and pyramidal neurons throughout the hippocampus (Figure 2-1G; a summary of the adult expression data is shown in Table 2-2). These expression patterns suggest that Notch1 may mark the stem cells of the postnatal brain.

We analyzed the expression of the HES genes in the postnatal brain to reveal activation of the Notch signal cascade. In contrast to Notch1 and Jagged1, Hes1 mRNA was not detected at P4 in the SVZ and dentate gyrus (Table 2-1). However, Hes5 mRNA was detected in the SVZ and lining of the lateral ventricles in a pattern similar to that of Notch1 (Figure 2-2A). Furthermore, expression of Hes5 at P4 showed a distribution similar to that of Notch1 in clusters of cells within the dentate gyrus (Figure 2-2B) as well as in scattered cells around the SVZ (arrows in Figure 2-2A) and within the stratum radialis (arrows in Figure 2-2B) and stratum oriens (arrowhead in Figure 2-2B) of the hippocampus. Hes3 mRNA was not detected in the forebrain of P4 animals (Table 1).

Notch receptor activity is modulated by a number of intracellular proteins (reviewed by Kimble and Simpson, 1997). Numb and Numblike are cytoplasmic proteins that bind to the intracellular domains of Notch receptors and negatively regulate signal transduction (Wakamatsu et al., 1999; Zhong et al., 1996). Numb transcripts were not detected in the SVZ at P4 and at low levels in neurons within the hippocampus and dentate gyrus (Table 2-1). Similarly, Numblike mRNA was found in cells throughout the forebrain but not in the SVZ (Table 2-1). Numblike expression in the hippocampus was restricted to the pyramidal cells of CA1-3 with low levels in the granule cells of the dentate gyrus.
Figure 2-1:

Notch1, Jagged1 and Jagged2 expression in the adult brain. A) Notch1 is expressed by cells in the subventricular zone (SVZ), lining of the lateral ventricles (VL) and the dentate gyrus (DG). B) Notch1 expressing cells in the SVZ are mainly associated with the subependymal layers of the SVZ, however, some cells outside the SVZ also express Notch1 mRNA (arrows). C) In the DG, Notch1 expression can be detected in the subgranule cell layer (SGL) and cells within the stratum radialis (sr; arrows) and stratum oriens (so, arrowheads). D) Jagged1 expression is maintained in the adult SVZ and E) is associated with the granule cells of the DG. F) Jagged2 is not expressed at detectable levels in the SVZ of adults but G) can be detected in the granule cells and pyramidal neurons (not shown) of the hippocampus. Co- cerebral cortex, CP- choroid plexus, RMS- rostromigratory stream.
We examined the expression of the *Hes* genes in the adult brain to reveal an active Notch signal. As in the early postnatal brain, Hes1 and Hes3 are not expressed at detectable levels in the SVZ or in the adult hippocampus (Figure 2-2C, D, and Table 2-2). However, Hes5 is expressed in clusters of cells within the adult SVZ and in the subgranule cell layer of the dentate gyrus in a pattern similar to that of Notch1 (arrows in Figure 2-2E, F). We also examined expression of Numb and Numblike in the adult brain. Numb expression was not detectable in the adult SVZ (Figure 2-2G) but was detected in the granule cells of the dentate gyrus and in the pyramidal neurons of the CA1-3 region of the hippocampus (Figure 2-2H and Table 2-2). Likewise, Numblike transcripts were not detected in the adult SVZ (Figure 2-2I) and only at low levels in cells throughout the dentate gyrus (Figure 2-2J).

**Figure 2-2:**
NUMB function in CNS

**Figure 2-2:**

Active Notch signaling in the forebrain. A) Hes5 expression was found in the SVZ and ventricular lining (VL) of the P4 brain as well as in cells more distant from the SVZ (arrows). B) In the DG, Hes5 is expressed at low levels by cells of the subgranule layer (SGL) and cells throughout the stratum radiatis (sr, arrows) and stratum oriens (so, arrowheads). Hes1 expression was not detected at prominent levels in the SVZ or DG of the P4 brain (see Table 1). C) Hes1 RNA is also not detectable in the SVZ or DG of the adult brain. E) Hes5 RNA on the other hand is expressed by clusters of cells in the adult SVZ (arrows). F) In the DG, Hes5 is associated with clusters of cells in the subgranule cells layer (SGL, arrows). G) Numb RNA was not detected in the adult SVZ, H) but expression was found in the granule cell layer (gcl) of the DG. I) Numblike RNA was not found in the adult SVZ J) but is expressed by the pyramidal neurons of the CA1-3 (not shown) and at low levels by the granule cells (gcl) of the DG. CP- choroid plexus.

In vitro gain-of-function experiments suggest that Notch signaling may play a role in neurons at later stages of development, particularly in the regulation of dendrite formation (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). Therefore, we examined the expression of Notch1, its ligands and downstream signaling components in the cerebral cortex. At P4, Notch1 mRNA levels in the cerebral cortex were low and only few Notch1 expressing cells could be detected by in situ hybridization (arrows in Figure 2-3A; insert shows a magnification of a Notch1 expressing cell in the cerebral cortex at P4). However, the ligands Dll1 (Figure 2-3B), Jagged1 (Table 2-1) and Jagged2 (data not shown) were all found to be expressed within the cerebral cortex. Although Notch1 is expressed at very low levels, Hes1 and Hes5 were detected in subpopulations of cells in all layers of the cerebral cortex (arrows in Figure 2-3C, D). Neither showed a layer specific expression pattern, and although some cells in the cerebral cortex displayed particularly high levels of Hes5 expression, most of the cells appeared to be labeled. Similarly, both Numb and Numblike mRNAs were detected throughout the P4 cerebral cortex with no layer-specific distribution (Table 2-1).

Notch1 was found to be expressed at low levels with only a few scattered cells in the adult cerebral cortex (arrows in Figure 2-3E). In addition, Notch3 showed a distinct distribution in putative blood vessels running radially in the adult cerebral cortex (Figure 2-3F) (Irvin et al., 2001). The blood vessel expression of Notch3 RNA in the adult is similar to that seen in the P4 brain (data not shown) and may reflect the described function of Notch3 mutations in the human stroke disorder CADISIL (Joutel et al., 1996). Dll1 expressing cells were detected throughout the cerebral cortex (Table 2-2), however, Jagged1 and Jagged2 were detected in a patchy expression pattern throughout the adult cerebral cortex (Figure 2-3G, H).
NUMB function in CNS

This reduced expression of Notch1 and its ligands was also associated with a reduction in the Hes gene expression. Only Hes5 could be detected in a subpopulation of cells scattered throughout the cortex in a distribution reminiscent of that of astrocytes (Figure 2-31). The reduced Notch signaling in the adult cortex was supported by the expression of Numb and Numblike mRNA in the cerebral cortex (Figure 2-3J and K).

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Notch1</th>
<th>Dll1</th>
<th>Dll3</th>
<th>Jagged 1</th>
<th>Jagged 2</th>
<th>HES1</th>
<th>HES3</th>
<th>HES5</th>
<th>Numb</th>
<th>Numblike</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SVZ</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DG (sgl)</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>nd</td>
<td>nd</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Forebrain</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>(+)</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+++(+)</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Hip</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>DG (gcl)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>PC</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 2-2:
Summary of expression patterns of Notch signaling components in the adult mouse brain. Expression in the subventricular zone (SVZ) and subgranule cell layer of the dentate gyrus (DG (sgl)) likely represent expression by or adjacent to putative postnatal neural stem cells. Forebrain region: cerebral cortex (Co), Hippocampus (Hip), dentate gyrus granule cell layer (DG (gcl)). Cerebellum: internal granule cell layer (IGL), Purkinje cells (PC). +++-prominent expression, ++-intermediate expression, +-low expression. (+)-low level expression in a subpopulation of cells, +++(+)-intermediate HES5 expression in most cells but prominent expression in a subpopulation. nd-not distinguishable.
Figure 2-3:
Active Notch signaling in the cerebral cortex. A) Notch1 RNA is expressed by a few cells at low levels scattered throughout the cerebral cortex of postnatal day 4 animals (arrows and insert). B) Dll1 RNA is also expressed at low levels throughout the cerebral cortex with slightly higher levels towards the superficial region of the cortex with the exception of the outer layer 1. C) Hes1 RNA was detectable in many cells of the cerebral cortex at P4 (arrows). D) Hes5 RNA is expressed by most cells of the cerebral cortex at P4 but at particularly high levels by a subpopulation. E) Notch1 expression in the adult cerebral cortex is restricted to a few cells scattered throughout the cortical layers (arrows and insert). F) Notch3 is prominently expressed by a subpopulation of cells in the cerebral cortex and by putative endothelial cells of blood vessels (bv). Jagged1 G) and Jagged2 H) RNAs are detectable in the inner cortical layers of the adult. I) As in the early postnatal brain Hes5 RNA is expressed at low levels by many cells in the adult cerebral cortex but at particularly high levels in a distinct subpopulation. Both Numb J) and Numblike K) show an extensive pattern of expression throughout the cerebral cortex but are spared from the outer cortical layer. L) A representative sense probe control (Numblike) on adult brain sections shows no specific staining.
The cerebellum is a region of the vertebrate brain where extensive neurogenesis takes place postnataally (reviewed by Hatten et al., 1997; Hatten and Heintz, 1995). Notch1 is prominently expressed in the developing cerebellum by the majority of cells in the developing Purkinje cell layer at P4 (Figure 2-4A). In addition, cells within the forming internal granule cell layer (IGL) and white matter also express Notch1. Based on their distribution it is possible that these cells represent immature astrocytes and oligodendrocytes. We also found Notch3 expression within the forming Purkinje cell layer (Figure 2-4B), however, it is unclear whether these cells are Purkinje cells or the Bergmann glia (Figure 2-4B). Furthermore, recent data indicate that Notch2 is expressed by granule cell precursors in the cerebellum and may regulate the onset of differentiation in the EGL (Irvin et al., 2001; Solecki et al., 2001).

In situ hybridization revealed a prominent expression of Jagged1 in cells of the external germinal layer (EGL) as well as the granule cells of the IGL (Figure 2-4D). However, ligands of the Dll-family were not detected in the cerebellum at P4 (Figure 2-4C and Table 2-1). The expression of Notch1, 3 and Jagged1 was associated with a prominent expression of Hes5 in the forming IGL and white matter as well as low levels of Hes1 in the IGL (Figure 2-4E, F). By contrast, Hes3 RNA was detected at low levels in putative Purkinje cells at P4 (Table 2-1). The putative reciprocal expression of Notch1 and Jagged1 in Purkinje cells and granule cells, respectively, suggests a bi-directional interaction between these two cell-types. Furthermore, expression of the regulatory molecules Numb and Numblike was detected in the EGL and, to a lesser extent in the IGL (Figure 2-4G, H). Whereas Numb displayed a patchy expression throughout the EGL, Numblike expressing cells were mainly located within the inner portion of the EGL where granule cells commence differentiation (arrows in Figure 2-4H).

Figure 2-4:
Notch signaling in the early postnatal cerebellum. A) Notch1 is expressed by cells of the Purkinje cell layer (PCL) and in the forming white matter (WM) at postnatal day 4 (P4). In addition Notch1 expression is associated with the blood vessels (bv) covering the surface of the cerebellum. B) Notch3 is also expressed by cells within the PCL and by blood vessels (bv). C) Dll3 expression was not detected in the cerebellum at P4. D) Jagged1 is prominently expressed in the external germinal layer (EGL) and cells of the forming internal germinal layer (IGL) but not by cells in the PCL. E) Hes1 was detectable at low levels in cells of the forming IGL but not within the EGL. F) Hes5 is prominently expressed by cells in the IGL and white matter (WM) (arrows) and may represent differentiating glia. G) Numb is expressed by cells in the EGL and at low levels in a punctate pattern in the IGL. H) Numblike expression is most prominent in cells at the inner aspect of the EGL (arrows). Arrowheads in G) and H) indicate the outer surface of the cerebellum.
Figure 2-4: Notch signaling in the early postnatal cerebellum
Finally, we examined the expression of Notch signaling molecules in the adult cerebellum. Notch1 expression was detected in the Purkinje cell layer (Figure 2-5A), but it cannot be excluded that cells in addition to the Purkinje cells such as Bergmann glia also express Notch1 (Figure 2-5A). In addition, cells within the IGL also displayed Notch1 expression (arrows in Figure 2-5A). Notch3 on the other hand showed a more diffuse pattern of staining within the Purkinje cell layer and did not appear to be associated with the large pyramidal neurons but rather with cells surrounding the Purkinje cells (Figure 2-5B). The ligands Dll3 and Jagged1 are also expressed in the adult cerebellum. Whereas Dll3 is expressed by Purkinje cells (arrow in Figure 2-5C), Jagged1 is restricted to the granule cells (Figure 2-5D).

Hes1 and Hes5 expression was not detected in the adult cerebellum (Figure 2-5E and Table 2-2), however, an active Notch signal was supported by the prominent expression of Hes3 by Purkinje cells (arrow in Figure 2-5F). The increased expression of Hes3 between P4 and adult coincides with the differentiation of the granule cells and their synaptogenesis with Purkinje cells (reviewed by Hatten et al., 1997; Hatten and Heintz, 1995). Interestingly, although Purkinje cells show an activation of Notch signaling with the expression of Hes3, they also express prominent levels of Dll3 (arrow in Figure 2-5C). The Purkinje cell-type Hes3 does not have a functional basic DNA binding domain (Hirata et al., 2000), and is incapable of binding to DNA. This may allow the expression of Dll3, which would normally be repressed by Hes proteins. Finally, both Numb and Numblike mRNAs were detected in the adult cerebellum. Whereas Numb is expressed by both Purkinje cells (arrow in Figure 2-5C) and granule cells (Figure 2-5G), Numblike was only detected in the granule cells (Figure 2-5H). Our data indicate an extensive expression of Notch1 and activation of downstream signaling components in defined regions of the postnatal brain.

Figure 2-5:

Notch signaling in the adult cerebellum. A) Notch1 expression can be detected in cells of the Purkinje cell layer (PCL) and a subpopulation of cells within the internal granule cell layer (IGL; arrows). B) Notch3 is expressed by cells in the PCL and blood vessels (bv) covering the surface of the cerebellum. C) Dll3 expression is restricted to Purkinje cells (PC; arrow) in the adult cerebellum and, D) Jagged1 is prominently expressed by granule cells in the IGL. E) Hes1 expression was not detectable in the adult cerebellum but, F) Hes3 is prominently expressed by Purkinje cells (PC; arrow). G) Numb was detected in the IGL and by Purkinje cells (PC; arrow) whereas H) Numblike is expressed by cells of the IGL.
Figure 2-5: Notch signaling in the adult cerebellum
2.4 OUTLOOK

Notch signaling is reported to regulate a large number of cellular decisions during development (reviewed by Artavanis-Tsakonas et al., 1999). Considering the expression pattern of Notch1, its ligands, downstream-effectors and modulators (see section 2.3), the importance of Notch signaling in the postnatal and adult brain must not be underestimated.

Indeed, Notch1 gain-of-function experiments in cultured primary cortical neurons and cell lines, suggested that Notch1, upon interaction with ligand-expressing cells, inhibits neurite outgrowth but increases dendritic branching (Berezovska et al, 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). In order to address the function of Notch1 in postmitotic neurons in vivo, it is necessary to analyze Notch ablation from transgenic mice carrying floxed Notch1 alleles (Lütolf et al., 2002) and the Cre-recombinase under the control of a late neuronal promoter, eg the Calbindin-Cre transgene (Stump and Taylor, unpublished data). Calbindin-Cre transgenic lines have been generated in our lab and were shown to express Cre-recombinase within the cortex, hippocampus and the Purkinje cells. These animals offer the necessary tool to analyze the effects of Notch1 ablation on dendrite formation and branching in vivo.

Based on the prominent expression of Notch1 and its ligands Jagged in germinal centres of the postnatal and adult brain, it is tempting to speculate that Notch signaling is associated with stem cells and ongoing neurogenesis in the adult brain. Recently, two independent groups did claim Notch signaling to be required for the self-renewal, but not for the generation of EGF-dependent, neural stem cells (Chojnacki et al., 2003; Hitoshi et al., 2002). A more direct study to analyze Notch1 function itself in adult-type neural stem cells was performed in our laboratory by Lütolf et al. (unpublished data). The floxed Notch1 alleles were inactivated in EGF-dependent neural stem cells in vitro by infection with a Cre-recombinase expressing Adenovirus. This study demonstrated that Notch1 influences maintenance of adult-type neural stem cells but does not affect cell lineage differentiation in vitro. In vivo, the relevance of Notch1 in stem cells could be addressed by targeted ablation in adult neural stem cells and by transplanting Notch1-deficient adult-type stem cells into an embryonic development using ultrasound backscatter biomicroscopy and following their proliferation and differentiation. This experimental setup allows to elucidate the influence of extrinsic and intrinsic factors on neural stem cell fate. Indeed, it is of great interest to study Notch function in the adult in view of potential therapeutics of neurodegenerative diseases, like e.g. Alzheimer's disease (see part 4).
In contrast to Notch1, Numb and Numblike, described as modulators of Notch signaling (Wakamatsu, 1999), are not expressed in the subventricular germinal zone; they are however prominently expressed in defined cortical layers and in the cerebellum of the postnatal and adult brain. Using a condition gene ablation approach, we were able to analyze the function of Numb during both embryonic and postnatal cerebellar development (see part 3).
3 MURINE NUMB REGULATES GRANULE CELL MATURATION IN THE CEREBELLUM

Published online: Anne-Laurence Klein, Olav Zilian, Ueli Suter and Verdon Taylor (2004)
Murine Numb Regulates Granule Cell Maturation in the Cerebellum, DevBiol

3.1 ABSTRACT

Notch is a key regulator of vertebrate neurogenesis and the cytoplasmic adaptor protein Numb is a modulator of the Notch signaling pathway. To address the role of murine Numb in development of the central nervous system we used a conditional gene ablation approach. We show that Numb is involved in the maturation of cerebellar granule cells. Although the specification of neural cell fates in the cerebellum is not affected in the absence of Numb, the transition from a mitotic progenitor to a mature granule cell is aberrant and migration of postmitotic granule cells to the internal granule cell layer is delayed. In some animals, this results in a complete agenesis of granule cells and a strong ataxia. We confirmed these findings in vitro and found that Numb-deficient cerebellar progenitor cells show a marked delay in granule cell maturation. Our results suggest that Numb plays a role in the transition of a mitotic progenitor to a fully differentiated granule cell in the cerebellum. In addition, the maturation of Purkinje cells is also delayed in Numb-deficient mice.

3.2 INTRODUCTION

Cells of the vertebrate nervous system are generated from the neuroepithelium during embryonic development. A subpopulation of cells within the neuroepithelium display stem cell-like properties: multipotency, the capacity for self-renewal and the ability to generate other cell-types through asymmetric cell division. The differentiation of these neural stem cells likely involves a precursor-like state before generation of the multiple cell-types of the brain (Mayer-Proschel et al., 1997; Rao and Mayer-Proschel, 1997). Neural precursor cells acquire a defined identity on the basis of their axial coordinates within the neuroepithelium. Thus, the development of stem cells into the various differentiated neural cell types of the adult nervous system must be strictly coordinated in a temporal and spatial manner. The mechanisms that control the development of the vertebrate nervous system are only partially
known. In *Drosophila*, Delta-Notch signaling regulates neurogenesis through lateral inhibition (Heitzler and Simpson, 1991; Muskavitch, 1994). However, cell-intrinsic mechanisms also play a role in the development of the *Drosophila* nervous system. *Drosophila* Numb (dNumb) has been found to be an important regulator of asymmetric cell division and fate determination in the nervous system, muscle and malpighian tubules of *Drosophila* (Frise et al., 1996; Guo et al., 1996; Spana and Doe, 1996; Wan et al., 2000). Genetic analyses have indicated that dNumb is involved in Notch signaling and that the two genes have reciprocal functions (Rhyu et al., 1994; Wang et al., 1997). Furthermore, dNumb/Notch signaling has been shown to regulate the choice between neuronal cell fate or programmed cell death (Lundell et al., 2003). Numb is a cytoplasmic, membrane-associated protein with an N-terminal phosphotyrosine-binding (PTB) domain, a proline-rich region (PRR) and an EH-binding site at the C-terminus (Mayer, 1999; Salcini et al., 1997; Dho et al., 1998). Numb has been claimed to bind to the intracellular domain (ICD) of Notch via its PTB domain in a phosphotyrosine-independent manner to prevent Notch signaling through an as yet unclear mechanism (Wakamatsu et al., 1999; Zhong et al., 1997). However, recent data suggest that dNumb regulates Notch activity by affecting the subcellular localization of the tetraspan protein *sanpodo* (O'Connor-Giles et al., 2003), and that mammalian Numb is involved in Notch1 receptor ubiquitination and proteasomal degradation but may not interact directly with Notch1 (McGill and Mc Glade, 2003).

During vertebrate central nervous system (CNS) development, neuroepithelial cells proliferate in the ventricular zone to enlarge the stem cell pool (Chenn and McConnell, 1995). Subsequently, stem cell progeny delaminate from the ventricular surface and start to differentiate. In the developing chick CNS, chicken Numb (cNumb) protein is reported to be localized to the basal pole of neuroepithelial cells during M-phase of the cell cycle (Silva et al., 2002; Wakamatsu et al., 1999). Symmetric cell division results in the formation of two identical daughter cells that acquire Numb. During asymmetric cell division cNumb is preferentially inherited by the basal daughter cell that differentiates, leaving the apical cNumb-depleted daughter cell as a progenitor. Retroviral overexpression of Numb in the chick neuroepithelium resulted in exit from the cell cycle and precocious neuronal differentiation. By contrast, retroviral overexpression of Notch1 ICD in the neuroepithelium resulted in an enlargement of the stem cell pool and a delay in neurogenesis (Wakamatsu et al., 1999). These results suggest that in the chicken cNumb is prominently involved as a cell-intrinsic cue in early developmental decisions made by neural progenitor cells. However, in mammals the situation is less clear. *In vitro* overexpression of mammalian mNumb in the
neural crest-derived MONC-1 cell line leads to the generation of neurons at the expense of
glia and smooth muscle cells and a bias in P19 cell differentiation into neurons instead of glia
(Verdi et al., 1996; Verdi et al., 1999). In addition, isoforms of human Numb (hNumb)
generated by alternative RNA splicing with a long PRR rather promote proliferation while
hNumb isoforms with a short PRR promote differentiation of P19 cells (Verdi et al., 1996;
Verdi et al., 1999).

Between E8.5 and E12.5, expression of Numb (mNumb) overlaps with that of Notch1
expression in neuroepithelial cells of the ventricular zone and it is localized to the apical pole
of mitotic cells (Zhong et al., 1996; Zhong et al., 1997). In cortical progenitors and
neuroblasts, asymmetric localization of mNumb during cell division affects the fate acquired
by the daughter cells (Shen et al., 2002). Furthermore, asymmetric distribution of Numb is
also seen in the rat retinal neuroepithelium (Cayouette and Raff, 2002). Asymmetric
distribution of Numb and the dynamic expression of different Numb isoforms at specific time
points during rat retinal development are associated with the temporally controlled generation
of the different retinal cell types (Dooley et al., 2003).

Two independent mNumb-deficient mouse lines have been generated that show severe cranial
neural tube closure defects and die around embryonic day 11.5 (E11.5), possibly as a result of
vasculature malformations (Zhong et al., 2000; Zilian et al., 2001). The in vivo role of
mNumb in mammalian neurogenesis and the fate of Numb-deficient neuroepithelial cells
remain unclear due to the early lethality of Numb-deficient mice (Zhong et al., 2000; Zilian et
al., 2001; Zhong, 2003). In order to address the function of mNumb during vertebrate CNS
development, we have conditionally ablated the mNumb gene from the mouse neural tube
using the Cre-lox system. Numb was inactivated specifically in neuroepithelial cells of the
midbrain hindbrain boundary by expression of Cre-recombinase from an engrailed2 promoter
to circumvent the lethal effects of mNumb loss-of-function. We followed the consequences of
mNumb ablation on neuroepithelial cells and their progeny through to adulthood and found
that mNumb is involved in the transition of cerebellar progenitors to mature granule cells.
3.3 MATERIALS AND METHODS

3.3.1 GENERATION OF MICE AND BREEDING

Mice carrying loxP-flanked Numb alleles have been described previously (Zilian et al., 2001) and mice carrying the cre-recombinase under the transcriptional control of the engrailed-2 promoter enhancer (line Tg3) were kindly provided by Dr. A. Joyner (Zinyk et al., 1998). The ROSA26-R Cre-reporter (R26R) transgenic line was provided by Dr. P. Soriano (Soriano, 1999). Embryos were generated by timed-mating, counting the morning after pairing as embryonic day 0.5. The animals were maintained on a mixed 129SV/C57BL6 background and sacrificed according to institutional guidelines.

3.3.2 IN SITU HYBRIDIZATION ANALYSIS OF GENE EXPRESSION

Embryos and brains were isolated and frozen in OCT (TissueTech) on dry ice. Frozen sections (20 μm) were thaw mounted onto Superfrost slides (Mettler), air-dried and fixed in 4% paraformaldehyde. The midline sagittal sections of the adult cerebellum were orientated between the peduncles. The embryos were mounted and cross-sections cut perpendicular to the neural tube at the level of the isthmic flexure. In situ RNA hybridization was performed with digoxigenin-labeled RNA probes for calbindin D28k, Mash1, Math1 (Atohl – Mouse Genome Informatics) and NeuroD, as described previously (Lutolf et al., 2002).

3.3.3 IMMUNOHISTOCHEMISTRY AND CELL FATE ANALYSIS

Histological analysis was performed by staining paraffin sections (5 μm) for 2 minutes in hematoxylin (Polysciences, Inc.) and 10 minutes in eosin (Polysciences, Inc.) followed by dehydration in alcohol and embedding in DePex (Serva). Indirect immunofluorescence was performed on frozen sections (20 μm) with antibodies against Nestin (monoclonal mouse antibody, 1:30; R401, Developmental Studies Hybridoma Bank, University of Iowa) and phosphohistone H3 (pH3) (polyclonal rabbit antibody, 1:300; Upstate Biotechnology). Sections were fixed in 4% paraformaldehyde and blocked in PBS 0.2% Tween20 containing 5% goat serum (blocking buffer). Antibodies were diluted in blocking buffer and incubated overnight at 4°C. Immunostaining against Calbindin D28k (monoclonal mouse antibody, 1:200; Sigma) and GABAa6 (polyclonal rabbit antibody, 1:1000; Chemicon) was performed on paraffin sections (5 μm) using standard protocols. Bound antibody was detected with Cy3-
or FITC-conjugated goat anti-mouse Ig (Jackson Labs) and goat anti-rabbit Ig (Jackson Labs). Bound GABAa6 antibody was visualized colorimetrically with HRP-conjugated anti-rabbit Ig using the DAKO Envision™+ HRP system (DAKO) according to the manufacturer’s instructions. b-galactosidase was detected by X-gal staining according to Zinyk et al. (Zinyk et al., 1998) or with anti-b-galactosidase antibodies (polyclonal rabbit antibody, 1:100; Cappel). Apoptotic cell death was detected by TUNEL staining using biotin-labeled UTP and a Cy3-conjugated streptavidin complex according to the manufacturer’s instructions (Roche Diagnostics). Images were collected using an Axiophot microscope (Zeiss) in conjunction with a ProgRes 3008 (Jenoptik) or Hamamatsu (Hamamatsu Photonics) CCD camera; image processing was performed with NIH Image and Adobe Photoshop 7.0 software.

3.3.4 ISOLATION OF CEREBELLAR NEUROEPITHELIAL CELLS AND IMMUNOCYTOCHEMISTRY

Embryonic day 10.5 embryos were sacrificed and the cerebellar primordium dissected under sterile conditions (figure 9A). The trunk region of the embryos was used for DNA isolation and genotyping by PCR. The cerebellar primordium of the individual embryos was dissociated and incubated in Dulbecco’s Modified Eagle Medium/F12 (DMEM/F12; Life Technologies) containing 1 mg/ml collagenase type IV (Sigma), 0.12 mg/ml hyaluronidase (Sigma) and 0.03 mg/ml trypsin inhibitor (Sigma) for 10 minutes at 37°C. The cells were collected by centrifugation and resuspended in differentiation medium consisting of DMEM/F12 containing 1% N2 (Gibco), 25 mM KCl, 10 ng/ml EGF (Peprotech), 10 ng/ml FGF-2 (Peprotech), 5 ng/ml NGF (Peprotech), 10 ng/ml BDNF (Peprotech) and 10 ng/ml IGF1 (Peprotech). The cells from the cerebellar primordium of one embryo were plated on 8 wells (18 mm) of a 24 well plate coated with 50 μg/ml poly-L-lysine and 20 mg/ml laminin. No obvious differences in the plating efficiency were observed between mutant and control cells. The cells were cultured for 6.5 or 13 days in differentiation medium and half of the medium was replaced every second day with medium containing double concentration of growth factors. The cells were fixed in 4% formaldehyde and immunostained with antibodies against β-TubulinIII (Tuj1) (monoclonal mouse antibody, 1:200; Sigma), Nestin (1:30; R401, Developmental Studies Hybridoma Bank, University of Iowa), Calbindin D28k (monoclonal mouse antibody, 1:200; Sigma), smooth muscle actin (SMA) (monoclonal mouse antibody, 1:500; Sigma) and O4 (monoclonal mouse antibody, 1:10; a kind gift of Dr. M. Schwab) in
conjunction with anti-β-galactosidase antibodies (polyclonal rabbit antibody, 1:100; Cappel). Additionally, cells were immunostained with antibodies against Math1 (polyclonal rabbit antibody, 1:200; a kind gift of Dr. J. Johnson). Bound antibody was detected with Cy3-conjugated goat anti-mouse Ig (Jackson Labs), FITC-goat anti-mouse IgM (Jackson Labs) or goat anti-rabbit Ig (Jackson Labs).

3.3.5 WESTERN BLOT ANALYSIS

E12.5 embryos were sacrificed, the cerebellar primordium dissociated and the isolated cerebellar neuroepithelial cells of individual embryos were plated on sister plates in differentiation medium (see section above). After five days, half of the cells of each embryo were fixed and stained with anti-b-galactosidase antibodies to determine the percentage of recombined cells. The other half of the cells was washed and protein extracted in 8 M Urea. 20 µg of protein of both control and mutant embryos were resolved on denaturing SDS-polyacrylamide gels. PonceauS staining was used to assess equal loading of proteins. The blots were incubated with a polyclonal rabbit anti-Numb antibody (1:800; kindly provided by Dr. H. Okano) at 4°C overnight and detected by ECL (Amersham) chemiluminescence. The blot was stripped and incubated with an anti-bactin antibody (monoclonal mouse antibody, 1:1000; Sigma) for normalization. Quantification was performed using 1D Image Analysis Software (Kodak Digital Science).

3.4 RESULTS

We have conditionally inactivated the Numb gene in neuroepithelial cells of the isthmus and cerebellar primordium at E9 using a Cre-loxP approach to circumvent the early embryonic lethality observed in Numb-deficient animals (Zhong et al., 2000; Zilian et al., 2001). Floxed Numb alleles were generated by introducing loxP sequences flanking the first coding exon of mNumb by homologous recombination (figure 1; Zilian et al., 2001). Cre-mediated recombination results in a null allele (Zilian et al., 2001). We restricted mNumb ablation to the midbrain hindbrain region of the neural tube by expression of Cre-recombinase under the transcriptional control of the engrailed2-promoter enhancer (En2-Cre) (figure 1). Mice homozygous for the floxed Numb allele show no detectable abnormal phenotype in the absence of Cre-recombinase. In addition, the ROSA26 Cre-reporter allele (R26R) was included in the analysis to follow the recombination and fate of Cre-expressing cells and their
NUMB function in CNS

progeny (Lutolf et al., 2002; Soriano, 1999). If Cre-recombinase is present, the \textit{loxP} sequences flanking the PGK neo cassette of the \textit{R26R} are recombined and b-galactosidase is constitutively expressed (Soriano, 1999).

![Diagram](image)

**Figure 3-1:**
Schematic structure of the \textit{mNumb} and ROSA26-reporter conditional alleles. The exon encoding for the translation initiation sequence and the first 42 amino acids of the \textit{mNumb} gene were flanked by \textit{loxP} sequences (triangles) to generate the floxed \textit{Numb} allele (1) and the null allele generated after Cre-induced recombination (2). Cre -recombinase was expressed from the Engrailed2 promoter enhancer sequences (\textit{En2-Cre}) (Zynik et al., 1998). The ROSA26-R Cre-reporter allele (\textit{R26R}) was used to follow the fate of recombined cells (see Soriano, 1999 for details). Cre-mediated recombination deletes the PGK neo cassette and results in constitutive expression of \textit{\beta}-galactosidase.

### 3.4.1 CONDITIONAL DISRUPTION OF \textit{mNUMB} DOES NOT AFFECT MIDBRAIN/HINDBRAIN FORMATION BETWEEN E10.5 AND E15

The ablation of \textit{mNumb} in the CNS was restricted to the midbrain hindbrain neuroepithelium by using the \textit{En2-Cre Tg3} transgenic line (Zynik et al., 1998). Cre-mediated recombination was detected at E10.5 by X-gal staining in the midbrain hindbrain neuroepithelium and in a restricted population of cells along the neural tube (figure 2 A, B). Animals carrying heterozygous and homozygous floxed \textit{Numb} alleles and an \textit{En2-Cre} transgene showed a similar pattern of recombination at E10.5 (figure 2A). To elucidate whether conditional \textit{Numb} ablation leads to changes in cell lineage determination and specification or differentiation, we analyzed the expression of the proneural genes \textit{Mash1} and \textit{Math1} at E12.5 by \textit{in situ} hybridization. \textit{Mash1} is expressed by putative Purkinje cell precursors starting in the lateral cerebellar primordium at around E12.5 (Guillemot and Joyner, 1993). Both control and mutant embryos showed a similar pattern of \textit{Mash1} expression with no aberrant distribution at E12.5 (figure 2 E, F), in contrast to findings made...
following conditional ablation of Notch1 from the same region of the neural tube (Lutolf et al., 2002). Mathl is a proneural bHLH transcription factor required for cerebellar granule cell specification and differentiation and the earliest known marker for granule cell precursors (Ben-Arie et al., 1997). Again and in contrast to the effects of Notch1 ablation, Mathl was detected in cells at the posterior edge of the cerebellar primordium forming the rhombic lip (figure 2 C, D). As Notch signaling has been proposed to regulate the onset of neuroepithelial cell differentiation in vivo (de la Pompa, 1997; Lutolf et al., 2002) and Numb may also regulate progenitor cell differentiation (Petersen et al., 2002), we analyzed the expression of Nestin, an intermediate filament protein in undifferentiated cells. Immunostaining with Nestin-specific antibodies did not reveal obvious changes in progenitor cell number or thickness of the neuroepithelium structure within the cerebellar anlage at E12.5 (figure 2 G, H). Lobulation of the cerebellum commences around E17.5 in mice, therefore, we analyzed the cerebellum of our mutants around this critical time point. At E15 and E18 the structure of the cerebellar anlage was similar in control and mutant embryos and in situ RNA hybridization analysis of neurogenic markers including Mash1, Math1 and NeuroD also failed to detect differences between the control and mutant animals (data not shown). In addition, few remaining Nestin-positive progenitor cells were detected in the ventricular zone of both mutant and control embryos (data not shown). Taken together, the analysis at E15 and E18 did not reveal alterations in the developing cerebellar anlage of mutant compared to control embryos.

Figure 3-2:
Embryonic analysis of conditional Numb-deficient mice. (A) E10.5 homozygous floxed Numb (\(\Lambda/\Lambda\)) animals carrying an En2-Cre and R26R allele showed recombined cells (blue) in the midbrain hindbrain region of the brain. (B) Heterozygous E10.5 floxed Numb (\(\Lambda/wt\)) animals carrying an En2-Cre and R26R allele revealed a similar restricted distribution of recombined cells (blue) in the midbrain hindbrain region of the developing brain. (C-F) In situ RNA hybridization on cross-sections through the neural tube, dorsal to the top and ventral to the bottom, of E12.5 mutant animals (\(\Lambda/\Lambda\)) showed normal expression of Math1 (C) and Mash1 (E) mRNA in the cerebellar primordium compared to control littermates (D, F: \(\Lambda/wt\)). (G, H) Immunostaining with anti-Nestin antibodies showed a similar expression pattern at E12.5 in the cerebellar neuroepithelium of mutant animals (G: \(\Lambda/\Lambda\)) compared to control animals (H: \(\Lambda/wt\)) (in G and H one half of the cerebellar primordium is depicted with dorsal to the top and ventral to the bottom). Scale bars: in C, 100 \(\mu m\) for C-F; in G, 50\(\mu m\) for G,H; cb.p. = cerebellar primordium, fp = floorplate, hb = hindbrain, mb = midbrain, ne = neuroepithelium, IV = fourth ventricle, sc = spinal cord.
3.4.2 CONDITIONAL ABLATION OF THE mNUMB GENE LEADS TO A DELAY IN GRANULE CELL PRECURSOR MATURATION

We have shown previously that Numb is expressed in the external and internal germinal layer (EGL, IGL) as well as in the Purkinje cell layer of the cerebellum of young postnatal (P4) mice (Stump et al., 2002). Thus, we analyzed granule cell differentiation in our mutants in more detail at this time point by examining the expression of Mathl and NeuroD. Mathl is expressed predominantly in the outer portion of the EGL by mitotic progenitors and is downregulated by postmitotic granule cell precursors, while NeuroD is expressed in the inner aspects of the EGL and in the developing IGL by postmitotic granule cells (Oberdick et al., 1998). Midsagittal sections revealed a reduction in the size of the mutant cerebellum at P4 (figure 3 A) when both granule cell precursors and Purkinje cells start to undergo terminal maturation. Mathl RNA could be detected in the outer EGL (figure 3 A,B) and NeuroD RNA in the inner EGL and IGL of both mutant and control littermates (figure 3 C,D). However,
NeuroD RNA levels were decreased in the IGL of mutant animals (figure 3 E,F). Concomitantly, histological staining revealed fewer cells located in the IGL of mutant mice, suggesting that less granule cell precursors had migrated into the mutant cerebellum compared to the controls. Moreover, the EGL of the mutant cerebellum is more densely packed compared to the EGL of the control littermates (figure 3 I,J). This suggests that terminal maturation and migration of granule cell precursors is delayed in the cerebellum of P4 mutant mice. In order to address whether the reduced cerebellar size was due to a loss of recombined cells in the mutant compared to littermates with one functional Numb allele, we performed X-gal staining. Mutant and control cerebella displayed a similar level of b-galactosidase activity at P4 indicating recombined cells in both groups of animals. Thus, Numb-deficient cells are able to give rise to neurons in vivo, confirming previous observations (Petersen et al., 2002; Zhong et al., 2000) (figure 3 G,H). Furthermore, our data suggest a developmental delay, which may account for the reduced cerebellar size observed in the early postnatal mutant mice.

Figure 3-3:
Figure 3-3:
Early postnatal cerebellar development is affected by *Numb* ablation. *In situ* RNA hybridization failed to reveal differences in Math1 mRNA expression in the outer portion of the external germinal layer (EGL) of mutant (A) and control (B) mice. *In situ* RNA hybridization for NeuroD showed expression restricted to the inner portion of the EGL and in the developing internal granule cell layer (IGL) in both mutant (C) and control (D) animals. Higher magnification pictures revealed reduced NeuroD mRNA expression in the developing IGL of the mutant (E) compared to the control (F) animals (arrowheads). Recombined cells (blue) were detectable in the developing cerebellum of mutant (G) as well as control (H) animals. Hematoxylin/Eosin staining revealed fewer cell bodies in the IGL of mutant (I) when compared to the control (J). Scale bars: in A, 500 μm for A-D; in E, 200 μm for E,F; in G, 100 μm for G,H; in I, 20 μm for I,J; Pcl = Purkinje cell layer

3.4.3 CONDITIONAL ABLATION OF mNUMB DOES NOT ALTER PROLIFERATION CHARACTERISTICS OF GRANULE CELL PRECURSORS AT P4

Granule cell precursors of the inner EGL leave the cell cycle and commence differentiation in the first three weeks of postnatal life in the mouse (Hatten and Heintz, 1995; reviewed in Hatten et al., 1997). In order to account for the small size of the mutant cerebellum, we examined the proliferation characteristics of granule cell precursors in the EGL of P4 mutant and control mice by immunostaining with anti-pH3 antibodies. Most of the proliferating cells were located in the outer EGL (figure 4 A,B). Quantification of the number of pH3-positive cells in the EGL revealed a similar number of proliferating cells per lobe length in the mutant animals (105.9 +/- 21.0 cells/mm lobe length, 4 animals analyzed) compared to controls (110.7 +/- 23.8 cells/mm lobe length, 3 animals analyzed) (figure 4 C). Moreover, to determine the cause of the reduction in the mutant cerebellum, we examined cell death by TUNEL analysis (figure 4 D,E). Quantification of TUNEL-positive cells in the mutant (3 animals) and control (3 animals) cerebella did not reveal detectable differences in the apoptotic rate (figure 4 F).
Figure 3-4:
Characterization of granule cell precursor proliferation and apoptosis in the early postnatal cerebellum. Immunostaining of P4 cerebellar sections (lobes VI, VII and VIII) with an anti-pH3 antibody to identify cells in the M-phase of the cell cycle revealed proliferating cells in the EGL (delineated by dotted line) of the mutant (A) and control (B) animals. Quantification using NIH Image software indicated that mutant and control animals have similar numbers of proliferating cells in the EGL per mm lobe length (C). TUNEL-staining (D, E) (white arrowhead) and quantification of apoptotic cells (F) per cerebellar area did not reveal differences in the apoptotic rate in the mutant when compared to the control animals at P4. (C, F: red bars = control; blue bars = mutant). Scale bars: in A and D, 100 μm for A, B, D, E.

Next, we addressed differentiation of the granule cells at later stages. At P10 histological staining revealed that the EGL of mutants is considerably thicker (figure 5 A,C) than that of control animals (figure 5 B,D). This is likely the result of an accumulation of granule cell precursors in the mutant EGL. In support of this interpretation, few Mathl-expressing cells were detected in the outer EGL of control animals, however, the EGL of mutants still contained an appreciable number of Mathl-positive cells (figure 5 E,D arrowheads). Moreover, an increase in NeuroD-expressing cells was detected in the EGL of mutants compared to control animals (figure 5 G,H arrowheads). Finally, GABAα6 receptor subunits, a marker of fully differentiated granule cells (Thompson and Stephenson, 1994), were clearly expressed in the IGL of control animals (figure 5J) while being hardly detectable in the IGL of mutant animals (figure 5I), thus strengthening the hypothesis of a delay in terminal maturation of Numb-deficient granule cell precursors.
Figure 3-5
Figure 3-5: Development of granule cell precursors is affected by Numb ablation. Hematoxylin/Eosin staining showed an accumulation of granule cell precursors in the EGL of mutant (A, C) compared to the control P10 animals (B, D). Purkinje cells in the mutant (black arrowhead, A) have a shorter, less extensive dendritic tree compared to control animals (black arrowhead, B). In situ RNA hybridization showed maintained Math1 mRNA expression in the outer EGL of P10 mutant (E) compared to control animals (F). The arrowhead in E points to the outer EGL of the mutant animal and shows the increased Math1 mRNA expression compared to the control littermate (F; arrowhead). In situ RNA hybridization revealed an increase in cells expressing NeuroD in the EGL of mutant animals (G) compared to controls (H). Arrowheads in G and H point to the differences in NeuroD mRNA in the mutant (G) compared to the control animals (H). GABAα6 receptor subunit as a marker of mature granule cells is virtually absent in the mutants (I) while it is restricted to the IGL of the control animal (J). Scale bars: in A, 20 μm for A, B, C, D, I, J; in E, 100 μm for E, F, G, H; WM = white matter, ML = molecular layer, IGL = internal granule cell layer, Pc 1 = Purkinje cell layer

3.4.4. CONDITIONAL ABLATION OF THE mNUMB GENE LEADS TO A DELAY IN THE MATURATION OF PURKINJE CELLS

Cerebellar Purkinje cell precursors cease proliferation around E13 in the mouse and migrate along the radial glial fibers to settle in a dense zone underlying the forming EGL. Terminal differentiation of Purkinje cells, however, occurs postnatally when they become arranged in a single cell layer and make contacts with the parallel fibers of the granule cells (Hatten and Heintz, 1995). To address the differentiation of Purkinje cells in the absence of Numb, we performed in situ hybridization with the Purkinje cell marker Calbindin D28k at P4 and P10 (figure 6). At P4, Calbindin D28k expressing Purkinje cells were arranged into a multi-cell layer in both the mutant and control animals (figure 6 A,B). However, at P10, while in the control animals the Purkinje cells had formed a relatively homogeneous single cell layer, the Purkinje cells of the mutants were often seen in multiple layers, an indication of delayed development (figure 6 C,D). As granule cells are partially responsible for the layered organization of the Purkinje cells, the reduced granule cell precursor development seen in the Numb-deficient animals may be partly responsible for the effects on the Purkinje cells. However, apart from the arrangement of Purkinje cell bodies into a single cell layer, Calbindin D28k immunostaining revealed a complex dendritic tree development in control animals, indicating fully mature Purkinje cells (figure 6 F, white arrowhead) while the architecture of the dendritic tree in the mutants was less elaborate (figure 6 E, white arrowhead). Confirming these data, histological staining illustrated reduced outgrowth of primary dendritic trees in the mutants (figure 5 A, black arrowhead) in comparison to the elongated primary dendritic tree in the control animals (figure 5 B, black arrowhead).
also evident that the molecular layer (ML), formed predominantly by Purkinje cell dendritic trees, is narrowed in the mutant (figure 4 A,C; figure 5 C). This reduced Purkinje cell development also explains the reduced size of the mutant cerebellum. Hence, these data indicate that terminal maturation of Purkinje cells in our mutants is affected and support the hypothesis of a delay in cerebellar development in Numb-deficient animals.

Figure 3-6
Figure 3-6: Development of Purkinje cells is aberrant following Numb ablation. *In situ* RNA hybridization on sagittal sections of P4 cerebellum with a Calbindin D28k probe showed the arrangement of Purkinje cell bodies in a multi-cell layer in both mutant (A) and control (B) animals within a similar region between lobes V and VI. However, in the control animals (B) Purkinje cells had started to be arranged in a single cell layer, particularly at the base of the lobes, whereas in the mutants the PC remained in multiple layers (arrowheads A, B). Calbindin D28k *in situ* RNA hybridization at P10 revealed that Purkinje cells were arranged in a single cell layer (arrowhead in D) in the control (D) animals while they remained partially as a multi-cell layer (arrowhead in C) in the mutant (C). The images in C and D are of comparable regions of lobe IX in both the mutant and control animals. Purkinje cell dendritic trees (arrowheads) were less complex in the mutant (E) than in the control animals at P10 (F) as shown by Calbindin D28k immunostaining in lobe IX. The molecular layer is thicker in the control than in the mutant animals (E, F). Scale bars: in A, 200 µm for A, B; in C, 200 µm for C, D; in E, 20 µm for E, F

3.4.5 CONDITIONAL ABLATION OF THE mNUMB GENE LEADS TO A REDUCTION IN ADULT CEREBELLAR FOLIATION AND PURKINJE CELL NUMBER

Histological staining and morphological analysis revealed that ablation of the mNumb gene resulted in a reduced cerebellar foliation (figure 7 A,B). In order to identify recombined cells in the brains of mutant and control animals we stained sagittal hemisected adult brains with X-gal. Recombined, b-galactosidase expressing cells were found in the molecular layer, granule cell layer and Purkinje cell layer of both control and mutant animals (figure 7 F,G). The presence of recombined cells in the cerebellum of the conditionally Numb-ablated animals indicates that mNumb is not absolutely required for survival and differentiation of granule cells and Purkinje cells *in vivo*. To quantify the changes in cerebellar formation as a consequence of Numb ablation, we measured the lobe length along the Purkinje cell layer on consecutive 20 µm midline sagittal cerebellar sections of mutant and control animals (figure 7 C,D). Some individual lobes of mutant cerebella were reduced in size (figure 7 E) and lobes VI and VII were most severely affected (*P*-value< 0.001, Student’s t-test) in the mutants (5.9 +/- 0.02 mm) compared to control animals (7.47 +/- 0.25 mm). In addition, these lobes were often fused. Although the fusion was already apparent in the cerebellum of mutant animals at P4, the overall organization of the cell layers within the adult cerebellum was not affected (figure 7 A,B).

We also performed Calbindin D28k *in situ* hybridization on sections of adult cerebellum to determine Purkinje cell number and distribution (figure 7 C,D). In both adult control and
mutant cerebella Purkinje cells were arranged into a single cell layer. However, Purkinje cell number was significantly reduced in the anterior lobes (control: 355 +/- 7; mutant: 310 +/- 23.5; P-value <0.001, Student’s t-test), in the central lobes (control: 235 +/- 1.1; mutant: 187 +/- 9.1; P-value <0.001, Student’s t-test) and in the posterior lobes (control: 212 +/- 47.4; mutant: 162 +/- 10.8; P-value <0.001, Student’s t-test) of the mutant, consistent with the observed reduction in lobe length (figure 7 H). However, the calculated density of Purkinje cells remained unaltered in mutant animals (23.9 +/- 0.9 cells/mm) compared to controls (25.5 +/- 1.7 cells/mm). In addition, the granule cell layer of mutants showed a normal thickness and density of cells compared with control animals. Taking into account the overall reduction in size of the cerebellum, this suggests that the number of granule cells is also reduced in mutants.

Figure 3-7
Figure 3-7: Morphological analysis of the adult cerebellum and quantification of the cerebellar size showing a reduction in adult cerebellar development. Hematoxylin staining of midline sagittal sections showed a marked reduction in the size of the mutant (A) cerebellum compared to control animals (B). Aberrant lobulation in the adult mutant cerebellum was also seen with fusion of lobes VI and VII (arrowheads in A, B). In situ RNA hybridization for Calbindin D28k on mutant (C) and control (D) cerebellar sections and quantification of lobe length revealed a reduction in the length of lobe 2/3, lobes 6/7 and lobe 10 in the mutant (** P<0.01, Student’s t-test) (E). Owing to the fusion of lobes 6 and 7, they were measured together, from the posterior end of lobe 5 to the anterior end of lobe 8. Lineage tracing using the Cre-reporter transgene R26R showed that Numb-deficient, β-galactosidase expressing cells (blue) were present in all layers (molecular layer (ML), Purkinje cell layer (Pcl) and the internal granule cell layer (IGL)) of the adult mutant cerebellum (homozygous floxed Numb En2-Cre and R26R) (F) comparable to control animals (heterozygous floxed Numb En2-Cre and R26R) (G). Analysis of Purkinje cell number and density (H; see text) in adult animals revealed a significant (* P<0.05, Student’s t-test) reduction in cell number within the anterior lobes (lobes 2-5) and the posterior lobes (lobes 9 and 10) and a highly significant (**) P<0.001, Student’s t-test) reduction in the Purkinje cell number within the central cerebellar lobes (lobes 6-8) of mutant animals (E, H: red bars = control; blue bars = mutant). Results shown are from a representative mutant animal and control littermate. Scale bars: in A, 1 mm for A, B; in E, 1 mm for E, F.

In a few rare cases we observed a strong phenotype in the mutant animals. In these mice the size of the cerebellum was dramatically reduced and the vermis was virtually absent (figure 8 A,B). The “typical” mutant (figure 8 C) vermis is slightly flattened but clearly distinguishable from the lateral hemispheres, which appear normal. Histological staining of adult midsagittal sections of the “severe” mutant cerebellum revealed a severely aberrant lobulation (figure 8D). Calbindin D28k in situ hybridization of midsagittal sections at P10 revealed a complete disorganization of the Purkinje cells (figure 8F) and X-gal staining showed that virtually all the cells in the cerebellum of these “severe” mutants had undergone recombination (figure 8E). We also examined the granule cell marker NeuroD by in situ RNA hybridization and found no detectable expression in the “severe” mutants (data not shown). Behaviorally, these animals showed ataxia and died at 2-3 weeks of age. It remains to be determined whether this strong phenotype reflects variations in the recombination efficiency or genetic background effects.
Figure 3-8: Some mutant animals show a severe reduction in cerebellar formation and ataxic movements. Whole mount preparations of adult brains showed a lack of vermis (ver) and reduced lateral hemispheres (hem) in the severely affected mutant (Δ/Δ*) (A) animals compared to the control (Δ/Δwt) (B) and “typical” mutant (Δ/Δ) (C) animals. The midbrain colliculi were more exposed in these mutants than in the controls. Histological staining revealed that the layered cerebellar cortical structure is completely absent at P10 (D). In situ RNA hybridization for Calbindin D28k on midline sagittal sections of severely affected P10 mutant animals showed the totally aberrant foliation of the cerebellum due to the complete disorganization of Purkinje cells (F). X-Gal staining on a consecutive section demonstrated a marked reduction in cerebellar size and a virtually complete recombination in all cells of the cerebellum (E). Scale bars: in A, 1 mm for A, B, C; in D, 100 μm for D-F; ic = inferior colliculus, sc = superior colliculus.

3.4.6 NUMB-DEFICIENT NEUROEPITHELIAL CELLS GENERATE REDUCED NUMBER OF NEURONS IN VITRO

To gain more insight into the role of Numb in the regulation of cerebellar neurogenesis we analyzed the effects of Numb gene ablation on neuroepithelial cells in culture. Cerebellar neuroepithelial cells were isolated from E10.5 embryos. The cells were cultured under defined conditions, which allow the generation of cerebellar Purkinje cells, granule cells and glia (G. Stump and V. T.; unpublished observation) (figure 9A). By removing the neuroepithelial cells from their normal context, we wanted to characterize their cell fate.
choice and differentiation potential. Cells were isolated from homozygous floxed Numb animals and littermates with wild-type Numb alleles carrying the En2-Cre transgene and R26R reporter. After 6.5 days in vitro β-galactosidase immunostaining revealed that 85% of the plated cells from both homozygous and control animals had undergone recombination. Western blot analysis performed on proteins isolated from sister cultures of neuroepithelial cells showed a 90% reduction in Numb protein levels in the mutant compared to control cells (figure 9B). The residual amount of Numb protein detected in the mutant cultures is likely due to the 15% of unrecombined cells. Next, we determined the percentages of recombined β-TubulinIII-positive granule cells, Calbindin D28k-positive Purkinje cells, Nestin-positive progenitor cells, O4-positive oligodendrocytes and smooth muscle actin (SMA)-positive smooth muscle-like cells by double-immunostaining with anti-β-galactosidase antibodies (figure 9 C-H). Similar numbers of Nestin-positive progenitors were generated by both Numb mutant (30.3 +/- 13.4% of 1298 recombined cells counted) and control (30.5 +/- 7.9% of 910 recombined cells counted) cells (P-value >0.05; Student’s t-test) (figure 9 I). Similarly, the number of Calbindin D28k-positive Purkinje cells (18.0 +/- 7.4% of 658 recombined cells counted) versus (11.9 +/- 3.2% of 953 recombined cells counted), O4-positive oligodendrocytes (5.3 +/- 1.4% of 694 recombined cells counted) versus (6.0 +/- 0.3% of 410 recombined cells counted) and smooth muscle actin (SMA)-positive smooth muscle cells (7.7 +/- 2.0% of 746 recombined cells counted) versus (8.8 +/- 2.0% of 973 recombined cells counted) was not significantly different (P-value >0.05; Student’s t-test) in cultures of mutant and control cells, respectively (figure 9 I). By contrast, the number of β-TubulinIII-positive putative granule cells was significantly reduced (P-value <0.001; Student’s t-test) in the mutant (14.7 +/- 6.3% of 3686 recombined cells counted) compared to control (34.6 +/- 7.0% of 1397 recombined cells counted) (figure 9 I). The average percentage of recombined cells was similar in cultures from control embryos (63.4 +/- 13.2 %) and mutant embryos (62.7 +/- 9.4%) indicating similar plating, survival and proliferation rates.

3.4.7 GENERATION OF GRANULE CELLS FROM NUMB-DEFICIENT PROGENITORS IN VITRO IS DELAYED

Combining the relative numbers of cells labeled, 92.7% of the cells in control cultures could be accounted for. However, 16.9% of recombined cells in the mutant cultures were not labeled by any of the differentiation markers analyzed. Since we did not observe an increase
in Nestin, Calbindin D28k, GFAP, O4 and SMA-expressing cells in the cultures of mutant cells after 6.5 days in vitro, it is unlikely that the unaccounted for recombined cells underwent a switch in cell fate. In addition, death of this population can also be excluded, otherwise, a proportional increase in the other cell types would have been observed. Hence, to determine whether the recombined granule cells were delayed in their development or arrested in a precursor-like state, we cultured the cerebellar neuroepithelial cells for 13 days. We have observed previously that, under defined cell culture conditions, most of the granule cells are born within the first 24 hours. However, the other cell types continue to be generated during the culture period (G. Stump and V. T., unpublished observation). In contrast to the 6.5 day cultures, the percentage of recombined granule cells generated in the mutant (16.4 +/- 5.5% of 1094 recombined cells counted) was no longer significantly different (P-value>0.05, Student’s t-test) to that observed in control cell cultures (25.0 +/- 5.2% of 1431 recombined cells counted) (figure 9 I). The increase in granule cells in mutant cell cultures after 13 days suggests that additional Numb-deficient granule cells were generated between day 6.5 and day 13 in vitro compared to the control cultures. Hence, it is likely that Numb-deficient progenitors go through a transitory precursor-like state to generate granule cells and that this transition is delayed in the mutant cells. In support of this hypothesis, when we performed immunostaining with antibodies against Math1, a marker of mitotic granule cell precursors in the EGL, an increased number of Math1-positive granule cell precursors was found in short-term cultures of mutant cells (7.9 +/- 1.2% of 2876 cells counted) compared to control cells (4.9 +/- 0.9% of 2801 cells counted) in short-term culture (P-value<0.05, Student’s t-test) (figure 9 I, insert). Although the number of Math1-positive cells declined after 13.5 days in culture, a significantly increased number of Math1-positive cells (P-value<0.05, Student’s t-test) was still present in the mutant (2.8 +/- 0.4% of 1282 cells counted) compared to control (1.5 +/- 0.4% of 1121 cells counted) (figure 9 I, insert). Together, these data support the hypothesis that Numb-deficient progenitor cells are delayed in the progression and maturation to granule cells.
Figure 3-9:
Figure 3-9: Analysis of cell fate and differentiation of Numb-deficient progenitors in vitro. (A) Schematic representation of the experimental procedure. Cerebellar neuroepithelial cells were isolated from the midbrain hindbrain region (indicated as b-galactosidase expressing cells (blue), excised region indicated by red quadrant) of E10.5 embryos. The cells were cultured for either 6.5d (short-term culture) or 13d (long-term culture) under defined conditions and subsequently fixed and stained with antibodies against b-galactosidase and, Nestin, b-TubulinIII, Calbindin D28k, O4 and SMA as well as Math1. (B) Western blot analysis performed on proteins isolated from cultured cerebellar neuroepithelial cells. Polyclonal anti-Numb antibodies recognized the 70 kDa Numb protein in cultures of control cells and revealed a major reduction in the amount of Numb protein in mutant cells. The residual amount of Numb protein detected in the mutant cell cultures is likely due to a number of unrecombined cells as b-galactosidase immunostaining revealed that approximately 85% of the plated cells underwent recombination. Anti-b-actin Western blot was performed to show equal loading of the proteins. Quantification and normalization of the Western blots to b-actin levels showed a 90% reduction in Numb protein in the mutant cell cultures. Nestin-positive progenitor cells (C), b-TubulinIII-positive granule cells (D), Calbindin D28k-positive Purkinje cells (E), SMA-positive smooth muscle cells (F), O4-positive oligodendrocytes (G) and Math1-positive granule cell precursors (H) were detected in both mutant and control cell cultures. (I) Quantification of marker expression by the recombined cells after 6.5 and 13 days in culture. Short-term culture revealed a highly significant (** P<0.01, Student’s t-test) reduction in the generation of recombined Numb-deficient b-TubulinIII-positive granule cells, while the proportion of the other cell types was not significantly affected (P>0.05, Student’s t-test) in the mutant compared to the control cell cultures. Long-term culture revealed a relative increase in the formation of recombined Numb-deficient b-TubulinIII-positive granule cells and suggested that additional granule cells are born between day 6.5 and 13 of the culture. After 13 days in vitro there was no longer a significant difference between mutant and control cell populations. Insert: Quantification of Math1-positive cerebellar neuroepithelial cells after 6.5 and 13 days in culture. There was a significant increase in Math1-positive cells in mutant compared to control cultures in both short-term and long-term conditions (P<0.05, Student’s t-test) (I: red bars = control; blue bars = mutant; n = number of embryos analyzed).
3.5 DISCUSSION

In this report we show that mNumb plays a role in regulating cerebellar granule cell differentiation, a function that is in agreement with the expression of mNumb in the EGL of the postnatal cerebellum. We also suggest that mNumb is involved in controlling the transition from a Nestin-negative neuroblast-like state to a terminally differentiated cerebellar granule cell. Additionally, we propose that mNumb is involved in terminal maturation of Purkinje cells.

We observed the first signs of delayed differentiation in the conditional Numb mutants in the early postnatal transition of proliferating EGL cells to postmitotic granule cells. Hence, early development of the cerebellum can proceed in the absence of mNumb. Furthermore, the expression of the proneural genes Mash1 and Math1 was not obviously affected in the conditional mNumb mutant mice indicating, that patterning and early cell fate decisions can occur in the absence of Numb. This may be due to a compensatory role of Numblike in early stages of cerebellar development. In situ RNA hybridization expression analysis confirmed that Numblike is expressed in the cerebellar neuroepithelium at E12.5 and overlaps with that of mNumb (data not shown). Hence, although the transition to a postmitotic granule cell is slowed in the absence of mNumb, Numblike may be able to functionally compensate for the mutation at all stages of cerebellar development. The delay, rather than a complete block of differentiation, may be the result of the time required for Numblike protein to accumulate in EGL cells. In the absence of reagents to unequivocally test this hypothesis, additional experiments will be required to substantiate this point further, including the regionalized inactivation of mNumb in the nervous system of Numblike-deficient animals. Targeting the ablation to a restricted portion of the neural tube should circumvent the early lethal effects observed in the mNumb, Numblike-double knockout animals (Petersen et al., 2002).

In contrast to the role of dNumb in cell fate decisions, mNumb does not seem to be involved in cell fate acquisition and patterning in the mouse central nervous system (Petersen et al., 2002). Taking advantage of the R26R reporter allele, we followed the fate of mNumb-ablated cells through to adulthood. The presence of recombined cells in all layers of the cerebellar cortex of mutant animals and the fact that cells were arranged in layers with apparently normal cytoarchitecture indicate that mNumb is also not absolutely required for the generation of the terminally differentiated cerebellar cell types.

Recent data analyzing cortical neuroepithelial cells in culture indicate that mNumb can be asymmetrically localized in dividing cells (Shen et al., 2002). In E10 mouse
neuroepithelial cells, asymmetric mNumb distribution does not correlate with cell fate acquisition. By contrast, in neural progenitors isolated during the peak neurogenic period (E13) mNumb shows a strong asymmetric localization in daughter cells and segregates to differentiating neurons while mNumb-lacking cells remain as progenitors (Shen et al., 2002). Hence, during early development of the nervous system, when cell divisions in the neural tube are thought to mainly expand the progenitor cell pool, mNumb may not directly affect cell fate choice. However, later in development mNumb might be part of an intrinsic program that regulates cell differentiation (reviewed by Cayouette and Raff, 2002).

Our findings of a delayed granule cell development in the absence of mNumb may well reflect its role in regulating Notch signaling. Previous data suggest that Notch signaling is important for granule cell development (Solecki et al., 2001). Gain-of-function experiments activating Notch2 signaling in the EGL resulted in extended precursor proliferation and a block in granule cell differentiation (Solecki et al., 2001). Our data raise the possibility that mNumb regulates granule cell differentiation, potentially by modulating Notch2 signaling in mitotic EGL cells and enabling transition to a differentiated granule cell. However, we cannot exclude that Numb has other functions independent of Notch. In particular, the role of Numb as an adapter molecule with several binding partners and a putative link to growth factor signaling through the EH-domain indicates a potential for functions in other pathways (McGill et al., 2003; Yogosawa et al., 2003; Nie et al., 2002). Similarly, as described for BDNF-/- mice, Numb-deficient granule cell precursors accumulate in the EGL; however, in contrast to BDNF-/- cells they fail to upregulate late differentiation markers in the EGL (Borghesani et al., 2002). Furthermore, Numb does not seem to act directly as a motogenic cue but may regulate granule cells responsiveness to migration cues such as BDNF. Other factors involved in regulating proliferation and maturation of granule cells such as Sonic hedgehog (Wallace, 1999), CyclinD2 (Huard et al., 1999), Zic1 (Aruga et al., 1998), Gas1 (Liu et al., 2001), p27/Kip1 (Miyazawa et al., 2000) and NSCL-1 (Uittenbogaard et al., 1999) have been described previously.

We have previously shown that mNumb is also expressed by postnatal Purkinje cells (Stump et al., 2002). Our findings here indicate that Numb-deficient Purkinje cells display an aberrant dendritic morphology, with a reduced outgrowth of the primary dendritic tree. This result is in concordance with previous in vitro studies demonstrating that Notch signaling inhibits dendritic outgrowth but promotes dendritic branching in cortical neurons (Redmond et al., 2000) and suggests that mNumb is involved in promoting terminal maturation processes in Purkinje cells.
The layered structure of the EGL, with mitotic cells in the outer region progressing to the inner layers upon differentiation, is reminiscent of the delamination seen in the neuroepithelium, where Notch signaling regulates differentiation. Based on its widespread expression in neuroepithelial cells of the neural tube, mNumb is likely to be involved in the development of other CNS cell types and structures. Conditionally ablating mNumb from the neuroepithelium of Numblike-deficient mice resulted in an increased neuronal differentiation within the forebrain (Petersen et al., 2002). Although these data do not directly reflect our findings, the differences may be due to the multiple roles of Numb in nervous system development (Verdi et al., 1996), allelic differences between the two Numb mutants or genetic background of the experimental animals. Furthermore, different isoforms of Numb can have different effects ranging from blocking differentiation of progenitor cells to promoting neurogenesis, *in vitro* (Verdi et al., 1999) and *in vivo* (Dooley et al., 2003). Thus, spatial and temporal differences in the role of mNumb throughout the CNS development are likely and may require precise tuning. Based on the expression of the En2-Cre transgene in all cerebellar cell types in our mutants, the analysis of cell autonomous and cell non autonomous functions of Numb will require lineage specific ablation.

In summary, we show that mNumb appears not to be involved in cell fate determination in the cerebellum but rather in promoting terminal differentiation. The task remains to integrate all signals and pathways with mNumb function in the formation of the cerebellum.
3.6 OUTLOOK

3.6.1 LINEAGE SPECIFIC REQUIREMENT OF NUMB

We analyzed the function of Numb in the developing cerebellum by conditional gene ablation and showed that Numb regulates terminal maturation of granule cells in vivo and in vitro. Additionally, we suggest that Numb is involved in the formation of Purkinje cell dendritic trees in vivo. Indeed, based on the expression pattern of Numb in the postnatal cerebellum, a function for Numb in both neuronal populations is probable.

Although the conditional ablation of Numb under the control of the Enl-Cre transgene is limited to neuroepithelial cells of the midbrain/hindbrain neural tube and their progeny, it is not confined to specific progenitor cells within this region. The En2-Cre recombination event occurs in neuroepithelial cells from the VZ as well as from the rhombic lip and can be detected through to adulthood in both Purkinje and granule cells. Hence, our approach did not allow cell autonomous or non autonomous effects on the loss and the delay in terminal maturation of both granule cells and Purkinje cells to be interpreted. Indeed, granule cells have been shown to regulate Purkinje cell development (Morrison and Mason, 1998) and to be dependent, themselves on secreted factors released from Purkinje cells. Thus, a cell lineage restricted ablation of Numb is necessary to distinguish between cell autonomous and cell non autonomous functions of Numb in granule cells and Purkinje cells.

In order to address whether the abnormal development of Purkinje cells is a primary or secondary effect, Numb ablation could be restricted to Purkinje cells. Transgenic mice carrying the Cre-recombinase under the control of the Calbindin D28K promoter were generated in our lab (Gila Stump and Verdon Taylor). One transgenic line shows specific expression of Cre-recombinase in postmitotic, immature Purkinje cells. Breeding of the Calbindin-Cre line with floxed Numb animals results in the ablation of Numb in Purkinje cells before the major onset of dendrite formation and branching. By including the R26R reporter transgene into the breeding, we will be able to identify Numb-deficient Purkinje cells. Biocytin labeling combined with immunostaining with dendritic markers such as Map2 and CalbD28K in cerebellar slice cultures will allow the 3 dimensional reconstruction of the dendritic tree. A putative function of Numb in dendrite formation and morphology could thus be addressed. Additionally, these mutant mice will reveal whether the effect on granule cell precursors is secondary due to the lack of Numb function in Purkinje cells.
Similarly, Numb ablation could be targeted to granule cell precursors in order to clearly reveal a cell autonomous function of Numb in granule cell precursors.

3.6.2 ISOFORM-SPECIFIC FUNCTIONS OF NUMB

Specific Numb isoforms were shown to be differentially and dynamically expressed in the nervous sytem and to mediate distinct functions (Dho et al., 1999; Verdi et al., 1999; Dooley et al., 2002). Our study did not address distinct functions of Numb isoforms during cerebellar development. Performing RT-PCR with primers flanking the alternatively spliced regions of Numb at different developmental time points and in different cell populations may reveal the dynamic expression pattern of different Numb isoforms during cerebellar development. Additionally, the use of isoform-specific antibodies (Dho et al., 1999) will not only allow the detection of distinct expression patterns, but also their subcellular localization and will reveal their ability to be asymmetrically distributed.

Loss-of-function studies of different isoforms may help to address their specific roles during development. To address this issue, one could perform *in vivo* knock-in experiments into the Numb locus. The knock-in constructs would contain a specific Numb isoform cDNA which would be expressed from the regulatory elements of the endogenous Numb promoter.

Moreover, gain-of-function experiments may clarify the function of the different Numb isoforms. Retroviral overexpression of Numb isoforms in postnatal cerebellar explant cultures may reveal that Numb PRR$^1$ isoforms promote granule cell proliferation. In contrast, Numb PRR$^5$ isoforms might block granule cell proliferation and induce premature differentiation of granule cell cells, based on the results obtained from overexpression experiments in a P19 cell line (Verdi et al., 1999). Alternatively, the function of the different Numb isoforms on proliferation and differentiation can be addressed by analyzing the effects of Numb overexpression in a medulloblastoma cell line. Medulloblastomas arise from the oncogenic transformation of granule cells and are described as one of the most aggressive childhood brain tumors. Since our data link Numb to proliferation and differentiation processes in granule cells, it is intriguing to speculate that Numb may be involved in medulloblastoma formation or be a potential target for treatment. In general, gain-of-function experiments, rather than loss-of-function studies, will help to get a quick overview of the function of the different Numb isoforms.
3.6.3 IS NUMB FUNCTION ESTABLISHED THROUGH THE NOTCH SIGNALING PATHWAY?

We were able to demonstrate in our model system that Numb regulates the transition of a granule cell precursor to a fully differentiated granule cell both in vivo and in vitro. Previously, it was shown that activation of Hes1 via Notch2, the only member of the Notch receptor family to be expressed in the EGL, leads to increased granule cell precursor proliferation and to a block in granule cell differentiation in vitro (Solecki et al., 2001). We hypothesize that Numb, which has been shown to antagonize Notch signaling, modulates Notch2 signaling in mitotic EGL cells and enables their terminal maturation. However, a few questions remain and need to be addressed.

First, a physical interaction between Numb and Notch2 has not been shown so far. Although the intracellular domain of the Notch family receptor is highly conserved, co-immunoprecipitation assays with Numb PTB domain and Notch2 ICD are required. Numb could act as an adapter molecule between Notch2 via its PTB domain and the ear-domain of α-adaptin via its C-terminus and initiate receptor-mediated endocytosis of Notch2. Additionally, Numb could act as adaptor protein for the recruitment of E3 ligases, mediate ubiquitination and degradation of the Notch2 ICD, as has been proposed for Notch1 (McGill and McGlade, 2003).

Second, the effects of Numb on the readout of the Notch signaling pathway remain to be described. If Numb function in the EGL is established via Notch2 signaling, we would expect an upregulation in the expression of Notch target genes as result of a prolonged Notch signal in our mutants. Till date, Hes1 and Hes5 are the only Notch target genes reported to be expressed in mitotic EGL cells (Solecki et al., 2001). The expression of Hes1 and Hes5 was detected by RT-PCR on purified granule neuron cultures from the postnatal cerebellum, containing both mitotic and differentiating cells. In contrast, we did not detect Hes1 or Hes5 expression in the EGL of postnatal mice by in situ hybridization (Stump et al., 2002). Further, in situ hybridization performed on postnatal cerebellar sections failed to show expression of Hes1 or Hes5 in the EGL of conditionally Numb ablated animals (own data, not shown). Quantitative RT-PCR represents now a valuable tool to detect expression and regulation of Notch target genes in both control and Numb-deficient granule cell precursors and will provide a possible link of Numb function through Notch signalling.

Further, Lütolf et al. showed that Notch1 ablation from the midbrain-hinbrain region induces promiscuous expression of the ligands DII1 and DII3 (Lütolf et al., 2002). Hence, it is
of interest to analyze the expression pattern and level of Jagged1, the only ligand to be expressed in the EGL, in postnatal Numb-deficient mice. Another interesting point to be considered is whether Numb can interact with the Jagged ICD, which was recently reported to be generated by a γ-secretase mediated cleavage, to translocate to the nucleus and to have transcriptional activity (LaVoie and Selkoe, 2003).

In addition to Notch signalling, secreted Shh is described as a mitogen for granule cell precursors (Wallace et al., 1999). It is possible that Numb does not only exert its function via regulating Notch signalling, but could also interact with components of the Shh signalling pathway to regulate granule cell proliferation. Since Numb has been described as an endocytic protein, one could speculate that Numb is involved in the receptor-mediated endocytosis of smoothened, the mediator of Shh signaling, thus making granule cell precursors unresponsive to the Shh signal and enabling them to undergo terminal maturation. A physical and functional interaction between Numb and Shh signaling pathway members still remains to be shown.

The fact that Numb acts as an endocytic protein and is able to target both receptor and cytoplasmatic proteins for degradation, increases the view on Numb functions, which till date was mostly restricted to the modulation of Notch signaling. Coimmunoprecipitation assays from primary cultures, pull-down-assays from transfected cell lines combined with blue native gel electrophoresis and microsequencing will identify further proteins able to complex and functionally interact with Numb (see also general discussion and outlook 4).

3.6.4 THE FUNCTION OF NUMBLIKE IN CNS DEVELOPMENT

In the granule cell lineage, we observe the first signs of delayed differentiation in the transition of a proliferating EGL cell to a postmitotic granule cell. Early specification and differentiation events in the developing cerebellum are not disturbed in the absence of Numb. This may be due to the compensatory role of Numblike, the expression of which overlaps with Numb in neuroepithelial cells of the midbrain-hindbrain neural tube (data not shown). Most Numb-deficient granule cell precursors, although delayed, reach the mature granule cell stage. The delay rather than the complete block of differentiation could again be a due to the compensatory role of Numblike. Indeed, Numblike is expressed in the inner portion of the EGL, a region containing postmitotic, differentiating granule cells.

Additional experiments are required to address this issue. Petersen et al. analyzed a conditional Numb/Numblike-double-knockout and describe Numb and Numblike to be
NUMB function in CNS

responsible for progenitor cell maintenance in the cortex (Petersen et al., 2002). However, these mutant embryos die around E11.5 which makes it impossible to address effects on late neurogenesis. Olav Zilian generated a second conditional floxed Numblike allele. Numblike-deficient mice develop normally (Petersen et al, 2002; Zilian, O; oral communication). It is still unclear whether the expression of Numblike veils effects of the Numb ablation in our Numb mutants. We, therefore, want to analyze the effects of Numb ablation in the developing CNS on a Numblike-deficient background. Breeding floxed Numb and Numblike-deficient mice with En2Cre-transgenic mice will clarify the function of both Numb and Numblike. Targeting the mutation to a specific cell lineage in the cerebellum will also be important to distinguish between cell-autonomous and cell-non autonomous defects in this experimental set-up (see section 3.6.1).

Alternatively, high resolution ultrasound backscatter microscopy (Turnbull, 2000) will allow to inactivate the floxed Numb/floxed Numblike alleles in utero with a Cre-recombinase expressing adenovirus. Embryos can be visualized in utero from E8 onwards and the injection of the adenovirus can thus be directed to the desired target, e.g. the neuroepithelium. By taking advantage of the R26R reporter transgene, we were able to identify infected, recombined cell clusters in the developing brain 2d after infection (fig.3-10). Depending on the viral titer, large areas of the embryo or only a small population of neuroepithelial cell can be infected. By creating a mosaic environment of infected, recombined Numb/Numblike-deficient and uninfected cells in the developing CNS, we will be able to distinguish between cell-autonomous and cell-non autonomous effects of Numb and Numblike ablation.

Although Numblike is able to bind to the Notch1 ICD, the biochemical mechanisms underlying the function of Numblike are still unclear. There is no evidence that Numblike acts as an endocytic protein as described for Numb, although it contains a DPF and an NPF motif, which are required for binding to the α-adaptin ear domain binding site and the Eps15 binding site respectively. Both subcellular localization and functional interaction partners of Numblike remain to be determined.
**Figure 3-10:** Recombination of R26R alleles is induced by Adeno-Cre infection *in utero* using UBM. (A) Representative image generated through ultrasound video microscopy of an E9 embryo. The site of viral injection is depicted by red lines (B) XGal whole mount staining performed on an E15 embryo isolated 6d after infection. Recombined cells can be found in the skin and in the forebrain (see red arrow) (C) Cross-section through the forebrain of an E15 embryo (6d after infection) reveals a clone of infected, recombined cells. ut=uterus, fbv=forebrain ventricle, hbv=hindbrain ventricle
A basic task in nervous system development is to generate the correct number and the immense variety of cell types that constitute the functional adult brain. In early vertebrate neurogenesis, the neuroepithelial cells undergo a certain number of symmetric divisions and generate identical daughter cells, thereby increasing the pool of neuroepithelial stem cells. In later neurogenesis, the neuroepithelial cells undergo asymmetric divisions and generate different daughter cells: one that remains as a neuroepithelial stem cell, thereby maintaining the progenitor pool while the second initiates differentiation along a certain neural lineage (McConnell, 1995; reviewed by Johnson, 2003).

Asymmetric divisions involve the unequal inheritance of fate determining proteins to the two daughter cells. The uneven distribution of these proteins is thought to confer distinct fates to both daughters. In Drosophila, Numb was identified as an asymmetrically distributed protein and was shown to be important in cell fate decisions (Jan and Jan, 2001; Spana et al., 1996; Rhyu et al., 1994). Experiments performed in Drosophila suggested that Numb functions through inhibition of the Notch signaling pathway, a pathway playing a crucial role in many developmental processes, including in the nervous system (Kimble and Simpson, 1997). Both vertebrate Numb and Notch have been identified. Given the high homology to their invertebrate counterparts, it was hypothesized that the mechanism of creating cell diversity through asymmetric division might be conserved in vertebrates. We have therefore analyzed the function of Numb during the development of the vertebrate CNS. In order to avoid the lethality observed in Numb mutant mice, we restricted our analysis of Numb function to the cerebellum by using a conditional gene approach.

We have shown that early proliferation and cell fate specification events in the embryo occur normally in the absence of Numb. However, we showed that Numb regulates later stages of neuronal maturation. In the absence of Numb, the arborization of Purkinje cell dendrites is reduced. This is in line with studies demonstrating that in vitro Notch1 is involved in the regulation of dendritic tree morphology (Berezovska et al., 1999b; Franklin et al., 1999b; Redmond and Gosh, 2001). Moreover, we have shown that Numb regulates the transition from a granule cell precursor to a mature, fully differentiated granule cell. Again, this result is in accordance with studies claiming that Notch signaling keeps granule cell precursors in a proliferative state (Solecki et al., 2001). In the absence of Numb, Notch signaling may act as an enhanced molecular ‘brake’ on differentiation (Lütolf et al., 2002).
and make granule cell precursors unresponsive to differentiation signals. The fact that Numb can act as an antagonist of Notch signaling, suggests that Numb can ‘release’ the molecular ‘brake’ imposed by Notch signaling and thus enables granule cell precursors to respond to extrinsic signals that are instructive or permissive for terminal maturation.

Recently, some biochemical mechanisms underlying the function of Numb were described. In vitro, Numb was described to mediate ubiquitination and proteasomal degradation of Notch1 (McGill and McGlade, 2003). Furthermore, Numb was reported to act as an endocytic protein. Not only is Numb staining associated with vesicles, but it also interacts via its DPF motif with the appendage domain of α-adaptin, a major component of clathrin-coated pits (Santolini et al., 2000; Berdnik et al., 2002). These data suggest that Numb could act as an adaptor protein between specific substrates and the endocytic machinery. In view of Numb's function in Notch signaling, Numb could thus be involved in receptor-mediated endocytosis of Notch.

A recent study provided more evidence for Numb being involved in endocytosis and identifies new interaction partners of Numb. Yeast two-hybrid analysis and co-immunoprecipitation experiments reveal an interaction of Numb and the collapsin response mediator protein-2 (CRMP-2) (Nishimura et al., 2003). CRMP-2 is critical for specifying axon/dendrite fate and axon growth in hippocampal neurons in vitro. Axon growth requires recycling of the neural cell adhesion molecule L1 via the endocytic machinery in the central growth cone (Kamiguchi et al., 2000). The authors were able to show that L1 co-immunoprecipitates with α-adaptin and Numb and thus hypothesized Numb to be required for L1 endocytosis at the growth cone. Since knock-down assays of CRMP-2 inhibited axon growth of hippocampal neurons in culture, the authors suggest that CRMP-2 is involved in Numb-mediated endocytosis of L1 and its subsequent recycling at the growth cone. These results shed new light on Numb function in granule cell maturation. The neural cell adhesion molecule L1 is expressed in postmitotic granule cell precursors as they migrate from the EGL past the Purkinje cell layer into the IGL (Rathjen et al., 1984). If Numb-mediated endocytosis of L1 is required for efficient migration, the delay in establishing the IGL in our Numb mutants could also be linked to a migration defect. Additionally, it would be interesting to establish a functional network between the transmembrane molecules L1 and Notch1, the ligand F3/contactin, a neural cell adhesion molecule of the immunoglobulin superfamily, and the cytoplasmatic protein Numb.
The increasing evidence that Numb binds to specific substrates on one hand and to the AP-2 complex of coated pits on the other hand, suggests that Numb could be involved as an adaptor protein in endocytosis of a variety of proteins (see also 3.6.3). A short while ago, it was shown that the endocytosis of the Notch ligand Delta is promoted by the activity of the E3 ubiquitin ligase Mindbomb in Xenopus or neuralized in Drosophila. Endocytosis of Delta seems to be required for efficient activation of Notch signaling (Itoh et al., 2003; Lai et al., 2001b; reviewed in LeBorgne and Schweisguth, 2003). Since Numb does not possess E3 ubiquitin ligase activity by itself, but is able to functionally interact with other ligases such as Itch (McGill and McGlade, 2003), it would be exciting to elucidate whether Numb is able to interact with E3 ubiquitin ligases in the Notch ligand expressing, signaling cells and thus may also participate in the endocytosis of Notch ligands. Hence, it is of great importance to identify additional interaction partners of Numb in order to clarify the function of Numb, which seemingly is not only restricted in modulating the Notch1 receptor.

In parallel to Notch signaling, the Wnt signaling pathway was described to regulate diverse developmental processes such as patterning of the body axis, nervous system development (Galceran et al., 2000; Brault et al., 2001) and endoderm induction (Lickert et al., 2002). Binding of the soluble Wnt ligand to the transmembrane receptor Frizzled leads, via the action of disheveled (Dsh), to the stabilization of β-catenin. β-catenin is the key element of the Wnt signaling pathway (reviewed by Hülsken and Behrens, 2000). In complex with LEF/TCF transcription factors, β-catenin translocates to the nucleus where it induces the transcription of target genes (reviewed by Miller et al., 1999). In the absence of Wnt, phosphorylation of β-catenin occurs in a multicomplex containing GSK-3β, APC, axin and axin2. Phosphorylation of β-catenin at specific residues induces its ubiquitination by βTrCP and its degradation in the proteasomes. Considering the widespread expression pattern of Numb and the important regulatory function of Wnt in the developing embryo, it is possible that Numb is involved in Wnt signaling. Numb may act together with βTrCP to regulate β-catenin degradation. A direct link between the Notch signaling and the Wnt has been proposed previously (Aulehla et al., 2002). On one hand, GSK-3β was proposed to phosphorylate the Notch1 ICD, thereby protecting the ICD from proteasomal degradation and modulating Notch signaling (Foltz et al., 2002). On the other hand, Dsh, when overexpressed, has been reported to suppress Notch signaling and to directly bind to the cytoplasmatic domain of Notch1 (Axelrod et al., 1996). The release of Dsh from the Notch ICD and the induction of Notch target genes were suggested to be mediated by Axin2, a direct target and
NUMB function in CNS

negative regulator of Wnt signaling cascade (Aulehla et al., 2002). Whether Numb interplays with Dsh to inhibit Notch signaling and whether the inhibitory function of Numb on Notch signaling may also be blocked via the scaffold protein Axin2 represents a challenging task to be resolved in future to further the understanding of the crosstalk between the Notch and the Wnt signaling pathway in embryonic development. Functional interaction studies between Numb and members of the Wnt signaling cascade that interfere with Notch signaling pathway must be addressed. Additionally, overexpression of Axin2 in a cell line expressing Numb and Notch may show whether Axin2 is able to release the inhibitory function of Numb on Notch signaling.

Beside the crucial role that Notch signaling and its modulators play in development, a lot of effort has been invested to clarify Notch function in neurodegenerative diseases, e.g. Alzheimer’s disease (AD). AD is characterized by the formation of amyloid plaques that lead to neuronal degeneration in brain regions involved in learning and memory. Amyloid plaques are mostly generated through aggregation of the 42 aa peptide form of amyloid-β (Aβ42) (Yankner, 1996). The Aβ42 peptide is generated through aberrant cleavage of the type I transmembrane amyloid precursor protein (APP). The proteolytic cleavage of APP is dependent on γ-secretase activity, which very likely is associated with presenelin (PS) activity. Indeed, mutations in PS1 and PS2 were shown to lead to an accumulation of the neurotoxic Aβ42 peptide thus linking presenilins to Alzheimer’s disease (Hardy et al., 1997; Lemere et al., 1996). Interestingly, PS1 was also shown to mediate proteolytic cleavage of the type I transmembrane protein Notch1 (DeStrooper et al., 1999; Fortini et al., 2001). The fact that a developmental pathway is associated to a neurodegenerative disease raised a lot of interest and furthermore suggests that modulators of Notch signaling might also be involved in the disease progress. In addition to the neurotoxic effect of amyloid plaques, the deregulation of cellular calcium homeostasis was described to mediate synaptic dysfunction and neuronal death in AD patients (Mattson et al., 2001). Numb isoforms with a short PTB were previously shown to sensitize cells for apoptosis upon elevation of intracellular calcium levels (Pedersen et al., 2002). These results suggested that Numb could be involved in the pathogenesis of AD by controlling Ca^{2+}-homeostasis. Indeed, it could be shown that cell death was promoted in neurons in the presence of Numb PTB₃ isoforms upon exposure to Aβ and increased cellular calcium levels (Chan et al., 2002). Furthermore, it was recently shown that Numb interacts with the γ-secretase-generated intracellular domain of APP (Roncarati et al., 2002). The APP intracellular domain (AIP) was able to bind to Numb and to
NUMB function in CNS

inhibit Notch activity \textit{in vitro}. Nevertheless, it still remains to be shown whether the effect of AIP on Notch is dependent on Numb. Alternatively, Numb could also interfere with the signaling of AID since AID was shown to possess transcriptional activity (Cao et al., 2001).

In conclusion, Numb likely does not act as a direct, instructive cell-fate determinant. Based on the widespread expression, Numb is likely to be involved in ‘housekeeping’ functions like endocytosis affecting many signaling pathways. In consequence, by regulating the expression of specific factors in a given cell, Numb indirectly allows the given cell to react differentially to both extrinsic and intrinsic signals and to adopt different fates. In CNS regeneration and treatment of neurological diseases, it will be a challenge to integrate Numb into the network of signals controlling proliferation, cell fate determination and differentiation.
REFERENCES


NUMB function in CNS


Dunwoodie, S. L., Henrique, D., Harrison, S. M., Beddington, R. S.(1997). Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo Development 124, 3065-3076


NUMB function in CNS


NUMB function in CNS


CURRICULUM VITAE

Personal Data

Name: Anne Laurence KLEIN
Date of birth: October 6, 1976
Nationality: Luxembourg (L)
Present position: Ph.D. student
Present address: Max-Planck-Institute of Immunobiology
Stubeweg 51, D-7901 Freiburg, Germany / Institute of Cell Biology, ETH Hönggerberg.
CH-8093 Zurich, Switzerland
Telephone No: ++49 (0)761 5108489 (D) / ++41 (0)1 6333307 (CH)
Fax No: ++49 (0)761 5108474 (D) / ++41 (0)1 6331069 (CH)
e-mail: klein@immunbio.mpg.de / anne.klein@cell.biol.ethz.ch

Professional Data

High school: Lycée classique de Diekirch (Luxembourg) 1988-1995
University: ETH Zurich, Biology studies 1995/96-2000
Graduation as Naturwissenschaftler “Dipl.Natw.ETH” 2000
PhD thesis, ETH Zurich, Institute of Cell Biology (under the supervision of Prof. Dr Ueli Suter and Dr Verdon Taylor) 2000-2002
PhD thesis continued, Max-Planck-Institute of Immunobiology, Freiburg, Germany (under the direct supervision of Dr Verdon Taylor) 2002-present
Courses in Center of Neuroscience Zurich (ZNZ) 2000-2003
Publications

Notch1 and its ligands Delta-like and Jagged are expressed and active in distinct cell populations in the postnatal mouse brain, Mechanisms of Development, 114, 153-159.

Murine Numb regulates granule cell maturation in the cerebellum, Developmental Biology, 266(1), 161-177

Oral Presentations

PhD retreat of the Center of Neuroscience Zurich, J. Kesselring, Valens 2001
Seminars at the Institute of Cell Biology, ETH Zurich 2000-2003

Poster Presentations

ZNZ Symposium (Center of Neuroscience Zurich), M.E. Schwab, Zurich 2001
D-BIOL Symposium ETH Zurich, Hans Hengartner, Davos 2002
ZNZ Symposium (Center of Neuroscience Zurich), M.E. Schwab, Zurich 2002
FENS Winterschool, Kitzbühel, 2003
ACKNOWLEDGEMENTS

My special thanks go to:

Dr. Verdon Taylor:
He was an excellent supervisor. He was always ready to support me with advice from the beginning of my scientific career.

Prof. Dr. Ueli Suter:
For his support during my PhD at the Institute of Cell Biology and the continued support provided during the time I spent at the Max-Planck-Institute in Freiburg, Germany

The Taylor Group, former members Gila and Simone and actual members Yves, Bettina, Frank, Bob, Matthias and Onur:
For being good labmates

The entire Suter group, especially Christoph, Guya and Johanna:
For their support and making my stay at the ICB most enjoyable

Prof. Dr Kemler and his unit:
For hosting and supporting me during the last year of my PhD thesis at the MPI

Dr. Dirk Junghans:
For all the help and discussions provided in- and outside of the lab

Further thanks go to:

My collaborator Dr. Olav Zilian

Prof. Dr. Isabelle Mansuy for having accepted to be a coreferee of my PhD thesis

These Projects were supported by an ETH grant to Verdon Taylor.