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Studying CNS Development Using The Cerebellum As A Model System

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SUMMARY

Thesis-Project I: Generation of Cerebellar Purkinje Cells and Granule Cells from Progenitors *in vitro*

Mechanisms that regulate the temporal and spatial formation of neurons and glia in the central nervous system (CNS) are poorly understood. Considerable effort is being invested into understanding the differentiation of multipotent CNS progenitor cells *in vitro* in order to gain insights into the formation and maintenance of neurons of the adult brain. We have focused on the cerebellum as an ideal model system to address the formation of defined populations of neurons and glial cells in the CNS. Here we describe a 2-dimensional cell culture system to maintain cerebellar progenitor cells in an undifferentiated state, and the induction of mature cerebellar neuron and glial cell differentiation *in vitro*. The cerebellar progenitor cells maintain their proliferative and differentiation capacity over many weeks *in vitro*. Furthermore, we show that a subpopulation of the cultured cerebellar neuroepithelial cells behave like stem cells and that the culture substrate plays a significant role in differentiation. We also show by expression of neurotransmitter receptors that the granule cells attain a high degree of terminal differentiation. Due to the adhesive nature of the culture, the cells are freely available to manipulation of the cell culture medium and substrate. We believe the cerebellar neuroepithelial cell culture system represents an excellent model to analyze factors that potentially regulate neurogenesis and determine CNS cell fate.

Thesis-Project II: Notch1 and the Ligands Delta and Serrate-Related are Expressed in Distinct Cell Populations in the Postnatal Brain

Notch1 signaling has been shown to play a pivotal role in the regulation of vertebrate neurogenesis. Recent experiments suggest that Notch signaling may also be involved in the regulation of later stages of differentiation particularly in postmitotic neurons of the brain. In order to address the putative role of Notch signaling in the developing CNS, we have examined the expression patterns of Notch1, Notch3 and components of the proposed Notch signaling cascade. *In situ* mRNA hybridization revealed distinct patterns of Notch and ligand expression in the postnatal and adult brain. Notch1 is associated with cells in the subventricular zone, the dentate gyrus and the rostromigratory stream, all regions of continued neurogenesis in the postnatal brain. In addition, Notch1 is expressed at low levels throughout the cortex and olfactory bulb at postnatal day 4 and shows striking expression in the cerebellar Purkinje cell layer. In the adult, Notch1 expression in the cortex is reduced but is maintained at elevated levels in the olfactory bulb and the cerebellar Purkinje cell layer. The ligands for Notch1 including Delta-like1 and 3 and the Serrate-related Jagged1 and Jagged2 proteins show distinct expression patterns in the developing and adult brain that overlap that of Notch1. The cellular distribution of Notch1 and its putative ligands suggest distinct roles for Notch1 signaling in specific subsets of cells in the postnatal brain. In addition the downstream targets of the Notch signaling cascade Hes1, Hes3 and Hes5 also show distinct patterns of expression. Hes5 coincides with the majority of Notch expression and can be detected in the cerebral cortex, cerebellum and putative germinal zones. Hes3 on the other hand shows a restricted expression in cerebellar Purkinje cells. In addition, we examined the expression patterns of intrinsic Notch regulatory proteins Numb and Numblike in the postnatal brain. The expression of Notch1 and ligands in the subventricular zone and in the sub-granule layer of the dentate gyrus suggest that Notch1 may regulate neurogenesis from putative stem cells in the postnatal brain and play role in differentiated cells of the adult brain.

Thesis-Project III: Calbindin-Cre Transgenic Mice as a Tool to Analyze Gene Function in the CNS

Analysis of gene function in the nervous system by targeted inactivation in ES cells can be problematic, particularly if the gene in question plays a vital role in other tissues. Hence, the ability to ablate genes specifically from particular cell types in a regulated manner assist in the evaluation of gene function. The Cre lox system of gene ablation has proven to be a powerful tool to address function *in vivo*. Therefore, we have generated mice that express Cre-recombinase from the Calbindin D28k promoter in an attempt to be able to ablate gene specifically from postmitotic Purkinje cells *in vivo*. The cerebellum and particularly Purkinje cells are an excellent system to address development of the mammalian CNS including cell fate determination, differentiation, migration and cell-cell interactions. We show that some of the Calbindin-Cre transgenic line generated exhibit recombinase activity in the cerebellar Purkinje cells as expected. In addition, a number of lines show widespread or restricted expression patterns of Cre-recombinase due to potential integration effects of the transgene promoter. These lines will be valuable for addressing gene function *in vivo*.

ZUSAMMENFASSUNG

Kurze Einführung

Unser Labor untersucht die Entwicklung des Zentralen Nervensystems (ZNS) auf zellulärer und molekularer Ebene. Dabei konzentrieren wir uns auf das Cerebellum als Modellsystem. Unter diesen Voraussetzungen hat diese Arbeit zum Ziel, Werkzeuge für die Analyse von Entwicklungsvorgängen im Kleinhirn zu etablieren und zu beschreiben. Das Cerebellum ist im Vergleich zum Cortex eine verhältnismässig einfache Struktur. Es besteht aus nur fünf verschiedenen Neuronentypen, wovon die Purkinjezellen und die Körnerzellen die wichtigsten sind. Sowohl die Struktur, als auch die Entwicklung des Kleinhirns sind gut dokumentiert. Für verschiedene Entwicklungsstadien von Körner- und Purkinjezellen sind molekulare Marker beschrieben, welche die Analyse von Differenzierungsvorgängen erleichtern. Die Kleinhirnanlage ist schon sehr früh in der Embryonalentwicklung erkennbar und kann in einem frühen Entwicklungsstdium isoliert werden. Zudem ist das Kleinhirn keine lebensnotwendige Struktur. Dies hat den Vorteil, dass Funktionsanalysen von Genen mittels zielgerichteter Mutagenese nicht durch sekundäre Effekte zum Tod des Versuchstieres führen, selbst wenn die Mutation zum Tod der betroffen Zellen führen würde. Dies erleichtert die Analyse von Genen, die während der Entwicklung eine Rolle spielen, enorm.

Im Gegensatz zum peripheren Nervensystem (PNS) ist die Regenerationsfähigkeit im Zentralen Nervensystem (ZNS) stark limitiert. Stammzellen sind aufgrund ihrer Plastizität zur Zeit die besten Kandidaten, Nervenzellen im alternden oder kranken Gehirn zu ersetzen. Über die letzten Jahre wurden bemerkenswerte Fortschritte

bezüglich des Verständnisses dieser Zellen erzielt. Neurale Stammzellen sind grob als multipotente Zellen, die sich selbst erneuern, die aber auch Neurone, Astrozyten und Oligodendrozyten bilden können, definiert. Im adulten Gehirn kann die Umgebung der Stammzellen nur sehr schlecht manipuliert werden. Aus diesem Grund werden Studien mit Stammzellen häufig *in vitro* durchgeführt. Reaktionen der Zellen auf Änderungen des Kulturmediums oder des Substrats geben Aufschluss über das Potenzial der Zellen und über die Beeinflussbarkeit der Zelldifferenzierung durch Faktoren im Medium. So können Signalsysteme ermittelt werden, welche für die Differenzierung der Zellen notwendig sind. Es hat sich gezeigt, dass aus Stammzellen *in vitro* auch Zelltypen generiert werden können, welche *in vivo* am Herkunftsort der Zellen nicht vorkommen. Dies ist aus medizinischer Sicht sehr interessant, macht aber klar, dass solche Daten in Bezug auf die Erforschung der Entwicklung im Organismus durch *in vivo* Experimente bestätigt werden müssen. Zudem können Fragestellungen, zum Beispiel bezüglich Dendritenbildung oder Axonprojektion, *in vitro* nur schlecht bearbeitet werden, da diese Prozesse auf eine intakte 3-dimensionale Struktur angewiesen sind.

In den Projekten I und III (Kapitel 2 und 4) dieser Dissertation werden Werkzeuge bereitgestellt, welche das Studium der Kleinhirnentwicklung auf zellulärer und molekularer Ebene, *in vitro* und *in vivo* ermöglichen sollen. Dies sind Fragestellungen mit denen sich die Grundlagenwissenschaft beschäftigt. Erkenntnisse über normale Entwicklungsabläufe bilden jedoch die Grundlage, abnormale Vorgänge im Krankheitsfall besser zu verstehen.

Projekt II (Kapitel 3) beschreibt die Expressionsmuster von Komponenten der Notch Signalkaskade. Beschreibende Experimente dieser Art bilden die Grundlage für die Interpretation von Phänomenen, welche bei *in vitro* oder *in vivo* Versuchen beobachtet werden.

Projekt I: Etablierung eines Kultursystems für neuroepitheliale Stammzellen der Kleinhirnanlage

In diesem Projekt wird ein 2-dimensionales Zellkultursystem für Vorläuferzellen aus der Kleinhirnanlage etabliert. Dabei können diese Zellen in undifferenziertem Zustand expandiert und die Differenzierung zu neuronalen und glialen Zellen des ZNS durch Veränderung der Kulturbedingungen induziert werden. *In vitro* behalten die

undifferenzierten Vorläuferzellen ihre Fähigkeit zu proliferieren und zu differenzieren über mehrere Wochen und Passagen. Mit diesem Kultursystem sind wir in der Lage aus undifferenzierten Progenitorzellen spezifische Neuronentypen des Kleinhirns, wie Purkinjezellen, Körnerzellen und Astrozyten zu generieren. Die Körnerzellen exprimieren den Neurotransmitterrezeptor GABAα6, was beweist, dass sich diese Neuronen in einem terminal differenzierten Zustand befinden. Zudem zeigen wir durch klonale Analyse, dass eine Subpopulation der undifferenzierten Zellen Stammzellcharakter aufweist und dass das Kultursubstrat einen wichtigen Beitrag zur Differenzierung leistet. Im Gegensatz zu Kultursystemen, bei welchen die Stammzellen als Aggregate in Suspension gehalten werden, sind hier sämtliche Zellen für Faktoren im Medium und für Substrate frei zugänglich. Wir glauben, dass dieses Kultursystem ein sehr gutes Modell für die Analyse von Faktoren ist, welche die Determinierung und Differenzierung von Stammzellen in der Neurogenese regulieren.

Projekt II: Komponenten der Notch Signalkaskade sind in unterschiedlichen Zellpopulationen des postnatalen Gehirns exprimiert

Während der Entwicklung eines Organismus wird immer wieder entschieden, welchen Weg eine Zelle einschlagen muss. Ein solcher Prozess findet auch in Keimzonen statt, wo aus einer Population von identischen Stammzellen einige zur Differenzierung ausgewählt werden. Dieses Auswahlverfahren findet über einen Prozess lateraler Inhibition statt, in welchem das Notch Signalsystem eine zentrale Rolle spielt. Die Signaltransduktion via Notch1 ist wichtig für die Regulation der Neurogenese in Vertebraten. Neuere Resultate von *in vitro* Experimenten schreiben dieser Signalkaskade aber auch Aufgaben im Bereich der terminalen Differenzierung von Neuronen, wie zum Beispiel der Dendritenbildung zu. Aufgrund dieser Daten haben wir die Expressionsmuster der Rezeptoren Notch1 und Notch3, sowie der Liganden Delta-like1, Delta-like3, Jagged1 und Jagged2 im postnatalen Gehirn mittels mRNA *in situ* Hybridisierung erörtert. Notch1 wird von Zellen in der subventrikulären Zone, im Dentate Gyrus, sowie in Zellen, die rostral in Richtung bulbus olfactorius wandern (rostromigratory stream) exprimiert. Dies sind alles Strukturen, in welchen nach der Geburt noch Neurogenese stattfindet. In vier Tage alten Mäusen konnten wir Notch1 mRNA auch in Zellen des Cortex, des bulbus olfactorius und der Purkinjezellschicht im

Cerebellum nachweisen. Im adulten Cortex wird dann die Notch1 Expression herunter reguliert, im bulbus olfactorius und in der Purkinjezellschicht bleibt die Expression aber gleichermassen erhalten. Die Liganden Delta-like1 und 3, sowie Jagged1 und 2 zeigen unterschiedliche Expressionsmuster, die mit dem Muster von Notch1 überlappen. Aufgrund der zellulären Verteilung von Notch1 und seinen Liganden kann vermutet werden, dass die Aktivierung der Notch Signalkaskade in verschiedenen Zellen unterschiedliche genetische Programme reguliert. Um die Aktivität der Notchkaskade besser abschätzen zu können, haben wir die Expressionsmuster von Hes1, Hes3 und Hes5 studiert. Aktiviert ein Ligand den Notchrezeptor, wird die Expression dieser Transkriptionsfaktoren durch die intrazelluläre Domäne von Notch1 induziert. Dabei überlappt das Expressionsmuster von Hes5 mehrheitlich mit demjenigen von Notch1 und konnte im Cortex, im Cerebellum und in den Zonen adulter Neurogenese nachgewiesen werden. Das Transkript von Hes3 wurde nur in den Purkinjezellen detektiert und Hes1 mRNA ist im Gehirn nicht in nachweisbarer Menge vorhanden. Neben den Hes Transkriptionsfaktoren, welche das Notchsignal in eine zelluläre Antwort umwandeln, haben wir die Expression der intrazellulären Notch-Inhibitoren Numb und Numblike studiert. Auch diese zeigen überlappende Expressionsmuster mit Notch1.
Die verschiedenen Expressionsmuster von Notch1 und 3 und ihren potenziellen Liganden in der subventrikulären Zone und der subgranulären Schicht im Dentate Gyrus lassen auf eine Rolle in der Regulation der Neurogenese im postnatalen Gehirn schliessen. Die Expression im Cortex und in den Purkinjezellen deuten auf eine Funktion von Notch1 in postmitotischen Neuronen hin.

Projekt III: Transgene Mäuse exprimieren Cre-Rekombinase in postmitotischen Neuronen.

Mittels knock-out Technologie lassen sich Funktionen von Genen *in vivo* analysieren. Direkte Geninaktivierung in embryonalen Stammzellen kann aber problematisch sein, wenn das betrachtete Gen eine lebenswichtige Rolle in einem anderen Gewebe spielt. Mittels dem Cre-lox System ist es möglich Gene lokal und zeitlich spezifisch auszuschalten. Dies erleichtert die Analyse der Genfunktion.
Mit dem Ziel, Gene aus postmitotischen Purkinjezellen zu eliminieren, haben wir transgene Mäuse hergestellt, welche die Cre-Rekombinase unter der Kontrolle eines

Promotorfragments des Calbindin D28k Gens exprimieren. Für dieses Promotorfragment wurde früher gezeigt, dass es in den Purkinjezellen transgener Mäuse zu einer Expression eines Reportergens führt.

In diesem Projekt beschreiben wir das Expressionsmuster der Cre-Rekombinase mit Hilfe des Reportergens LacZ. Wie erwartet, weisen die Tiere einiger transgener Mauslinien eine Cre Aktivität in den Purkinjezellen auf. In einigen Linien wurden auch Rekombinationsvorgänge in postmitotischen Neuronen anderer Hirnstrukturen nachgewiesen. Die verschiedenen Cre Expressionsmuster sind vermutlich auf Positionseffekte bei der Transgenintegration zurückzuführen.

Die verschiedenen transgenen Mauslinien werden die Analyse von Genfunktionen in Purkinjezellen und in anderen postmitotischen Neuronen ermöglichen und so einen Beitrag zum Verständnis der Kleinhirn- und ZNS-Entwicklung leisten.

Schlussfolgerungen und Ausblick

Die Reparatur des beschädigten oder kranken Gehirns ist ein wichtiges und ehrgeiziges Ziel. Über die letzten Jahre haben Experimente mit embryonalen und adulten Stammzellen gezeigt, dass Stammzellen viele der Anforderungen erfüllen, die für eine Regeneration des Nervensystems nötig sind. Schon seit mehreren Jahren ist es möglich, Neuronen aus Stammzellen zu generieren. Doch die Differenzierung in spezifische Neuronentypen konnte erst vor kurzem, anhand der Produktion von dopaminergen Nervenzellen, gezeigt werden. Hier beschreiben wir die Etablierung eines Zellkultursystems, in welchem aus multipotenten, neuralen Vorläuferzellen der Kleinhirnanlage, Purkinjezellen und Körnerzellen produziert werden können. Doch um das Nervensystem erfolgreich zu reparieren, reicht es nicht, verschiedene Zelltypen zu generieren. Die Zellen müssen einerseits nach der Implantation überleben und sich andererseits richtig in das bereits bestehende, neurale Netzwerk einfügen. Aus diesem Grund ist es notwendig, Prozesse der terminalen Differenzierung in postmitotischen, bereits determinierten Zellen zu studieren. Die dabei betrachteten Prozesse sind von der dreidimensionalen Struktur des Nervensystems abhänging und können somit *in vitro* nur bedingt analysiert werden. Mittels konditionaler knock-out Technologie kann die Funktion von Genen in bestimmten Zellen, zu bestimmten Zeitpunkten *in vivo* untersucht werden. Aus diesem Grund haben wir transgene Mäuse hergestellt, welche

die Cre-Rekombinase in postmitotischen Neuronen exprimieren. Mit Hilfe dieser Calbindin-Cre Mäuse kann die Genfunktion in Purkinjezellen, aber auch in postmitotischen Neuronen anderer Hirnstrukturen analysiert werden. Um die Funktion von Notch1 in Purkinjezellen zu untersuchen haben wir Mäuse, bei welchen das erste kodierende Exon von Notch1 mit loxP Sequenzen flankiert ist, mit den Calbindin-Cre Tieren gekreuzt. Zudem haben wir die Expressionsmuster verschiedener Komponenten der Notch Signalkaskade beschrieben, was die Analyse der doppeltransgenen Tiere erleichtern soll.

1 INTRODUCTION

Most tissues in the adult have a potential for repair and replacement of dead or injured cells. Two main strategies are used by these tissues for the generation of new cells. The simplest form is by proliferation of cells within or around the damaged area which remained intact. In this clonal form of tissue repair daughter cells with identical genotype and phenotype are produced. This type of regeneration is used by hepatocytes and endothelial cells. The second strategy to replace differentiated cells is via cell genesis similar to the process used during development. An undifferentiated multipotent cell divides to generate a copy of itself and a cell which undergoes differentiation. The haematopoetic system and epithelial cells are archetypes of this stem cell derived regeneration. Stem cells with the potential to produce differentiated progeny exist throughout the life of the animal in these systems.

The term "neural stem cell" is used loosely to describe cells that (i) can generate neural tissue or are derived from the nervous system, (ii) have some capacity for self-renewal, and (iii) can give rise to cells other than themselves through asymmetric cell division (Gage, 2000). The existence of stem cells in the developing brain has been proposed for many years, however, their isolation and characterization has been elusive (Gage et al., 1995a; Morshead et al., 1994; Stemple and Mahanthappa, 1997). In the adult brain the existence of cells that are able to proliferate and produce neuronal cell types was proven by radiolabelling and retroviral infection studies, at least for certain brain areas like the olfactory system or the hippocampus (Altman, 1962; Gage, 2000; Graziadei and Graziadei, 1979; Graziadei et al., 1978; Price et al., 1987; Rakic, 1985). Even though recent publications report a small amount of neurogenesis after brain damage (Alvarez-

Buylla et al., 2000; Dash et al., 2001; Magavi et al., 2000) it is known that in most areas of the brain dead or damaged cells are replaced mainly by astrocytes resulting in astrocytic scars. Therefore, the identification and isolation of multipotent and self renewing cells from the brain is of major interest for the treatment of neurodegenerative disorders. Much effort has been directed into isolating cells from the adult CNS which may retain neurogenic potential (Gage et al., 1995b; Weiss et al., 1996a; Weiss et al., 1996b).

The manipulation of stem cells seems to be the most promising way to succeed in tissue repair (Anderson et al., 2001; Gage, 2000). A crucial step towards nervous system repair is to promote the generation of new neural cells. However, to repair a tissue that contains hundreds of diverse, highly specialized cells will be a challenging task. Success will depend on rigorously defining the developmental potential of these stem cells (Fig. 1-1) and finding conditions to produce specific cell types (Temple, 2001).

Many organs use similar genetic programs for repair processes to those used during development. During embryogenesis the epithelial cells of the neural ectoderm give rise to the cells of the adult nervous system. However, the mechanisms that control the development of the central nervous system are largely unknown and difficult to examine due to the large diversity of cell types and the migration of maturing cells away from their birth place. These neuroepithelial cells are proposed to be neural stem cells. It is suggested that the neural stem cells produce precursor cells, so called neuroblasts and glioblasts, showing a restricted potential and a fate commitment which then eventually differentiate into multiple cell types of the brain (Fig. 1-1). The ordered and exact differentiation of the stem cells into progenitors and then precursor cells and finally to the various neural cell types is not understood. The timing of such events is highly controlled and results in the organized arrangement of differentiated cells in distinct layers of the brain. This regulated generation and differentiation of the multiple cell types is probably controlled to a major extent by local cell-cell interactions and signalling pathways.

The development of the CNS does not end with the generation of specific cell types that reach a certain position in the brain or the spinal cord after migration. The functionality of the CNS is obtained by the interactions of the various neuronal cell types among each other and with glial cells. Information processing is achieved by communicating neurons

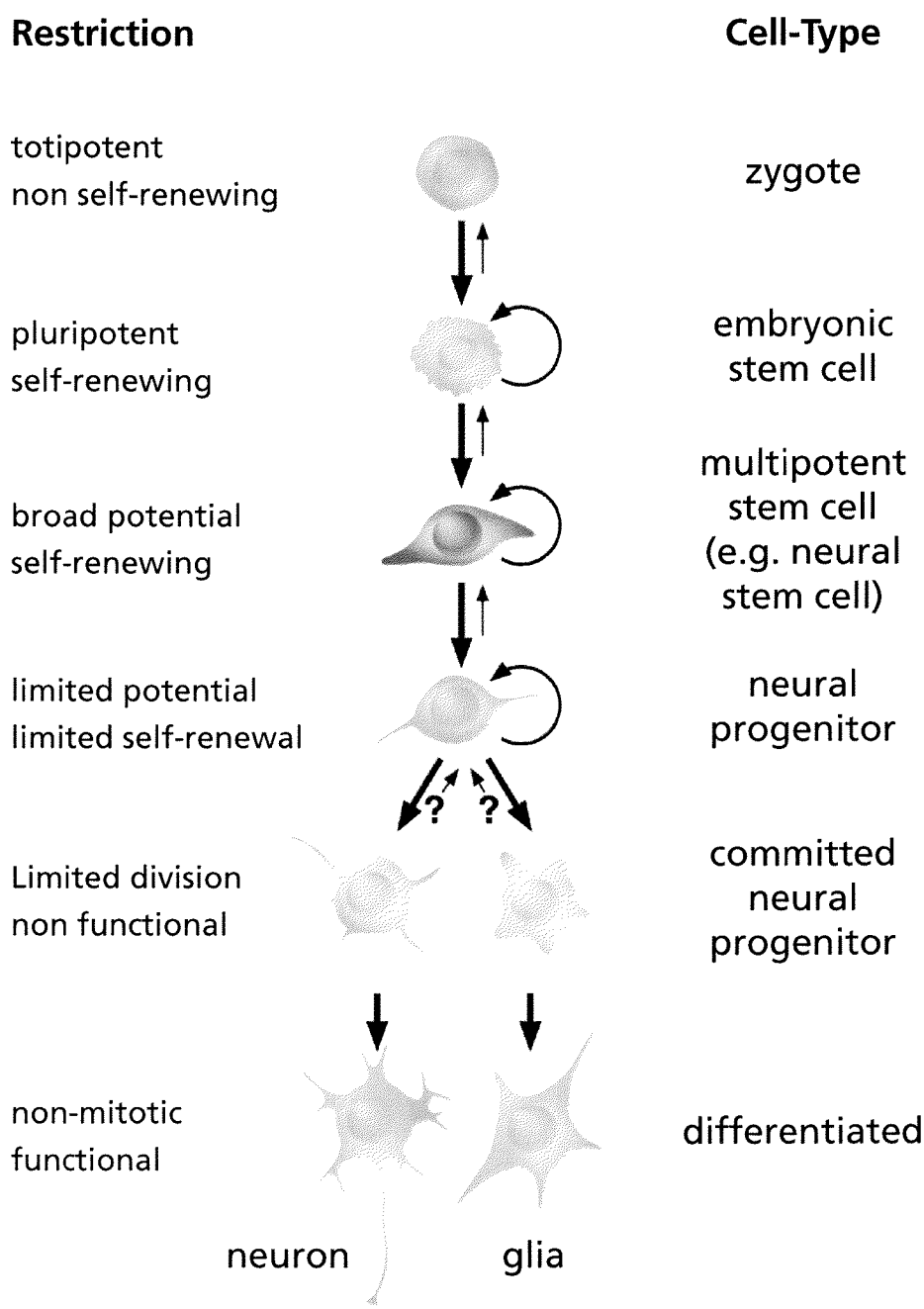

Figure 1-1: Potential and restriction of stem cells

A model showing the ongoing restriction of stem cells and precursors during development, starting with the most primitive and multipotent stem cell and progressing to the most restricted differentiated cell. The small arrows pointing up suggest that cells are also capable to dedifferentiate under certain conditions. However, this process is not well documented or understood. (Modified after Gage, 2000)

contacting each other via synapses. Therefore, it is not only important to have the right cell types at the right place but also that these cells project their axons and dendrites correctly in order to form a functional network. During the last years some mechanisms involved in axonal guidance (Bernhardt, 1999; Ghysen and Dambly-Chaudiere, 2000;

Kamiguchi and Lemmon, 2000; Stoeckli and Landmesser, 1998), dendrite formation and synaptogenesis (Cline, 2001; Redmond and Ghosh, 2001; Zervas and Walkley, 1999) have been revealed. However, it is still largely unclear how the complex circuitry in the brain is established, which genes are regulating these processes and how they are influenced by electric activity.

1.1 Cerebellum as a Model to Study CNS Development

A major challenge to analyze CNS stem cell development is the immense complexity of the brain. The cerebral cortex is particularly difficult to study as its stem cells *in vivo* give rise to numerous neuronal and all glial cell types. Furthermore, the cerebral cortex is complexly patterned from an early stage (E9-10) in the mouse suggesting that cells from cortical areas may be restricted in their fate very early. This complicates lineage analysis and results in potential heterogeneity in the analysis of lineage differentiation.

The cerebellum represents an ideal system to study neurogenesis due to the relatively limited number of neuronal cell types which are located within a highly organized structure (Fig. 1-2). The cells are generated from the cerebellar primordium which is already clearly defined at E10 (Fig. 1-3 on page 17). Cerebellar development is a well characterized process for which stage and cell type-specific molecular markers are known (Hatten et al., 1997 and references therein; Hatten and Heintz, 1995) (Fig. 1-3 on page 17). The cells giving rise to the cerebellar primordium are fated early in murine development (E9) (Hatten and Heintz, 1995), however, the differentiation of the cerebellar neuroepithelial cells takes place during late embryogenesis between E14 and 21 and terminal differentiation of certain cell types is even postnatal. The Purkinje and the granule cells are the most prominent neurons of the cerebellum. Purkinje cells are located in a single cell layer (Purkinje cell layer; PCL) and are the only efferent neurons innervating mainly structures in the deep cerebellar nuclei. The granule cells are the most abundant neurons in the whole brain and are interneurons connecting Golgi cells and mossy fibers with Purkinje cells. However, the neuroepithelial cells of the IV ventricle region not only give rise to all neurons of the cerebellum they are also the source of all types of glia including astrocytes and oligodendrocytes. The IV ventricle region is particularly important for the development of the white matter of the brain as a

substantial proportion of the oligodendrocytes of the CNS are derived from the IV ventricle region of the neonatal brain *in vivo*.

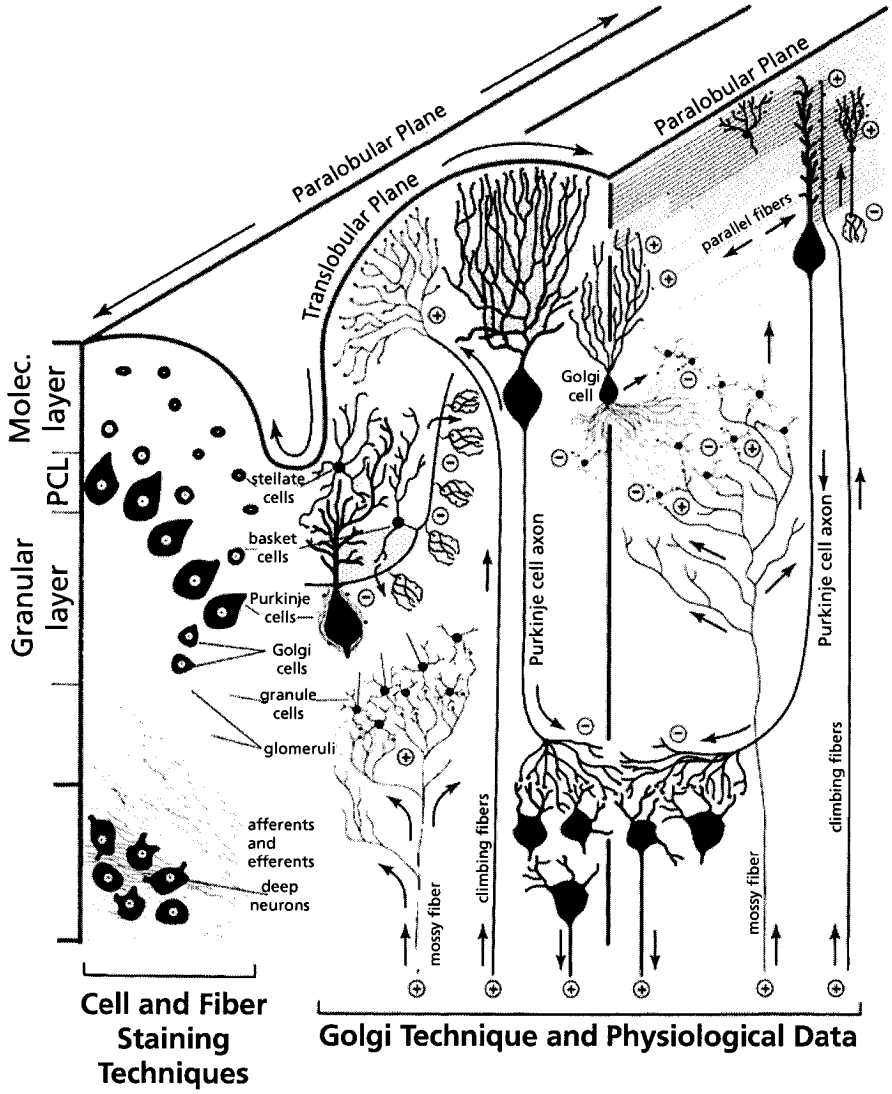

Figure 1-2: Structure and cell types of the adult cerebellum

The cerebellum is a highly organized structure containing three layers. In the Purkinje cell layer (PCL) the Purkinje cells are localized. These are the only efferent neurons in the cerebellar cortex synapsing on the neurons of deep cerebellar nuclei. Their dendritic trees project into the molecular layer where they contact the parallel fibers of the granule cells that are found in the inner granular layer. Information from various region of the CNS is transported by mossy fibers and climbing fibers. Afferents and efferents are myelinated by oligodendrocytes and, therefore, form the so called white matter of the cerebellum. (From J. Altman and S. A. Bayer: Development of the cerebellar system)

Although the cerebellum is involved in important functions like balance, fine motor control, eye movements, and as reported more recently, also in learning and memory (Okano et al., 2000; Thach, 1998; Thompson and Krupa, 1994) the cerebellum is not a

vital structure. This simplifies the analysis of gene function using conditional gene ablation or overexpression strategies.

In the cerebellum, cell fate choices and cell differentiation, as well as radial glia dependent and independent migration can be studied. The developing EGL provides a model for the formation of secondary germinal matrices as found in the subventricular zone of the cerebral cortex or the lateral ganglionic eminence. Terminal differentiation and synaptogenesis can even be analyzed postnatally. These features make the cerebellum a complete and ideal model to study CNS development.

1.2 THE DEVELOPMENT OF THE CEREBELLUM

The cerebellum develops through a program of gene expression that includes (i) genes that specify the cerebellar territory, (ii) genes that specify dorso-ventral polarity, and (iii) genes that mark specific cell types generated in the dorsal region of the tissue (Hatten and Heintz, 1995). Genetic analysis indicates that the engrailed genes (En1, En2) are indispensable for the formation of the cerebellar region (Davis and Joyner, 1988; McMahon, 1993; McMahon et al., 1992; Wurst et al., 1994) and that the bone morphogenetic proteins (BMPs) dorsalize cells within this region (Lee and Jessell, 1999) resulting in a patterned cerebellar primordium.

After the designation of the cerebellar anlage at embryonic day 9.5 in the mouse a phase of strong proliferation follows in the cerebellar neuroepithelium finishing at E14 (Davis and Joyner, 1988; McMahon et al., 1992; Miale and Sidman, 1961; Wurst et al., 1994). At embryonic day E10-11 the first cells leave the presumptive stem cell pool around the ventricular zone, migrate into the cerebellar anlage and may differentiate into neuroblasts of the deep cerebellar nuclei (Altman and Bayer, 1985b; Altman and Bayer, 1985c). Between embryonic day 12 and 14, Purkinje cell precursors are generated from the ventral portion of the cerebellar neuroepithelium and migrate along the radial glial system to form a rudimentary zone overlying the germinal zone thereby providing a scaffold for the formation of the other neuronal layer of the cerebellar cortex, the internal granule cell layer (Altman and Bayer, 1985a; Alvarado-Mallart and Sotelo, 1992; Hallonet and Le Douarin, 1993). During early stages of Purkinje cell development transcription factors like Mash1 and Prox1 (Oliver et al., 1993) are expressed and the calcium binding protein calbindin D28 is upregulated as soon as the Purkinje cell

precursors leave the cell cycle (Fig. 1-3). The generation of granule cells originating from the dorsal portion of the cerebellar anlage, the rhombic lip, is controlled by transcription factors Math1 (Ben-Arie et al., 1997; Helms et al., 2001), Zic1, Zic2 (Nagai et al., 1997) and RU49 (Yang et al., 1996) (Fig. 1-3). Targeted disruption of Math1 leads to a cerebellar cortex devoid of granule cells (Ben-Arie et al., 1997). Overexpression of RU49, a transcription factor of the zinc finger family leads to more granule cells and a larger cerebellar cortex (Hatten, 1999; Yang et al., 1999). The neuroepithelial cells from the rhombic lip have been shown to be a separate population of cerebellar neuroepithelial cells by *in vitro* experiments (Alder et al., 1996). At embryonic day 14 in the mouse the first granule cell precursors start to migrate over the surface of the developing cerebellum (Cajal, 1889) using a radial glia independent mechanism, so called morphogenetic movements. These cells form a second proliferative region on the surface of the cerebellum called the external germinal layer (EGL) (Fugita, 1967; Fugita et al., 1966). The major burst of proliferation forming the multi-cell layer of the EGL occurs peri- and postnatally. During this period cells of the inner most layer of the EGL leave the cell cycle and start to migrate into the cerebellum. At this point Math1 is down-regulated and NeuroD seems to control this step in development (Miyata et al., 1999). For migration into the cerebellum the granule cells follow the Bergmann glial cells, pass the Purkinje cells and form the internal granule layer (IGL) where they differentiate into mature granule neurons during the first three postnatal weeks (Fig. 1-3)(reviewed in Hatten and Heintz, 1995).

1.2.1 Signaling through Receptor Tyrosine Kinases is Important for CNS Development

From previous studies, a number of peptide growth factors have been found to be able to regulate or modulate lineage differentiation in the PNS and CNS. Experiments using the neural crest stem cell culture system as well as *in vivo* studies have identified factors which may play a role in generation of the peripheral nervous system (PNS) by modulating the differentiation of neural progenitor cells (Dong et al., 1995; Gavrilovic et al., 1995; Shah et al., 1994; Sommer et al., 1996; Stemple and Anderson, 1992). More recent work using cell culture systems for CNS derived stem cells has also identified peptide growth factors important for survival and differentiation processes in the CNS

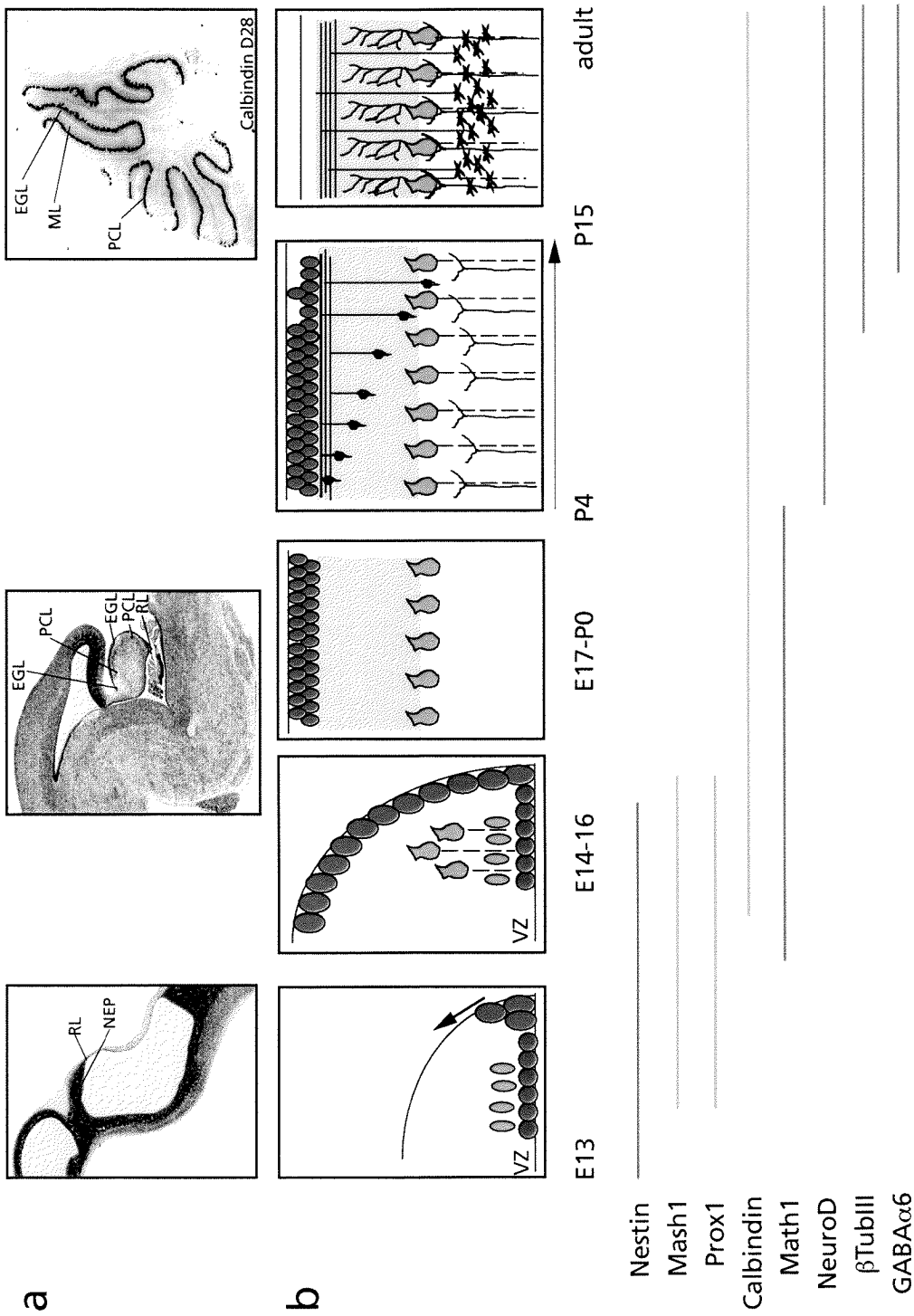

Figure 1-3: Cerebellar development

a) Photographs of the developing mouse cerebellum at E12, E15 and adult stages.
b) Schematic representation of the developing cerebellum showing the migration pathways of Purkinje cell precursors (red) and granule cell precursors (blue). The neuroepithelial cells are represented in green. On the bottom the expression phases of molecular makers for the different cell types are shown.

(Bottenstein et al., 1979). Insulin was shown to be important for Schwann cell survival (Gavrilovic et al., 1995) Insulin-like growth factor 1 (IGF1) has been reported to promote the proliferation, survival, and maturation of sympathetic neuroblasts (Caroni and Grandes, 1990; Mill et al., 1985), the genesis of retinal neurons (Drago et al., 1991; Hughes et al., 1993), and the survival of CNS projections and motor neurons (Hughes et al., 1993; Neff et al., 1993). It has also been reported that IGF-1 acts as a differentiation factor on postmitotic CNS stem cell-derived neuronal precursors (Arsenijevic and Weiss, 1998). Fibroblast growth factor (FGF2) has been shown to induce proliferation of multipotent stem cells *in vitro* and seems to be an important factor for their survival (Gritti et al., 1996; Murphy et al., 1990; Qian et al., 1997; Vescovi et al., 1993). Recent injury studies revealed that FGF2 also influences proliferation of adult neural progenitor cells (Yoshimura et al., 2001). FGF2 and thyroid hormone also modulate proliferation and survival of the oligodendrocyte/type-2 astrocyte O2A progenitor cell *in vitro* (Barres and Raff, 1994; Raff et al., 1988; Temple and Raff, 1986). Epidermal growth factor (EGF) has been reported to be a particularly potent mitogen for a subset of CNS stem cells and has been used to isolate multipotent cells from the adult mouse and human brain (Gage et al., 1995b; Qian et al., 2000; Reynolds et al., 1992; Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Vescovi et al., 1993). EGF receptor knock-out mice display background specific phenotypes. On a CD-1 or a 129/Sv x C57Bl/6 background abnormalities in skin, liver, kidney and the brain were found. In the cerebellum, Purkinje cell number was affected resulting in a smaller cerebellum and in the cerebral cortex cell migration was impaired (Sibilia and Wagner, 1995; Threadgill et al., 1995). Additionally, EGF was shown to influence gliogenesis (Lillien, 1995; Lillien and Wancio, 1998). Growth factors from the bone morphogenetic protein (BMP) family such as Tumor Growth Factor alpha (TGF-α) have been used to isolate multipotent cells from the brain and the BMPs seem to be important in generation of the astrocyte lineage (Gross et al., 1996; Reynolds et al., 1992; Reynolds and Weiss, 1992). This role in astrocyte generation in the CNS highlights the striking differences to the PNS where BMP-2 and -4 are involved in neurogenesis from the neural crest stem cells (Shah et al., 1994). Finally, Platelet Derived Growth Factor (PDGF) has been shown to induce O-2A progenitor cell proliferation although only for a limited time and number of cycles (Durand and Raff, 2000; Tang et al., 2000). After this defined response the O2A

progenitors lose their mitogenic response and differentiate. Furthermore, oligodendrocyte precursors express PDGF receptor alpha during their migration period (Raff et al., 1988; Temple and Raff, 1986). More recently, Williams et al. have described an ability of PDGF to induce neuronal differentiation in isolated cortical neuroepithelial cells grown in adhesive cultures (Williams et al., 1997). However, it is likely that even CNS derived stem cells from different areas of the brain react differently to certain growth factors.

The aim of one project was to determine peptide growth factors involved in cerebellar development. Since peptide growth factors are not necessarily expressed by their target cells a screening strategy for their receptors, the receptor tyrosine kinases, was chosen. We used a PCR based screening approach (Lai and Lemke, 1991; Taylor et al., 1994; Wilks et al., 1991; Wilks et al., 1989) to determine which subfamilies of RTKs are present during cerebellar development (for material and methods see Appendix 1). The results summarized in table 1 show the 11 found RTKs expressed during cerebellar development and their ligands.

Table 1-1: RTKs expressed during cerebellar development

RTK	Ligands
erbB4	Neuregulins 1, 2 and 3, EGF[a]
FGFR-1	FGF-1, FGF-2[b]
flt-1	VEGF[c]
IR	Insulin
trkB	BDNF[d], NT4/5[e]
PDGFRα	PDGFA[f], PDGFB[f]
DDR-1[g]	Matrix molecules, Collagens
DDR-2[e]	Matrix molecules, Collagens
Axl	Gas6, protein S
Tyro3	Gas6, protein S

a.epidermal growth factor
b.fibroblast growth factor 1 and2
c.vascular endothelial growth factor
d.brain derived neurotrophic factor
e.neurotrophin 4/5
f.Platelet Derived Growth Factor
g.discoidin domain containing receptors 1 and 2

This screening showed that RTKs from several families are involved in cerebellar development.

The family of the erbB receptors has been studied extensively (reviewed by Buonanno and Fischbach, 2001; Gassmann and Lemke, 1997). Knock-out studies revealed that the erbB receptors are involved in the development of heart as well as the peripheral and CNS (Britsch et al., 1998; Carraway, 1996; Erickson et al., 1997; Riethmacher et al., 1997). Most obvious is the implication of the neuregulin-erbB signaling pathway in heart development. Loss of function mutants for neuregulins and the receptors erbB2 and erbB4 die between E10 and E11 due to a defect in the heart myocardium. Targeted mutations in the erbB3 gene lead to embryonic death at E13.5 which also is caused by a heart defect. However, the knock-outs also display interesting defects in neural development, where this signaling mechanism is involved in the generation of the PNS and the CNS (reviewed in Adlkofer and Lai, 2000; Davies, 1998; Garratt et al., 2000; Gassmann and Lemke, 1997). In rescued erbB2 knock-outs where the heart defect is circumvented by heart specific erbB2 overexpression, Schwann cells are lacking (Woldeyesus et al., 1999). Neuregulin has also been reported to regulate neural precursor growth and oligodendrocyte generation (Calaora et al., 2001) and together with erbB4 they seem to be involved in synaptic plasticity (Huang et al., 2000). We and others (Gilbertson et al., 1998; Ozaki et al., 1998) have shown that erbB4 is expressed in the granule cell lineage in the cerebellum (Chapter 1-4 on page 21 A, B). Knock-out analysis of the erbB3 gene revealed that this receptor is required for normal cerebellar development at early stages (Erickson et al., 1997). Later, erbB3 receptors start to be expressed in myelinating oligodendrocytes (Chapter 1-4 on page 21 C, D). Additionally neuregulin is involved in the generation of radial glial cells (Anton et al., 1997) and therefore is important for the scaffold building of the cerebellum.

TrkB is the receptor for Brain derived neurotrophic factor (BDNF) and Neurotrophin 4/5 (NT-4/5). By loss-of-function mutations of the BDNF gene it was shown that this growth factor is involved in the development and maturation of Purkinje cells in the cerebellum. In these knock-out mice Purkinje cells display distorted dendritic trees with multiple but very small primary dendrites (Schwartz et al., 1997). Furthermore, BDNF signaling is involved in the regulation of dopamine D3 receptor expression in the nucleus accumbens (Guillin et al., 2001).

DDR-1 and DDR-2 are receptors which are characterized by a discoidin I domain. This domain has not been found in other RTKs but was first described in the Discoidin I

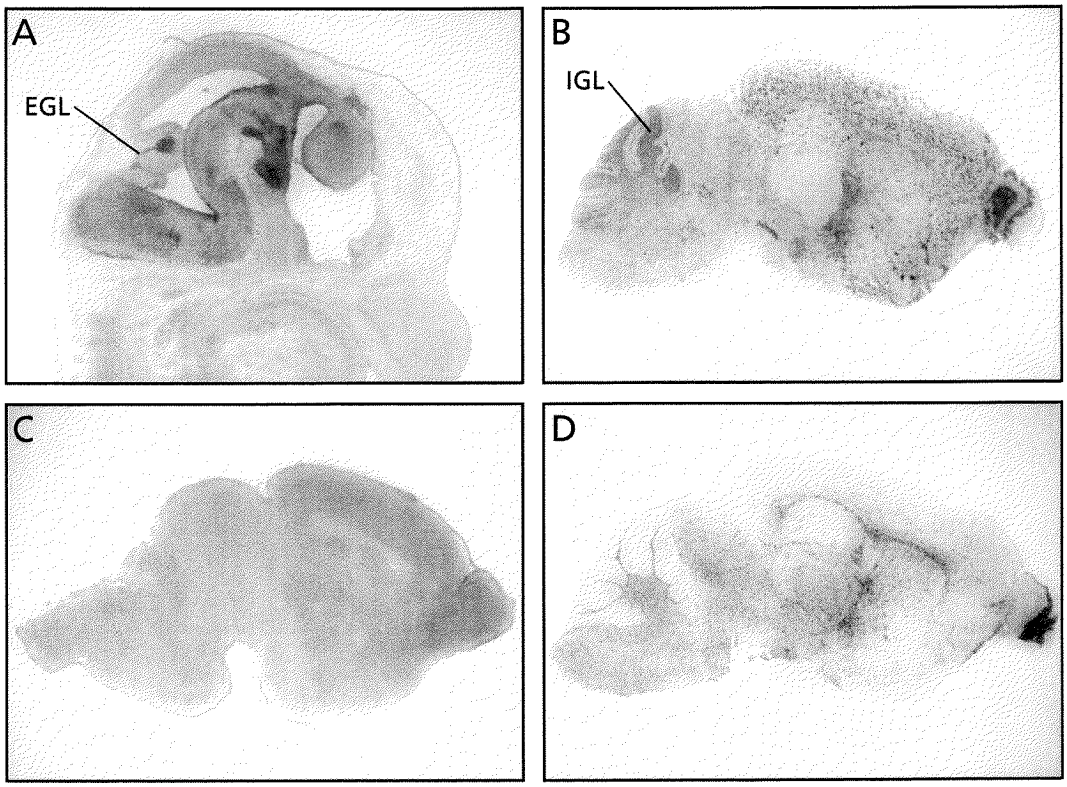

Figure 1-4: Expression of erbB3 and erB4 in the developing brain

Expression patterns of erbB3 and erbB4 by *in situ* hybridisation. A, B) erbB4 expression in E13 and P14 mouse brain. The staining of the granule cell precursors in the EGL (A) and later in development in the IGL (B) is clearly visible. C, D) erbB3 staining in P3 and P14 mouse brain. ErbB3 expression in cells of the Purkinje cell layer at P3 (C). When myelination in the brain starts at P14 erbB3 is expressed in oligodendrocytes (D).

protein in the slime mold Dyctiostelium descoideum. Further analysis indicated DDR-1 (also named MCK-10, EDDR1, NEP, Cak, trkE, Ptk-3, NTRK4 and RTK6) and DDR-2 (also named CCK-2, Tyro10 and TKT) to be nonintegrin collagen receptors that are able to transduce signals directly from the extracellular matrix (Shrivastava et al., 1997; Vogel et al., 1997). These receptors are also widely expressed during development and adulthood (Alves et al., 1995; Johnson et al., 1993; Lai and Lemke, 1991; Lai and Lemke, 1994; Sanchez et al., 1994; Zerlin et al., 1993). DDR2 seems to be involved mainly in cell proliferation as shown by a loss-of-function mutation (Labrador et al., 2001). Bhatt et al. have reported that DDR1 functions in axon extension of cerebellar granule cells (Bhatt et al., 2000).

Tyro3, Axl and Mer are three related receptor protein-tyrosine kinases characterized by an extracellular domain exhibiting significant amino acid sequence similarity to neural cell adhesion molecules (Lai and Lemke, 1991; Nagata et al., 1996; O'Bryan et al.,

1991). Protein S and Gas6 (growth arrest-specific gene-6) activate each of these receptors (Prieto et al., 1999; Stitt et al., 1995). Gas6 is expressed extensively in the CNS (Prieto et al., 1999) and has implications in several cellular functions like growth, survival, adhesion, and chemotaxis (Avanzi et al., 1998; Fridell et al., 1998; Goruppi et al., 1996). Tyro3 is the only family member expressed at embryonic stages and also shows the widest expression in adult animals. In the cerebellum all three receptors have been found in Purkinje cells and Tyro3 is also expressed by granule cells and Bergmann glia. Gas6 is expressed by the Purkinje cells suggesting a autocrine and paracrine signaling mechanism. The receptors were also detected in cerebellar white matter, primarily during the time of myelination (Prieto et al., 2000). Furthermore, signaling through these receptors is also involved in tumorigenesis, in the haematopoetic system (reviewed in Crosier and Crosier, 1997) and in vascular cell function (Melaragno et al., 1999).

The receptor tyrosine kinase flt-1 is implicated in the development and the maintenace of the vasculature and is part of a complex signaling system including several receptors and ligands of which VEGF is the most important (reviewed in Ferrara, 2001; Robinson and Stringer, 2001; Shibuya, 2001; Zachary and Gliki, 2001).

1.3 Analysis of Cerebellar Development *in vitro* and *in vivo*

Chapters 2 to 4 describe three independent projects. In chapter 2 and 4 two tools were generated to examine developmental processes of the CNS *in vitro* and *in vivo* using the cerebellum as a model system. In chapter 3 the expression patterns of the Notch signaling pathway components in the postnatal brain are described.

In vitro culture experiments are often used to study the potential of CNS stem cells and to investigate the differentiation pathways along which these cells generate neurons and glial cells. Currently, two popular approaches for the culture of multipotent cells from the CNS are used. Neurospheres involve growing cells in suspension, and this technique has been used successfully to identify multipotent cells from the ventricular zone of the cerebral cortex. Stem cells isolated from adult brains are EGF-dependent whereas when isolated from embryos FGF2 acts as a mitogen and survival factor (Reynolds et al., 1992;

Reynolds and Weiss, 1992; Reynolds and Weiss, 1996; Vescovi et al., 1993; Weiss et al., 1996a). Neurosphere differentiation into neuronal and glial cells is induced by plating onto various substrates (Gritti et al., 1996; Vescovi et al., 1993).

For adherent cultures, cells are isolated from the embryonic CNS and plated onto a substrate. In practice this system is more difficult but has the advantage that the experiments are easier to control. A prototype of an adherent culture for multipotent neuroepithelial cells was published by Kalyani et al. in 1997 using cells from the developing spinal cord. These cells could be kept in presence of FGF2 and chicken embryo extract (CEE) in an undifferentiated stage expressing the intermediate filament Nestin, which is a molecular marker for undifferentiated cells. Fibronectin was used as a substrate which was shown to restrict their differentiation. These adherent cells could generate all neural cell types including putative motorneurons by plating onto different substrates (Kalyani et al., 1997; Mayer-Proschel et al., 1997; Mujtaba et al., 1998; Rao and Mayer-Proschel, 1997; Rao et al., 1998). The analysis of differentiation relied on the expression of antigens previously reported to be associated with the differentiated cell types. Furthermore, Kalyani et al. showed that neurospheres of the spinal cord display the same phenotype when plated onto fibronectin as cells initially isolated onto the substrate (Kalyani et al., 1997; Mayer-Proschel et al., 1997; Mujtaba et al., 1998; Rao and Mayer-Proschel, 1997; Rao et al., 1998). However, neurospheres do not require components of the CEE needed to keep the adherent cultures in an undifferentiated state suggesting that these factors may be produced by neurospheres in an autocrine fashion (Kalyani et al., 1997; Mayer-Proschel et al., 1997; Mujtaba et al., 1998; Rao and Mayer-Proschel, 1997; Rao et al., 1998). Due to the advantages of the cerebellum as a model to study CNS development and in order to minimize the effects of intrinsically produced trophic factors (Kalyani et al., 1997; Mayer-Proschel et al., 1997; Mujtaba et al., 1998; Rao and Mayer-Proschel, 1997; Rao et al., 1998), we established an adherent cell culture system for rat cerebellar neuroepithelial cells. This system is described in chapter 2. However, cell culture experiments may not be suitable to study complex processes such as neurite formation or synaptogenesis, particularly, if these are dependent on the three-dimensional structure of the developing brain and on the factors they find within this scaffold. Therefore, the study of gene function *in vivo* is important and necessary. In our laboratory we have analyzed the function of Notch1 during early cerebellar development

by conditional Notch1 ablation (Lütolf et al., in press) and now want to asses the function of this gene during its second wave of expression in postmitotic Purkinje cells using a similar approach. In order to be able to interpret the results of this loss-of-function experiment later on, we first focused on the description of the expression patterns of Notch signaling components in the postnatal brain. In chapter 3 we show the expression patterns of Notch1 & 3, of the ligands Jagged 1 & 2 and Delta-like 1 & 3 as well as of the downstream targets Hes1, Hes 3 and Hes 5. Furthermore, two Notch-inhibitors Numb and Numblike were included in the study. Having analyzed the expression of ligands, receptors and downstream targets it is possible to draw some conclusions about the locations where Notch signaling is active in the postnatal brain. The presence of Notch1 and the expression of Hes3 in Purkinje cells suggest that the Notch signaling pathway is active in these cells. In order to inactivate Notch1 signaling in the Purkinje cells by conditional gene ablation we generated transgenic mice expressing Cre-recombinase under the transcriptional control of the proximal region of the Calbindin D28 promoter. These Calbindin-Cre transgenic mice were bred with Rosa26 reporter mice. In chapter 4 the lacZ expression patterns of 4 double-transgenic mouse lines are described. These mice will be used to ablate Notch1 from postmitotic Purkinje cells, as well as from other postmitotic neurons.

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2 GENERATION OF CEREBELLAR PURKINJE CELLS AND GRANULE CELLS FROM PROGENITORS *in vitro*

2.1 INTRODUCTION

Development and maintenance of the vertebrate nervous system requires precise regulation of cell proliferation, differentiation and survival. Although neural stems cells have been identified in a number of adult brain regions, neurogenesis in the adult nervous system is limited both spatially and in the types of neuron generated (reviewed by Temple, 1999; Temple and Alvarez-Buylla, 1999). It is likely that the neurogenic potential of these putative adult neural stems cells is restricted by the environment of the adult brain as *in vitro* these cells are able to generate multiple cell types. The processes that regulate the differentiation of adult neural stem cells are likely to be similar to those active during embryonic development of the nervous system. Embryonic neural progenitors have been used as means to study neurogenic mechanisms that may also have relevance in the adult nervous system (reviewed by Alvarez-Buylla and Temple, 1998; Gage et al., 1995). The central nervous system (CNS) of vertebrates is derived from a pool of progenitor cells that lie within the neuroepithelium of the neural tube. These progenitor cells can be isolated and show stem cell-like properties *in vitro* with an ability to proliferate and self-renew and to generate neurons and glial cells in culture (Davis and Temple, 1994; Kalyani et al., 1997; Murphy et al., 1990; Qian et al., 1997; Reynolds et al., 1992; Reynolds and Weiss, 1996; Vescovi et al., 1993).

During embryonic development, progenitor cells of the mammalian nervous system

proliferate and differentiate to produce the neurons and glial cells of the different brain regions. In general, differentiation of the mammalian CNS proceeds through a period of neurogenesis in mid to late embryogenesis followed by extensive gliogenesis starting around birth (Bayer and Altman 1991). The mechanisms that regulate the proliferation and differentiation of the embryonic neural progenitors in the various brain regions is not known. Much of the work to elucidate factors that can modulate neurogenesis of neural stem cells has focused on cells isolated from the presumptive forebrain, hippocampal and spinal cord regions of the neural tube (reviewed by Gage, 1998; Gage, 2000; McKay, 2000b; Stemple and Mahanthappa, 1997). Using these culture models, fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) have been identified as trophic factors that identify two independent, putative stem-population (Davis and Temple, 1994; Murphy et al., 1990; Reynolds et al., 1992; Vescovi et al., 1993). These stem cells proliferate in response to high levels of their respective mitogen and can be maintained in an undifferentiated state *in vitro*. Withdrawal of the trophic factors results in a rapid onset of differentiation and the generation of neurons and glial cells including oligodendrocytes (Johe et al., 1996).

Subsequently, it has been proposed that FGF-2 is not only a mitogen for neural progenitors, but at low concentrations, also induces neurogenesis (Murphy et al., 1990; Qian et al., 1997). Similarly, platelet-derived growth factor has also been shown to promote neurogenesis of FGF-2-dependent progenitor cells *in vitro* (Williams et al., 1997). By contrast, gliogenesis can be induced with ciliary neurotrophic factor (CNTF) (Johe et al., 1996). It has been proposed that the switch from neurogenesis to gliogenesis that occurs *in vivo* may be regulated by the responsiveness of multipotent progenitors and their progeny to EGF signaling (Burrows et al., 1997). Increased EGF receptor expression and activation by TGFα *in vitro* correlate with an onset of gliogenesis at the expense of neurogenesis (Burrows et al., 1997). Conversely, recent data suggests an intrinsic differentiation pathway within embryonic neural progenitors with a conversion from neurogenesis to gliogenesis (Morrison, 2000; Qian et al., 2000).

However, the factors which regulate the differentiation of neurogenic progenitors into the different neurons of the specific brain regions is not known. Recently, it has been demonstrated that multipotent cells isolated from the brain as well as embryonic stem cells (ES cells) can be induced to generate midbrain dopaminergic neurons *in vitro*

(Kawasaki et al., 2000; Lee et al., 2000). Utilizing combinations of trophic factors it has been possible to generate neuronal enriched culture in which 30% of the cells express tyrosine hydroxylase as a marker of dopaminergic neurons. We have focused on the mechanisms that may regulate neurogenesis in the CNS using the cerebellum as a model system. The cerebellum consists of only five different neuronal subtypes as well as macroglia. The two major neuron types in the cerebellum, the Purkinje cell and the cerebellar granule cell, have well defined developmental pathways and display an intimate relationship with each other (Altman and Bayer, 1997; Hatten et al., 1997; Hatten and Heintz, 1995).

The cerebellum is derived from the neuroepithelial cells forming the roof of the fourth ventricle, posterior to the midbrain hindbrain organizer (isthmus) (Wassef and Joyner, 1997). In rats, the precursors of the Purkinje cells leave the cell cycle around embryonic day (E) 15 and start to migrate radially along radial glial cells towards the surface of the cerebellum (Altman and Bayer, 1997; Hatten et al., 1997; Hatten and Heintz, 1995). These precursors can be identified by their expression of the proneural gene Mash-1 (Guillemot and Joyner, 1993) and the postmitotic Purkinje cells upregulate expression of the calcium binding protein Calbindin D28k (reviewed in Hatten et al., 1997).

Granule cells are derived form the neuroepithelial cells at the posterior edge of the cerebellar primordium, the so called rhombic lip (Altman and Bayer, 1997; Hatten et al., 1997; Hatten and Heintz, 1995). These precursors express the basic helix-loop-helix transcription factor Math-1 (Akazawa et al., 1995). They proliferate extensively and migrate rostrally over the surface of the cerebellum starting around E15. By birth, the granule cell precursors have formed a second germinal layer (external germinal layer; EGL) over the surface of the cerebellum where they continue to proliferate. Granule cells differentiate from this germinal layer and migrate into the cerebellum, forming the internal granule cell layer (IGL) (reviewed by Hatten et al., 1997; Hatten and Heintz, 1995). Upon terminal differentiation the cerebellar granule cells express the a-subunit of the gamma-aminobutyric acid (GABA) receptor (Hatten et al., 1997).

In order to address the mechanisms that regulate the differentiation of Purkinje cells and granule cells from the neuroepithelium of the cerebellar primordium, we have established an adhesive cell culture system that allows us to maintain cerebellar progenitors in an undifferentiated state over many weeks and passages. In addition, we

are able to induce differentiation of these progenitor cells under defined conditions into putative Purkinje cells and granule cells as well as astroglia. The neurons generated reach terminal differentiation as indicated by upregulation of specific markers including neurotransmitter receptors. We also show the dynamics of the differentiation of Purkinje cells, granule cells and glial cells from mitotic precursors. Clonal experiments provided evidence that a significant proportion of the neuroepithelial cells behave like cerebellar stem cells *in vitro*. This system provides a model to study the generation of two independent neuronal subtypes and may give insights into the factors that regulate cerebellar development.

2.2 Materials and Methods

2.2.1 Animals and Culture Medium

Time mated Sprague Dawley rats were obtained from RCC Ltd (BRL Fullingsdorf, Switzerland). Female rats were mated overnight and 14:00 hours the following day counted as embryonic day 0.5. Pregnant females were sacrificed and E14.5 embryos excised into L-15 medium (Gibco). The embryos were freed from extra-embryonic tissue and the neural tube excised. The cerebellar primordium forming the roof of the fourth ventricle, posterior to the midbrain was isolated and incubated in 1ml of enzyme mix (2mg/ml collagenase typeIV (Worthington Biochemical Corp.), 1.2mg/ml hyaluronidase type IV-S (Sigma) and 0.3mg/ml trypsin inhibitor (Sigma) diluted in DMEM) for 10min. at 37°C. Subsequently, 9ml of DMEM/F12 medium containing 20% FCS (Sera-Tech) was added, the cells collected by centrifugation at 80g for 5min. and 9ml of the supernatant were aspirated and the cells resuspended into the remaining 1 ml of medium and triturated with a fire polished pipette. The cells were collected by centrifugation at 80g for 5min. and resuspended in 5ml of DMEM/F12 medium containing N2 supplement, 20ng/ml FGF2, 20ng/ml human EGF, 25mM KCl.

2.2.2 Cerebellar Progenitor Cell Isolation and Culture

Cells isolated form the E14.5 rat cerebellar primordium were plated in 5ml of serum-free, defined medium (DMEM/F12, N2 supplement, 20ng/ml FGF2,20 ng/ml human EGF, 25mM KCl) onto uncoated tissue culture dishes (Corning). After 2 hours an equal

volume of DMEM/F12 medium containing 20% FCS was added. During the first week of culture the medium was exchanged every second day with DMEM/F12 medium containing 20% FCS, the cells adhered to the uncoated plastic and proliferated to form an epithelial monolayer. The cells were passaged by trypsinization, resuspended in DMEM/F12 medium containing 20% FCS and plated on to poly-L-lysine/laminin coated culture dishes and cultured in DMEM/F12 medium with 20% FCS or differentiation medium (DMEM/F12, N2 supplement, 20ng/ml FGF2, 20ng/ml human EGF, 25mM KCl, 100ng/ml IGF-1, 10ng/ml NGF, 10ng/ml BDNF, 40ng/ml NT3). The medium was exchanged every second day.

Embryo Preparation and Sectioning.

Time-mated females were sacrificed and embryos excised into cold PBS. Embryos were freed of extra-embryonic tissue, mounted in OCT (TissueTech) and stored at -70°C. Twenty µm sections were thaw mounted onto Superfrost slides (Mettler) and air dried. Sections were fixed for 15min. in 4% paraformaldehyde and washed in PBS. Sections were incubated in PBS containing 10% goat serum (blocking buffer). Sections were stained according to the immunocytochemistry protocol described below.

2.2.3 Antibodies and Immunocytochemistry

For analysis of differentiation, cells were fixed after 5 days for 10min. in 4% formaldehyde washed in PBS and blocked for 1 hour in PBS containing 0.2% Triton-X100, 10% goat serum (blocking buffer). Primary antibodies were diluted in blocking buffer, incubated either 1-2 hours at room temperature or overnight at 4°C washed in PBS 0.2% Tween20 and detected by incubation for 1 hour at room temperature with Cy3 conjugated goat anti-mouse Ig antibodies (Jackson ImmunoResearch Labs) or FITC conjugated goat anti-rabbit Ig antibodies (Jackson ImmunoResearch Labs) diluted 1:100-1:400 in blocking buffer. For immunocytochemical triple-staining two monoclonal antibodies (anti-β-TubulinIII & anti-Calbindin D28k or anti-Calbindin D28k & anti-nestin) together with a polyclonal anti-GFAP (Dako, 1:500) were used. In order to separate the staining of the two monoclonal antibodies the procedure was performed in two steps. First the staining with one monoclonal antibody was done using an anti-IgG-HRP secondary antibody (Dako 1:500). For colorimetric detection of the peroxidase

AEC (Sigma) was used as a substrate. The colorimetric reaction was developed to complete saturation. Then the cells were washed 5 times in PBS and reblocked in PBS, 0.2% Triton-X100, 5% normal goat serum. The second step of the staining procedure was performed as described before using the Cy3 or FITC-coupled secondary antibodies. After washing with PBS 0.2% Tween20 coverslips were mounted with AF1 (Citifluor) containing Hoechst dye. Pictures were taken with a Hamamatsu CCD camera and image processing was performed with Adobe Photoshop (Adobe) software. Cells were quantified by counting random fields. Total cell number was assessed by Hoechst staining of nuclei and specific antibodies used for cell type identification. Primary antibodies included the monoclonal antibodies, anti-nestin (R401, Developmental Studies Hybridoma Bank, 1:30), anti-Calbindin D28k (Sigma, 1:100), anti-β-TubulinIII (Sigma, 1:300), anti-GFAP (Sigma, 1:200), O4 (provided by Dr. M. Schwab, Brain Research Institute, Zurich University, 1:10, cells were stained with O4 in the absence of detergent) and the polyclonal antibodies, anti-Map2 (Sigma, 1:100) and anti-GABAα6R (1:200) (provided by Dr. F.A. Stephenson, London School of Pharmacy).

2.2.4 Analysis of Cell Proliferation by BrdU Incorporation

Cerebellar progenitor cells were treated with BrdU (Roche Diagnostics) for the stated period of time. At the end of the labeling period the cells were washed twice with fresh medium and either fixed or chased with medium without BrdU. Cells were fixed in ethanol -20°C for 30min., washed in PBS and detected according to manufacturers instructions (Roche Diagnostics) using AEC (Sigma) as substrate. The cells were subsequently fixed with 4% formaldehyde and incubated with primary antibody and stained as described above.

2.2.5 PCR Analysis of Purkinje Cell Differentiation

Cells were lysed in 500µl 5M Guanidinium Isothiocyanate buffer and RNA prepared according to the acid-phenol chloroform method (Chomczynski and Sacchi, 1987). cDNA was generated from 1µg of total RNA using mouse MLV reverse transcriptase according to the manufacturers instructions (Roche Diagnostics). The cDNA was standardized by 25 cycles of linear GAPDH PCR: annealing 53°C 1 minute, extension

72°C 1 minute and denaturation 95°C 1 minute. Rat Mash-1 and Calbindin D28k were amplified using 40 cycles: annealing 58°C 1 minute, extension 72°C 1 minute and denaturation 95°C 1 minute. Primer sequences were: Mash-1 5' primer 5'-agcagctgctggacgagca-3', 3' primer 5'-cctgcttccaaagtccattc-3'; Calbindin D28k 5' primer 5'-ctgcaccatggcagaatccc-3', 3' primer 5'-gccactgtggtcagtgtcatac-3' and GAPDH 5' primer 5'-tccatgacaactttggcatcgtgg-3', 3' primer 5'-gttgctgttgaagtcacaggagac-3'. Amplified cDNA fragments were separated on 1.5% agarose gels and visualized by ethidium bromide staining.

2.2.6 Clonal Analysis of NEP Cells Differentiation Potential

Cells were expanded as described in presence of 20% FCS. At 80% confluency the cells were replated on PLL/laminin coated dishes at clonal density (10-30 cells per 30mm dish). Single cells were marked after 6h and cultured for additional 10 days. The cells were washed several times with DMEM/F12 before differentiation medium (N2 supplement, EGF (20ng/ml), bFGF (20ng/ml), NGF (20ng/ml), BDNF (20ng/ml), NT-3 (40ng/ml), IGF1 (100ng/ml)) was applied. Half of the differentiation medium was exchanged every second day. Immunocytochemical triple stainings were performed after 8 days of differentiation. Statistical analysis was performed on 240 clones of 3 independent experiments.

2.3 Results

2.3.1 Differentiation within the Cerebellar Primordium *in vivo*

The cerebellar primordium can be identified as a clearly defined structure of the developing rat nervous system as early as E9 where it forms the roof of the fourth ventricle, caudal to the isthmus at the midbrain hindbrain flexure (Altman and Bayer, 1997; Wassef and Joyner, 1997). Although extensive birth dating studies have been performed to determine the temporal and spatial differentiation of the cells that form the cerebellum (Altman and Bayer, 1997), we have used immunocytochemistry to confirm

the earliest appearance of differentiated neurons and glia in the rat cerebellum. We have used antibodies against the neuronal specific isoform III of β-Tubulin (TuJ1) as an early marker of differentiated neurons and Calbindin D28k to identify Purkinje cells in the developing cerebellar primordium. In addition, we have analyzed the appearance of glial fibrillary acid protein (GFAP) as a marker of glial cells and the intermediate filament protein nestin, which is expressed at high levels by undifferentiated cells within the neuroepithelium of the neural tube.

From E10 through to, and including E14.5, the rat cerebellar primordium consists primarily of undifferentiated nestin-positive cells (data not shown and Fig. 2-1A). At E14.5 few β-TubulinIII-positive cells were found in the cerebellar priomordium and Calbindin D28k expressing putative Purkinje cells are also absent (Fig. 2-1B and C). In addition, GFAP expression was undetectable at E14.5 confirming the late onset of gliogenesis in the cerebellum (Fig. 2-1D). These observations are in accordance with the birth dates of the neurons and glial cells in the rat cerebellum (Altman and Bayer, 1997). Hence, we isolated cells from the E14.5 rat cerebellar primordium as a source of cerebellar progenitor cells.

2.3.2 Cerebellar Progenitors can be Maintained in an Undifferentiated State *in vitro*

Cells isolated from the E14.5 rat cerebellar primordium were plated in serum containing medium onto uncoated tissue culture dishes. The cells adhered to the culture plastic and proliferated to form tight epithelial-like clusters that covered the entire culture dish. The subconfluent cells could be passaged onto fresh uncoated culture dishes and maintained in a proliferative state for many weeks (data not shown). The homogeneity of the cultures was assessed by immunostaining with antibodies against nestin, β-TubulinIII, Map2, Calbindin D28k, GFAP and O4. Twenty four hours after passaging the majority of the cells attached to the culture dish and expressed high levels of nestin protein (Fig. 2-2 on page 41A). After 5 days of culture in serum containing medium almost all (>90%) of the cells still expressed nestin (Fig. 2-2B). Less than 1% of the total cells were found to express β-TubulinIII and Map2 (Fig. 2-2C and D). This was also the case for GFAP expressing cells (Fig. 2-2F) and Calbindin D28k-positive, putative Purkinje cells were not detected in these serum containing cultures (Fig. 2-2E).

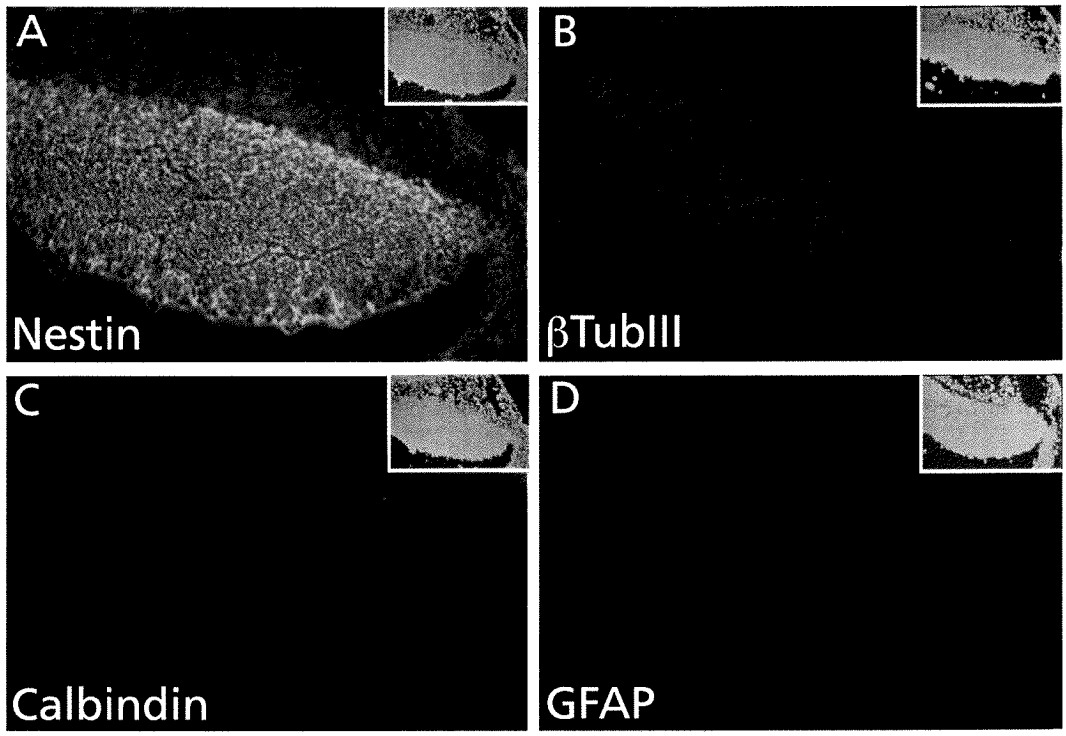

Figure 2-1: Analysis of differentiation in the embryonic day 14.5 cerebellar primordium

Sagittal sections of E14.5 rat cerebellar primordium were immunostained with antibodies against neural differentiation markers. A) Nestin antibodies recognize undifferentiated neuroepithelial cells that comprises the majority of the cerebellar primordium. B) The neuronal marker β-TubulinIII is not detected in the cerebellum at E14.5, however, cells at the isthmus and outside the cerebellum do show expression (not shown). C) Calbindin D28k is a marker for Purkinje cells in the cerebellum. Calbindin D28k-positive cells were not detected in the cerebellum of the E14.5 rat in agreement with previously published data. D) Gliogenesis is mainly postnatal in the CNS and GFAP expressing cells were not detected in the E14.5 cerebellar primordium. Inserts show the corresponding Hoechst staining of the sections.

2.3.3 Culture Substrate Affects Cerebellar Progenitor Cell Differentiation

We addressed the possible effects of culture substrate on cerebellar progenitor cell differentiation. When maintained on uncoated tissue culture plastic the cells remained adherent and expressed nestin as a marker of undifferentiated cells (Fig. 2-3 on page 42A). However, when plated on laminin and cultured in serum containing medium, a marked increase in the number of β-TubulinIII-positive neurons was observed after 5 days (Fig. 2-3B) without affecting the low numbers of Calbindin D28k-positive cells and GFAP-positive glia (data not shown). By contrast, when plated onto poly-L-lysine coated dishes and cultured in serum containing medium, the number of β-TubulinIII-

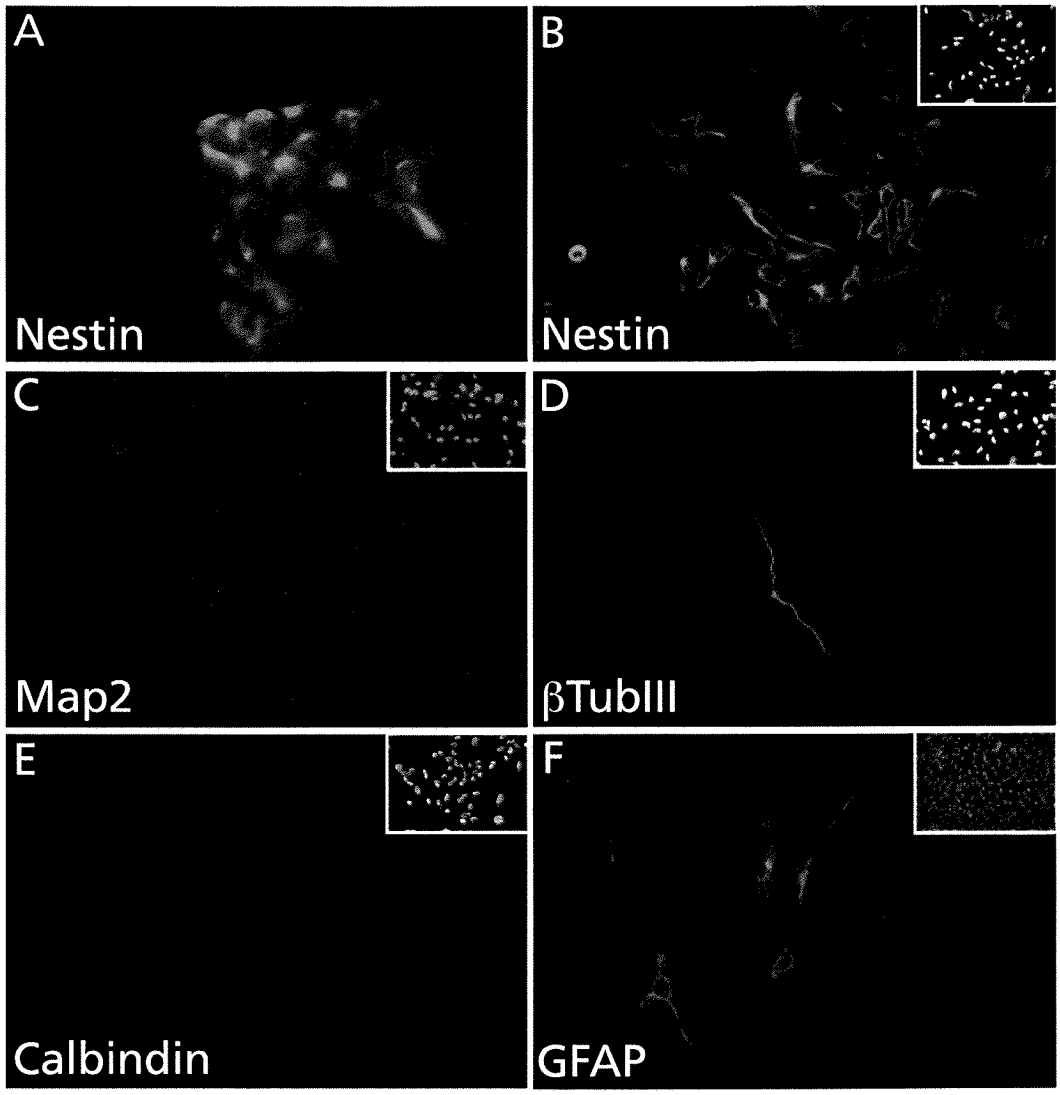

Figure 2-2: Cerebellar progenitor cells remain undifferentiated in serum containing culture medium when grown on uncoated plastic dishes

The majority of the cerebellar progenitor cells are nestin-positive 24 hours after replating (A), and remain nestin-positive *in vitro* even after many days in culture and multiple passages (B shows cells after passaging and 5 days of culture in serum containing medium). In serum containing medium, few β-TubulinIII (C) or Map2 (D)-positive cells were detected after 5 days. E) Calbindin D28k-positive putative Purkinje cells were also absent under these conditions. F) A few GFAP-positive astrocytes were detected but accounted for >1% of the cells. Inserts show the corresponding Hoechst staining of the cells in the field.

positive neurons was unaffected but the number of GFAP-positive cells increased (data not shown).

2.3.4 Cerebellar Progenitor Cell Differentiation is Potentiated by Serum Withdrawal

We assessed the effects of serum on the progenitor cell differentiation. When serum was

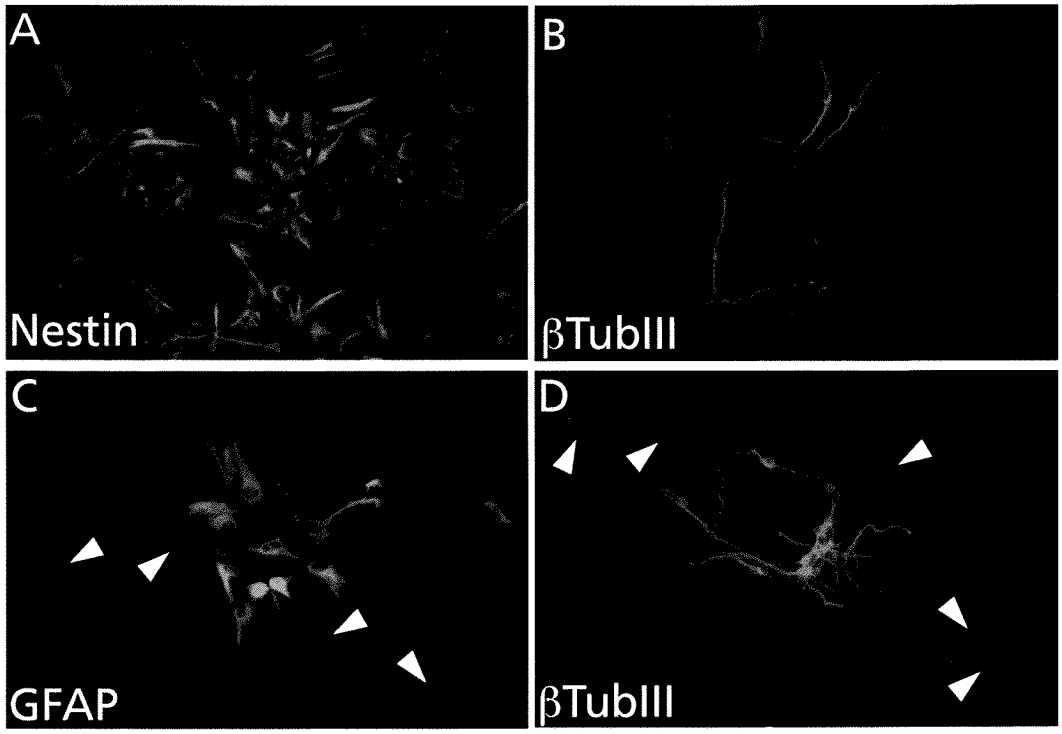

Figure 2-3: The culture substrate and medium affect the differentiation of cerebellar neuroepithelial cells

Cerebellar cells were cultured for 5 days on uncoated (A), laminin (B and D) or poly-L-lysine (C) coated dishes in the presence (A and B) or absence (C and D) of serum. A) In the presence of serum and grown on uncoated dishes >90% of the cells retained expression of nestin with little differentiation. B) When grown on laminin coated plates 3% of the cells expressed the early neuronal marker β-TubulinIII after 5 days. C) Glial differentiation increased in the absence of serum on poly-L-lysine coated plates. D) When cultured in serum free medium on laminin coated plates up to 20% of the cells expressed β-TubulinIII after 5 days. Arrowheads indicate GFAP-negative cells (C) and β-TubulinIII-negative cells (D) under defined medium conditions.

withdrawn and the cells cultured in defined medium the substrate effects became more prominent with increase β-TubulinIII-positive neurons on laminin coated dishes and GFAP-positive cells on poly-L-lysine coated plates (Fig. 2-3C and D). Hence, we plated cells on poly-L-lysine/laminin coated plates to assess differentiation. After 5 days of culture in serum containing medium the cells had proliferated and spread on the poly-L-lysine/laminin coated plates. The cells were fixed and immunostained to identify differentiated and undifferentiated cells. In three independent experiments nestin-positive cells accounted for 90 +/- 1.2% of all cells (Fig. 2-4 on page 43A). Anti-β-TubulinIII and anti-Map2 antibodies identified the same, small population of neurons (3 +/- 0.6%) and Calbindin expressing cells were found at a frequency of 1 +/-0.3% (Fig. 2-4A). GFAP-positive, putative astrocytes made-up 6.6 +/-1.1% of the cells (Fig. 2-4A) and O4-positive cells were not detected (data not shown).

Upon withdrawal of serum and 5 days of culture under defined conditions on poly-L-lysine/laminin coated plates the number of nestin-positive, putative progenitor cells fell to an average of 49 +/-7% in three independent experiments (Fig. 2-4B). The number of β-Tubulin-positive neurons increased on average to 15 +/-8.9% and GFAP-positive cells to 26 +/-8% in five independent experiments (Fig. 2-4B and Fig. 2-5A and G, respectively). Interestingly, Calbindin D28k-positive Purkinje cells only accounted for 1% of the cells in serum containing cultures (Fig. 2-4A) but made-up 25 +/-6.6% of the cells in the defined cultures (Fig. 2-4B and Fig. 2-5D). Comparing the differentiation of the cerebellar progenitors in the absence and presence of serum showed a significant increase in β-TubulinIII-positive neurons ($p<0.05$; Student's t-test, * in Fig. 2-4C) and a highly significant ($p<0.01$; Student's t-test, ** in Fig. 2-4C) increase in the number of Calbindin D28k and GFAP expressing cells in the absence of serum. These increases in differentiated cells was associated with a highly significant decrease in the number of nestin-positive putative progenitor cells in the absence of serum (Fig. 2-4C).

A **Differentiation in 20% FCS containing medium**

exp.	βTubulinIII	Calbindin	GFAP	Nestin
1	2.4% (n=532)	0.8% (n=490)	5.6% (n=558)	88.8% (n=484)
2	3.6% (n=1620)	1.2% (n=1424)	7.8% (n=1464)	90.4% (n=1480)
3	3.0% (n=1602)	1.4% (n=1400)	6.4% (n=1412)	91.2% (n=1399)

B **Differentiation in defined medium**

exp.	βTubulinIII	Calbindin	GFAP	Nestin
1	18.3% (n=1082)	32.4% (n=1027)	17.4% (n=1134)	55.1% (n=1121)
2	5.6% (n=3199)	19.6% (n=1483)	22.8% (n=2091)	nd
3	6.4% (n=513)	28.8% (n=611)	38.5% (n=556)	nd
4	19.9% (n=1007)	19.3% (n=945)	27.6% (n=1376)	41.3% (n=1691)
5	25.8% (n=859)	nd	22.1% (n=1329)	50.3% (n=1359)

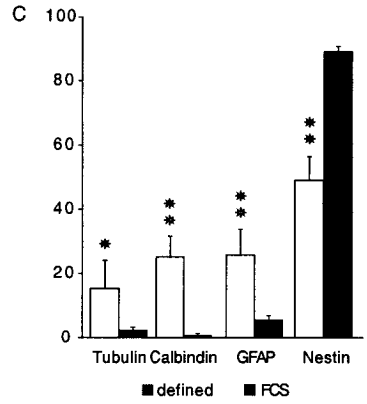

Figure 2-4: Statistical analysis of cerebellar progenitor cell differentiation *in vitro*

A) Results of three independent experiments to estimate the number of β-TubulinIII, Calbindin D28k, GFAP and nestin positive cells in the presence of serum. Numbers are given as the percent positive cells of a total on n cells counted. B) Results of 5 independent differentiation experiments to estimate the number of β-TubulinIII, Calbindin D28k, GFAP and nestin positive cells generated after 5 days of differentiation under defined conditions. Numbers are given as the percent positive cells of a total on n cells counted (n.d.-not determined). C) Graphical representation of the averaged results from A and B indicating an increase in β-TubulinIII and Calbindin D28k expressing neuronal populations and GFAP expressing glial cells at the expense of nestin-positive progenitor cells.

2.3.5 Cerebellar Progenitors Generate Differentiated Granule Cells

In order to identify the type of neurons labeled by the β-TubulinIII and Map2 antibodies and address their differentiation state, we double immunostained cells cultured under defined conditions for 5 days with anti-β-TubulinIII and anti-GABAα6 receptor subunit antibodies (Thompson et al., 1992). The majority of the β-TubulinIII-positive neurons were costained with GABAα6 receptor subunit antibodies (Fig. 2-5A, B and C). This suggested that the β-TubulinIII-positive neurons likely represented granule cells that had progressed to terminal stages of differentiation (Hatten et al., 1997). By contrast, Calbindin D28k-positive putative Purkinje cells were not stained with GABAα6 receptor subunit antibodies (Fig. 2-5D, E and F). In defined medium GFAP expressing cells represented an average of 26% of the cells in cultures after 5 days. Even in the absence of serum, O4-positive putative oligodendrocytes were not detected in the cultures plated on poly-L-lysine/laminin after 5 days of differentiation (Fig. 2-5H).

2.3.6 Undifferentiated Cerebellar Progenitors are Mitotically Active *in vitro*

In order to address the mitotic activity of the progenitors that give rise to the differentiating cells and the birth date of the cells in the defined cultures, we performed BrdU labeling experiments. The cells were exposed to BrdU that was added to the defined differentiation medium either for the first 12 hours after plating or in the last 12 hours of culture after 4.5 days. The cells were subsequently fixed after a total of 5 days in defined culture medium and immunostained with lineage specific antibodies and BrdU incorporation assessed with an anti-BrdU antibody.

When the cells were exposed to BrdU during the last 12 hours of culture starting 4.5 days after replating, 52 +/-7.2% of the nestin-positive cells had incorporated BrdU (Fig. 2-6). This indicated that the majority of the nestin-positive cells in the cultures are mitotically

Continued **Fig. 2-5:** D) Calbindin D28k immunostaining identifies a population of cells with highly branched processes (arrow indicates Calbindin D28k-negative, GABAα6 receptor-positive neurons, arrowhead indicates a negative cell). E) These cells are not stained with GABAα6 receptor (arrow indicates a GABAα6 receptor-positive neuron, arrowhead indicates a negative cell) or β-TubulinIII antibodies (not shown). F) Overlay of Calbindin D28k and GABAα6 receptor immunostaining in D and E showing that the antibodies recognize different cell populations. G) GFAP-positive astrocytes represent a substantial proportion of cells after 5 days of differentiation. H) O4-positive putative oligodendrocytes are not detected in differentiated cerebellar progenitor cell cultures.

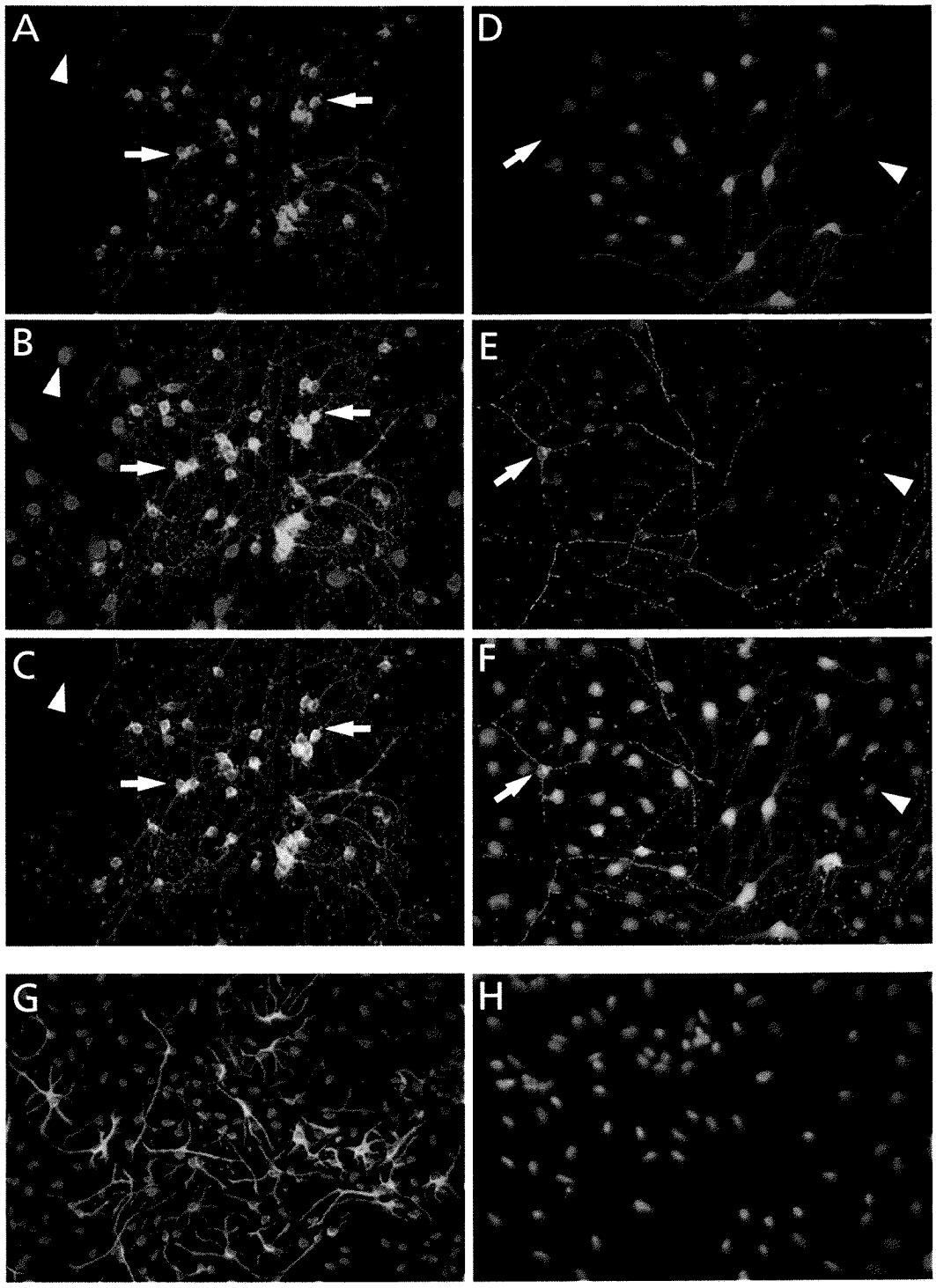

Figure 2-5: Cerebellar progenitor cells generate differentiated granule and Purkinje cells *in vitro*

A) After 5 days under differentiation conditions a population of neurons are identified with β-TubulinIII antibodies (arrows indicate β-TubulinIII-positive neurons, arrowhead indicates negative cells). B) Immunostaining with anti-GABAα6 receptor antibodies identifies the same population of neurons (arrows indicate GABAα6 receptor-positive neurons, arrowhead indicates negative cells). C) Overlay of β-TubulinIII and GABAα6 receptor immunostains in A and B (arrows indicate β-TubulinIII, GABAα6 receptor double positive neurons, arrowhead indicates negative cells). *Continued on previous page.*

active after 5 days (Fig. 2-6). However, when pulsed with BrdU in the first 12 hours of culture and analyzed after 5 days only 14.5 +/-5.3% of the nestin-positive cells showed a strong incorporation of BrdU (Fig. 2-6) whereas the other cells labeled with the nestin antibody displayed a weak incorporation of BrdU. We interpret this to indicate the rapid proliferation of the undifferentiated cells in these cultures. The BrdU incorporated during the first 12 hours is likely diluted between the progeny during cell divisions over the subsequent 4.5 days.

A

	βTubulinIII		Calbindin		GFAP		Nestin	
BrdU pulse	0-12h	4.5-5d	0-12h	4.5-5d	0-12h	4.5-5d	0-12h	4.5-5d
exp.1	42	5	5.5	1.5	6	10	22	47
exp.2	43	7	9	2	6	15	7	57
average	42.5	6	7.25	1.75	6	12.5	14.5	52

B

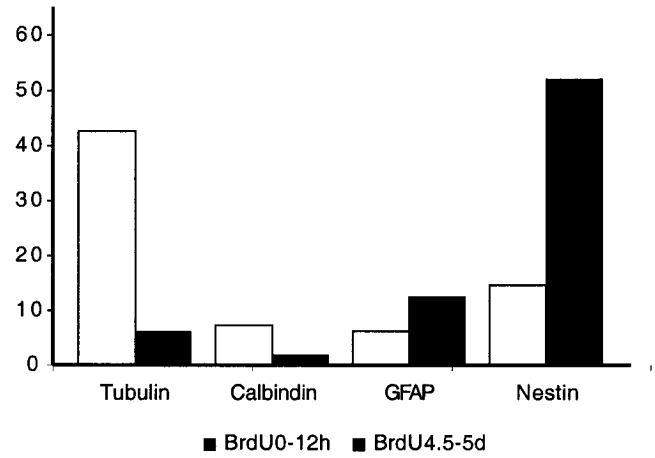

Figure 2-6: BrdU analysis of the differentiating cerebellar progenitor cell cultures

Cerebellar progenitor cells were transferred to defined differentiation medium and exposed to a 12 hour pulse of BrdU during the first 12 hours of culture (0-12h) or after 4.5 days (4.5-5d). The cells were subsequently fixed after 5 days and differentiation and BrdU incorporation analyzed. A) The results of two independent experiments showing the percentage of β-TubulinIII, Calbindin D28k, GFAP or nestin-positive cells that had incorporated BrdU during the first 12 hours (0-12h) or last 12 hours (4.5-5 days) of differentiation. B) Graphic representation of the averaged results shown in A indicate that the majority of the nestin expressing cells are mitotically active throughout the culture (the low levels of BrdU positive cells after BrdU exposure at 12 hours may represent dilution of the stain due to continued cell proliferation). Over 40% of the β-TubulinIII expressing neurons are derived from cells which pass through the cell cycle in the first 12 hours after onset of differentiation. The majority of Calbindin D28 and GFAP expressing cells do not incorporate BrdU during the first or last 12 hours of culture.

2.3.7 Granule Cells Differentiate from Mitotic Progenitors *in vitro*

Analysis of BrdU incorporation into β-TubulinIII-positive, putative granule cells showed

that an average of 6 +/-1.4% passed through the cell cycle during the last 12 hours in culture (Fig. 2-6). However, 42.5 +/-0.4% of the β-TubulinIII-positive cells incorporated BrdU when exposed during the first 12 hours after plating (Fig. 2-6). As β-TubulinIII is a differentiation marker it is unlikely that these neurons are entering the cell cycle but rather are derived from progenitors that replicate during the BrdU treatment period. Extrapolating from the 4.5-5 day BrdU treatment where approximately 6% of the β-TubulinIII expressing neurons incorporated BrdU, it is likely that the majority of granule cells are derived from progenitors that are mitotically active through the culture period. In addition, 42.5% of the granule cells are derived from cells that divide in the first 12 hours.

By contrast, only 1.75 +/-0.4% of the Calbindin D28k-positive cells incorporated BrdU during the last 12 hours of culture (Fig. 2-6). Furthermore, only 7.25 +/-1.2% of the putative Purkinje cells incorporated BrdU during the first 12 hour labeling period. One interpretation of these results is that the Purkinje cells are derived from progenitors that are mitotically inactive. Another possibility could be that Purkinje cells are derived from mitotic precursors during a restricted time window and that these precursors lose the BrdU incorporated during the first 12 hours due to dilution in a similar fashion to that seen with the nestin-positive cells.

2.3.8 Differentiated Purkinje cells are Generated late in the Cultures

In order to address the putative time point at which Purkinje cells are generated from the cerebellar progenitor cells *in vitro*, we used RT-PCR to analyze the appearance of Mash-1 and Calbindin D28k transcripts. Mash-1 is a proneural gene of the basic helix-loop-helix family of transcription factors that is expressed in putative Purkinje cell precursor cells (Guillemot and Joyner, 1993). RT-PCR revealed a basal expression of Mash-1 in the cultures maintained in DMEM/F12 containing 20% FCS (Fig. 2-7). This basal expression was also evident during the first 1.5 days of culture in defined differentiation conditions. Subsequently, Mash-1 levels increased on day 3 and 5, respectively. A similar pattern was also observed for the expression of Calbindin D28k, which was detected at low levels in the presence of serum and during the first 3 days in defined medium, but increased dramatically by day 5 (Fig. 2-7). This would suggest that

Purkinje cell precursors increase in the cultures after 3 days under defined conditions and differentiated Calbindin D28k-positive Purkinje cells are generated mainly between day 3 and 5. Linear GAPDH RT-PCR analysis of expression was used to monitor cDNA levels in the reactions and was comparable in each sample (Fig. 2-7).

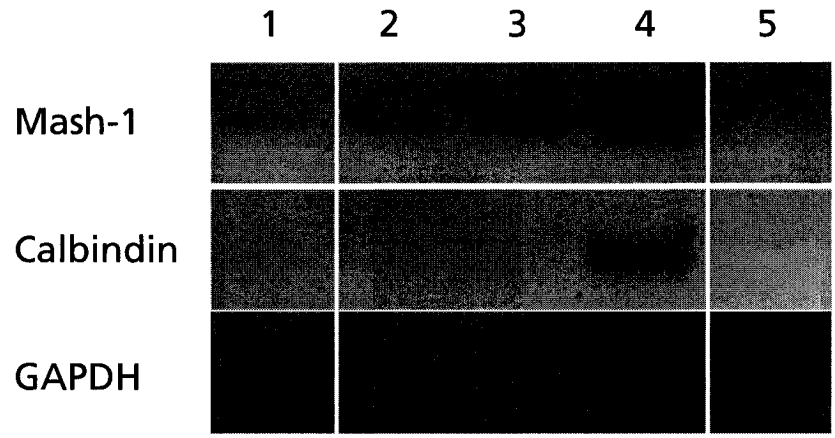

Figure 2-7: RT-PCR analysis of Purkinje cell differentiation from cerebellar progenitor cells *in vitro*

Cerebellar progenitor cells were passaged from serum containing medium into either fresh serum containing medium or defined differentiation medium. RNA was isolated after 1.5, 3 and 5 days and cDNA generated and used as a template for PCR reactions to amplify Mash-1, Calbindin D28k and GAPDH. Mash-1 expression was detected at low basal levels in serum containing medium (lane 1) and after 1.5 days in defined medium (lane 2). Mash-1 expression increased on day 3 and day 5 under defined differentiation conditions (lane 3 and 4, respectively). Calbindin D28k expression was maintained at very low levels in serum containing medium (lane 1) and during the first 3 days of culture in defined differentiation medium (lane 2 - 1.5 days, lane 3 - 3 days). Between day 3 and day 5 of differentiation, Calbindin D28k expression increased dramatically (lane 4). Linear GAPDH PCR indicated that similar amounts of cDNA were used in each reaction. Control reactions were performed on RNA without reverse transcription (lane 5 -RT).

2.3.9 Glial Cells Incorporate BrdU during the 5 day Culture

The cerebellar neuroepithelial cells generate not only neurons but also glial cells *in vitro* (Fig. 2-2 and Fig. 2-3). Therefore, we analyzed the incorporation of BrdU into GFAP expressing cells. When exposed to BrdU during the last 12 hours of culture 12.5 +/-3.5% of the glial cells had passed through the cell cycle (Fig. 2-6), suggesting that these glia were either derived from a mitotic precursor during this period or that these GFAP cells were themselves mitotically active. When pulsed with BrdU during the first 12 hours of culture 6 +/-0% of the GFAP-positive cells had incorporated BrdU (Fig. 2-6). These data

indicate that the glial cells are either generated from progenitors that are not proliferating, or, more likely, that the BrdU incorporated during this period is diluted during subsequent cell divisions.

2.3.10A Significant Proportion of NEP Cells Display Stem Cell-like Properties

In order to determine the differentiation potential of a single NEP cell, clonal analysis experiments were performed. After an initial expansion period the cells were replated on PLL/laminin coated dishes at clonal density, single cells marked, and cultured in FCS containing medium for 10 days. Differentiation was induced after this period by withdrawal of serum. Analysis revealed that 90% of the clones contained nestin expressing cells (Fig. 2-8C). Around 70% of the clones contained Calbindin-positive cells and about 20% also contained β-TubulinIII-positive putative granule cells (Fig. 2-8C). However, the proportion of neuronal cells within the clones was relatively low compared to the bulk culture experiments. Interestingly β-TubulinIII expressing granule cells were only found in clones that formed tight epithelia-like structures. Putative astrocytes were found in approximately 40% of the clones (Fig. 2-8C). The immunocytochemical analysis revealed that most of the clones contained at least 2 different cell types (96%). We also show that 10% of the clones contained nestin-, GFAP- and β-TubulinIII-expressing cells (Fig. 2-8C). Furthermore, 12% of the clones contained putative Purkinje and granule cells as well as astrocytes (Fig. 2-8A-C). Hence, as more than 70% of the clones contained Calbindin expressing cells and over 90% of the total clones contained nestin-positive cells, it is likely that more than 10% of the clones contained undifferentiated cells, Purkinje cells, granule cells and astrocytes. This indicates that at least 10% of the cultured NEP cells maintain the ability to produce multiple neuronal-types and glial cells *in vitro*.

2.4 Discussion

Elucidation of the mechanisms regulating cell fate determination and differentiation in the vertebrate nervous system are central to understanding development and potential therapeutic approaches for diseases of the nervous system (McKay, 2000a; McKay,

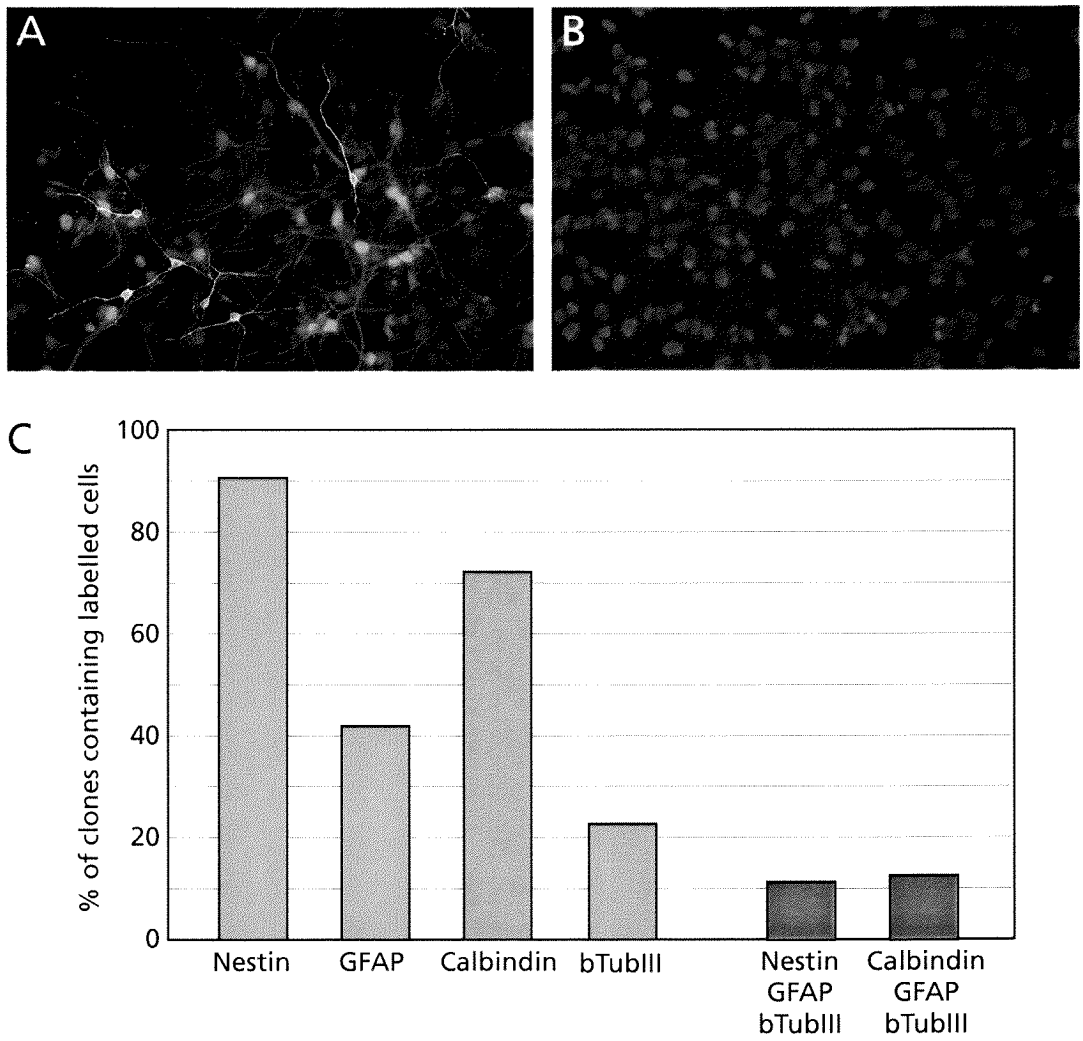

Figure 2-8: Clonal analysis of cerebellar NEP differentiation potential *in vitro*

Cerebellar progenitor cells were replated at clonal density onto PLL/laminin coated dishes and clones were expanded in presence of 20% FCS. After 10 days differentiation was induced by withdrawal of serum. After 10 days of differentiation the clones were analyzed by immunocytochemistry. A) Triple-staining for Calbindin D28 (red), β-TubulinIII (white) and GFAP (green) showing a clone derived from a multipotent progenitor. B) DAPI staining of clone in A. C) Bar diagram showing the percentage of clones containing cells that express indicated markers. 90% of the clones contain nestin-positive cells. 10% contain undifferentiated and all of the differentiated cell types.

2000c). We have focused on the cerebellum as a model to understand the processes of cell fate determination and differentiation that generate the vertebrate brain. The cerebellum is a highly organized structure consisting of five neuronal types that are arranged into well defined layers. The development of the cerebellum has been extensively studied and detailed patterns of cell differentiation, migration and gene expression have been determined (reviewed in Hatten et al., 1997; Hatten and Heintz, 1995). The cerebellar primordium can be identified early in vertebrate development as

the structure anterior to the point where the neural tube fails to close above the fourth ventricle and posterior to the midbrain hindbrain flexure.

Detailed studies have shown that the isthmic organizer at the midbrain hindbrain boundary is a source of FGF8 signaling that is required to induce cerebellar formation (Martinez et al., 1999). We have shown that Notch1 signaling plays a central role in regulating the onset of neurogenesis in the cerebellar neuroepithelium (Lütolf et al., in press). However, the genes that regulate neuroepithelial cell fate and Purkinje cell versus granule cell differentiation are not clear. Recent data indicate that the basic helix-loop-helix transcription factor Math-1 is required for granule cell formation in the cerebellum and Math-1 gene ablation results in a lack of EGL and granule cells in the mouse (Ben-Arie et al., 1997). It is currently unclear which factors regulate the expression of Math-1 and hence determine granule cell fate in the cerebellar neuroepithelium *in vivo*. However, it has been shown that members of the Bone Morphogenetic Protein (BMP) family can induce Math-1 expression in ventral midbrain neuroepithelial cells *in vitro* and that these treated cells can generate cerebellar granule cells when transplanted into the postnatal EGL of host mice (Alder et al., 1999). Although, BMPs are expressed by the roof plate cells and the meningeal cells adjacent to the rhombic lip (Alder et al., 1999), it is unclear whether BMP family members regulate granule cell fate *in vivo*.

Therefore, we have established a cell culture system where undifferentiated progenitor cells can be maintained in adhesive culture in order to study potential factors that regulate neuronal differentiation in the cerebellum. We show that cerebellar progenitor cells can be isolated and maintained in culture over many weeks and passages and that they retain expression of the intermediate filament protein nestin. These progenitor cells are mitotically active and do not express markers of differentiated neural cells in subconfluent cultures. By contrast, if the cells are maintained in a confluent state, spontaneous differentiation is often observed, with neurons appearing as clusters on top of the confluent progenitor cells (data not shown). These findings indicate that these cells retain their neurogenic potential *in vitro*.

Therefore we have examined the neurogenic and gliogenic potential of these cells and their potential as a system to analyze cerebellar differentiation. We have shown that these progenitor cells can be induced to differentiate by changing the culture environment and medium. Many of the previous experiments in other cell culture systems have indicated

that various growth factors are able to regulate the differentiation of neural progenitors (reviewed by Gage, 1998; Gage, 2000; McKay, 2000b). We show that the substrate on which the cells are grown can also influence the differentiation of progenitor cells into either neuronal, in the case of laminin, or glial lineages in the case poly-L-lysine. This is intriguing and suggests that extracellular matrix interactions in combination with paracrine growth factors may be involved in neural differentiation and cell fate.

In addition, the cerebellar progenitor cells can be induced to differentiate into different neuron types and glial cells. Previous studies examining neurogenesis from CNS progenitor cells have mainly addressed neuronal versus glial differentiation and only recently have different neuronal populations, generated *in vitro*, been analyzed (Vicario-Abejon et al., 2000). We have been able to identify neuronal subtypes including β-TubulinIII, GABAα6 receptor subunit-positive, putative granule cells and Calbindin D28k-positive Purkinje cells in our cultures. Hence, the cerebellar progenitor cells retain the potential to generate the two major neuronal population of the cerebellum. It is envisaged that these cells will be useful to determine the factors that modulate cell fate and potentially neuronal and glial lineage determination. It is currently unclear whether Purkinje cells and granule cells are derived from multipotent cerebellar progenitors that become independently determined to a specific fate *in vivo* or whether the two neurons are generated from fate-restricted precursors. However, we provide evidence that at least a subpopulation of the cerebellar NEP cells display stem cell like properties and are able to generate both neuronal cell types as well as glial cells. It remains to be established whether there is a relationship between the differentiation of granule cells and Purkinje cells in these cultures, or between neurogenesis and gliogenesis. To address such questions further clonal as well as co-culture experiments of NEP cells with Purkinje cells, granule cells or astrocytes will be required.

We were unable to detect O4-positive cells in our culture suggesting that the cerebellar progenitor cells isolated from the dorsal neural tube are unable to produce oligodendrocytes. Direct support for this finding comes from data indicating that oligodendrocytes are generated from neuroepithelial cells of the ventral neural tube (Pringle et al., 1998; Pringle et al., 1996; Richardson et al., 1997). This suggests that the oligodendrocytes found in the white matter of the cerebellum may migrate in from the ventral midbrain hindbrain region of the neural tube or from more distal regions.

The cerebellum is an ideal system to study neurogenesis in the CNS, however, little is known about the factors that determine Purkinje cell and granule cell fate of the neuroepithelial cells. We have established a cell culture system from which we can generate Purkinje cells and granule cells as well as glia. A large proportion of these NEP cells show multipotency and over 10% retain the ability to generate putative Purkinje cells, granule cells and astroglia *in vitro*. This system opens up the possibility to evaluate neurogenic factors and gene function *in vitro*. The results of these experiments may be relevant not only for cerebellar development but also for differentiation in other regions of the CNS.

2.5 OUTLOOK

It is envisaged that the cerebellar NEP cell culture system will be useful to determine the mechanisms involved in cell fate and lineage determination, differentiation, proliferation and survival. These processes can be analyzed by the application of various growth factors or by overexpression studies using constitutively active or dominant negative forms of growth factor receptors or transcription factors. We could show earlier that cerebellar NEP cells are highly infectable by retroviruses and that they are susceptible to DNA transfection methods (data not shown). In order to analyze these developmental processes bulk and clonal culture experiments will be necessary.

2.5.1 TESTING GROWTH FACTORS IN THE NEP CULTURE

The cells will be treated with candidate factors already during expansion in order to "prime" the multipotent precursors before the induction of differentiation. During differentiation the cells then will be cultured in presence or absence of the candidate factors. Analysis of the experiments will be mainly by immuno-histochemistry using specific antigenic markers described above but also by RT-PCR where specific antibodies are not available. In the following paragraphs some candidate growth factors are described.

Bone Morphogenetic Proteins

BMP2 and BMP4 have been shown to be important for the dorsalization of the neural

tube (Barth et al., 1999; Liem et al., 1995; Nguyen et al., 1998) and that they can promote neural crest cell formation from CNS stem cells (Molne et al., 2000; Mujtaba et al., 1998). There is recent evidence that BMP2 and BMP4 are involved in proliferation and fate determination of neural precursor cells when signalling via the BMP receptor 1a (Bmpr-1a). Sequential signaling through Bmpr-1b then leads to mitotic arrest and terminal differentiation (Panchision et al., 2001). Whether a neural progenitor proliferates or leaves the cell cycle seems to be dependent on the ratio of Bmpr-1a and Bmpr-1b. Panchision et al. also showed that in transgenic mice overexpressing a constitutively active form of Bmpr-1a cells from the anterior alar plate were transformed into choroid plexus precursor cells.

It would be interesting to investigate the effects of BMP2 and BMP4 on the cerebellar NEP cells in a dose dependant manner. Currently three hypothesis can be postulated: As mentioned these factors could have a dorsalizing effect on cerebellar NEP cells. Similar to the results published by Mujtaba et al., the NEP cells could be pushed towards neural crest which could be monitored by the molecular marker p75. A second possibility would be that the cells would turn into choroid plexus cells as it has been described previously (Panchision et al., 2001). This switch could be determined by RT-PCR for Foxj1. The third hypothesis is that the NEP cells still give rise to cerebellar cell types but that the differentiation rate of the different cells is altered. This could be analyzed by the same set of markers already used to describe the cell culture system. Preliminary experiments have shown that when BMP2 is added to the differentiation medium, differentiation into cerebellar cell types is decreased. However, it is not yet clear which cell types are produced, since the population of Nestin-expressing cells is also reduced (data not shown).

Alder et al. (1996) have shown that the granule cell precursors located at the rhombic lip belong to a distinct population of cells. Furthermore they provided evidence that BMP7 is important for the upregulation of Math1 in these cells (Alder et al., 1999). On the other hand, we have shown by clonal analysis that more than 10% of the cultured cells are multipotent and give rise to putative granule cells and Purkinje cells indicating that these neurons are generated by only one type of neural progenitor in the neuroepithelium. Treating clonal neuroepithelial cells with BMP7 will reveal whether this factor is able to push multipotent cells towards the granule cell lineage. If so, more β-Tubulin III

expressing cells would be expected compared to control cultures. This increase in granule cell number may be generated in expense of Purkinje cells or astrocytes which will be assessed by using the molecular markers Calbindin D28 and GFAP.

Sonic hedgehog (Shh)

Shh has been demonstrated to be produced by migrating and settled Purkinje cells and to act as a mitogen for granule cell precursors (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999). Furthermore, Shh is able to induce differentiation of Bergmann glia (Dahmane and Ruiz-i-Altaba, 1999). Therefore, the application of Shh to the cerebellar NEP cells may lead to a larger fraction of granule cells within the culture, which may be shown by immunohistochemical stainings for β-Tubulin III and GABAα6 receptor subunit in conjunction with a quantification of proliferation by BrdU incorporation analysis. An increase in radial glial cells could be monitored using RC-2 antibodies.

Ciliary Neurotrophic Factor (CNTF)

CNTF has been shown to increase Purkinje cell survival *in vitro* and also to promote the generation of astrocytes (Larkfors et al., 1994). Preliminary results using our culture system confirm the results of this study. However, it is not yet known whether CNTF acts as a mitogen for certain progenitor cells or whether it influences differentiation of uncommitted NEP cells at the expense of another cell population.

Factors for Receptors Identified in the RTK-Screening

In the receptor tyrosine kinase screening project we identified several receptors expressed during cerebellar development (see Table 1.1). Therefore, the application of factors binding to receptors from the erbB family, the PDGF receptors or Axl and Tyro3 could reveal interesting functions of these signaling pathways.

For the differentiation of the NEP cells we use poly-L lysine and laminin as substrates and we have shown that these substrates influence the differentiation of cerebellar NEP cells. In the RTK screening we have found DDR-1 and DDR-2, both of which are activated by collagens. In order to show the full potential of the cerebellar NEP cells it will be necessary to plate the cells on substrates other than PLL and laminin.

2.5.2 Infection or Transfection with Transcription Factors or Growth factor Receptors

Basic helix loop helix transcription factors like Math1, Mash1 and NeuroD are known to influence cerebellar development at various stages (see chapter 1.2). In our laboratory we have identified Mist-1, another member of this family which is expressed by granule cell precursors in the external germinal layer. Currently no function of Mist-1 has been reported in the CNS. Infection of the cerebellar NEP cells with a Mist-1 expressing retrovirus could provide first hints about the function of this transcription factor in the granule cell lineage. Double transfection experiments in cerebellar NEP cells suggest an interplay between Mist-1, Math-1 and NeuroD which show overlapping expression patterns (Diploma thesis S. Brack 2000, ETH Zürich).

In a similar approach to the one used by Panchision et al., dominant active and negative forms of receptor tyrosine kinases could be used to study the effects of different signaling cascades during the lineage determination and cell differentiation of cerebellar NEP cells. Again interesting candidates have been identified in the screening project for receptor tyrosine kinases. Additional pathways like the BMP or the Shh signaling mechanism have already been mentioned (see 2.5.2). Furthermore, overexpression of Notch signaling pathway components could reveal interesting functions of these factors in cell differentiation and lineage determination (see chapter 3).

2.5.3 Establish the Cerebellar NEP Culture for Mouse Cells

A third way to investigate the function of signaling pathways in the cerebellar NEP cell culture system is to culture cells from mice that carry a targeted mutation within these pathways. Therefore, it will be important to establish the cerebellar NEP cell culture system for mouse NEP cells. Preliminary results have shown that it is possible to isolate the neuroepithelium from mouse embryos as early as E10.5. Culturing these cells in defined medium leads to differentiation in a similar manner to that seen in the rat cerebellar NEP cell cultures. Conditions to keep the cells in an undifferentiated state but still competent to differentiate after induction remain to be identified.

2.5.4 Cocultures with Choroid Plexus Cells

The fact that the cells at the rhombic lip belong to a distinct cell population giving rise to the granule cells (Alder et al., 1996) leads to the question which signals are involved in specifying these cells and from where these signals originate. One could imagine that the signals are only provided by the cells of the cerebellar primordium and consist of contact dependent and independent mechanisms. However, another source of signals could be provided by the choroid plexus (CP) a structure that is attached to the rhombic lip and to date has been poorly investigated. In preliminary experiments, we have isolated choroid plexus cells from E14.5 rat embryos and cultured them under undefined conditions in presence of 20% fetal calf serum. These cells survived and proliferated, however, passaging seemed to be problematic. The application of conditioned medium from CP cells or the co-culture of cerebellar NEP cells together with these cells could reveal whether signals from this source can influence the differentiation of cerebellar NEP cells *in vitro*.

With the experiments described in sections 2.51-2.5.4 it may be possible to analyze the roles of several signaling cascades in cellular processes like fate determination, differentiation, survival and proliferation. Furthermore, they may lead to the identification of factors that are involved in the generation of granule cell or Purkinje cell precursors from multipotent NEP cells.

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3 NOTCH1 AND THE LIGANDS DELTA AND SERRATE-RELATED ARE EXPRESSED IN DISTINCT CELL POPULATIONS IN THE POSTNATAL BRAIN

3.1 INTRODUCTION

Notch signaling has been shown to play a pivotal role in the regulation of differentiation in the nervous system of both invertebrates and vertebrates (reviewed by Artavanis-Tsakonas et al., 1999). Through the process of lateral inhibition Notch signaling in cells of the Drosophila proneural cluster in the ectoderm selects one of the multipotent progenitors to differentiate into the sensory organ precursor while repressing differentiation of the neighboring cells (reviewed by Kimble and Simpson, 1997). Although Drosophila has a single Notch, mammalians have four Notch-related genes (Notch1-4) (Lardelli et al., 1994; Weinmaster et al., 1991; Weinmaster et al., 1992; reviewed by Weinmaster, 1997) that display a high degree of structural conservation to each other and to their Drosophila and C. elegans homologues (Fig. 3-1A). They also are structurally related to their ligands belonging to the Delta-like and the Jagged family in the sense that Notch receptors are single transmembrane proteins containing a large number of tandemly arranged extracellular EGF-repeats. However, Notch receptors in addition contain a LNR (Lin Notch Repeat) region on the extracellular side and on the intracellular side, six ankyrin repeats, an OPA, and a PEST (proline, glutamine, serine threonine) domain are located. Notch1-3 expression has been shown to be associated with mitotic and neurogenic regions of the developing nervous system (Irvin et al., 2001). Notch1 (Conlon et al., 1995; de la Pompa et al., 1997; Swiatek et al., 1994) and

Notch2 (Hamada et al., 1999) are critical for embryonic development and Notch3 is involved in the human disease CADASIL (Joutel et al., 2000; Joutel et al., 1996). Other Notch related diseases are T-cell leukemia (Ellisen et al., 1991), Allgaille's syndrome (Li et al., 1997; Oda et al., 1997), and syndactylism (Sidow et al., 1997). Additionally, Notch processing seems to be related to Alzheimer's disease mechanisms since it has been shown that Notch activation is dependent on Presinilin1 protease (Kopan and Goate, 2000; Soriano et al., 2001). Notch4-deficient mice suggest that Notch4 and Notch1 may play overlapping roles in embryonic vascular morphogenesis (Krebs et al., 2000). Recently, we have shown that signaling through Notch1 performs a similar regulation of differentiation within the midbrain hindbrain neuroepithelium of embryonic mice (Lütolf et al., in press). Hence, in multipotent progenitor cells of the invertebrate and vertebrate nervous systems, Notch signaling may perform a conserved function to regulate differentiation (reviewed by Artavanis-Tsakonas et al., 1999).

A family of six Delta, Serrate-related proteins has also been found in vertebrates (Chitnis et al., 1995; Henrique et al., 1995; Lindsell et al., 1995; Nye and Kopan, 1995) (Fig. 3-1B). Ligands of both families contain a DSL (Delta/Serrate/Lag-2) domain which seems to be involved in receptor recognition and binding. The principle difference between the two types of ligands is that those of the Serrate-type harbour cysteine-rich (CR) domains, where as the others do not. Furthermore, the short intracellular domains display little structural conservation. Naturally occurring and targeted mutations in the genes of the Delta-like (Dll) family show embryonic lethality or severe skeletal abnormalities (Bulman et al., 2000; Hrabe de Angelis et al., 1997; Kusumi et al., 1998). Furthermore, the results of other studies including targeted gene ablations of the Serrate-related homologues Jagged1 and Jagged2 confirm a crucial role for Notch signaling in many steps of embryonic development (Jiang et al., 1998; Lanford et al., 1999; Li et al., 1997; Oda et al., 1997; Sidow et al., 1997; Xue et al., 1999).

In addition to a regulatory role in modulating onset of differentiation, further experiments have shown that, as in the *Drosophila* sensory organ, Notch signaling may also be involved in regulating cell fate in the vertebrate nervous system (Chambers et al., 2001; Furukawa et al., 2000; Gaiano et al., 2000; Lundkvist and Lendahl, 2001; Morrison et al., 2000; Tanigaki et al., 2001). Activation of Notch signaling results in promotion of glial cell formation from multipotent progenitors in the telencephalon,

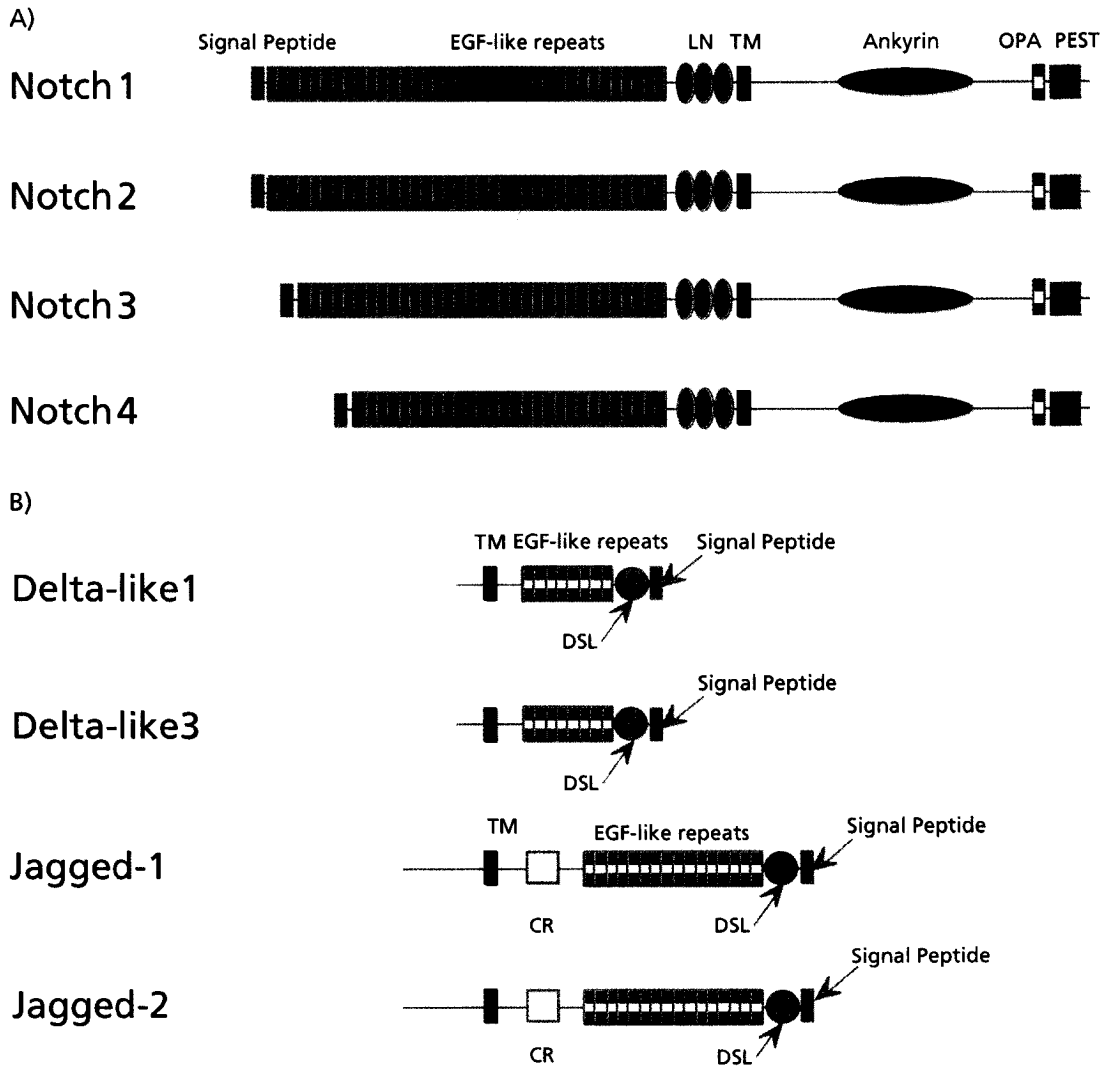

Figure 3-1: The mammalian Notch receptors and their ligands from the DSL-family

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 and Jagged2) have an additional cystein-rich region in front of the transmembrane domain (TM). The short cytoplasmic domains show only little structural conservation.

retina and neural crest (Chambers et al., 2001; Furukawa et al., 2000; Gaiano et al., 2000; Morrison et al., 2000; Tanigaki et al., 2001). In addition, conditional ablation of Notch1 from neuroepithelial cells *in vivo* results in a loss of glial cells suggesting an inherent requirement of Notch1 signaling for gliogenesis (Lütolf et al., in press).

The mechanisms involved in activation of Notch signaling are also conserved from invertebrates to vertebrates (reviewed by Chan and Jan, 1998; Kopan and Goate, 2000; Lendahl, 1998). Interaction of Notch and its transmembrane ligands in invertebrates

constitutes a reciprocal signaling pathway between neighboring cells (Heitzler et al., 1996; Heitzler and Simpson, 1991; Kunisch et al., 1994; Simpson et al., 1992). The Notch protein is post-translationally modified by cleavage in the extracellular domain by a Furin-like protease found in the endoplasmic reticulum/Golgi compartments of cells. The two portions of the Notch receptor associate and are expressed at the cell surface as a heterodimer. Activation of the receptor by binding to a ligand presented by a neighboring cell results in two additional proteolytic cleavages including a final activation by a γ-secretase that may include members of the Presenilin family (reviewed by Kopan and Goate, 2000; Selkoe, 2000) and the release of the intracellular domain (ICD) of Notch into the cytoplasm. The known components downstream of Notch have been elucidated by molecular biological and genetic approaches in insects and worms, and involve a number of transcriptional regulators (reviewed by Bray, 1998; Kimble and Simpson, 1997; Kopan and Goate, 2000; Lewis, 1996) (Fig. 3-2). The ICD of Notch translocates to the nucleus and associates with members of transcription regulator family CSL. This complex activates the transcription the Hairy, Enhancer of split-related (HES) genes which belong to the basic helix-loop-helix transcription factors (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). In heterodimeric complexes with TLE proteins (Chen and Courey, 2000) they down-regulate the expression of Achaete-Scute-complex (AS-C) genes including neurogenin and Mash1. Since the AS-C proteins regulate the expression of a number of neural genes, this is thought to be a mechanism by which Notch signaling suppresses neurogenesis in flies and also in mammals (Yao et al., 2000).

Members of the Hes gene family have been described to be expressed in the nervous system during embryonic development and are associated with the proliferating neuroepithelium (Ohtsuka et al., 1999). Loss-of-function mutations demonstrated that both Hes1 and Hes5, the predominantly expressed Hes genes in the neural tube, negatively regulate the onset of neurogenesis *in vivo* (Cau et al., 2000; Ohtsuka et al., 2001). Loss of Hes1 and Hes5 results in a reduction in neural stem cells in the compound mutant (Ohtsuka et al., 2001). By contrast, Hes3-deficient mice do not show and obvious phenotype, however, Hes1/Hes3 double mutants show a patterning defect in the neural tube with ablation of the midbrain hindbrain region (Hirata et al., 2000). Due to the central role of the proneural genes in the induction of neurogenesis and regulation of cell

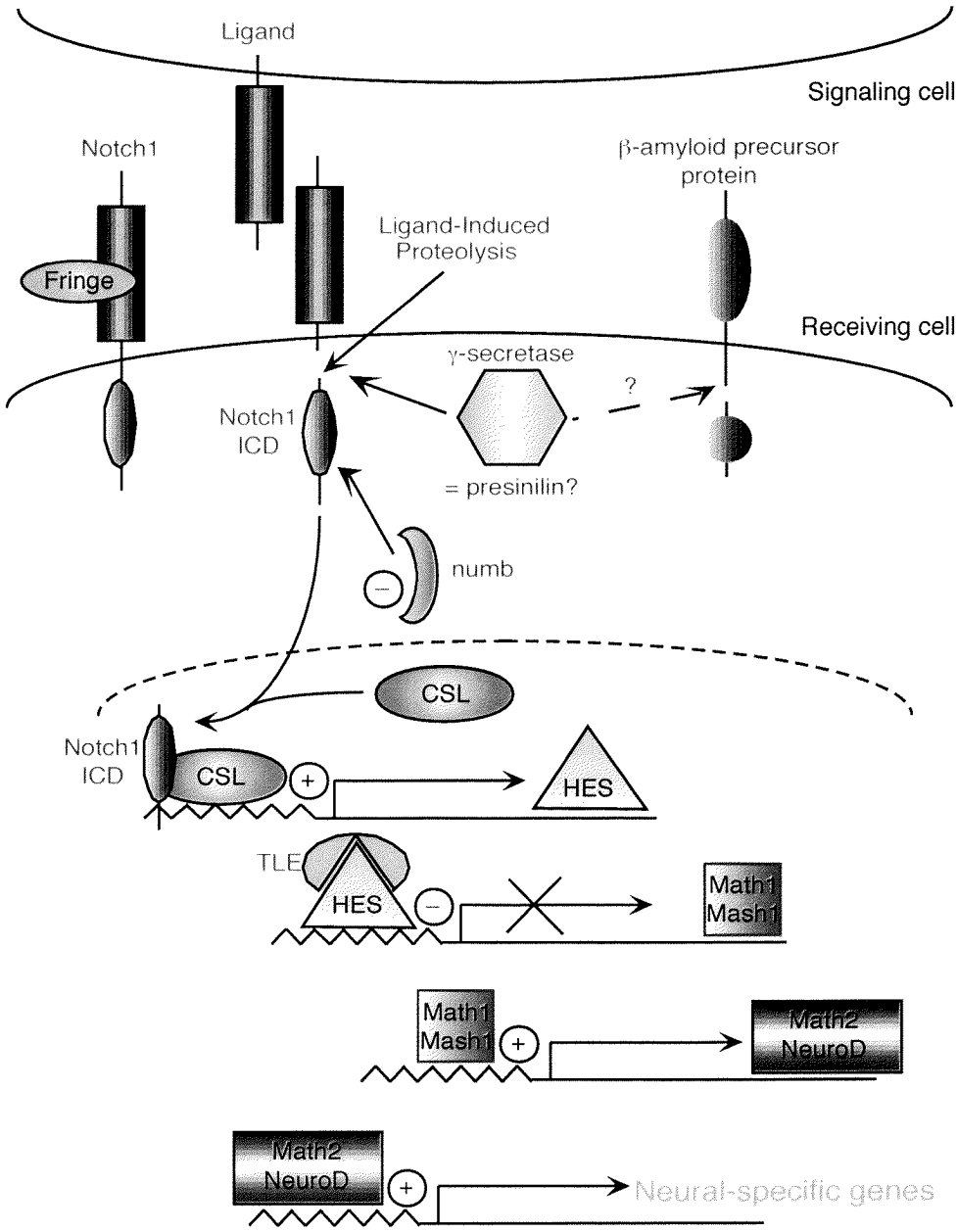

Figure 3-2: The Notch signaling cascade and the hypothetical role of Presinilin1

Notch is activated by its ligands on neighbouring cells. Activation of Notch results in cleavage of the intracellular domain (ICD) by a γ-secretase (Presinilin1 ?), which also processes β-amyloid precursor protein. The Notch ICD then 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 aquire a neuronal fate.

fate in the nervous system, activated Notch signaling represses neural differentiation and promotes secondary cell fate (Cau et al., 2000; Lee, 1997; Nieto et al., 2001; Ohtsuka et al., 2001; Satow et al., 2001).

The Notch signaling pathway is connected with several other signaling cascades like the BMP or the LIF signaling pathway (reviewed in (Morrison, 2001). Furthermore, the Wnt-signaling pathway inhibits Notch signaling via desheveld that blocks the intracellular domain of Notch1 (Axelrod et al., 1996; De Strooper and Annaert, 2001). In addition the intracellular proteins Numb and Numblike are able to regulate the intracellular signal by binding to the cytoplasmic domain of active Notch thus preventing interaction with CSL protein. Numb and Numblike are widely expressed throughout the embryonic nervous system with overlapping patterns. Recent data have shown that the ablation of Numb results in embryonic lethality before E10.5 (Zhong et al., 2000; Zilian et al., 2001). The embryos show defects in the generation of the vasculature and aberrant anterior neural tube formation (Zhong et al., 2000; Zilian et al., 2001).

Gain-of-function experiments in CNS neurons *in vitro* suggest that Notch signaling may also play an important role in later stages of neural development (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). Overexpression of an intrinsically active form of Notch1 in primary cortical neurons and a neuroblastoma cell line resulted in altered neurite formation (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). Activation of Notch signaling suppresses dendrite extension and promotes branching *in vitro* (Franklin et al., 1999). These experiments suggest that the interaction of Notch expressing dendrites with ligand expressing neuronal processes may regulate dendritogenesis *in vivo* (Berezovska et al., 1999; Redmond et al., 2000; Sestan et al., 1999).

In order to gain a better understanding of the functions of Notch signaling in the brain, we have examined the expression patterns of Notch1, Notch3 and their ligands in early postnatal and adult animals. In addition, we have analyzed the activation of the classical Notch pathway by addressing the expression of Hes genes in the postnatal brain. We show that Notch1 is extensively expressed at the mRNA level throughout the early postnatal brain. Notch1 is particularly prominent in the subventricular zone and sub-granule layers of the dentate gyrus throughout postnatal development as is its down-stream 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 expression of ligand.

3.2 Materials and Methods

In situ Hybridization Analysis of Gene Expression

Embryos and brains were isolated and frozen in OCT (TissueTech) on dry ice. Twenty µm frozen sections were thaw mounted onto Superfrost slides (Mettler), air-dried, and fixed in 4% paraformaldehyde. The midline parasagital sections of the adult brain were orientated between the cerebellar peduncles. In situ RNA hybridization was performed with digoxigenin-labeled RNA probes for Notch1, Notch3, Dll1, Dll3, Jagged1, Jagged2, Hes1, Hes3, Hes5, Numb and Numblike overnight at 72°C in buffer containing 50% formamide, and detected using an anti-DIG-AP antibody according to manufacturer's instructions (Roche Diagnostics). Expression was detected by colorimetric reaction with NBT (340mg/ml) and BCIP (165mg/ml) as reaction products. Coverslips were mounted in glycerol and images taken using an Axioplan microscope in conjunction with and Axiocam CCD camera. Image analysis was performed with Photoshop 5.0 software.

3.3 Results

3.3.1 Expression of Notch1 Transcripts in the Early Postnatal Brain

Currently little is known about the roles of Notch signaling in neural development besides the regulation of embryonic progenitor cell differentiation. Recent experiments indicate that Notch signaling may play a role in postmitotic neurons by regulating neurite formation. Therefore, we addressed the expression patterns of Notch1 and its putative ligands in the postnatal brain of mice. At P4 Notch1 transcripts are prominently expressed in the mouse brain. High levels of Notch1 transcripts are detected in the subventricular zone (SVZ), the region that gives rise to the olfactory neurons and contains putative stem cells of the postnatal brain (Fig. 3-3A). Notch1 expression is also

associated with cells lining the ventricular zone overlying the SVZ and the lateral ventricles over the hippocampal structure (Fig. 3-3B). Punctate expression of Notch1 mRNA is evident in the dentate gyrus but is not associated with the majority of granule cells (Fig. 3-3C). Notch1 transcripts are also evident in cells along the rostromigratory stream (RMS) towards the olfactory bulb (Fig. 3-3A). Notch1 coincides with expression of the ligand Jagged 1 in the SVZ and dentate gyrus where Jagged1 transcripts are mainly localized to granule neurons and neurons of the CA3 region (Fig. 3-3D, E). Deltalike1 as well is expressed by cells in the ventricular zone (Fig. 3-3F) however, in the hippocampus its localization is mainly restricted to the CA1 region and in the dentate gyrus no expression was detected (Fig. 3-3G). By contrast, Jagged 2 mRNA is only detectable in the pyramidal neurons of the P4 hippocampus (data not shown). The other ligands for Notch1, Dll1, Dll3 and Jagged2 are not detectable in the SVZ at P4 (data not shown).

3.3.2 NOTCH1 EXPRESSION IN THE ADULT BRAIN

In the adult Notch1 transcripts remain prominently expressed in the germinal zones as well as in the RMS (Fig. 3-4A). Within the SVZ, clusters of cells lying adjacent to the ventricle lining express Notch1 mRNA (Fig. 3-4A, B). A few cells lining the lateral ventricles above the hippocampus also retain expression of Notch1 (Fig. 3-4A). Within the hippocampus, small clusters of Notch1 expressing cells can be detected in the subgranule cell layer, another region of continued neurogenesis in the adult brain (Fig. 3-4C). In addition, scattered cells can also be found in the brain outside the SVZ as well as in the hippocampus (Fig. 3-4B, C). As in the early postnatal brain, Jagged1 expression can also be detected in cells scattered in and around the SVZ (Fig. 3-4D) and by the granule cells of the dentate gyrus (DG) (Fig. 3-4E). As in the P4 brain expression of the Dll ligands were not detected in the SVZ or DG and Jagged2 transcripts are restricted to the granule cells and pyramidal neurons throughout the hippocampus (Fig. 3-4F).

Fig. 3-3 on page 68: Notch1, Jagged1, Deltalike1 expression in the P4 brain

A) Notch1 is expressed in the subventricular zone (SVZ), dentate gyrus (DG) and rostromigratory stream (RMS). B) Notch1 expressing cells in the SVZ are associated with the ventricle lining and the subependymal layer. C) Notch1 expressing cells in the DG are associated with the granule cell layer, subgranule cell layer and the stratum radialis. D) Jagged1 is expressed in the ventricular lining and subependymal cells of the SVZ and by the granule cells of the DG (E). F, G) Deltalike1 is expressed in the early postnatal SVZ and the CA1 region of the hippocampus but not in the dentate gyrus.

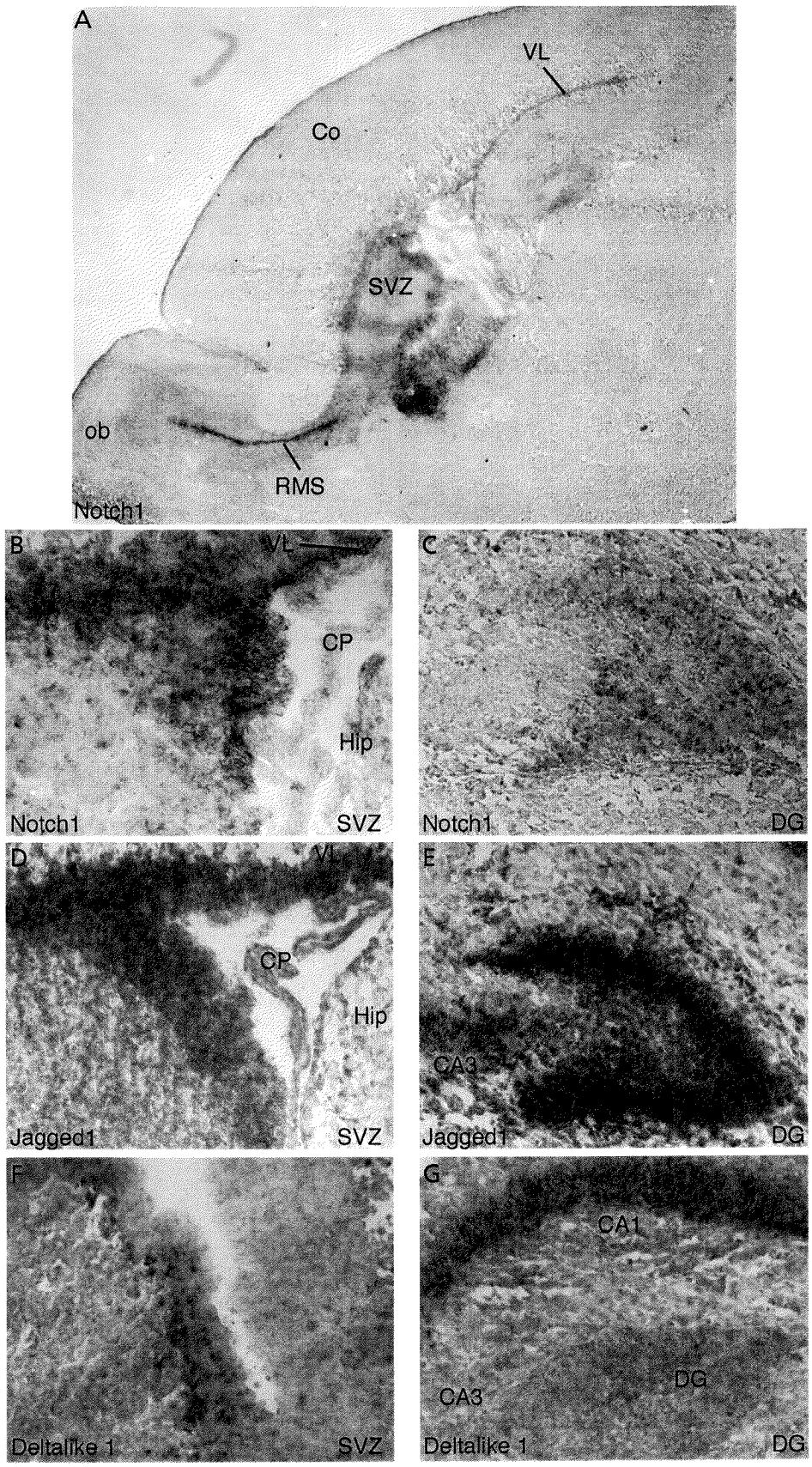

Figure 3-3: Notch1, Jagged1 and Deltalike1 expression in the P4 brain

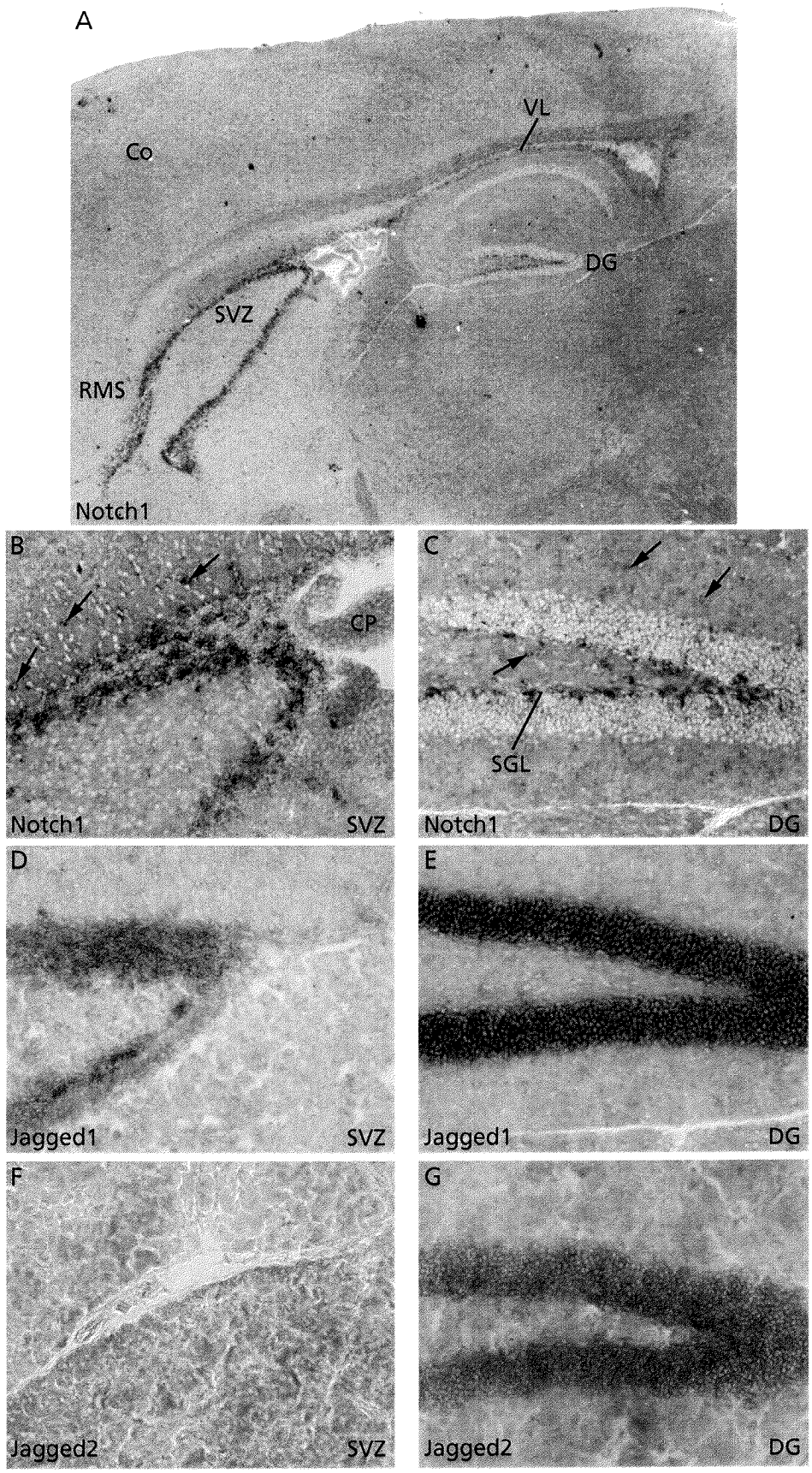

Figure 3-4: Notch1, Jagged1 and Jagged2 expression in the adult brain

3.3.3 HES mRNA Expression Indicates Active Notch Signaling in the Postnatal Brain

In order to address whether the expression of Notch1 and its ligands within the postnatal brain is associated with activation of the Notch signal cascade, we analyzed the expression of the HES genes. The Hes genes are the first known downstream targets of the Notch cascade. By contrast to the expression of Notch1 and Jagged1 in the subventricular zone and dentate gyrus, Hes1 mRNA was not detected in the P4 forebrain (Fig. 3-5A, B). However, Hes5 mRNA was detected in the SVZ and lining of the lateral ventricles is a similar pattern to that of Notch1 (Fig. 3-5C and Fig. 3-3B). Furthermore, expression of Hes5 in the dentate gyrus showed a similar distribution to that of Notch1 in clusters of cells under the granule cells of the dentate gyrus (Fig. 3-5D). In addition, as with Notch1 expression, Hes5 mRNA was found in cells scattered throughout the tissue surrounding the SVZ and within the hippocampus. Hes3 mRNA was not detected in the forebrain of P4 animals confirming the restricted expression described previously (data not shown).

Notch signaling has been shown to regulate the activity of a number of intrinsic factors of the basic helix-loop-helix family (reviewed by Robey, 1997). However, activity of the Notch receptor itself is also modulated by a number of intracellular proteins (reviewed by Kimble and Simpson, 1997). Numb and Numblike are cytoplasmic proteins that bind to the intracellular domains of Notch receptors and negatively regulate transduction of the signal to the nucleus (Wakamatsu et al., 1999; Zhong et al., 1996). In addition, it has

Fig. 3-4 on page 69: Notch1, Jagged1 and Jagged2 expression in the adult brain

A) Notch1 is expressed in the subventricular zone (SVZ), lining of the lateral ventricles and the dentate gyrus (DG). B) Notch1 expressing cells in the SVZ are mainly associated with the subependymal layers of the SVZ. C) In the GD Notch1 expression can be detected in the subgranule cell layer (SGL) and cells within the stratum radialis. D) Jagged1 expression is maintained in the adult SVZ and is associated with the granule cells of the DG (E). F) Jagged2 is not expressed at detectable levels in the SVZ of adults but can be detected in the granule cells and pyramidal neurons of the hippocampus.

Fig. 3-5 on page 71: Active Notch signaling in the adult forebrain

Hes1 expression was not detected in the SVZ (A) or DG (B) of the postnatal day 4 brain. Hes5 was detected in the SVZ and ventricular lining of the postnatal brain (C). D) In the DG Hes5 is expressed by cells of the subgranule layer (SGL) and cells throughout the stratum radialis and stratum oriens. Numb was not detected in the SVZ but was expressed by cells of the choroid plexus. F) In the DG Numb was detected in the forming granule cell layer and pyramidal cells of the hippocampus. G) Numblike was not found in the SVZ but was detected in cells of the cortex outside the germinal zone. H) In the hippocampus Numblike is expressed in the pyramidal neurons of the CA1-3 and at low levels in the granule cells of the DG.

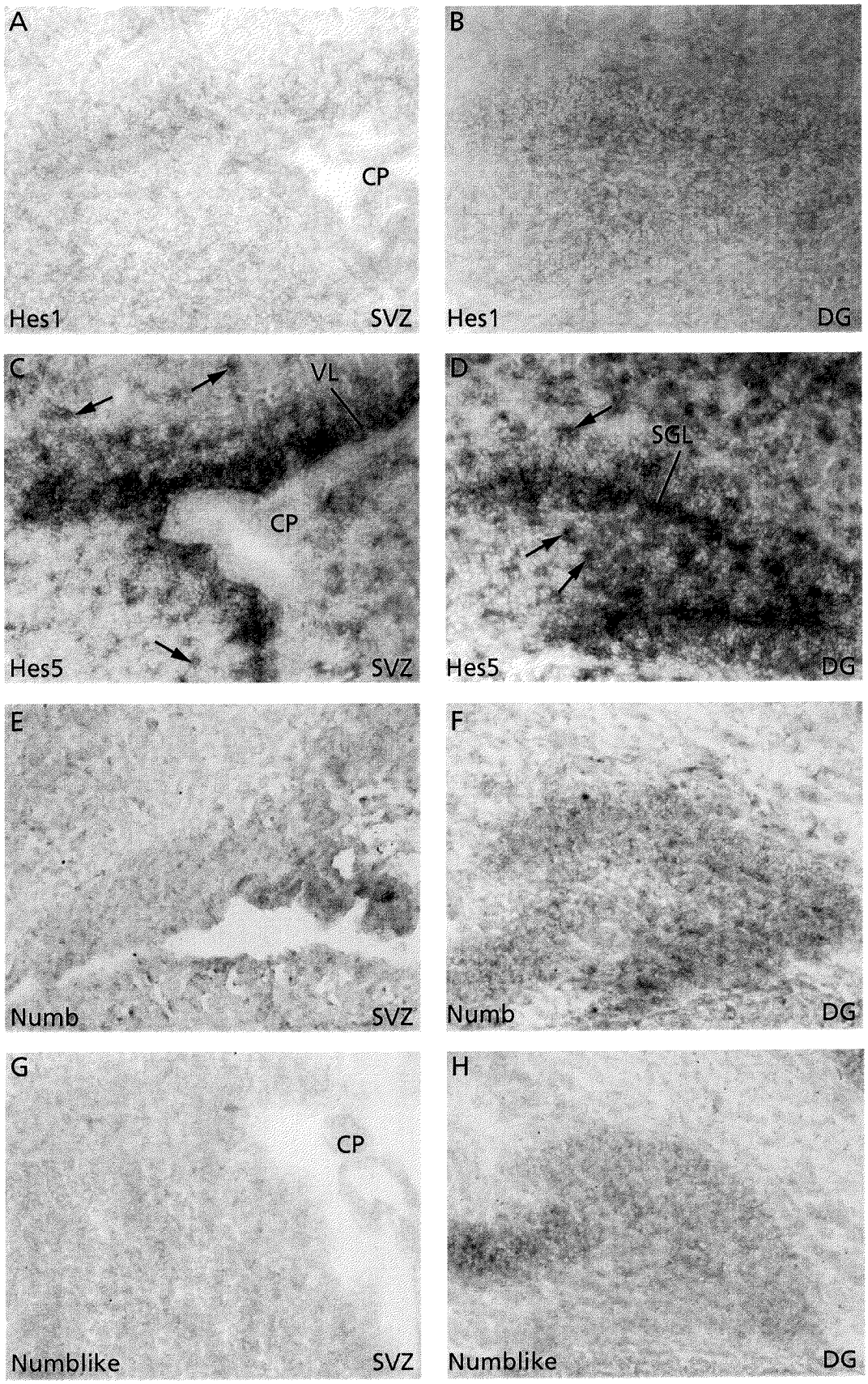

Figure 3-5: Active Notch signaling in the postnatal forebrain

been proposed that Numb and Numblike may be involved in the induction of neurogenesis and cell fate. We examined the expression of Numb and Numblike in the forebrain at P4. Numb transcripts were not detected in the SVZ at P4 and only low level, neuronal associated expression was observed in a punctate pattern within the hippocampus and dentate gyrus. Similarly, Numblike mRNA was found in cells throughout the forebrain but not in the cells of the SVZ (Fig. 3-5G). Numblike expression in the hippocampus was restricted to the pyramidal cells of the CA1-3 with very low levels in the granule cells of the dentate gyrus.

3.3.4 Active Notch Signaling Remains in the Germinal Zones of the Adult Brain

We have shown that notch1 and its ligands are expressed in the potential germinal zones of the adult brain. We examined the expression of the Hes genes in the adult to confirm an active Notch signal in the adult. As in the early postnatal brain Hes1 and Hes3 are not expressed at detectable levels in the SVZ (Fig. 3-6A) or in the adult hippocampus. However, Hes5 is expressed in clusters of cells within the SVZ and in the subgranule cell layer of the dentate gyrus in a similar pattern to that of Notch1. Therefore, we also examined expression of the regulatory molecules Numb and Numblike in the adult forebrain. As in the P4 brain Numb mRNA was not detectable in the SVZ (Fig. 3-6E) and only a very low levels in cells scattered throughout the dentate gyrus. Similar, to our findings at P4 Numblike expression was absent from the SVZ (Fig. 3-6G) but was prominent in the pyramidal neurons of the CA1-3 region of the hippocampus (Fig. 3-6H).

Fig. 3-6 on page 73: Active Notch signaling in the adult brain

Active Notch signaling in the adult forebrain. Hes1 is not detectable in the SVZ (A) or DG (B) of the adult brain. C) Hes5 is expressed by clusters of cells in the SVZ. D) In the DG Hes5 is associated with clusters of cells in the subgranule cells layer (SGL). Numb was not detected in the SVZ (E) and was detected at low levels in the granule cell layer of the DG (F). Numblike was not found in the adult SVZ (G) but is expressed in the pyramidal neurons of the CA1-3 and at low levels in the granule cells of the DG (H).

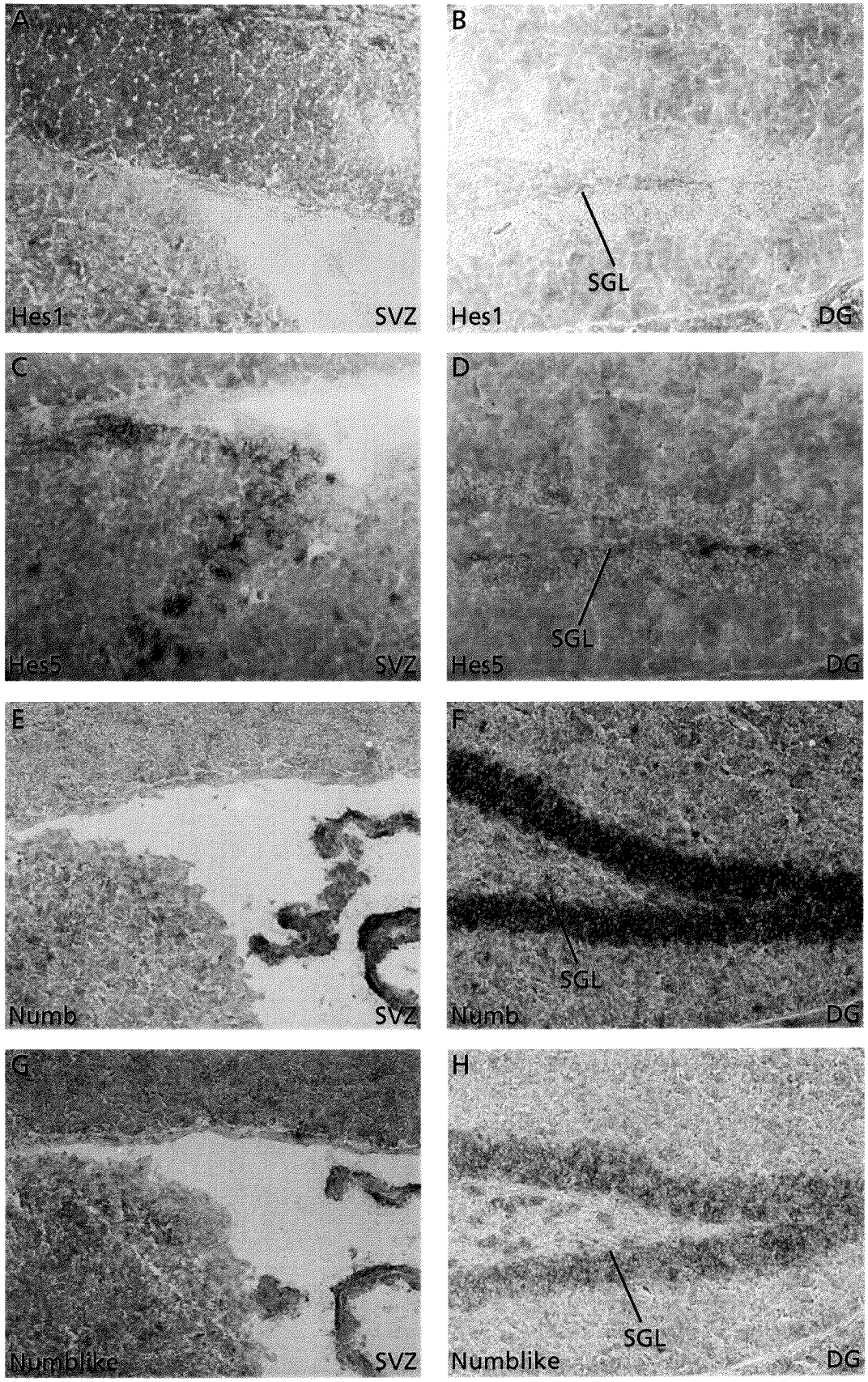

Figure 3-6: Active Notch signaling in the adult brain

3.3.5 Notch Signaling Components are Detectable throughout the Cortex

In vitro gain of function data suggest that Notch signaling may play a role in neurons at later stages of development, particularly in the regulation of dendrite formation. Therefore, we examined the expression of Notch1, its ligands and downstream signaling components in the early postnatal brain. At P4 Notch1 mRNA levels in the cortex were very low and only few scattered cells could be detected by in situ hybridization (Fig. 3-7A).

However, the ligands Dll1, Jagged1 and Jagged2 were all expressed within the cortex. Dll1 shows a rather ubiquitous low level expression throughout the cortical layers with slightly higher levels towards the superficial region of the cortex with the exception of layer 1 (Fig. 3-7B). Similarly Jagged1 is expressed throughout the cortex with a more prominent localization in the outer cortex (Fig. 3-7C). By contrast, Jagged2 is expressed in a homogenous manner in all layer of the cortex (Fig. 3-7D). Although Notch is expressed a very low levels Hes1 and Hes5 were detected in subpopulations of cells in all levels of the cortex (Fig. 3-7E, F). Neither showed a layer specific expression pattern and although some cells in the cortex showed particularly high levels of Hes5 expression most of the cells seemed to be labeled. Similarly both Numb and Numblike mRNAs were detected throughout the cortex with no layer-specific distribution (Fig. 3-7G, H).

3.3.6 Notch Signaling is Active in the Cortex

We examined Notch receptor and signaling component expression in the adult brain to addressed whether Notch signaling remained active. Notch1 was found to be expressed a very low levels with only a few scattered cells in the adult cortex expressing detectable levels of mRNA (Fig. 3-8A). Although Notch1 was not detected to high levels in the adult cortex Notch3 showed a distinct distribution in putative blood vessels running

Fig. 3-7 on page 75: Active Notch signaling in the early postnatal cortex

A) Notch1 is expressed at low levels in the cerebral cortex of postnatal day 4 animals. B) The ligand Dll1 shows an extensive expression throughout the cortex and particularly in the outer layers. C) Jagged1 was detected in all cortical layers in a punctate pattern with major expression in the outer cortical layers. D) Jagged2 shows a wide spread expression pattern throughout the cortex. E) Hes1 was detectable in a few cells of the cerebral cortex. F) Hes5 is expressed by most cells of the cortex and at high levels by a subpopulation of cells (arrow). Both Numb (G) and Numblike (H) show an extensive pattern of expression throughout the cerebral cortex.

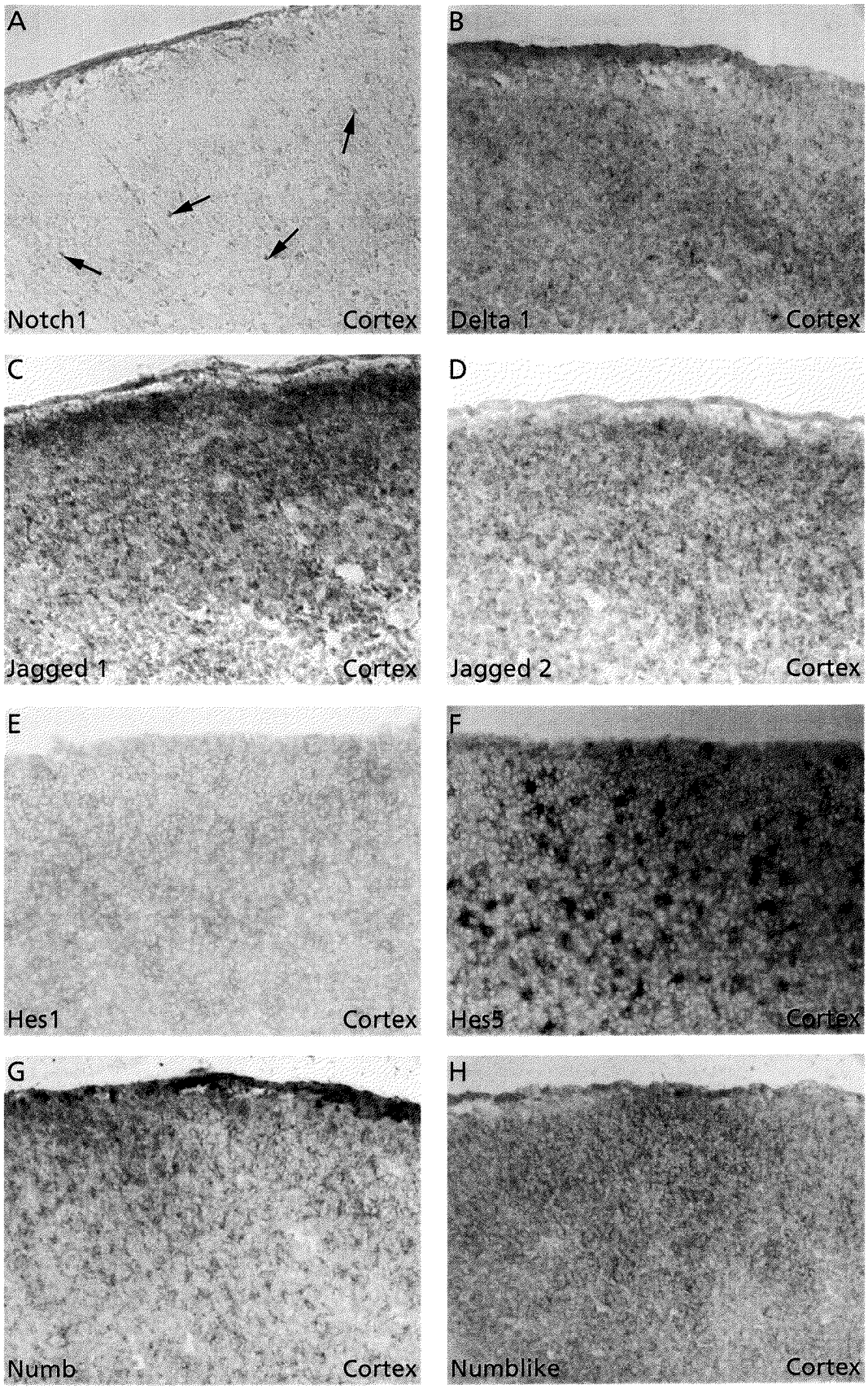

Figure 3-7: Active Notch signaling in the early postnatal cortex

radial in the cortex (Fig. 3-8B). The expression of Notch3 RNA in the blood vessels of the adult is similar to that seen in the P4 brain (data not shown) and reflects the described function of Notch3 mutations in the human stroke disorder CADISIL (Joutel et al., 1996). The ligands for Notch, Dll1, Jagged1 and Jagged2 are also down-regulated in the adult brain compared to early developmental stages but all can be detected in a patchy like expression throughout the cortex (Fig. 3-8C-E). This reduced expression of Notch1 and its ligands was also associated with a reduction in the Hes gene expression and Hes5 could only be detected in scattered cells in the cortex (Fig. 3-8F). The reduced active Notch signaling in the adult cortex was also supported by the expression of Numb and Numblike mRNA in cells of the deeper cortical layers (Fig. 3-8G, H).

3.3.7 Notch Signaling in the Postnatal Cerebellum

The cerebellum is one region of the vertebrate brain where extensive neurogenesis takes place postnatally (reviewed by Hatten et al., 1997; Hatten and Heintz, 1995). We analyzed the expression of Notch signaling components in the developing cerebellum at P4. Notch1 is prominently expressed in the developing cerebellum by the majority of cells in the developing Purkinje cell layer (Fig. 3-9A). In addition, cells within the forming internal granule cell layer and white matter are also express Notch1. It is possible, based on their distribution, that these cells represent immature glial cells of the astrocytic and oligodendrocytic lineages. We also examined the expression of Notch3 and, like Notch1 cells within the forming Purkinje cell layer also expressed Notch3 RNA. However, it is unclear whether these cells are the Purkinje cells or Bergmann glia (Fig. 3-9B). In situ hybridization with probes to the Notch ligands revealed a prominent expression of Jagged1 in the cells of the EGL as well as the granule cells of the IGL(Fig. 3-9C), whereas Jagged2 was expressed by cells in the Purkinje cell layer (Fig. 3-9D). However, it is unclear which cells in the Purkinje cell layer are positive for

Fig. 3-8 on page 77: Active Notch signaling in the adult cerebral cortex

A) Notch1 expression in thee adult cortex is restricted to a few cells scattered throughout the cortical layers. B) Notch3 shows a prominent expression in blood vessels (bv) of the cortex. C) Dll1 is widely expressed in the cerebral cortex and Jagged1 (D) and Jagged2 (E) are detectable a low levels in the inner layers of the cortex. F) Hes5 is expressed at low levels by many cells in the cortex but at particularly high levels in a subpopulation of cells. Both Numb (G) and Numblike (H) show an extensive pattern of expression throughout the cerebral cortex but are spared from the out cortical layer.

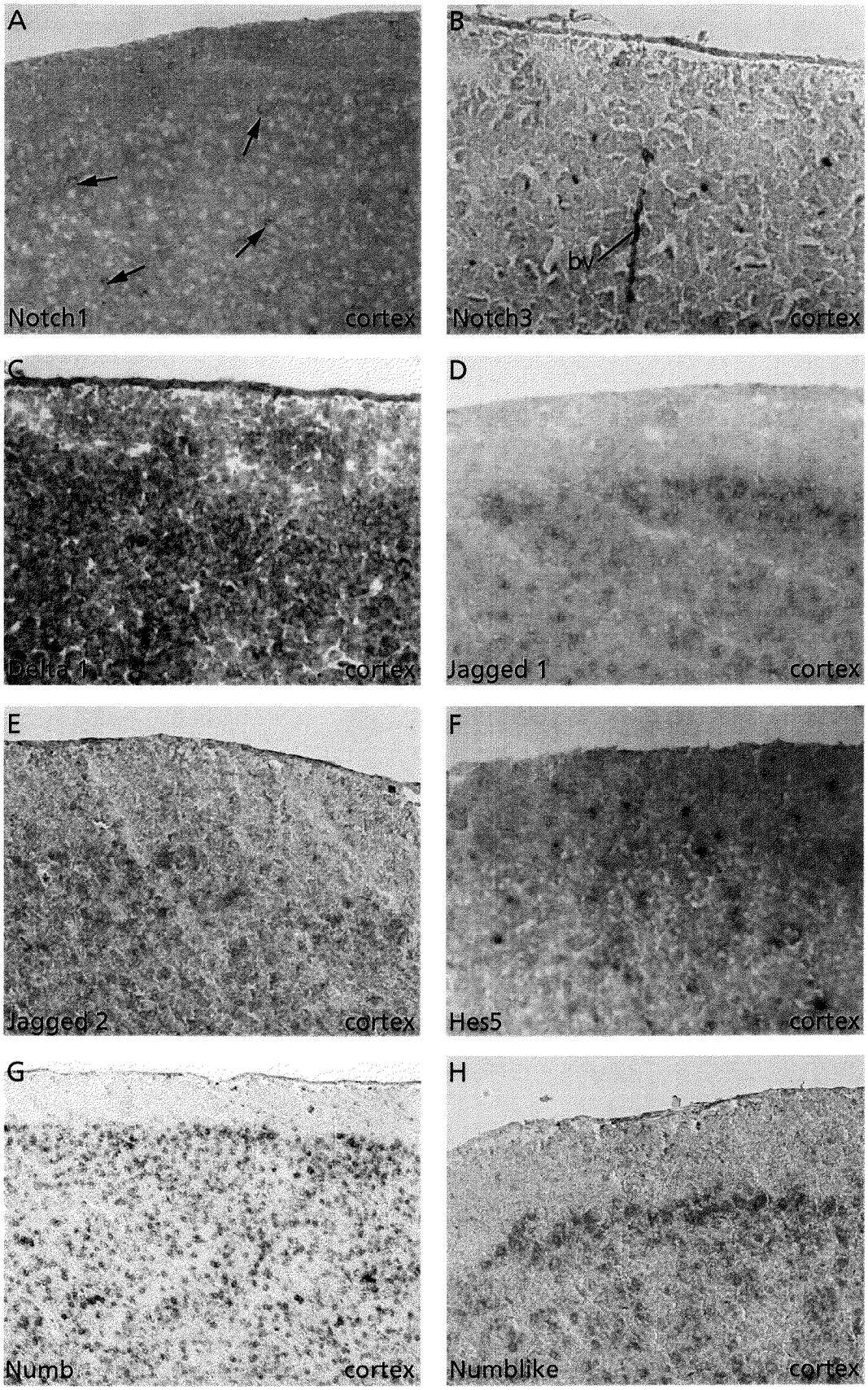

Figure 3-8: Active Notch signaling in the adult cerebral cortex

this ligand. Dll1 was expressed by cells in the IGL whereas Dll3 was not detectable at all (data not shown). The expression of Notch1, 3 and Jagged1 was associated with a prominent expression of Hes5 in the forming IGL and white matter as well as low levels of Hes1 in the IGL(Fig. 3-9F). By contrast Hes3 RNA was detected at low levels in putative Purkinje cells. Furthermore, expression of the regulatory molecule Numb was detected in the EGL and, to a lesser extent in the IGL. This expression overlapped with that of the related molecule Numblike which was mainly found in the EGL(Fig. 3-9G, H).

3.3.8 Notch Signaling in the Adult Cerebellum

Finally, we examined the expression of Notch signaling molecules in the adult cerebellum. Notch1 expression was detected in Purkinje cell layer. Expression was associated with the Purkinje cells but it can not be excluded that cells adjacent to the Purkinje cells such as Bergmann glia also express Notch1 (Fig. 3-10A). Notch3 on the other hand showed a more diffuse pattern of staining within the Purkinje cell layer and did not seem to be associated with the large pyramidal neurons but rather cells surrounding the Purkinje cells (Fig. 3-10B). We also found that the ligands Dll3 and Jagged1 remained expressed in the adult cerebellum. Where as Dll3 is expressed by Purkinje cells (Fig. 3-10C), Jagged1 is restricted to the granule cells (Fig. 3-10D). Hes1 expression was not detected (Figure 8E), however, the presence of an active Notch signal in the adult cerebellum was supported by the prominent expression of Hes3 by the Purkinje cells (Fig. 3-10F). However, both Numb and Numblike RNA were detectable in the adult cerebellum. Where as Numb is expressed by both Purkinje cells and granule cells (Fig. 3-10G), Numblike was only detected in the granule cells (Fig. 3-10H).

Fig. 3-9 on page 79: Notch signaling in the postnatal cerebellum

A) Notch1 is expressed in cells of the Purkinje cell layer (PCL) and in the forming white matter. In addition Notch1 expression is associated with the blood vessels (bv) covering the surface of the cerebellum. B) Notch3 is also expressed by cells within the PCL and bv. C) Jagged1 is prominently expressed in the external germinal layer (EGL) and cells in the forming internal germinal layer (IGL) but not by cells in the PCL. D) Jagged2 is detected in cell of the PCL in the cerebellum at postnatal day 4. E) Hes1 was detectable at low levels in the forming IGL but not within the EGL. F) Hes5 is prominently expressed in the IGL. G, H) Both Numb and Numblike are expressed in the EGL and at low levels in a punctate pattern in the IGL.

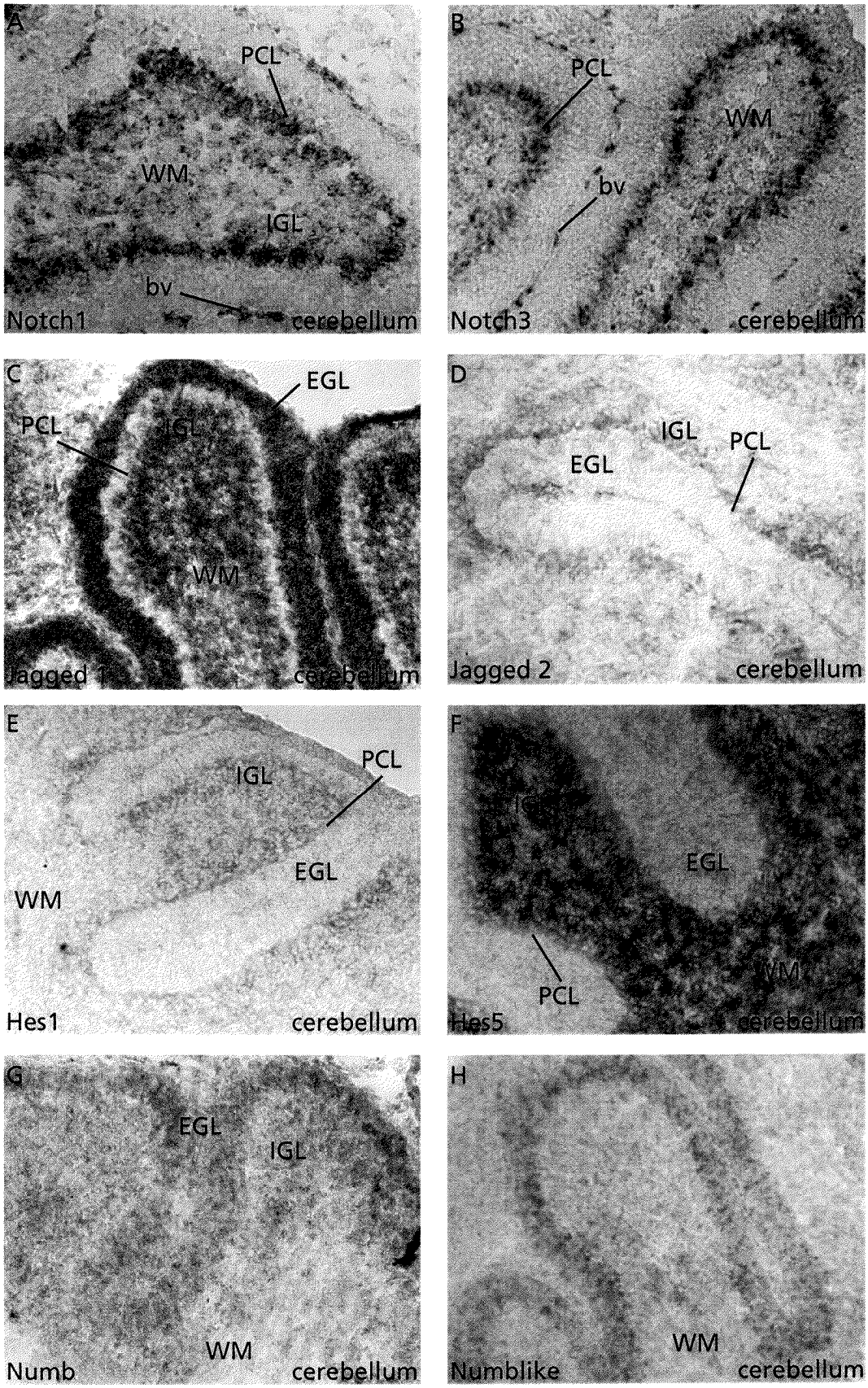

Figure 3-9: Notch signaling in the postnatal cerebellum

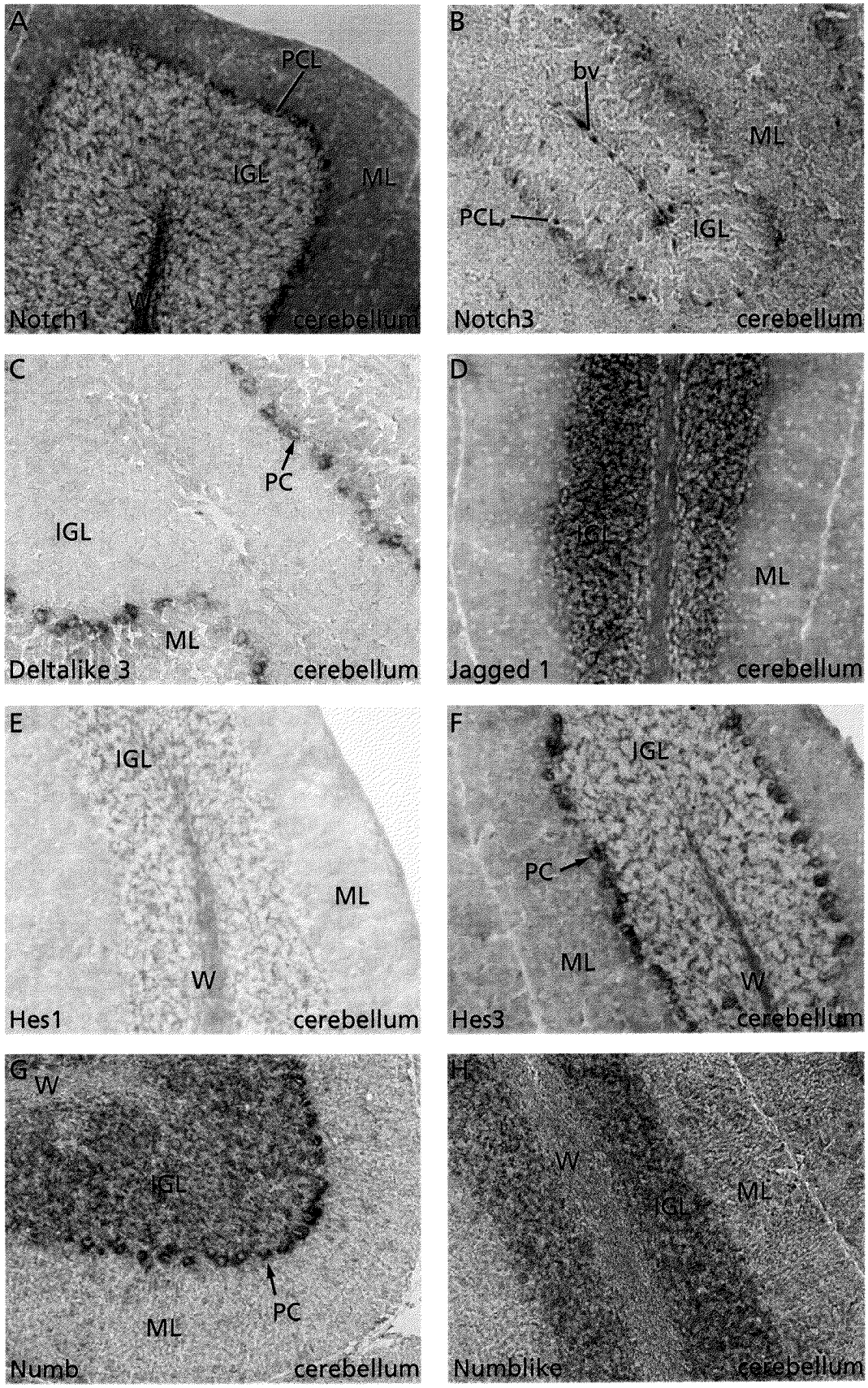

Figure 3-10: Notch signaling in the adult cerebellum

3.4 DISCUSSION

Over recent years Notch signaling has received considerable interest due to its putative roles in regulating differentiation and fate in various tissues (reviewed by Kimble and Simpson, 1997). The function of Notch signaling in the nervous system has been particularly intensively studied, mainly in the regulation of progenitor cells differentiation and lineage fate (Chitnis et al., 1995; Dorsky et al., 1997; Furukawa et al., 2000; Gaiano et al., 2000; Henrique et al., 1997; Lanford et al., 1999; Morrison et al., 2000; Muskavitch, 1994; Nieto et al., 2001; Scheer et al., 2001; Tanigaki et al., 2001; Wakamatsu et al., 2000). The majority of these studies have focussed on development and differentiation of the embryonic nervous system and little evidence has been presented for a role of Notch signaling in the regulation of neural development at later stages.

More recently it has been proposed that Notch signaling may be involved in neural development independent of regulating progenitor cell differentiation (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). Gain-of-function experiments and activation of Notch signaling in cultured neurons results in changes in dendrite morphology. Paracrine interactions between neighboring neurons is suggested to block dendrite extension and stimulate branching. Therefore, Notch signaling may be an important regulator of neuron-neuron interactions and synapse formation. However, little is known about the expression patterns of the Notch receptors, their ligands and activation of downstream targets in the postnatal CNS.

We have focussed on analysis of Notch1 and its ligands Dll1, Dll3, Jagged1 and Jagged2 in the postnatal brain. We show that at P4 Notch1 is prominently expressed in the germinal zones of the forebrain. Within the SVZ Notch1 transcripts are localized to clusters of cells lining the ventricles and in the sub-ependymal layer. The SVZ is one of the few regions in the postnatal brain where neurogenesis continues with the generation

Fig. 3-10 on page 80: Notch signaling in the adult cerebellum

A) Notch1 expression can be detected in cells of the Purkinje cell layer (PCL). B) Notch3 can be detected in the PCL and blood vessels (bv) covering the surface of the cerebellum. C) Dll3 is expression is restricted to Purkinje cells (PC) in the adult cerebellum. D) Jagged1 is prominently expressed by granule cells in the IGL. E) Hes1 expression was not detectable in the adult cerebellum. F) Hes3 is prominently expressed by Purkinje cells in the adult cerebellum. G, H) Both Numb and Numblike are expressed in the IGL and Numb is also expressed by Purkinje cells.

of olfactory bulb neurons that migrate rostrally along the RMS. In addition, Notch1 transcripts are clearly detected in the dentate gyrus of the postnatal mouse brain, another region of continued neurogenesis. The expression of Notch1 RNA continues in these two regions into adulthood. Therefore, we propose that Notch1 may mark the stem cells of the postnatal brain and, as in the developing CNS, regulate differentiation and cell fate even in the adult nervous system. In addition, the ligand Jagged1 is prominently expressed by cells within the SVZ and dentate gyrus, thus providing the necessary activation of the receptor. In order to check this activation possibility, we analyzed the expression of the downstream targets of Notch signaling the Hes genes. Both in the SVZ and in the subgranule layer of the dentate gyrus Hes genes were active indicative of a functional Notch signal. Interestingly, Hes5 was the most prominently expressed Hes gene in these germinal zones. This parallels the extensive expression of Hes5 in the embryonic neuroepithelium.

However, we also analyzed the expression of Notch signal molecules outside the germinal zones of the postnatal brain. Although Notch1 has been claimed to be involved in the regulation of neurite formation, Notch1 mRNA levels were very low in the postnatal brain. A few cells within the cortex express Notch1 transcripts, however, it is unclear what type of cells these represent. By contrast, the ligands Dll1, Dll3, Jagged1 and Jagged2 are all expressed in the cerebral cortex and many show layer specific expression patterns. This suggests that Notch ligands may identify populations of neurons within the cortical layers. Based on the proposed function of Notch signaling in the regulation of dendrite formation it is possible that the combination of Notch and ligand expression also regulate which neurons interact with each other.

We have shown previously that Notch signaling is pivotal in the regulation of cerebellar development during embryogenesis (Lütolf et al., in press). The expression of Notch receptors in the cerebellum at later stages indicates a potential role postnatally. We show that Hes3 is expressed by Purkinje cells at P4 and in the adult. Although the precise regulation of the Hes3 gene has not been studied, the expression in Purkinje cells suggests an active Notch signal in Purkinje cells. We show that Jagged1 is highly expressed in the developing cerebellum and is associated with both undifferentiated granule cell precursors in the EGL and differentiated granule cells, which are the major afferent connection to the Purkinje cells. It has been shown that Purkinje cells regulate

the proliferation of granule cell precursors by secreting the mitogen Sonic Hedgehog (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). The putative reciprocal expression of Notch and Jagged1 in Purkinje cells and granule cells, respectively suggests an additional bidirectional interaction. We show that the expression of the putative downstream regulator Hes3 increases between P4 and adult in the Purkinje cells. This coincide with the differentiation of the granule cells and their synaptogenesis with Purkinje cells (reviewed by Hatten et al., 1997; Hatten and Heintz, 1995). This suggests that activation of Notch signaling in Purkinje cells is progressive with age and corresponds to the increased interaction with granule cells. Importantly, the early postnatal period in the cerebellum is associated with the extension and elaboration of the Purkinje cell dendritic tree which has been shown to be dependent upon granule cell interactions (reviewed by Hatten et al., 1997; Hatten and Heintz, 1995).

Interestingly, Purkinje cells show an activation of Notch signaling with the expression of Hes3 however, they also express prominent levels of Dll3. Evidence from other systems suggests that Notch signaling and ligand expression are involved in a negative feedback loop (reviewed by Kimble and Simpson, 1997) leading to a down regulation of the ligand in a cell expressing Notch receptor. Therefore, finding an active Notch signal and the expression of Dll3 in the same cells seem contradictory, however, it has been shown that Hes3 can be expressed as different isoform and that the Purkinje cell type Hes3 does not have a functional basic, DNA binding domain (Hirata et al., 2000). Hence, the Purkinje cell expression of an isoform of Hes3 that is incapable of binding DNA may allow the expression of genes normally repressed by Hes proteins. The function of Dll3 expressed by Purkinje cells is intriguing but may be involved in the interaction of Purkinje cells and their target neurons or in forming the boarders to maintain the parallel organization of the Purkinje cell dendrites.

Due to the early lethality and complex phenotypes of mutants of Notch signaling components, conditional gene inactivation will be required to address their roles at later stages of development. Due to the pronounced expression of many of the Notch signaling molecules in the cells of the cerebellum and the non vital function of the cerebellum for embryonic and postnatal development this, region of the brain represents an ideal system structure to address Notch signaling in the postnatal brain.

3.5 OUTLOOK

3.5.1 ANALYSIS OF CALBINDIN-CRE/LOXNOTCH1 TRANSGENIC MICE

In order to understand Notch signaling in postmitotic neurons it will be necessary to generate a number of transgenic animals that ablate Notch1 in a conditional manner. In our laboratory mice carrying a floxed Notch1 allele were crossed with the described Calbindin-Cre mice (see chapter 4). The expression data in chapter 3 is envisaged to support the analysis of these transgenic mice as well as of other Notch related transgenics.

3.5.2 DETERMINE THE EXPRESSION PATTERNS OF OTHER NOTCH SIGNALING MODULATORS

Notch signaling has been shown to be modulated by molecules of the Fringe family of secreted molecules (Hicks et al., 2000; Johnston et al., 1997; Ju et al., 2000; Mikami et al., 2001; Moloney et al., 2000; Panin et al., 1997). Three *Drosophila* Fringe homologues have been identified in vertebrates to date; Lunatic Fringe, Manic Fringe and Radical Fringe. Fringe has been shown in *Drosophila* to inhibit Serrate-Notch signaling but to potentiate Delta-Notch signaling (Panin et al., 1997). This seems to be achieved by post-translational modification of Notch by Fringe which shows glycosyltransferase activity (Moloney et al., 2000). Furthermore it has been suggested that Fringe is able to form a complex with Notch intracellularly, before it gets secreted (Ju et al., 2000). These findings may explain how Fringe acts cell-autonomously to modulate the ligand preference of Notch in *Drosophila*. However, in order to understand the functions of the three Fringe homologues in vertebrates the determination of their expression patterns will be necessary.

The Notch signaling pathway is connected with other signaling cascades which positively or negatively interact with it (De Strooper and Annaert, 2001; Solecki et al., 2001). Wnt signaling has been shown to influence the Notch signaling pathway via *desheveld* which acts negatively on the intracellular domain of Notch1 and may block interactions with CSL proteins (De Strooper and Annaert, 2001; Hing et al., 1994; Uyttendaele et al., 1998). Recent studies provided evidence that Sonic Hedgehog can

also induce Hes1 expression (Solecki et al., 2001). In order to fully understand the functions of Notch signaling during development and at postnatal stages it will be important to evaluate the exact expression patterns of all molecules that are able to suppress or potentiate the effects of Notch.

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4 CALBINDIN-CRE TRANSGENIC MICE TO ANALYZE GENE FUNCTION IN THE CNS

4.1 INTRODUCTION

Our understanding of the genes that regulate development of the mammalian central nervous system is limited. *In vitro* experiments using cultured neurons, glia and progenitors have identified a number of candidate genes that may play a role in cell proliferation and differentiation *in vivo* (Johe et al., 1996; Temple and Qian, 1995; Williams et al., 1997). However, in order to address the function of these molecules during neuronal and glial formation, loss of function approaches are required. Conventional gene inactivation by targeted mutation of a gene by homologous recombination in embryonic stem (ES) cells has inherent problems (Gu et al., 1993; Sauer, 1993). Inactivation of a gene with an expression pattern that is not restricted may result in secondary effects or even lethality, complicating analysis of gene function in neural development. Tissue-specific temporal gene ablation techniques have been described that allow gene inactivation in a temporally and spatially restricted manner (Brocard et al., 1997; Kellendonk et al., 1999; Tsien et al., 1996). The most common method of conditional gene ablation in the mouse utilizes the specific activity of the bacteriophage P1-derived Cre-recombinase (Gu et al., 1993). Cre-recombinase recognizes tandem repeats of the 34 basepair loxP sequence and mediates site-specific recombination. Hence, loxP sequences can be introduced by homologous recombination in mouse ES cells, to flank an entire gene or vital exon of a gene. Careful positioning of the loxP sequences in non-crucial or intronic regions results in the generation of a

functional allele that, upon expression of Cre-recombinase, can be temporally and spatially inactivated (Brocard et al., 1997; Gu et al., 1993; Kellendonk et al., 1999; Sauer, 1993; Tsien et al., 1996). Tissue specificity of the ablation event is controlled by expression of the Cre-recombinase from a promoter expressed in the cells where the gene to be inactivated is functional. This technique has been used successfully to address gene function in the nervous system (Gu et al., 1994; Kuhn et al., 1995; Lütolf et al., in press; Sakai et al., 2001)

We have focused on the cerebellum as a model to address gene function in vertebrate neurogenesis and gliogenesis as well as in cell fate decisions due to the clearly defined anatomical structure and restricted neuronal complexity (Altman and Bayer, 1985a; Altman and Bayer, 1985b; Altman and Bayer, 1985c). Unlike the cerebral cortex, which contains hundreds of different neuronal types, the cerebellum comprises only five types of neuron. These neurons are arranged in a specific, regular pattern and networks that have been well defined and that are contained within the cerebellar structure (Altman and Bayer, 1997). The Purkinje cells are large pyramidal GABAergic neurons that form the only efferent from the cerebellar cortex. The activity of Purkinje cells is activated by incoming signals via the climbing fibers. The major targets of the Purkinje cells are the deep cerebellar neurons that lie in nuclei within the white matter of the cerebellum. The second input to the cerebellar cortex is from the mossy fibers that synapse on and activate the major interneuron of the cerebellum the granule cell. The granule cells synapse on and activate the Purkinje cell dendrites via the parallel fibers within the molecular layer. The parallel fibers of the granule cells also activate the three other interneurons in the cerebellum. The stellate and basket cells reside in the molecular layer and the Golgi cells are interspersed between the granule cells in the IGL. Both stellate and basket cells are interneurons receiving inputs from the parallel fibers and regulating the cerebellar output signal. The stellate cell axons contact the Purkinje cells on the dendrites whereas the inhibitory basket cells synapse directly on the soma of the Purkinje cells. The GABAergic Golgi cells act in a negative feedback loop back to the activated granule cells. In addition to the clearly defined cytoachitecture and neuronal connections, the development of the cerebellum has been well defined and markers are available to address different stages of Purkinje and granule cell differentiation (reviewed by Hatten et al., 1997; Hatten and Heintz, 1995).

We aim to address gene function in Purkinje cells of the developing cerebellum. Purkinje cell precursors leave the dorsal neuroepithelium posterior to the midbrain hindbrain boundary between E12.5 and E15 in the mouse (Hatten and Heintz, 1995). These immature Purkinje cells are postmitotic and upregulate the expression of the calcium binding protein Calbindin D28k, which is the earliest known lineage marker of cerebellar Purkinje cells (Hatten and Heintz, 1995). They retain expression of Calbindin D28k throughout their development and into adulthood and are the only neuron type of the cerebellum to express the gene. Calbindin D28k is proposed to function as a calcium buffer in the dendrites of the Purkinje cells (Airaksinen et al., 1997; Heizmann, 1992; Heizmann, 1993) and Calbindin D28k-deficient animals show defects in fine motor control, one of the prime regulatory functions of the cerebellum (Airaksinen et al., 1997; Pavlou et al., 1996).

In order to obtain mice that can be used for analysis of gene function in the nervous system *in vivo*, we have generated transgenic animals that express Cre-recombinase from Calbindin D28k promoter elements. It has been shown that 3 kbp of sequence 5' to the Calbindin D28k gene contain a number of putative regulatory elements (Arnold and Heintz, 1997; Pavlou et al., 1996). The Purkinje cell element (PCE1) at position -85 bp to -45 bp is crucial for expression of the Calbindin D28k gene in cerebellar Purkinje cells (Arnold and Heintz, 1997). The Calbindin D28k promoter also contains two Vitamin D-responsive elements (DREs) at -2295 bp and -1160 bp that mediate transcription in the kidney (Kuwano and al., 1994). In addition, other elements of the Calbindin-D28k promoter include three putative TATA-boxes (-1630 bp, -354 bp and -29 bp), a cAMP-responsive element (-330 bp) and a Myc-binding site (-480 bp). Therefore, we have used the proximal 1.1 kbp of the Calbindin D28k promoter to drive expression of Cre-recombinase in the mouse as this region has been shown to be required and sufficient to drive transgene expression in Purkinje cells (Pavlou et al., 1996). We show that of the nine transgenic lines generated, two exhibit recombinase activity in Purkinje cells. In addition, some of the lines also show expression of Cre-recombinase in the cortex and hippocampus in a pattern that potentially reflects a fate map of the cells that express Calbindin D28k during development. Finally, some of the transgenic lines express Cre-recombinase in a pattern that is ectopic to the endogenous Calbindin D28k, likely due to integration effects on the transgene. These transgenic animals will be a valuable system

to address gene function in different neuronal populations in the CNS.

4.2 MATERIALS AND METHODS

4.2.1 CONSTRUCT AND GENOTYPING

A genomic PstI fragment containing the proximal 1.1kb of the Calbindin D28k promoter was isolated from CALZSV2 (Pavlou et al., 1996) and subcloned into pBluescript. The SpeI/SalI promoter fragment was cloned into the pNuKCre plasmid (kindly provided by Dr. Pedro Herrara) upstream of the coding sequence for a nuclear targeted Cre-recombinase and 2.2kb of the human growth hormone mini-gene. This construct was linearized by NotI digestion and used for pronuclear injection into fertilized mouse eggs. Founder animals were identified by PCR and back crossed onto Bl6/D2 F1 animals. The offspring were analyzed by transgene specific PCR. Transgenic offspring were mated with wild-type (Bl6/C57) or Rosa26R reporter mice (Soriano, 1999). Calbindin-Cre genotyping was by genomic PCR on DNA isolated from tail biopsies. The primers used were: Cre sense 5'-ACC AGG TTC GTT CAC GCA TGG -3' and Cre anti-sense 5'-AGG CTA AGT GCC TTC TCT ACA C -3'. PCR reaction conditions were: 1min. 94°C, 1min. 53°C and 1min. 72°C.

4.2.2 X-GAL ANALYSIS OF RECOMBINATION ACTIVITY

Adult and early postnatal brains were isolated, cooled to 4°C for 5 min. and hemisected. One half was frozen on dry ice in OCT (Sakura) and stored for cryosectioning at –80°C. The other brain half was used for whole-mount X-Gal staining. The wholemount tissue was fixed in 2% paraformaldehyde, 0.2% glutaraldehyde in PBS for 30min. on ice. After fixation brains were washed 3 times 30min. in PBS, 0.002M MgCl2, 0.02% NP-40. The wholemount tissue was transferred to pre-warmed X-Gal solution and incubated at 37°C for 2 to 12 hours. The staining solution was 1mg/ml X-Gal (Axonlab), 2.12mg/ml potassium hexacyanoferrate(II) trihydrate and 1.64mg/ml potassium hexacyanoferrate(III) in PBS. The staining reaction was stopped by fixation in 4% paraformaldehyde/PBS for 30 minutes. 20µm sagital cryosections were thaw-mounted onto superfrost slides, and fixed in 0.4% glutaraldehyde for 10min. at 4°C. X-Gal staining on cryosections was performed as above. To stop the staining reaction the slides

were placed in TE for 10min. and coverslips mounted with 87% glycerol.

4.2.3 MICROSCOPY AND IMAGE PROCESSING

Sections were analyzed on a Zeiss Axioplan microscope and images taken using a ProgRes 3008 camera or Zeiss AxioCam. Wholemounts were photographed using a ProgRes 3008 camera in conjunction with a Zeiss Stemi 2000-C Binocular. Image processing was performed using Adobe Photoshop 5.0 software.

4.3 RESULTS

In order to generate transgenic mice for temporal and spatial gene ablation in postmitotic neurons of the CNS, we used the Calbindin D28k promoter to drive expression of Cre-recombinase. A cDNA encoding Cre-recombinase with an N-terminal nuclear localization signal (Nuk) was placed into the 5' untranslated region of Calbindin D28k gene, downstream of 1.1 kbp of the proximal promoter (Fig. 4-1). Sequences of the human growth hormone mini-gene were cloned downstream of the Cre cDNA to enhance transgene expression (Fig. 4-1). Here we describe detailed analysis of four lines (655, 658, 660 and 663) that express Cre recombinase in various neuronal cell types in the brain.

4.3.1 CALBINDIN-CRE LINE 655 LINES SHOWS A SPECIFIC RECOMBINATION PATTERN IN THE ADULT BRAIN

In order to assess the expression pattern of Cre in the individual transgenic lines we intercrossed animals of each line with animals carrying a Cre-reporter (Fig. 4-1) (Soriano, 1999). The presence and distribution of recombined cells was monitored in adult offspring of these breedings by X-Gal staining of 2mm thick sagittal brain sections. Animals of transgenic line 655 showed a restricted expression of β-galactosidase in cerebellar Purkinje cells (Fig. 4-2A). At higher magnification, X-Gal precipitate can be seen in structures extending into the molecular layer of the cerebellum (Fig. 4-2B). Cyrosections of the adult cerebellum stained with X-Gal confirmed expression of β-galactosidase activity in Purkinje cells, which extends into the putative dendritic tree (Fig. 4-2C, arrows). However, not all of the Purkinje cells of the adult cerebellum had

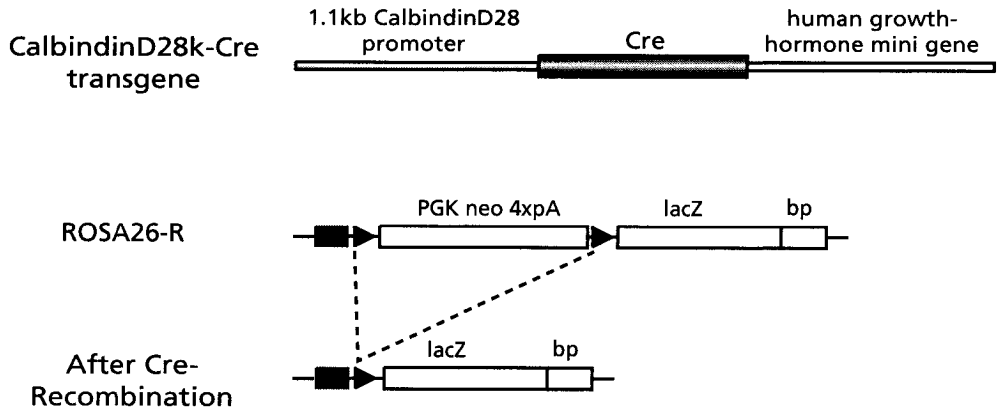

Figure 4-1: Schematic representation of the CalbindinD28k-Cre construct

The proximal 1.1kb of the Calbindin D28k promoter was used to drive expression of Cre-recombinase with a N-terminal nuclear localization signal. Sequences from the human growth hormone mini-gene were placed downstream of the Cre coding region. Transgenic animals carrying this construct were crossed with Rosa26R reporter animals that contain stop-transcription cassette (PGK neo 4xpA) flanked by loxP sites in front of the LacZ reporter gene, knocked-into the ubiquitously active Rosa26 locus (Soriano, 1999). After recombination in the Cre-expressing cells the LacZ gene comes under the transcriptional control of the ubiquitous promoter.

undergone recombination and a pronounced Zebra-like stripe pattern of recombination was evident from a dorsal view of a whole mount adult cerebellum (Fig. 4-2D). Few β-galactosidase expressing cells were detected outside the cerebellum of adult 655 animals. However, cells within the putative ventromedial hypothalamic nucleus (VMH) and pontine reticular (PRN) and gigantocellular nuclei (GCN) of the hindbrain had undergone recombination (Fig. 4-2A). In addition, scattered cells within the deep layers of the cerebral cortex (arrows) and caudate putamen (CaP) (arrowhead) showed Cre-recombinase activity (Fig. 4-2E).

4.3.2 Recombination in cerebellar Purkinje cells of the Calbindin-Cre line 655 occurs before postnatal day 4

In order to address the onset of Cre-recombinase expression in the cerebellar Purkinje cells of Calbindin-Cre line 655, we analyzed β-galactosidase expression in postnatal day 4 (P4) animals. Recombined cells were detected in the Purkinje cells of the cerebellum and in the pontine region of the hindbrain in a pattern reflecting the later distribution of β-galactosidase expressing cells in the adult (Fig. 4-3A, C). At P4 the zebra-like stripes

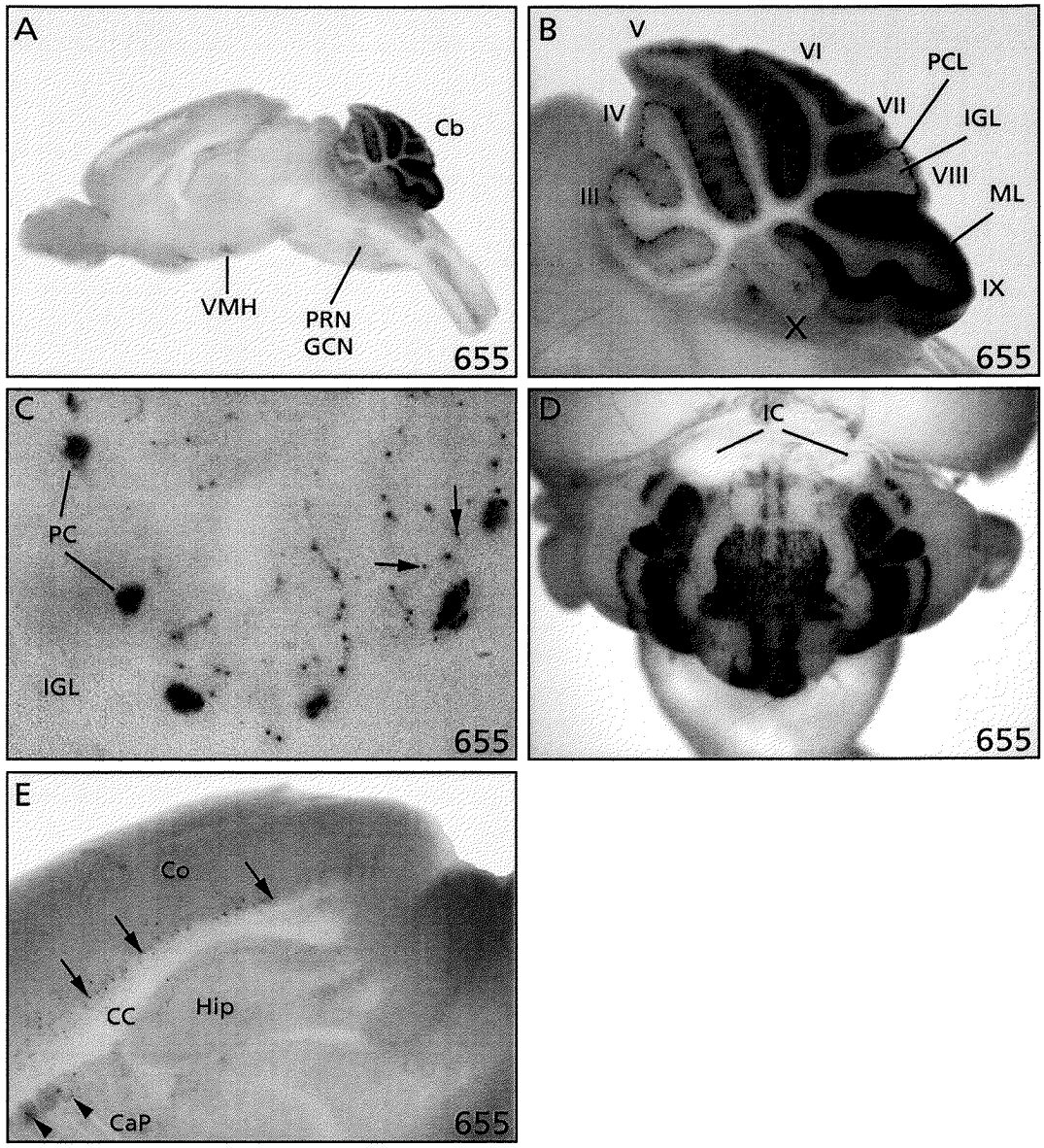

Figure 4-2: X-Gal staining of adult brains of mice from transgenic line 655

A) On wholemount preparations of brains LacZ is detectable in the cerebellum (Cb) and in neurons of the pontine region of the brain stem. B) Recombined Purkinje cells showing β-galactosidase staining extending into the molecular layer (ML) (arrow). C) On sections X-Gal precipitate can be detected in putative branching points of the Purkinje cell (PC) dendrites (arrows). D) Dorsal view of a wholemount preparation showing a subpopulation of recombined Purkinje cells that are organized in a defined stripe pattern in the cerebellum. E) Few cells in the basal layer of the cerebral cortex (Co, arrows) and anterior caudate putamen (CaP, arrowheads) are positive for reporter-gene expression. IGL Internal granule layer, PCL Purkinje cell layer, VMH ventromedial hypothalamic nucleus, PRN pontine reticular nuclei, GCN gigantocellular nuclei, CC corpus callosum, I-X cerebellar lobes.

of recombined Purkinje cells were clearly detectable (Fig. 4-3B). At this stage the dendritic trees of the Purkinje cells are not fully formed. Therefore, X-Gal staining of the forming molecular layer is less prominent than in the adult cerebellum (Fig. 4-3C and Fig. 4-2C). Few β-galactosidase expressing cells were detected in the cerebral cortex at

this stage (not shown). As expected from the adult expression data, no recombined cells were detected in the hippocampus of line 655 at P4 (data not shown). We also analyzed the expression of β-galactosidase at embryonic stages to determine the onset of Cre-activity. Recombined cells could be detected in the putative Purkinje cells of the E18 cerebellum (Fig. 4-3D) and neurons of the ventral hindbrain (not shown), confirming that the recombination seen in the adult animals had taken place early in development. Furthermore, the analysis at E18 revealed that cells in the intestine also expressed Cre-recombinase at early developmental stages (Fig. 4-3E). However, X-Gal staining of sections of E14 embryos, which is the time point when endogenous Calbindin D28k expression starts, revealed no β-galactosidase activity in the cerebellum (data not shown).

4.3.3 TRANSGENIC LINE 660 SHOWS AN EXTENSIVE PATTERN OF RECOMBINATION

In contrast to the restricted distribution of recombined cells in adult 655 animals, animals of line 660 showed an extensive pattern of β-galactosidase activity throughout the brain, particularly in the olfactory bulb, cerebral cortex, hippocampus, midbrain and cerebellum (Fig. 4-4A). In the cerebellum, intense expression of β-galactosidase was visible in the internal granule cell layer (IGL) (Fig. 4-4B, C). Recombined cells were detected in all but the outermost layers of the cerebral cortex but not in the white matter of the corpus callosum (Fig. 4-4D). In the hippocampus, pyramidal cells of all CA regions and the granule cells of the dentate gyrus had undergone recombination in the adult as had cells of the caudate putamen (Fig. 4-4D).

We analyzed early postnatal animals and found extensive recombination with β-galactosidase expressing cells in the cerebral cortex, hippocampus, and throughout the basal forebrain and brain stem (Fig. 4-5A). In the cerebellum, β-galactosidase expressing cells were detected, in the forming IGL (Fig. 4-5B). Granule cell precursors of the inner most layer in the EGL also had undergone recombination whereas the mitotically active precursors in the EGL do not express β-galactosidase (Fig. 4-5B, arrows). β-galactosidase activity was also detected in the deep cerebellar neurons (Fig. 4-5C). This early postnatal pattern of recombination coincides with the distribution of recombined cells in the adult mice and suggests that granule cells express the transgene only after

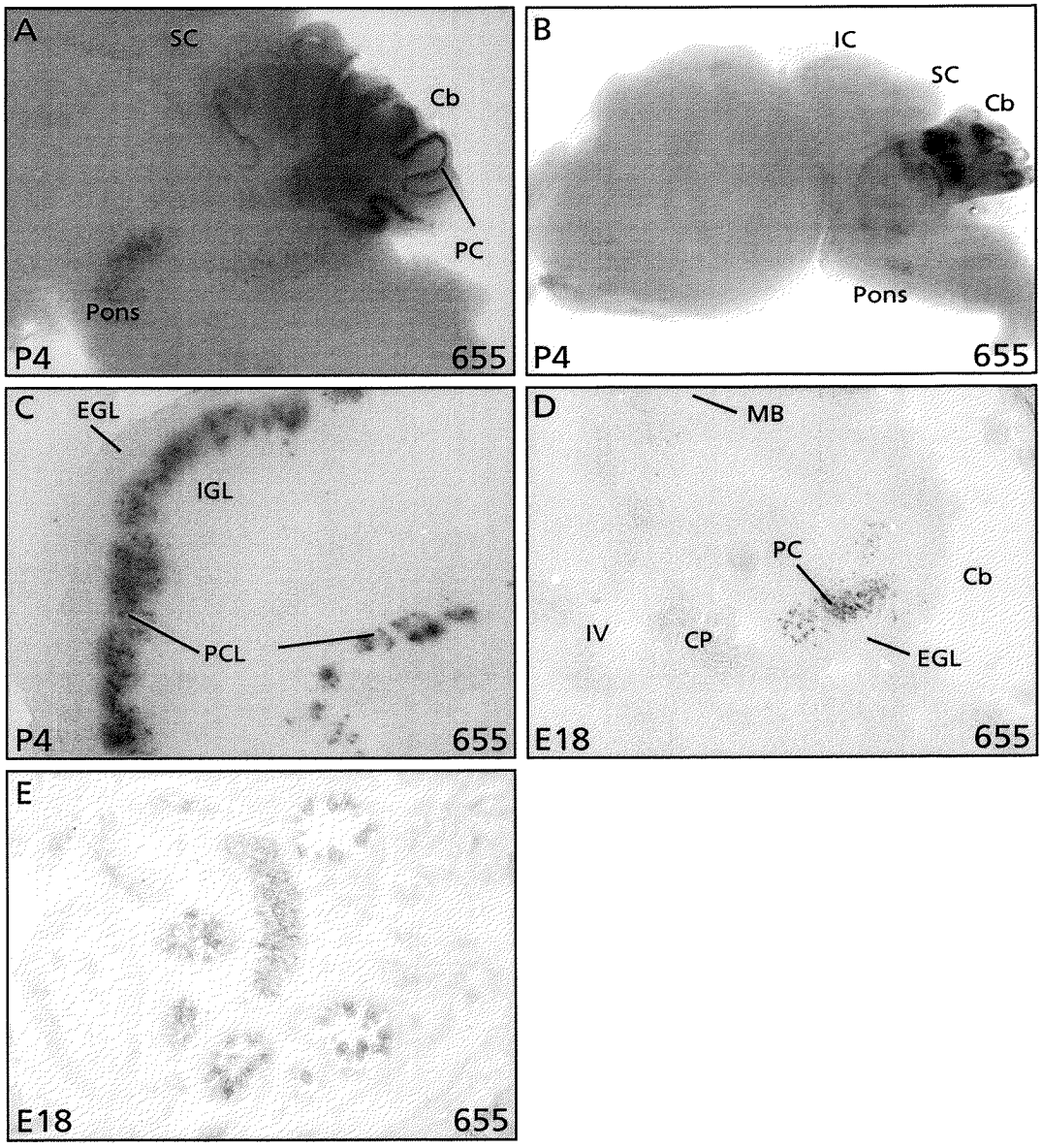

Figure 4-3: Cre expression in postnatal day 4 brain and E18 embryos of line 655

A) Purkinje cells (PC) and neurons in the pons show signs of recombination due to the expression of the LacZ reporter on wholemount preparations. B) At P4 recombined Purkinje cells are organized in a stripe pattern in the cerebellum (Cb). C) On sections cells within the Purkinje cell layer (PCL) show extensive b-galactosidase expression. D) Sections of E18 mice reveal that Purkinje cells have already undergone recombination. E) Epithelial cells in the intestine of E18 transgenic mice have also undergone recombination and express b-galactosidase. CP choroid plexus, EGL external germinal cell layer, IC inferior colliculi, IGL internal granule layer, MB midbrain, SC superior colliculi, IV fourth ventricle.

they have left the cell cycle in the EGL and commenced terminal differentiation. To further analyze the onset of recombination in line 660 we analyzed β-galactosidase expression in E18 embryos. Recombined cells could be detected in the outer layers of the forming cerebral cortex (Fig. 4-5D). However, β-galactosidase expressing cells were not found in other regions of the brain (data not shown). Importantly, putative progenitor

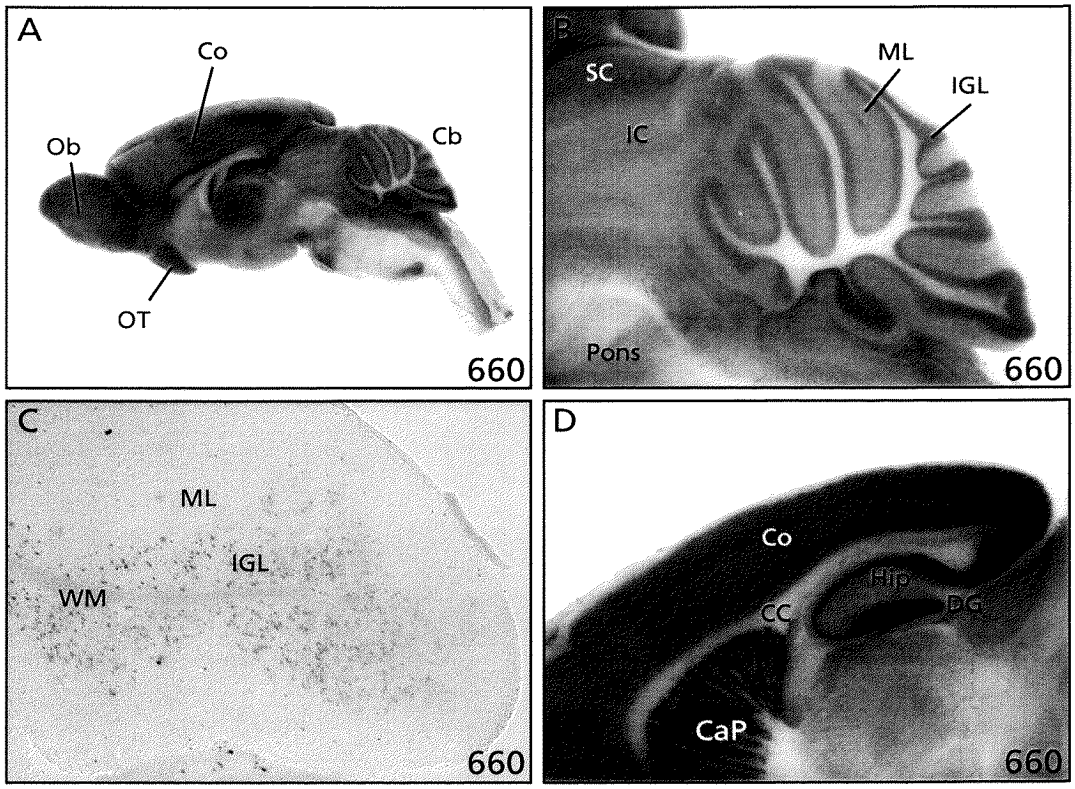

Figure 4-4: X-Gal staining of adult brains of transgenic line 660

A) Extensive b-galactosidase expression can be detected in the cortex (Co), basal forebrain, olfactory bulb (Ob), cerebellum (Cb) and pons. B) The proportion of recombined cerebellar granule cells is higher in the posterior lobes relative to anterior lobes. C) Sections of the cerebellum also reveals heterogeneic expression of Cre activity. D) In the forebrain recombination occurs in the cerebral cortex, caudate putamen (CaP), dentate gyrus (DG) and all CA regions of the hippocampus (Hip). CC corpus callosum, IC inferior colliculi, IGL internal granule layer, ML molecular layer, SC superior colliculi, WM white matter.

cells within the ventricular zone had not undergone recombination suggesting that the transgene is only expressed by postmitotic neurons at that stage. Recombination in the rest of the embryo was restricted to the epithelium of the intestine as described for line 655 (Fig. 4-5E and 3E). Recombination activity could not be detected in E12 embryos (data not shown).

4.3.4 Transgenic line 658 shows a similar pattern of Cre expression to that of line 660

Similar to the transgenic line 660, line 658 shows an extensive pattern of β-galactosidase activity throughout the adult brain (Fig. 4-6A). The cerebral cortex, olfactory bulb, hippocampus and granule cells of the cerebellum all show Cre reporter gene activity (Fig. 4-6A, B). Furthermore, recombined cells could be detected within the basal

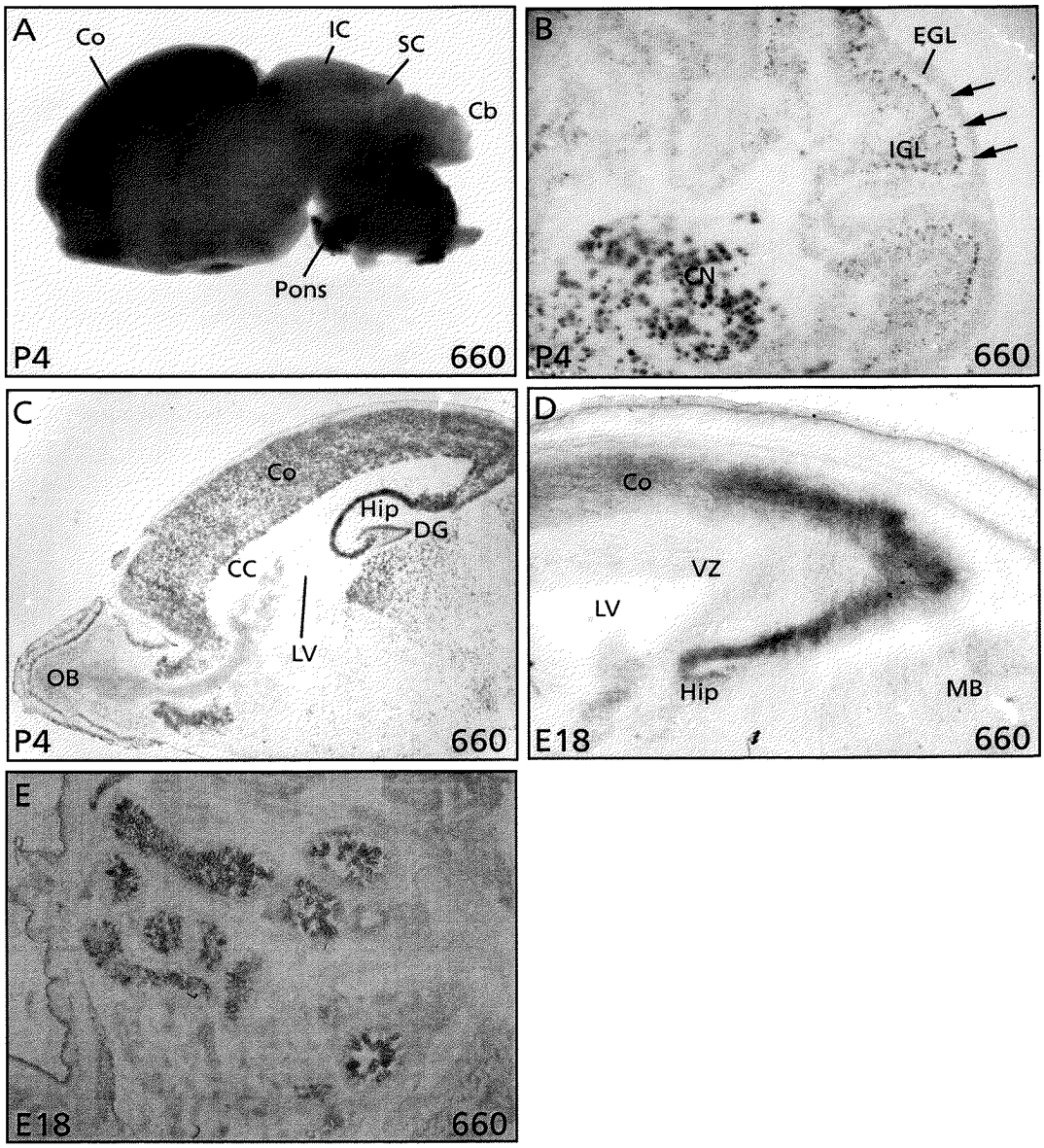

Figure 4-5: Cre expression in P6 brain and E18 embryo of line 660

A) At P4 b-galactosidase is strongly expressed in the forebrain and the cerebellum (Cb) on wholemount preparations. B) In the cerebellum recombination occurred in the deep cerebellar nuclei (CN) and the first granule cells reaching the internal granule layer (IGL). Furthermore, granule cell precursors at the innermost layer of the external germinal layer (EGL) (arrows) have undergone recombination. C) In the forebrain extensive reporter expression is found in the cortex (Co), hippocampus (Hip), dentate gyrus (DG) and olfactory bulb (OB). D) At E18 embryos of line 660 X-Gal precipitate was only detected within the cortex where it was not associated with the ventricular zone (VZ). E)) Epithelial cells in the intestine of E18 transgenic mice have also undergone recombination. CC corpus callosum, IC inferior colliculi, LV lateral ventricle, MB midbrain, SC superior colliculi.

forebrain (Fig. 4-6A). In the cerebral cortex, the reporter gene was expressed through all the layers whereas no recombination was found in the white matter of the corpus callosum (Fig. 4-6C). In addition, in transgenic line 660, Cre activity was detected in neurons of the caudate putamen. In the cerebellum, granule cells in the IGL had

undergone recombination in the adult (Fig. 4-6B). The distribution of X-Gal precipitate suggests that more granule cells in the posterior part of the cerebellum had undergone recombination than in the anterior lobes and that Purkinje cells do not express the Cre transgene (Fig. 4-6B and data not shown). Similar to transgenic line 660, neurons of the deep cerebellar nuclei also expressed β-galactosidase in the adult (Fig. 4-6B).

At P4 the staining in the cerebral cortex, hippocampus and olfactory bulb was already prominent (Fig. 4-6D). Cells in all layers of the cerebral cortex and both the pyramidal neurons of the hippocampus and granule cells of the dentate gyrus showed β-galactosidase activity (Fig. 4-6E). In the cerebellum, the deep cerebellar neurons showed strong expression of the reporter gene whereas neither the EGL nor granule cells had undergone recombination (Fig. 4-6F). The lack of expression of β-galactosidase in the granule cells of line 658 at P4 suggests a later activation of the transgene in the cerebellar interneurons compared to line 660.

4.3.5 Recombination in Purkinje and granule cells in transgenic line 663

A similar pattern of recombination to that of lines 660 and 658 was detected in the forebrain of animals from line 663 (Fig. 4-7A). Cells throughout the cerebral cortex, basal forebrain, olfactory bulb and hippocampus of adult animals express β-galactosidase (Fig. 4-7A and data not shown). In the cerebellum, granule cells throughout the IGL had undergone recombination (Fig. 4-7B). In addition, and in contrast to lines 660 and 658, Purkinje cells had also showed recombination activity (Fig. 4-7C, arrow). The presumptive dendritic trees of recombined Purkinje cells extending into the molecular layer of the cerebellum were detected by X-Gal staining (Fig. 4-7C, arrowheads). Furthermore, neurons from the deep cerebellar nuclei also expressed β-galactosidase (data not shown). In the cerebral cortex a high degree of recombination was detected (Fig. 4-7D), however, cells in layer I and in the white matter of the corpus callosum did not show β-galactosidase expression (Fig. 4-7D).

At P4 the pattern of recombined cells in the forebrains of 663 animals resembles that found in animals of lines 660 and 658 (data not shown). β-galactosidase is expressed in all but the outermost layer of the cerebral cortex, as well as in the olfactory bulb (data not shown). Many cells in basal forebrain had not undergone recombination at this stage. In

the cerebellum, putative Purkinje cells and the first granule cells entering the IGL have undergone recombination of the reporter allele at P4 (data not shown). In addition, β-galactosidase was detected in neurons laying in the brainstem coinciding with the expression pattern in the adult brain (data not shown). The staining pattern appeared to

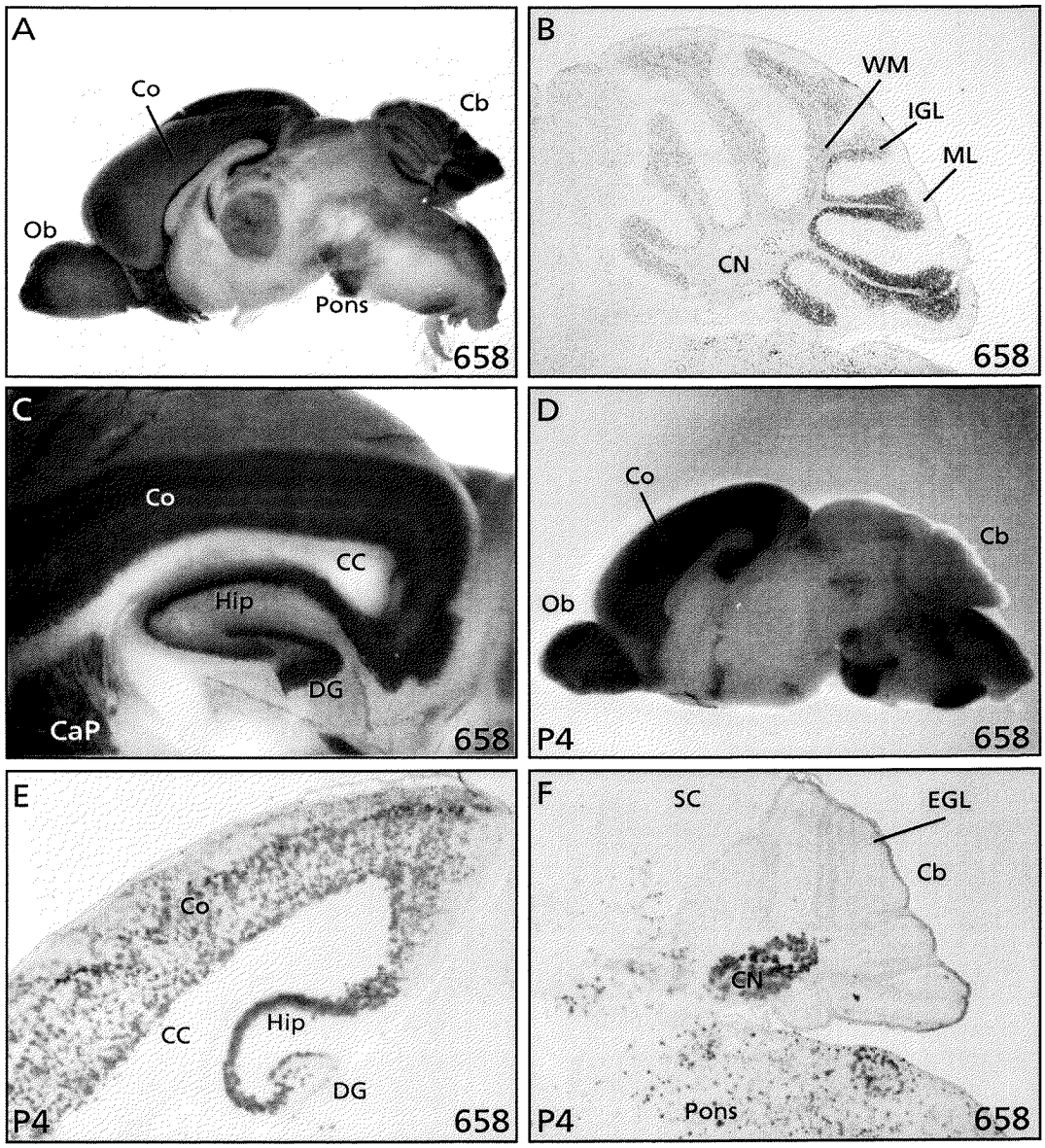

Figure 4-6: X-Gal stainings on adult and P4 brains of line 658

A) Wide Cre activity was detected in the forebrain, olfactory bulb (Ob), midbrain, cerebellum (Cb) and poms. B) The proportion of recombined cerebellar granule cells is higher in the posterior lobes relative to anterior lobes. Neurons from the deep cerebellar nuclei (CN) also express b-galactosidase. C) In the forebrain recombination is detected in the cortex (Co), hippocampus (Hip), dentate gyrus (DG) and the caudate putamen (CaP). D) The detected recombination activity at P4 is consistent with what is seen in the adult brain. E) In the forebrain the hippocampus (Hip), dentate gyrus (DG) and cerebral cortex have already recombined at P4. F) In the cerebellum mainly cells of the deep cerebellar nuclei have undergone recombination. CC corpus callosum, EGL external germinal layer, IGL internal granule layer, ML molecular layer, SC superior colliculi,s WM white matter.

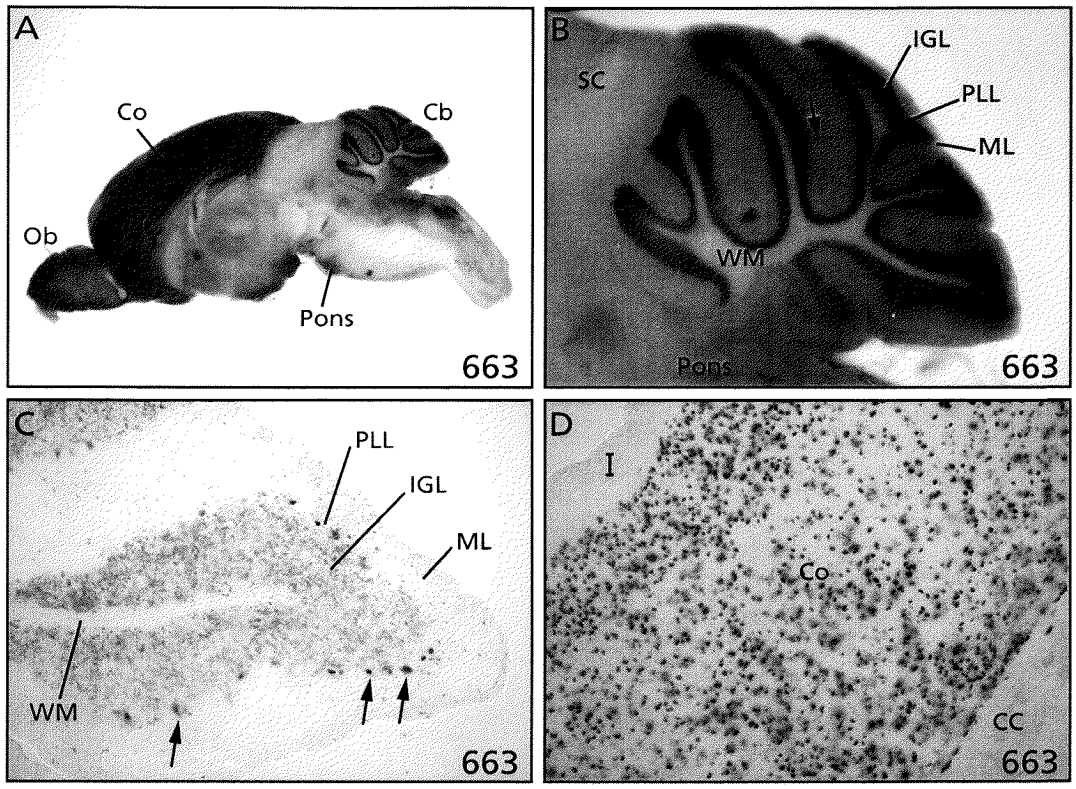

Figure 4-7: X-Gal stainings on adult brains of line 663

A) Wide recombination activity is detected in the forebrain, pons, olfactory bulb (OB) and cerebellum (Cb) as well as in some cells within the basal forebrain. B) In the cerebellum the cells in the internal granule layer (IGL) and a proportion of the Purkinje cells have undergone recombination. C) Similar to line 655 the precipitate is visible in the putative dendritic tree of the Purkinje cells (arrow) extending into the molecular layer (ML). D) In cerebral cortex (Co) the cells show extensive reporter activity. However, cortical layer I (I), as well as the corpus callosum (CC) contains no recombined cells. PLL Purkinje cell layer, SC superior colliculi, WM white matter.

be a combination of the patterns seen in lines 655 and 658 at this stage.

4.4 Discussion

Conventional gene knockout techniques generate animals that inherit genetic mutations in all cells. This regionally and temporally unrestricted genetic manipulation often lead to severe developmental defects or premature death, which can preclude the analysis at later developmental stages. In order to analyze gene function specifically in a chosen tissue or developmental stage it is important to establish temporally and spatially restricted gene ablation strategies. The Cre/lox system has been shown to be a powerful way reach this goal (Gu et al., 1994; Kuhn et al., 1995; Lütolf et al.,in press; Sakai et al., 2001)

The cerebellum regulates fine motor control, but also plays roles in learning and cognitive functions (Thach, 1998; Thompson and Krupa, 1994). Purkinje cells form the sole output from the cerebellar cortex, therefore, these neurons are not only interesting to study developmental processes of neurogenesis and differentiation, but also to investigate dynamic processes such as long term depression (LTD) or long term potentiation (LTP). Hence, the possibility to generate cell-specific mutations in Purkinje cells is of interest to a wide range of developmental and behavioural neurobiologists.

We have established transgenic mouse lines expressing Cre recombinase in postmitotic neurons in the CNS using the proximal 1.1kb of the Calbindin D28k promoter. Fortuitous integration effects in the different lines has resulted in a small zoo of animals that can be utilised to study gene function in many regions of the brain. Transgenic lines 658, 660 and 663 all displayed strong recombination activity in the forebrain, the hippocampus, the striatum and the olfactory bulb. This Cre expression potentially reflects a fate map of the cells in the brain that express Calbindin D28k during certain stages of development (Christakos et al., 1987; Frantz and Tobin, 1994; Jande et al., 1981; Rabie et al., 1985). In the developing cortex, the subventricular zone cells do not undergo recombination allowing analysis of gene function specifically in postmitotic neurons commencing during mid-embryogenesis.

We show that the transgenic lines 655, 658, 660 and 663 will be valuable to study gene function in the cerebellum. Line 655 shows highly specific recombination in Purkinje cells in a distinct stripe-pattern. Recombination was detected as early as E18 and suggests that Cre expression in these animals may parallel the expression of the endogenous Calbindin D28k. Although Calbindin D28k has been reported to display a stripe-like expression pattern during early cerebellar development (Oberdick et al., 1998) the fact that not all Purkinje cells expressed Cre during their development is likely due to an integration artifact. However, this offers the possibility to compare mutant and wildtype Purkinje cells within the same tissue and may restrict the severity of any behavioral phenotype. Hence, line 655 provides a tool to perform loss-of-function studies specifically in Purkinje cells within the cerebellum. Lines 658 and 660, as well as 663 show strong recombinase activity in the cerebellar granule cell layer. The granule cell precursors in the EGL of lines 658 and 660 do not undergo recombination until they leave the cell cycle and started to migrate through the molecular layer to reach the final

position in the IGL. Using these lines it may be possible to analyze genes involved in terminal differentiation and radial migration of the granule cells or in axon extension without affecting proliferation in the EGL. We also show that line 663 expresses Cre-recombinase in granule cells and in a population of Purkinje cells, opening up the possibility to delete genes from both neuronal cell types in the cerebellum.

In summary, the transgenic animals we have generated allow the analysis of gene function by conditional gene ablation in specific cell populations of the brain and cerebellum. By utilizing different Cre-lines it will be possible to adress gene function in both, granule and Purkinje cells. Our demonstration of recombination in cells outside the ventricular zone and putative postmitotic neurons makes these lines well suited to study genes involved in neuronal migration and terminal differentiation throughout the brain. Furthermore, they will facilitate the analysis of neurotransmitter signaling and other molecules involved in synapse formation as well as neuronal plasticity and maintenance.

4.5 OUTLOOK

4.5.1 ANALYSIS OF NOTCH1 FUNCTION IN POSTMITOTIC NEURONS

The cerebellum is a model system to study postnatal neurogenesis as well as terminal differentiation of postmitotic neurons. Purkinje cells are derived from neuroepithelial cells of the cerebellar primordium that are known to express Notch1 (Reaume et al., 1992; Weinmaster et al., 1991). However, when the Purkinje cell precursors leave the cell cycle and start to migrate into the cerebellar anlage Notch1 is down regulated (Reaume et al., 1992; Weinmaster et al., 1991). Notch1 is reexpressed by Purkinje cells postnatally (Irvin et al., 2001; and Chapter 4) but so far the function of Notch1 at this late stage of development is known. *In vitro* experiments implicate Notch1 in dendrite formation (Berezovska et al., 1999; Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). In order to analyze Notch1 function in postmitotic neurons Cre-recombinase expressing lines 655, 660 and 663 were bread to lox/loxNotch1, Rosa 26 mice. The β-Galactosidase gene inserted into the rosa 26 locus can be used as a lineage tracer to facilitate the analysis of a phenotype. Triple transgenic animals based on the line 655 will be useful to determine Notch1 function in postmitotic Purkinje cells. The lineage tracer β-Galactosidase can be used to check the survival of cells that have deleted

Notch1. In order to determine differentiation of Notch1 deficient Purkinje cells markers such as Calbindin D28k, Map2, Kv3.3b and synaptophysin can be used (Hatten et al., 1997; Hatten and Heintz, 1995). These markers will visualize the dendritic tree of the Purkinje cells (Calbindin D28, Map2) and reveal synapse formation (Kv3.3b and synaptophysin). With the floxed Notch1 mice generated with transgenic lines 660 and 663 the effects of Notch1 in neurons of other brain regions can be analyzed. Since Cre-recombinase is not expressed in structures like the subventricular zone in these animals one can circumvent early differentiation defects and focus on later stages of neuronal differentiation including dendrite formation. However, we propose in chapter 4 that Notch signaling may influences the formation of neuronal contacts between specific neuronal sub-types. The loxNotch1 transgenics based on Cre-expressing lines 660 and 663 could be interesting models to study this hypothesis in the cerebellum, the cerebral cortex and the hippocampus.

4.5.2 Determining Gene Function During Cerebellar Development

The fact that the three lines 655, 660 and 663 show different Cre expression patterns in the cerebellum makes them good candidates to analyze the function of any gene involved in cerebellar development or in its maintenance by conditional gene ablation. Thereby, the gene can be specifically ablated in postmitotic Purkinje cells or in the granule cells of the IGL. In combination with line 663 a loxP flanked gene can be ablated in both cell types. This will allow to address detailed questions of gene function. Transgenic line 655 can be used to study genes involved in radial migration, in dendrite formation and synaptogenesis of Purkinje cells, as well as for the analysis of genes encoding paracrine factors that are important for granule or glial cell generation. Therefore, genes for cell-cell interaction molecules, transcription factors or growth factors will be interesting targets for gene ablation studies.

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5 General Conclusions and Outlook

Replacement of neurons in the aging and damaged nervous system is an important goal. A crucial step towards nervous system repair is promoting the generation of new neural cells. This is a great challenge since the nervous system contains hundreds of diverse and highly specialized cells. Over the last few years, remarkable progress has been made towards identifying and understanding neural stem cells. These cells can be isolated from embryonic structures as well as from adult tissues and have been shown to display a high degree of plasticity which is a prerequisite to generate the variety of neural cell types found in the adult nervous system. However, only recently has the generation of dopaminergic neurons from brain-derived stem cells and from embryonic stem cells been described (Kawasaki et al., 2000; Studer et al., 2000). These were the first reports where specific neuronal cell types were generated from stem cells *in vitro*. However, to generate differentiated neural cells *in vitro* will not be enough to succeed in nervous system repair. Reimplanted cells need to survive in the environment of the adult nervous system and not less important, they need to integrate into the existing network of the nervous system. Therefore, processes in postmitotic neurons like axon projection or dendrite formation need to be investigated. We have chosen the cerebellum as a model to study neurogenesis as well as later stages of development in postmitotic neurons. With only five neuronal cell types the cerebellum is a relatively simple structure that has already been well characterized (Hatten and Heintz, 1995). Known molecular markers describe discrete differentiation stages of the major neurons and provide a good basis to study differentiation, fate decisions and environmental requirements of cerebellar progenitor cells from the neuroepithelium.

In order to study these processes we have established a 2-dimensional cell culture system (see chapter 2). The culture system shows the advantage that both substrate and medium composition can easily be modulated. With this system we are able to expand the cells in an undifferentiated state where they maintain the expression of the intermediate filament protein Nestin and to induce differentiation to cerebellar neurons and glial cells. The fact that Purkinje cells and mature granule cells can be generated from multipotent neuroepithelial cells *in vitro* is remarkable and will enable us to investigate differentiation and fate determination processes in more detail. Furthermore, we have shown that cells in this system are infectable by retroviruses. This system enables us to investigate the influences of growth factors by changing the composition of the medium, of cell adhesion molecules by plating the cells onto different substrates, and of transcription factors and intracellular signal transducers by retroviral infection. These experiments will be used to identify candidate genes potentially involved in cell proliferation, differentiation and fate determination. However, processes such as dendrite formation or synaptogenesis occur after the different neuronal cell populations have been generated and may be dependent on an intact 3-dimensional structure. Therefore, these processes are less suitable for analysis *in vitro*. Furthermore, cell culture experiments can result in the production of *in vitro* artefacts. Hence, *in vitro* results must be supported and corroborated by *in vivo* experiments

Conditional gene ablation has proven to be a powerful tool to investigate gene function *in vivo* (Brocard et al., 1997; Kellendonk et al., 1999; Lütolf et al., in press; Tsien et al., 1996). In order to analyze gene function in specific cell types at specific developmental stages in the cerebellum, Cre-recombinase expressing transgenic mice will be necessary. Furthermore, mice carrying inducible versions of the Cre-transgenes (Gao et al., 1999; Gossen et al., 1994) will support the analysis of gene function during specific developmental periods. We have generated transgenic mice expressing Cre-recombinase under the control of a 1.1kb fragment of the Calbindin D28k promoter. These mice express Cre-recombinase in postmitotic neurons of the CNS (see chapter 4). The different transgenic lines show diverse expression patterns. We are currently using these animals to determine the function of Notch1 in postmitotic neurons. However, these Calbindin-Cre mice can also be used to analyze the function of other genes expressed in postmitotic neurons, if a loxP flanked allele has been generated. However, even

conditional gene ablation is not resistant to compensatory effects that may block or mask an observable phenotype. Thus, it will be valuable to isolate and culture the neuroepithelial cells from mutant mice in order to analyze developmental phenotypes in simplified *in vitro* systems. Such NEP cultures of mutant mice enable the analysis of changes in factor dependence and substrate requirements as well as the determination of developmental processes by transgene overexpression via retroviral infection.

In summary, the neuroepithelial cell culture system and Calbindin-Cre transgenic mice provide two complementary tools to investigate molecules involved in cerebellar and cortical neurogenesis. The cell culture will be useful to study the genesis of cerebellar cells and to test their plasticity, whereas the transgenic mice will help to analyze developmental processes at later stages. In both areas considerable progress has to be made towards the goal of understanding nervous system repair.

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6 APPENDIX: MATERIALS AND METHODS OF THE RECEPTOR TYROSINE KINASE SCREENING

6.1 SUMMARY OF THE SCREENING APPROACH

We have isolated cerebellar RNA from mice at different developmental stages. The stages E10.5, E13, E15, E18, P3, P14, P20 and adult reflect important time points for the differentiation of at least one of the cerebellar cell lineages (Hatten and Heintz, 1995). The RNA was reverse transcribed into cDNA by random primed reverse transcription. This cDNA then was used as a template in radioactive PCR reaction with degenerate primers raised against kinase domains V and IX of Tyrosine Kinases (Lai and Lemke, 1991; Wilks, 1989). To separate the PCR products corresponding to receptor tyrosine kinases (RTKs) from those of other tyrosine kinases the PCR reactions were run over a high resolution polyacrylamide gel. The PCR products corresponding to the RTKs (201bp) were isolated and reamplified in a nonradioactive PCR using the same primers as before. Then the products were cloned into pBluescript plasmid vector. 500 colonies were analyzed by sequencing and dot blotting with southern hybridization.To further analyze the found RTKs RNA *in situ* hybridization on mouse brains of different developmental stages was performed.

6.2 METHODS

6.2.1 RNA ISOLATION

Cerebellar tissue from different developmental stages was homogenized in presence of 2M guanidinium isothiocyanat (GT) buffer. After addition of 1/10 vol. of 2M NaOAc (pH4.5) RNA was extracted using water saturated phenol and chloroform. After two phenol/chloroform extractions the RNA was precipitated with 1 vol. of isopropanol and resuspended in diethyl pyrocarbonate (Sigma) treated water.

6.2.2 REVERSE TRANSCRIPTION

25, 50 and 100ng of RNA were used to generate reverse transcribed cDNA. The reaction volume was 10µl and contained reverse transcription buffer (Stratagene), 0.1M DTT, 2.5mM deoxyribonucleotides (Perkin Elmer), 2U/µl RNase inhibitor (Roche), 16ng/µl random hexamers (Pharmacia) and 1.25U/µl reverse transcriptase (Stratagene). After incubation for 60min. at 37°C the enzyme was heat inactivated at 68°C for 10 minutes. Then 30µl water were added to reach a final volume of 40µl. Relative concentrations of different samples were compared by GAPDH PCR and same amounts were then used for further analysis.

6.2.3 AMPLIFICATION OF RECEPTOR TYROSINE KINASE DOMAINS V-IX

The cDNA was used in a PCR with degenerate primers that were shown to amplify the receptor tyrosine kinase domains V-IV (Lai and Lemke, 1991; Wilks, 1989). For the first round of PCR 32P-labelled dCTP was added to the reaction. The reaction conditions were: 35 cycles of 1min. 95°C, 1min. 37°C and 1min. 72°C. Products then were separated on a denaturing 8M urea, 6% polyacrylamide gel. The PCR products were detected by exposure to X-ray film (Kodak). The bands corresponding to the RTKs (201bp) were eluted from the gel (in TE at 55°C for 3h) and reamplified with 12 cycles in a nonradioactive PCR using the same primers and conditions as before.

6.2.4 CLONING THE PCR PRODUCTS INTO PBLUESCRIPT

The degenerate primers contained an EcoRI and a PstI restriction site. Therefore, the

PCR products were cloned into pBluescript vector digested with EcoRI/PstI. XL-1 blue bacteria were transformed with the ligation products by electroporation or calcium phosphate. 50 clones were sequenced and for each receptor tyrosine kinase that was found a radioactive hybridization probe was prepared. With these probes the remaining 450 clones were tested by dot blotting with southern blot hybridization. Clones that didn't give a signal then were sequenced.

6.2.5 *In situ* HYBRIDIZATION

Embryos and brains were isolated and frozen in OCT (TissueTech) on dry ice. Twenty μm frozen sections were thaw mounted onto Superfrost slides (Mettler), air-dried, and fixed in 4% paraformaldehyde. In situ RNA hybridization was performed with digoxigenin-labeled RNA probes overnight at 72°C in buffer containing 50% formamide, and detected using an anti-DIG-AP antibody according to manufacturer's instructions (Roche Diagnostics). Expression was detected colorimetric reaction with NBT (340mg/ml) and BCIP (165mg/ml) as reaction products. Coverslips were mounted in glycerol and images taken using an Axioplan microscope in conjunction with a Hamamatsu camera.

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