Conditional ablation of Notch1 from the vertebrate central nervous system

Author(s):
Lütolf, Simone

Publication Date:
2002

Permanent Link:
https://doi.org/10.3929/ethz-a-004370521

Rights / License:
In Copyright - Non-Commercial Use Permitted
Conditional Ablation Of Notch1 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

Simone Lütolf
dipl.sc.nat.
Swiss Federal Institute of Technology (ETH), Zürich, Switzerland
Born May 9, 1973 in Zürich, Switzerland
Citizen of Switzerland

Accepted on the recommendation of
Prof. Dr. Ueli Suter, examiner
Prof. Dr. Martin Schwab, coexaminer
Dr. Verdon Taylor, coexaminer

2002
ACKNOWLEDGEMENTS

My special thanks go to:

Dr. Verdon Taylor
He was a great supervisor and a big help during the last three years in the lab but also outside the lab when I needed some advice and an honest opinion. He always impressed me with his indefatigable and continuous power to fight for whatever it was.

Prof. Dr. Ueli Suter:
For his continuous support during my PhD in his group.

Gila Stump:
For his support in the lab and outside the lab.

The Taylor group:
Lobo, Gila, Anne-Laurence and Matthias for the interesting scientific discussions and nice chats we had.

The whole Suter group:
For the nice atmosphere.

Further thanks go to:

My collaborators Freddy Radtke and Ivan Radovanovic.
Martin Schwab for beeing my coreferee.

These Projects were supported by an ETH grant to Verdon Taylor.
# Table of Content

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Introduction</strong></td>
<td>6</td>
</tr>
<tr>
<td>1.1</td>
<td>CNS DEVELOPMENT</td>
</tr>
<tr>
<td>1.1.1</td>
<td>CEREBELLAR CORTEX: A MODEL FOR CORTICAL LAMINATION</td>
</tr>
<tr>
<td>1.1.2</td>
<td>CNS DAMAGE AND REPAIR</td>
</tr>
<tr>
<td>1.2</td>
<td>NOTCH INTRODUCTION</td>
</tr>
<tr>
<td>1.2.1</td>
<td>NOTCH SIGNALING IN NERVOUS SYSTEM DIFFERENTIATION</td>
</tr>
<tr>
<td>1.2.2</td>
<td>NOTCH SIGNALING IN DROSOPHILA SENSORY ORGAN PRECURSOR CELL DEVELOPMENT</td>
</tr>
<tr>
<td>1.2.3</td>
<td>THE VERTEBRATE NOTCH FAMILY AND ITS LIGANDS</td>
</tr>
<tr>
<td>1.2.4</td>
<td>THE NOTCH SIGNALING PATHWAY</td>
</tr>
<tr>
<td>1.2.5</td>
<td>NOTCH FUNCTION IN VERTEBRATE CNS</td>
</tr>
<tr>
<td>1.2.6</td>
<td>NOTCH1 EXPRESSION IN MAMMALIAN CNS</td>
</tr>
<tr>
<td>1.2.7</td>
<td>NOTCH1 SIGNALING IN POSTMITOTIC NEURONS</td>
</tr>
<tr>
<td>1.2.8</td>
<td>NOTCH SIGNALING AND PRESENILINS</td>
</tr>
<tr>
<td>1.2.9</td>
<td>NOTCH1 DEFICIENT ANIMALS</td>
</tr>
<tr>
<td>2</td>
<td>NOTCH1 IS REQUIRED FOR NEURONAL AND GLIAL DIFFERENTIATION IN THE CEREBELLUM</td>
</tr>
<tr>
<td>2.1</td>
<td>INTRODUCTION</td>
</tr>
<tr>
<td>2.2</td>
<td>MATERIALS AND METHODS</td>
</tr>
<tr>
<td>2.2.1</td>
<td>GENERATION OF MICE AND BREEDING</td>
</tr>
<tr>
<td>2.2.2</td>
<td>IN SITU HYBRIDIZATION ANALYSIS OF GENE EXPRESSION</td>
</tr>
<tr>
<td>2.2.3</td>
<td>IMMUNOFLOUORESCENCE AND CELL FATE ANALYSIS</td>
</tr>
<tr>
<td>2.2.4</td>
<td>ISOLATION AND CULTURE OF CEREBELLAR GLIAL CELLS</td>
</tr>
<tr>
<td>2.3</td>
<td>RESULTS</td>
</tr>
<tr>
<td>2.3.1</td>
<td>CRE-MEDIATED RECOMBINATION IS RESTRICTED TO THE NEUROEPITHELIAL CELLS OF THE MIDBRAIN HINDBRAIN REGION</td>
</tr>
<tr>
<td>2.3.2</td>
<td>ABLATION OF NOTCH1 INDUCES PROMISCUOUS DELTA-LIKE LIGAND EXPRESSION IN THE CEREBELLUM</td>
</tr>
<tr>
<td>2.3.3</td>
<td>ABLATION OF NOTCH1 INDUCES EARLY ONSET OF NEUROGENESIS IN THE CEREBELLUM</td>
</tr>
<tr>
<td>2.3.4</td>
<td>NOTCH1-DEFICIENT CELLS LEAVE THE PROGENITOR CELL POOL BUT FAIL</td>
</tr>
</tbody>
</table>
# Table of Content

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TO GENERATE DIFFERENTIATED NEURONS</td>
<td>31</td>
</tr>
<tr>
<td>2.3.5 Notch1 ablation from the neuroepithelium results in increased cell death</td>
<td>33</td>
</tr>
<tr>
<td>2.3.6 Notch1 ablation from the neuroepithelium results in cell loss</td>
<td>36</td>
</tr>
<tr>
<td>2.3.7 Purkinje cell number is reduced in the early postnatal cerebellum</td>
<td>37</td>
</tr>
<tr>
<td>2.3.8 Conditional ablation of Notch1 results in a reduction in neurons in the adult cerebellum</td>
<td>39</td>
</tr>
<tr>
<td>2.3.9 Notch1 ablation leads to a reduced number of Purkinje cells in the adult cerebellum</td>
<td>42</td>
</tr>
<tr>
<td>2.3.10 Notch1-ablated cells are absent from the adult cerebellum of mutant mice</td>
<td>42</td>
</tr>
<tr>
<td>2.3.11 Notch1-ablation results in a loss of cerebellar glial cells</td>
<td>43</td>
</tr>
<tr>
<td>2.4 Discussion</td>
<td>46</td>
</tr>
<tr>
<td>2.5 Outlook</td>
<td>49</td>
</tr>
<tr>
<td>2.5.1 Intrinsic requirement for Notch1 and survival</td>
<td>49</td>
</tr>
<tr>
<td>2.5.2 Does Notch1 actively promote gliogenesis?</td>
<td>50</td>
</tr>
<tr>
<td>2.5.3 Notch1 plays a role in postmitotic Purkinje Cells</td>
<td>51</td>
</tr>
<tr>
<td>3 Notch1 regulates adult neural stem cell potential</td>
<td>52</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>52</td>
</tr>
<tr>
<td>3.2 Materials and Methods</td>
<td>54</td>
</tr>
<tr>
<td>3.3 Results and discussion</td>
<td>55</td>
</tr>
<tr>
<td>3.3.1 Mutant and Control adult stem cells have the same ability to form primary infected neurospheres</td>
<td>55</td>
</tr>
<tr>
<td>3.3.2 Mutant cells show a reduced capacity for self-renewal</td>
<td>56</td>
</tr>
<tr>
<td>3.3.3 Notch1 does not influence cell fate and is not required for gliogenesis</td>
<td>57</td>
</tr>
<tr>
<td>3.4 Outlook</td>
<td>59</td>
</tr>
<tr>
<td>3.4.1 Proliferation</td>
<td>59</td>
</tr>
<tr>
<td>3.4.2 Functional conservation of Notch1 in adult and embryonic neural stem cells</td>
<td>60</td>
</tr>
<tr>
<td>3.4.3 Experiments to address the role of Notch1 in postnatal neural stem cells in vivo</td>
<td>61</td>
</tr>
<tr>
<td>4 General discussion and outlook</td>
<td>62</td>
</tr>
<tr>
<td>4.1 References</td>
<td>66</td>
</tr>
<tr>
<td>Curriculum vitae</td>
<td>73</td>
</tr>
</tbody>
</table>
Central nervous system (CNS) development is difficult to study because of the immense complexity of the brain. The cerebellum is an excellent model system to study CNS neurogenesis due to its well defined developmental and anatomical structure, and because of the stage-specific differentiation markers that are available (Hatten et al., 1997 and references therein; Hatten, 1995). Furthermore, the fact that major cerebellar defects are compatible with life facilitates the use of conditional gene ablation techniques to target gene manipulations to the cerebellar primordium.

Notch signaling molecules have been shown to be involved in developmental decisions in many tissues during invertebrate development through a process of lateral inhibition (Kimble and Simpson, 1997). Members of the Notch family are transmembrane EGF-domain containing receptors that interact with transmembrane ligands Delta or Serrate, which are expressed by neighboring cells. During development of the Drosophila peripheral sensory organ, Notch-Delta signaling selects a single precursor cell from the equivalent group of proneural cluster cells (Heitzler and Simpson, 1991; Muskavitch, 1994). This cell downregulates expression of Notch, which relieves a repression and the cell will adopt the neuronal fate and initiate neurogenesis (reviewed in (Simpson, 1990). In the mouse four Notch-related genes have been identified with wide ranging expression patterns. Three Notch genes are expressed in the developing mouse CNS, Notch1-3. However, Notch1 is the most prominently expressed in undifferentiated cells of the embryonic neural tube. Conventional gene ablation by targeted gene inactivation experiments have been performed in order to address the role of Notch1 in mouse development (Conlon et al., 1995; Swiatek et al., 1994). However, the mutation results in
Summary

a fatal phenotype at embryonic day 9.5. Notch1 starts to be expressed at around E9 in the neural tube neuroepithelial cells. Hence, limited information was derived from the Notch1-deficient mouse concerning the role of Notch1 in neurogenesis and the fate of the Notch1-deficient neuroepithelium. We have generated a conditional ablation of the Notch1 gene from the mouse neural tube neuroepithelial cells using the Cre-Lox system to address the role of Notch1 in neurogenesis and the fate of the Notch-deficient cells. Our results show that Notch1 regulates the onset of neurogenesis in the vertebrate CNS and Notch1-deficient neuroepithelial cells induce aberrant neurogenic programs. Contrary to expectations, the Notch1-deficient differentiating cells are unable to enter the neuronal lineage and undergo programmed cell death. The consequence of the mutation, in contrast to loss of Notch function mutations in Drosophila, is a reduction in the number of neurons in the adult CNS.

Not only the neuroepithelial cells of the midbrain/hindbrain region are under the control of Notch1, but also the adult neural stem cells isolated from the subventricular zone of the adult mouse brain. We have shown that Notch1 is important for the maintenance of adult neural stem cells and unlike previous reports where Notch1 has been claimed to promote gliogenesis (Furukawa et al., 2000; Gaiano et al., 2000), we demonstrate that Notch1 is not intrinsically required for the formation of glial cells. We hypothesise that Notch1 acts as a molecular "brake" on differentiation. Notch1 keeps the multipotent cells in an undifferentiated state and thereby controls their response to differentiation signals. To be able to force stem cells to adopt a neuronal fate rather than a glial fate by downregulating Notch1 might be a beneficial option in CNS repair.
ZUSAMMENFASSUNG

Das Zentralnervensystem (ZNS) entwickelt sich durch die kontrollierte Abfolge verschiedener komplexer Prozesse. Da der laminare Aufbau des Cortex mit seinen zahlreichen Zelltypen sehr kompliziert ist, hat sich unser Labor entschlossen, sich auf die Entwicklung des Cerebellums (Kleinhirn) zu konzentrieren. Diese Struktur hat einige Vorteile für das Forschen an Prozessen in der Entwicklung des ZNS auf molekularer und zellulärer Ebene im Vergleich zum Cortex. Das Kleinhirn ist eine relativ einfache Struktur mit laminarem Aufbau, bestehend aus nur fünf neuronalen Zelltypen, wovon Körnerzellen und Purkinje Zellen die wichtigsten sind (Aider et al., 1996; Altman and Bayer, 1985b). Molekulare Marker, die die verschiedenen Zelltypen in unterschiedlichen Entwicklungsstadien beschreiben, sind bekannt und erleichtern die Analyse von Differenzierungsvorgängen (Hatten et al., 1997; Hatten and Heintz, 1995). Hinzu kommt, dass das Kleinhirn keine lebensnotwendige Struktur ist und somit auch Gene, die in der Entwicklung eine wichtige Rolle spielen, abgeschalten werden können, ohne dass dies zum Tode des Versuchstiers führen würde, selbst wenn sich das Kleinhirn nicht mehr entwickeln könnte.


Diese Resultate unterstützen unsere Hypothese, dass Notch1 in Vertebraten, im Gegensatz zur Funktion in der Drosophila, nicht für das Auswahlverfahren der undifferenzierten Zellen verantwortlich ist. Die Differenzierungssignale in der Umgebung entscheiden, was aus den jeweiligen Zellen geschieht, sobald Notch1 zulässt, dass die Zellen auf die extrinsischen Faktoren reagieren können.
1 INTRODUCTION

1.1 CNS DEVELOPMENT

Formation of the vertebrate CNS requires a precise control of dorsal/ventral and anterior/posterior patterning. To establish the different regions of the future brain the initial step is to define the anterior-posterior (AP) axis and the subdivisions of the neural tube into brain vesicles (Rubenstein et al., 1998). The restricted expression of a set of transcription factors mark domains that will become the forebrain (Rubenstein et al., 1998), midbrain and cerebellum (Joyner, 1996), hindbrain (Guthrie, 1996), and spinal cord (Lee and Jessell, 1999). The onset of axial patterns within the nervous system is closely linked to the onset of neural induction. Thus, as the expression of specific transcription factors commences within different territories of the emerging CNS, subsets of neurons begin to acquire a dorsal or ventral identity (Hatten and Heintz, 1995). In some regions of the central nervous system, for example in the prospective spinal cord, Sonic hedgehog acts both as a ventralizing signal released from the underlying axial mesoderm or notochord and an inducer of floorplate and motorneuron identity (Ericson et al., 1997). Conversely, dorsalizing signals such as members of the Bone Morphogenetic Protein family are released from the roofplate or ectoderm overlying the neural tube and induce neural crest and interneuron cell fate (Lee and Jessell, 1999). Cell specification and the control over migration then leads to the laminar architecture of the brain (Middleton and Strick, 1998).
1.1.1 Cerebellar cortex: a model for cortical lamination

CNS development is difficult to study because of the immense complexity of the brain. The cerebellum is an excellent model system to study CNS neurogenesis due to its well-defined developmental and anatomical structure, and because of the stage-specific differentiation markers that are available (Hatten et al., 1997 and references therein; Hatten and Heintz, 1995). Furthermore, the fact that major cerebellar defects are compatible with life facilitates the use of conditional gene ablation techniques to target gene manipulations to the cerebellar primordium.

At E9.5 the murine cerebellar primordium consists of the neuroepithelium (NEP). NEP cells show stem cell-like properties and are the precursors for all the different cell types of the cerebellum. Neurons of the deep nuclei are the first cerebellar neurons to be generated at around E10-11 (Altman and Bayer, 1985a), followed by precursors of the Purkinje cell (Altman and Bayer, 1985b). Purkinje cells migrate along the radial glial fibers out of the NEP and settle into a broad zone where they remain until the early postnatal period. In the mouse cerebellum, Purkinje cell precursors are generated between embryonic day 11-13 (E11-13). This broad zone of Purkinje cells provides a scaffold for the formation of the other neuronal layer of the cerebellar cortex, the internal granule cell layer (Altman and Bayer, 1985b). As development proceeds the Purkinje cells align to form a single cell layer and terminal differentiation of Purkinje cells occurs at postnatal day 20 (P20) when they make contact with the parallel fibers of the granule cells and with afferent axons of the climbing fibers. The Purkinje cells are the only efferent neurons of the cerebellar cortex, innervating mainly the deep cerebellar nuclei (Herrup and Kuemerle, 1997)(Fig. 1-1).

The other major neuronal cell type of the cerebellum are the granule cells. The precursors of granule cells first appear in a defined region of the neuroepithelium called the rhombic lip (Alder et al., 1996). Around E14 the cells in this domain separate from the adjacent NEP, cross the lip, and migrate over the cerebellar anlage, thereby generating a second germinal layer, called the external germinal layer (EGL). Within the EGL, the precursors proliferate rapidly in the peri- and postnatal period until they form a 7-8 cell thick layer. During the second and third postnatal week the differentiating granule cells migrate along radial glia into the cerebellum, pass the layer of Purkinje
cells, and finally differentiate to form the internal granule cell layer (IGL) (Hatten, 1993).

Figure 1-1:
The cerebellum is a highly organized structure containing three layers: The Purkinje cell layer (PCL). The Purkinje cells aligne to form a single cell layer and are the only efferent neurons in the cerebellum, innervating mainly the deep cerebellar nuclei. They make contact with the parallel fibers of the granule cells located in the internal granular layer. The molecular layer contains mostly interneurons and the parallel fibers. (From J. Altman an S. A. Bayer: Development of the cerebellar system)

The granule cells are the most abundant neurons in the mammalian brain and are excitatory interneurons providing the connection between the afferent mossy fibers and Purkinje cells.

1.1.2 CNS DAMAGE AND REPAIR

Diseases and injury of the brain are particularly debilitating due to the reduced potential of the adult CNS to replace or repair damaged axons and neurons. Hence, until recently it was believed that the adult brain and spinal cord do not regenerate after injury, however, recent discoveries have forced a reconsideration of this accepted principle (Gritti et al., 1996; McKay, 2000; Palmer et al., 1997; Reynolds and Weiss, 1992). Advances in our
understanding of how the brain develops and further studies in developmental neurobiology, intracellular signaling and neuro-immunology are bringing the regeneration field closer to success. Neural stem cells, cells which are mitotically active, can self-renew and have the potential to generate all of the three major cell types of an adult brain (neurons, glia and oligodendrocytes) have now been found in the adult CNS (Gage et al., 1995). Neural stem cells have been proposed to exist in two main regions of the adult brain, in the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus of the hippocampal formation (SGZ) (Doetsch et al., 1999; Temple, 1999) and in the spinal cord (Gage et al., 2002; Yamamoto et al., 2001). However, recent data claim that the source of newly generated dentate gyrus neurons are neuron-specific progenitors with limited self-renewal capacity and not multipotential stem cells (Seaberg et al., 2002). Hence, new effort is being put into CNS regeneration. Currently, dead or damaged cells in the adult brain are mainly replaced by astrocytes resulting in astrocytic scars (Dusart et al., 1991; Hatten et al., 1991). Therefore, it is of major interest for the treatment of neurodegenerative disorders to elucidate the signals involved in cell fate decisions and neuronal differentiation. It is not only important to have the correct cell types at the right place but also to project their axons and dendrites to form a functional network. Work in Drosophila has indicated that the receptor molecule Notch is involved in cell fate decision and neuronal differentiation in the invertebrate nervous system. Hence, Notch is a good candidate to play a role in CNS development in mammals (see section 1.2.6). Therefore, we have address the role of Notch signaling in the formation of the mammalian brain.

### 1.2 NOTCH INTRODUCTION

#### 1.2.1 NOTCH SIGNALING IN NERVOUS SYSTEM DIFFERENTIATION

Cell-cell interactions play a crucial role in specifying cell fate and pattern formation during development. Notch is a transmembrane receptor that is activated through contact with its ligand expressed on a neighboring cell and is involved in important developmental decisions in various invertebrate tissues including the nervous system (Kimble and Simpson, 1997). Notch signaling has been most extensively studied in invertebrates and particularly in the peripheral nervous system (PNS) in Drosophila.
1.2.2 Notch Signaling in Drosophila Sensory Organ Precursor Cell Development

The surface of the ectoderm of Drosophila is covered with sensory bristles that are generated under the influence of Notch. Notch is required to generate the correct number of neuronal precursor cells of the sensory organs and regulates their fate, either neuronal or epidermal, by interacting with the transmembrane ligands Delta and Serrate expressed on neighboring cells (Heitzler and Simpson, 1991; Muskavitch, 1994).

During Drosophila neurogenesis, a single neural precursor of a proneural cluster, a group of cells with equal developmental potential, is singled out through a small stochastic fluctuation in Notch signaling. The initial fluctuation is then reinforced by a feedback loop to down-regulate receptor expression in the cell which initially has slightly more ligand expression. This cell will then become the sensory organ precursor (SOP) cell and its progeny will give rise to the sensory bristle organ or sensillum. Through a process, called lateral inhibition, the emerging neural precursor SOP cell prevents its neighbors from adopting the same fate (reviewed in Simpson, 1990). The transmembrane protein Notch is the receptor for a signal that diverts cells from a primary neuronal fate and directs them towards a secondary epidermal fate.

Notch signaling is involved not only in the initial stages of sensillum formation by generation of the SOP cell, but also later in the asymmetric cell divisions that the SOP cell undergoes to generate the SpIIa and SpIIb cell (reviewed in Hawkins and Garriga, 1998 and Jan and Jan, 1998). The SpII cells undergo a secondary asymmetric division to generate the four cells of the sensillum, the bristle, socket, neuron and sheath (glial) cell. Notch has been shown to be involved in the determination of the SpII cells and their progeny. In Drosophila embryos that lack Notch function (Hartenstein and Posakony, 1990), two SpIIb cells are produced from the SOP cell instead of one SpIIa and a SpIIb cell. Furthermore, the secondary asymmetric division is ablated and all precursor cells become neuronal at the expense of sheath cells. Whereas in embryos with a gain-of-function Notch mutation (Hawkins and Garriga, 1998), two SpIIa cells are generated and subsequently socket cells at the expense of bristles (reviewed in Artavanis-Tsakonas et al., 1995; Greenwald and Rubin, 1992; Simpson, 1995).

Unlike Notch, Numb, an inhibitor of Notch, does not play a role in the formation of the SOP from the proneural cluster, but rather regulates Notch signaling during the...
asymmetric cell divisions of later stages of sensillum formation (Rhyu et al., 1994). Ablation of Numb has the same phenotype as overexpression of Notch and overexpression of Numb has the same effect as ablation of Notch signaling. This data suggest that Notch and Numb may have reciprocal functions in the formation of the sensory organ.

1.2.3 The vertebrate Notch family and its ligands

Currently, four members of the Notch family have been identified in mammals (Notch1-4), and at least six ligands, Delta-like1-4 and Jagged 1 and 2 (Fig. 1-2). Notch belongs to a conserved family of transmembrane receptors. All of the members of this family are single-pass transmembrane proteins that respond to ligands of the Delta-Serrate-Lag2 (DSL) family and contain several conserved sequence motifs. The large extracellular domain contains many tandem repeats of an epidermal growth factor (EGF) motif as well as three copies of a “Lin-12/Notch repeat” (LNR). In addition, Notch proteins contain intracellular domains that include a block of six CDC10 (ankyrin) repeats. These are important for the level of activation of the Hes-1 promoter, a downstream target (Beatus et al., 2001). The intracellular domain of the receptor also contains two nuclear localization signals (NLS), a glutamine-rich C-terminal region (OPA), and a proline-glutamate-serine-threonine “PEST” sequence that regulates protein turnover and degradation. Vertebrate Notch homologs have a high degree of amino-acid similarity suggesting a conserved and crucial role for Notch in vertebrate development (Coffman et al., 1990; Del Amo et al., 1992; Lardelli et al., 1994; Lardelli and Lendahl, 1993; Stifani et al., 1992; Weinmaster et al., 1991).

1.2.4 The Notch signaling pathway

Evidence suggests that the proteolytic processing of Notch proteins is important for their function (see section 1.2.8). During processing Notch gets cleaved extracellularly and the N-terminal fragment remains associated with the C-terminal transmembrane portion by a disulfide-bridge. The function of this processed form is not known but Notch proteins which have the extracellular domain or both the extracellular and the transmembrane domain deleted encode constitutively active receptors and are found to be localized to the nucleus (reviewed in Artavanis-Tsakonas et al., 1995). These
A) The Notch receptor family members have the same conserved structural motifs. The extracellular domains contain multiple tandemly arrayed EGF-like repeats and a cystein-rich LNR region. Following the transmembrane domain (TM) there are 6 ankyrin repeats, an OPA and a PEST domain. B) Like the Notch receptors, the ligands display a high degree of structural conservation. The extracellular domain of the ligands also include multiple copies of the EGF-like sequence. The Serrate-related ligands (Jagged1/2) have an additional cystein-rich region in front of the transmembrane domain (TM). The short cytoplasmic domains show only little structural conservation.

observations led to the hypothesis that ligand activation brings about proteolytic cleavage of Notch, so that the intracellular portion of Notch itself moves to the nucleus and collaborates with the nuclear factor RBP-Jk (belonging to the CSL family). RBP-Jk is a mammalian homolog to the Drosophila Suppressor of Hairless (Su(H)) protein, a sequence specific DNA-binding protein, which appears to be responsible for activating the transcription of downstream target genes in response to Notch receptor activity (reviewed in Honjo, 1996; Weinmaster, 1997). RBP-Jk is a transcriptional repressor in
the absence of Notch signaling. Direct targets for the binding by the RBP-Jk protein (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995) are genes of the Hes family, homologs of the Enhancer of split complex (E(spl)) in Drosophila. The Hes genes negatively regulate the activation of different proneural genes like Mash-1, a mammalian homolog to the Drosophila achaete-scute complex (AS-C), which encodes a basic helix-loop-helix (bHLH)-type transcription factor (Johnson et al., 1990). Mash-1 has been found to be expressed in the early developing brain, and plays key roles in multiple steps of neurogenesis (Guillemot and Joyner, 1993; Jan and Jan, 1993). Downregulating Mash-1 by Notch activity leads to suppression of neurogenesis (Fig. 1-3).

1.2.5 Notch function in vertebrate CNS

The first member of the vertebrate Notch family that has been cloned was the Xenopus Xotch (Chitnis et al., 1995). Overexpression experiments with the dominant active intracellular domain of Xotch (XICD) or with the secreted or wild-type form of Xotch ligand showed that Notch can also regulate neurogenesis in vertebrates. Activation of the Xotch pathway leads to inhibition of neurogenesis whereas suppression of the pathway with a dominant negative form of the ligand results in enhanced neurogenesis (Chitnis et al., 1995; Coffman et al., 1993).

Different groups have claimed, that Notch1 plays a role not only in neurogenesis but also in regulating gliogenesis in vertebrates. Constitutively activate truncated forms of Notch receptors inhibit oligodendrocyte differentiation (Wang et al., 1998). These results provide strong evidence that the ligand Jagged1, which is expressed on axons, activates Notch1 on the oligodendrocyte precursors (OPC) and inhibits oligodendrocyte differentiation. Jagged1 does not inhibit oligodendrocyte differentiation by stimulating proliferation of the OPCs, but by uncoupling proliferation from differentiation. The Jagged1 activated OPCs are arrested at an early stage of differentiation (Wang et al., 1998). As development proceeds in vivo, Jagged1 is down-regulated with a time course that parallels myelination and oligodendrocytes reduce expression of Notch1 and start to mature.

More recently, another function of Notch1 in gliogenesis has been suggested. Gain-of-function experiments using activated Notch1 in the rat retina and in the embryonic
Figure 1-3:
Ligand-induced proteolysis leads to activation of the intracellular domain of Notch (ICD). The ICD translocates to the nucleus where it interacts with members of the CSL-family to activate the expression of the HES genes. The HES genes repress the transcription of proneural genes like Mash1 or Math1. Therefore, activation of Notch results in a block of neural-specific gene expression and thus prevents the cell to adopt a neuronal fate.

mouse forebrain have revealed a gliogenic potential for the Notch signal (Furukawa et al., 2000; Gaiano et al., 2000).
1.2.6 NOTCH1 EXPRESSION IN MAMMALIAN CNS

Notch1 is expressed in a wide variety of tissues during early mouse development. It starts at embryonic day 8 (E8) in the primitive streak during gastrulation and in the presomitic mesoderm during the process of somitogenesis. At E9-9.5 Notch1 is highly expressed in mitotic cells in the neuroepithelium in the midbrain/hindbrain region of the neural tube (Del Amo et al., 1992; Reaume et al., 1992). These high levels of Notch1 expression remain in the cells of the ventricular zone through E16.

In the cerebellum, Notch1 expression first becomes detectable at E9-9.5 in the cells of the neuroepithelium of the primordium. Notch1 is slowly down-regulated by the differentiating Purkinje and granule cell progenitors as they migrate out of the ventricular zone at around E13-14. Later, a second wave of Notch1 expression by Purkinje cells is detected starting around postnatal day 4 (P4). Notch1 is prominently expressed in the subventricular zone and subgranule layers of the dentate gyrus of the hippocampus throughout postnatal development as are its ligand Jagged1 and downstream target Hes5. Notch1 is also expressed by cortical, olfactory, and hippocampal neurons and by cells in the cerebellar Purkinje cell layer. In the adult, Notch1 expression is down-regulated but can be detected in the germinal zones, and in the Purkinje cell layer of the cerebellum where it is associated with the expression of the ligand Jagged1 (Stump et al. 2002, in press). The expression pattern of Notch1 postnatally suggests a putative role in late stages of cell differentiation.

1.2.7 NOTCH1 SIGNALING IN POSTMITOTIC NEURONS

As described above, recent studies indicated that mammalian Notch receptors continue to be expressed in postmitotic and mature neurons in the developing and adult mouse brain, although at relatively low levels (Sestan et al., 1999; Stump et al. 2002, in press). The Notch signal transduction pathway is active in postmitotic primary neurons, suggesting a physiological role for Notch in regulating developmental events subsequent to specification of cell fate in the CNS (Berezovska et al., 1999b). Overexpression of the active form of Notch1 in vitro resulted in premature inhibition of neurite growth but induced elaborate dendritic branching (Berezovska et al., 1999b; Franklin et al., 1999; Redmond et al., 2000). Hence, it was concluded that neurite growth and the final size of the neuronal dendritic field depend on Notch activity. Notch activity,
however, increases with the number of interneuronal contacts. High density cultures have shown that cell-cell contacts blocked dendritic growth and increased branching. This phenotype could be rescued by overexpressing Numb, the physiological inhibitor of Notch signaling, and led to significant recovery of neurite outgrowth in high density cultures (Berezovska et al., 1999b; Sestan et al., 1999). As neurons express both receptor and ligand (Stump et al. 2002, in press) Notch signals may act in a paracrine and autocrine fashion (Sestan et al., 1999). However, it is not known whether Notch regulation of dendritic development involves the same effectors that regulate cell fate decisions.

An alternate mechanism for the effects of Notch signaling on process outgrowth in Drosophila has been proposed by Giniger (Giniger, 1998). Abl, a tyrosine kinase, is, like Notch, concentrated in axons. Notch can interact with the Abl accessory protein, disabled (Dab). Dab binds directly to NICD with its PTB domain. Dab may act as an adaptor protein that links Notch to Abl in response to a signal from Delta. Furthermore, Abl kinase activity is strictly dependent on integrin-mediated cell adhesion for activation (Lewis et al., 1996). Since both Abl and integrins are thought to influence the actin cytoskeleton, they could provide a link between Notch activation and cytoskeletal changes, thus mediating the effects of Notch signaling on axonal and dendritic morphology. These biological changes are also mediated through activation of multiple other signal transducers, like for example Rho GTPases.

Rho family GTPases are central regulators of axonal growth and dendritic morphology. The best studied family members are Rho, Rac, and Cdc42. On the basis of the effects of individual Rho-family GTPases in dendritic development, one would predict that the effects of Notch on growth inhibition may involve activation of Rho, whereas the effects on dendritic branching may involve activity of Rac1 and Cdc42 (Redmond and Ghosh, 2001). Extracellular signals might influence dendritic development specifically by activating particular Rho-family GTPases at different time points and in different environments. In this way Notch signaling is involved in the transition from growth to stability by regulating the capacity of neurons to extend and elaborate neurites.
1.2.8 **NOTCH SIGNALING AND PRESENILINS**

Notch is synthesized as a 300 kDa precursor protein that undergoes subsequent proteolytic processing (reviewed by Chan and Jan, 1998). Notch receptors are cleaved three times. The first is an extracellular furin-mediated cleavage, N-terminal to the transmembrane domain, and occurs in the trans-Golgi network. Such a mature Notch receptor is transported to the cell surface, where it is presented as a heterodimeric molecule, covalently linked by disulfide bonds (Blaumueller et al., 1997; Chan and Jan, 1998; Logeât et al., 1998). The receptor is then cleaved once more in the extracellular domain, closer to the membrane, by a Kuzbanian-like metalloprotease (Pan and Rubin, 1997). This second cleavage is ligand dependent and a prerequisite for the third and last signal transducing proteolysis, which takes place in the intracellular domain. This intracellular cleavage, which is necessary for the release of the intracellular domain of Notch to translocate to the nucleus where Notch exerts its function, can be blocked by γ-secretase inhibitors.

The main hallmark of familial Alzheimer’s disease are the elevated levels of β-amyloid protein 42 (Aβ42), a 42 amino acid long peptide, aberrantly generated from the amyloid precursor protein (APP) by β- and γ-secretases (Berezovska et al., 1998). This subspecies of Aβ is highly amyloidogenic and is the primary protein deposited in senile plaques. Mutations in the Presenilin (PS) genes have been shown to play a role in the accumulation of these peptides. In addition to the role of PS in APP processing, *in vitro* studies suggest that the same enzyme might be responsible for the intracellular cleavage of Notch (Berezovska et al., 1999a; De Strooper et al., 1999; Selkoe, 2000; Struhl and Greenwald, 1999; Wolfe et al., 1999).

There are two PS family members known, presenilin 1 and 2 (PS1/2). Different reports have demonstrated that ligand induced cleavage of Notch1 occurs via a PS1-dependent γ-secretase enzymatic activity identical to that having APP γ-secretase activity. PS1 has eight transmembrane domains. Two functionally important aspartate residues in domain TM6 and 7. A loss of PS1, by deleting the aspartate residues, has been shown to prevent ligand induced Notch1 nuclear translocation and accumulation (Berezovska et al., 2000; Struhl and Greenwald, 1999; Ye et al., 1999). It is still unclear, if presenilins are indeed γ-secretases, or facilitators that directly traffic APP and Notch1 to the right cellular compartment for γ-secretase processing. It is also not fully understood if Notch1 binds
directly to PS1 or via a protein called Nicastrin which has been recently found to interact with Notch1 (Chen et al., 2001).

For the treatment of Alzheimer's disease it is important to understand the exact function of presenilins in the processing of APP and Notch. If the regulation of APP and Notch are identical processes, the use of γ-secretase inhibitors in a therapeutical approach may lead to severe side effects in Notch function. However, one group has recently succeeded in separating the APP and Notch functions of PS1. Kulic et al. have shown that specific mutations in PS1 could simultaneously cause a loss of function in Notch signaling but a gain of pathological misfunction in Aβ production. The two mutations may affect the conformation of PS1 in a way that would specifically allow PS1 interaction with APP but not with Notch1. It seems that different domains of PS1 are required for distinct substrate interactions (Kulic et al., 2000).

1.2.9 Notch1 deficient animals

Notch1-deficient mice have been generated (Conlon et al., 1995; Swiatek et al., 1994). However, these animals die at embryonic day 9.5 (E9.5). Examination of the embryos did not reveal any obvious relation between the abnormalities found due to the loss of Notch1 and the cause of death. In Notch1-deficient embryos the initial stages of somitogenesis proceeded normally. The first 6 to 8 somites form correctly but become arrested in development and failed to reach the 14 to 16 somite stage (Conlon et al., 1995; Swiatek et al., 1994) and die. Increased apoptosis and necrosis were found especially in the neural tube. In contrast to the studies in Drosophila and Xenopus, where premature differentiation and increased neuronal cell number were observed, nothing could be detected in Notch1-deficient embryos. In addition, no abnormalities could be found in the CNS, with the exception of a slight increase in Mash1 expression. However, it has to be taken into account that the animals die before the major onset of neurogenesis in the mouse CNS and before the major upregulation of Notch1 in the neural tube. To study the function of Notch1 in the developing CNS and to circumvent the early lethality, we have used conditional gene ablation to delete Notch1 in a tissue- and time-specific manner from the neuroepithelium of the midbrain/hindbrain region, starting around E9.
2 NOTCH1 IS REQUIRED FOR NEURONAL AND GLIAL DIFFERENTIATION IN THE CEREBELLUM


2.1 INTRODUCTION

The nervous system of vertebrates is derived from the neuroepithelial cells of the neural tube. The early embryonic neuroepithelium contains cells that are considered to have stem cell-like properties in that they are mitotically active, can self renew, and have the potential to generate all of the differentiated cells of the adult central nervous system (CNS) (Gage et al., 1995; Kalyani et al., 1997; McKay, 1997; Qian et al., 1997; Vescovi et al., 1993). The neural tube is patterned early in development into defined regions that will develop into the vesicles and structures of the more mature CNS. Within these vesicles the fate of the neuroepithelial cells is regulated to generate neurons and glial cells in a spatially and temporally well defined manner. The midbrain hindbrain boundary is an important organizer in the anterior region of the developing CNS (Wassef and Joyner, 1997). This so called isthmic structure gives rise to signals that include FGF8 and which regulate anterior posterior patterning and formation of the caudal midbrain and the cerebellum (Martinez et al., 1999).

The differentiation of multipotent neural progenitor cells isolated from the embryonic nervous system can be modulated by growth factor treatment in vitro (Johe et al., 1996;
Notch1 Function in CNS Development

Qian et al., 1997; Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Williams et al., 1997). However, the molecules that regulate neuroepithelial cell differentiation and determine cell fate in the mammalian neural tube are poorly defined. In invertebrates such as Drosophila, the signaling molecule Notch is involved in developmental decisions in many tissues, including the nervous system, through a process of lateral inhibition (Kimble and Simpson, 1997). Notch is required to generate the correct number of neuronal precursor cells of the sensory organs and also regulates their fate by interacting with transmembrane ligands Delta and Serrate expressed on neighboring cells (Heitzler and Simpson, 1991; Muskavitch, 1994).

Notch regulates the expression of a cascade of transcription factors belonging to the basic helix-loop-helix (bHLH) family (reviewed by Artavanis-Tsakonas et al., 1999; Kalyani et al., 1997; Robey, 1997). Recent data have shown that Notch receptor signaling is regulated by a complex process of proteolysis (reviewed by Chan and Jan, 1998; Kopan and Goate, 2000; Lendahl, 1998). Multiple extracellular cleavages are rapidly followed by a ligand-induced intracellular, juxtamembrane proteolysis via a γ-secretase that may include members of the Presenilin family (reviewed by Kopan and Goate, 2000; Selkoe, 2000). The cleaved intracellular domain of the Notch activates Enhancer of Split-related (E(Spl)) genes (the Hes family in vertebrates) in a complex including Suppressor of Hairless-like (Su(H)) nuclear factors (the vertebrate CSL family). Subsequently, the Hes proteins suppress expression of the proneural bHLH transcription factors related to the Drosophila Achaete-Scute Complex genes. These proneural genes are pivotal in the induction of neurogenesis and regulation of neuronal differentiation (Cau et al., 2000; Nieto et al., 2001; Ohtsuka et al., 2001; Satow et al., 2001; reviewed by Lee, 1997).

Vertebrate neuroepithelial cells require precise regulation of differentiation and cell fate determination. Hence, it has been proposed that Notch signaling may play a role in vertebrate neurogenesis (Artavanis-Tsakonas et al., 1999). Gain-of-function experiments performed in Xenopus laevis and chicken have provided strong indications that Notch signaling can maintain neural progenitor cells in an undifferentiated state (Austin et al., 1995; Chitnis, 1995b; Coffman et al., 1993; Wakamatsu et al., 1999). Overexpression of an intrinsically active form of Notch or the ligand Delta blocked progenitor cell differentiation and reduced neurogenesis at early stages (Ahmad et al., 1997; Austin et...
al., 1995; Chitnis, 1995b; Coffman et al., 1993; Wakamatsu et al., 1999). In other studies,
strong overexpression of Delta in chick retinal and *Xenopus* otic vesicle progenitors mimicked a reduction in Notch signaling, resulting in increased neuronal differentiation of isolated progenitor cells (Dorsky et al., 1997; Henrique et al., 1997). Furthermore, overexpression of a dominant negative Delta blocked Notch signaling in clustered cells and resulted in premature differentiation of retinal and otic vesicle progenitors (Austin et al., 1995; Dorsky et al., 1997; Henrique et al., 1997). These findings have been supported by antisense oligonucleotide experiments to reduce *Notch1* expression in the retina of chicken embryos, which caused an increase in neurons without affecting neuroepithelial cell proliferation (Austin et al., 1995). Gain-of-function experiments in the rat retina and mouse telencephalon have also indicated that Notch signaling in neural progenitors may regulate neuronal versus glial differentiation of multipotent progenitors (Furukawa et al., 2000; Gaiano et al., 2000; reviewed by Lundkvist and Lendahl, 2001). This hypothesis has recently been supported by overexpression of a constitutively active Notch in the Zebrafish retina resulting in an instructive signal for gliogenesis (Scheer et al., 2001). A similar gliogenic effect can also be induced in cultured murine neural progenitors in vitro by overexpression of intrinsically active Notch3 (Tanigaki et al., 2001).

*Notch1* expression in the mouse neuroepithelium starts around embryonic day 9 (E9) and continues in the ventricular zone and subventricular zone cells at later developmental stages (Del Arno et al., 1992; Reaume et al., 1992; Weinmaster et al., 1991). Inactivation of the *Notch1* gene in mice resulted in a developmental arrest at the 4-6 somite stage and death around E9.5 (Conlon et al., 1995; Swiatek et al., 1994). In addition, a potential precocious expression of the neurogenic bHLH transcription factor Mash-1 was observed (de la Pompa et al., 1997). However, the *in vivo* role of Notch1 in mammalian neurogenesis and the fate of Notch1-deficient neuroepithelial cells remain unclear due to the early lethality of Notch1-deficient animals. Thus, we have conditionally ablated the *Notch1* gene from the mouse neural tube neuroepithelium using the Cre-Lox system. *Notch1* was inactivated specifically in neuroepithelial cells of the midbrain hindbrain boundary and the consequences of *Notch1* ablation on the neuroepithelial cells and their fate was followed through to adulthood. Our approach allowed us to analyze the effects of the loss of Notch1 function on a restricted population of neuroepithelial cells, and
revealed a pivotal role for Notch1 in the differentiation of mouse cerebellar progenitor cells.

2.2 MATERIALS AND METHODS

2.2.1 GENERATION OF MICE AND BREEDING

Mice carrying LoxP-flanked Notch1 alleles have been described previously (Radtke et al., 1999) and mice carrying the Cre-recombinase under the transcriptional control of the engrailed-2 promoter enhancer were kindly provided by Dr. A. Joyner (Zinyk et al., 1998). The R26R transgenic line was kindly provided by Dr. P. Soriano (Soriano, 1999). Embryos were generated by timed-mating counting the morning after pairing as embryonic day 0.5. As ablation of the engrailed-2 gene also results in cerebellar foliation defects (Millen et al., 1994), we addressed the possibility that the abnormalities seen in our homozygous Floxed Notch1 En2-Cre animals may be caused by effects of the En2-Cre transgene on endogenous engrailed-2 activity. Therefore, we analyzed heterozygous Floxed Notch1 En2-Cre animals and En2-Cre transgenic animals but could not find defects in cerebellar development. Hence, we exclude that the En2-Cre transgene induced the reduced cerebellar vermis seen in our mutants. Furthermore, we have observed an identical phenotype using a second line of En2-Cre animals that shows a similar expression pattern in the cerebellum (Zinyk et al., 1998). This indicates that the reduction in cerebellar neurons is caused by the loss of Notch1 rather than an integration effect of the En2-Cre transgene. The analysis was performed with animals carrying the En2-Cre transgene and homozygous Floxed Notch1 alleles. We have also observed an identical phenotype in En2-Cre transgenic animals hemizygous for Floxed and null-Notch1 alleles.

2.2.2 IN SITU HYBRIDIZATION ANALYSIS OF GENE EXPRESSION

Embryos and brains were isolated and frozen in OCT (TissueTech) on dry ice. Twenty \(\mu\)m frozen sections were thaw mounted onto Superfrost slides (Mettler), air-dried, and fixed in 4% paraformaldehyde. Histological analysis was performed by staining sections for 30 seconds in hematoxylin (Sigma) followed by dehydration in alcohol and embedding in Eukitt (Plano). The midline sagital 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 (Fig. 2-2A). The constriction of the neural tube at the isthmus between the midbrain and the cerebellum was used as the landmark for the anterior aspect of the presumptive cerebellum, and the point of maximum opening of the roof of the fourth ventricle as the posterior margin of the cerebellar primordium. In situ RNA hybridization was performed with digoxigenin-labeled RNA probes for Calbindin D 28k, Dll1, Dll3, Hes1, Hes3, Hes5, Jagged1, Jagged2, Mash-1, Math-1, and Notch1, overnight at 72°C in buffer containing 50% formamide, and detected using an anti-DIG-AP antibody according to manufacturer’s instructions (Roche Diagnostics). Consecutive sections covering the entire cerebellar primordium were analyzed with three different probes, Xgal staining or antibodies. Whole mount in situ RNA hybridization was performed according to Wilkinson (Wilkinson, 1992).

2.2.3 IMMUNOFLUORESCENCE AND CELL FATE ANALYSIS

Immunofluorescent analysis of protein expression was performed with antibodies against β-TubulinIII (TuJ1)(1:200; Sigma), neurofilament 160 (1:100; Sigma), nestin (1:30; R401, Developmental Studies Hybridoma Bank, University of Iowa), and Calbindin D 28k (1:200; Sigma). Sections were fixed in 4% paraformaldehyde and blocked in PBS 0.2% Tween20 containing 5% goat serum. Antibodies were diluted in blocking buffer and incubated overnight at 4°C. Bound antibody was detected with Cy3- or FITC-conjugated goat anti-mouse Ig (Jackson Labs) and goat anti-rabbit Ig (Jackson Labs). Analysis of β-galactosidase activity was performed by Xgal staining according to Zinyk et al. 1998. β-galactosidase expression was detected with anti-β-galactosidase antibodies (1:100; Cappel). BrdU was injected into time-mated females and the embryos excised after 3 hours. BrdU detection was performed with an FITC-conjugated anti-BrdU antibody according to the manufacturer’s instructions (Roche Diagnostics). Apoptotic cell death was analyzed by TUNEL staining using biotin-labeled UTP and an FITC-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.
5.0 software. Confocal microscopy was performed with a Leica confocal microscope and Imaris software (Bitplane AG, Technopark Zurich, Switzerland).

2.2.4 ISOLATION AND CULTURE OF CEREBELLAR GLIAL CELLS

Postnatal day 4 (P4) mice were sacrificed by decapitation, the cerebellum removed and the vermis region separated from the hemispheres under sterile conditions. The tails of the sacrificed animals were used for DNA isolation and the genotype of the animals analyzed by PCR. The vermis of the individual animals were dissociated and incubated in Ringer’s Solution containing 0.25% trypsin for 30 minutes at 37°C. The cells were collected by centrifugation and resuspended in 6 ml of BME (Gibco) containing 10% FCS. The cells were triturated with a fire-polished Pasteur pipette and passed through a 50 μm cell sieve. The cells from the vermis of one animal were plated on six 35 mm dishes coated with poly-L-lysine. There were no obvious differences in the plating efficiency between mutant and control cells. The cells were cultured for 5 days in BME, 10% FCS, and half of the medium was replaced every second day. The cells were fixed and immunostained with anti-GFAP antibodies in conjunction with anti-β-galactosidase antibodies or Xgal staining. The cultures from mutant animals contained considerably less cells than those from control animals, which likely reflects the 50% reduction in the size of the cerebellar vermis of the mutants compared to control littermates at P4 (Fig. 8A,B).

2.3 RESULTS

To address the role of Notch1 in the development and maintenance of the CNS we have conditionally inactivated the Notch1 gene in midbrain hindbrain neuroepithelial cells from E9. The Notch1 gene encodes a type-1 membrane receptor of approximately 300 kDa with an N-terminal signal peptide, 36 epidermal growth factor repeats and conserved Lin/Notch repeats forming the extracellular, ligand binding domain (Fig. 2-1). The intracellular portion of Notch1 contains Ankyrin-like motifs as well as a polyglutamine (Opa) region and a PEST protein stability sequence. The Floxed Notch1 allele was generated by introducing LoxP sequences upstream and downstream of the first translated exon encoding the signal peptide (Fig. 2-1). Recombination between the
LoxP sites ablates the first coding exon of the Notch1 gene and results in a null allele (Radtke et al., 1999) (Fig. 2-1). Mice homozygous for the Floxed Notch1 allele show no abnormal phenotype and were used to analyze the function of Notch1 by temporal and spatial gene ablation.

Figure 2-1:
Schematic representation of Notch1 and the transgenic constructs. Notch1 is a type-I transmembrane protein with a cleavable signal peptide followed by 36 EGF-like repeats and three Lin/Notch domains (LN). The intracellular domain contains Ankyrin-like repeats and a polyglutamine stretch (Opa) and a protein stability PEST sequence. The first coding exon of the Notch1 gene was flanked by LoxP sequences (triangles) to generate the Floxed Notch1 allele (1) and the null allele for Notch1 generated after Cre-induced recombination (2). EcoRI (Rl) restriction sites flanking the Floxed coding exon are shown. The Cre-recombinase was driven from the engrailed-2 promoter enhancer (En2-Cre) which restricted expression to the neuroepithelium of the midbrain hindbrain boundary (Zinyk et al., 1998). The ROSA26-R Cre-reporter allele (R26R) was used in combination with the En2-Cre transgene and Floxed Notch1 alleles for cell fate tracing. The R26R comprises a Floxed PGK promoter driving a neomycin cassette containing 4 polyadenylation sequences (4XpA) and a b-galactosidase coding region (lacZ) with polyadenylation site (bpA) introduced into the ROSA26 locus by homologous recombination (Soriano, 1999). Cre-mediated recombination deletes the PGK neo cassette and results in constitutive expression of b-galactosidase.

The ablation of Notch1 from the neuroepithelial cells of the developing CNS was restricted by combining homozygous Floxed Notch1 alleles and a Cre-recombinase transgene expressed under the control of the engrailed-2 promoter enhancer (En2-Cre) (Fig. 2-1). This approach circumvents the early embryonic lethality observed in Notch1-deficient animals (Conlon et al., 1995; de la Pompa et al., 1997; Swiatek et al., 1994). In addition, we followed the fate of Cre-recombinase expressing cells and their progeny by
Notch1 Function in CNS Development

including the ROSA26 Cre-reporter allele \((R26R)\) in the analysis (Soriano, 1999). The \(R26R\) allele consists of a Floxed PGK neo cassette containing 4 polyadenylation sites and a \(\beta\)-galactosidase gene downstream of the distal LoxP site (Fig. 2-1). In the absence of Cre-recombinase the expression of \(\beta\)-galactosidase mRNA is blocked by the PGK neo polyadenylation sites. In the presence of Cre-recombinase the LoxP sequences flanking the PGK neo cassette are recombined and \(\beta\)-galactosidase is expressed (Soriano, 1999).

2.3.1 **CRE-MEDIATED RECOMBINATION IS RESTRICTED TO THE NEUROEPITHELIAL CELLS OF THE MIDBRAIN HINDBRAIN REGION**

We have used the \(En2-Cre\) transgene to drive expression of Cre-recombinase in the embryonic nervous system. Previous experiments revealed that the Tg22 line of \(En2-Cre\) animals shows recombinase activity within the midbrain hindbrain region of the neural tube, starting at E9 (Zinyk et al., 1998). We have confirmed this restricted expression by combining the \(En2-Cre\) and \(R26R\) alleles. At E10, \(\beta\)-galactosidase expressing cells can be detected by whole-mount preparations in the midbrain hindbrain neuroepithelium and progenitor cells within the medial aspect of the cerebellar primordium of control animals (Fig. 2-2A).

Analysis of triple transgenic mutant animals carrying homozygous Floxed \(Notch1\) alleles, the \(En2-Cre\) transgene and the \(R26R\) reporter showed a similar, restricted appearance of \(\beta\)-galactosidase expressing cells in the midbrain hindbrain region at E10.

Fig. 2-2 on page 27

Determination of the \(En2-Cre\) expression domain using the \(R26R\) reporter and restricted ablation of Notch1 signaling. (A). Heterozygous Floxed Notch1 \((\Delta/\Delta\Delta)\) animals carrying an \(En2-Cre\) and \(R26R\) allele showed a restricted distribution of Cre-expressing cells (blue staining) and their progeny in the midbrain hindbrain region of the developing brain at E10. The plane of the sections used in the subsequent analysis is depicted in red. (B). Homozygous Floxed \(Notch1\) \((\Delta/\Delta\Delta)\) littermates carrying an \(En2-Cre\) and \(R26R\) allele showed a similar distribution of \(\beta\)-galactosidase (blue) expressing cells in the midbrain hindbrain region of the brain to control embryos at E10. (C). In situ RNA hybridization on cross-sections through the neural tube, dorsal to the left and ventral to the right, of E10 mutant animals \((\Delta/\Delta\Delta)\) showed normal expression of Notch1 mRNA in the ventral region of the hindbrain neural tube but loss of expression in the medial cerebellar primordium (arrow). (D). Control Floxed \(Notch1\) \((lox/lox)\) animals without an \(En2-Cre\) transgene showed normal distribution of Notch1 transcripts throughout the dorsal and ventral region of the hindbrain neural tube including the cerebellar primordium (arrow). (E). The down-regulation of Notch1 expression in the medial portion of the cerebellar primordium of the mutants coincides with the region of recombination as indicated by expression of \(\beta\)-galactosidase from the \(R26R\) allele on adjacent sections (F). (G). Hes5 expression was strongly reduced in the medial cerebellar primordium of mutant embryos \((\Delta/\Delta\Delta)\) (arrow). By contrast, control embryos \((lox/lox)\) showed homogeneous expression of Hes5 in the cerebellar primordium (H). Scale bars in C for C, D, G, H and E for E, F are 100 µm.
Figure 2-2:
Notch Function in CNS Development

Notchi Function in CNS Development

(Fig. 2-2B). Anatomical changes in midbrain hindbrain boundary formation were not observed in the mutant embryos and expression of the caudal midbrain rostral hindbrain domain marker Engrailed-1 in the mutants was indistinguishable from that in control embryos (Fig. 2-2A,B and data not shown). To confirm the ablation of the Notch1 gene we analyzed the expression of Notch1 mRNA on cryosections of mutant and control embryos at E10. Sections of embryos were prepared perpendicular to the axis of the neural tube at the level of the midbrain hindbrain region and cerebellar primordium (plane of section is indicated on the whole-mount preparation shown in Fig. 2-2A). Sections hybridized with the Notch1 probe revealed a strong reduction in Notch1 expression within the cerebellar primordium in the dorsal aspect of the neural tube of mutant compared to control animals (arrows in Fig. 2-2C,D). The reduced expression of Notch1 in the mutants was restricted to the medial portion of the cerebellar neuroepithelium and overlapped with the expression of β-galactosidase in the triple transgenic mutants (Fig. 2-2E,F). Based on these data, we consider β-galactosidase expressing cells in homozygous Floxed Notch1, En2-Cre, R26R animals as being Notch1-ablated. However, different recombination events even in the same cell may differ in their efficiency (Vooijs et al., 2001).

Notch signaling in invertebrates regulates the expression of a cascade of bHLH transcription factors. The first genes of this cascade in mammals are the E(Spl)-related Hes genes. Activation of Notch signaling induces the expression of Hes genes and particularly Hes1 and Hes5 in the nervous system. We have analyzed the expression of Hes1, Hes3 and Hes5 in the cerebellar primordium by in situ RNA hybridization following ablation of Notch1. Whereas no marked changes in Hes1 and Hes3 expression were observed in the mutant compared to control embryos at E10 (data not shown), expression of Hes5 was drastically reduced in the cerebellar primordium compared to control animals (arrows in Fig. 2-2G,H). These data are consistent with Notch1 gene ablation in the cerebellar primordium resulting in down-regulation of the Notch1 signal and the loss of Hes5 gene expression.
2.3.2 Ablation of Notch1 induces promiscuous Delta-like ligand expression in the cerebellum

Based on data from other experimental systems, Notch and its ligands are thought to be reciprocally regulated within the same cell (Kimble and Simpson, 1997). An increased Notch signal results in down-regulation of ligand expressed by the receiving cell. By analogy, reduced Notch activity induces ligand expression by the signaling cell. Therefore, we examined the fate of the Notch1-ablated cells within the neuroepithelium of the cerebellar primordium by *in situ* RNA hybridization with probes for the Notch1 ligands Delta-like 1 and 3 (Dll1 and Dll3) and Jagged 1 and 2 on sections of E10 embryos. Mutant embryos showed an upregulation of Dll1 within the neuroepithelium of the medial portion of the cerebellar primordium (arrow in Fig. 2-3A,B). In addition, cells expressing high levels of Dll3 were observed in the dorsal aspect of the medial cerebellum of E10 mutants (arrows in Fig. 2-3C). However, in contrast to the cells with increased Dll1 expression that resided within the ventricular zone of the cerebellar primordium, the cells precociously expressing Dll3 were found towards the basal aspect of the neuroepithelium in a position analogous to differentiating cells (Fig. 2-3C,D) (Dunwoodie et al., 1997). Ectopic expression, upregulation or abnormal distribution of the ligands Jagged 1 and Jagged 2 were not observed at E10 (data not shown). Taken together, the increased expression of Notch ligands observed in the mutants supports the spatially-restricted reduction of Notch signaling in the neuroepithelium of the cerebellar primordium.

2.3.3 Ablation of Notch1 induces early onset of neurogenesis in the cerebellum

To determine the identity of the cells that had initiated precocious differentiation within the cerebellum, we analyzed the expression of the proneural genes Mash-1 and Math-1.
Figure 2-3:
Mash-1 is a mouse orthologue of the Drosophila Achaete-Scute genes and has been shown to be expressed in the cerebellar primordium commencing around E12 (Guillemot and Joyner, 1993). This Mash-1 expression is likely to be associated with putative Purkinje cell precursors. At E10, we observed precocious expression of Mash-1 mRNA in cells within the dorsal aspect of the medial cerebellum of mutant embryos in positions similar to those of the Dll1 and Dll3 expressing cells (Fig. 2-3E,F). These cells likely represent Notch1-ablated neuroepithelial cells that have prematurely differentiated into neuronal precursors. In addition, we also observed an increased expression of Math-1 in E10 mutants (Fig. 2-3G,H). Math-1 in the cerebellum is the earliest known marker for granule cell precursor cells and is required for their determination (Ben-Arie et al., 1997). It remains open whether the observed expression represents an expansion of the Math-1 expression domain within the neuroepithelium or whether the granule cell precursor cells have started rostral migration over the cerebellar primordium prematurely. Whole mount in situ RNA hybridization using Mash-1 (data not shown) and Math-1 (Fig. 2-3I,J) confirmed the medial and rostral appearance of differentiating cells in the mutant compared to control animals. These data are in agreement with the precocious expression of Mash-1 observed in Notch1-deficient mice and validate our conditional gene ablation approach (de la Pompa et al., 1997).

2.3.4 NOTCH1-DEFICIENT CELLS LEAVE THE PROGENITOR CELL POOL BUT FAIL TO GENERATE DIFFERENTIATED NEURONS

To determine the fate of the cells that initiate premature differentiation, we analyzed the expression of neural differentiation markers. Consecutive sections stained for Mash-1 mRNA (Fig. 2-3E,) and nestin (Fig. 2-4A,B), an intermediate filament protein expressed by multipotent neuroepithelial cells, revealed that the differentiating cells had down-regulated nestin (arrow in Fig. 2-4A) and left the neuroepithelial cell pool at E10. Immunofluorescence with antibodies against pan-neuronal markers such as β-TubulinIII (TuJ1) (Fig. 2-4C,D) and neurofilament 160 kDa (Fig. 2-4E,F) identified differentiating cells outside the cerebellar primordium of both mutant and control animals, but did not reveal aberrant neurons within the mutant animals. We then analyzed the expression of Calbindin D 28k, which is upregulated in postmitotic Purkinje cells between E14 and E16 and serves as a cell-type marker in the cerebellum. Calbindin D 28k expression
Figure 2-4:
Differentiation state of cells within the cerebellar primordium. Immunofluorescent analysis of cross-sections through the neural tube at E10, dorsal to the left and ventral to the right, shows a reduction in the expression of the intermediate filament protein nestin in the medial aspect of the mutant (Δ/Δ) cerebellar primordium (arrow in A) compared to control (lox/lox) littermates (B). Examination of neuronal differentiation genes such as β-TubulinIII (TuJ1) (C,D), and neurofilament 160 (E,F) failed to reveal ectopic formation of differentiated neurons in the cerebellar primordium between E10 mutant and control animals. Calbindin D 28k expression could not be detected in E10 mutant animals at the protein (G) or mRNA (H) levels, indicating the absence of precociously differentiated postmitotic Purkinje cells. Scale bars in A for A,B and in C for C-H are 100 μm.

could not be detected at the protein (Fig. 2-4G) or mRNA (Fig. 2-4H) levels within the dorsal aspect of the cerebellar primordia of mutant or control embryos (data not shown) at E10. These data suggest that the Notch1-deficient cells had initiated differentiation by E10 but had not progressed to immature neurons or Calbindin D 28k expressing Purkinje
cells. Due to the previously demonstrated role of Notch proteins in the regulation of cell fate in invertebrates, we also analyzed the expression of other markers such as glial fibrillary acidic protein (GFAP), A2B5, and RC2, which is expressed by radial glial cells during late embryogenesis. We did not find increased expression of these markers in the mutant compared to control animals, suggesting that the neuroepithelial cells had not undergone an apparent neuronal to glial lineage fate switch (data not shown).

At E12.5, increased expression of DI13 was no longer evident (Fig. 2-5A,B). Mash-1 expression within the medial cerebellum was less pronounced since normal Mash-1 expression had started in the lateral domains of the cerebellar primordium (Fig. 2-5E,F). The altered expression of DI13 seen at E10 was still detectable at E12.5 although less prominent than at earlier ages (arrow in Fig. 2-5C,D). Analysis of Calbindin D 28k (Fig. 2-5G,H) and β-TubulinIII (TuJ1) (Fig. 2-5I,J) expression at E12.5 did not reveal increased or promiscuous neuronal or Purkinje cell differentiation. Furthermore, analysis of glial markers also did not identify aberrant glial differentiation at E12.5 (data not shown). Hence, although the Notch1-ablated cells had initiated differentiation within the cerebellum at E10 they had not progressed to express markers of differentiated neural cells by E12.5.

### 2.3.5 Notch1 ablation from the neuroepithelium results in increased cell death

To elucidate the fate of Notch1-ablated cells, we examined cell proliferation and cell death. BrdU was injected into time-mated females and the embryos excised 3 hours later. The expression of Mash-1 mRNA and the incorporation of BrdU into proliferating cells was analyzed on consecutive sections covering the entire cerebellar primordium of mutant and control embryos at E10 and E12.5. Although the Mash-1 expressing cells had

---

**Fig. 2-5 on page 34**

Expression patterns of Notch signaling cascade components and neuronal markers Calbindin D 28k and β-TubulinIII (TuJ1) at E12.5. In situ RNA hybridization on cross-sections through the neural tube, dorsal to the left and ventral to the right, of E12.5 mutant (Δ/Δ) (A,C, and E) and control embryos (lox/lox) (B,D and F) show that the extended expression of DI11 (A,B) observed in E10 mutants compared to controls was no longer seen at E12.5 and the induction of DI13 (arrow in C,D) and Mash-1 (E,F) was detectable but less pronounced than at E10. In situ RNA hybridization did not reveal precocious expression of Calbindin D 28k (G,H) in the mutant compared to control animals. Expression of the pan-neuronal marker β-TubulinIII (TuJ1) (I,J) revealed differentiated cells in the lateral aspects of the cerebellum (arrows in I,J) but no precocious neurogenesis in the mutants. Scale bar in A for A-J is 100 μm.
initiated a premature differentiation program, there were no obvious differences in BrdU incorporation within the medial cerebellar primordium at E10 or E12.5 (data not shown).
TUNEL analysis for apoptotic cells within the neuroepithelium failed to reveal significant differences between mutant and control embryos at E10 (Fig. 2-6A,B). This supports the equivalent distribution and numbers of β-galactosidase expressing cells observed in E10 mutant and control embryos (Fig. 2-2,B). However, when TUNEL staining was performed on sections of E12.5 embryos, a strong increase in the number of apoptotic cells was observed within the mutant cerebellum compared to control embryos.
(arrow in Fig. 2-6C,D). The TUNEL-positive cells were mainly restricted to the medial, presumptive ectopically differentiating cells, with few TUNEL-positive cells within the lateral domains of the cerebellum in all mutants examined. Double immunofluorescence with nestin antibodies and TUNEL staining showed that the apoptotic cells in the medial cerebellar primordium of the mutant lie outside the neuroepithelium in the differentiation zone and express low levels of nestin (Fig. 2-6C,D). Additional TUNEL analysis of embryos between E10 and E12.5 indicated that increased apoptosis starts in the mutants around E11 and peaks at E12 with approximately six-fold more TUNEL-positive cells per section in the mutants (43 TUNEL-positive cells/section; an average from 3 embryos) than in controls (7 TUNEL-positive cells/section; an average from 3 embryos). Furthermore, in contrast to control animals where apoptosis was similar in medial and lateral regions of the cerebellar primordium (7 and 10 TUNEL-positive cells/section, respectively), the increased apoptosis seen in the mutants was restricted to the medial domain of the cerebellum (43 versus 4 TUNEL-positive cells/section, medial and lateral respectively).

### 2.3.6 NOTCH1 ABLOCATION FROM THE NEUROEPITHELIALM RESULTS IN CELL LOSS

The increased cell death observed in the mutant embryos at E12.5 suggests that Notch1 ablated cells are eliminated before undergoing terminal differentiation. Therefore, we analyzed the distribution of β-galactosidase expressing cells in E12.5 embryos. In contrast to E10 where mutant and control embryos were indistinguishable (Fig. 2-2A,B), at E12.5, mutant embryos displayed fewer β-galactosidase expressing cells in the midbrain hindbrain region (Fig. 2-6E,F). These data support the interpretation that the

**Fig. 2-6 on page 35**

Cell survival and fate analysis in the mutant cerebellum. TUNEL analysis (green) for dying cells and nestin (red) for neuroepithelial progenitor cells in the cerebellum failed to show a difference between mutant and control animals (A,B) at E10. However, at E12.5, mutant animals contained a greatly increased number of TUNEL-positive cells (green; arrow in C) within the medial aspect of the cerebellar primordium compared to control animals (D). These TUNEL-positive cells were in a position analogous to differentiating neuroblasts and had down-regulated expression of nestin (C). The lineage tracing Cre-reporter transgene R26R revealed an extensive number of β-galactosidase expressing cells in the midbrain hindbrain region of control animals carrying heterozygous Floxed Notch1, En2-Cre, and R26R transgenes (A/wt) (E). A substantial reduction in the number of β-galactosidase expressing cells was observed in the E12.5 mutant embryos (genotype: homozygous Floxed Notch1, En2-Cre, and R26R (Δ/Δ)) (F), compared to control embryos (E). Scale bar in A for A,B is 100 µm and in C for C,D is 50 µm.
ablation of Notch1 from neuroepithelial cells induces premature initiation of a differentiation program and subsequent cell death. Due to the apparent loss of Notch1-deficient cells in the cerebellum of mutant embryos at E12.5, we addressed the consequences of the Notch1 ablation in E15 embryos. Immature Purkinje cells start to express Calbindin D 28k between E14 and E16 (Hatten et al., 1997). Normal differentiation progresses in a lateral to medial fashion (Altman and Bayer, 1985a). In E15 Notch1 mutant embryos, the expression of Calbindin D 28k reflected that of control embryos with a pronounced lateral to medial distribution and no obvious increase in the medial aspects of the mutant cerebellum (Fig. 2-7 A,B). Analysis of E12.5 embryos had revealed a highly reduced population of β-galactosidase expressing cells in the region of the midbrain hindbrain boundary of mutant compared to control embryos (Fig. 2-6 E,F). Therefore, we addressed whether the remaining β-galactosidase positive cells at E12.5 are insensitive to the loss of Notch1 by analyzing E15 embryos (Fig. 2-7 C,D). Control embryos (heterozygous Floxed Notch1, En2-Cre, R26R) showed extensive expression of β-galactosidase by most of the cells throughout the medial cerebellum and isthmic region of the brain (Fig. 2-7 D). By contrast, β-galactosidase expression in the mutants was restricted to a few cells within the isthmus and posterior midbrain with no β-galactosidase expressing cells detectable in the developing cerebellum (Fig. 2-7 C). This suggests that the residual Notch1-ablated cells found in the cerebellum at E12.5 had been eliminated by E15. TUNEL analysis of the cerebellar primordia of E15 embryos showed no difference between mutant animals (16 TUNEL-positive cells/section; an average from 20 sections of 3 embryos) and control animals (14 TUNEL-positive cells/section; an average from 15 sections of 2 embryos) (arrows in Fig. 2-7 E,F). Thus Notch1-deficient cells within the cerebellar primordium are eliminated by apoptosis before E15.

2.3.7 Purkinje cell number is reduced in the early postnatal cerebellum

Due to the apparent loss of Notch1-ablated cells in the embryonic brain, we analyzed cerebellar formation in early postnatal animals. At P4, the cerebellum is undergoing active lobulation in the mouse. The Purkinje cells are arranged into a dense cell layer that becomes extended in the first three postnatal weeks as the number of granule cells in the
Figure 2-7:
Differentiation and fate of Notch1-ablated cells in the cerebellar primordium at E15. In situ RNA hybridization for Calbindin D 28k expression on sagittal sections of E15 mutant (homozygous Floxed Notch1 En2-Cre; Δ/Δ) (A) and control embryos (heterozygous Floxed Notch1 En2-Cre; Δ/wt) (B) show no differences in the expression of the Purkinje cell marker. Cell fate analysis of mutant (Δ/Δ) and control animals (Δ/wt) at E15 using the lineage tracer Cre-reporter transgene R26R revealed an absence of b-galactosidase expressing cells from the cerebellum (CB) of mutant (C) compared to control embryos where most of the cerebellar cells had undergone R26R recombination (D). Some b-galactosidase expressing cells were detected in the isthmus (IST) and posterior midbrain (pMB) region of the E15 mutant brain (C). However, the control embryos (Δ/wt) also showed substantially more b-galactosidase expressing cells in these brain regions (D). TUNEL analysis (green and arrows in E,F) for dying cells and nestin (red) for neuroepithelial progenitor cells on cross-sections of the hemi-cerebellum (midline to the right and dorsal to the top) at E15 showed no significant difference between mutant (Δ/Δ) and control animals (Δ/wt) (E,F). Scale bars in A for A-D and E for E,F are 100 μm.

internal granule cell layer increases. Hematoxylin analysis of brains of P4 mutant animals revealed a marked reduction in the size of the cerebellum (data not shown). Therefore, we performed in situ RNA hybridization with a Calbindin D 28k probe to
Notch1 Function in CNS Development

identify Purkinje cells and to reveal the extent of cerebellar lobulation. The cerebellum of P4 mutant animals showed a severe reduction in the extent of lobulation compared to control littermates (Fig. 2-8A,B). As in the control animals the Purkinje cells of the mutants were arranged into a dense multicellular layer, however, the total length of the layer was dramatically reduced. Although the Purkinje cell layer is approximately 50% shorter in the mutants, the thickness and hence the density of the cells appeared not to be affected compared to control littermates (Fig. 2-8A,B). This finding reflects the increased apoptosis observed in the cerebellum of the mutants during embryonic development that is likely due, at least in part, to a loss of Purkinje cells.

2.3.8 **CONDITIONAL ABLATION OF NOTCH1 RESULTS IN A REDUCTION IN NEURONS IN THE ADULT CEREBELLM**

Mutant animals carrying homozygous Floxed Notch1 alleles and the En2-Cre transgene survived to adulthood and showed no obvious abnormalities compared to control littermates even beyond one year of age. Macroscopic examination of adult mutant brains revealed a severe reduction in size and foliation of the cerebellar vermis compared to littermates devoid of the En2-Cre transgene or heterozygous for the Floxed Notch1 allele (Fig. 2-8C,D) consistent with the observations made at P4. Histological examination of sagittal brain sections confirmed that the cerebellar lobes of the mutant

---

**Fig. 2-8 on page 40 and 41**

Morphological analysis of the postnatal cerebellum and determination of cell loss. Examination of the cerebellum of P4 animals by in situ RNA hybridization with a Calbindin D 28k probe (A,B) showed a significant reduction in the length of the Purkinje cell layer in mutant (A) compared to control (B) animals (reduced by 50%) without obvious changes in cell density. Morphological analysis of the cerebellum from adult homozygous Floxed Notch1 En2-Cre mutant (Δ/Δ) (C,E) and control, homozygous Floxed Notch1, littermates (lox/lox) (D,F) revealed a marked reduction in the size and foliation of the medial cerebellum (arrow in C). Histological examination of midline sagittal sections showed aberrant lobulation of the adult mutant cerebellum (E) with a loss of lobe VII compared to control (F) littermates. In situ RNA hybridization with a Calbindin D 28k probe (G,H) and quantification of lobe length (I), revealed a significant (**; p< 0.05 Student’s t-test) reduction in the length of lobe 2/3 and a highly significant (***; p< 0.01 Student’s t-test) reduction in the other lobes of the mutant (black bars) with the exception of lobe 10. Due to the loss of lobe 7, lobe 6 and 7 were measured together, from the posterior end of lobe 5 to the anterior end of lobe 8. Analysis of Purkinje cell number and density (J and data not shown) in adult animals showed a highly significant (**) reduction in cell number within the anterior (A, lobes 2-5) and central (C, lobes 6 to 8) lobes of the mutant (black bars) as well as over the total cerebellum, but no significant difference in the posterior lobes (P, lobes 9 and 10). Lineage tracing using the Cre-reporter transgene R26R confirmed that few Notch1-deficient, b-galactosidase expressing Purkinje cells were apparent in the medial cerebellum of adult mutants (genotype: homozygous Floxed Notch1 En2-Cre and R26R (Δ/Δ)) (arrow in K), compared to control animals carrying heterozygous Floxed Notch1 En2-Cre and R26R transgenes (Δ/wt)(L). GL- granule cell layer, ML- molecular layer, PL- Purkinje cell layer, C- inferior colliculus. Scale bar in A for A,B and in E for E-H and in K for K,L are 1 mm.
Figure 2-8:
animals were severely reduced in length and degree of foliation (Fig. 2-8E). The major abnormalities were apparent in the anterior (II-V) and central (VI-VIII) lobes. Cerebellar lobes IV, V and VI were reduced in length (approximately 40%) and lobe VI showed no secondary foliation at the distal extremity (compare Fig. 2-8E,F). Lobe VII was either absent or fused to the posterior aspect of lobe VI. Lobes VIII and IX were moderately reduced in length but were less affected than anterior structures (Fig. 2-8G,I). However,

measuring the thickness of the molecular and internal granule cell layer failed to reveal significant differences between mutant and control animals. Hence, the overall structure and organization of the cell layers within these lobes were not affected (Fig. 2-8E).

The phenotypes ranged from animals with reduced lobe length and number to some rare cases where the vermal region of the cerebellum was virtually absent (data not shown). This spatially restricted phenotype mirrors the En2-Cre activation pattern, which has been shown to be expressed from E9 onwards in a population of progenitors that give rise to the vermis of the cerebellum (Zinyk et al., 1998). Lineage tracing experiments with the En2-Cre transgenic animals (Tg22) and a Cre-reporter allele revealed recombination activity in all cells of the medial cerebellar vermis, with little activity detected in the hemispheres (Zinyk et al., 1998). This expression pattern does not reflect
the entire expression domain of the endogenous *engrailed*-2 and might be due to integration effects on the transgene or a lack of specific elements in the transgene promoter. However, it enabled us to focus on a subpopulation of cells within the developing cerebellum.

### 2.3.9 **NOTCH1 ABLATION LEADS TO A REDUCED NUMBER OF PURKINJE CELLS IN THE ADULT CEREBELLUM**

Purkinje cells are important regulators of cerebellar lobulation and cell number. Thus, we determined the number and density of Purkinje cells in the vermis of mutant (Fig. 2-8G) and control (Fig. 2-8H) mice using *in situ* RNA hybridization with a Calbindin D 28k probe. We counted Calbindin D 28k-positive cell bodies per lobe and length of the Purkinje cell layer on ten consecutive 20μm midline sagittal sections. Purkinje cell density in mutants (22.94 +/-0.94 cells/mm) was slightly, but significantly (p<0.05; Student’s t-test) lower than in control animals (25.08 +/-0.75 cells/mm). Furthermore, the length of the individual lobes (Fig. 2-8I) and the number of Purkinje cells per lobe (Fig. 2-8J) were strongly reduced in the mutants explaining the reduced size and lobulation of the cerebellum (Fig. 2-8E). The granule cell layer of the mutants showed normal thickness and density of cells compared to control animals (Fig. 2-8E,F). Due to the overall smaller size of the medial cerebellum, this suggests that the number of granule cells is also reduced. However, this reduction may be secondary to the loss of Purkinje cells, which regulate granule cell precursor proliferation by secretion of the mitogenic factor sonic hedgehog (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999).

### 2.3.10 **NOTCH1-ABLATED CELLS ARE ABSENT FROM THE ADULT CEREBELLUM OF MUTANT MICE**

The increased cell death observed in the mutant cerebellar primordium up to E12.5, and the reduced size of the medial cerebellum at postnatal stages support the hypothesis that cells from which Notch1 expression had been ablated were eliminated by apoptosis. This interpretation was corroborated by the obvious reduction in the number of β-galactosidase expressing cells in the hindbrain region of mutant E12.5 and E15 embryos carrying the *R26R* allele (Fig. 2-6E,F and Fig. 2-7c,d). In order to address whether
Notch1-deficient cells were present in the adult cerebellum, we analyzed the expression of β-galactosidase in the cerebellum of the mutants carrying the R26R allele compared to control animals. As predicted, Purkinje cells within the medial cerebellum of control animals carrying the En2-Cre and R26R transgenes expressed β-galactosidase (Fig. 2-8 L). By contrast, only occasional β-galactosidase expressing cells were detected in the cerebellum of adult homozygous Floxed Notch1 animals carrying the En2-Cre and R26R transgenes (Fig. 2-8K). These cells may represent either Notch1-ablated cells that escaped apoptosis during embryonic development, or incomplete Cre-recombination of the two Floxed Notch1 alleles. Interestingly, the prevalence of β-galactosidase positive, putative granule cells was also reduced in the cerebellum of mutants compared to control animals (Fig. 2-8K,L). Thus, the dying cells observed in the mutant embryonic primordium may not only be immature Purkinje cells but also putative granule cell precursors.

2.3.11 NOTCH1-ABLATION RESULTS IN A LOSS OF CEREBELLAR GLIAL CELLS

Notch signaling in the developing CNS may also regulate neuronal versus glial cell fate choices of putative stem cells (Furukawa et al., 2000; Gaiano et al., 2000). Therefore, we addressed whether ablation of Notch1 from the cerebellar neuroepithelium results in changes in glia formation. Initially, we analyzed GFAP expressing cells in the cerebellum of adult mutant and heterozygous animals. Anti-GFAP antibodies labeled the radial processes of Bergmann glia in the molecular layer as well as astroglial cells in the internal granule cell layer (Fig. 2-9A,B). No obvious differences were observed in GFAP expression or glial cell distribution in the cerebellum of mutant compared to control animals. Therefore, we assessed whether the En2-Cre transgene is expressed in cells that generate the glia of the cerebellum. We immunostained midline sagittal cerebellar sections from adult control animals, carrying En2-Cre and R26R alleles and one wild-type Notch1 gene, with antibodies against GFAP and β-galactosidase. Anti-GFAP antibodies labeled Bergmann glia in the cerebellum with their processes in the molecular layer and cell bodies intermingled with Purkinje cells within the Purkinje cell layer (arrowhead in Fig. 2-9C). Immunostaining for the β-galactosidase enzyme showed a distinct, punctate pattern within Purkinje cells (asterisk in Fig. 2-9C) similar to the
distribution of the Xgal precipitate seen in the cerebellum by detection of β-galactosidase activity. A large proportion of the GFAP-positive Bergmann glia also showed β-galactosidase immunoreactivity when analyzed by confocal microscopy (arrowhead in Fig. 2-9C). We quantified the percentage of GFAP β-galactosidase double-positive cells on midline sagittal sections from adult triple mutant and control animals. In control animals with one wild-type *Notch1* allele 66 +/- 6% of the GFAP-positive cells had undergone recombination compared to only 7 +/- 3% of the GFAP-positive cells in
the mutants indicating a highly significant reduction in recombined GFAP-positive cells in the mutants (P<0.01; Student's t-test; Fig. 2-9D).

In order to support the quantification of the GFAP β-galactosidase double-positive cells on cerebellar sections, we isolated cells from the cerebellar vermis of early postnatal control (heterozygous Floxed Notch1, En2-Cre, R26R) and mutant (homozygous Floxed Notch1, En2-Cre, R26R) animals and assessed GFAP and β-galactosidase expression in vitro. In agreement with the in vivo data, 49 +/- 9% of the GFAP-positive cells from control animals also expressed β-galactosidase (arrowheads in Fig. 2-9E) but only 4 +/- 4% of the GFAP-positive cells expressed β-galactosidase in cultures from mutant littermates (Fig. 2-9E). These data confirm the substantial and highly significant (P<0.01; Student's t-test) reduction in the percentage of glial cells derived from putative Cre-expressing progenitors in the mutant compared to control animals (Fig. 2-9F). In addition, we quantified the proportion of all of the cells isolated from the control and mutant animals that had undergone recombination. Whereas 74 +/- 18% of cells isolated from control animals expressed β-galactosidase only 2 +/- 1% of the cells from the mutant animals had undergone recombination. These data confirm the reduction in β-galactosidase expressing cells observed on sections of the cerebellum from mutant compared to control animals.

Fig. 2-9 on page 44

Effects of Notch1 ablation on glial cell formation. Immunofluorescent analysis of GFAP expression in the vermis of adult mutant (A) and control (B) animals revealed no obvious differences in the glial cell population or density of Bergmann glia fibers (A,B), suggesting that although the size of the vermis is reduced, the proportion and distribution of glial cells is not substantially affected in the mutants. Confocal analysis of midline sagittal sections of adult control (heterozygous Floxed Notch1, En2-Cre, R26R (A/wt)) cerebellum immunostained with anti-GFAP (green) and anti-b-galactosidase (red) antibodies revealed co-expression in a population of Bergmann glial cells (arrowhead in C). The soma of a Purkinje cell expressing β-galactosidase but negative for GFAP is also indicated (asterisk in C). (D). Quantification of GFAP b-galactosidase double-positive cells on midline sagittal sections through the cerebellum of adult control (heterozygous Floxed Notch1, En2-Cre, R26R (A/wt)) and mutant (homozygous Floxed Notch1, En2-Cre, R26R (A/A)) showed a highly significant (**P<0.01; Student's t-test) reduction in recombined glial cells in the mutant (7 +/- 3%; homozygous Floxed Notch1, En2-Cre, R26R (A/A)) compared to the control animals (66 +/- 6%; heterozygous Floxed Notch1, En2-Cre, R26R (A/wt)). Isolation of glial cells from the vermis region of the cerebellum of P4 control (A/wt) and mutant (A/A) animals. The percentage of GFAP b-galactosidase double-positive cells is shown as is the total number of GFAP-positive cells in each culture (n=) and the average of all four experiments (Avr.). Note that the total number of cells in the cultures from mutants was lower than that from control animals due to the 50% reduction in size of the mutant vermis at P4. GL- granule cell layer, ML- molecular layer, W- white matter.
2.4 Discussion

Using a conditional loss-of-function approach we have demonstrated that Notch1 plays a central role in the mouse neuroepithelium to maintain cerebellar neural precursor cells in an undifferentiated state and to regulate the onset of their differentiation. Based on the expression pattern of Notch1 in the neuroepithelial cells throughout the neural tube, it is likely that it also plays a similar role in other regions of the developing mammalian CNS. Down-regulation of Notch1 appears to be sufficient to induce neurogenesis in the developing cerebellar neuroepithelium but does not allow the progression to immature neurons. Instead, the affected cells are eliminated by apoptotic cell death, which leads to a reduction in the number of cerebellar neurons later in development.

Ablation of Notch1 from cerebellar neuroepithelial cells results in increased Dll1 andDll3 expression and neural progenitor cell differentiation, comparable to the effects of loss of Notch function in the Drosophila sensory organ. Based on the patchy distribution of Dll1 and the low expression levels of other Notch ligands in the normal cerebellar primordium at E10 (Fig. 3B, data not shown), it is plausible that lateral signaling between cerebellar neuroepithelial cells may involve Dll1 activation of Notch1. Precocious Dll3 expression is also induced in the mutant cells between E10 and E12.5, similar to the normal upregulation of Dll3 by differentiating cells later in development. It remains to be clarified whether progenitor cells switch Notch ligand expression from Dll1 to Dll3 as differentiation progresses or if these ligands mark precursors of independent cell lineages in the cerebellum.

The precocious neurogenesis in conditional Notch1 mutants leads to early upregulation of the proneural genes Mash-1 and Math-1, but does not affect the spatially restricted expression pattern of these genes normally seen later in development (Ben-Arie et al., 1997; Guillemot and Joyner, 1993). Mechanistically, these findings can be interpreted to indicate that neuroepithelial cells of the cerebellum are patterned before the ablation of Notch1, or that Purkinje cell and granule cell determination factors are acting on the mutant neuroepithelial cells at E10. The identities of molecules that may regulate neuroepithelial cell fate in the cerebellum in vivo are not known. However, targeted gene ablation studies implicate Math-1 as a potential granule cell determination factor in vivo (Ben-Arie et al., 1997). Furthermore, in vitro experiments suggest that bone morphogenetic proteins (BMPs) derived from the roof plate may induce Math-1
expression and granule cell fate in the cerebellum (Alder et al., 1999). Hence, a coordinated action of Notch1 and BMP signaling may regulate cerebellar neuroepithelial cell differentiation and granule cell fate determination.

Using TUNEL and immunostaining in combination with the R26R allele, we have shown that Notch1-ablated cells undergo apoptosis before differentiating into immature neurons. This may be due to the aberrant, premature differentiation of Notch1-deficient neuroepithelial cells. Alternatively, the apoptotic cell death of putative neuroblasts may also be caused by a continuous requirement for Notch1. Indeed, recent evidence suggests that Notch signaling may be required by postmitotic cortical neurons to regulate dendrite formation (Berezovska et al., 1999b; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). However, as the Notch1-deficient cells fail to generate β-TubulinIII or neurofilament 160-positive neurons in our animals, aberrant neurite formation cannot explain the observed cell death. It appears more likely that a lack of crucial trophic support for the precociously differentiating cells leads to their elimination.

The loss of neurons in the medial aspect of the cerebellar vermis reflects the expression pattern of the En2-Cre transgene (Tg22) as determined by recombination-mediated lineage tracing (Zinyk et al., 1998). This restricted pattern of the En2-Cre transgene expression circumvents the early embryonic lethality of the Notch1-deficient mice and, by utilizing the R26R reporter allele, allowed us to trace the Notch1-ablated cells in the mutant cerebellum in the context of non-ablated cells. Although the vermis of the mutant animals was strongly reduced in size, the remaining cells were organized into layers with apparently normal cytoarchitecture. In addition, the cerebellar hemispheres formed normally. This restricted phenotype was reflected in the lack of obvious behavioral abnormalities in the mutants. The origin of the remaining, non Cre-recombined cells within the cerebellar vermis of the mutants is unknown.

It has been proposed that the developing CNS contains a population of multipotent progenitor cells that give rise to neurons during early development and then switch to a gliogenic fate later (Qian et al., 2000). During late embryonic and early postnatal development of the cerebellum, glial progenitors proliferate and differentiate into the astroglial cells of the cerebellum, including the Bergmann glia (Altman et al., 1997). We show by fate tracing experiments with the R26R reporter allele that the En2-Cre transgene is expressed by cerebellar glia or their precursors. Our results indicate that,
although the distribution and density of glial cells in the mutant cerebellum was not obviously affected, Notch1-deficient neuroepithelial cells may be unable to generate mature glial cells. The apparent reduction in Notch1-deficient glia in the cerebellum of our mutant animals reflects the dramatic under representation of Notch1-deficient cells in the cerebellum of mutant animals. Our loss-of-function data is also supported by recent gain-of-function experiments showing that increased Notch activity can suppress neurogenesis in the mouse embryonic cortex and both the rat and Zebrafish retina and promote glial formation (Furukawa et al., 2000; Gaiano et al., 2000; Scheer et al., 2001). Thus, ablation of Notch1 from the cerebellar neuroepithelium may restrict multipotent progenitors to a non-glial cell fate. Indeed, we have shown that Notch1-ablated cells in the cerebellar primordium commence aberrant neuronal differentiation and are subsequent removed by apoptosis. As a consequence, the population of gliogenic cells in the cerebellum of our mutants may also be diminished. On the other hand, loss of Notch1-deficient glial cells in the mutants may be the result of a specific block of differentiation or an indirect effect of neuronal loss. Hence, Notch1 signaling is required, directly or indirectly, for the generation of mature glial cells in the cerebellum. Overexpression of Notch signaling components in isolated neural crest cells indicates that regulation of Notch function is important for the formation of the peripheral nervous system (PNS). Notch1 activation suppresses neurogenesis and can induce gliogenesis in the PNS (Morrison et al., 2000; Wakamatsu et al., 2000) whereas overexpression of the antagonistic protein Numb results in apoptosis (Wakamatsu et al., 2000). Interestingly, the Numb-induced apoptosis in avian neural crest cells could be rescued by neurotrophins (Wakamatsu et al., 2000). By analogy, the apoptotic death of precociously differentiating Notch1-deficient cells in our mutants may also be due to a lack of trophic support in the early cerebellar primordium. In conclusion, we show that Notch1 regulates the differentiation of neuroepithelial cells in the cerebellum and that precociously differentiating Notch1-deficient cells are eliminated by apoptosis. In addition, Notch1-deficient progenitor cells fail to generate differentiated glial cells in vivo. Our findings have wide-ranging implications for Notch functions in other regions of the developing CNS and suggest that the mechanisms regulating the differentiation of putative stem cells in the adult CNS may also be under the control of Notch signaling pathways.
2.5  **OUTLOOK**

### 2.5.1 INTRINSIC REQUIREMENT FOR NOTCH1 AND SURVIVAL

We have studied the function of Notch1 in the developing mouse cerebellum using conditional gene ablation. However, our results raised a number of new questions. We have shown *in vivo* that Notch1-deficient cells undergo precocious differentiation but are eliminated by apoptosis between E11 and E15. These cells never reach an immature neuron state resulting in a loss of cerebellar Purkinje and granule cells. Why do these Notch1-deficient neural progenitors die? It will be important to test whether Notch1 also plays directly a role in the survival of these precursor cells and is intrinsically required for their progression to a mature neuron or whether it is the environment of the midembryonic cerebellum that is not permissive for neurogenesis. However, the apoptotic cell death of putative neuroblasts may also be the result of lacking crucial trophic support for the precociously differentiated cells. This may be more likely than the intrinsic requirement since over-expression of the Notch antagonising protein Numb results in apoptosis in avian neural crest cells *in vitro* which can be rescued by neurotrophins (Wakamatsu et al., 2000).

These questions could be answered with cell culture experiments. We have established in our lab a culture system to maintain neuroepithelium cells in an undifferentiated state. The medium used contains important growth factors that have been shown to support neuronal and glial differentiation and survival (Stump and Taylor, manuscript in preparation). By isolating neuroepithelial cells from the midbrain/hindbrain region of En2-Cre/FloxedNotch1/R26R embryos at E10, a timepoint before the onset of apoptosis *in vivo*, we show that Notch1-deficient cells are able to generate Purkinje (PC) and granule cells (GC) strongly suggesting that Notch1 is not intrinsically required for the generation and survival of cerebellar neurons (M. Bodmer semester work). This supports the hypothesis that the environment of the cerebellar primordium at mid-embryogenesis is likely not permissive for the survival of precociously differentiating Purkinje cells and granule cells.

These so called bulk cultures have the advantage that after the dissociation of the midbrain/hindbrain region all of the cells are accessible for manipulations with different growth factors. However, the dissociation may also be a disadvantage since Notch1 is a...
transmembrane receptor and acts via ligands on neighboring cells. Therefore, an explant culture of embryonic midbrain/hindbrain neural tube may be more appropriate to address the effects of Notch1 ablation in vitro.

These midbrain/hindbrain neural tube explants of En2-Cre/FloxedNotch1/R26R animals at E10 could be allowed to develop in vitro and subsequently analyzed with differentiation and recombination markers to study the differentiation potential of Notch1-deficient cells. With this same experimental set-up one could also address the growth factor requirements of the precociously differentiating Notch1-deficient neurons by supplementing the culture medium with trophic factors using a candidate approach. The results may indicate which families of growth factors may be responsible for cerebellar neuronal survival. Subsequent expression analysis of these factors in the developing cerebellum would strengthen such findings. In order to identify the time window where these cells are dependent upon trophic support and Notch1 function we could use the same midbrain/hindbrain explant system isolated from FloxedNotch1/R26R animals. Deletion of Notch1 could then be induced at different time points using an adenovirus carrying the Cre-recombinase as described in section 3.2. Analysis of recombination, cell death and differentiation may reveal important insights into cerebellar neuron formation.

### 2.5.2 Does Notch1 actively promote gliogenesis?

We have shown a dramatic under-representation of Notch1-deficient glia in the cerebellum of our mutant animals. However, we could not distinguish whether this was the result of a specific differentiation block or an indirect effect of neuronal loss. Hence, Notch1 might be required directly or indirectly for the generation of mature glial cells in the cerebellum. We have used the NEP culture to show that Notch1-deficient neuroepithelial cells can give rise to glial cells in vitro and is therefore not intrinsically required for the generation of glia. However, in vivo precocious differentiation of glial precursors is not observed when Notch1 is ablated from the NEP cells at E9. The likely explanation is that the multipotent cells within the NEP are exposed to a neurogenic environment early in development and, therefore, have the potential to react to neurogenic factors when the neurogenic block by Notch1 is removed. In vitro the culture
medium is permissive for neurogenesis and gliogenesis (see also general discussion and outlook 4).

2.5.3 NOTCH1 PLAYS A ROLE IN POSTMITOTIC PURKINJE CELLS

More recently, Notch1 has been proposed to have functions in the regulation of dendrite morphology (Berezovska et al., 1999b; Franklin et al., 1999; Redmond and Ghosh, 2001). A role in dendrite growth and branching is based on \textit{in vitro} experiments overexpressing gain-of-function Notch mutants (see Introduction 1.2.7). However, overexpression of Notch1 or its ligands is difficult to control and might lead to miss-interpretation of the results. Therefore, a conditional \textit{knock-out} has been generated in our lab to study the function of Notch1 in postmitotic Purkinje cells \textit{in vivo}. Animals carrying the Cre-recombinase under the transcriptional control of the Calbindin D 28k promoter show expression of Cre-recombinase in postmitotic neurons (Stump et al., submitted). Interbreeding of these Calbindin-Cre lines and floxed Notch1 animals carrying the Rosa 26 reporter transgene results in loss-of-Notch1 function in specific neuronal populations including one line where Cre-recombinase is specifically expressed in Purkinje cells. Purkinje cells of the adult cerebellum are an ideal neuron to study dendrite formation and branching due to their well defined and organized arborizations. The combination of recombination and Purkinje cell dendritic markers including Calbindin D28k and MAP-2 will enable a 3D reconstruction of the Notch1-deficient Purkinje cells to study morphology. In addition, some of the Calbindin-Cre lines express Cre-recombinase within the cerebral cortex and the hippocampus. A putative role for Notch1 in dendrite and synapse formation may manifest itself by conditional ablation of Notch1 from the hippocampus and analysis of behavioral abnormalities.
3 Notch1 Regulates Adult Neural Stem Cell Potential

3.1 Introduction

Mammalian embryonic stem cells of the blastocysts inner cell mass are totipotent. The various cell fates they make are determined as the embryo develops and cellular diversification is largely complete at or shortly after birth (Gage, 2000). However, many adult tissues such as epidermis, hair and the hematopoietic system are in a natural state of dynamic flux and show ongoing regeneration (Fuchs and Segre, 2000). These tissues contain adult stem cells that are often localized to specific niches with a complex microenvironment, which provides the appropriate determination and differentiation signals. These signals may restrict these adult stem cells to a few possible differentiation pathways in vivo although recent evidence indicates a neuronal potential of stem cells isolated from a number of adult tissues (reviewed by Clarke and Frisen, 2001; Toma et al., 2001; Vescovi et al., 2001; Watt and Hogan, 2000). In addition, adult stem cells have a self-renewal capacity but usually show a long cell-cycle time (Fuchs and Segre, 2000). When choosing a fate, stem cells often pass through a transient state of fast proliferation and then differentiate into the appropriate cell types (Fuchs and Segre, 2000).

During embryonic development the nervous system is derived from the neural tube. Multipotent neural crest stem cells detach from the most dorsal tip of the neural tube before mid-embryogenesis and migrate on different pathways to specific regions where they differentiate into axially determined tissues including the neurons and glial cells of the PNS (Le Douarin, 1986; Stemple and Anderson, 1992). The fate of a neural crest cell
is influenced by local cues in the neural tube and by molecules along the specific migration pathways (Anderson, 2001). However, the multipotent neural crest stem cells are derived from progenitors in the embryonic central nervous system (CNS). These so-called neuroepithelial stem cells are found in the germinal ventricular zone of the embryonic neural tube. The identity of the CNS stem cell is still debated (Gage, 2000) but radial glia or related cells within the neuroepithelium generate the neurons and glia of the CNS through a period of neurogenesis followed by a predominantly peri- and postnatal period of gliogenesis (Qian et al., 2000). Hence, radial glia potentially have a duel function as a scaffold for migrating neurons in early development and later as potential precursor cells which can produce cortical neurons (Alvarez-Buylla et al., 2001; Malatesta et al., 2000; Noctor et al., 2001).

The adult CNS displays a limited capacity for neuronal regeneration, however, adult neural stem cells have been found in various regions of the brain including the subventricular zone (SVZ) and subgranule cell layer of the hippocampus (reviewed by Alvarez-Buylla and Temple, 1998; Temple, 1999). In the SVZ the precise location of the neural stem cells in the ependymal (Johansson et al., 1999; Momma et al., 2000) or subependymal layer (Chiasson et al., 1999) is unclear (review by Seaberg and van Der Kooy, 2002; Temple, 1999). Adult mammalian neural stem cells were initially identified by isolating the SVZ of the lateral ventricle walls of adult mouse brain (Reynolds and Weiss, 1992). The stem cells aggregated into structures referred to as neurospheres in the presence of epidermal growth factor (EGF) (Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Vescovi et al., 1993). More recently, it has been shown that the neural stem cells residing in the adult SVZ have astrocyte-like features (Alvarez-Buylla et al., 2001; Doetsch et al., 1999). The differentiation of these multipotent cells can be influenced in vitro by exposure to exogenous growth factors. Ciliary neurotrophic factor (CNTF) drives differentiation towards a gliogenic fate, whereas Plateled derived growth factor (PDGF) and Brain derived neurotrophic factor (BDNF) lead to an increased number of neurons (Johe et al., 1996; reviewed by Kintner, 2002).

We and others have shown that Notch1 is expressed in the SVZ and subgranule cell layer of the dentate gyrus in putative neural stem cells (Stump et al. 2002; Irvin et al., 2001). Furthermore, we showed by conditional gene ablation that Notch1 regulates neural progenitor cell differentiation in the developing embryo (Lutolf et al., 2002).
examine the role of Notch1 in the maintenance of adult neural stem cells and in the regulation of their differentiation potential. We show by conditional gene ablation that Notch1 plays an important role in maintenance of the EGF-dependent adult-type neural stem cells. In addition, we show that loss of Notch1 function does not directly regulate the cell lineage differentiation of neural stem cells in vitro.

3.2 Materials and Methods

EGF-responsive cells were isolated from the adult mouse CNS and allowed to form free-floating aggregates of proliferating cells (neurospheres) in the presence of EGF (Chiasson et al., 1999). Initial adult neural stem cell enrichment was accomplished by dissociating the primary neurospheres after six days in vitro to a single cell suspension and culturing to form secondary neurospheres. These enriched neural stem cells were then dissociated to single cells (controlled by microscopy and cell counting) and infected at a multiplicity of infection of 1:50 with an Adenovirus carrying the Cre-recombinase under the control of the CMV promoter (kind gift from Dr. M. Giovannini). The ability of Notch1-deficient and control (heterozygous Floxed Notch1 R26R or wild-type Notch1 R26R) neural stem cells to form neurospheres 6 days post-infection was assessed. The infected spheres were fixed in 2% paraformaldehyde, 0.2% glutaraldehyde 30 mins in neurosphere medium. The fixed neurospheres were subsequently stained with X-Gal (Lutolf et al., 2002) to identify spheres where Cre-recombinase had been active. The proportion of X-Gal-positive spheres was compared to the total number of spheres. In addition, the differentiation capacity of mutant and control spheres was assessed by plating the infected neurospheres 6 days after infection onto poly-L-lysine/Laminin coated culture dishes. Recombination was assessed with anti-β-galactosidase antibodies and differentiation with antibodies against the lineage markers; β-TubulinIII (Sigma) and neurofilament 160 (Sigma) for neurons, and glial fibrillary acidic protein (Sigma) to identify astroglia and anti-Nestin antibodies (Rat-401, DSHB) to identify undifferentiated cells. The self-renewal and hence stem cell potential of the Notch1-deficient neural stem cells was assessed by passaging the infected cells 6 days post-infection and allowing reformation of neurospheres (secondary neurospheres after infection). The spherogenic potential of Notch1-deficient and control cells infected with Adeno-Cre was assessed by X-Gal staining.
3.3 RESULTS AND DISCUSSION

3.3.1 MUTANT AND CONTROL ADULT STEM CELLS HAVE THE SAME ABILITY TO FORM PRIMARY INFECTED NEUROSPPHERES

We have inactivated the Notch1 gene in EGF-dependent neural stem cells to address the role of Notch1 signaling during neurogenesis within the adult brain. The Floxed Notch1 allele (Lutolf et al., 2002; Radtke et al., 1999) was inactivated in cultured neural stem cells in vivo by infection with a Cre-recombinase expressing Adenovirus (Adeno-Cre). The recombination efficiency was followed by including the ROSA26 Cre-reporter allele (R26R) in the analysis (Soriano, 1999). To explore the function of Notch1 in adult-type neural stem cells, we isolated the SVZ of the lateral ventricle walls of postnatal mouse brains (Seaberg and van Der Kooy, 2002). Putative neural stem cells residing within this region of the brain express Notch1 (Stump et al. 2002; Irvin et al., 2001) and can be maintained as neurospheres in vitro in an undifferentiated state (Chiasson et al., 1999). These EGF-dependent neural stem cells self-renew and continue to form neurospheres after trituration (Chiasson et al., 1999). It is this ability to be passaged and maintain multipotency in vitro that distinguishes the stem cells from other progenitors in the brain (Chiasson et al., 1999). Hence, we infected passaged primary neurosphere cells with Adeno-Cre and analyzed secondary neurosphere formation as a means of focusing on neural stem cells (see methods section 3.2). Infected neural stem cells isolated from homozygous Floxed Notch1 and heterozygous Floxed Notch1 animals as well as mice carrying wild-type Notch1 alleles were allowed to form neurospheres for 6 days. The efficiency of Cre-mediated recombination was addressed by X-Gal staining of the spheres. Only cell aggregates larger than 12μm in diameter were considered to be neurospheres. The recombination efficiency in the mutant, heterozygous and wild-type neurospheres was assessed by counting the proportion of the spheres stained with X-Gal. Spheres from all three genotypes showed a similar recombination efficiency of 93, 94 and 96%, respectively indicating that neural stem cells are able to generate neurospheres even in the absence of Notch1. The number of spheres generated from the dissociated, infected primary neurospheres was also comparable between mutant, heterozygous and wild-type (Fig. 3-1; Table 1 and data not shown). Suggesting that absence of Notch1 signaling does not prevent sphere formation.
**Figure 3-1:**
Adeno-Cre infected neural stem cells retain the potential to generate neurospheres. X-Gal staining shows neurospheres derived from recombined neural stem cells. Table showing the quantification of recombined neurospheres derived from wild-type (wt), heterozygous Floxed Notchi (Δ/Δ) and homozygous Floxed Notchi (Δ/Δ) animals six days post-infection.

### 3.3.2 Mutant Cells Show a Reduced Capacity for Self-Renewal

To examine the ability of Notch1-deficient cells to maintain stem cell-like characteristics, we examined the ability of the cells to generate neurospheres after dissociation of the infected spheres (correspond to secondary neurospheres after infection). The wild-type cells infected with Adeno-Cre maintained their ability to form neurospheres as 95% of the dissociated spheres were derived from recombined stem cells (preliminary data not included in Fig. 3-2; Table 2). However, the spherogenic potential of the Notch1-deficient cells had drastically reduced. 54% of the heterozygous Notch1-deficient cells and only 28% of the mutant spheres were derived from a recombined stem cell (Fig. 3-2; Table 2). These data reveal a highly significant reduction in stem cell potential between homozygous and heterozygous cells (T-test; p< 0.01). Hence, Notch1-deficient neural stem cells lose their self-renewal capacity upon down-regulation of Notch1.
Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Δ/Δ Rec.</th>
<th>Δ/Δ Non.</th>
<th>Δ/Δ Rec.</th>
<th>Δ/Δ Non.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75</td>
<td>36</td>
<td>362</td>
<td>1144</td>
</tr>
<tr>
<td>2</td>
<td>795</td>
<td>539</td>
<td>317</td>
<td>998</td>
</tr>
<tr>
<td>3</td>
<td>782</td>
<td>474</td>
<td>369</td>
<td>972</td>
</tr>
<tr>
<td>4</td>
<td>451</td>
<td>433</td>
<td>676</td>
<td>1098</td>
</tr>
<tr>
<td>5</td>
<td>505</td>
<td>445</td>
<td>381</td>
<td>1139</td>
</tr>
<tr>
<td>6</td>
<td>426</td>
<td>573</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>571</td>
<td>745</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>3605</td>
<td>3245</td>
<td>2105</td>
<td>5351</td>
</tr>
<tr>
<td>Total Rec. + Non.</td>
<td>6850</td>
<td>7456</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% recom.</td>
<td>54</td>
<td>28</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3-2:

Notchi deficient stem cells lose the ability to self-renew and generate neurospheres. Table showing the quantification of six day old recombined neurospheres derived from dissociated heterozygous Floxed Notchi (Δ/Δ) and homozygous Floxed Notchi (Δ/Δ) animals six days post-infection (correspond to secondary neurospheres after infection).

3.3.3 NOTCH1 DOES NOT INFLUENCE CELL FATE AND IS NOT REQUIRED FOR Gliogenesis

Notch1 has been proposed to regulate the fate choices made by neural stem cells and active Notch1 to induce CNS glial fate (Furukawa et al., 2000; Gaiano et al., 2000). To study the role of Notch1 in adult neural stem cell fate choices, we analyzed the differentiation potential of the cells within Notch1 expressing and deficient neurospheres. Six days post-Adeno-Cre infection, neurospheres were differentiated by plating on poly-L-lysine/Laminin coated culture dishes. The untriturated spheres were differentiated for 7 days, and analyzed with antibodies against, the pan-neuronal markers Neurofilament 160 and β-TubulinIII, the astroglial marker GFAP and Nestin as a marker of undifferentiated cells. Recombination was assessed with an anti-β-Galactosidase antibody. A similar proportion of recombined clones from homozygous, heterozygous and wild-type animals contained neurons, glia and undifferentiated cells (Fig. 3-3; Table 3). In addition, the proportion of neurons and glia within the clones was not different.
Table 3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Neurofilament</th>
<th>GFAP</th>
<th>Nestin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δ/Δ m&lt;sub&gt;α=4&lt;/sub&gt;</td>
<td>35</td>
<td>36</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>49%</td>
<td></td>
<td>69%</td>
</tr>
<tr>
<td>Δ/Δ m&lt;sub&gt;α=4&lt;/sub&gt;</td>
<td>13</td>
<td>33</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>28%</td>
<td></td>
<td>83%</td>
</tr>
</tbody>
</table>

Figure 3-3:

Analysis of differentiation potential of recombined neurospheres derived from heterozygous Floxed Notch1 (Δ/Δm) and homozygous Floxed Notch1 (Δ/Δm) animals. Neurospheres were plated six days post-infection onto poly-L-lysine coated plates and differentiated for seven days. Neurofilament, β-TubulinIII (red) expressing neurons within a single neurosphere clone (A). GFAP expressing astroglia (red) within a clone (B) and Nestin expressing cells (red) in a neurosphere clone (C). Table showing quantification of recombined clones containing neurons (neurofilament 160 and β-TubulinIII), astroglia (GFAP) and undifferentiated cells (nestin). In green we show the recombination event with β-Galactosidase (A,B,C). Inserts show the corresponding Hoechst staining of the cells in the field.

between the Notch1-deficient and control cells (data not shown). Hence, we show that Notch1-deficient cells can give rise to neurons and glia and that loss of Notch1 does not influence neural stem cell fate choices in vitro. In vivo we and others have shown that altering Notch1 signal in embryonic neural progenitors affects the formation of glia. Our current experiments indicate that active Notch1 is required for the maintenance of neural stem cells but their fate choices are likely controlled by other extrinsic signals. We propose that Notch1 may function as a molecular modulator of extrinsic neurogenic signals and prevent neural stem cells entering the neuronal fate without affecting gliogenesis. Hence, activated Notch1 is required by neural stem cells in order that they maintain their self-renewal capacity and their ability to respond to the correct differentiation signals. This suggests that the regulation of stem cell differentiation and the fates chosen by undifferentiated cells are connected but regulated by independent processes.
These findings have important implications for the regulation of neurogenesis in the adult brain and the role of Notch1 in this process. Further, we show that the ability to regulate neural stem cell maintenance and differentiation in the postnatal brain likely requires the modulation of Notch signaling. The identities of the differentiation factors that regulate cell fate choices in vivo remain to be determined.

3.4 OUTLOOK

3.4.1 PROLIFERATION

Using conditional gene ablation we have been able to show conclusively for the first time that Notch1 is important for the maintenance of neural stem cells. Notch1-deficient neural stem cells are unable to maintain a self-replicating, multipotent potential in vitro. However, it is unclear whether Notch1 also plays a role in regulating neural stem cell proliferation. To address this point BrdU incorporation assays would need to be performed on isolated neural stem cells. Adult neural stem cells could be pulsed with BrdU for different periods of time during neurosphere formation. Combined with the conditional ablation of Notch1 from mutant cells and the control infection of ROSA26R Notch1 wildtype cells with Adeno-Cre changes in cell cycle length due to loss of Notch1 function could be assessed.

Proliferation could also be addressed in vivo by injecting BrdU directly into the ventricle of the forebrain or by administer it in the drinking water for different periods of time. Sacrificing the animals at different time points to check for BrdU incorporation and β-Gal expression would reveal changes in recombined cell proliferation. The animals used for these experiments would be the FloxedNotch1/R26R animals infected with the retrovirus carrying Cre recombinase (see 3.4.3) or the Nestin-CreERT2/FloxedNotch1/R26R (see 3.4.2) and as controls the same animals without the FloxedNotch1 allele. We would compare heterozygous and homozygous FloxedNotch1 animals with the mentioned controls since our preliminary data (see Fig. 3-2) suggest haploinsufficiency. Beside looking at proliferation we would also perform Tunel assay to check for increased cell death in heterozygous and homozygous Notch1-deficient cells.
3.4.2 Functional conservation of Notch1 in adult and embryonic neural stem cells

The conditional Notch1 ablation experiments were all performed on adult-type neural stem cells. The results show that Notch1 ablation does not affect cell fate choices and that Notch1-deficient adult neural stem cells retain their ability to generate neurons and glial cells. The potential of adult and embryonic stem cells should be compared and the effects of Notch1 ablation in these two cell types addressed. For example: Do adult neural stem cells lose some of the potential that embryonic neural stem cells have? Adult neural stem cells appear to have a wider differentiation potential than previously thought but how do they differ from embryonic neural stem cells? To study some of these points one could repeat the condition ablation experiments with embryonic-type neural stem cells. This can be achieved by substituting EGF in the medium with bFGF and using embryonic brains as a source of stem cells.

To further analyze the differentiation potential of adult neural stem cells compared to embryonic neural stem cells in vivo we could assay their ability to contribute to the formation of various tissues by introducing them into the early embryonic environment and observing the fate of their progeny. We could isolate the adult neural stem cells as described in our experiments from our FloxedNotch1/R26R animals and from R26R animals as controls. We would take these cells into culture, induce the deletion of Notch1 with the Adeno-Cre virus described earlier and transplant the cells in the embryonic forebrain. The neural stem cells derived from R26R mice express β-galactosidase if recombination has occurred, enabling identification of their progeny by X-Gal histochemistry or with antibodies against β-galactosidase. However, this method with those tools has some disadvantages. The transplanted cells will most likely be a mixture of neural stem cells and already restricted multipotent precursor cells. And the passaging in vitro could alter their differentiation potential. Therefore, one could generate transgenic animals, where the nestin promoter, used in the CNS as a marker for undifferentiated progenitor cells (Reynolds and Weiss, 1992; Zimmerman et al., 1994), would drive expression of a Cre-ER fusion protein (Cre-ERT2) (Zimmerman et al., 1994). This protein is inactive and can be induced in vivo by the administration of an estrogen receptor ligand such as estradiol or Tamoxifen. To follow up the progeny of the Notch1-deficient nestin expressing cells the R26R-reporter animals or R26RGFP-
reporter alleles would be used. The latter would enable the isolation of a pure population of nestin-positive progenitor cells by FAC sorting. With these tools it should be possible to isolate embryonic nestin-positive and adult nestin-positive cells and analyze the gene expression pattern of those different populations with the help of the microarray technology. Plans for the future would be to also characterize the proteome in order to compare embryonic with adult neural stem cells. This knowledge could be important for future therapeutic approaches to repair brain damages.

3.4.3 EXPERIMENTS TO ADDRESS THE ROLE OF NOTCH1 IN POSTNATAL NEURAL STEM CELLS IN VIVO

Our in vitro analysis strongly suggests that Notch1 is required by EGF-dependent neural stem cells in order to maintain their fate. This hypothesis should be addressed in vivo. The more elegant way to study the role of Notch1 in adult neural stem cells in vivo would be to use the Nestin-CreERT2 mice together with our FloxedNotchl/R26R mice. However, since the Nestin-CreERT2 mice are not yet available, another possibility would be to perform retroviral infections. One could inject retrovirus carrying the Cre-recombinase into the lateral ventricles of the forebrain of adult FloxedNotchl/R26R mice. The retrovirus will infect surrounding proliferating cells. Although the retrovirus will also infect proliferating precursor cells we can still compare our FloxedNotchl/R26R mice with control mice (R26R) by performing X-Gal stainings together with stainings of different differentiation markers to account for their differentiation potential.
4 GENERAL DISCUSSION AND OUTLOOK

Notch signaling is involved in many important processes during early development (Heitzler and Simpson, 1991; Kimble and Simpson, 1997; Muskavitch, 1994). Considerable effort has been put into understanding the function of Drosophila Notch (Kimble and Simpson, 1997). Genetic analysis of gain and loss of Notch function in the fly has revealed Notch as a key component in the process of lateral signaling during organogenesis (reviewed in Simpson, 1990). Hence, as mammalian Notch homologues are highly related to Drosophila Notch, this lead to the hypothesis that members of the vertebrate Notch family may be involved in the regulation of vertebrate development. We have analyzed the function of Notch1 in vertebrate neurogenesis by combining conditional gene ablation in vivo and ex vivo with analysis of cell fate determination in vitro. We focused on the role of Notch1 in development of the cerebellum early in embryogenesis and also in adult neural stem cells. Our approach not only circumvented the early lethality of the Notch1 mutation in mice but also allowed us to make a hypothesis on the molecular role of Notch1 signaling in multipotent progenitor cells of the nervous system.

We have shown that Notch1 regulates the onset of differentiation of multipotent neural cells. Both neuroepithelial cells from the midbrain/hindbrain region of the neural tube and neural stem cells isolated from the subventricular zone of the adult mouse brain are under the control of the Notch1 signaling pathway. However, unlike Notch in invertebrates, Notch1 does not directly influence the fate chosen by mammalian neural progenitor cells. Previous reports have suggested that Notch signaling in the mammalian central nervous system promotes gliogenesis at the expense of neurogenesis (Furukawa
et al., 2000; Gaiano et al., 2000). We show that Notch1 is not required for gliogenesis by either embryonic or adult neural stem cells. As previous experiments addressing the role of Notch as an instructive signal for gliogenesis were performed by overexpression of dominant gain-of-function NotchICD it is difficult to interpret the significance of these findings with regards to the function of endogenous Notch1. Hence, if Notch signaling is required for promoting glial cell fate from multipotent progenitors either Notch1 is redundant in this processes, potentially compensated for by another member of the family, or gliogenesis in vitro is regulated by an alternative pathway. The cells could be influenced by other gliogenic factors in the environment for example by the LIF/ciliary neurotrophic factor (CNTF) signaling which activates the GFAP transcription by binding a complex of STAT1/3 to the STAT binding site.

Wnt signaling has also be found to be involved in regulating cell fate decisions by altering the transcriptional program of target cells in an instructive fashion (Brannon et al., 1997; Riese et al., 1997). Binding of the soluble ligand Wnt to the transmembrane receptor frizzled inhibits GSK-3β (glycogen-synthase-kinase) via a pathway involving the protein disheveled (Dsh). Dsh has also been reported to suppress Notch signaling when overexpressed and to directly bind to the cytoplasmic domain of Notch (Axelrod et al., 1996). GSK-3β, however, is responsible for the phosphorylation of β-catenin in the Wnt/β-catenin signaling pathway but also for the phosphorylation of the intracellular domain of Notch1 (NICD). This process alters the degree to which NICD and β-catenin are subjected to proteasome-mediated degradation.

Cell fate decisions seem to depend upon a complex interplay of many molecules influencing the Notch cascade directly on different levels of signaling like the Wnt signaling pathway but also upon molecules in the close environment like LIF/CNTF which direct the cells towards a certain fate. Hence, the function of Notch1 may be to act as a molecular “brake” on differentiation rather than directly regulating cell fate decisions. Notch1 is not instructive in the way that it promotes gliogenesis and inhibits neurogenesis but maintains progenitor cells in an undifferentiated state by controlling their response to differentiation signals. Hence, downregulation of Notch1 signaling in progenitor cells during the neurogenic period of mid to late embryogenesis leads to the adoption of neural cell fate. However, cells destined to become glia are maintained in an undifferentiated state by an active Notch1 until extrinsic signals are permissive or
instructive for the formation of glial cells. This hypothesis is supported by all in vivo and
in vitro data concerning Notch1 function in neural development. Even the NotchICD
experiments indicate that Notch maintains progenitor cells fate at the expense of
neurogenesis but does not affect the formation of glia. Hence towards the end of
embryogenesis and in the postnatal brain progenitor cells respond to the gliogenic
signals around them. The source of the Notch1 activating signal that maintains
progenitor cells in the embryonic and adult nervous system remains to be shown.
However, based on the extensive expression of the Notch1 ligands including Dll3 and
Jagged1 by neurons surround the putative neural stem cells of the embryonic and adult
brain it is likely that the neurons themselves regulate subsequent progenitor cell
differentiation. The nature and source of the gliogenic signal is unknown but neurons are
also a potential candidate as the driving force in gliogenesis.

We have shown that Notch1 is important for the maintenance of adult neural stem cells
and that Notch1 is not intrinsically required for the formation of glial cells. These
findings could have important implications for CNS repair. Glial scar formation, which
also includes gliogenesis, is a common tissue reaction after injury of the adult CNS. This
scar environment impairs the regeneration process. Hence, the ability to drive neural
stem cells in the adult brain towards neuron formation rather than gliogenesis may be
very valuable. Furthermore, recent evidence suggests that newly made neurons in the
adult brain may be able to integrate into an existing network, possibly contributing to
functional recovery of the damaged brain (van Praag et al., 2002). Finally, it would be
interesting to find out if the mitotic cells induced after brain injury, which usually form
the glial scar, reexpress Notch1. If so, these cells may reenter a progenitor cells like state
and be able to assist in replacing lost neurons if provided with the correct neurogenic
signals and a concomitant downregulation of Notch1 signaling.

Other studies have already surprised by showing how similar adult SVZ-derived cells are
to embryonic neural stem cells in their response to growth and differentiation factors in
vitro (Johe et al., 1996). If adult neural stem cells are not as restricted in their
differentiation potential as previously believed, these findings would also help regarding
the ethical aspect of stem cell replacement therapy. One could isolate, expand and
partially differentiate neural stem cells from a patients own brain with experimental
difficulties or from other own tissues like skin or bone marrow and transplant these cells back into the lesioned area avoiding the need for embryonic stem cells. 

Beside the important role Notch plays in developmental processes, Notch has gained much attention when it was suggested to be involved in stem cell biology and in Alzheimer’s disease. Most of the mutations associated with familial Alzheimer's disease (AD) have been identified in the genes encoding presenilin (PS) proteins 1 and 2 and the amyloid precursor protein (APP) (Haass et al., 1994). Disease-linked mutations in PS1/2 and APP result in an increase in the production of the 42-amino acid peptide form of amyloid-β (Aβ42) (Borchelt et al., 1996; Lemere et al., 1996; Scheuner et al., 1996), which is a major component of the amyloid plaques deposited in the brains of AD patients. The mutated presenilins, which are cofactors stimulating the catalytic activity or represent the γ-secretase activity itself (Wolfe et al., 1999), do not block the proteolytic processing of the βAPP but lead to an aberrant processing that results in a neurotoxic 42-amino acid instead of an 40-amino acid peptide. The disease leads then to a degeneration of mature neurons and a loss of memory. Recent findings have shown a direct role for PS1 in Notch proteolytic cleavage and release from the membrane (De Strooper et al., 1999; Song et al., 1999). Notch was found to be involved in the regulation of mammalian neurite development (Franklin et al., 1999) by regulating the capacity of neurons to extend and elaborate neurites. A mutation in the PS1 gene would not allow the release of the NICD and therefore inhibit the translocation of NICD to the nucleus in order to exert its function. A disruption in neurite development and subsequent loss of synapses could lead to reduced memory seen in AD patients. The relationship of Notch and Presenilins suggests that Notch signals might be important in the maintenance and plasticity of the synapses.

A lot of energy has been invested into the research of the Notch field and from the perspective of treatment it is important to get a better understanding of how this important signaling molecule functions.
4.1 REFERENCES

4 - General Discussion and Outlook


in the adult hippocampus, Nature 415, 1030-4.
# Curriculum Vitae

## Personal Data

<table>
<thead>
<tr>
<th>Name</th>
<th>Simone Lütolf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of birth</td>
<td>May 9, 1973</td>
</tr>
<tr>
<td>Nationality</td>
<td>CH</td>
</tr>
<tr>
<td>Present position</td>
<td>Ph.D. student</td>
</tr>
<tr>
<td>Present adress</td>
<td>Institut für Zellbiologie, ETH Hönggerberg, CH-8093 Zürich, Switzerland</td>
</tr>
<tr>
<td>Telephone No</td>
<td>++41 (0)1 633 33 07</td>
</tr>
<tr>
<td>Fax No</td>
<td>++41 (0)1 633 10 69</td>
</tr>
<tr>
<td>e-mail</td>
<td><a href="mailto:luetolf@cell.biol.ethz.ch">luetolf@cell.biol.ethz.ch</a></td>
</tr>
</tbody>
</table>

## Professional Data

<table>
<thead>
<tr>
<th>High school</th>
<th>Kantonsschule Hohe Promenade ZH 1985-1992</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eidg. Matura Typus B (Latin/English)</td>
</tr>
<tr>
<td>University</td>
<td>University Irvine (USA), Praktikum 1992-1993</td>
</tr>
<tr>
<td></td>
<td>ETH Zürich, Studium in Biologie 1993-1998</td>
</tr>
<tr>
<td></td>
<td>Diplom als Naturwissenschaftler mit Titel</td>
</tr>
<tr>
<td></td>
<td>&quot;Dipl. Natw.ETH&quot; 1998</td>
</tr>
<tr>
<td></td>
<td>PhD thesis, ETH Zürich, Institut für Zellbiologie 1998-2002</td>
</tr>
<tr>
<td></td>
<td>Courses in Center of Neuroscience Zurich (ZNZ), 1998-2002</td>
</tr>
<tr>
<td></td>
<td>Certificate in Neurobiology 2002</td>
</tr>
</tbody>
</table>
Publications
Gila Stump, André Durrer, Anne-Laurence Klein, Simone Lütolf, Ueli Suter and Verdon Taylor (2002). Notch1 and the ligands Delta and Serrate-related are expressed and active in distinct cell populations in the postnatal brain, in press.

Oral Presentations
PhD retreat of the Center of Neuroscience Zurich, J. Kesselring, Valens 1999
Seminars at the Institute of Cell Biology, ETH Zurich 1998-2001
Data Blitz Session at the Symposium of the Center of Neuroscience Zurich, 2001
UC Davis Molecular and Cell Biology Graduate Seminar Series, P. Armstrong, USA 2001
UC Davis Chondrocyte Biology Seminar Series, H. Benton, USA 2001

Poster Presentations
D-BIOL Symposium ETH Zurich, K. Wüthrich, Davos 2000
ZNZ-Symposium (Center of Neuroscience Zurich), M.E. Schwab, Zurich 2000
ISDN Heidelberg, Germany 2000
ZNZ-Symposium (Center of Neuroscience Zurich), M.E. Schwab, Zurich 2001
SFN San Diego, Neuroscience meeting, USA 2001

Additional education
D-BIOL Symposium ETH Zurich, K. Wüthrich, Davos 1998 /1999
ZNZ-Symposium (Center of Neuroscience Zurich), M.E. Schwab, Zurich 1999