Regionalization of adult neurogenesis:

The role of the transcription factors Dlx2 and Pax6 in the murine subependymal zone

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Ehrenwörtliche Versicherung

Ich versichere hiermit ehrenwörtlich, dass die Dissertation von mir selbständing, ohne unerlaubte Beihilfe angefertigt ist.

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Für meine Eltern

For my parents

Table of Content

1 Abstract

The vast majority of neurons in the murine brain are generated during embryonic neurogenesis. However, at least two neurogenic niches continue to produce specific types of neurons throughout life. The adult dentate gyrus harbours stem cells that generate dentate granule neurons and the subependymal zone produces distinct types of olfactory interneurons. The adult neurogenic subependymal zone is derived from the embryonic dorsal and ventral subventricular zone of the telencephalon, i. e. progenitor domains which generate both the ventral and dorsal glutamatergic and GABAergic neurons, respectively. While a cascade of transcription factors beginning with Pax6 governs the generation of glutamatergic cortical neurons, transcription factors of the Dlx family are crucial for the embryonic neurogenesis of GABAergic neurons. Notably, Pax6 and Dlx transcription factors factors are expressed in the adult subependymal zone. In this study I investigated the regionalization of the adult subependymal neurogenic niche in regard to Pax6 and Dlx and I examined the role of these factors in neuronal subtype specification.

Consistent with their embryonic origin progenitors in the adult brain express Dlx1 and Dlx2 in the lateral, but not the dorsal subependymal zone. Using retroviral vectors I demonstrated that Dlx2 is necessary for neurogenesis of virtually all olfactory interneurons arising from the lateral subependymal zone. Beyond its function in generic neurogenesis, Dlx2 plays a crucial role in neuronal subtype specification in the adult olfactory bulb promoting specification of dopaminergic interneurons. Strikingly, Dlx2 requires interaction with Pax6, as Pax6 deletion blocks Dlx2 mediated neuronal specification. Of note, however, Pax6 protein is expressed in a gradient being especially abundant in dorsal regions of the adult subpenedymal zone. While playing obviously a role in the genesis of GABAergic interneurons, I also investigated whether the dorsal subependymal zone could give rise to glutamatergic neurons which have so far been overlooked. Surprisingly, progenitors located mainly in dorso-rostral regions of the subependymal zone express transcription factors previously linked to glutamatergic neurogenesis like Pax6 → Neurogenin2 → Tbr2 → Tbr1. These neurons migrate along the rostral migratory stream and integrate into the glomerular layer of the olfactory bulb. Finally, I provide evidence that these Tbr2-positive cells could become recruited following cortical lesions where callosal projection neurons are depleted.

2 Introduction

It has been believed that no new neurons in the adult brain are generated and that therefore neuronal loss cannot be compensated. Consequently, the adult brain is devoid of neuronal stem cells, and thus of the ability to make new nerve cells and regenerate after injuries (S. Ramón y Cajal, 1928). Thus, most of the common brain diseases accompanied by neuronal loss cannot be cured. Amongst them are well known ones: stroke where a certain brain area lacks oxygen supply followed by neuronal death, Parkinson's disease accompanied by the loss of dopaminergic neurons in the substantia nigra, and Alzheimer's disease with a general neuronal degeneration. According to this dogma, the vast majority of neurons in the mammalian brain is generated during embryonic development. However, this doctrine ended in 1969 (J. Altman, 1969) when adult generated neurons were found in two regions of the adult mammalian forebrain that receive a constant supply of new neurons: the **olfactory bulb** and the **dentate gyrus** (Fig. **1**).

2.1 An abstract of the history of adult neurogenesis and basic principles

The phenomenon of **postnatal and adult neurogenesis** had been first described by (J. Altman, 1969) although proliferation in the adult central nervous system had been suggested previously (E. Allen, 1912). Because techniques were not available for tagging these proliferating cells, these investigators could only speculate about their fate. This problem was solved when autoradiographic labelling techniques were introduced. Proliferating cells could be labelled by injection of [3H]-thymidine which is incorporated into the genome during DNA synthesis. This demonstrated on the one hand the persisting proliferation in the postnatal and adult forebrain, on the other hand tagged cells could be tracked and their fate observed (J. Altman, 1969; M. S. Kaplan and J. W. Hinds, 1977). Altman's study suggested that the rostral extension of the rat ventricle is a mitotically active region and contains progenitor cells which later differentiate into short-axoned neurons, or microneurons in the olfactory bulb (J. Altman, 1969). The same labelling technique also suggested neurogenesis in the dentate gyrus of the hippocampus in rodents (J. Altman and G. D. Das, 1965; S. A. Bayer, 1982; S. A. Bayer et al., 1982; S. A. Bayer, 1983).

Although Altman was rather cautious in stating cell replacement in the olfactory bulb: "Considering the large number of immigrating cells and the relatively small size of the receiving area, it is conceivable that the addition of these cells does not have a growth but a renewal function a." [reviewed by (F. Nottebohm, 2002a)].

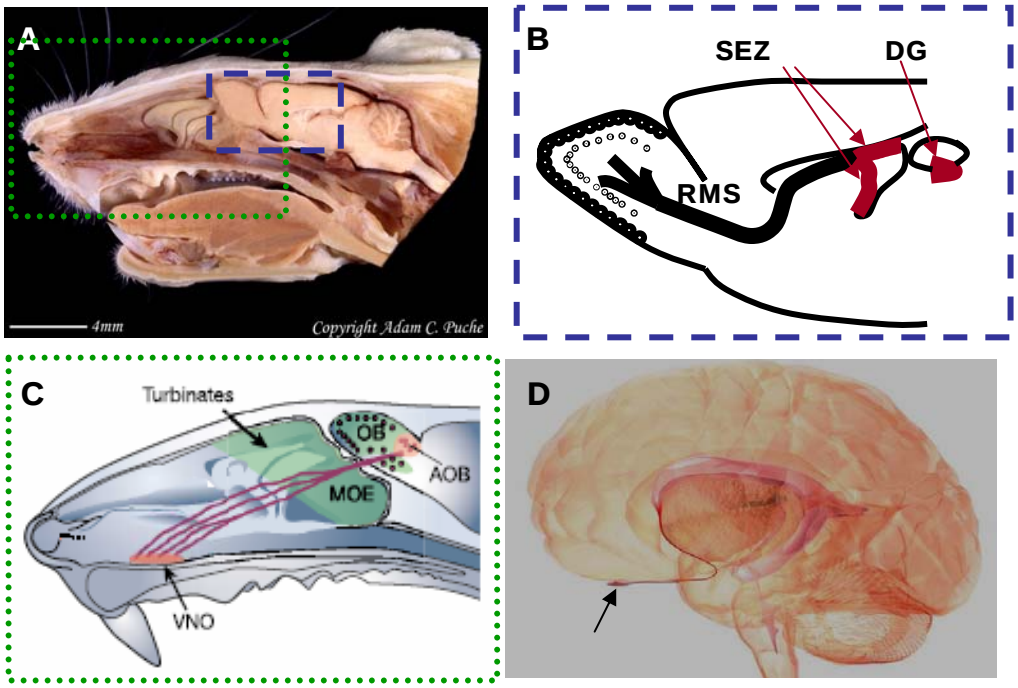


Fig. 1: Overview of adult neurogenic regions in the mammalian forebrain

Medial view of the whole adult rat head dissected away to show the brain and other structures present at the midline. Photograph by Adam C. Puche, copyright Adam C. Puche from The Olfactory Image Archive. The boxed area is shown in a schematic drawing in (B).
(B) Schematic sagittal overview of the adult rat/mouse brain at more lateral levels. The neurogenic areas (DG, SEZ) are indicated in red. Cells generated within the subependymal zone migrate along the rostral migratory stream to the olfactory bulb.
DG = dentate gyrus, SEZ = subependymal zone, RMS = rostral migratory stream, OB = olfactory bulb
(C) Schematic drawing of the rat forehead indicating the vomeronasal organ (VNO) that projects into the accessory olfactory bulb (AOB). The main olfactory epithelium (MOE) is located closely to the main olfactory bulb (OB). It contains G-Protein couple odorant receptors connected to olfactory sensory neurons (OSN) that project into the outer region of the olfactory bulb (S. Firestein, 2001).
(D) The human olfactory bulb (red; indicated by arrow) is in relation to brain size much smaller compared to the rodent brain. It also receives neurons from a subventricular zone overlying the ventricles (red) that migrate via a rostral migratory stream (outlined in red) towards the anterior olfactory cortex (arrow).

Although Altman's findings were clearly suggesting neurogenesis, it took many years until his hypothesis was proved and accepted. Scientists were not sure that the cells he called labelled neurons were, in all cases, neurons. They were concerned that neurogenesis was greatest at postnatal ages arguing for a delayed development in these brain regions. Furthermore, the incorporation of [3H]-thymidine during DNA repair in

neurons could have been observed by Altman (F. Nottebohm, 1985, 2002a). Altman's studies were confirmed when new labelling techniques for proliferating cells gave the same results, e. g. by the DNA analogue BrdU and retroviral labelling (H. A. Cameron et al., 1993; F. S. Corotto et al., 1993; M. B. Luskin, 1993; C. Lois and A. Alvarez-Buylla, 1994; H. G. Kuhn et al., 1996). New methods for lineage tracing substantiated the available tracking methods by transplantation experiments (S. De Marchis et al., 2007; M. Kohwi et al., 2007a; F. T. Merkle et al., 2007) and Cre-mediated fate mapping (S. Willaime-Morawek et al., 2006; M. Kohwi et al., 2007a; J. Ninkovic et al., 2007; R. E. Ventura and J. E. Goldman, 2007; K. M. Young et al., 2007; I. Imayoshi et al., 2008).

The mitotically active region was named after its location under the ventricular ependyma "subependymal layer" (I. Smart, 1961; P. D. Lewis, 1968) and is called nowadays **subependymal zone** (SEZ) or subventricular zone (SVZ) (Fig. **1**). Newly generated young neurons or neuroblasts travel along the ventricular wall in rostral direction towards the olfactory bulb and form at the ventricle's anterior extension the **rostral migratory stream** (RMS) migrating into the olfactory bulb. These neuronal cells integrate upon their arrival in the olfactory bulb as specific subtypes of interneurons. As the size of the olfactory bulb does not substantially change throughout life these newly generated neurons must replace previously existing cells (L. Rosselli-Austin and J. Altman, 1979; M. S. Kaplan et al., 1985; M. Biebl et al., 2000; L. Petreanu and A. Alvarez-Buylla, 2002). However, only specific types of neurons are generated during adulthood and replaced.

Notably, adult neurogenesis is not only found in the rodent brain but recently also in primates and humans (P. S. Eriksson et al., 1998; E. Gould et al., 1998; D. R. Kornack and P. Rakic, 1999; M. A. Curtis et al., 2007) as well as in non-mammalian vertebrates and insects (S. A. Goldman and F. Nottebohm, 1983; M. Cayre et al., 1996; B. Adolf et al., 2006). Although the **human olfactory bulb** (**anterior olfactory cortex**) is relatively small in size compared to the rest of the human brain (Fig. **1**). Strikingly even the human olfactory bulb might be a region of endogenous ongoing neurogenesis and consequently there are adult human brain stem cells present. Young immature neuroblasts migrate also from a subventricular zone that overlies the caudate nucleus via a kind of descending rostral migratory stream (Fig. **1**) that takes a turn and then passes towards the anterior olfactory cortex which gives rise to the olfactory tract that leads finally to the olfactory bulb (M. A. Curtis et al., 2007). The human rostral

migratory stream has an amazing length of 17 mm whereas the mediolateral extent is 2.7 mm (M. A. Curtis et al., 2007); in contrast the rodent rostral migratory steam is only a few millimiters long.

However, two interesting questions still remain to be fully clarified: wyh do the olfactory bulb and the dentate gyrus need new neurons throughout life and why is adult neurogenesis retained and restricted to few neurogenic regions in the adult brain?

2.2 The neurogenic niche: why is adult neurogenesis retained and restricted to few neurogenic regions?

A neurogenic niche requires not only specialized stem cells but also needs a special microenvironment that enables stem cells to generate progeny and to maintain themselves. This process is highly regulated by extracellular factors and signalling pathways that are described in more detail later. Consequently, the environment and the cells located in the niche allow neurogenesis to occur (I. Ortega-Perez et al., 2007). Precursors,, astrocytes, vasculature, microglia, extracellular matrix and the basal membrane are also present in non-neurogenic brain areas but they seem to possess specific properties in the subependymal zone and dentate gyrus (D. A. Lim et al., 2007; Z. Mirzadeh et al., 2008; Q. Shen et al., 2008; M. Tavazoie et al., 2008). Other non-neurogenic regions might contain stem cells that cannot act without the according niche factors (A. Buffo et al., 2008). They may contain precursor cells, but lack the permissive microenvironment. The neurogenic potential of a niche can be tested by transplantiation studies. When grafted into a neurogenic niche a precursor should be able to develop into a neuron and upon insertion into a non-neurogenic area a precursor should develop a glial cell or die (R. Seidenfaden et al., 2006; I. Ortega-Perez et al., 2007). Nevertheless non-neurogenic regions can exhibit reactive neurogenesis upon introduction of neurogenic factors (A. Buffo et al., 2005) and contain an endogenous stem cell population after injury (A. Buffo et al., 2008).

The rate of neuronal production in the adult brain is by far lower than during embryonic development. If the neurons generated during adulthood would have the same properties compared to embryonic neurogenesis then adult neurogenesis can be considered insignificant (F. Nottebohm, 2002b). Furthermore, only few organs receive or need new neurons throughout life: the olfactory bulb and the dentate gyrus.

2.3 Why do the olfactory bulb and the dentate gyrus need new neurons throughout life?

Neurogenesis responds to intrinsic and extrinsic factors and is a highly dynamic process (G. L. Ming and H. Song, 2005). Notably, it decreases upon aging in both the subependymal and subgranular zone (H. G. Kuhn et al., 1996; K. Jin et al., 2003b; E. Enwere et al., 2004). Numerous studies reported enhanced neurogenesis in the dentate gyrus upon learning, running and enriched environment (G. Kempermann et al., 1997; E. Gould et al., 1999; M. Nilsson et al., 1999; H. van Praag et al., 1999b; H. van Praag et al., 1999a; J. Brown et al., 2003; B. Leuner et al., 2004; J. Ninkovic et al., 2007). Neurogenesis is related to the performance on a hippocampal dependent task. A decrease in the number of newly generated granule neurons is correlated with impaired performance (G. Kempermann, 2002; F. Nottebohm, 2002b; I. Ortega-Perez et al., 2007). Odour enrichment enhanced neurogenesis in the subependymal zone (C. Rochefort et al., 2002) and olfactory discrimination learning increases the survival of adult-born neurons (M. Alonso et al., 2006; A. Mouret et al., 2008). In summary, numerous studies support the notion that adult neurogenesis is a dynamic, plastic process that respond to many extrinsic and intrinsic stimuli.

On the contrary, the functional significance regarding behavioural studies is still under debate. Various theories suggested potential roles of adult neurogenesis. Ongoing genesis of periglomerular neurons may reflect a basic mechanism for rewiring that accommodates learning (C. Rochefort et al., 2002), and continuously born neurons are temporarily immature with unique physiological properties (G. Gheusi and P. M. Lledo, 2007). New neurons may contribute to environmental adaptation, learning and may maintain these organs especially plastic (F. Nottebohm, 1985; A. Alvarez-Buylla et al., 1990; J. R. Kirn and F. Nottebohm, 1993). Notably, also adult generated olfactory granule neurons undergo distinct experience-dependent modifications of their olfactory responses (S. S. Magavi et al., 2005). Evidence for the functional significance of adult neurogenesis could be achieved by ablation of newly born neurons by irradiation, treatment with antimitotic drugs or expression of a fragment of the diphtheria toxin in newly born neurons (G. Kempermann et al., 1998; T. J. Shors et al., 2002; J. Raber et al., 2004; M. D. Saxe et al., 2006; I. Imayoshi et al., 2008). Whereas treatment with antimitotic drugs or irradiation did not alter hippocampal depedent spatial memory formation (T. J. Shors et al., 2002; M. D. Saxe et al., 2006), other studies suggested that ablated adult neurogenesis in the dentate gyrus results in impaired spatial memory

formation (G. Kempermann et al., 1997; G. Kempermann et al., 1998; J. Raber et al., 2004; J. S. Snyder et al., 2005; I. Imayoshi et al., 2008). The efficiency of killing newborn neurons and strain or species dependent differences may contribute to these discrepancies. Interestingly, ablation of newborn neurons for 6 months in the olfactory bulb did not alter the ability of odor discrimination (I. Imayoshi et al., 2008). However, other studies suggest a role for newly generated neurons in the olfactory bulb which examined mice that ablated neurogenesis for one year (G. Gheusi et al., 2000).

Deletion of TrkB in adult hippocampal progenitors and subsequent cell death in the dentate gyrus of the hippocampus do not affect learning. In contrast deletion of TrkB increases anxiety-like behaviour and these mice are insensitive to antidepressive treatment in depression paradigms (M. Bergami et al., 2008; Y. Li et al., 2008).

In summary, olfactory and hippocampal neurogenesis may play an important role in odor discrimination, the formation of spatial memories, or anxiety and depression but it is difficult to prove the functional significance in behavioural tests. More studies with behavioural tests may contribute to the clarification of this issue. Since adult neurogenesis replaces only specific types of neurons, ablation or knock-down of this process is not comparable with an acute lesion which destroys the whole olfactory bulb or the dentate gyrus functionally (J. B. Aimone et al., 2006). Newly generated neurons may contribute to a rewiring process (C. Rochefort et al., 2002), however the output creating neurons of the olfactory bulb are generated during embryonic neurogenesis and do not undergo replacement during adulthood (G. Shepherd, 2004; V. Egger and N. N. Urban, 2006; G. M. Shepherd et al., 2007).

2.4 The olfactory bulb: function and anatomical structure

The olfactory bulb belongs to the central nervous system (CNS), is an outgrowth of the forebrain, specialized for processing the molecular signals derived from odour receptors that give rise to the sense of smell (S. Firestein, 2001; G. Shepherd, 2004). Based on the size of the olfactory bulb compared to the size of the rest of the brain, animals can be classified as (i) macrosmatic, such as rodents with large olfactory bulbs, (ii) microsmatic, such as primates with relatively small OBs, and (iii) anosmatic animals with no or vestigial olfactory bulbs (J. I. Johnson et al., 1994; T. D. Smith and K. P. Bhatnagar, 2004). An indication of the importance of olfactory systems is that a significant proportion (~ 4%) of the genome of many higher eukaryotes encodes the proteins of smell (L. Buck and R. Axel, 1991; S. Firestein, 2001; X. Zhang and S.

Firestein, 2002). Most mammals and reptiles have two distinct parts of their olfactory system: a main olfactory system and an **accessory olfactory system** (Fig. **1**) (A. C. Allison, 1953; H. H. Hoffman, 1963; D. G. Moulton and L. M. Beidler, 1967). The latter is important in the specific task of finding a receptive mate (Fig. **1**).

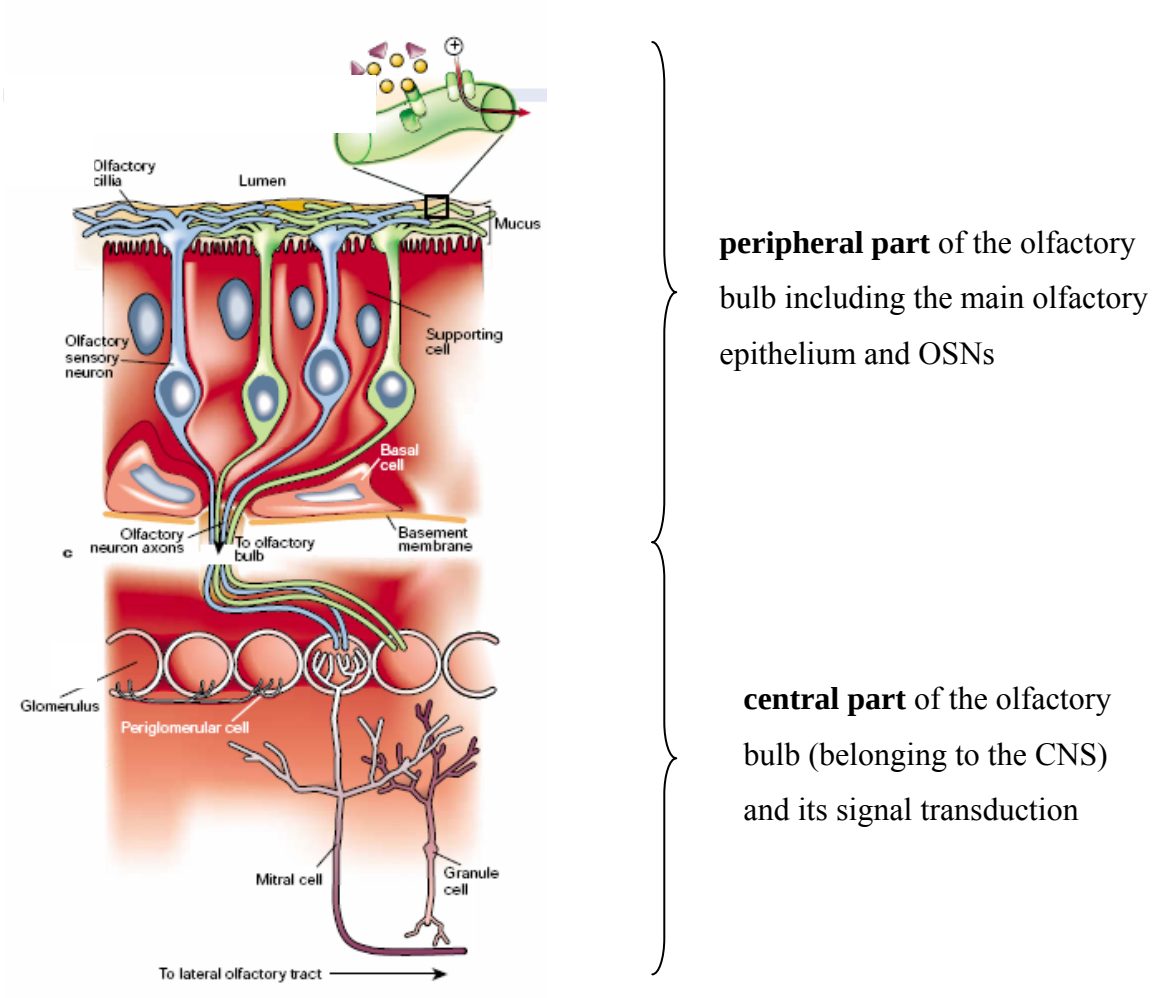


Fig. 2: Overview of the peripheral and central part of the olfactory system

Olfactory sensory neurons (OSNs) are the primary sensing cells and harbour odour receptors located in their cilia. They send axons to the main olfactory bulb which is part of the central nervous system. The incoming signals from terminals of OSNs are processed in the outer layer of the olfactory bulb that is organized in glomeruli. Mitral cells are the main class of cells in the olfactory bulb that sends axons to other brain centres such as the piriform cortex, the amygdale and the entorhinal cortex (S. Firestein, 2001).

Known as the **vomeronasal system**, it specializes in recognizing species-specific olfactory signals produced by one sex and perceived by the other. These signals contain information not only about location but also reproductive state and availability. Behavioural evidence indicates that most often, the stimuli detected by the accessory olfactory system are **pheromones** which are believed to interact with the endocrine

system. In addition to its role in sexual behaviours, it is important in influencing other social behaviours such as territoriality, aggression and suckling (L. B. Buck, 2000; S. Firestein, 2001). The vomeronasal system projects into the accessory olfactory bulb where information is processed and sent to higher brain centers.

However, this work focuses on **the main olfactory system** which is responsible for the sense of smell. The olfactory bulb receives input from the olfactory sensory neurons, integrates and processes incoming signals, and sends output directly to the olfactory cortex. It can be divided into two parts: (i) a **peripheral part**, where olfactory receptors detect an external stimulus in the main olfactory epithelium (MOE) belonging to the peripheral nervous system, sensing an external stimulus derived from an odour and encode this as an electric signal; (ii) the central part where all incoming signals are integrated and output is created (Fig. **2**).

The odorants are inhaled through the nasal cavities during breathing and reach the **main olfactory epithelium** (MOE) which is located in the peripheral part of the olfactory system. It contains several millions of olfactory sensory neurons (OSNs) that express a variety of **odour receptors** (ORs). They belong to an evolutionary old, sophisticated chemical-detecting system of G-protein coupled receptors and represent probably the largest family in the genome with more than 1,000 genes for ORs (S. Firestein, 2001). A remarkable amount of compounds is detected by the ORs, amongst them are aliphatic and aromatic molecules with diverse functional groups, including aldehydes, esters, ketones, alcohols, alkenes, carboxylic acids, amines, imines, thiols, halides, nitriles, sulphides and ethers (S. Firestein, 2001). ORs are harboured by **olfactory sensory neurons** (OSNs) which are bipolar cells with a single dendrite that reaches up to the surface of the tissue and ends in about 20–30 very fine cilia up to 200 µm length. These cilia, which lie in the thin layer of mucus covering the tissue, are the site of the sensory transduction apparatus. Thin axons from the proximal poles of the cell are bundled. However, upon reaching the olfactory bulb they defasciculate and begin to reorganize (P. Mombaerts et al., 1996; S. Firestein, 2001; W. W. Au et al., 2002; H. B. Treloar et al., 2002) (Fig. **2**).

2.4.1 The basic circuitry of the main olfactory bulb

Axons from olfactory sensory neurons terminate in a superficial area of the main olfactory system (olfactory bulb) which is organized in several layers and signals are processed in an outward-inward fashion. Notably, the synaptic architecture of neuronal

circuits in the olfactory bulb is conserved across vertebrate classes (A. C. Allison, 1953; R. Tabor and R. W. Friedrich, 2008).

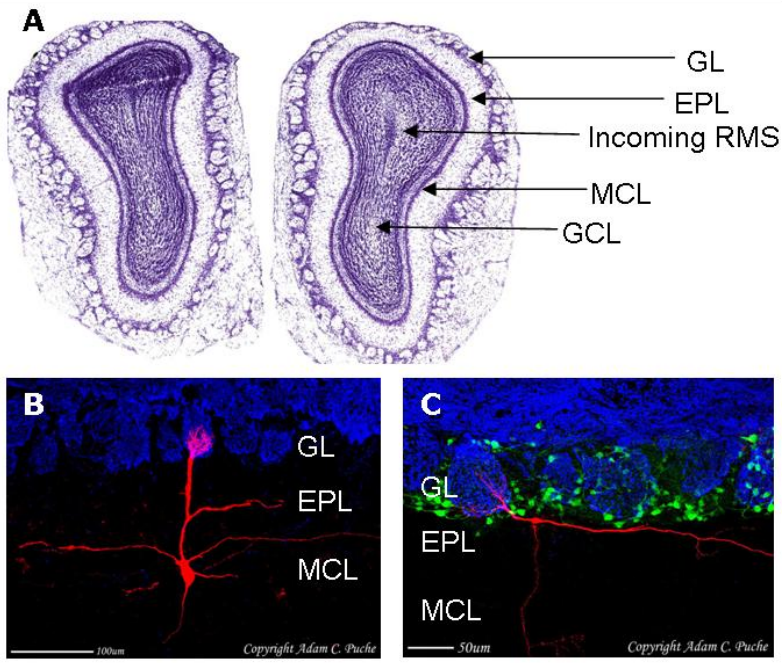


Fig. 3: Overview of a coronal section of the olfactory bulbs

(A) Coronal section from the adult olfactory bulb indicating the composition of the bulbs in different layers: the glomerular layer (GL) is organized in glomeruli and OSNs synapse onto mitral cells here; the external plexiform layer (EPL) where only few neurons are located and where some dendrites of mitral cells end; the mitral cell layer (MCL) contains only one layer of output creating cells called mitral cells; the granule cell layer (GCL) is a dense layer of interneurons synapsing on mitral cell and external tufted cell axons; and the deepest layer of the olfactory bulb where newly generated neuroblasts enter from the rostral migratory stream.

(B) Postnatal day 8 mouse olfactory bulb section with a single mitral cell filled with biocytin (red) and olfactory receptor axons stained with olfactory marker protein (blue). Photography by Adam C. Puche, cell biocytin fill by Philip Heyward, copyright Adam C. Puche. Picture taken from The Olfactory Image Archive

(C) Adult mouse olfactory bulb section with a single superficial tufted cell filled with biocytin (red), dopaminergic neurons stained with tyrosine hydroxylase (green) and olfactory receptor axons stained with olfactory marker protein (blue). Photography by Adam C. Puche, cell biocytin fill by Sergei Karnup, copyright Adam C. Puche. Picture taken from The Olfactory Image Archive

Axons of olfactory sensory neurons terminate in the outer layer which comprise **glomeruli** (glomerular layer), spherical regions of neuropil which serve as input modules for the olfactory bulb (Fig. **2**, Fig. **3**, Fig. **4**). The **glomerular layer** is followed by the **external plexiform layer** constituted predominantly of dendrites of mitral and tufted cells, the **mitral cell layer** which contains the output creating mitral cells. The corresponding cell bodies are located in a thin sheet (mitral cell layer) in case of the mitral cells around 200 – 450 µm under the glomerular cell layer or in case of the tufted cells in the external plexiform layer [reviewed in (G. Shepherd, 2004; V. Egger

and N. N. Urban, 2006; G. Gheusi and P. M. Lledo, 2007)] (Fig. **3**, Fig. **4**). The inner part of the olfactory bulb is called **granule cell layer** and harbours interneurons connected with mitral cells (Fig. **3**). These interneurons act mostly inhibitory and mediate synaptic interactions across glomeruli or output creating mitral cells (G. Shepherd, 2004; R. Tabor and R. W. Friedrich, 2008).

The most prominent inter-glomerular synaptic pathway is the mitral cell – interneuron - mitral cell pathway, where periglomerular or granule cells are excited by the glutamatergic mitral cell via interneuron synapses and feed back **GABAergic inhibition** onto the same and other mitral cells (G. Shepherd, 2004; R. Tabor and R. W. Friedrich, 2008). This might shape spatio-temporal patterns of the olfactory bulb output activity and may thereby optimize odour representations for processing in higher brain regions.

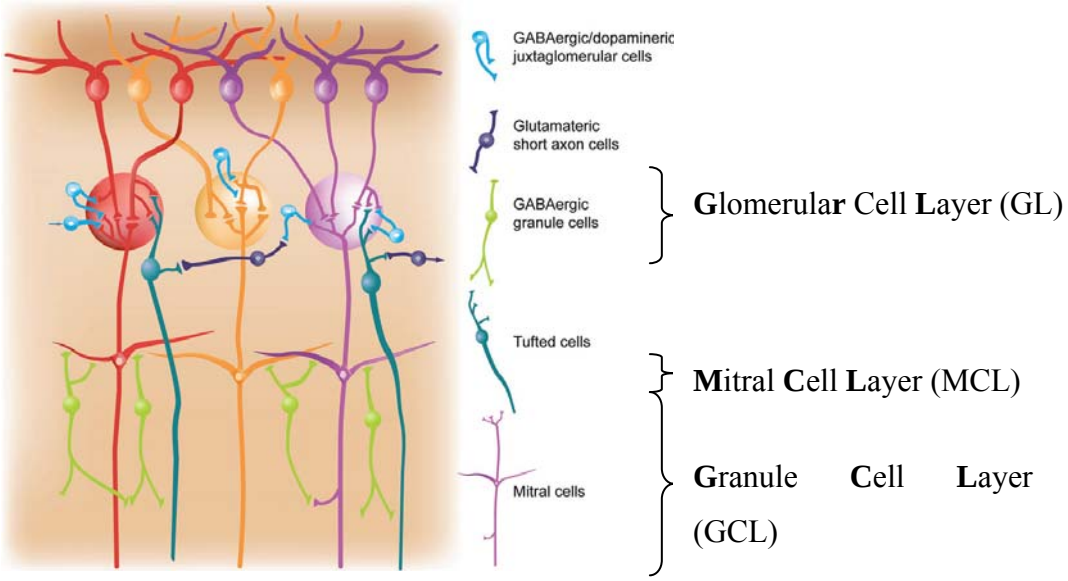


Fig. 4: Basic circuitry of the main olfactory bulb

Olfactory sensory neurons that express the same odorant receptor gene project their axons to either of two glomeruli in the olfactory bulb. Three populations of sensory neurons, each expressing a different odorant receptor gene, are depicted in different colors. Their axons converge on specific glomeruli, where they synapse with the dendrite of local interneurons (periglomerular neurons) and second-order neurons (mitral cells). The lateral dendrites of mitral cells contact the apical dendrites of granule cells. Short axon cells are bulbar interneurons that contact both apical and lateral dendrites of mitral cells (G. Gheusi and P. M. Lledo, 2007).

The glomerulus is the basic unit in the odour map and there are about 1,800 glomeruli in the rodent olfactory bulb (A. C. Allison, 1953). In a remarkable example of convergence, glomeruli receive input from between 5,000 and 10,000 olfactory receptor cells but output onto only 50 neurons, the mitral cells (P. Mombaerts et al., 1996; G.

Shepherd, 2004; V. Egger and N. N. Urban, 2006; A. Walz et al., 2006). By combining so much input, the olfactory system is able to detect even very faint odours. Inside the glomerulus, the axons of OSNs contact the dendrites of several types of cells; amongst them are mitral and tufted cells, short-axon cells and periglomerular interneurons (Fig. **2,** Fig. **3,** Fig. **4**) which are described in detail in the following paragraphs.

Only two cell types create output from the olfactory bulb to other brain regions: **mitral cells and external tufted cells.** They use glutamate as their primary neurotransmitter in contrast to most other cell types in the olfactory bulb. While these cell types differ in their axonal and dendritic projection patterns, they both form **dendro-dendritic synapses** with interneurons that exert predominantly inhibitory effects (J. M. Christie et al., 2001; G. Shepherd, 2004; V. Egger and N. N. Urban, 2006; M. Wachowiak and M. T. Shipley, 2006) (see also 2.4.2 Intrinsic neurons: Periglomerular and granule neurons). Interestingly, the dendritic tree of a mitral or tufted cell belongs to one single glomerulus (Fig. **2**, Fig. **3**, Fig. **4**). Axonal projections of mitral cells gather at the posterolateral surface to form the **lateral olfactory tract** (K. Kishi et al., 1984; G. Shepherd, 2004; A. Walz et al., 2006) and target a number of brain areas, including the piriform cortex (main part of the olfactory cortex), the medial amygdala, and the entorhinal cortex (J. A. Dusek and H. Eichenbaum, 1997). The piriform cortex is probably the area most closely associated with identifying the odour. The medial amygdala is involved in social functions such as mating and the recognition of animals of the same species. The entorhinal cortex is associated with memory, e.g. to pair odours with proper memories (J. E. Schwob and J. L. Price, 1984; A. Walz et al., 2006).

Only few neurons are located in the **external plexiform layer**, as it is mostly constituted of dendrites from mitral and external tufted cells. Some cell bodies of external tufted cells are located here as well as intrinsic neurons expressing parvalbumin (K. Toida et al., 2000). Interestingly, most of the granule neurons establish reciprocal dendro-dendritic contacts with secondary dendrites of mitral/tufted cells in the externalplexiform layer, while the primary dendrite has hardly synapses and is therefore responsible for the transmission of the incoming signal to the cell body (G. Shepherd, 2004).

As mentioned above, other neurons synapse in the glomerular layer besides mitral cells and olfactory sensory neurons. These are known collectively as juxtaglomerular or periglomerular neurons and are mainly GABAergic (for details see chapter: 2.4.2 Intrinsic neurons: Periglomerular and granule neurons). In addition **short axon cells**

have been suggested to be glutamatergic and to contact both apical and lateral dendrites of mitral cells (J. L. Price and T. P. Powell, 1970; G. Gheusi and P. M. Lledo, 2007).Their dendrites branch in the interglomerular spaces (G. Shepherd, 2004). A second type of short axon cells exist in deeper layers of the olfactory bulb (deep short axon cells) which exhibit different functions: (i) intrabulbar projections from deep to superficial layers, (ii) projection into the olfactory bulb and into higher olfactory areas, and (iii) influence of olfactory bulb activity by selectively innervating GABAergic interneurons (M. D. Eyre et al., 2008).

2.4.2 Intrinsic neurons: Periglomerular and granule neurons

Periglomerular neurons are located in the outer layer of the olfactory bulb (glomerular layer) where the mitral cells receive input from the olfactory sensory neurons. In contrast, granule neurons exhibit their function on the output creating axonal parts of mitral cells. Therefore, one glomerulus connected to specific mitral cells and granule neurons can be seen as one basic "olfactory colum" and might be analogue with orientation columns in the visual cortex (G. Shepherd, 2004).

Periglomerular neurons (PGNs) are located in the glomerulus and belong to one of the smallest neurons in the brain and were originally described by Cajal as glial cells. Their cell body is only around 6 – 8 μm in diameter (A. J. Pinching and T. P. Powell, 1971b) and their bushy dendrites arborize mostly within one glomerulus intermingling with the terminals of OSNs and the dendritic branches of mitral and external tufted cells (Fig. **5**) (G. Shepherd, 2004). Axons of periglomerular neurons function as information transmitter to neighbouring glomeruli and act either inhibitory or excitatory (G. M. Shepherd, 1963; W. J. Freeman, 1974; T. V. Getchell and G. M. Shepherd, 1975a, b). Notably, olfactory sensory neuron axons do not terminate randomly within one glomerulus but rather occupy distinct intraglomerular parts (L. J. Land et al., 1970; L. J. Land and G. M. Shepherd, 1974; H. Treloar et al., 1996). They synapse onto mitral and external tufted cells, but are also connected to periglomerular neurons via axodendritic synapses (A. J. Pinching and T. P. Powell, 1971a, b). Furthermore, PGNs form dendro-dendritic synapses onto mitral and tufted cells and vice versa.

Notably, periglomerular neurons are **heterogeneous** in regard to their neurochemical identity; however the majority is GABAergic demonstrated by the presence of GABA and the GABA synthesizing enzyme GAD65 and GAD67 in the corresponding GFP knock-in mice (P. Panzanelli et al., 2007; S. Parrish-Aungst et al., 2007) (Fig. **5**).

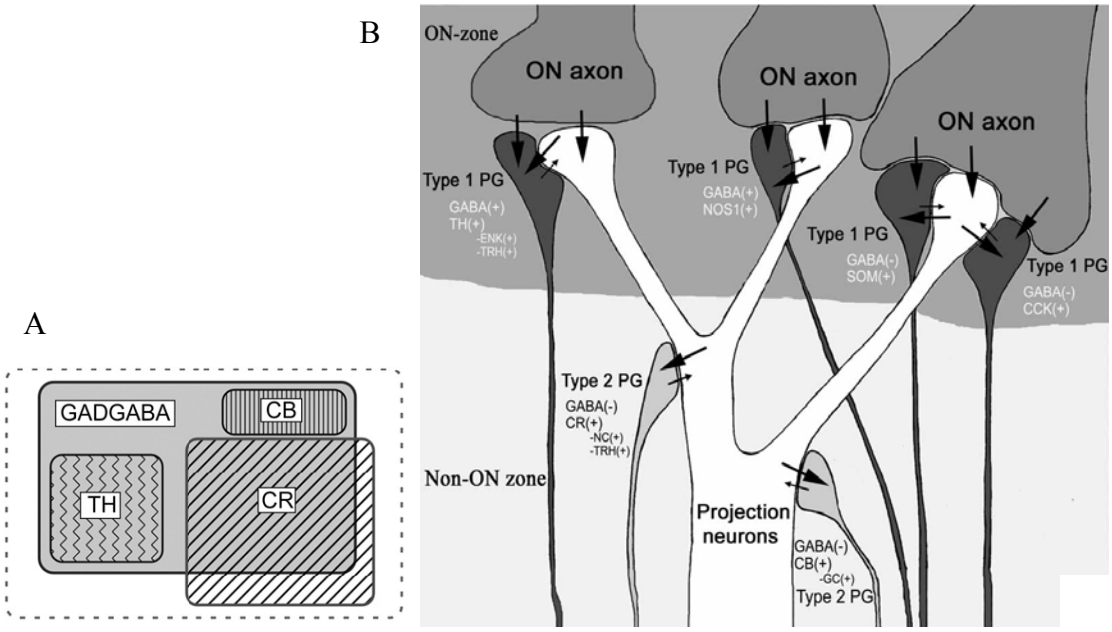


Fig. 5:Schematic overview of the neurochemical identity of periglomerular neurons in the adult mouse olfactory bulb.

(A) Most of the glomerular neurons are of GABAergic identitiy as confirmed in the GAD65 and 67 GFP knock-in mice. Whereas granule neurons are homogeneous, periglomerular neurons can be subdivided according to their neurochemical identity. Calretinin (CR), Calbindin (CB) and Tyrosine hydroxlyase (TH). Adapted from (K. Kosaka and T. Kosaka, 2007).
(B) At least two different types of cells periglomerular neurons can be classified: **Type 1** extends its dendrites throughout the sensory and the synaptic compartments of the glomerular neuropil and receive synapses from the olfactory sensory neurons. This type is composed of at least two groups of neurons; the first is GABAergic including the dopaminergic/TH positive ones, and the second is GABA negative. **Type 2** periglomerula neurons restrict their dendrites to the synaptic subcompartment of the glomerular neuropil, so they do not receive synapses from the olfactory sensory neurons. This type includes two subgroups; the first expresses the calcium-binding protein calbindin D-28K and the second calretinin. Big arrows represent symmetrical synaptic contacts, and small arrows represent asymmetrical synaptic contacts. ON = olfactory sensory neuron; PG = periglomerular neuron; adapted from (M. Gutierrez-Mecinas et al., 2005)

Amongst them are tyrosine hydroxylase-positive, calretinin-positive and calbindin-positive cells (K. Kosaka et al., 1995; K. Toida et al., 2000; P. Panzanelli et al., 2007; S. Parrish-Aungst et al., 2007). Interspecies differences in the neurochemical composition of periglomerular cells could indicate different modes in the modulation of olfactory information. Based on their connectivity two subpopulations can be distinguished: (i) **type 1** periglomerular neurons, which receive excitatory synaptic input from the olfactory sensory neurons, and (ii) **type 2** cells, which establish few or no synapses with olfactory sensory neuron axons (K. Kosaka et al., 1997; K. Toida et al., 1998; K. Toida et al., 2000; P. Panzanelli et al., 2007). Notably, both classes are dendritically interconnected with those of the principal neurons in the glomerular neuropil (Pinching and Powell, 1971a; Toida et al., 1998, 2000).

The majority of periglomerular neurons is generated postnatally (S. A. Bayer, 1983) and these cells undergo **replacement during adult neurogenesis** (J. Ninkovic et al., 2007; I. Imayoshi et al., 2008). Upon integration into the network periglomerular neurons have been reported to maintain structural plasticity partially and newly born neurons provide a source for wiring plasticity in the olfactory bulb (A. Carleton et al., 2003; P. M. Lledo et al., 2006; A. Mizrahi, 2007; M. S. Grubb et al., 2008).

Granule neurons (GNs) are as small as periglomerular neurons (6 - 8 μm) and are grouped in horizontal clusters (G. Shepherd, 2004; I. Imayoshi et al., 2008). Their name originates from their grainy appearance to early microscopists in the inner parts of the olfactory bulb. Their processes are extended radially and perpendicular and terminate in the external plexiform layer. According to their depth several classes of granule neurons have been identified and suggested that may have different functions (G. M. Shepherd, 1972): Superficial GNs sending their dendrites mainly to the superficial external plexiform layer, deep GNs send their dendrites mainly to the deep external plexiform layer amongst the dendrites of mitral cells and intermediate GNs that all extend dendrites into the external plexiform layer. Granule cells do not possess axons and are exclusively connected to mitral cells by dendro-dendritic synapses. As a result of its bi-directionality, the dendro-dendritic synapse can cause mitral cells to inhibit themselves (auto-inhibition), as well as neighbouring mitral cells (lateral inhibition). This circuit is the main output controlling circuit of the olfactory bulb.

Like periglomerular neurons also these neurons are **generated during postnatal development and throughout adulthood**. Upon migration and maturation granule neurons have been classified into five different developmental stages: (1) tangentially migrating neuroblasts (days 2–7); (2) radially migrating young neurons (days 5–7); (3) GCs with a simple unbranched dendrite that does not extend beyond the mitral cell layer (days 9–13); (4) GCs with a nonspiny branched dendrite in the external plexiform layer (days 11–22); and (5) mature GCs (days 15–30) (L. Petreanu and A. Alvarez-Buylla, 2002). Interestingly, shortly after the development of spines around half of the granule neurons undergo apoptosis. The survival might depend on the level of activity that they received as suppression of activity results in increased cell death (L. Petreanu and A. Alvarez-Buylla, 2002). In contrast to periglomerular neursons, granule neurons are relatively **homogeneous** in their neurochemical identity: the great majority is GABAergic and few calretinin-positive cells can be detected, but most of the cells do not stain for Tyrosine Hydroxylase or Calbindin (R. Batista-Brito et al., 2008).

In summary, intrinsic neurons of the olfactory bulb are important for signal transduction and for shaping output acitivity. This may thereby optimize odour representations for processing in higher brain regions. But how are these specific neurons generated from neural stem cells?

2.5 The dentate gyrus compared to the subependymal zone

The second neurogenic niche beside the subependymal zone in the adult forebrain is located within the hippocampus: the **dentate gyrus**. The hippocampus is an organ involved in the long-term storage of memories and receives most of its input from the entorhinal cortex which fibres end in the outer molecular layer of the dentate gyrus. The CA3 region is targeted by the mossy fibres originating from the granule neurons in the dentate gyrus (Fig. **6**). The pyramidal neurons of the CA3 region are connected to those of the CA1 region via the Schaffer collaterals. The hippocampus signals back to the cortex, and other brain regions, e.g. hypothalamic areas, and the amygdala. Notably, even in the human dentate gyrus and olfactory bulb new neurons appear after treatment with the DNA analogue BrdU which is incorporated into the genome during DNA synthesis (P. S. Eriksson et al., 1998).

In contrast to the subependymal zone, the production of these adult generated neurons occurs locally in the **subgranular layer** and young neurons integrate functionally only a few cell layers away in the granular cell layer a couple of weeks after birth (F. H. Gage, 2000; G. Kempermann et al., 2004; G. L. Ming and H. Song, 2005; R. F. Hevner et al., 2006; N. Toni et al., 2008). Unlike the subependymal zone, the dentate gyrus generates mostly **glutamatergic granule neurons** (G. Kempermann et al., 2004; G. L. Ming and H. Song, 2005; R. F. Hevner et al., 2006; N. Toni et al., 2008), even though a small proportion of newly generated GABAergic interneurons have been reported (S. Liu et al., 2003). Hence the conserved transcription factor sequence in the dentate gyurs is distinct from the subependymal zone and matches cortical development, cerebellum, and dentate gyrus (Fig. **7**) (R. F. Hevner et al., 2006): Pax6 → Tbr2 → NeuroD → Tbr1. Also the bHLH transcription factors Mash1 and Neurogenin2 are expressed in the dentate gyrus in proliferating progenitors (B. Seri et al., 2004; I. Ozen et al., 2007; M. Uda et al., 2007).

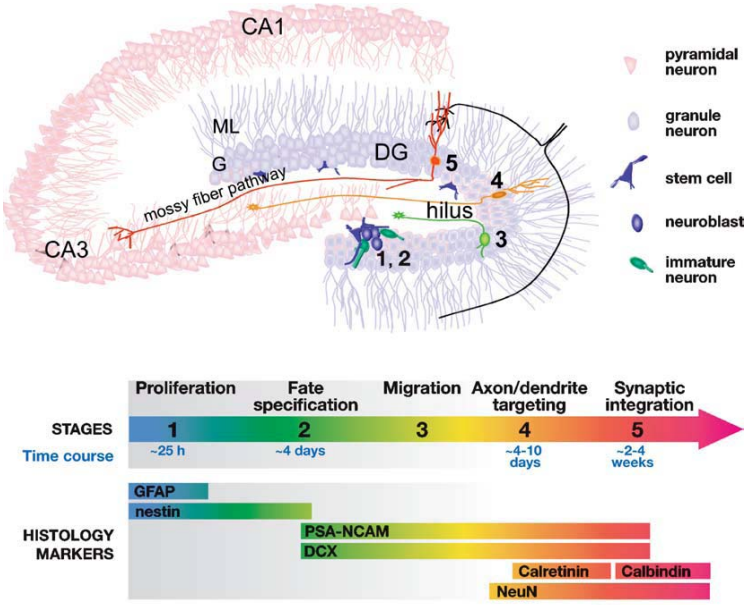


Fig. 6: Generation of granule neurons in the dentate gyrus (G. L. Ming and H. Song, 2005).

Adult neurogenesis in the dentate gyrus of the hippocampus undergoes five developmental stages. Stage 1. Proliferation: Stem cells (blue) with their cell bodies located within the subgranular zone in the dentate gyrus have radial processes that project through the granular cell layer and short tangential processes. These stem cells give rise to transient amplifying progenitors (light blue). Stage 2. Differentiation: transient amplifying progenitors differentiate into immature neurons (green). Stage 3. Migration: Immature neurons (light green) migrate a short distance into the granule cell layer. Stage 4. Axon/dendrite targeting: Immature neurons (orange) extend their axonal projections along mossy fiber pathways to the CA3 pyramidal cell layer. They send their dendrites in the opposite direction towards the molecular layer. Stage 5. Synaptic integration: New granule neurons (red) receive inputs from the entorhinal cortex and send outputs to the CA3 and hilus regions. DG = dentate gyrus region; ML = molecular cell layer; GL = granular cell layer.

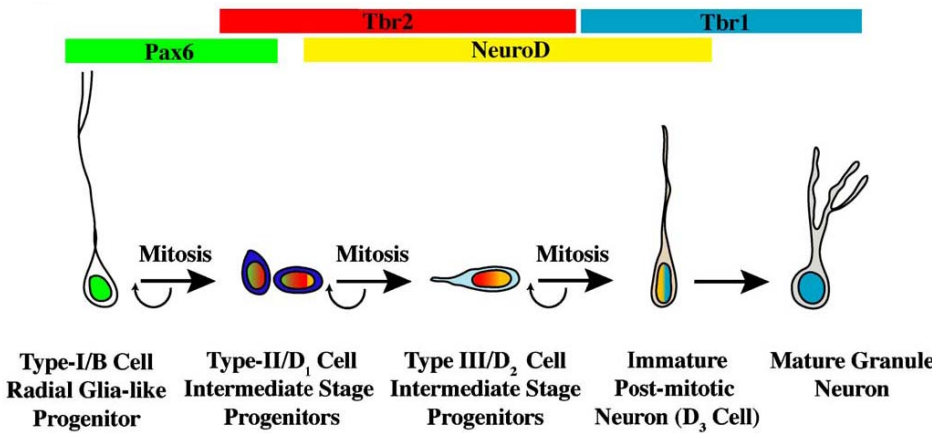


Fig. 7: Transcription factor expression during neurogenesis in the adult mouse dentate gyrus (R. F. Hevner et al., 2006).

At least three progenitor and two post-mitotic stages are identified during the development of granule neurons. Colored bars at the top of the figure represent the transcription factors expressed during each stage of development. Nuclei of individual cells are color coded to signify the transcription factors that they express. Curved arrows indicate that these progenitor cell types may be capable of self-renewing divisions.

The subependymal zone differs from the dentate gyrus in several aspects: (i) so far only GABAergic interneuron production has been reported from the subependymal zone and hence the transcriptions factor sequence differs from the dentate gyrus; (ii) neuroblasts destined for the olfactory bulb migrate away forming the rostral migratory stream until they reach the olfactory bulb; (iii) the subependymal zone underlies the lateral ventricular wall and neural stem cells of the subependymal zone contact the ventricle. The cellular architecture of the subependymal zone includes therefore ependymal cells besides migrating neuroblasts, neural stem cells and transit-amplifying precursors.

2.6 The cytoarchitecture of the subependymal zone

The subependymal zone is located at the lateral wall of the lateral ventricle and generates neurons destined for the olfactory bulb, which migrate through the rostral migratory stream. The neurogenic zone is only a few cell layers thick. The "**niche**" is defined as "a specific location in a tissue whose microenvironment enables stem cells to reside for an indefinite period of time and produce progeny cells while self-renewing" (I. Ortega-Perez et al., 2007).

The below listed cell types are located in the subependymal neurogenic niche (Fig. **8**):

1.- ependymal cells, lining the ventricular wall

2.- astrocytes, amongst them are the neurogenic, **astrocytic neural stem cells** (NSCs)

3.- transit-amplifying progenitors (TAPs) that are the immediate progeny of stem cells with a very fast cell cycle

4.- neuroblasts generated from transit amplifying progenitors, which migrate away from the subependymal zone forming the rostral migratory stream that leads to the olfactory bulb

1. The **ependymal cells** are mostly multiciliated and form tight-junctions thereby separating the cerebrospinal fluid circulating in the ventricles and spinal cord from the brain tissue (Fig. **8**). At the end of neurogenesis radial glia transform into astrocytes but also form the ependymal layer (P. Malatesta et al., 2003; F. T. Merkle and A. Alvarez-Buylla, 2006). They cover most of the apical surface (**ventricular surface**), connected by tight junctions and cilia arise from basal bodies (γ-Tubulin+) (F. Doetsch et al., 1997; Z. Mirzadeh et al., 2008). These cilia beating has been linked to

the movement of the cerebrospinal fluid in the ventricular lumen (K. Sawamoto et al., 2006). Moreover, this study suggested that the orientation of neuroblasts correlates with the flow of the cerebrospinal fluid (K. Sawamoto et al., 2006). At least two types of ependymal cells can be distinguished according to the number of cilia (Z. Mirzadeh et al., 2008). Ependymal cells have previously been considered as stem cells (C. B. Johansson et al., 1999), however proliferation of this cell type had not been confirmed under physiological conditions in the telencephalon (F. Doetsch et al., 1999b; Z. Mirzadeh et al., 2008). However, after injury in the spinal cord ependymal cells proliferate (K. Meletis et al., 2008). In neurogenic zones, ependymal cells are grouped in **pinwheel-structures** around one cluster of stem cells that only cover a small area in the middle of the pinwheel. Notably, in non-neurogenic zones, i. e. the third ventricle, ependymal cells are shaped more regularly (Z. Mirzadeh et al., 2008).

2. Neural Stem Cells (NSCs) of the supendymal zone are a subpopulation of GFAP (glial fibrillary acidic protein) expressing astrocytes that generate neuronal progeny destined for the olfactory bulb (F. Doetsch et al., 1999b; T. Imura et al., 2003; A. D. Garcia et al., 2004; N. Sanai et al., 2004; S. Ahn and A. L. Joyner, 2005; J. Ninkovic et al., 2007; I. Imayoshi et al., 2008). Different types of astrocytes are found in the subependymal zone: (i) mature differentiated astrocytes containing a small soma and numerous processes are usually located further away from the ventricular surface; (ii) neurogenic neural stem cells are located in close vicinity to the ependymal cells lining the ventricular wall, contacting the ventricle with a single endfoot; (iii) astrocytes with processes parallel to the ventricular surface (tangential neural stem cells) had been identified and suggested to resemble radial glia or translocating astrocytes in the embryonic forebrain (Q. Shen et al., 2008). Neural stem cells exhibit a rather **slow cell cycle** as demonstrated by the use of the DNA-base analogue BrdU. After BrdU administration, label-retaining cells (neural stem cells) retain BrdU for extended periods due to their relatively long cell-cycle (M. Carlen et al., 2002; D. Colak et al., 2008; M. Tavazoie et al., 2008).

Notably, label-retaining neural stem cells divide **close to blood vessels** (K. L. Baker et al., 2006; Q. Shen et al., 2008; M. Tavazoie et al., 2008) and express the laminin receptor a6b1 integrin (VLA6), which is lost upon differentiation, and this receptor allows direct adhesion of neural stem cells to vascular cells (Q. Shen et al., 2008). Furthermore, neural stem cells are exposed to an extracellular matrix that is thought to

trap niche growth factors; this matrix includes basal lamina structures that contain laminin (F. Mercier et al., 2002; A. Kerever et al., 2007; Q. Shen et al., 2008).

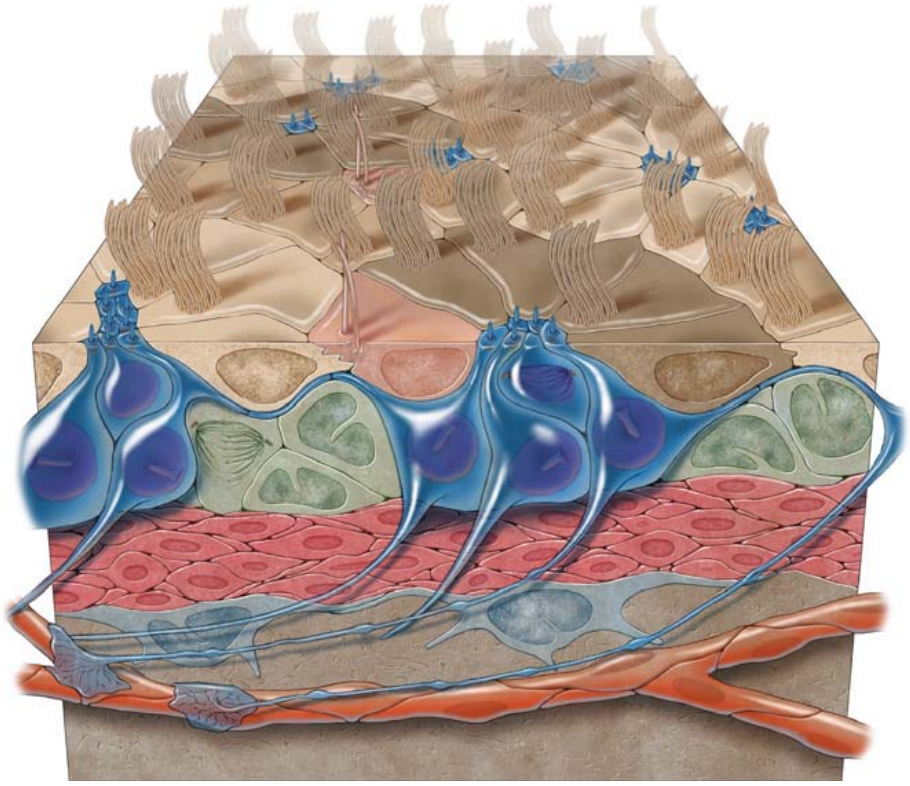


Fig. 8: Three-dimensional model of the adult subependymal neurogenic niche (Z. Mirzadeh et al., 2008).

Three-dimensional model of the adult subependymal neurogenic niche illustrating stem cells (blue), transit-amplifying progenitrors (green), and neuroblasts (red). GFAP+ (blue) neural stem cells have a long basal process that terminates on blood vessels (orange) and an apical ending at the ventricle surface. Note the pinwheel organization (light and dark brown) composed of ependymal cells (brown and peach) encircling stem cells apical surfaces.

Only one **primary cilium** arises from a single basal body of stem cells that cover very little parts of the ventricular surface and group together in a donut-like structure surrounded by a pinwheel of ependymal cells (Z. Mirzadeh et al., 2008). Notably, the number of stem cells is nowadays estimated to nearly **one-third of all cells** touching the ventricular wall (Z. Mirzadeh et al., 2008), in contrast to previous suggestions (F. Doetsch et al., 1997). **Two hot-spots** have been claimed according to the presence of the number of stem cells accessing the ventricle: the anterior-ventral wall and the posterior-dorsal region of the ventricular wall (Z. Mirzadeh et al., 2008).

One common in vitro method to prove the multipotency of neural stem cells is the **neurosphere assay** (see methods for details). The ventricular wall is isolated, dissociated, and single cells are kept in a suspension culture under the addition of

growth factors (EGF/FGF2) (B. A. Reynolds and S. Weiss, 1992; C. M. Morshead et al., 1994; C. B. Johansson et al., 1999; B. Berninger et al., 2007). After some days cells start to proliferate and neurospheres form which can be passaged for a long time (self-renewal). After plating in adherent, differentiation conditions neurosphere cells differentiate into astrocytes, neurons and oligodendrocytes (multipotency). A second method to demonstrate the ability of stem cells is an in vivo method. After elimination of transit-amplifying cells and neuroblasts with the antimitotic drug cytosine-b-D-arabinofuranoside (Ara-C), stem cell astrocytes divide to rapidly regenerate the subependymal zone (F. Doetsch et al., 1999a; M. Tavazoie et al., 2008).

Neural stem cells and ependymal cells can be distinguished by the expression pattern of **different antigens**: stem cells are **GFAP**+, S100b-negative, CD24-negative, Vimentin-negative, whereas ependymal cells were GFAP-negative, CD24+, Vimentin+, S100ß+ (Z. Mirzadeh et al., 2008). Apical processes of stem cells were **nestin+** and **CD133+** (prominin+). Notably, all these proteins are not exclusively expressed in neural stem cells, like **Sox2** or GFAP, and cannot be used as exclusive neural stem cell marker (A. D. Garcia et al., 2004; M. Tavazoie et al., 2008). Furthermore, the radial glia marker brain lipid-binding protein (**BLBP**) (E. Hartfuss et al., 2001) and the **LeX** antigen, which is the trisaccharide 3-fucosyl-*N*-acetyllactosamine (H. C. Gooi et al., 1981; A. Capela and S. Temple, 2002), is not exclusively expressed in GFAP-positive astrocytes but also includes the EGF receptor positive cells (TAPs) (J. C. Platel et al., 2008b). Neural stem cells can be distinguished from non-dividing and/or non-neurogenic astrocytes by the incorporation of the DNA base analogue BrdU. However, due to the slow cell-cylcle BrdU has to be given for 1 – 2 weeks in the drinking water followed by a chase with BrdU-free water to allow dilution of BrdU in fast dividing cells.

The adult subependymal zone retains many developmental characteristics, e. g. the expression of specific extracellular matrix components and growth factors which regulate the expression of transcription factors promoting either the neurogenic or the oligodendrogliogenic lineage. A range of external signal cascades have been identified over the last years. The primary cilium of adult neural stem cells exhibits an antenna like function (J. J. Breunig et al., 2008; Y. G. Han et al., 2008), but also receptors on the cell surface of transit-amplifying progenitors and neuroblasts detect external signals. Various cell types respond differently when exposed to external signals depending on their receptor cocktail, the presence or absence of a primary cilium, as

well as their contact to blood vessels. Some prominent signalling patways are shortly introduced in the following paragraphs.

Notch signalling is regulating stem cell survival through Notch receptor activation which induces the expression of the specific target genes amongst them are hairy and enhancer of split 3 (Hes3), Hes5, and Sonic hedgehog (Shh) (G. Stump et al., 2002; A. Androutsellis-Theotokis et al., 2006). Notch encodes a transmembrane receptor that is cleaved to release an intracellular domain (Nicd) that is directly involved in transcriptional control (A. Goriely et al., 1991; S. Artavanis-Tsakonas et al., 1999; A. Androutsellis-Theotokis et al., 2006). This cleavage occurs after binding of its ligands Delta-like 4 (Dll4) and Jagged 1 (Jag1) (A. Chitnis et al., 1995; K. K. Johe et al., 1996).

Sonic hedgehog signaling components like Patched (Ptc), Smo, Suppressor of fused and Gli transcription factors concentrate in primary cilia (S. Ahn and A. L. Joyner, 2005; K. C. Corbit et al., 2005; C. J. Haycraft et al., 2005; R. Rohatgi et al., 2007) and have a critical role in the expansion and establishment of progenitors. Furthermore, loss of primary stem cell cilia leads to abrogated Shh activity which in turn leads to increased cell cycle exit, and morphological abnormalities in the adult dentate gyrus (J. J. Breunig et al., 2008; Y. G. Han et al., 2008). In olfactory neurogenesis Hedgehog signalling is required for maintenance of neural stem cells (S. Ahn and A. L. Joyner, 2005; F. Balordi and G. Fishell, 2007b, a).

Bone morphogenic protein (BMP) mediated signalling is active in adult neural stem cells of the SEZ and is crucial to initiate the neurogenic lineage (D. Colak et al., 2008). The BMP pathway is locally enriched in the venricular wall of the adult mouse brain: BMP ligands 2, 4, 6, and 7, the feedback inhibitor Noggin, the BMP specific type II receptor, the transcription factor Smad4 that mediates BMP signaling, and Id1 and Id3, which act as downstream targets of the BMP pathway, are present in the adult subependymal zone (D. A. Lim et al., 2000; P. Peretto et al., 2002; X. Fan et al., 2003; P. Peretto et al., 2004; D. Colak et al., 2008). Adult deletion of the transcription factor Smad4 in adult neural stem cells and their progeny does not affect self-renewal of neural stem cells (D. Colak et al., 2008). However, a great reduction of neuroblasts was observed already ten days after ablation of Smad4 (D. Colak et al., 2008). Concomitantly the transcription factor Olig2 was upregulated strongly in transit-amplifying progenitors and results in migration of cells to the corpus callosum and increased oligodendrogliogenesis (D. Colak et al., 2008). Thus, BMP signalling is active in neural stem cells and transit-amplifying progenitors and required for

progression into the neuronal lineage. Deletion of Smad4 and infusion of Noggin into the lateral ventricle resulted in an increase in Olig2-positive oligodendrocytes precursors at the expense of neurogenesis (D. Colak et al., 2008). Notably, blockade of the BMP signalling in the dentate gyrus, e. g. by Noggin infusion, expands the neural stem cell pool (M. A. Bonaguidi et al., 2008).

Besides the above described signalling pathways there are many more growth factors and proteins involved in maintaining neural stem cells and influencing neurogenesis. Amongst them is **PEDF** (pigment epithelium-derived factor) which is secreted by components of the murine subependymal zone. Intraventricular PEDF infusion resulted in activation of slowly dividing neural stem cells, whereas a blockade of endogenous PEDF decreased their proliferation (C. Ramirez-Castillejo et al., 2006). The soluble carbohydrate-binding protein **Galectin-1** is expressed in astrocytes of the SEZ including the neural stem cells. Infusion of Galectin-1 into the ventricle suggested that Galectin-1 is an endogenous factor that promotes the proliferation of NSCs in the adult brain (M. Sakaguchi et al., 2006). Platelet-derived growth factor (**PDGF**) affects astrocytic neural stem cells expressing the PDGF receptor (PDGFR α) (E. L. Jackson et al., 2006) but rather promotes oligodendrogliogenesis than self-renewal.

3. Transit-amplifying progenitors (or transit-amplifying precursors; TAPs) are the **immediate progeny** of neural stem cells with a **very fast cell cycle** as demonstrated by short BrdU labelling. A single pulse of the thymidine analogue BrdU for one hour before sacrifice labels transit-amplifying progenitors and some neuroblasts. Neural stem cells are hardly labelled according to their long cell cycle. Recent studies suggested that also transit-amplifying progenitors like neural stem cells divide in the close vicinity of blood vessels and often contact the vasculature at sites devoid of Aquaporin 4(AQP4) staining (M. Tavazoie et al., 2008) and express the **EGF receptor** (C. M. Morshead et al., 1994; K. B. Seroogy et al., 1995; C. S. Weickert et al., 2000; F. Doetsch et al., 2002; M. Tavazoie et al., 2008). At least two lineages of transit-amplifying progenitors had been identified according to the generated progeny: (i) transit-amplifying progenitors that express the transcription factors **Mash1 and Olig2** generate oligodendrocytes migrating towards the white matter (M. A. Hack et al., 2005; C. A. Marshall et al., 2005; B. Menn et al., 2006; D. Colak et al., 2008); (ii) neurogenic transit-amplifying progenitors that generate granule and periglomerular neurons express transcription factors in various combinations, amongst them are **Pax6**, members of the

Dlx-gene family and **Mash1** (F. Doetsch et al., 2002; C. M. Parras et al., 2004; M. A. Hack et al., 2005; B. Menn et al., 2006; D. Colak et al., 2008).

As mentioned above, **BMP-mediated signalling** is necessary for the progression of transit-amplifying precursors towards the neuronal lineage. Ablation of the transcription factor Smad4 which is present in neural stem cells and transit-amplifying precursors blocks BMP-signalling and leads to aberrant expression of Olig2, even in transit-amplifying precursors that express Dlx2 (D. Colak et al., 2008).

Transit-amplifying progenitors in the subependymal zone express the **epidermal growth factor receptor** (EGFR) on their cell surface (C. M. Morshead et al., 1994; K. B. Seroogy et al., 1995; F. Doetsch et al., 2002; M. Tavazoie et al., 2008). The EGFR is activated by binding of its specific ligands including epidermal growth factor, heparin-binding EGF-like growth factor, and transforming growth factor α (TGFα). Upon activation by its growth factor ligands the EGFR dimers stimulate intracellular tyrosine kinase activity. Infusion of EGF into the murine lateral ventricle increased proliferation in the subependymal zone and concomitantly decreased the number of cells migrating towards the olfactory bulb (C. G. Craig et al., 1996; H. G. Kuhn et al., 1997; J. Fallon et al., 2000). The responding cellular population to EGF infusion includes transit-amplifying progenitors (F. Doetsch et al., 2002).

Another important regulator in mammalian neural development has been implicated with adult neurogenesis: The **canonical Wnt signalling** pathway of which ß-catenin is an important downstream component (A. Patapoutian and L. F. Reichardt, 2000). Wnt-signalling plays an important role in neurogenesis of the developing and adult dentate gyrus (J. Galceran et al., 2000; S. M. Lee et al., 2000; D. C. Lie et al., 2005). Forced expression of ß-catenin with retoviral vectors promotes the proliferation of transit-amplifying progenitors in the subependymal zone and inhibits their differentiation into neuroblasts (K. Adachi et al., 2007).

4. Several 10,000 **neuroblasts** are newly generated each day and form **chain-like structures** during their journey along the ventricular wall. Finally, they form the rostral migratory stream at the subependymal zone's rostral extension between the white matter and the striatum. Upon their entrance in the core of the olfactory bulb these chains detach and the neuroblasts start to migrate radially and finally integrate into the olfactory bulb network. However, only few neuroblasts can manage to fully

mature and find their place in the mature network. Most of them fail and die after a few weeks (L. Petreanu and A. Alvarez-Buylla, 2002; G. Kempermann et al., 2004).

Neuroblasts still divide as demonstrated by a short BrdU pulse during their journey to the olfactory bulb (M. B. Luskin, 1993). Notably, **blood vessels** seem to act as guide for their migration and ran parallel in the RMS whereas this is not the case to such extent along the ventricular wall (M. Tavazoie et al., 2008). Reelin acts as detachment signal for chain-migrating neuroblasts (I. Hack et al., 2002), as well as the extracellular matrix glycoprotein tenascin-R, which in addition promotes their radial migration in the olfactory bulb (A. Saghatelyan et al., 2004). Overlapping antigens expressed by these young migrating neurons are **doublecortin (DCX)** (J. G. Gleeson et al., 1999), **PSA-NCAM** and **mCD24** (V. Calaora et al., 1996; F. Doetsch et al., 1997).

Many components of **GABAergic signalling** have been identified in the subependymal zone: GABA is synthesized and released by neuroblasts (R. R. Stewart et al., 2002; A. J. Bolteus and A. Bordey, 2004; X. Liu et al., 2005; J. C. Platel et al., 2008a). Neuroblasts and neural stem cells express $GABA_A$ receptors that are activated by GABA (R. R. Stewart et al., 2002; D. D. Wang et al., 2003; A. J. Bolteus and A. Bordey, 2004; X. Liu et al., 2005). GABA levels are regulated by GABA transporters expressed in neural stem cells but not in neuroblasts (A. J. Bolteus and A. Bordey, 2004; J. C. Platel et al., 2007). Tonic $GABA_A$ receptor activation reduces proliferation and the migration speed of neuroblasts (L. Nguyen et al., 2003; A. J. Bolteus and A. Bordey, 2004; X. Liu et al., 2005). Migration of neuroblasts is a process that is also tightly regulated by several molecules and signalling pathways. Brain derived neurotrophic factor (BDNF) as a very prominent representative is expressed in the SEZ-RMS-OB system as well as its receptor TrkB and p75 (T. Zigova et al., 1998; A. Mackay-Sima and M. I. Chuahb, 2000; E. Gascon et al., 2005; S. Chiaramello et al., 2007). The **hepatocyte growth factor** (HGF) and its receptor Met protein are expressed in the olfactory bulb and throughout the migratory pathway (D. Garzotto et al., 2008). HGF promotes migration of RMS neuroblasts, acting both as an inducer and attractant (D. Garzotto et al., 2008). Furthermore, directional migration towards the olfactory bulb is controlled by chemorepulsion (Slit-Robo signalling; (W. Wu et al., 1999; K. Sawamoto et al., 2006)) and molecules acting as attractants by the olfactory bulb, such as Netrin-1, and GDNF (S. Murase and A. F. Horwitz, 2002; G. Paratcha et al., 2006).

Taken together, neural stem cell maintenance, transit-amplifying precursor divisions, neurogenic lineage decisision, and migration of neuroblasts from the subependymal zone towards the olfactory bulb are tightly controlled processes with diverse signalling pathways involved in this regulation. Signalling pathways often result ulitmately in the change of transcriptional profiles. Some of these pathways are exhibiting their function via gradient expressions like in development. Recent reports suggest that also the adult subependymal zone is regionalized (W. Kelsch et al., 2007; F. T. Merkle et al., 2007; K. M. Young et al., 2007) what will be described in more detail in the following chapter.

2.7 Comparison of the architecture of embryonic and adult subependymal zone

Adult neural stem cells are derived from embryonic neuroepithelial cells, which form an apical layer of ventricular zone precursors. At early developmental stages neuroepithelial cells function as primary progenitors and divide symmetrically to expand the stem cell pool. Consequently only few neurons are produced; amongst them are the Cajal-Retzius cells. Neuroepithelial cells have an elongated shape spanning from the ventricular to the pial surface. At later stages they either transform directly into neurons or divide asymmetrically, generating a radial glia cell that remains in the ventricular zone (radial glia cell) and a daughter cell that migrates radially outward (P. Malatesta et al., 2003; T. E. Anthony et al., 2004; W. Haubensak et al., 2004; M. Gotz and W. B. Huttner, 2005; F. T. Merkle and A. Alvarez-Buylla, 2006). Radial glia are present in the **dorsal and ventral telencephalon** and divide to generate striatal neurons and oligodendrocytes either directly or via an intermediate progenitor (basal progenitors) forming a subventricular zone that gives rise to cortical neurons (M. Gotz and W. B. Huttner, 2005; F. T. Merkle and A. Alvarez-Buylla, 2006).

Radial glia cells exist until neonatal stages where they generate oligodendrocytes, and olfactory bulb interneurons. Soon after birth they retract their processes and transform into ependymal cells or astrocytes, few forming later the adult neual stem cell pool (A. D. Tramontin et al., 2003; F. T. Merkle and A. Alvarez-Buylla, 2006). Notably, the adult subependymal zone has essentially a similar structure like the embryonic neurogenic zone. Neural stem cells in the adult brain are intercalated into the ependymal layer and have direct access to the ventricle via a single endfoot (Q. Shen et al., 2008). Beneath is a subventricular zone of active proliferation and differentiation of

transit-amplifying progenitors that give rise to neuroblasts and oligodendrocytes (Q. Shen et al., 2008). Neuroblasts migrate along the lateral wall of the lateral ventricle towards the olfactory bulb (M. B. Luskin, 1993), whereas in the embryo they migrate along radial glia processes (S. C. Noctor et al., 2004; S. C. Noctor et al., 2007).

Taken together, the embryonic and adult neurogenic niches are similar in their cellular architecture. However, different types of neurons are generated during embryonic neurogenesis and this raises the question which brain regions contribute to the adult subependymal zone and do they produce the same neurons as in development?

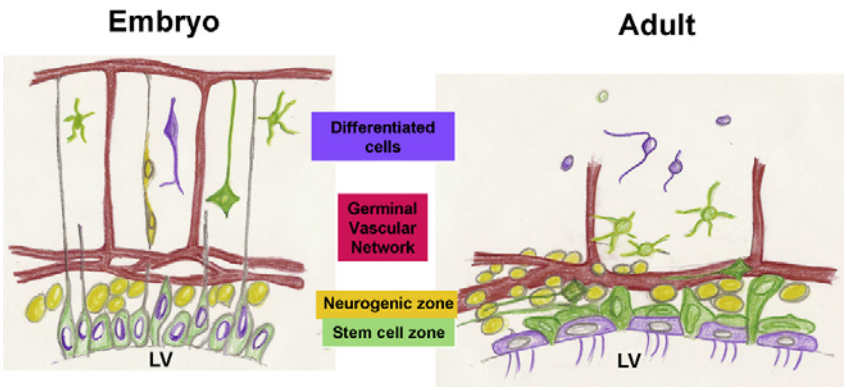


Fig. 9: The embryonic versus the adult neurogenic niche (Q. Shen et al., 2008).
In the embryo, an apical layer of stem cells produces an actively proliferating subventricular zone that generates neurons that migrate toward the pia guided by radial glia. Coincident with neurogenesis, the vasculature grows from the pial surface toward the germinal cells.
The adult subependymal zone has an essentially similar structure: apical neural stem cells, believed to include the subependymal zone stem cells, are intercalated into the ependymal layer and directly contact the ventricle. Just subjacent is an subventricular zone of active proliferation, differentiation, and migration, including Mash1+ and Olig2+ transit-amplifying progenitors, PSA-NCAM+/DCX+ neuroblasts. The adult germinal zone is intimately associated with a subependymal zone vascular plexus. LV = lateral ventricle.

2.8 Regionalization of the embryonic telencephalon and adult subependymal neurogenic niche

The origin of dorsal and ventral radial glia forming later the adult subependymal zone may contribute to the regionalization of this structure. Although the lateral wall of the lateral ventricle (the lateral subependymal zone) appears as the major source of adult olfactory neurogenesis, injections of permanent lineage tracers into the lateral subependymal zone hardly labelled any progeny migrating to the glomerular layer of the olfactory bulb, while more such cells were observed following injection into the rostral migratory stream (M. A. Hack et al., 2005; M. Alonso et al., 2008; J. G. Mendoza-Torreblanca et al., 2008). Different regions of origin for distinct types of

periglomerular neruons were recently further substantiated by transplantation experiments (S. De Marchis et al., 2007; M. Kohwi et al., 2007a; F. T. Merkle et al., 2007) and recent studies suggest the restriction of precursors to generate neurons with a pre-determined pattern, i. e. dendritic targeting or neurochemical identity was independent of the host environment and therefore a cell-autonomous effect (W. Kelsch et al., 2007; F. T. Merkle et al., 2007).

2.8.1 Regionalization in the embryonic telencephalon

In order to give evidence for the regionalization of the adult subependymal zone, Cre-mediated fate mappings were performed. Reporter activity of an Emx1-Cre line would demonstrate the contribution of the dorsal telencephalon to the adult subependymal neurogenic niche (S. Willaime-Morawek et al., 2006; M. Kohwi et al., 2007a; R. E. Ventura and J. E. Goldman, 2007; K. M. Young et al., 2007; R. Batista-Brito et al., 2008). Indeed, fate mapping confirmed that the dorsal Emx1-derived telencephalon contributes to the adult subependymal zone. During embryonic development Pax6 is expressed in the **doral telencephalon** in the Emx1-expressing territory where mainly **glutamatergic neurons** arise (J. A. Gorski et al., 2002; P. Malatesta et al., 2003; T. T. Kroll and D. D. O'Leary, 2005) (Fig. **10**). However, the Gsh2-derived brain region (**ventral telencephalon**) generates **GABAergic neurons** (J. C. Szucsik et al., 1997; H. Toresson et al., 2000; H. Toresson and K. Campbell, 2001).

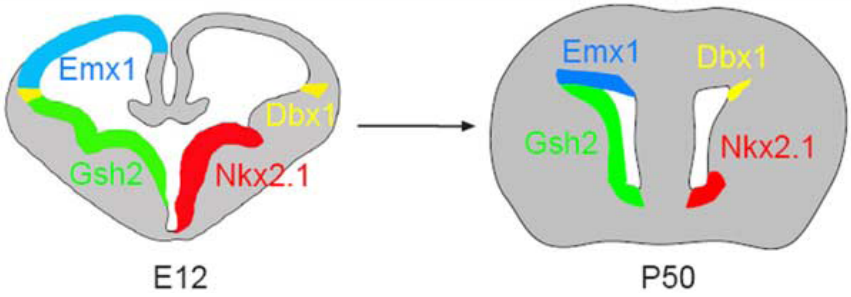


Fig. 10: Neural stem cells and their progeny in the developing forebrain (adapted from (K. M. Young et al., 2007).

All regions of the telencephalic neuroepithelium contribute to the adult subependymal zone. Schematic depicting the different embryonic neuroepithelial domains targeted by Cre mice crossed with a GFP reporter and their relative contribution to generating the adult subependymal zone.

In the adult olfactory bulb the Emx1-derived area generates around 25 % of all new BrdU+ neurons in the olfactory bulb compared to 70 % of the Gsh2-derived lineage (K. M. Young et al., 2007). Therefore the lateral ventricular wall is the main source of

olfactory interneurons. Interestingly, no Calbindin+ neurons were detected from the Emx1-derived lineage, but dopaminergic, tyrosine hydroxylase+ and even more Calretinin+ interneurons were observed (K. M. Young et al., 2007).

Notably, different transcription factors pattern these territories thereby specifying progenitors and are introduced shortly in the following paragraphs.

2.8.2 Patterning during embryonic development

The dorsal and ventral telencephalon generate glutamatergic and GABAergic neurons, respectively, as well as Olig2+ oligodendrocytes arising from ventral regions. Pax6 and Tbr1&2 are important transcription factors for the gernation of glutamatergic neurons, whereas Gsh1&2, Mash1 and Dlx transcription factors drive the generation of GABAergic striatal projection and cortical interneurons.

In the Emx1-derived brain region, Pax6 is present in radial glia cells and governs the generation of glutamatergic neurons (M. Gotz et al., 1998; N. Heins et al., 2002; T. T. Kroll and D. D. O'Leary, 2005; V. Nikoletopoulou et al., 2007). **Pax6** belongs to the paired-like class of transcription factors with two highly conserved motifs: a **homeodomain and a paired domain**, (D. Bopp et al., 1986; S. Cote et al., 1987; C. Walther and P. Gruss, 1991) and classified in vertebrates as the Pax multi-gene family (U. Deutsch et al., 1988; G. R. Dressler et al., 1990; B. Jostes et al., 1990; D. Plachov et al., 1990; M. D. Goulding et al., 1991; C. Walther and P. Gruss, 1991; C. Walther et al., 1991).

Fig. 11: mRNA scheme indicating domains of the transcription factor Pax6 (N. Haubst et al., 2004). Pax6 exhibits two DNA binding sites, namely the homeodomain and the paired domain, which is in turn subdivided into the PAI and RED domain both able to bind DNA independently. Exon5a is 14 amino acids long and is inserted in the Pax6(5a) splice variant. HD = homeodomain, TA = transactivation domain, PD = paired domain

Several splice variants are known in the Pax6 gene: a truncated form lacking the paired domain (paired-less) and Pax6(5a) with a 14 amino acid insertion into the PD (J. A. Epstein et al., 1994; Z. Kozmik et al., 1997; M. K. Duncan et al., 2000; T. R. Anderson et al., 2002; R. Mishra et al., 2002; N. Haubst et al., 2004). In addition several point mutations of the Pax6 gene are described, amongst them the very well studied Sey mice (**Small eye**) which lack the transactivation domain of Pax6 due to a point mutation

inserting a stop codon (B. L. Hogan et al., 1986; B. L. Hogan et al., 1988; R. E. Hill et al., 1991). This functional null mutant does not posess eyes and nasal cavities, dies perinatally and shows severe defects in forebrain development (B. L. Hogan et al., 1986; B. L. Hogan et al., 1988; R. E. Hill et al., 1991; M. Gotz et al., 1998; J. Briscoe et al., 1999; T. T. Kroll and D. D. O'Leary, 2005).

In contrast, the homeodomain transcription factor **Gsh2** is expressed in the medial and lateral **ganglionic eminence** and drives the generation **of GABAergic** neurons, such as cortical interneurons and striatal GABAergic projection neurons (Fig. **10**) (J. C. Szucsik et al., 1997; D. D. Eisenstat et al., 1999; H. Toresson et al., 2000; H. Toresson and K. Campbell, 2001). Gsh2 is expressed in both of the ganglionic eminences while Gsh1 is largely confined to the medial ganglionic eminence (J. C. Szucsik et al., 1997; H. Toresson et al., 2000; H. Toresson and K. Campbell, 2001). Gsh2 function is essential for the molecular identity of early striatal progenitors and in its absence the ventral telencephalic regulatory genes Mash1 and Dlx are lost from most of the striatal germinal zone (J. C. Szucsik et al., 1997; H. Toresson et al., 2000; H. Toresson and K. Campbell, 2001).

The transcription factor **Dlx2** belongs to the **homeobox** containing family which consists of Dlx1, 2, 5, and 6. Distal-less (Dll) has been originally described in drosophila distal limb and antenna development (J. R. Whittle et al., 1986; S. M. Cohen et al., 1989; S. M. Cohen and G. Jurgens, 1989). Vertebrate Dlx genes play developmental roles of ears, nose, mandible and maxilla (G. W. Robinson et al., 1991; G. Q. Zhao et al., 1994; D. Acampora et al., 1999; M. J. Depew et al., 1999; K. S. Solomon and A. Fritz, 2002).

In addition, Dlx transcription factors function in forebrain development (S. A. Anderson et al., 1997a; S. A. Anderson et al., 1997b; O. Marin et al., 2000; S. J. Pleasure et al., 2000). The vast majority of telencephalic interneurons originate in the **ventral telencephalon** – the medial and lateral ganglionic eminences where Dlx genes are predominantly present (Fig. **12**) (S. W. Wilson and J. L. Rubenstein, 2000; J. G. Corbin et al., 2001; O. Marin and J. L. Rubenstein, 2001; S. A. Anderson et al., 2002). The Dlx gene family specifies **GABAergic interneurons** migrating to the cortex during embryonic neurogenesis, but controls also the generation of GABAergic striatal projection neurons (S. A. Anderson et al., 1997b; S. A. Anderson et al., 2001; G. Panganiban and J. L. Rubenstein, 2002; M. A. Petryniak et al., 2007; G. Colasante et

al., 2008); thereby **repressing Olig2** and consequently an oligodendrocyte fate (M. A. Petryniak et al., 2007).

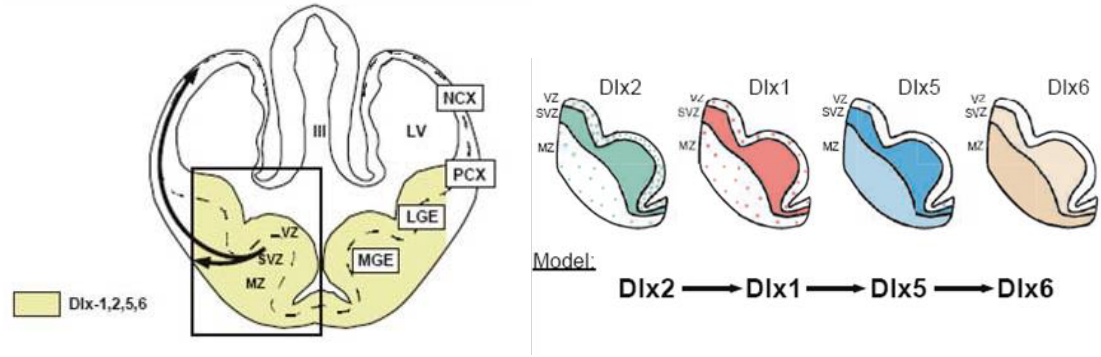


Fig. 12: Dlx transcription factors in the developing forebrain (adapted from (G. Panganiban and J. L. Rubenstein, 2002)).

The left schematic shows the appearance of GABAergic interneurons from the medial ganglionic eminence (MGE) and lateral ganglionic eminence (LGE) migrating to the neocortex (NCX). Dlx transcription factors (yellow) appear to be present in the same regions.
The right panel shows the current model of the sequential appearance of Dlx transcription factors: Dlx2 and Dlx1 are both present in the subventricular zone (SVZ) but only weakly expressen in the ventricular zone (VZ) where the stem cells are located. Upon maturation of GABAergic neuroblasts they start to migrate and to express the transcription factors Dlx5 and Dlx6. Notably, Dlx5 is expressed in the SVZ and mantle zone (MZ) whereas Dlx6 is prominent in the outer mantle zone.

Furthermore, Dlx 1& 2 down-regulate Notch signalling during neurogenesis and promoting differentiation of progenitors (K. Yun et al., 2002). Notably, Dlx transcription factors appear sequentially during embryonic neurogenesis starting with Dlx1 and 2 expression in the subventricular zone (SVZ) and weak expression in the ventricular zone (VZ). Upon maturation Dlx5 and 6 are expressed in in the subventricular zone and in the mantle zone (MZ) (Fig. **12**).

The mouse homologue of the transcription factor mouse achaete scute homologue 1 (**Mash1**) carries a DNA binding basic helix-loop-helix motif (**bHLH**) (L. C. Lo et al., 1991; F. Guillemot and A. L. Joyner, 1993; F. Guillemot et al., 1993). Mash1 belongs to the family of proneural genes (N. Bertrand et al., 2002). During murine embryonic development Mash1 is expressed in the **ventral telencephalon** (L. C. Lo et al., 1991; F. Guillemot and A. L. Joyner, 1993; M. H. Porteus et al., 1994; C. Fode et al., 2000) where it is involved in the generation of GABAergic neurons, modulation of Notch signalling, and specifiying oligodendrocytes (S. Casarosa et al., 1999; S. Horton et al., 1999; C. Fode et al., 2000; K. Yun et al., 2002; C. M. Parras et al., 2007; M. A. Petryniak et al., 2007).

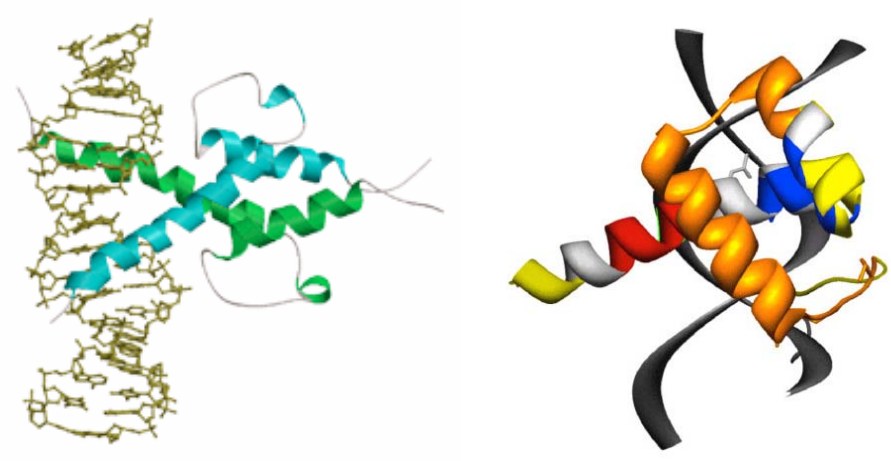


Fig. 13: The basic helix-loop-helix motif (left) and the homeodomain (right) binding to DNA (adapted from (T. Shimizu et al., 1997; V. J. Lynch et al., 2006))

The transcription factor **Olig2** also carries a basic helix-loop-helix (**bHLH**) motif and is essential for the generation of all oligodendrocytes in the central nervous system (Q. R. Lu et al., 2000; H. Takebayashi et al., 2000; J. Wang et al., 2000; Q. Zhou et al., 2000; L. Dimou et al., 2008). In contrast to most other proneural genes Olig2 acts not as activator, but as **repressor** instead (R. Mizuguchi et al., 2001; B. G. Novitch et al., 2001; M. A. Hack et al., 2005). During embryonic development Olig2 is expressed in ventral regions from which **oligodendrocytes** arise which migrate later to the dorsal telencephalon (J. A. Alberta et al., 2001; S. Nery et al., 2001; N. Tekki-Kessaris et al., 2001; D. H. Rowitch et al., 2002; M. A. Petryniak et al., 2007). Notably, Olig2 function is also required for the generation of motorneurons in the spinal cord (D. J. Anderson, 2001; R. Mizuguchi et al., 2001; B. G. Novitch et al., 2001).

As adult neurogenesis originates from stem or progenitor cells not only in the lateral, but also medial and dorsal subependymal zone (F. T. Merkle et al., 2007), this raises the question whether distinct molecular determinants mediating patterning at earlier developmental stages may still be active in the adult and influence the subtype specification of olfactory bulb interneurons.

2.8.3 Patterning in the adult subependymal zone

Generation of progeny from adult neural stem cells, maturation of neuroblasts, and their differentiation into distinct subpopulations reflects a complex network of transcription factors which act as intrinsic fate determinants (J. Ninkovic et al., 2007). Since the dorsal and ventral telencephalon both contribute to the adult subependymal zone, distinct subsets of interneurons may originate from different sets of progenitors that are

specified by transcription factors depending on their respective region of origin. In this chapter, some of the known transcription factors in adult olfactory neurogenesis will be briefly introduced: Pax6, Mash1, and Olig2.

In adult olfactory neurogenesis, **Pax6** plays a **neurogenic role in the subependymal zone** and is important for GABA and **dopaminergic periglomerular neuron specification** (T. L. Dellovade et al., 1998; M. A. Hack et al., 2005; M. Kohwi et al., 2005). In the subependymal zone Pax6 is present in a subset of transit-amplifying progenitors and in neuroblasts of the rostal migratory stream (M. A. Hack et al., 2005). When neurosphere cells derived from the adult subependymal zone are transduced with retroviral vectors encoding Pax6 and GFP, a potent neurogenic effect could be observed (M. A. Hack et al., 2004). Consistently, over-expression in vivo led to up-regulation of DCX, promoting the neurogenic lineage, while dominant-negative approaches or Cre-mediated ablation of Pax6 blocked neurogenesis significantly (M. A. Hack et al., 2005).

Upon arrival in the olfactory bulb Pax6 is down-regulated in most of the newly arriving neuroblasts except in a subpopulation destined to become dopaminergic periglomerular neurons (M. A. Hack et al., 2005). Importantly, over-expression of Pax6 by retroviral vectors into the rostral migratory stream which is a niche for periglomerular neurons led to a prominent increase in the generation of dopaminergic periglomerular neurons (M. A. Hack et al., 2005). In summary, Pax6 plays a crucial role in neurogenesis and in dopaminergic periglomerular neuron fate.

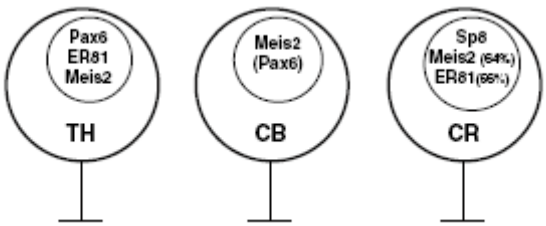


Fig. 14: Dlx5/6-Cre derived periglomerular neurons and their transcriptional profiles (adapted from (Z. J. Allen, 2nd et al., 2007a)).

The vast majority of the mature TH-positive population express the transcription factors Pax6, ER81 and Meis2. The majority of the calbindin-positive population express the transcription factor Meis2 while a small population, indicated by parenthesis, has been shown to express Pax6 (M. A. Hack et al., 2005). Nearly all of the calretinin-positive population expresses the transcription factor Sp8 (R. R. Waclaw et al., 2006) while slightly more than half express Meis2 and/or ER81.

Besides Pax6 other transcription factors are expressed in **dopaminergic periglomerular neurons**, such as **ER81** and **Meis2** (Z. J. Allen, 2nd et al., 2007a). The

transcription factor **Sp8** is involved in the generation of calretinin-positive periglomerular neurons and is only present in a subset of neuroblasts in the rostral migratory stream (R. R. Waclaw et al., 2006; Z. J. Allen, 2nd et al., 2007a). Notably, Pax6 presence in the adult dentate gyrus has also been reported in radial glia-like progenitors that label only with Pax6 and in early intermediate stage progenitors that label with both Pax6 and Tbr2 (R. F. Hevner et al., 2006).

In the adult murine brain **Mash1** is present in both neurogenic niches, the dentate gyrus and the subependymal zone extending into the rostral migratory stream (C. M. Parras et al., 2004). **Transit amplifying cells of the subependymal zone** express Mash1 that is required for at least two lineages, the neuronal and oligodendrocyte lineage (C. M. Parras et al., 2004). Retroviral over-expression of Mash1 in the adult dentate gyrus leads to the generation of oligodendrocytes in this region (S. Jessberger et al., 2008). In the subependymal zone Mash1 colocalizes partially with Olig2 which is crucial for the generation of oligodendrocytes.

The bHLH transcription factor **Olig2** which acts as **repressor** is present in the **adult subependymal zone** and is localized in a subset of **transit amplifying progenitors** (M. A. Hack et al., 2005; B. Menn et al., 2006). Retroviral over-expression of Olig2 in this brain region leads to the massive generation of oligodendrocytes migrating towards the white matter (M. A. Hack et al., 2005). Over-expression of a dominant-negative form of Olig2 which triggers activation of Olig2 target genes abolishes generation of oligodendrocytes from the subependymal zone (M. A. Hack et al., 2005).

Furthermore, expression of Olig2 in neurogenic transit-amplifying progenitors is suppressed by **BMP-mediated signalling**. When BMP-signalling is blocked, e. g. deletion of the transcription factor Smad4 or infusion of Noggin, Olig2 is aberrantly expressed in transit-amplifying precursors generating now Olig2-positive oligodendrocytes precursors. These cells migrate into the white matter and corpus callosum forming later mature oligodendrocytes (D. Colak et al., 2008).

2.9 Candidate genes

Notably, expression of Dlx2 in the adult subependymal zone had been reported previously (M. H. Porteus et al., 1994; F. Doetsch et al., 2002). However, little is known about the expression profile of Dlx2-positive cells in the subependymal zone and about their fate in the olfactory bulb. I therefore investigated the function of Dlx2

in the adult subependymal zone and in neuronal subtype specification for olfactory interneurons.

2.9.1 The role of Dlx2 in adult neurogenesis in regard to Pax6

Dlx1 & Dlx2 belong to the key molecular regulators for GABAergic neuron specification during development besides Gsh1 & Gsh2 since the respective double mutant mice loose virtually all GABAergic telencephalic interneurons prior to birth (S. A. Anderson et al., 1997a; D. D. Eisenstat et al., 1999; H. Toresson et al., 2000; H. Toresson and K. Campbell, 2001). However, the function of these transcription factors in adult neurogenesis is yet unknown as most of the mouse mutants die perinatally. We therefore aimed to determine here whether Dlx transcription factors maintain their region-specific expression in the adult forebrain and whether they still act to determine all or only a specific subset of olfactory interneurons. Expression of Dlx2 had been observed in transit-amplifying progenitors and neuroblasts (M. H. Porteus et al., 1994; F. Doetsch et al., 2002). Furthermore, Pax6 had been shown to be present in virtually all neuroblasts and Olig2 as a marker for transit-amplifying progenitors (M. A. Hack et al., 2005). Given this co-existence in the same cell types I examined whether Pax6 is required for Dlx2 function and if these transcription factor would interact on the molecular level.

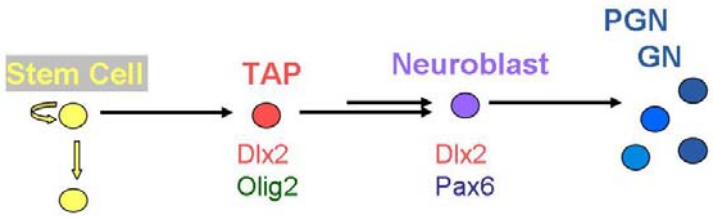


Fig. 15: Schematic summarizing the known network of some transcription factors in the adult subependymal zone.

The transcription factors Dlx2 and Olig2 are both present in transit-amplifying progenitors (TAPs), whereas Pax6 is expressed in neuroblasts. The aim of the study was to study the function of the transcription factor Dlx2 in regard to Pax6.

2.9.2 Tbr1 & Tbr2

Since previous reports demonstrated the contribution of the Emx1-derived, Pax6 expressing dorsal telencephalon to the adult subependymal zone (K. M. Young et al., 2007; S. Willaime-Morawek and D. van der Kooy, 2008), I investigated the regionalization of the subependymal zone in regard to the presence of transcription factors of the embryonic dorsal telencephalon. Furthermore, I examined if the dorsal

wall would generate glutamatergic neurons destined for the olfactory bulb. Consequently this would suggest that neural stem cells of the dorsal subependymal zone recapitulate their developmental program. In the adult subependymal zone Pax6 is expressed in a gradient from dorsal to ventral, whereas Dlx transcription factor are exclusively expressed in the lateral ventricular wall. During embryonic development, dorsal progenitors generate predominantly glutamatergic neurons, in a Pax6-dependent manner, including those of the olfactory bulb (T. Nomura and N. Osumi, 2004; T. T. Kroll and D. D. O'Leary, 2005; V. Nikoletopoulou et al., 2007). Thus, the entire population of olfactory projection neurons is thought to derive from a Pax6-expressing territory and Pax6 is crucial for the formation of the olfactory bulb (T. L. Dellovade et al., 1998; D. Jimenez et al., 2000). In the developing cerebral cortex, Pax6 regulates Neurogenin1 and **Neurogenin2** which are crucial to specify at least deep cortical layer neurons towards a glutamatergic fate (C. Schuurmans et al., 2004). In addition, in the cerebral cortex and hippocampus Pax6 and its target Neurogenin2 regulate expression of the T-box transcription factors **Tbr1 and Tbr2** expressed in early postmitotic glutamatergic neurons and their intermediate progenitors (R. F. Hevner et al., 2006).

Given the glutamatergic progeny of the dorsal telencephalon during development and its contribution to the adult subependymal zone, I searched for progenitor cells in the dorsal part of the subependymal zone that may generate glutamatergic neurons in the adult olfactory bulb.

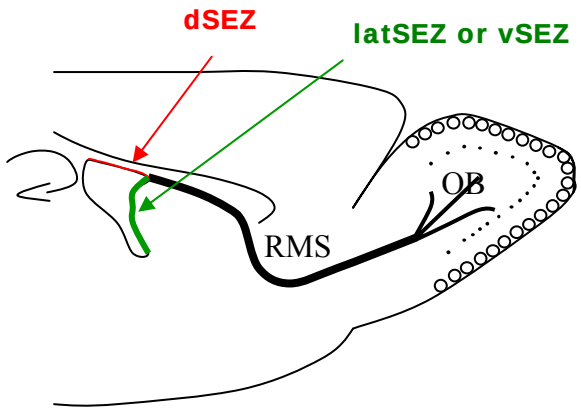


Fig. 16: Schematic depicting the dorsal region (dSEZ) and lateral region (latSEZ) of the adult subependymal zone.

Previous study demonstrated the regionalization of the adult subependymal zone. However, during development glutamatergic neurons are generated in the dorsal telencephalon forming later the dorsal wall of the subependymal zone (dSEZ). Therefore, we investigated if transcription factors would be expressed in the dSEZ that governs the generation of glutamatergic olfactory neurons. OB = olfactory bulb; RMS = rostral migratory stream; dSEZ = dorsal subependymalzone; latSEZ = lateral subependymal zone, vSEZ = ventral subependymal zone.

3 Aims of this study

The postulated regionalization of the adult subependymal zone and the transcriptional network specifying olfactory interneurons is still debated and should be addressed in this study.

In my thesis, the regionalization of the adult subependymal zone should be clarified. The candidate gene Dlx2 should be studied in regard to regionalization, expression pattern and function in adult neurogenesis and subtype specification in the olfactory bulb. The function of Dlx2 should be examined in vivo by the construction and injection of retroviral vectors for gain- and loss-off-function experiments. Furthermore, the in vivo results should be supported by in vitro models (neurosphere assay and direct plated subependymal zone progenitors) and in vivo time-lapse imaging should be employed to further elucidate the function of Dlx2.

In addition, the contribution of the dorsal subependymal zone which is derived from the embryonic dorsal telenecpehalon should be elucidated in regard to glutamatergic olfactory neurogenesis. Transcription factors involved in cortical glutamatergic neurogenesis should be identified in the dorsal adult subependymal zone. If these factors would be present in these regions their progeny in the rostral migratory stream and in the olfactory bulb should examined using labelling methods like the DNA base analogue BrdU, retroviral injections, as well as transgenic mouse lines

.

4 Results

4.1 A Dlx2- and Pax6-dependent transcriptional code for periglomerular neuron specification

4.1.1 Region-specific expression of Dlx1, 2, 5&6 in the adult telencephalon

In order to examine the expression patterns of Dlx genes in the adult telencephalon we used *in situ* hybridization with probes specific for Dlx1, 2, 5 and 6 mRNAs. Dlx 1 and Dlx 2 mRNAs were abundantly expressed in the lateral - but not the dorsal or medial – wall of the lateral ventricle, in the rostral migratory stream (RMS) and the olfactory bulb (OB) (Fig. **17** A, B). In contrast neither Dlx5 nor Dlx6 mRNAs were detected within the subependymal zone, but started to be expressed at low levels within the rostral migratory stream, and became abundant in the olfactory bulb (Fig. **17** C, D) (G. Levi et al., 2003). This is consistent with the developmental profile of higher expression levels of Dlx5& 6 at later stages of cell maturation (D. D. Eisenstat et al., 1999). Note that the weak signal present in the white matter of the cerebral cortex is also seen in the sense control and hence reflects background (Fig. **17** E, E', E''). In addition, in the dentate gyrus neither Dlx2 mRNA nor protein of Dlx transcription factors was found in immunohistochemistry using a pan-Dlx antibody (which is directed against the homeodomain of Dlx2 and therefore recognizes also Dlx1, 5 and s) (G. Panganiban et al., 1995; R. J. Kohtz et al., 2001; J. Feng et al., 2004) (Fig. **17** F, G), reminiscent of the absence of these transcription factors in this dorso-medial region during development.

The exclusive expression of Dlx1 & 2 all along the ventral-lateral, but not the dorsal wall of the lateral ventricle is consistent with the expression pattern during embryonic development and is pronouncedly different from the localisation of Pax6-positive cells (Fig. **18** D). Dlx transcription factors are present in the ganglionic eminences whereas Pax6 is prominent in the dorsal telenecephalon. In the adult subependymal zone only some Pax6-positive cells are located along the lateral ventricular wall and most are detected dorsally and in the rostral migratory stream (Fig. **18** D, E; Hack et al., 2005).

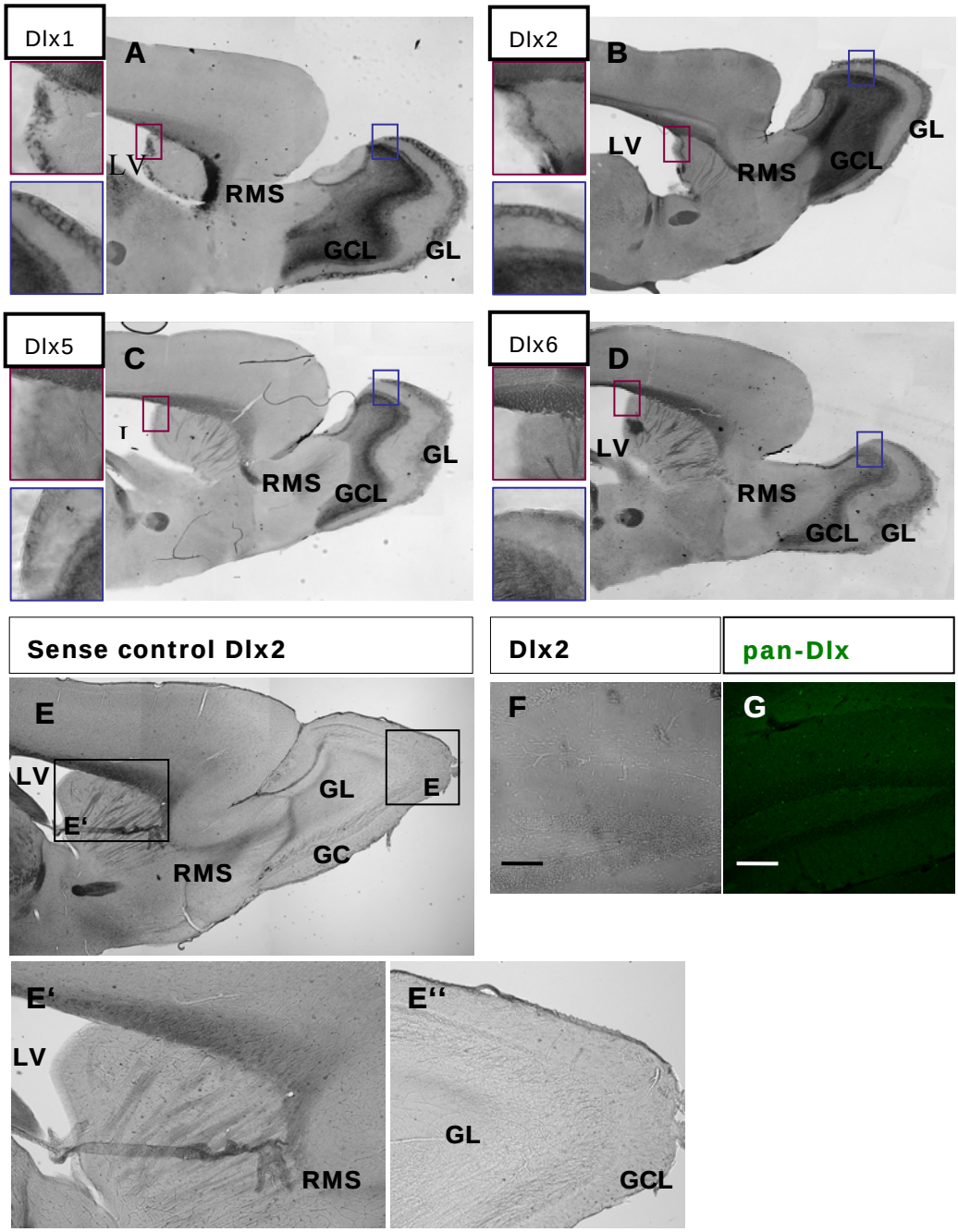


Fig. 17: Expression pattern of the Dlx transcription factors in the adult murine brain.

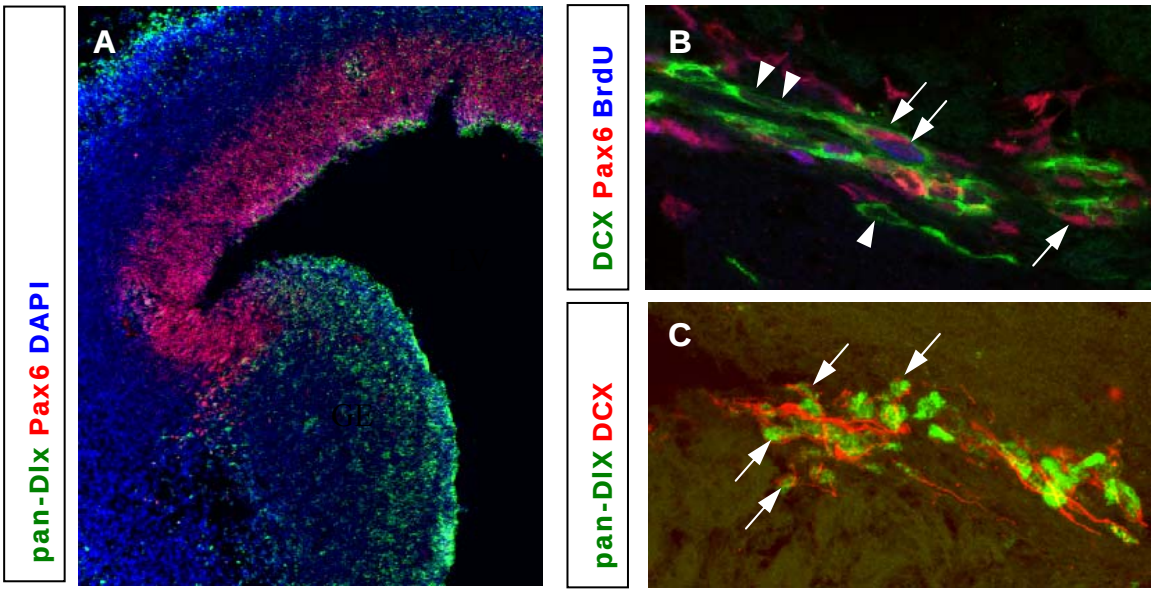
In situ hybridization for (A) Dlx1, (B) Dlx2, (C) Dlx5 and (D) Dlx6 mRNA. Note the intense mRNA signal for Dlx1 and Dlx2 in the adult subependymal zone in contrast to Dlx5 and Dlx6. In the RMSrostral migratory stream and olfactory bulb, Dlx1 and Dlx2, as well as Dlx5 and Dlx6 mRNA are present. Boxed areas are shown in higher magnifications

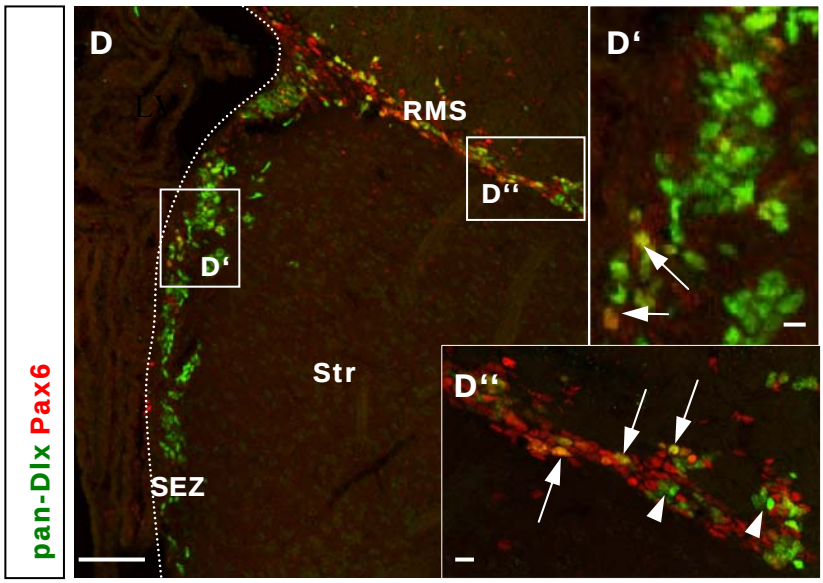
.(E) Sense control for Dlx2 in situ hybridization shows no labelling in the subependymal zone and olfactory bulb. (E'-E'') higher magnifications of boxed areas.

(F) In situ hybridization for Dlx2 mRNA shows no labelling in the dentate gyrus

(G) Immunostaining for pan-Dlx (K. Jin et al.) in the dentate gyrus shows absence of all transcription factors of the Dlx family on the protein level. LV = Lateral Ventricle, GCL = granule cell layer, GL = glomerular cell layer, RMS= rostral migratory stream; Scale bar: 100µm

Next we performed double-immunohistochemistry for Pax6 in combination with the pan-Dlx antibody which recognizes Dlx1 & 2 in the subependymal zone (given the absence of Dlx5 & 6 mRNA in this brain region). Sections from an embryonic day E14 mouse brain demonstrate that Pax6 is prominent in dorsal regions giving rise to cortical neurons. In contrast Dlx transcription factors are expressed in the ventral telencephalon (Fig. **18** A). In the adult rostral migratory stream both Pax6 and Dlx transcription factors are present in neuroblasts (Fig. **18** B, C). But are these transcription factors also co-expressed in the adult forebrain? I observed that the majority of cells were only Dlx-immunoreactive (80 ± 3%) but Pax6-negative in the adult subependymal zone. Only 1 in 10 Dlx-positive (Dlx+) cells also displayed Pax6 immunoreactivity and a similar proportion of cells within the subependymal zone expressed only Pax6 (Fig. **18** D, E). In contrast, within the rostral migratory stream the majority of cells expressed Pax6 and 20% co-expressed Pax6 and Dlx (Fig. **18** D'', E). The high number of the only Pax immunoreactive cells in dorsal regions of the subependymal zone and the beginning of the rostral migratory stream may suggest that these cells may give rise to another separate lineage that is Dlx-negative. Consistently, Pax6 expression was detected in DCX-negative cells suggesting that they are progenitors. Interestingly, amongst the doublecortin-positive (DCX+) neuroblasts in the rostral migratory stream Dlx-positive cells predominated (90 %) and an even higher proportion of double-positive cells (about 40 %) was observed. Taken together, the expression patterns of Dlx and Pax6 genes in the adult telencephalon resemble their regionalization during development.





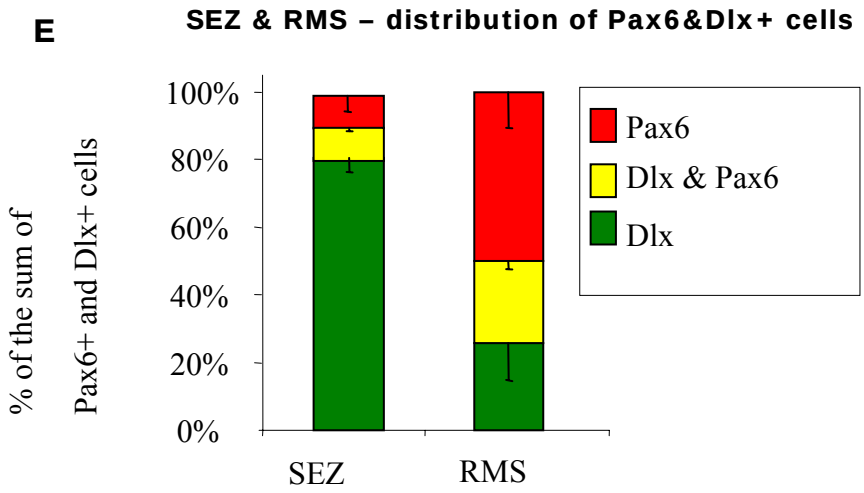


Fig. 18: Pax6 and Dlx immunoreactive cells in the adult subependymal zone and rostral migratory stream

(A) Micrograph depicting a sagittal section of an embryonic day 14 section. Immunofluorescence for Pax6 (red) and pan-Dlx (green) demonstrates that Dlx transcription factors are present in ventral regions. In contrast, Pax6 expression is restricted to the dorsal telencephalon. GE = ganglionic eminence; LV = lateral ventricle.

(D) Overview of the lateral wall of the lateral ventricle (LV) depicting the subependymal zone (SEZ) and rostral migratory stream (RMS) double stained for Dlx and Pax6 proteins. High magnification images of boxed areas in (A') show the subependymal zone and (A") the rostral migratory stream. Arrows indicate double-positive, arrowheads single-positive cells.

(E) Histogram depicting the proportion of cells immunoreactive for only one or both of these transcription factorss. Notably, the proportion of Pax6+ cells and cells immunoreactive for both Dlx and Pax6 increases from the subependymal zone to the rostral migratory stream (comparison between SEZ and RMS for Dlx+, Pax6+ and Dlx+/Pax6+, $P < 0.001$ (ANOVA), number of cells analysed = 332 for SEZ and number of cells analyzed = 363 for RMS, n = 3 animals).

4.1.2 Identity of Dlx-immunoreactive cells in the subependymal zone and olfactory bulb

Next we characterized the identity of cells expressing Dlx transcription factors. While astroglia-like stem cells divide rather slowly, they give rise to rapidly dividing transient-amplifying progenitors (TAPs), a large proportion of which then generates DCX-positive neuroblasts that continue to divide and commence migrating towards the olfactory bulb. Dlx-immunoreactivity was absent in GFAP-positive cells that comprise the neural stem cell compartment (Fig. **19** A).

In order to label fast proliferating cells like transit-amplifying progenitors we gave a short pulse of the DNA base analogue 5-Bromo-2′-deoxy-Uridine (BrdU) which is incorporated into the genome of all dividing cells during DNA synthesis. Since transit-amplifying progenitors divide much faster compared to neural stem cells, a single BrdU pulse before sacrifice labels only these progenitors including a proportion of still dividing neuroblasts. However, if BrdU is given into the drinking water for longer periods (2 – 3 weeks) dividing cells including neural stem cells are labelled by the DNA base analogue. If the BrdU period is followed by a chase of BrdU-free water labelled transit-amplifying progenitors differentiate into neuroblasts and migrate towards the olfactory bulb, alternatively they further proliferate, thereby diluting the BrdU again. Neural stem cells remain in the subependymal zone and their slow cell cycle does not allow prominent dilution of the BrdU. Therefore these slowly proliferating cells can be visualized after long BrdU pulses followed by a BrdU-free chase.

One hour before sacrifice we injected the DNA base analogue BrdU to label fast proliferating cells. We observed Dlx-positive (Dlx+) cells amongst the BrdU labelled compartment consistent with previous data (F. Doetsch et al., 2002). To distinguish Dlx expression in transit-amplifying progenitors from neuroblasts, we performed triple-immunohistochemistry with Dlx, BrdU and DCX (Fig. **19** B) and classified transient-amplifying progenitors as BrdU+/DCX-negative and neuroblasts as BrdU+/DCX+ cells (transit-amplifying progenitors are white and light grey and neuroblasts black and dark grey in Fig. **19** E). Two thirds of all transit-amplifying progenitors were Dlx-positive (Fig. **19** E), whereas the remaining third was BrdU-positive/Dlx-negative. Consistent with the presence of Dlx transcription factors in transit-amplifying progenitors, most of the DCX-negative/Dlx-positive cells were colablled with Mash1, a transcription factor present in transit-amplifying progenitors (Fig. **19** C) (C. M. Parras et al., 2004).

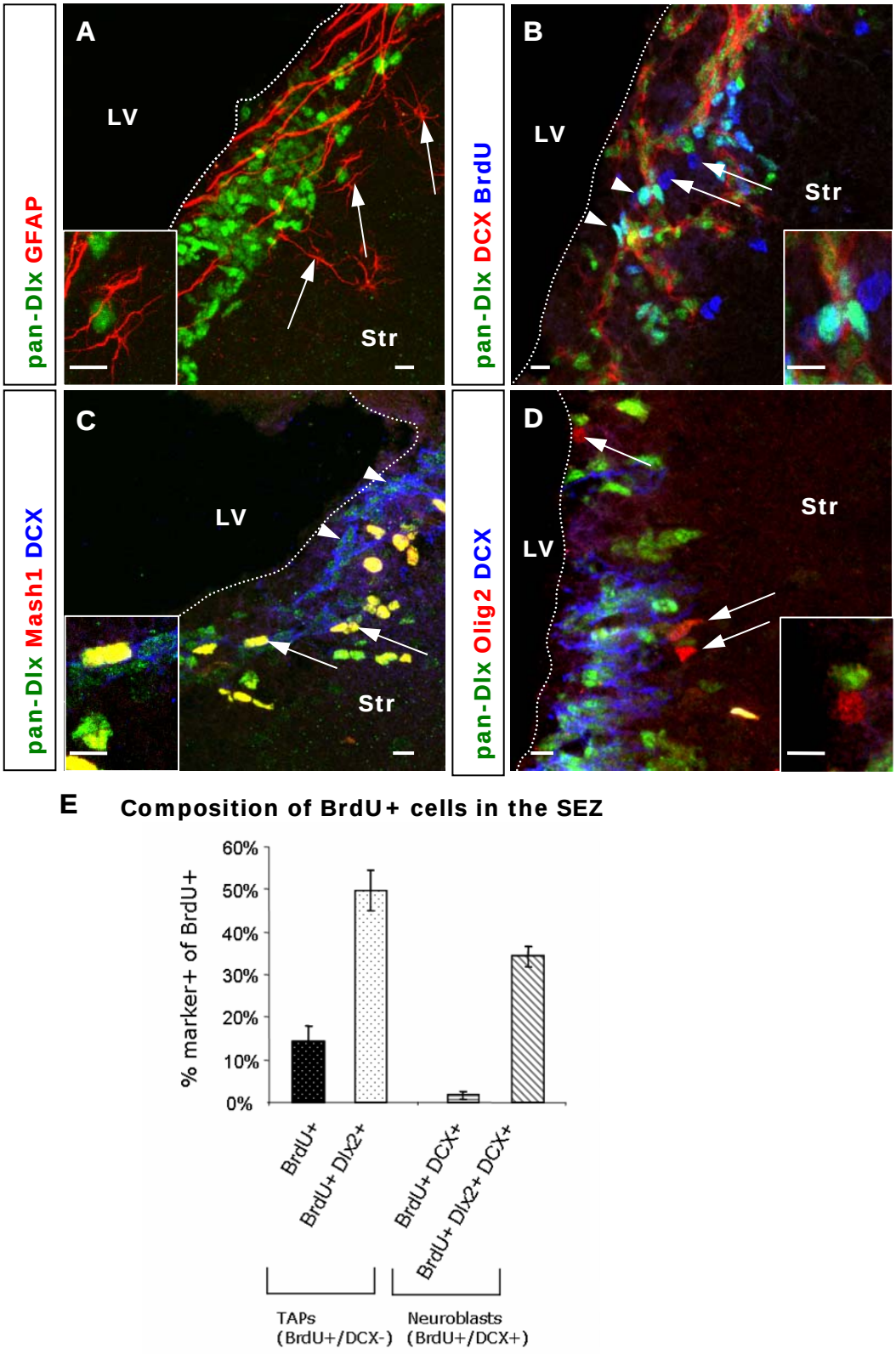


Fig. 19: Dlx-immunoreactive cells in the adult subependymal zone and rostral migratory stream.

The panels in (A – D) depict example micrographs to identify the cell types expressing Dlx as indicated in the panels. Histogram in (E) depicts the composition of BrdU-positive cells comprising transit-amplifying progenitor cells (TAPs; white and light grey bars) and neuroblasts (dark grey and black bars) in the subependymal zone. Note that virtually all neuroblasts are Dlx-positive, while about a forth of all transit-amplifying progenitors are Dlx-negative (number of cells analyzed in total = 295, n = 3 animals). Scalebars: (A) 100µm, (A'-G) 10µm, insets in (C-G) 10µm; LV = Lateral Ventricle, Str = Striatum, SEZ = subependymal zone, RMS = rostral migratory stream

Previous reports demonstrated the presence of the transcription factor Olig2 in the adult subependymal zone and its role in the generation of oligodendrocytes (M. A. Hack et al., 2004; M. A. Hack et al., 2005; B. Menn et al., 2006; D. Colak et al., 2008). Indeed we found that the BrdU+/Dlx-negative cells express Olig2 (Fig. **19** D) as Dlx-immunoreactivity was not co-localized with Olig2 (Fig. **19** D) (D. Colak et al., 2008). While not all transit-amplifying progenitors express Dlx virtually all neuroblasts were Dlx-immunopositive in the ventral subependymal zone (Fig. **19** D) as described also for Dlx2 protein (Doetsch et al., 2002).

Upon maturation neuroblasts migrate along the lateral wall of the lateral ventricle and form the rostral migratory stream before entering the olfactory bulb. Notably, Dlx protein was still present in the young neurons reaching the stream and within the olfactory bulb in neurons that had already down-regulated DCX upon maturation. Interestingly, we noted a particularly high Dlx-immunoreactivity in the glomerular layer (Fig. **20** A). Interestingly, pan-Dlx immunostaining was confined to most of the calbindin-positive and virtually all tyrosine hydroxylase-positive (TH+) periglomerular neurons (TH+/Dlx+: 99 ± 1 %, n = 3 animals, 340 cells; Fig. **20** B, C), i.e. the GABA-immunoreactive subtypes, while calretinin-positive periglomerular neurons were characteristically devoid of pan-Dlx-immunoreactivity (Fig. **20** D).

The dopaminergic subpopulation accounted for 31 ± 8 % (n = 3 animals, 841 cells counted) of all Dlx-positive cells. Given that Pax6 is involved in the generation of dopaminergic periglomerular neurons (M. A. Hack et al., 2005) and Dlx1 & 2 are co-expressed with Pax6 in the subependymal zone, we further tested if this would be the case in the dopaminergic subpopulation. Immunohistochemistry with a Dlx2 specific antibody and Pax6 demonstrated that virtually all Pax6-positive periglomerular neurons also co-expressed Dlx2 (Fig. **20** E). Amongst the Dlx2-positive cells in the glomerular layer 41 ± 6 % (n = 3 animals, 518 cells counted) displayed Pax6 immunoreactivity. Thus this data suggest that these factors may interact to specify the dopaminergic neuron fate as implicated in the developing ventral thalamus (G. S. Mastick and G. L. Andrews, 2001; G. L. Andrews et al., 2003).

Since only Dlx1 and Dlx2 are expressed in the subependymal zone, we focussed our functional analysis in the remainder of this study on one of these, namely Dlx2.

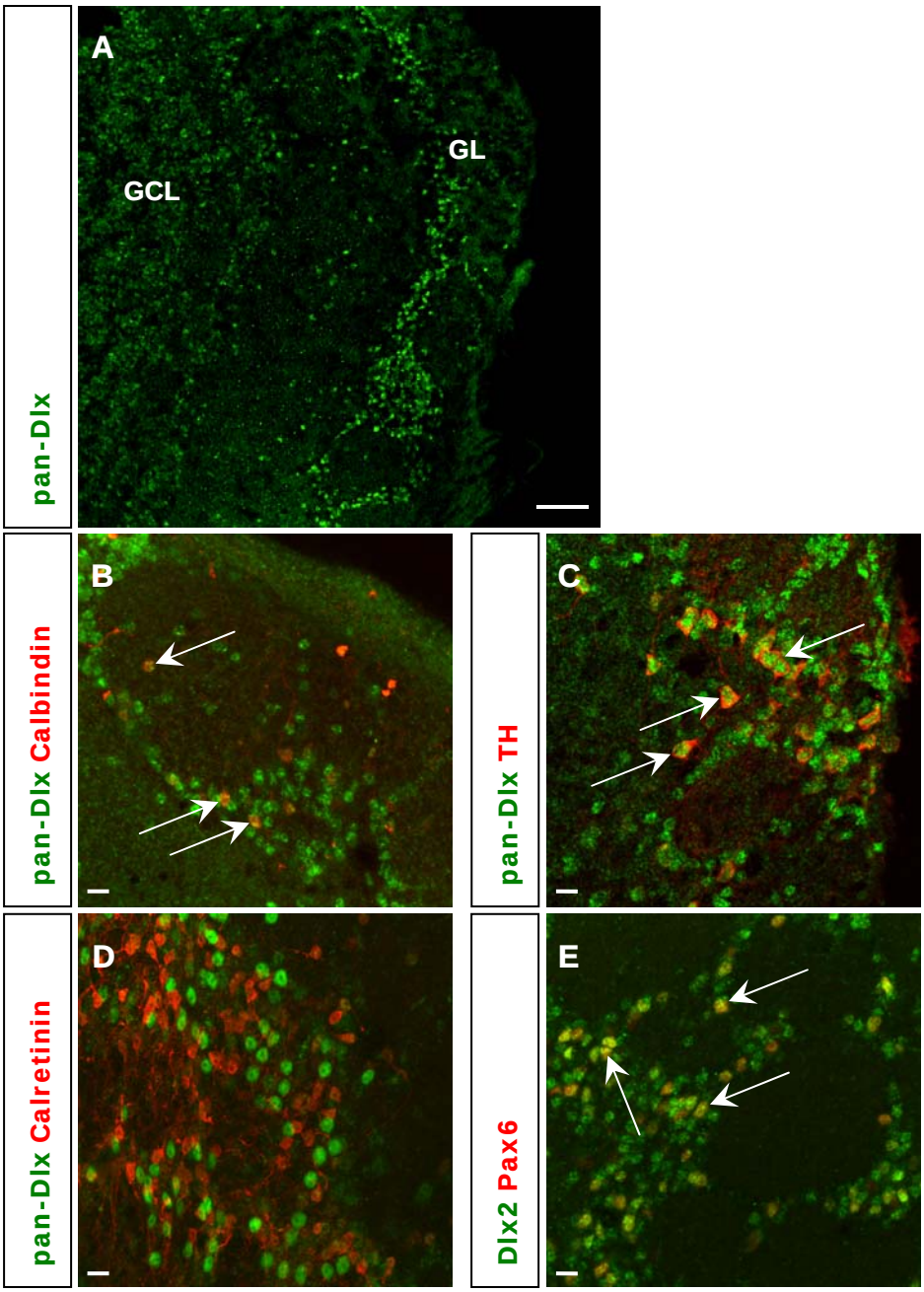


Fig. 20: Dlx-immunoreactive cells in the adult olfactory bulb.

(A-E) Fluorescent micrographs depicting pan-Dlx (A-D) or Dlx2 (E) immunoreactivity in an overview of the OB (A) or within the GL (B-E) in double-stainings as indicated in the panels. Note that some Dlx+ cells are also immunoreactive for calbindin or TH as well as the transcription factor Pax6 (arrows indicating double-positive cells for Dlx and marker). (A) Scalebar: 100µm, (B-E) Scalebar: 10µm. OB = olfactory bulb, GCL = granule cell layer, GL = glomerular layer, TH = Tyrosine Hydroxylase.

4.1.3 Dlx2 acts potently neurogenic on adult subependymal zone derived cells in vitro

4.1.3.1 Dlx2 over-expression in neurosphere derived cells leads to neurogenesis

We first performed gain-of-function studies in vitro, by transducing adult subependymal zone derived neurosphere cells with pseudotyped retroviral vectors encoding GFP behind an IRES sequence for control and Dlx2-IRES-GFP for Dlx2 over-expression (Fig. **21** A). Reliable co-expression of Dlx2 and GFP was confirmed (Fig. **21** B - E).

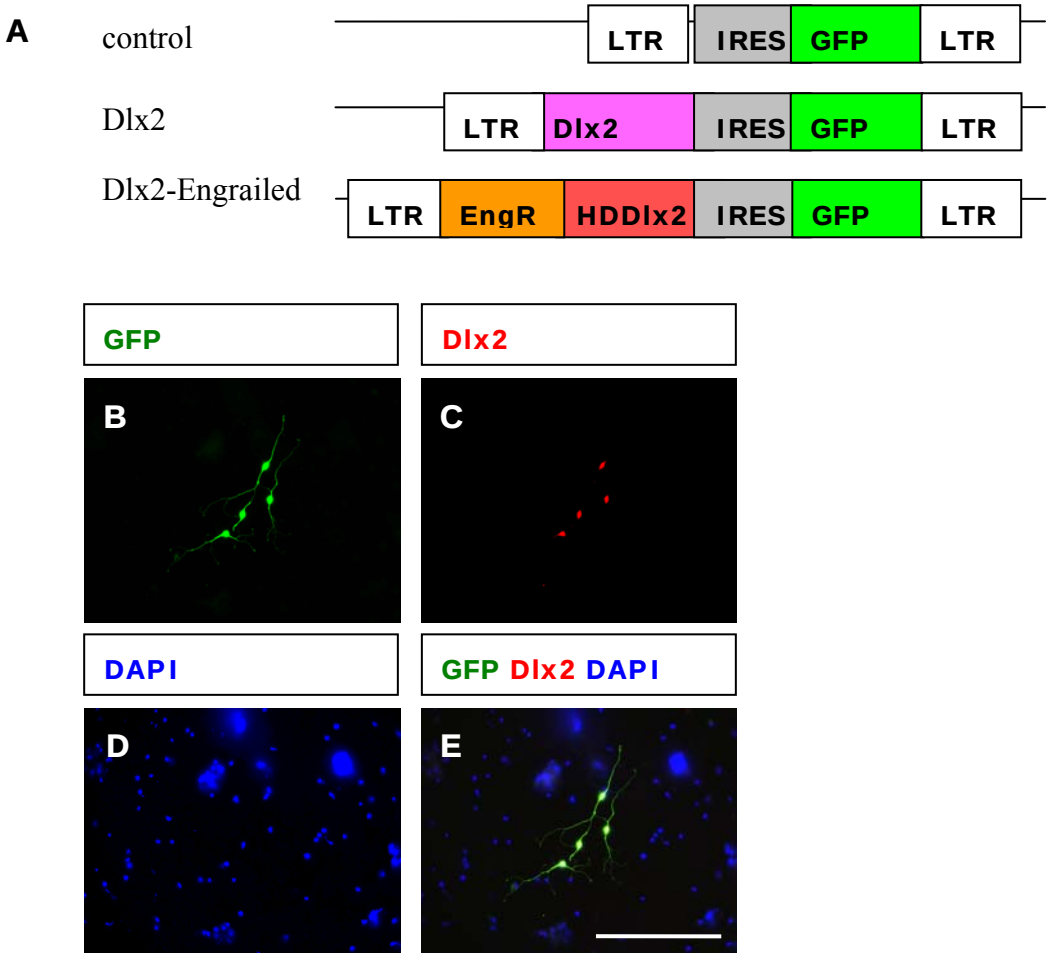


Fig. 21: Expression of Dlx protein following retroviral transduction with Dlx2.
(A) Schematic drawing of the retroviral constructs used for control and manipulation of Dlx2. Immunofluorescence for GFP, Dlx2 and DAPI as indicated on neurosphere cultures 7 days after retroviral over-expression of Dlx2. Microcraphs showing (B) GFP, (C) Dlx2, (D) DAPI and (E) overlay. Scalebar: 100µm.

Retroviral vectors transduce exclusively dividing cells. Retroviruses cross the cell membrane, but lack any complex to overcome the nuclear membrane. When the transduced cell is dividing at the same time following transduction the nuclear

membrane is not existent during that time window and retroviral vectors are incorporated into the genome.

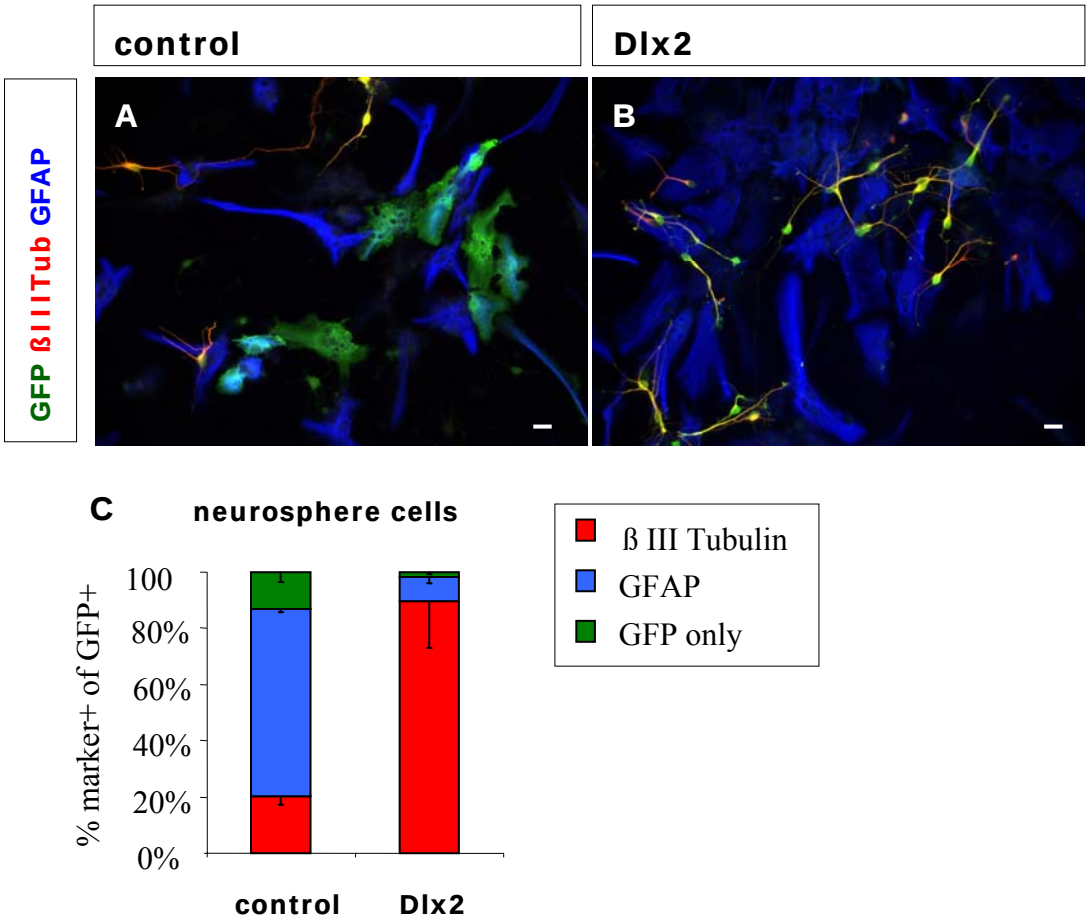


Fig. 22: Expression of Dlx protein following retroviral transduction with Dlx2

(A, B) Fluorescence micrographs of subependymal zone derived neurosphere cells after 7 days differentiation immunostained for ßIIITubulin (red), GFAP (blue) and GFP (green). Note the vastly increased number of transduced (GFP+) cells co-localising with the neuron-specific antigen ßIIITubulin following Dlx2 over-expression (B) compared to control (A). Scale bars: 10µm

(C) Histogram depicting the proportion of transduced cells (GFP+) acquiring an astroglial (GFAP+, blue), neuronal (ßIIITubulin+, red) or none of these fate following viral transduction in subependymal zone dervived neurosphere cells (D; P = 0.03 (GFAP comparison, t-Test), P = 0.004 (ßIIITubulin comparison, t-Test), control number of cells analyzed in total = 979; Dlx2 number of cells analyzed in total = 1192, n = 3 independent experiments each).

When control transduced neurosphere cells were examined seven days later, the majority of GFP-positive cells were GFAP-positive astrocytes, while only one fifth were ß-III-Tubulin-positive neurons (Fig. **22**). In pronounced contrast, the vast majority of neurosphere-derived cells transduced with Dlx2 acquired a neuronal fate (Fig. **22**), at the expense of the astroglial population. This data suggests that Dlx2 is a potent inducer of a neuronal fate in this in vitro model of adult neural stem cells, reminiscent of its function during development (G. Panganiban and J. L. Rubenstein, 2002).

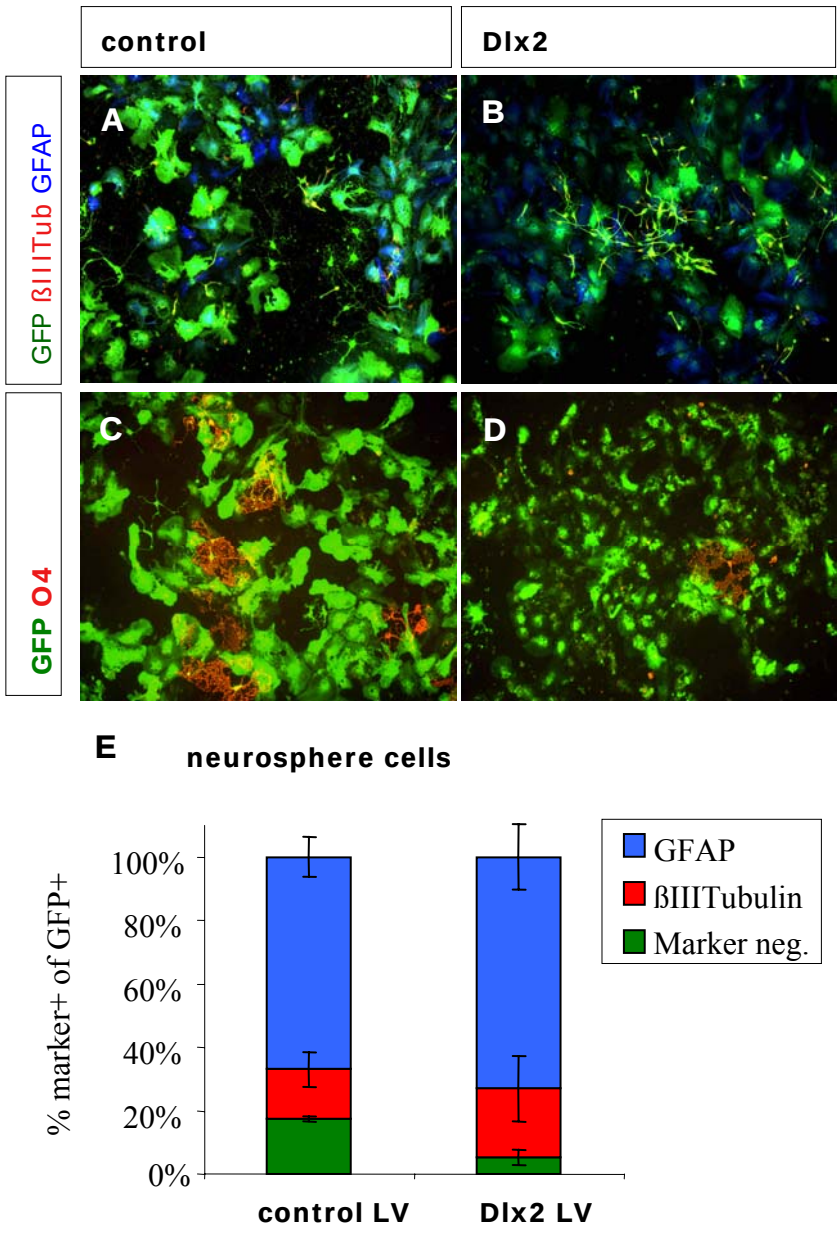
Next, we performed the same experiment but using lentiviral vectors. Lentiviruses are able to cross the nuclear membrane by the use of integrase complexes and hence are able to transduce non-dividing cells (L. Naldini, 1998). Interestingly, the neurogenic effect of Dlx2 over-expression was less pronounced by lentiviral vectors. We still observed a small increase in the number of ß-III-Tubulin-positive neurons, mostly at the expense of marker negative cells (Fig. **23** A, B, E). Additionally, the number of oligodentrocyte marker O 4 immunoreactive cells decreased (Fig. **23** C, D), whereas the proportion of GFAP-positive astrocytes remained stable in both conditions. Dlx2 over-expression may therefore prevent an oligodendrocytes fate, but could not force destined astrocytes to switch their fate into the neuronal lineage. As the effect of Dlx2 retroviral over-expression was by far more prominent than with lentivirus we chose the former for the following in vivo experiments.

Fig. 23: Dlx2 lentiviral over-expression in neurospheres exhibits only small neurogenic effects

(A - B) Fluorescence micrographs of subependymal zone derived neurosphere cells after 7 days differentiation immunostained for ß-III-Tubulin (young neurons), GFAP (astrocytes, blue) and GFP (green).

(C – D) Immunohistochemistry on differentiated neurospheres for the oligodendrocytes antigen O4 shows decreased expression upon Dlx2 lentiviral transduction.

(E) Histograms depicting the proportion of transduced neurosphere cells (GFP+) expressing the neuronal marker ß-III-Tubulin (red), the astrocytes marker GFAP (blue) or none of these (green). Dlx2 seems to act mostly on the marker negative population that comprises oligodendrocytes and progenitor cells.



4.1.3.2 Primary non-expanded adult subependymal zone progenitors

Next we aimed to examine whether Dlx2 was not only sufficient to instruct neurogenesis, but was also required for neurogenesis from adult neural stem cells.

Neurosphere-derived cells are expanded in growth factors like epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2) under non-adherent conditions (B. A. Reynolds and S. Weiss, 1992; M. A. Hack et al., 2004; B. Berninger et al., 2007; A. Chojnacki and S. Weiss, 2008). Following differentiation and retrieval of growth factors neurosphere derived cells generate intrinsically few neurons and differentiate predominantly into GFAP+ astrocytes (Fig. **22**). Additionally, they up-regulate transcription factors of the oligodendroglial lineage like Olig2 (M. A. Hack et al.,

2004). Therefore this culture system is less suitable for a loss of function analysis of Dlx2.

However, we developed a culture system for primary adult subependymal zone derived cells cultured in the absence of EGF and where the cells are directly plated after preparation FGF2 (for details see Material and Methods: 6.9.2 Non-expanded adult progenitor cultures). Notably, most cells (except GFAP+ astroglia) in the cultures not treated by EGF and FGF2 were Dlx-immunoreactive (Fig. **24**).

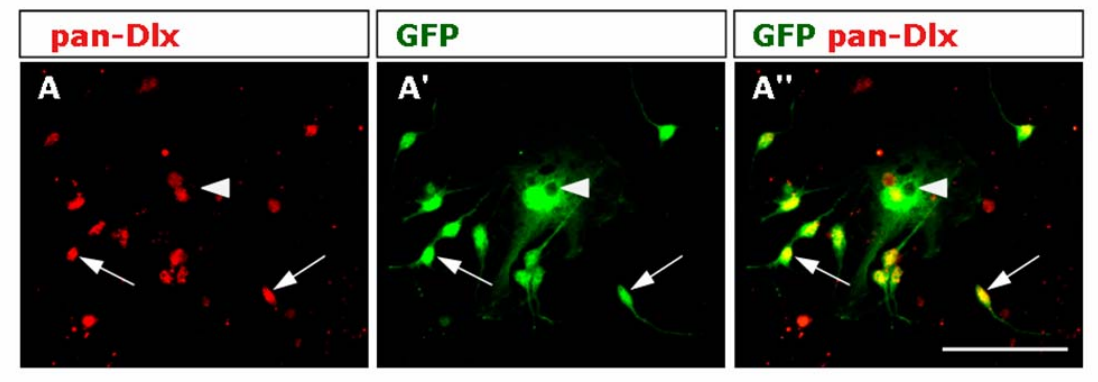


Fig. 24: Expression of Dlx protein in adult primary non-expanded adult progenitors.
(A-A'') Immunohistochemistry 7 days after transduction with control viral vectors. Micrographs show immunohistochemistry for (A) Dlx, (A') GFP and (A") overlay. Arrows indicate Dlx+ cells with neuroblast morphology and arrowhead indicateds Dlx-negative cell with astrocytic morphology.

When I quantified the number of intrinsically generated neurons I found that the cell type composition was distinct from neurosphere derived cultures. The proportion of neurons and astrocytes in vitro 3 days and 7 days after plating was identified byr immunohistochemistry for ß-III-Tubulin, O4 or GFAP and DAPI (Fig. **25**). Notably more than half of the cells adopted a neuronal identity similar to the in vivo situation. Only around 10 % of the DAPI-positive cells expressed the astrocyte marker GFAP and after 7 days in vitro a similar number of O4-positive oligodendrocytes was observed. Notably, also in vivo a lot of neuroblasts are present in the subependymal zone, whereas differentiated neurosphere generate only 10 – 20 % neurons. This data suggest that primary subependymal zone cultures comprise progenitors that preferentially give rise to cells with a neuronal identity similar to the in vivo situation.

In order to better characterise these progenitor cultures I next analysed the composition of individual clones. I took advantage of retroviral vectors encoding GFP to follow the progeny of single transduced cells that had divided at the time of transduction. Cultures were transduced 2 – 4 hours after seeding and coverslips were fixed seven days after transduction and analysed when less than 40 clones were present.

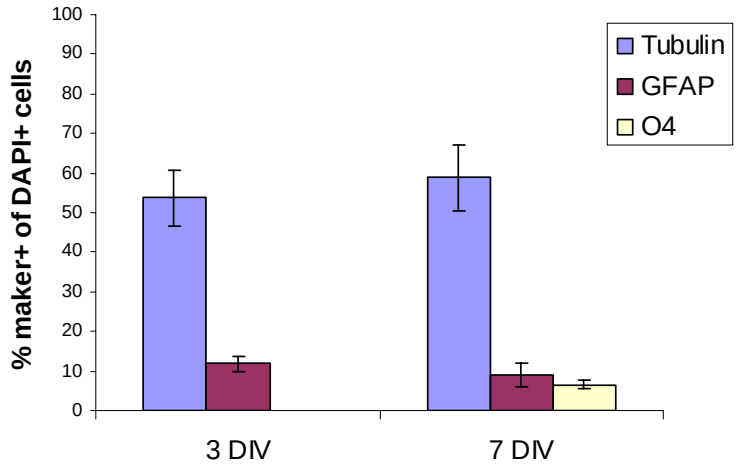


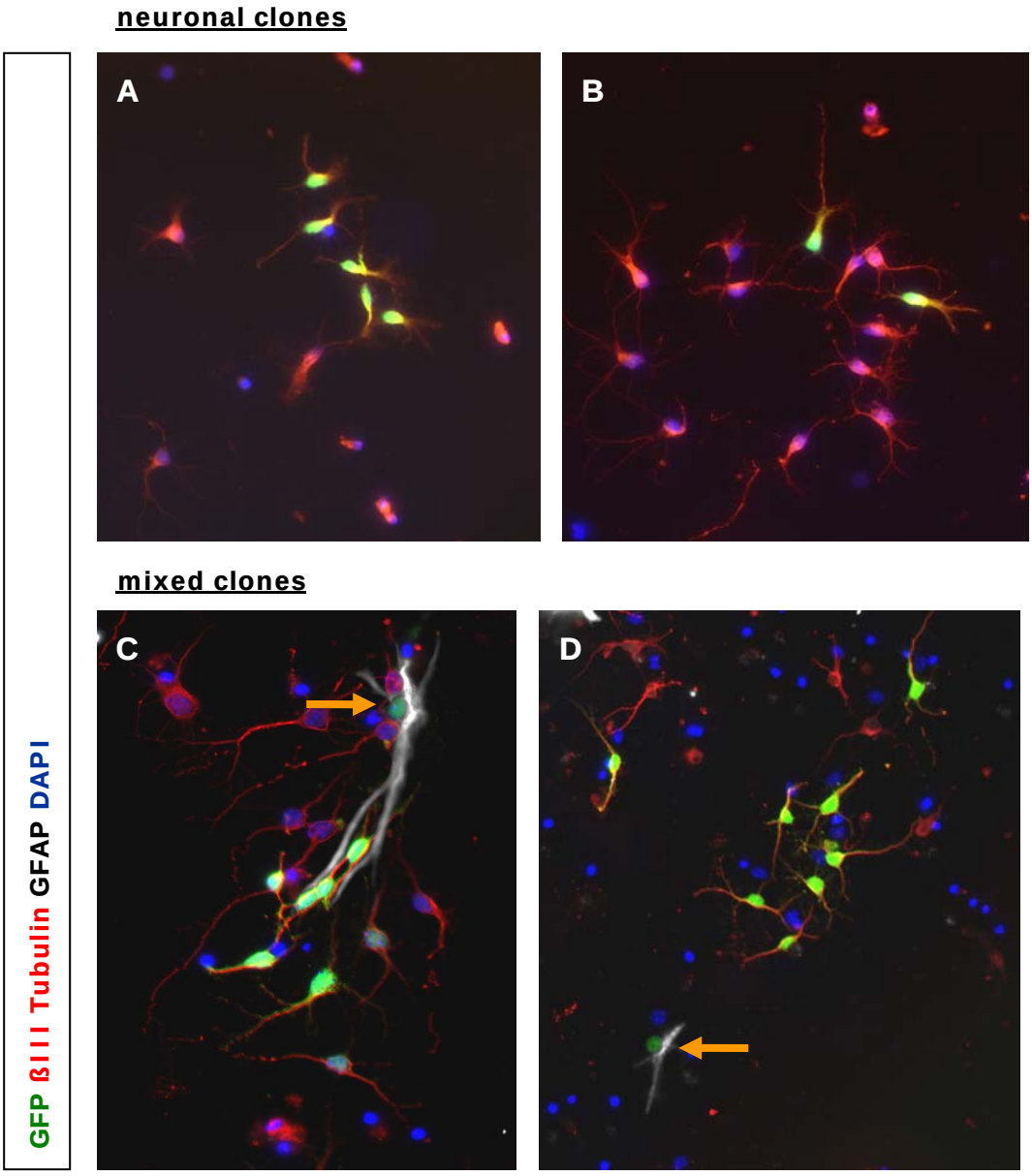
Fig. 25: Identity of the cultured cells 3 days and 7 days in vitro.
Immunohistochemistry for ßIIITubulin (violet) or GFAP (red) or O4 (yellow) counterstained with DAPI demonstrated that the majority of the cells is of neuronal identity 3 days and 7 days in vitro. Oligodendrocytes could be only observed 7 days after in vitro and to a similar extent than astrocytes.

Just considering the size of the clones most of them were rather small in size (1 - 2) and often only a single cell was observed suggesting that the sister cell died. We detected only few clones with more than 6 cells. Immunohistochemistry for the neuronal marker ß-III-Tubulin, the astrocytic marker (GFAP), and GFP (green) was performed to identify transduced cells. Most of the clones were of neuronal identity (ß-III-Tubulin). Only few non-neuronal clones and mixed clones were observed.

In order to correlate the size and the identity of the clones we further analysed the size and the identitfied as follows. Clones were classified into three types: purely neuronal clones, in the case that all cells were found immunoreactive for GFP and the neuronal marker ß-III-tubulin; purely non-neuronal or glial clones, if all cells comprised within were ß-III-Tubulin-negative, but expressed the glial markers GFAP or the oligodendrocytes marker O4; and mixed clones, for the case that at least one of the cells was ß-III-Tubulin reactive while at least another cell was found positive for GFAP or O4. As expected, most of the small clones were of neuronal identity (Fig. **26** A, B). Interestingly, non-neuronal clones were also small in size. Mixed clones were typically larger in size and even when clones were mixed most of the transduced cells were of neuronal nature (Fig. **26** C, D, F).

In summary, clonal analysis showed that a great proportion of neurons are generated from precursors in vitro. Interestingly, few mixed clones could be identified. Like in vivo where most of the astrocytes are quiescent and only few function as neural stem

cell with a slow cell cycle this situation is perfectly mimicked in this culture system with only few mixed clones. The retroviral clonal analsysis indicates that cultured adult subependymal zone progenitors are distinct with respect to their proliferative properties, differing either in cell divison cycle length or their capacity to undergo several rounds of cell divisions.



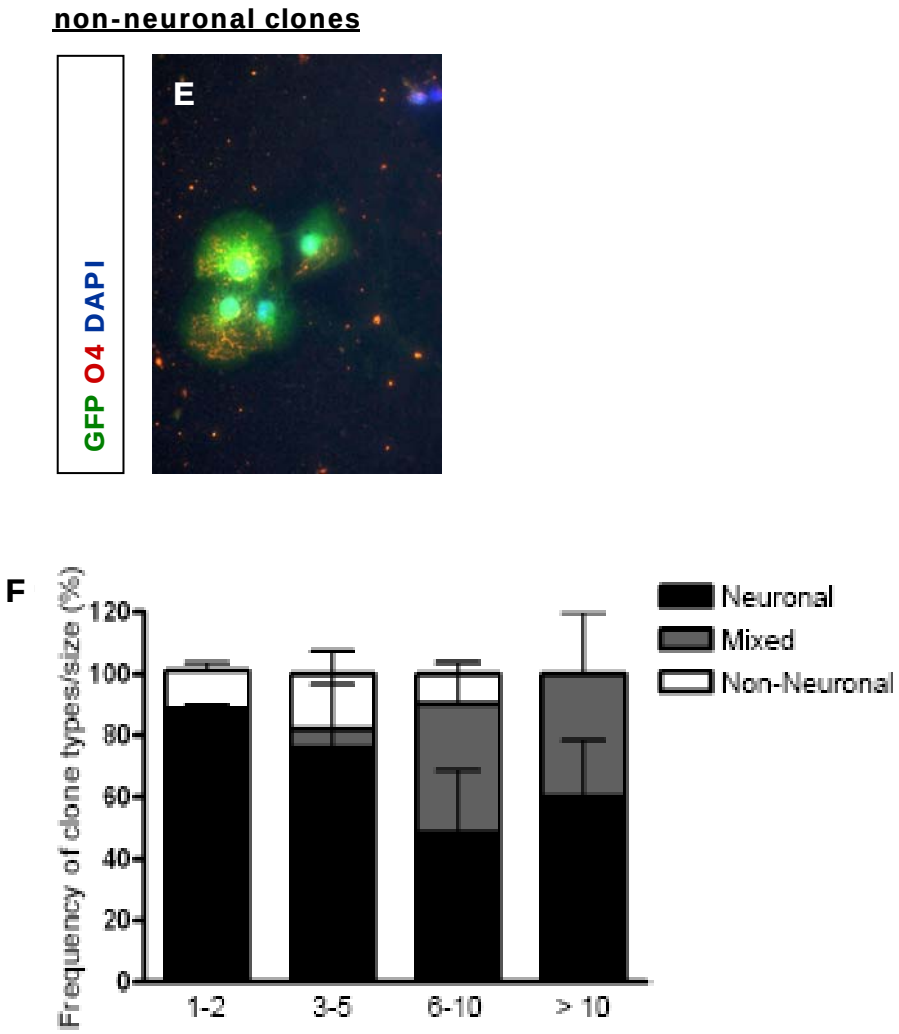


Fig. 26: Clonal analysis 7 days post retroviral transduction in subependymal progenitor cultures.
Immunohistochemistry for GFP (green), GFAP (white), GFP (green) , ßIII Tubulin (red) and DAPI (blue).
(A, B) Example of neuronal and (C, D) mixed clones. Orange arrows indicate astrocytes in mixed clones.
(E) Relatively few oligodendrocyte clones were found in the retroviral clonal analysis which were positive for the oligodendrocyte marker O4.
(F) Clonal size plotted additionally with their identity demonstrated again that most clones were of neuronal identity. Pure non-neuronal clones were rare and even mixed clones contained mainly neurons.

4.1.3.3 Dlx2 and Dlx2-Engrailed over-expression in adult subependymal zone progenitors

We took advantage of the above described culture system to test the role of endogenous Dlx2, using a retroviral vector encoding a chimeric protein in which the Dlx2 homeodomain had been fused to the Engrailed repressor domain (Fig. **29** A and Material & Methods). The homeodomain of Dlx2 was amplified by PCR from a mouse Dlx2 cDNA containing plasmid (gift from G. Mastick) and inserted into a pCDNA3 vector. The Engrailed repressor domain (plasmid kindly provided by Mineko Kengaku) was also amplified by PCR and inserted into the pCDNA3 vector. The whole cassette

was digested and ligated into the pmxig retroviral vector between the LTR and the IRES followed by GFP for visualization. This construct is converting Dlx2-mediated transactivation into repression (S. E. Harris et al., 2003; J. M. Woda et al., 2003; T. Kaji and K. B. Artinger, 2004) (Fig. **29** A).

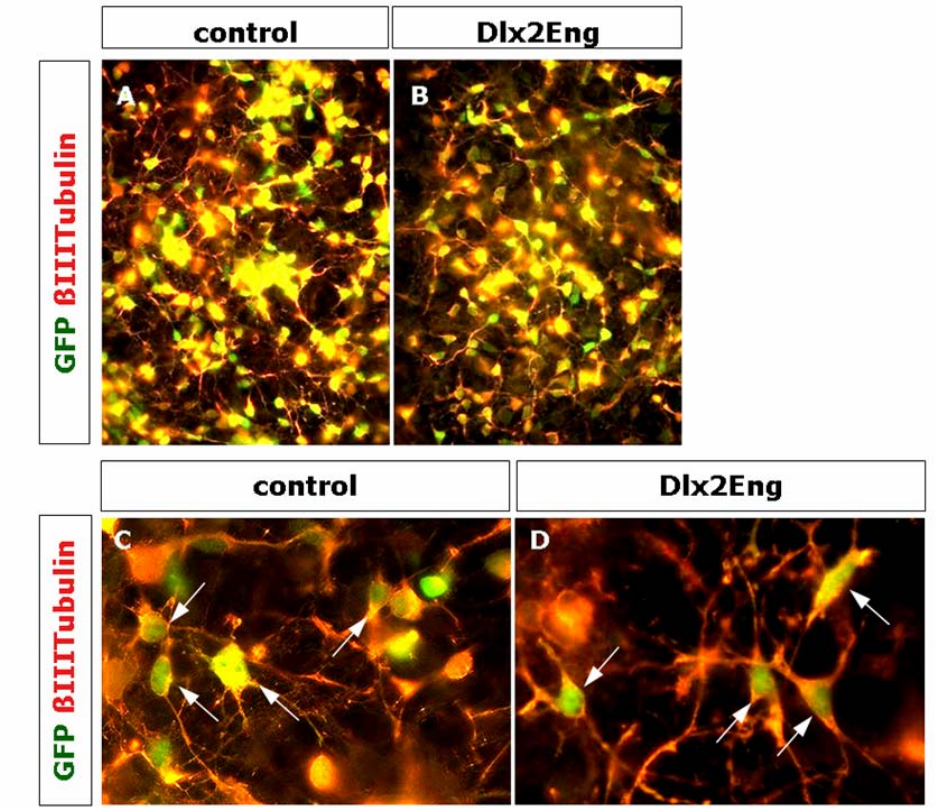


Fig. 27: Dlx2-Engrailed transduction does not alter neurogenesis in embryonic E 13 cultures.
Cultures of embryonic day E 13 transduced with control (A, C) or Dlx2-Engrailed (B, D) viral vectors and kept for 3 days in vitro (DIV). The number of ß-III-Tubulin+ cells did not alter, suggesting that Dlx2-Engrailed did not interfere with neurogenesis in cortical progenitors from embryonic day E13.Arrows indicated double-positive cells for GFP and ß-III-Tubulin.

In order to confirm the specificity of the Dlx2-Engrailed construct we further tested its specificity independently in the present study, beyond the previous data on the function of Dlx2-Engrailed fusion proteins as repressors and antagonists of the endogenous Dlx2 function (S. E. Harris et al., 2003; J. M. Woda et al., 2003; T. Kaji and K. B. Artinger, 2004). As cells isolated from the embryonic day 13 (E 13) cerebral cortex that contain very few, if any Dlx2+ progenitors cells at this stage, we used them to determine potential off-target effects of Dlx2-Engrailed. E 13 cortical progenitors infected with Dlx2-Engrailed still differentiated normally into neurons (control GFP+/ß-III-Tubulin+ 94.5 %; Dlx2-Eng 97 %, one experiment, number of cells analyzed for control = 618 and Dlx2-Eng = 355), suggesting that Dlx2-Engrailed transduction does not interfere with neurogenesis in cells that do not express Dlx2 (Fig. **27**). Therefore we conclude

that the effect of Dlx2-Engrailed is specific and reveals the need of endogenous Dlx2-regulated targets to be up-regulated for neurogenesis to proceed normally.

As a second test in vivo, we injected the above described construct of Dlx2-Engrailed into the dentate gyrus of the hippocampus. Neurogenesis in this region leads to the generation of glutamatergic granule neurons and Dlx-proteins are not present in this region (Fig. **20**). Injection of Dlx2-Engrailed containing retrovirus did not prohibit the generation of DCX-positive neuroblasts seven days later (Fig. **28**), further supporting the specificity of this construct.

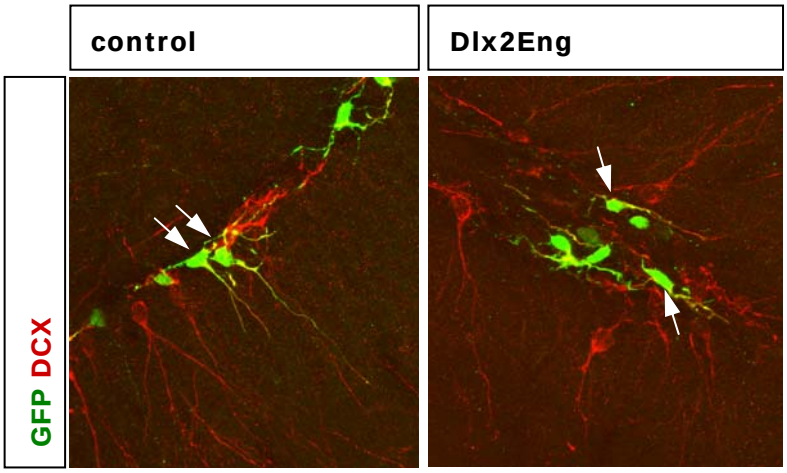
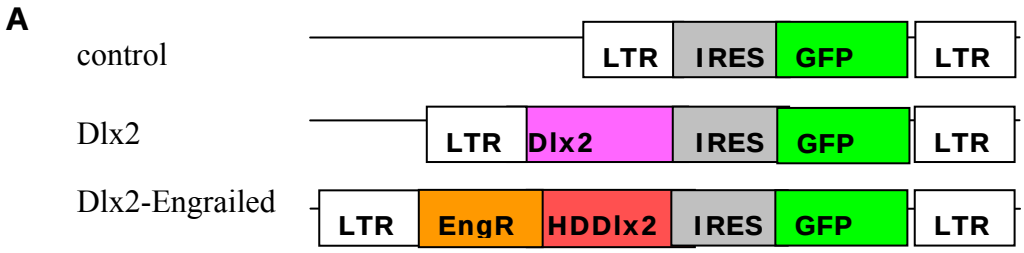


Fig. 28: Injection of control and Dlx2Eng retroviral vector into the dentate gyrus.
Immunohistochemistry for GFP (green) and neuroblasts (DCX, red) after injection of control and Dlx2Eng retroviral vectors into the dentate gyrus 7 days later. Neuroblasts were present following injections of control and Dlx2Eng retroviral vectors in an equal manner. Arrows indicate double-positive cells for GFP and DCX.

Given the specificity of the Dlx2-Engrailed construct we transduced direct plated subependymal cultures to turn Dlx2 mediated activation into repression. In general upon retroviral transduction of these cultures control virus transduced cells generate a much higher proportion of neurons (about 60 %) than cells derived from neurospheres (about 20 %) that have been expanded in the presence of EGF and FGF2 (Fig. **22**).



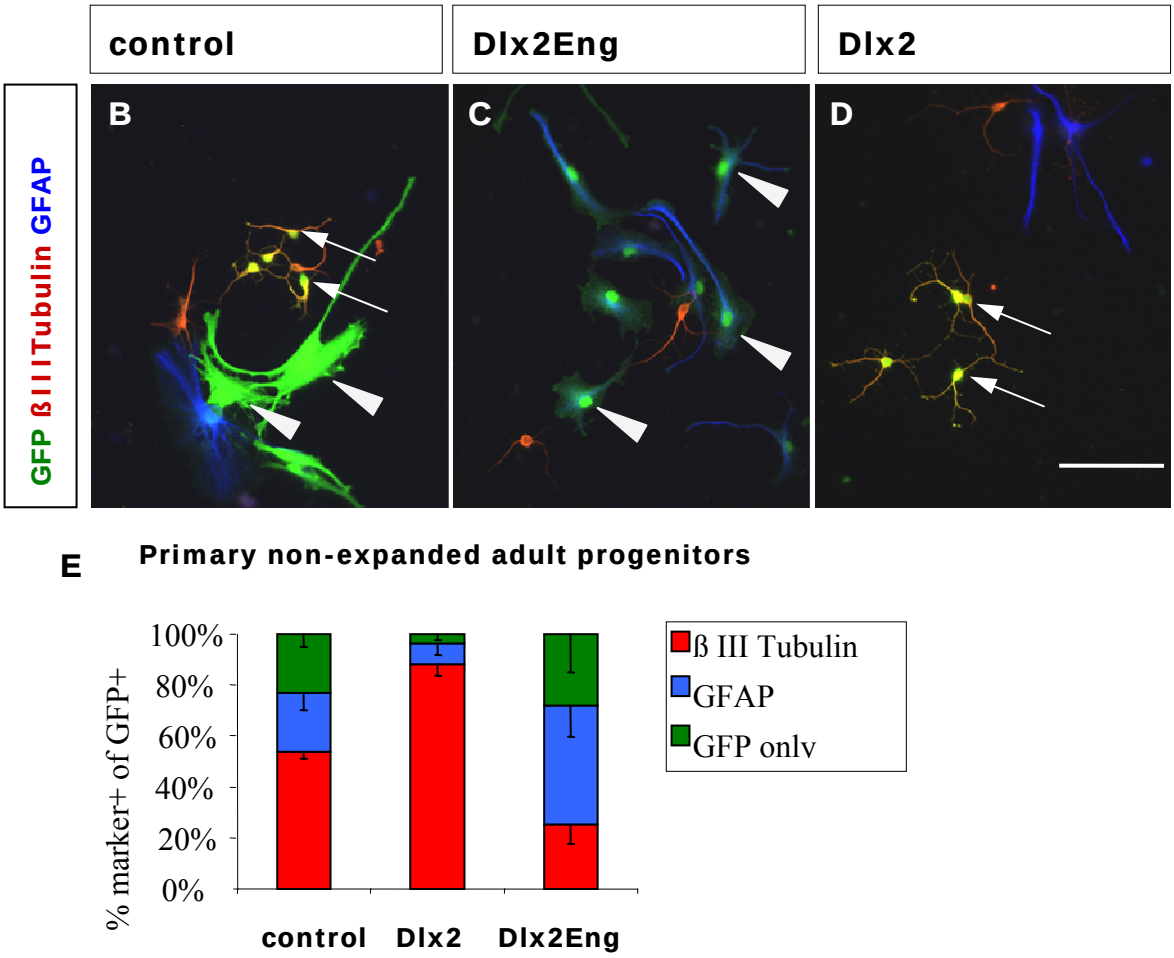


Fig. 29: Dlx2 acts potently neurogenic in adult subependymal zone cells in vitro.
(A) Schematic drawing of the retroviral constructs used for control and manipulation of Dlx2.
(D – F) Retroviral transduction of primary non-expanded adult progenitor cultures with (B) control, (C) Dlx2 and (D) Dlx2-Engrailed and double-stained for neurons (TuJ1, red) and astrocytes (GFAP, blue) as indicated. Representative examples for ß-III-Tubulin+ neuroblasts are indicated by arrows. Arrowheads indicate GFAP+ astrocytes. Scalebar: 100µm.
Histograms depicting the proportion of transduced cells (GFP+) acquiring an astroglial (GFAP+, blue), neuronal (ß-III-Tubulin+, red) or none of these fate following viral transduction in primary non-expanded adult progenitors (E, ß-III-Tubulin+ $P < 0.001$ (ANOVA), control number of cells analyzed in total = 1002; Dlx2 number of cells analyzed in total = 850; Dlx2-Eng number of cells analyzed in total = 525, n = 3 independent experiments each) 7 days after differentiation. Note the potent neurogenic effect of Dlx2 in these adult progenitors.

Following forced expression of Dlx2-Engrailed in primary subependymal zone cells, neurogenesis was drastically reduced, while GFAP-positive astrocytes were increased in number (Fig. **29**). Conversely, over-expression of Dlx2 resulted in a further increase in neurogenesis with nearly 90 % of GFP-positive cells acquiring a neuronal (ß-III-Tubulin-positive) fate (Fig. **29**). These data therefore suggest a powerful neurogenic role of Dlx2 in adult stem and progenitor cells.

4.1.4 Dlx2 acts potently neurogenic in adult subependymal zone cells in vivo

To examine whether Dlx2 transcriptional activity is also required for adult neurogenesis in vivo, we performed stereotactic injections of the above described retroviral vectors into the lateral subependymal zone (M. A. Hack et al., 2005; D. Colak et al., 2008) (for details see chapter: 6.2.2 Stereotactic injections). The majority of the control transduced cells analysed 3 days post injection (dpi) into the subependymal zone were DCX-positive neuroblasts (Fig. **30** A, E; for description of the quantification see Methods 6.7 Quantitative analysis and statistics) and increased even further following forced expression of Dlx2 (Fig. **30** B, E). Conversely, the number of DCX-positive cells was drastically reduced to about one third of the control numbers following transduction with the retrovirus encoding Dlx2-Engrailed (Fig. **30** C, E).

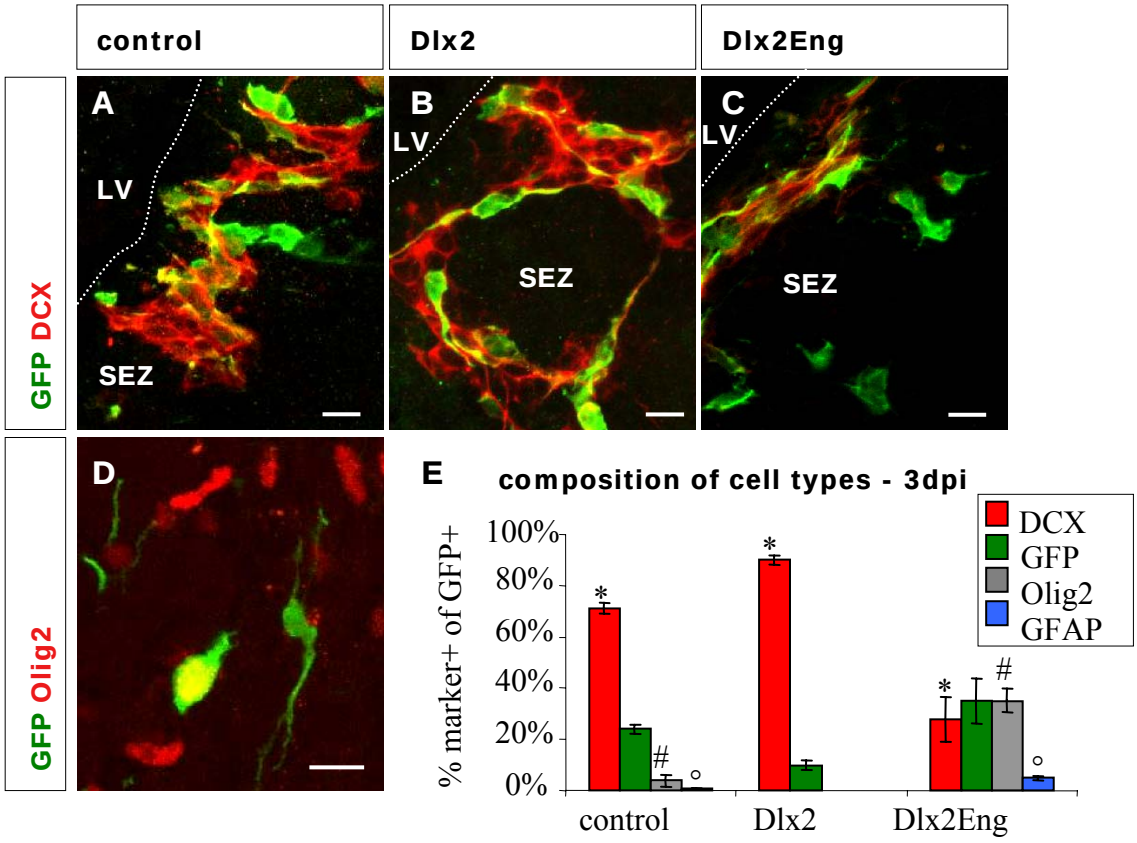


Fig. 30: Dlx2 acts potently neurogenic in adult subependymal zone cells in vivo.

(A, B, C; E) Representative examples of micrographs depicting transduced (GFP+) cells following stereotactic injections of (A) control, (B) Dlx2 and (C) Dlx2-Engrailed retroviral vectors into the adult subependymal zone and double-stained for the neuroblast-specific antigen DCX (Doublecortin) or the transcription factor Olig2 (D). The histogram in (E) depicts the proportion of transduced cells with different fates 3 days post injection (dpi): neuroblasts (DCX+, red), astroglia (GFAP+, blue) or oligodendroglial precursors (Olig2+, grey). (DCX+, $P < 0.001$ (ANOVA), Olig2+, $P = 0.0013$ (ANOVA), GFAP+, $P = 0.0020$ (ANOVA), total cells analysed: control = 243, Dlx2 = 229; n = 4 animals each, Dlx2-Eng number of cells analyzed in total = 349; n = 3 animals; significance is indicated by the following symbols: *, °, #). Scalebar: 10µm; LV = lateral ventricle, SEZ = subependymal zone

Dlx2 is required for fast proliferation of subependymal zone progenitors, but not sufficient to elicit a further increase

To further understand Dlx2 may affect the number of neuroblasts in the adult subependymal zone, we considered either a role in neuronal fate specification, given the fast alterations in progenitor fate observed already by three days post injection (3 dpi), or possible effects on neuroblast survival and proliferation, respectively. To examine cell death, sections were stained for activated caspase3, but virtually no caspase3+/GFP+ cells could be detected, in contrast to the effects seen for Dlx transcription factors in the retina (J. de Melo et al., 2007). To examine the proliferation of the transduced cells, we injected BrdU at 3 dpi, one hour prior to perfusion of the animals (see Material & Methods). Following Dlx2 transduction no difference was detectable in the labelling index, as given by the proportion of BrdU+ cells amongst all GFP-positive cells (control 30 ± 6 %, cells counted in total = 243; Dlx2 24 ± 11 %, cells counted in total = 229, n = 4 animals each group; comparison of control and Dlx2, $P > 0.05$ (Bonferroni's multiple comparison test). However, after Dlx2-Engrailed transduction the proportion of BrdU-positive cells had markedly decreased (8 ± 2 %, cells counted in total = 140, n = 3 animals, comparison of control, Dlx2 and Dlx2-Eng $P = 0.0203$ (ANOVA) and $P < 0.05$ (Bonferroni's multiple comparison test)), suggesting that Dlx2-mediated activation of target genes is required for the high proliferative rate of transit-amplifying progenitors and neuroblasts. As, however, the increase in neuroblasts caused by Dlx2 over-expression was not accompanied by alterations in proliferation Dlx2 seems to affect the neuronal fate decision directly, while at the same time also being required for regulating progenitor proliferation.

To further support this hypothesis we examined the expression of the transcription factor Mash1 which is present mostly in transit-amplifying progenitors of both the neuronal and oligodendroglial lineage (C. M. Parras et al., 2004). Mash1 is colocalized with Dlx transcription factors in DCX-negative transit-amplifying progenitors (Fig. **19** D), however a substantial portion of Mash1 coexpresses Olig2 predominantly in dorsal regions of the subependymal zone close to the white matter (Fig. **31**). Upon Dlx2 over-expression this transcription factor was down-regulated and following transduction with Dlx2-Engrailed Mash1 presence was unaffected compared to the control (Fig. **32**). Therefore Dlx2 over-expression promotes differentiation to the neuronal lineage, while Dlx2-Engrailed promotes proliferation of the same number of transit-amplifying progenitors. Next we examined the longterm fate of the Dlx2-Engrailed transduced

cells. As Mash1 is present in these cells but these cells do not proceed to the neuronal lineage, we examined the transcription factor Olig2 which is important for the generation of oligodendrocytes (Q. R. Lu et al., 2002; D. H. Rowitch, 2004).

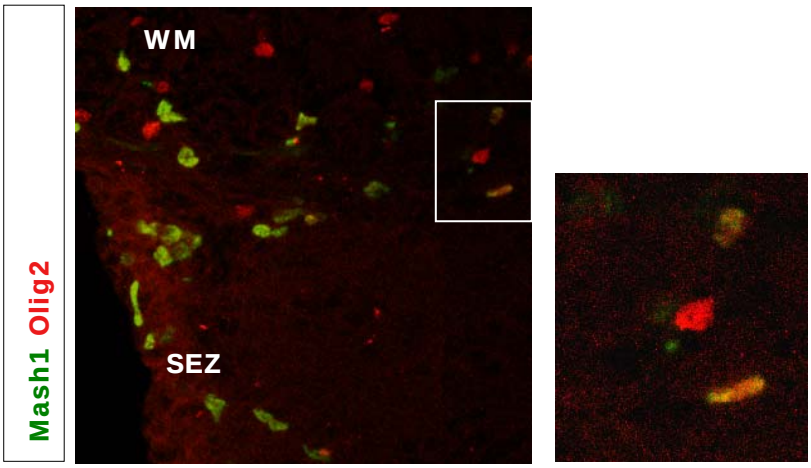


Fig. 31: Mash1 and Olig2 co-expression in the adult dorsal subependymal zone

Immunohistochemistry for Mash1 (green) and Olig2 (red) shows colocalization of both transcription factors in some cells. Inset is shown at higher magnification.

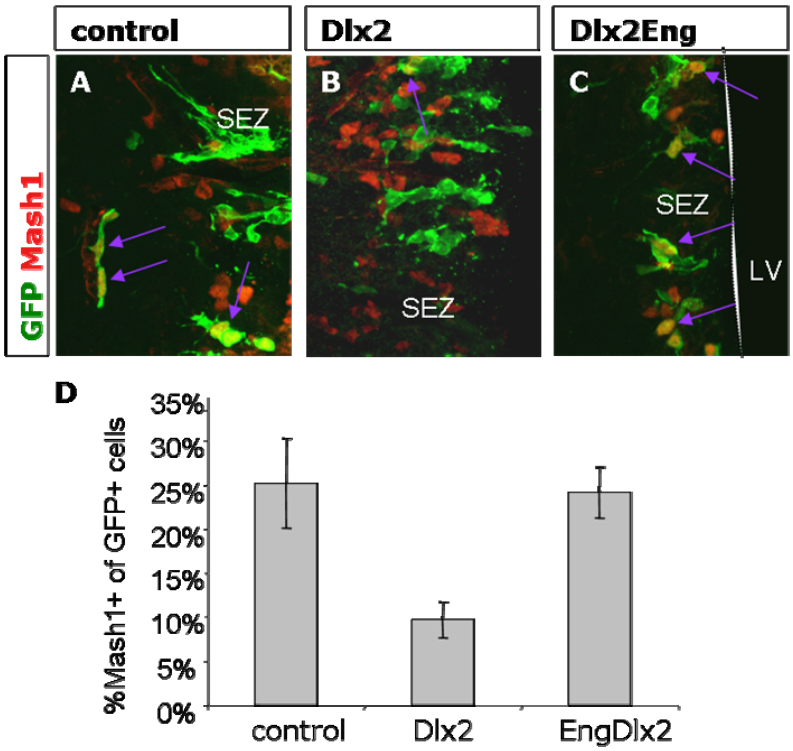


Fig. 32: Mash1 expression in transduced cells 3 days post injection into the adult subependymal zone

(A-C) Immunohistochemistry for GFP (green) and Mash1 (red) in representative examples of control, Dlx2 over-expression and Dlx2Eng mediated functional repression. Note that upon Dlx2-Engrailed over-expression, many cells are Mash1 positive. Violett arrows indicated double-positive cells for Mash1 and GFP.

(D) Histogram depicting the percentage of Mash1+/GFP+ cells amongst all GFP+ cells in the adult subependymal zone three days post injection; total cells analysed: control = 488, Dlx2 = 188, EngDlx2 = 191; n = 3 animals each.

4.1.5 Increase in astrocyte fate following blockade of Dlx2-mediated transcriptional activation in the adult subependymal zone

Already at 3 dpi we observed an 8 fold increase in the number of GFP+/Olig2-positive (Olig2+) cells (Fig. **30** E), as well as in the number of cells co-expressing NG2, an antigen commonly detected on oligodendrocyte progenitor cells (NG2+: control: 0.7 ± 0.2%, Dlx2-Eng: 12 ± 5%, P = 0.017 (t-Test), control n = 4 animals, Dlx2-Eng n = 3 animals), suggestive of an apparent increase in oligodendroglial fate decision. However, also the number of cells co-expressing GFP and GFAP was increased by 5 fold (Fig. **30** E).

Indeed, the astrocytic progeny derived from Dlx2-Engrailed transduced cells continued to increase in number when assessed at later stages (21 dpi). Three weeks after Dlx2-Engrailed injection the subependymal zone became virtually devoid of GFP-positive neuroblasts, while now the population labelled by GFAP had become predominant amongst all Dlx2-Engrailed transduced cells (Fig. **33** A, B). Compared to the 3-day analysis the proportion of Olig2+ and NG2-positive cells amongst the GFP-positive cells had remained relatively constant (Fig. **33** A, C; 5 E) (Olig2+: Dlx2-Eng 3 dpi 35 ± 5 %, Dlx2-Eng 21 dpi 31 ± 6 %, P = 0.8048 (t-Test); NG2+: Dlx2-Eng 3 dpi 12 ± 5 %, Dlx2-Eng 21 dpi 8 ± 2 %, P = 0.2526 (t-Test), n = 3 animals for 3 dpi and n = 4 animals for 21 dpi). However, a small but significant increase in the number of oligodendrocytes, identified by the antigen Adenomatous polyposis coli (APC) (D. M. McTigue et al., 2001; H. Ding et al., 2003; P. Malatesta et al., 2003), could be observed following interference with Dlx2-mediated transactivation (Fig. **33** A, D, E), suggestive of some increase in oligodendrogliogenesis that had been initiated already 3 days after transduction as visible by the increase in olgiodendroglial progenitors and then these cells further proceeded to mature into APC-positive oligodendrocytes. Intriguingly, however, the proportion of oligodendrocyte progenitors did no longer increase between 3 and 21 days after transduction with Dlx2-Eng, but rather GFAP-positive cells then increased in number.

Normally, oligodendrocyte progenitors leave the subependymal zone and migrate towards the white matter tract forming the corpus callosum in medial regions where they differentiate into mature oligodendrocytes (M. A. Hack et al., 2005; B. Menn et al., 2006; D. Colak et al., 2008). To examine whether these cells are increased in number after Dlx2-Engrailed transduction, we monitored the position of GFP-positive cells by quantifying the proportion of all GFP-positive cells (21 dpi) located in the

olfactory bulb, rostral migratory stream, subependymal zone or corpus callosum as described before (M. A. Hack et al., 2005; D. Colak et al., 2008). While most cells derived from subependymal zone cells transduced with the control virus had reached the olfactory bulb 21 dpi and their number further increased after Dlx2 transduction, only 42 ± 6 % of cells transduced with the Dlx2-Engrailed virus had reached the (Fig. **33** F). Thus, the majority of Dlx2-Engrailed transduced cells fail to migrate towards the OB and most of these remain in the subependymal zone (Fig. **33** F). Some of the Dlx2-Engrailed transduced cells had also migrated to the white matter, and even though their proportion was small it was significantly increased compared to that of the control virus transduced cells (Fig. **33** F).

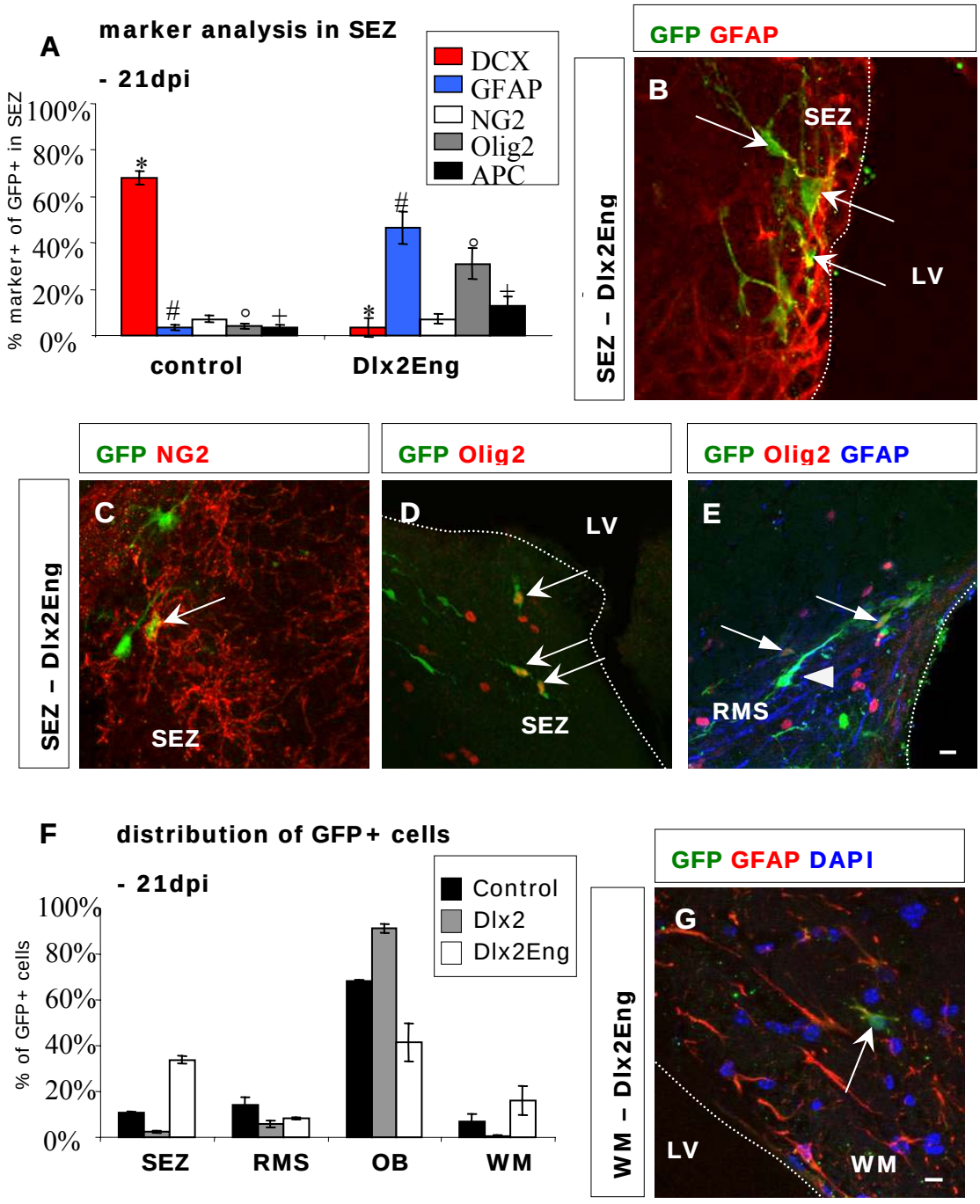
Fig. 33: Increase in astrocyte fate following blockade of Dlx2-mediated transcriptional activation in the adult subependymal zone

(A) Quantification of transduced cells within the subependymal zone after a survival time of 3 weeks (21 dpi) following injection with viruses encoding GFP or Dlx2-Engrailed. The proportion of GFP+/GFAP+ (blue) cells increased strongly after Dlx2-Engrailed injection, mostly at the expense of GFP/DCX+ neuroblasts. The number of Olig2+ cells (grey) remained similar and a slight increase in the number of NG2+ and APC+ (black) cells was observed. (DCX+, $P < 0.001$ (t-Test), NG2+, $P = 0.7825$ (t-Test), Olig2+, $P < 0.001$ (t-Test), APC+, $P = 0.0218$ (t-Test), number of cells analyzed in total: control = 68, Dlx2-Eng = 98, n = 4 animals each, significance is indicated by the following symbols: *, #, °, +).

(B-E; G) Cellular identities of the progeny after Dlx2-Engrailed transduction shown in immunohistochemistry for GFP and specific markers as indicated in the panels; of note, GFP+/Olig2+ cells indicated by arrows do not co-localise with GFP+/GFAP+ cells shown by arrowheads in (E); (B-D) arrows indicate GFP+/marker+ cells. Scalebar: 10µm

(F) Quantification of the distribution of cells three weeks after injection into the subependymal zone. The proportion of transduced GFP-positive cells located in the subependymal zone, rostral migratory stream, olfactory bulb, and white matter was quantified. The majority of control transduced cells had reached the olfactory bulb with their number further increased following Dlx2 transduction. Conversely, only 42 % of Dlx2-Eng transduced cells reached the olfactory bulb. Of note, the proportion of transduced cells remaining in the subependymal zone increased following Dlx2-Engrailed transduction. $P < 0.001$ (2-way ANOVA comparison of all regions and groups); control number of cells analyzed in total = 376, n = 4 animals; Dlx2 number of cells analyzed in total = 344, n = 3 animals Dlx2-Eng number of cells analyzed in total = 195, n = 3 animals).

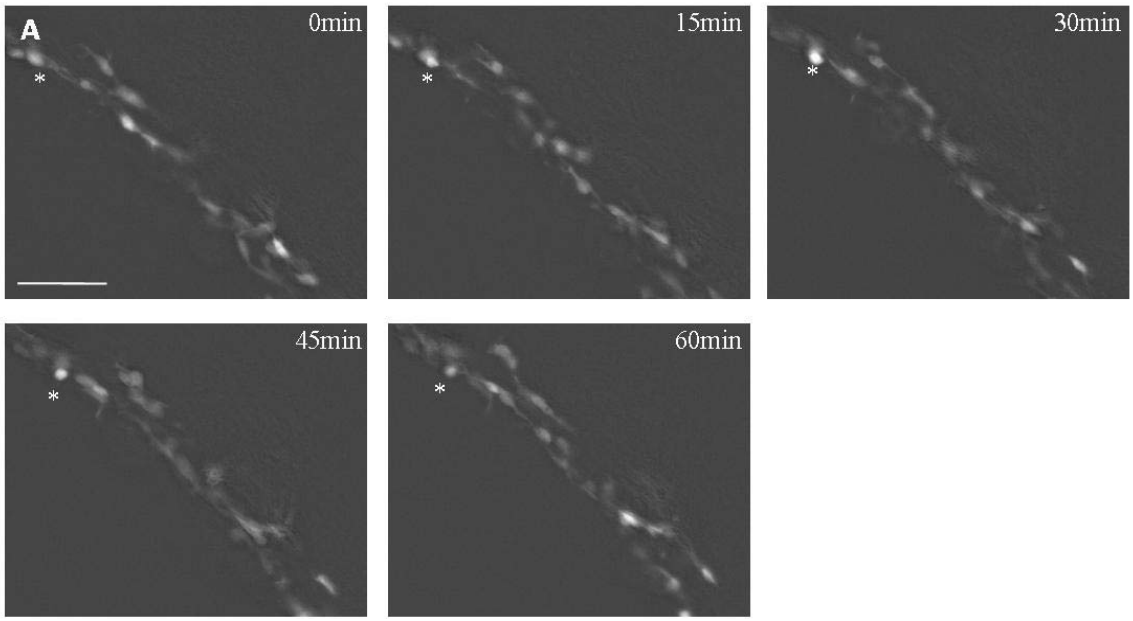
Scalebar: 10µm; LV = lateral ventricle, SEZ = subependymal zone, RMS = rostral migratory stream, OB = olfactory bulb, WM = white matter



Interestingly, however, this does not mean that all of these cells became oligodendrocytes, as even amongst the cells that had reached the white matter following Dlx2-Engrailed transduction GFAP-positive astrocytes were detected (Fig. **33** G). Thus, interference with Dlx2-mediated transcripitional activation results in virtual absence of neuroblast generation by 3 weeks, but favours mostly the generation of GFAP-positive cells – a notable difference to the effect of Dlx2 deletion during embryonic development (M. A. Petryniak et al., 2007).

4.1.6 Dlx2 promotes, but is not required for migration of adult subependymal zone and rostral migratory stream progenitors

As Dlx2-Engrailed transduced cells remained largely in the subependymal zone, and Dlx transcription factors regulate migration of embryonic neuroblasts (I. Cobos et al., 2007; T. N. Le et al., 2007), the migration of transduced cells were monitored by live time-lapse microscopy (see Material & Methods:6.4 Time-lapse videoimaging; collaboration with Armen Saghatelyan). Only cells exhibiting migratory behaviour were included in the analysis, thereby excluding both dead and non-migrating cells (S. C. Nam et al., 2007). Control, Dlx2 and Dlx2-Engrailed containing retroviral vectors were injected into the subependymal zone and rotral migratory stream and acute slices of the adult mouse forebrain were prepared five days later. Time-lapse videoimaging of transduced cells in the subependymal zone and rostral migratory stream revealed that Dlx2 transduction increased the velocity of migration by about 30 % compared to controls (Fig. **34** A, B, for statistics and cell numbers see Figure legend; collaboration with Armen Saghatelyan & Marina Snapyan). As a consequence of the increased velocity the mean distance that cells propagated increased after Dlx2 over-expression (Fig. **34** C).



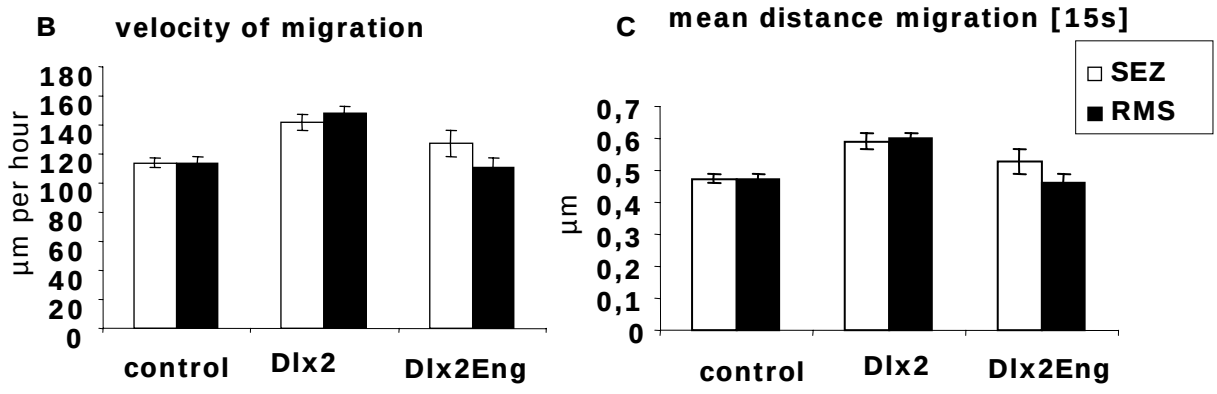


Fig. 34: Migration analysis with time lapse videoimaging

Control, Dlx2 and Dlx2-Engrailed containing retroviral vectors were injected into the subependymal zone and rostral migratory stream and slices were prepared five days later.

(A) Example for migrating cells following transduction with Dlx2. Arrows indicate migrating cells, asterisk indicates non-migrating cell. Scale bar: 50um

(B) Micrograph depicting velocity of migrating cells in µm per hour. In both sets of experiments Dlx2 transduction increased the velocity of migration by about 30 % compared to controls. (SEZ control 114 ± 3 µm/h, n= 87 cells, Dlx2 142 ± 6 µm/h, n= 22 cells, Dlx2Eng 127 ± 9 µm/h, n = 12 cells; 2 - 4 slices each condition; $P < 0.001$ (ANOVA); RMS control 114 ± 4 µm/h, n = 29 cells, Dlx2 148 ± 5 µm/h, n = 43 cells, Dlx2Eng 111 ± 6 µm/h, n = 22 cells, 2 - 3 slices, $P = 0.0025$ (ANOVA)).

(C) Micrograph showing the mean distance migration in 15s. As a consequence of the increased velocity the mean distance that cells propagated increased after Dlx2 over-expression. (SEZ control 0.47 ± 0.013 µm/15 sec, n = 86 cells, Dlx2 0.59 ± 0.024 µm/15 sec, n = 22 cells, Dlx2Eng 0.53 ± 0.039 µm/15 sec; n = 11 cells, 2 - 4 slices each condition; $P = 0.0401$ (ANOVA); RMS control 0.47 ± 0.017 µm/15 sec, n = 29 cells, Dlx2 0.60 ± 0.016 µm/15 sec, n = 43 cells, Dlx2Eng 0.46 ± 0.025 µm/15 sec, n = 22 cells, 2 - 3 slices each condition, $P < 0.001$ (ANOVA))

Thus, Dlx2 over-expression promotes migration of cells in both, rostral migratory stream and subependymal zone. In contrast, GFP+ cells transduced with Dlx2-Engrailed were not significantly altered in their speed of migration (Fig. **34** B). These data therefore suggest that Dlx2 promotes migration of neuroblasts and that the high number of cells remaining in the subependymal zone following Dlx2-Engrailed transduction is not due to migration deficits, but rather alterations in fate and proliferation.

4.1.7 Dlx2 promotes a dopaminergic periglomerular neuron fate in the adult olfactory bulb

As mentioned above, Dlx2 expression is maintained at particularly high levels in periglomerular neurons prompting us to examine its later role in neuronal subtype specification. As we had previously observed that viral injections into the subependymal zone do not result in substantial numbers of neuronal progeny populating the glomerular layer, we injected the viral vectors into the rostral migratory stream (Fig. **35** A) (M. A. Hack et al., 2005). After Dlx2 over-expression, we observed

an approximately 3 fold increase in the proportion of neurons populating the glomerular layer compared to control injections (Fig. **35** B).

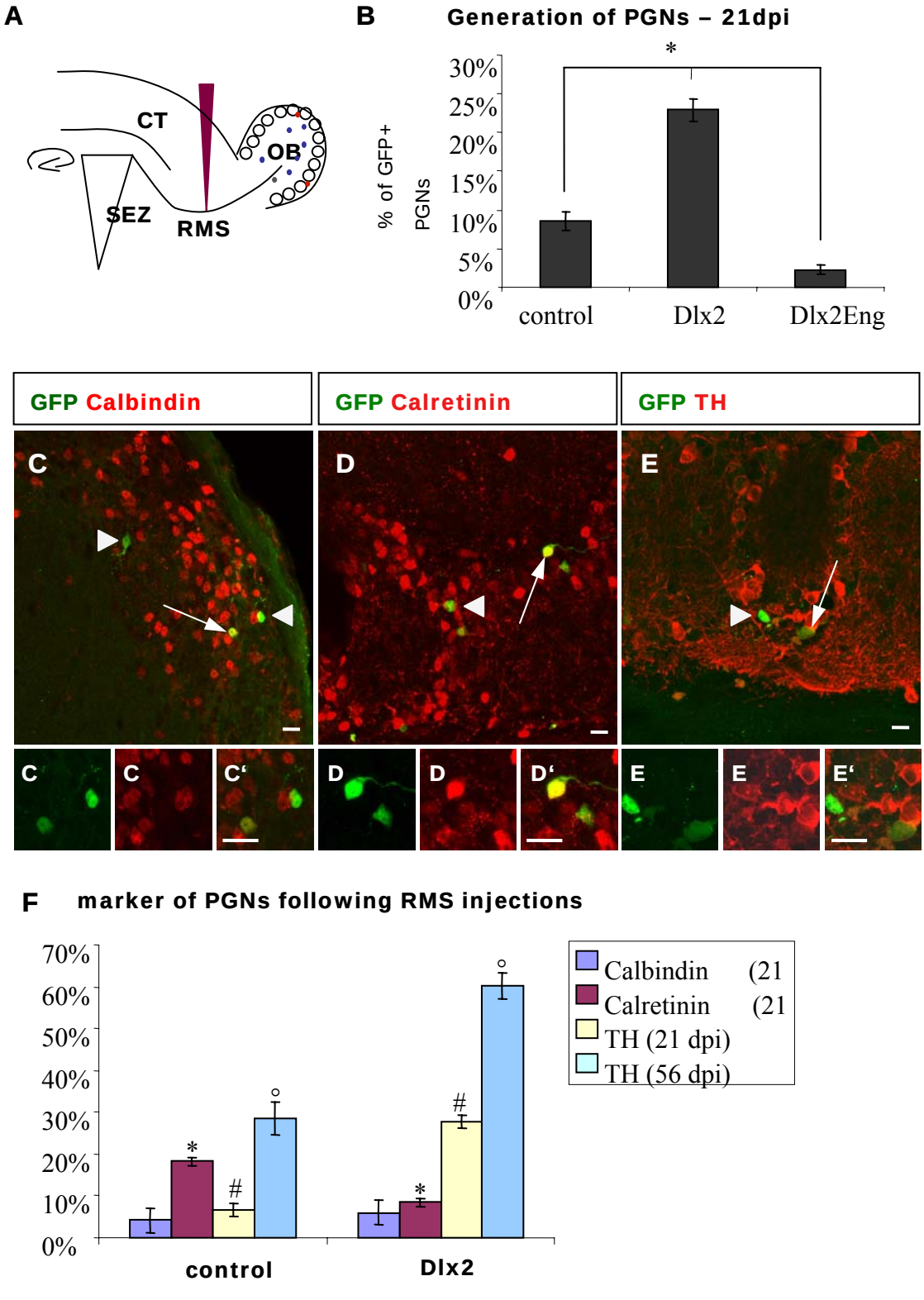


Fig. 35: Dlx2 promotes a dopaminergic periglomerular neuronal fate in the adult olfactory bulb

(A) Schematic drawing of a sagittal mouse brain section with a red arrow indicating the injection site.
(B) Histogram depicting the proportion of newly generated periglomerular neurons amongst the GFP+ cells transduced with control, Dlx2 or Dlx2-Engrailed viral vectors injected into the rostral migratory stream. Significantly more periglomerular neurons are generated following Dlx2 transduction whereas their number decreased following Dlx2-Engrailed transduction. (control n = 5 animals, Dlx2 n = 4 animals, Dlx2-Eng n = 2 animals, number of cells analyzed in total: control = 3158, Dlx2 = 2185, Dlx2-Eng = 148; $P < 0.001$ (ANOVA) indicated by * in the panel).
(C, D, E) depict fluorescent micrographs showing representative examples of transduced periglomerular neurons: GFP (green) and (C) calbindin (red), (D) calretinin (red) and (E) tyrosine hydroxylase (TH) (red). Note that all three types of PGNs are generated following retroviral transduction. Arrows highlight positive cells; arrowheads indicate marker-negative cells. Higher magnifications of the accordant markers are shown in C'-C''', D'-D''' and E'-E'''.
(F) Histogram showing composition of GFP+ periglomerular neurons. Calbindin+/GFP+ periglomerular neurons remained constant in the control and Dlx2 transduction. However, the proportion of tyrosine hydroxylase + periglomerular neurons following Dlx2 transduction increased stongly, mostly at expense of the calretinin+/GFP+ periglomerular neurons. (21d: calbindin+, $P = 0.3669$ (t-Test); calretinin+, $P = 0.0235$ (t-Test); TH+, $P< 0.001$, n = 3 animals each group, number of cells analyzed in total: control = 336, Dlx2 = 412; 56d: TH+, $P < 0.001$ (t-Test), number of cells analyzed in total: control = 174, Dlx2 = 76, n = 4 animals each group; significance is indicated by the following symbols: *, #, °)
Scale bar: 10µm, CTX = Cortex, SEZ = Subependymal Zone, RMS = Rostral migratory stream, OB = olfactory bulb, PGN = periglomerular neuron, TH = Tyrosine Hydroxlase

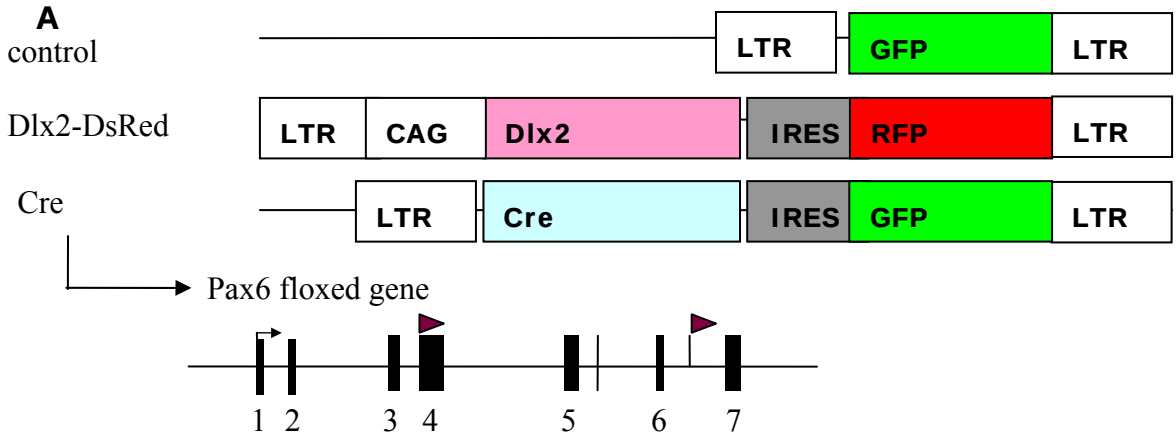
Next, we examined whether the increased number in periglomerular neurons was biased towards a specific subtype. After injection of the control virus into the rostral migratory stream, 4 ± 1 % of all GFP-positive periglomerular neurons were calbindin+, 18 ± 0.5 % calretinin+ and 7 ± 0.75 % tyrosine hydroxylase+.

After Dlx2 over-expression we detected a profound increase (4-fold) in the proportion of tyrosine hydroxylase+ periglomerular neurons, mostly at the expense of the calretinin-positive periglomerular neurons (Fig. **35** C – F). Notably, no tyrosine hydroxylase expressing periglomerular neurons could be observed amongst the few cells detectable after transduction with Dlx2-Engrailed. Given the fact that tyrosine hydroxylase expression is known to increase with maturation (P. C. Brunjes, 1994; B. Winner et al., 2002; M. A. Hack et al., 2005), we examined whether Dlx2 over-expression had only accelerated the expression of tyrosine hydroxylase, or had indeed permanently increased the proportion of this neuronal subtype. Consistent with the gradual maturation of tyrosine hydroxylase expression, eight weeks after control injections into the rostral migratory stream, the proportion of tyrosine hydroxylase+ neurons had further increased to 29 ± 3 % (from 7 % after 3 weeks). However, following Dlx2 over-expression a still larger number of periglomerular neurons expressed tyrosine hydroxylase indicating that Dlx2 has not merely accelerated maturation, but had permanently altered neuronal subtype acquisition (Fig. **35** F). Thus, Dlx2 over-expression promotes the acquisition of a periglomerular neuronal fate with a strong bias towards the dopaminergic subtype.

4.1.8 Dlx2 requires Pax6 to promote dopaminergic periglomerular neurons fate

As the above results obtained with Dlx2 manipulation were highly reminiscent of our previous results with Pax6 (M. A. Hack et al., 2005), we were prompted to ask whether Dlx2 indeed requires Pax6 in olfactory bulb neuronal subtype specification or whether it acts redundantly with Pax6, in particular because both transcription factors are co-expressed in periglomerular neurons (Fig. **20**). To clarify this issue, we examined the effect of Dlx2 over-expression in the absence of Pax6 protein. To delete Pax6 we injected a virus encoding Cre recombinase (Cre-IRES-GFP) into mice in which the Pax6 gene had been flanked with loxP sites (before exon 4 and at an intron between exon 6 and 7 (R. Ashery-Padan et al., 2000; M. A. Hack et al., 2005). As previously shown, injection of Cre encoding virus allows the efficient deletion of Pax6 in adult progenitors (Fig. **36** B - E) (R. Ashery-Padan et al., 2000; M. A. Hack et al., 2005).

To examine the effect of Dlx2 over-expression on the fate of periglomerular neurons following Cre-mediated deletion of Pax6 we performed co-injections of the Dlx2 encoding virus (Dlx2-IRES-DsRed) together with either a control virus or the Cre-encoding virus into the rostral migratory stream and examined their respective progenies with immunostainings for GFP and RFP 3 weeks later. Labelled cells were (i) only green for control or Cre expression, (ii) only red upon Dlx2 over-expression or (iii) yellow when neuroblasts were transduced by both viruses (Fig. **37** A, B). Transduction with the Dlx2 encoding virus reproducibly resulted, as expected, in a profound increase in the proportion of periglomerular neurons (Fig. **37** A – E; for overview see panels C, D). Strikingly, however, the effect of Dlx2 over-expression was totally abrogated in the absence of Pax6, i.e. in cells double-infected with the Cre and Dlx2 encoding viruses (Fig. **37** E). Thus, Dlx2 requires Pax6 to instruct periglomerular neuron fate.



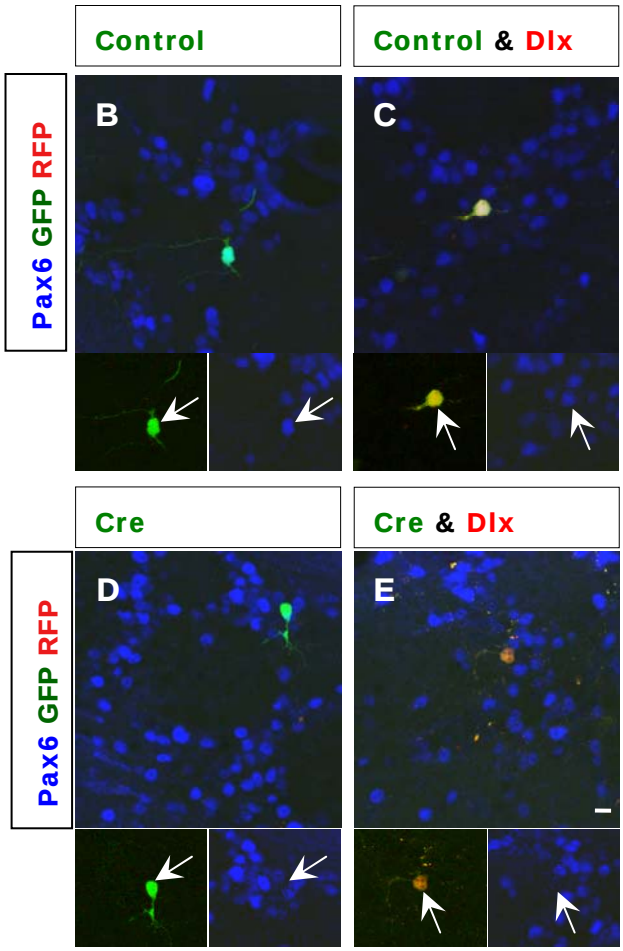
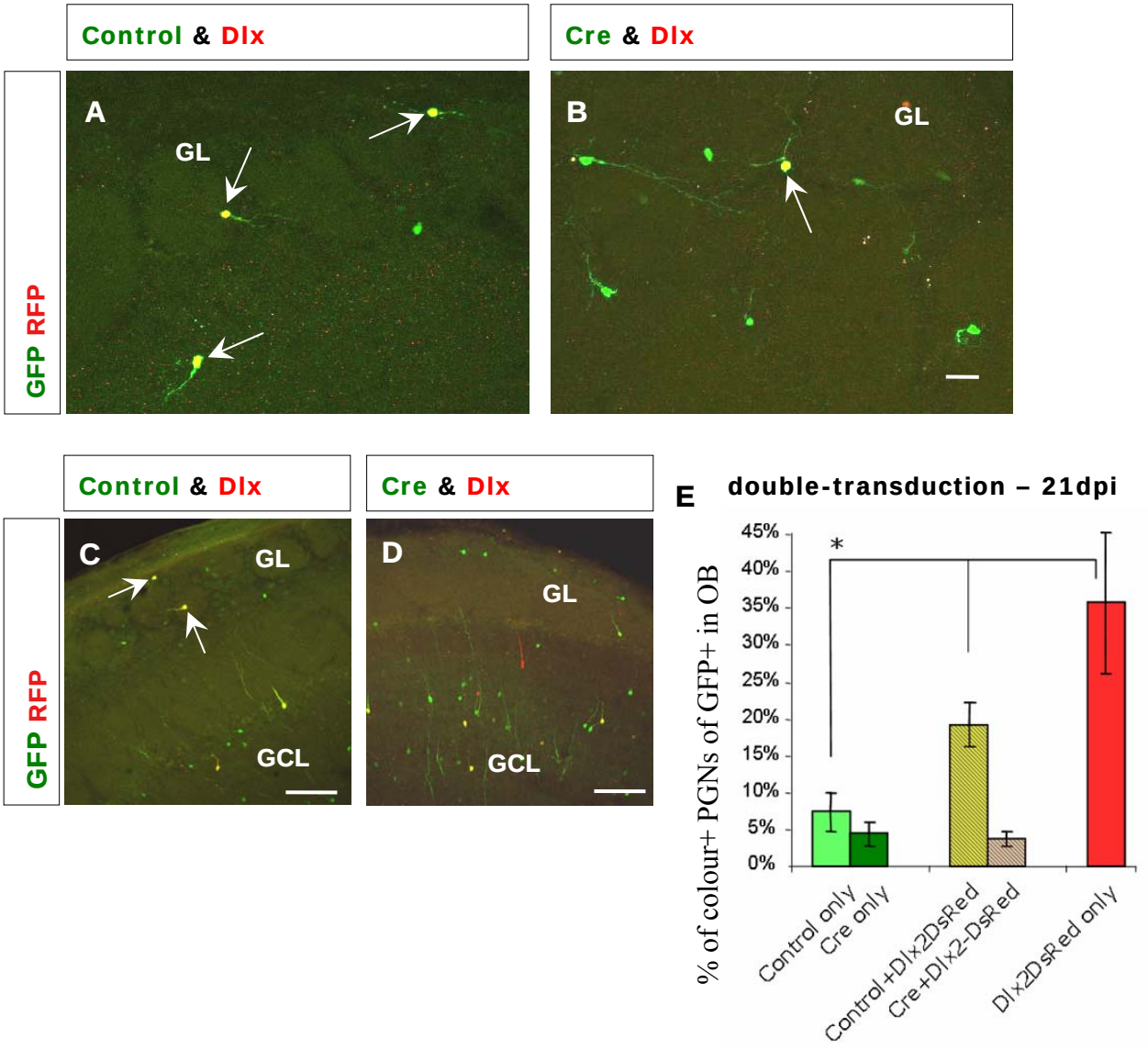


Fig. 36: Cre-mediated deletion of Pax6 results in loss of Pax6 protein.

(A) Schematic drawing of the retroviral constructs used for control, over-expression and Cre-mediated deletion of Pax6 in mice in which exon 4 - 6 of the Pax6 gene had been flanked by loxP sites (construct indicated on the bottom with violet triangles indicating loxP-sites and black rectangles for exons). Note that the construct for Dlx2 over-expression is followed by an IRES-DsRed cassette.
(A-D) Immunohistochemistry for GFP (green), DsRed (red) and Pax6 (blue) 21 days after injection of viral vectors encoding for either GFP (control) and Dlx (red) or Cre and Dlx (red) into the RMS. All examples are taken from the glomerular layer.

Fig. 37: Dlx2 requires Pax6 to promote a dopaminergic periglomerular neuron fate.

(B-E) Injections of the above constructs (A) into the rostral migratory stream (RMS) resulted in green (Control or Cre) and yellow (co-transduced with Dlx2-DsRed) cells in the olfactory bulb. Note the decreased generation of yellow periglomerular neurons (depicted by arrows) following loss of Pax6 protein in the glomerular layer. Arrowheads depict only green periglomerular neurons.
(F) Quantification of newly generated periglomerular neurons 21 days post injection (dpi) after injection into the RMS of either control and red Dlx2 virus or Cre and red Dlx2 virus into homozygous Pax6 floxed mice. ($P < 0.001$ (ANOVA), comparison of control + Dlx2DsRed with Dlx2DsRed only $P > 0.05$ (Bonferroni's multiple comparison test), Control and Dlx2-Red number of cells analyzed in total = 4678; Cre and Dlx2-Red number of cells analyzed in total = 3024; n = 4 animals each). Notably, the generation of periglomerular neurons could not be rescued by Dlx2 over-expression following Cre-mediated deletion of Pax6.



Similar results were obtained when periglomerular neuronal subtypes were examined. Following transduction with the Cre encoding virus the proportion of tyrosine hydroxylase+ neurons amongst all periglomerular neurons decreased (control 7 ± 1 %, Cre 1 ± 0.8 %, control cells counted in total = 126, Cre cells counted in total = 111, n = 3 animals each, P = 0.035 (t-Test)), whereas the relative contribution of calretinin+ cells to the total population of periglomerular neurons increased (from 16 ± 4 % after control virus transduction to 76 ± 4 % after Cre virus transduction, P < 0.001 (t-Test), control cells counted in total = 189, Cre cells counted in total = 206, n = 4 animals each, Fig. **38** A - C). This data suggest that in the absence of Pax6 periglomerular neurons fail to adopt a dopaminergic fate switching predominantly to a calretinin-positive phenotype instead. Of note, the proportion of calbindin+ cells was hardly

affected (control 3 ± 2 %, Cre 4 ± 2 %, Cre+Dlx2 2 ± 1.5 %, P = 0.3679 (ANOVA), control cells counted in total = 92, Cre cells counted in total = 138, Cre+Dlx2 cells counted in total 63, n = 4 animals each, Fig. **38** A). Notably, the increase in calretinin+ neurons amongst the periglomerular neurons upon Cre-containing virus transcuction and Pax6 deletion could no longer be reverted by Dlx2 over-expression when it had co-infected Cre-transduced cells (76 ± 5 % Calretinin+ cells amongst the double-infected cells, cells counted in total = 105, n = 4 animals), and no tyrosine hydroxylase expressing neurons were detected either (Cre+Dlx2 cells counted in total = 68, n = 4 animals). Thus, Dlx2 requires Pax6 to promote the dopaminergic periglomerular neuron subtype identity and to prevent the acquisition of a calretinin-positive subtype (Fig. **38**).

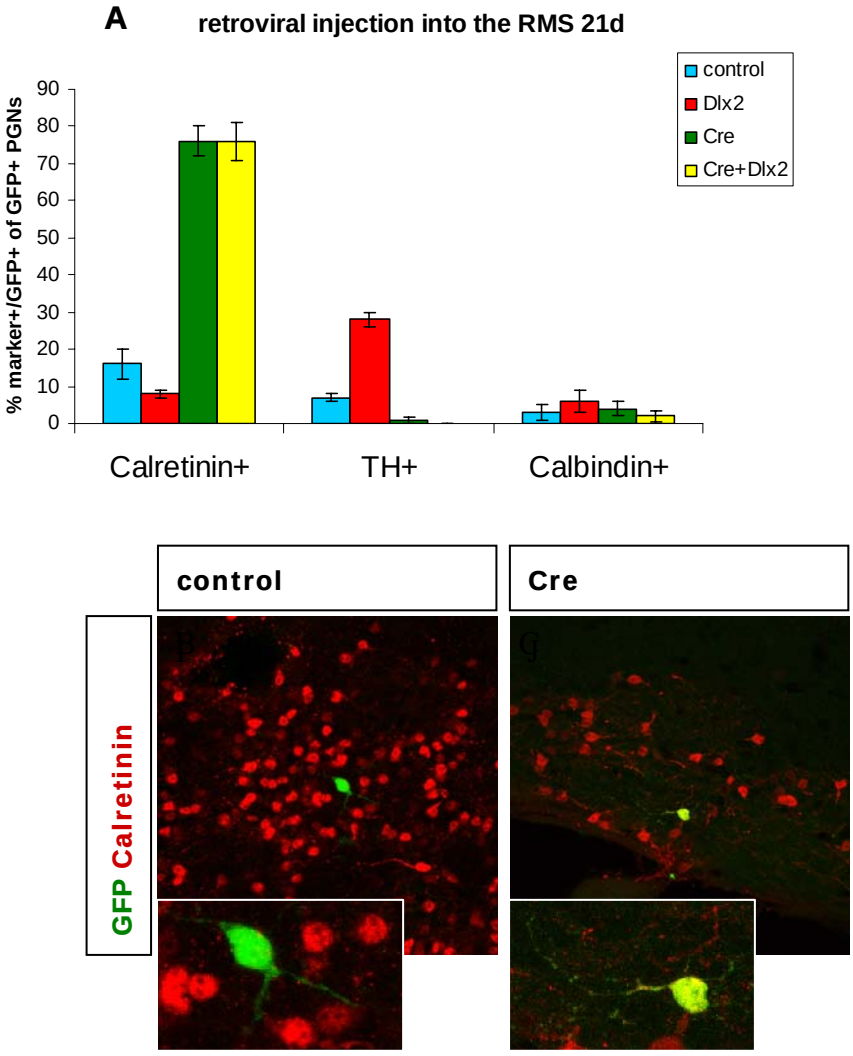
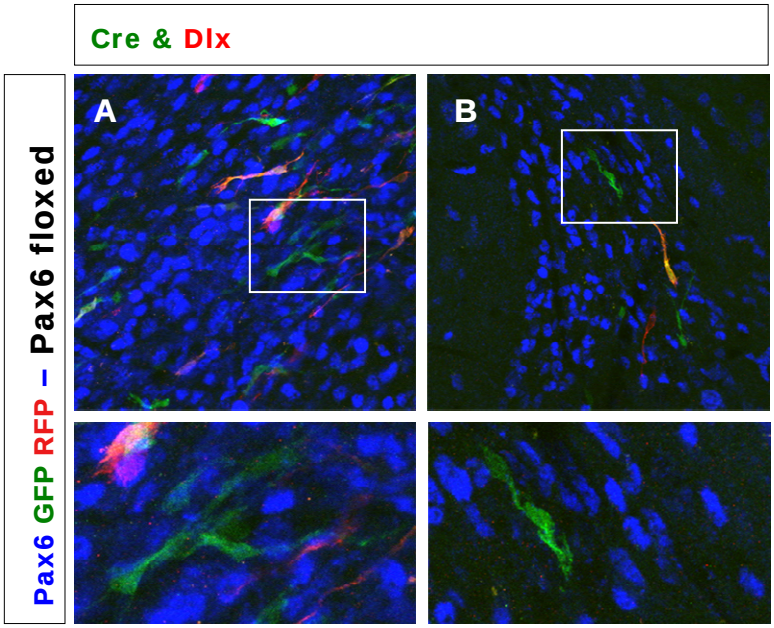


Fig. 38: Absence of Pax6 leads to the generation of calretinin-positive+ periglomerular neurons.
(A) Quantification of the different periglomerular neuron subtypes generated upon control, Dlx2, Cre and Cre+Dlx2 injection. TH-positive neurons amongst all periglomerular neurons decreased (control 7 ± 1 %, Cre 1 ± 0.8 %, control cells counted in total = 126, Cre cells counted in total = 111, n = 3 animals each, P = 0.035 (t-Test)). Calretinin-positive cells to the total population of periglomerular neurons increased from 16 ± 4 % after control virus transduction to 76 ± 4 % after Cre virus transduction, P < 0.001 (t-Test), control cells counted in total = 189, Cre cells counted in total = 206, n = 4 animals each.

(B – C) Generation of calretinin-positive periglomerular neurons was much more frequent upon Cre injection into Pax6 floxed mice. Insets show higher magnification of transdcuced cells.
SEZ = Subependymal zone, OB = Olfactory bulb, IP = immuno precipitates, GL = glomerular layer, GCL = granule cell layer, CTX = Cortex, PGN = periglomerular neuron.

4.1.9 Dlx2 acts neurogenic in the absence of Pax6 in the adult subependymal zone

Given that the absence of Pax6 leads to a shift of periglomerular neuron fate towards the generation of the Calretinin-positive subtype and that Dlx2 over-expression at the same time could not rescue this effect, we examined the neurogenic effects Dlx2 over-expression in the absence of Pax6 in the subependymal zone. We injected the Cre recombinase encoding virus (Cre-IRES-GFP) and the Dlx2-IRES-DsRed at the same time into the subependymal zone of Pax6 floxed mice (Fig. **39**). Absence of Pax6 protein was confirmed in Cre-GFP transduced cells (Fig. **39** A, B). Preliminary results indicate that predominantly Cre-GFP expressing cells reside in the subependymal zone 18 days post injection. In contrast, the majority of Dlx2 over-expressing cells and most double-transduced cells were found in the rostral migratory stream (Fig. **39** C). This data might suggest that Dlx2 over-expression promotes subependymal neurogenesis in the absence of Pax6.



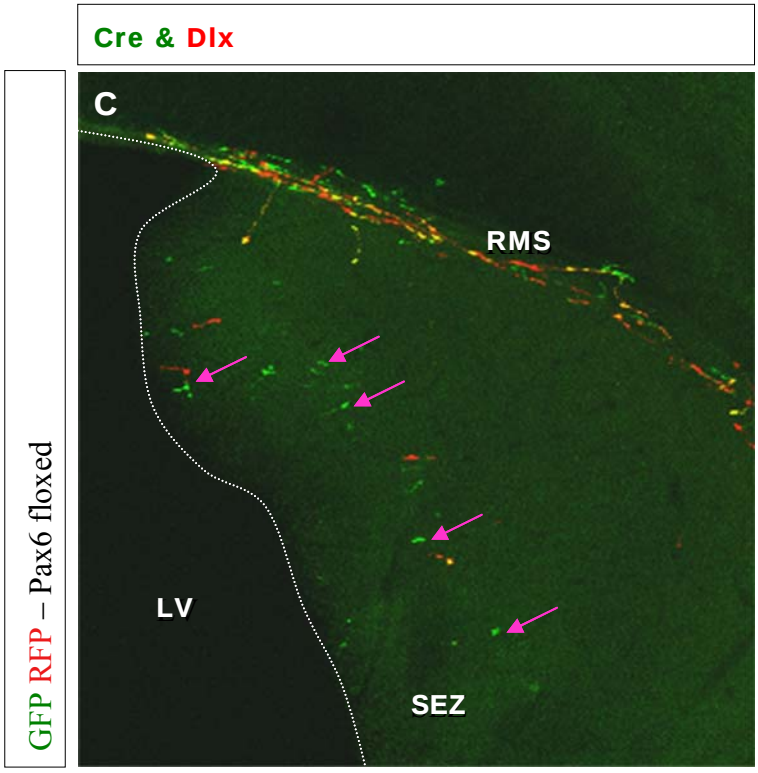


Fig. 39: Dlx2 retroviral mediated neurogenesis in the subependymal zone in the absence of Pax6.
Injection of Dlx2-DsRed (red) and Cre-GFP (green) retrovirus into the subependymal zone of Pax6 floxed mice. (A, B) Immunostaining for Pax6 protein indicated that almost all of the Cre-GFP expressing cells were negative for Pax6.
(C) Cre-GFP positive cells resided predominantly in the subependymal zone 18 days post injection (violoett arrows highlight Cre-GFP positive cells). Most of the double transduced cells were observed in the rostral migratory stream.

4.1.10 Molecular interaction between Pax6 and Dlx2

At the molecular level this result could be explained by two scenarios: (i) both transcription factors may cooperate by mutually regulating their expression, albeit otherwise controlling a specific set of different target genes; (ii) they may interact directly, for instance by partaking in the same transcriptional complex.

In order to investigate whether Pax6 is a target of Dlx2, we over-expressed Dlx2 in neurosphere cells. Of note, similar results were obtained when analyzing the Dlx2-GFP transduced cells in vivo – neither in the subependymal zone nor in the olfactory bulb were all Dlx2-transduced cells Pax6+ as expected if Dlx2 would up-regulate Pax6. Thus, it appears that at least in this cellular context these transcription factors do not cross-regulate each other's expression – a finding also consistent with the heterogeneity of Dlx only, Pax6 only and Dlx/Pax6-double-positive cells in vivo (Fig. **19**).

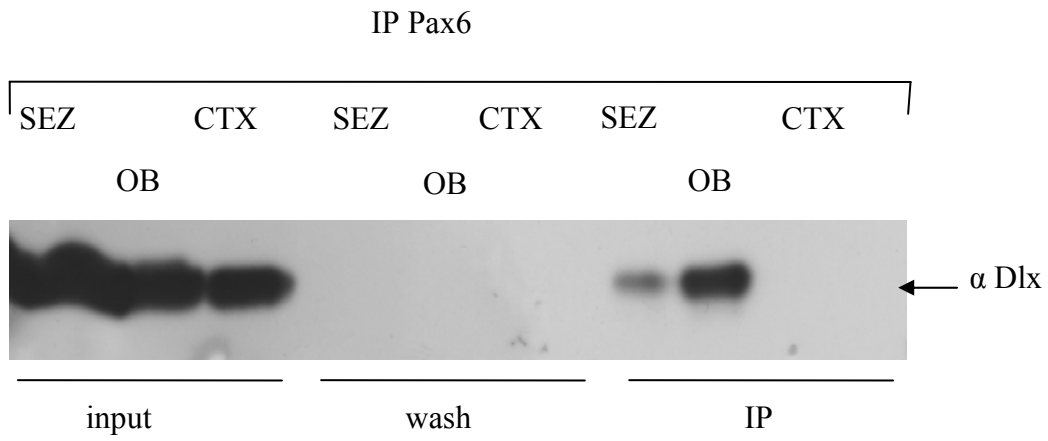


Fig. 40: Co-Immunoprecipitation of Dlx by Pax6.

Western Blot for pan-Dlx on Pax6 precipitated total lysates of subependymal zone (SEZ), olfactory bulb (OB) and Cortex (CTX). No signal for Dlx proteins was detected in the wash fraction or immunoprecipitates from the cerebral cortex, whereas Dlx transcription factors were pulled down by Pax6 antibody in lysates prepared from both the subependymal zone and, even more strongly, the olfactory bulb.

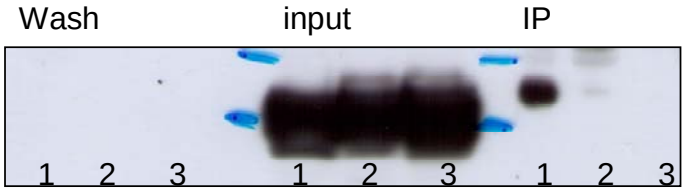


Fig. 41: Controls for Co-Immunoprecipitation of Dlx by Pax6.

Control Western Blot for pan-Dlx on Pax6 precipitated total lysates of olfactory bulb. Input of total olfactory bulb lysate is in the middle, whereas washing after precipitation is on the left, immunoprecipitates (IP) are on the right side.
Number 1 is indicating normal immunoprecipitation with Pax6 antibody and protein-G coupled agarose beads.
Number 2 did not contain immunoprecipitates of Dlx protein. No agarose beads were used, but Pax6 antibody was added.
Number 3 indicates control immunoprecipitation without antibody, but agarose beads were added.

Thus, Pax6 and Dlx transcription factors appear to be regulated independently, but may act concertedly when contained in the same cells. To test this hypothesis and in particular the possibility that these transcription factors partake in the same transcriptional complex, we performed an immunoprecipitation analysis. Total lysates were prepared from adult mouse subependymal zone, olfactory bulb, and cerebral cortex and were immunoprecipitated with a mouse monoclonal anti-Pax6 antibody (Developmental Studies Hybrdimoa Bank, purified and concentrated by Dr. Dorothea Schulte) and Dlx protein was then revealed by western blot analysis of these precipitates (Fig. **40**). While no signal for Dlx proteins was detected in the wash

fraction or immunoprecipitates from the cerebral cortex (where Pax6 and Dlx2 do not co-localize in the same cells), Dlx transcription factors were found in western blots of lysates immunoprecipitated with the Pax6 antibody prepared from both the subependymal zone and, even more strongly, the olfactory bulb. This data therefore suggests that Dlx proteins and Pax6 physically interact and require each other to exert some of their key functions in adult neurogenesis.

4.2 The generation of glutamatergic neurons in adult olfactory neurogenesis

4.2.1 Tbr1 and Tbr2 expression in a dorsal subregion of the rostral subependymal zone

During development, progenitors of GABAergic neurons in the ventral telencephalon express several members of the Dlx transcription factor family (Dlx1,2,5,6) and the proneural transcription factor Mash1, while progenitors of glutamatergic neurons in the dorsal telencephalon express Pax6 and different members of the proneural transcription factor family, Neurogenin1 and Neurogenin2 (Ngn2) (N. Bertrand et al., 2002). I have described in the first part of my studies the persistence of such spatial distribution in the adult subependymal zone. Dlx expressing progenitors are mostly located in the lateral ventricular wall derived from the ventral telencephalon and Pax6-positive progenitors prominently expressed in dorsal regions of the subependymal zone (dSEZ) (Fig. **19**) (M. A. Hack et al., 2005).

According to embryonic development, we further investigated the persistence of a dorso-ventral organization of the adult subependymal zone. Consequently we hypothesized that the ventral-lateral wall would give rise to GABAergic olfactory interneurons as described previously (M. Kohwi et al., 2007a; K. M. Young et al., 2007; R. Batista-Brito et al., 2008; M. S. Brill et al., 2008), but that the dorsal wall might maintain progenitor/stem cells for the generation of glutamatergic neurons. As the transcription factor sequence generating glutamatergic neurons is conserved in embryonic neocortical neurogenesis, adult dentate gyrus and developing cerebellum (R. F. Hevner et al., 2006) and includes Pax6, Neurogenin,2, Tbr2 and Tbr1, we started by examining the existence and expression of T-box transcription factors Tbr1 and Tbr2.

Immunohistochemistry for Tbr2 and Tbr1 in the adult mouse forebrain showed that indeed, Tbr1-positive and Tbr2-positive cells were detected in the dorsal wall of the subependymal zone and rostral migratory stream (RMS) (Fig. **42** A - C). The expression was most abundant in medial sections with a gradient to lateral sections where I could observe only very few Tbr1 and Tbr2 expressing cells. Next I examined the identity of these Tbr immunorreactive cells. Expression of these transcription factors in mature cortical neurons in the adult forebrain and olfactory bulb has been described previously (R. F. Hevner et al., 2006; Z. J. Allen, 2nd et al., 2007a; B. J.

Molyneaux et al., 2007), but so far no Tbr expressing progenitors have been observed in the adult subependymal zone.

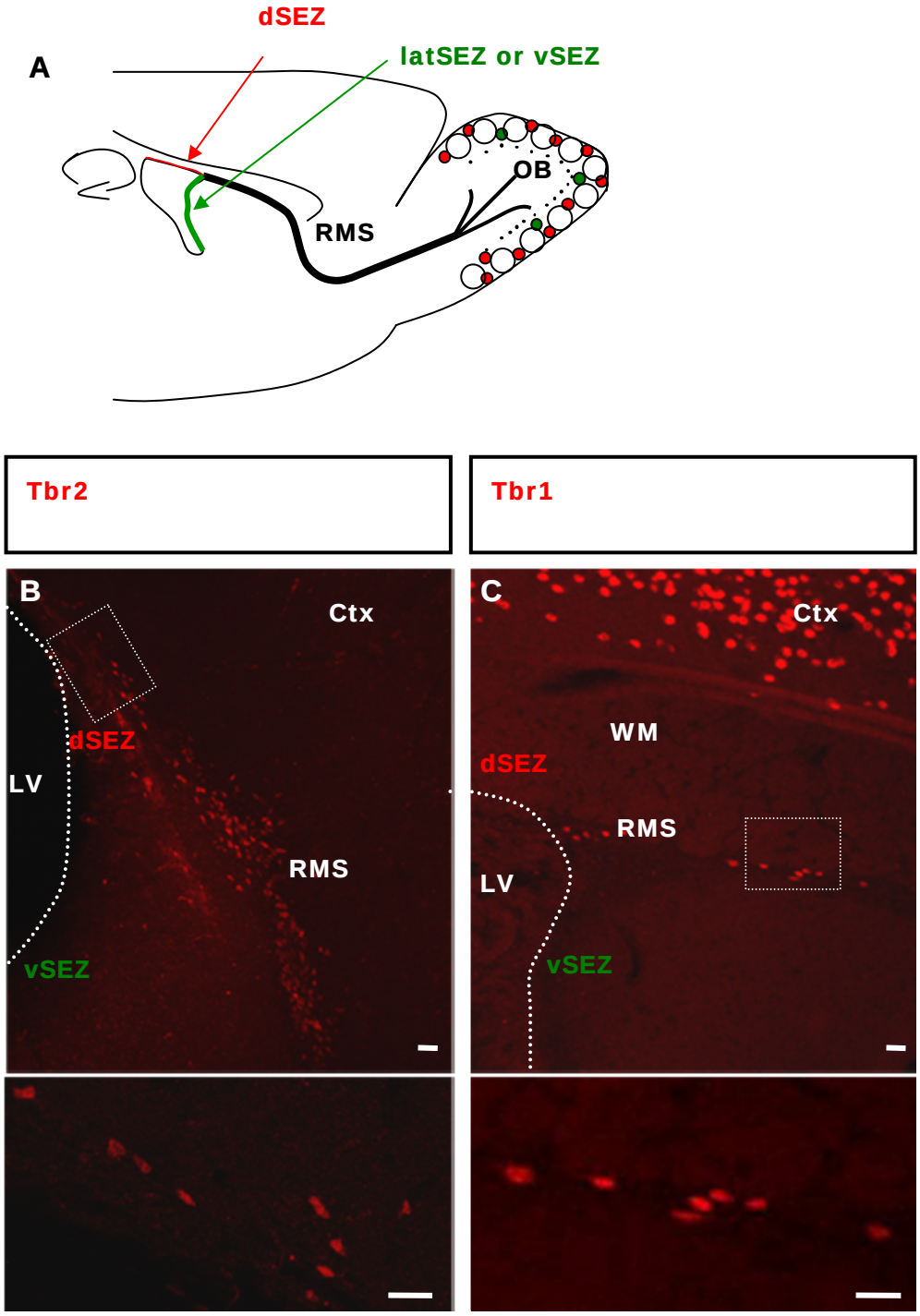


Fig. 42: Tbr1 and Tbr2 transcription factors are present in the adult subependymal zone and rostral migratory stream.

(A) Schematic drawing depicting the dorsal (dSEZ, red) and the lateral or ventral wall (latSEZ; vSEZ, green) of the subependymal zone. Periglomerular neurons derived from the dorsal and ventral lineage are depicted in green and red, respectively.
Both, Tbr2 (B) as well as Tbr1 (C) protein is only detectable in dorsal regions of the subependymal zone and rostral migratory stream but not in the lateral wall of the lateral ventricle. WM = white matter, Ctx = Cortex, LV = lateral ventricle, RMS = rostral migratory stream

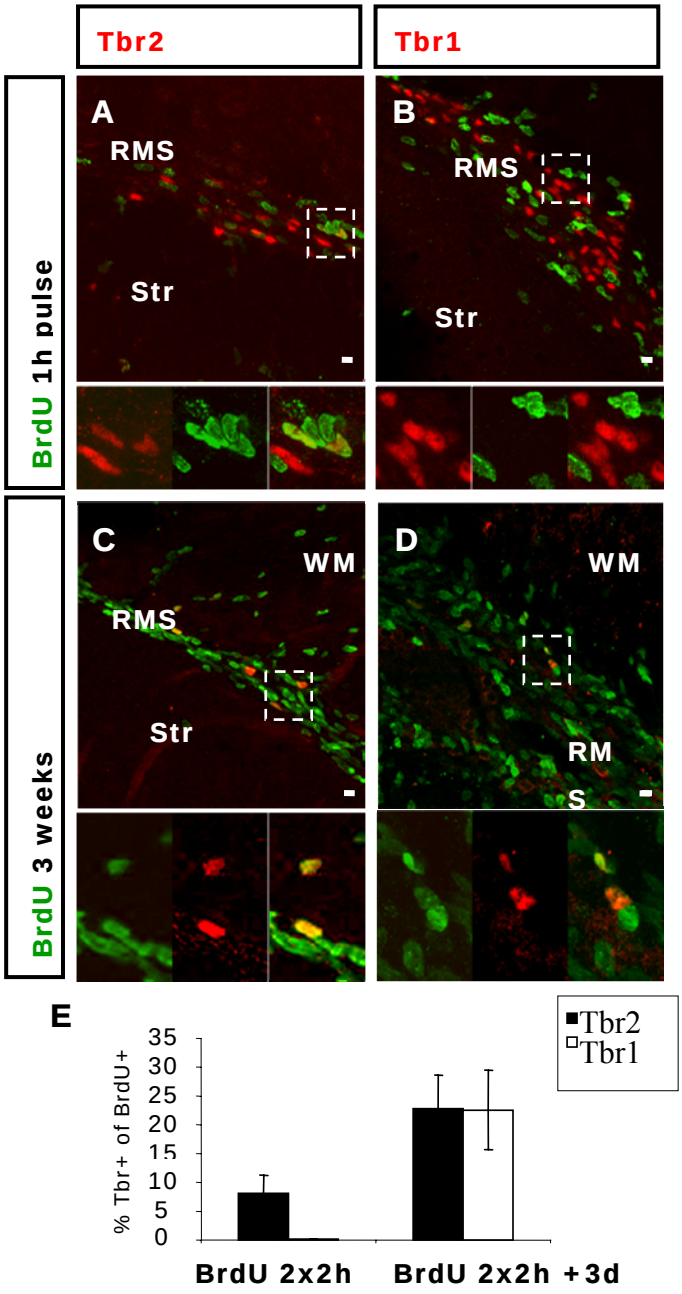
4.2.2 Tbr2- and Tbr1-expressing cells define a subset of GAD67-negative proliferating neuroblasts

In order to examine whether that Tbr1 and Tbr2 expressing cells in the rostral migratory stream are indeed derived from neural stem cells in the supependymal zone we used several approaches: (i) labelling of proliferating cells with the DNA base analogue BrdU; (ii) in vivo genetic fate mapping in adult mice with the inducible form of Cre under the Glast promotor turning on a GFP reporter in adult neural stem cells that is then inherited to their progeny; and (iii) retroviral vector injections into the rostral migratory stream which transduces only dividing cells.

We first gave BrdU in the drinking water for three weeks to adult animals (over 8 weeks) in order to label neural stem cells and their progeny. Indeed I observed BrdU birth dated cells in the adult rostral migratory stream by immunohistochemistry for Tbr1 or Tbr2 (Fig. **43** C, D). Next, we tested if one of the Tbr expressing populations would also be labelled by a short BrdU pulse. Upon a single BrdU pulse one hour before sacrifice, a small proportion of Tbr2-positive cells (7 ± 1%, n(cells)=285) had incorporated BrdU (Fig. **43**, while no Tbr1 and BrdU colocalization (1 ± 1%, n(cells)=201) was detectable. However, when we examined cells three days or longer after the BrdU injection, Tbr1-positive cells had become double-positive, suggesting that they are the immediate progeny of Tbr2-positive progenitors (Fig. **43**).

Fig. 43: Tbr2 expression in proliferating progenitors and Tbr1 expression in postmitotic neuroblasts.

(A) Tbr2+ (red) cells are fast proliferating as shown by a short BrdU pulse (BrdU immunohistochemistry (green)).
(B) In contrast, Tbr1+ (red) cells are not labelled by a short BrdU (green) pulse suggesting that Tbr1 is expressed in later maturation stages. Boxed areas are shown at higher magnifications.
(C, D) Proliferating Tbr1&2+ cells shown in immunohistochemistry for BrdU (green) and Tbr (red) following a BrdU pulse of three weeks in drinking water.
(E) Histogram depicting the number of proliferating Tbr1+ and Tbr2+ cells in the rostral migratory stream 2h, and 3days after 2 BrdU pulses with 2h interval. Tbr2 expression is detected first and only after 3 days Tbr1 is present demonstrating the presence of these transcription factors in the chronology Tbr2, Tbr1.
Scalebar: 20 μm; Str = Striatum, WM = white matter



Since Tbr1 and Tbr2 positive cells proliferate and are present in the rostral migratory stream I performed immunohistochemistry for the neuroblast marker DCX. The majority of Tbr2 expressing cells (70 ± 8%, n(cells)=255) and virtually all Tbr1-positive cells (97 ± 2%, n(cells)=265) were DCX-positive neuroblasts or immature neurons (Fig. **44** A - B). While Tbr2-positive neuroblasts proliferate themselves, we next asked whether these are derived from adult neural stem cells of the subependymal zone we performed fate mapping analysis using GLAST::CreERT2 mice crossed with a GFP reporter line (Z/EG; see material&methods for detail) (A. Novak et al., 2000; T. Mori et al., 2006).Indeed one week after end of tamoxifen induction the reporter line

under the GLAST-promotor at the age of eight weeks we confirmed the origin of Tbr-expressing cells from an astrocyte-like neural stem cell (Fig. **44** D).

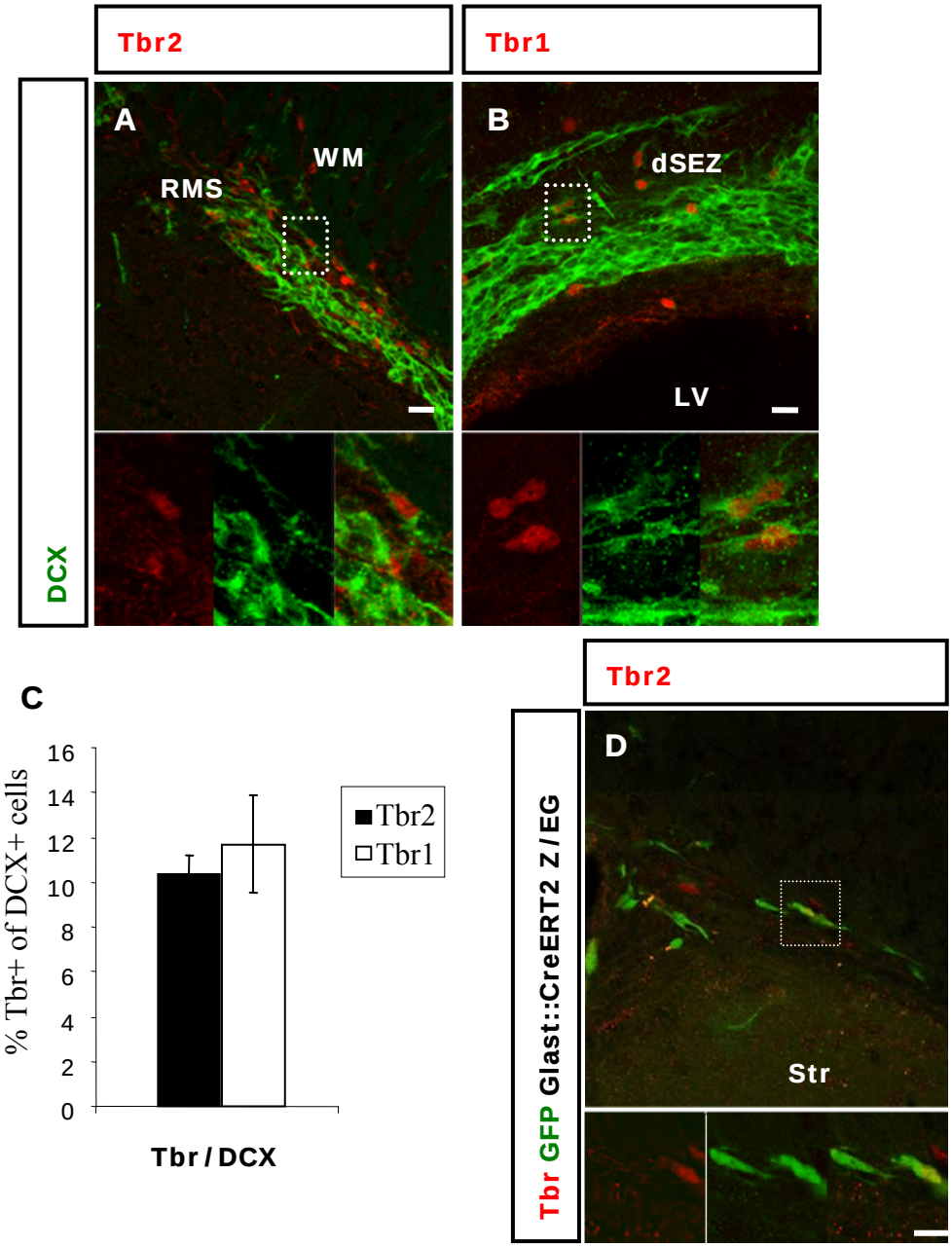


Fig. 44: Tbr expressing neuroblasts are derived from adult neural stem cells.

(A, B) Tbr2+ (A, red) and Tbr1+ (B, red) cells both colocalize with the neuroblast marker DCX (green) in the rostral migratory stream. Boxed areas represent higher magnifications.
(C) Histogram depicting the number of doubleocrtin (DCX) positive neuroblasts expressing Tbr2 (black) or Tbr1 (white), respectively. (n = 3 animals, n(cells; Tbr1) = 315, n(cells; Tbr2) = 289).
(D) Adult recombination of a GFP reporter (Z/EG) under the Glast promoter at the age of eight weeks resulted in Tbr2+/GFP+ cells migrating away from the subependymal zone. Induction was performed for five days, followed and animals were sacrificed after one week at the end of tamoxifen induction. Scale bar = 20 µm. LV = lateral ventricle, Str = Striatum, WM = white matter, dSEZ = dorsal subependymal zone, RMS = rostral migratory stream

Additionally, proliferation of progenitors in the adult forebrain which give rise to Tbr2-positive and Tbr1-positive migrating neuroblasts was also observed following retroviral transduction in the rostral migratory stream. Retroviruses transducing exclusively dividing cells and encoding GFP were injected into the rostral migratory stream. Seven days after the injection I observed double-positive cells for GFP and Tbr1 or Tbr2 close to the olfactory bulb confirming that Tbr1 and Tbr2 expressing cells originate from proliferating progenitors and migrate towards the olfactory bulb (Fig. **45** A - B). Both, Tbr2 and Tbr1 expressing cells co-localize with GFP in equal proportions (Tbr2: 5 ± 1 %, n(cells)=160; Tbr1: 3.5 ± 2 %, n(cells)=116; Fig. **45** C).

So far all neuroblasts generated in the subependymal zone and rostral migratory stream have been thought to differentiate into GABAergic granule neurons and and periglomerular neurons. The latter can be classified into distinct subpopulations according to their expression of tyrosine hydroxylase, calbindin or calretinin (S. Parrish-Aungst et al., 2007). However, only a small calretinin-positive population had been postulated to be GABA-negative (K. Kosaka and T. Kosaka, 2007). Since almost all neuroblast are destined to become GABAergic olfactory neurons they already express glutamic acid decarboxylase (GAD) and synthesize GABA in the rostral migratory stream (J. C. Platel et al., 2007). I examined the presence of GAD in Tbr1 and Tbr2 immunoreactive cells in the mouse line GAD67::GFP where a GFP cassette is knocked in the GAD67 locus (N. Tamamaki et al., 2003). First, we confirmed the reliable co-expression of GFP or GAD67 on mRNA level in GAD67::GFP mice (Fig. **46**). Virtually all GFP expressing cells were also positive for GAD mRNA. Accordingly, in-situ hybridization for GFP mRNA confirmed the reliable co-expression of the protein GFP in GAD67::GFP mice. Immunohistochemistry for GAD67-GFP and Tbr1 or Tbr2 demonstrated that T-box transcription factor containing neuroblasts in the rostral migratory stream were negative for GAD67, while virtually all other neuroblasts were GFP-positive as expected (Fig. **45** D - G). Thus, Tbr1 and Tbr2 expressing neuroblasts / young neurons express none of the enzymes required for the synthesis of the neurotransmitter GABA, suggesting that they may be biased towards a different neurotransmitter fate, supposedly glutamatergic fate.

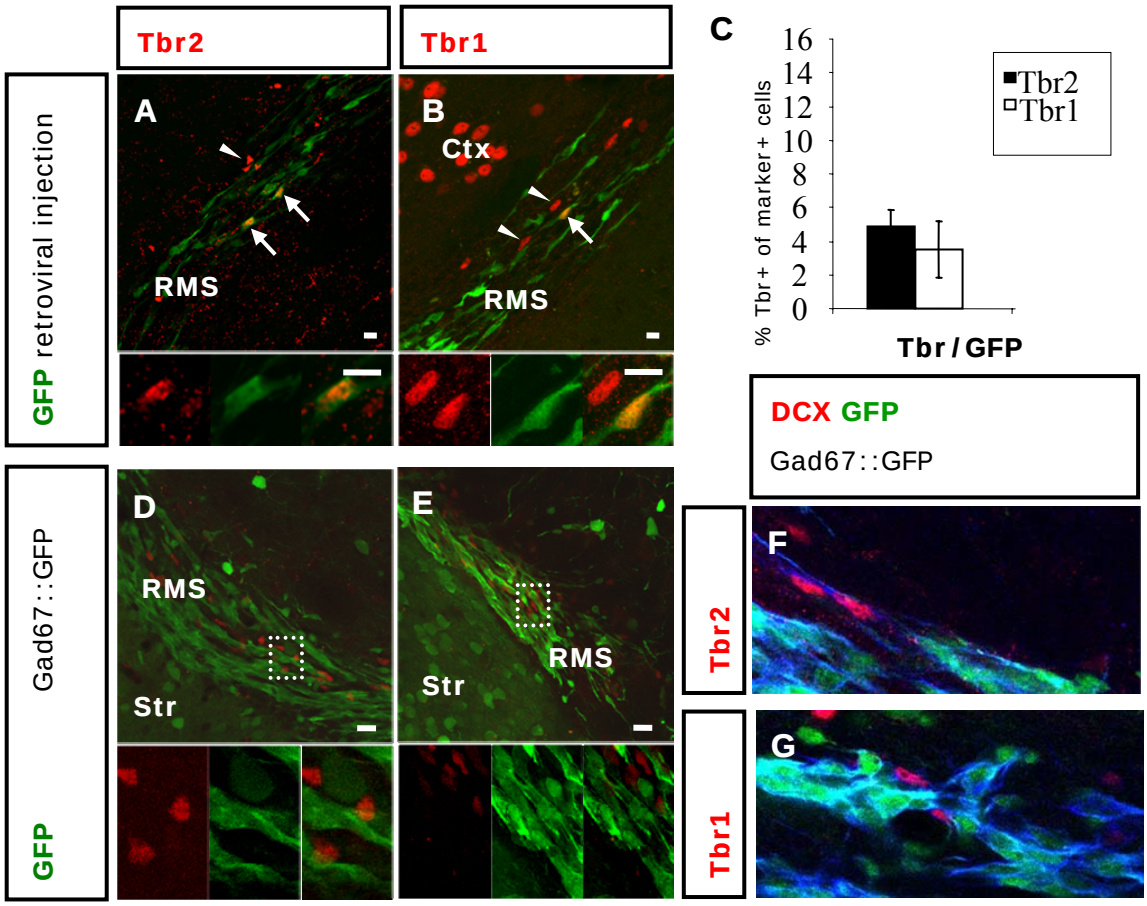


Fig. 45: Tbr1 and Tbr2 define a subset of GAD67-negative migrating neuroblasts.

(A, B) Retroviral transduction demonstrated presence of GFP-positvie and Tbr1 or Tbr2 positive neuroblasts seven days after injection into the rostral migratory stream. Arrows indicate double-positive cells expressing GFP and Tbr1 or Tbr2; arrowheads indicate Tbr1 or Tbr2 positive cells that were not GFP transduced.

(C) Quantification of double positive cells for GFP and Tbr1 or Tbr2, respectively, shows that equal subpopulations of Tbr1 and Tbr2 are present. When Tbr-positive neuroblasts in the rostral migratory stream were quantified higher numbers were observed as retroviral vectors transduce only dividing cells. n = 3 animals.

(D, E) Tbr2-positive (D, red) and Tbr2-positive (E, red) cells in the rostral migratory stream are GFP-negative in GAD67::GFP mice. Inserts show higher magnifications of the boxed areas.

(F, G) Triple immunohistochemistry for DCX (blue), GFP, and Tbr2 (F, red) or Tbr1 (G, red) in GAD67::GFP mice. Tbr expressing cells colocalize with DCX in the rostral migratory stream, however they are negative for Gad67-GFP.

Scalebar: 20µm, Str = Striatum, WM = white matter, dSEZ = dorsal subependymal zone, latSEZ = lateral subependymal zone, RMS = rostral migratory stream, Ctx = Cortex, OB = olfactory bulb

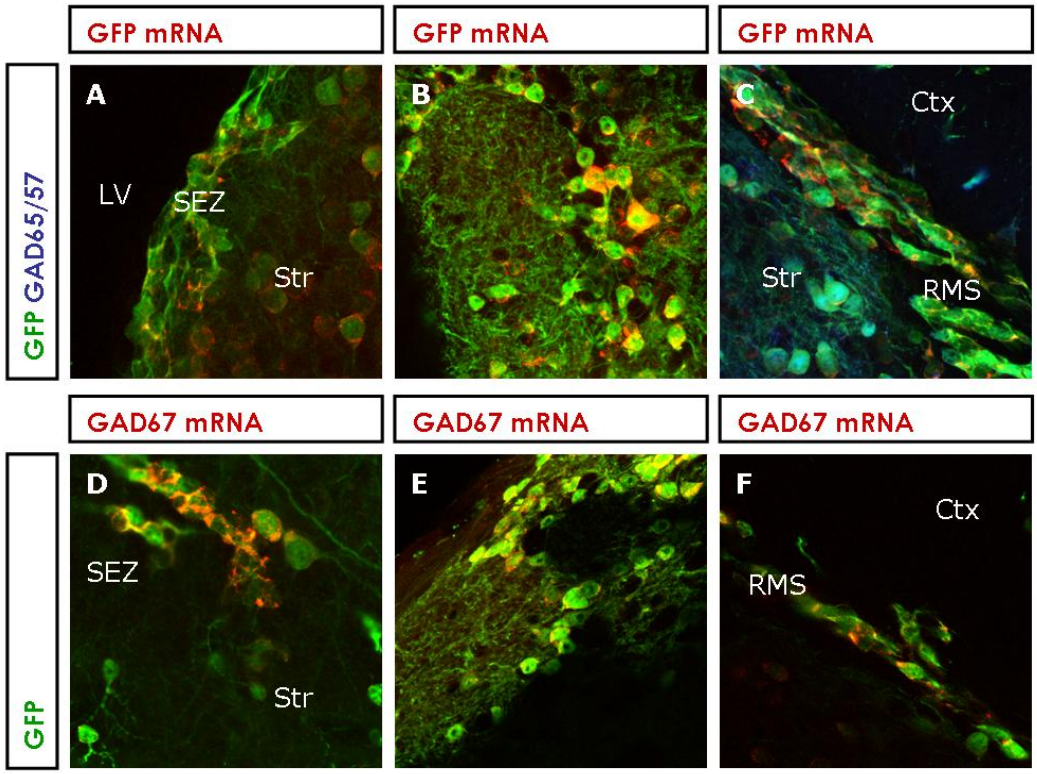


Fig. 46: GFP and GAD67 mRNA colocalize in GAD67::GFP mice.
Reliable expression of GFP in the GAD67 genomic locus was confirmed on mRNA level for GFP and GAD67. (A – C) In-situ hybridization for GFP combined with immunohistochemistry for GFP and (C) GAD67.
(D – F) GAD67 mRNA was present in nearly all GFP-positive cells. Very few GFP-positive cells did not contain GAD67 mRNA, but mRNA for GAD67 always colocalized with GFP.
SEZ = subependymal zone; Ctx = Cortex; RMS = rostral migratory stream; Str = Striatum.

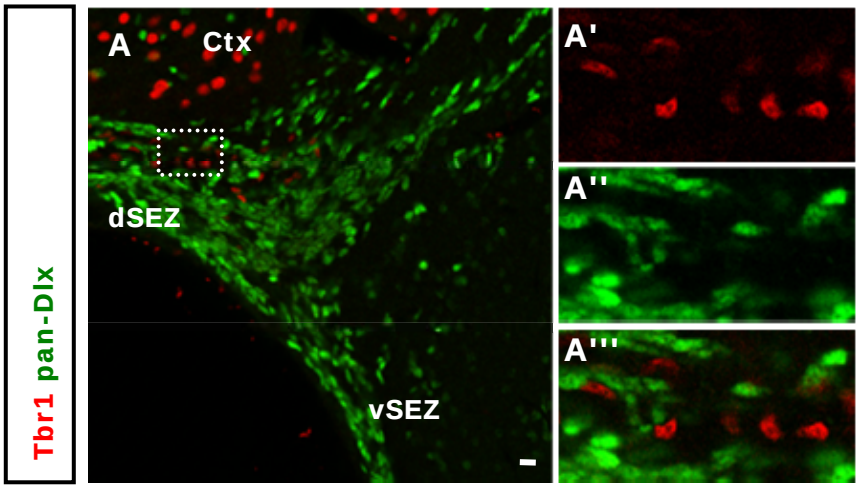
4.2.3 Tbr2 and Tbr1 expressing cells are distinct from Dlx expressing progenitors

Given that Tbr1 and Tbr2 expressing cells define a GAD-negative subset of neuroblasts we hypothesized that they should not express Dlx transcription factors. Indeed, no co-expression between Dlx proteins and either Tbr1 was observed (Fig. **47** A): the Dlx-positive lineage arising from ventral regions, whereas the dorsal lineage would express Tbr transcription factors.

Are other transcription factors present in the Tbr lineage that could suggest that these progenitors give rise to olfactory neurons with a glutamatergic identity? Glutamatergic hippocampal neurogenesis has been suggested to arise from a Pax6 and/or Mash1 positive progenitor that would up-regulate only at later stages T-box transcription factors, first Tbr2 and later Tbr1. In the dorsal telencephalon, Pax6 and Neurogenin2

are up-stream of T-box genes (C. Englund et al., 2005; P. Mattar et al., 2008). Notably, a large fraction of Mash1-positive cells expresses Dlx proteins. Hence I performed immunohistochemistry for Pax6 or Mash1 in combination with Tbr2 and Tbr1.

Interestingly, a minor population of Tbr2 expressing progenitors and virtually no Tbr1-positive cells contained Mash1 (Tbr2: 11 ± 2%, n(cells)=375; Tbr1: 3 ± 1.5%, n(cells)=321; Fig. **47**). This data are consistent firstly, with the colocalization of Tbr proteins and the neuroblast marker DCX and secondly, with previous reports which demonstrate that Mash1 is mostly contained in transit-amplifying progenitors (C. M. Parras et al., 2004). Accordingly, a minor population of Tbr2 positive cells would be also expressed in transit-amplifying progenitors whereas Tbr1 expression is exclusively restricted to positmitotic neuroblasts. These data are consistent with the short BrdU pulse where only Tbr2, but not Tbr1-positive cells are labelled. In contrast to Mash1 expression, Pax6 protein was present in Tbr2 and Tbr1 positive cells (Tbr2: 34 ± 3%, n(cells)= 692; Tbr1: 30 ± 5%, n(cells)=420; 3 animals each) (Fig. **47** D, E). Interestingly, Pax6 and Mash1 protein colocalize partially in the adult subependymal zone (Fig. **47** F).



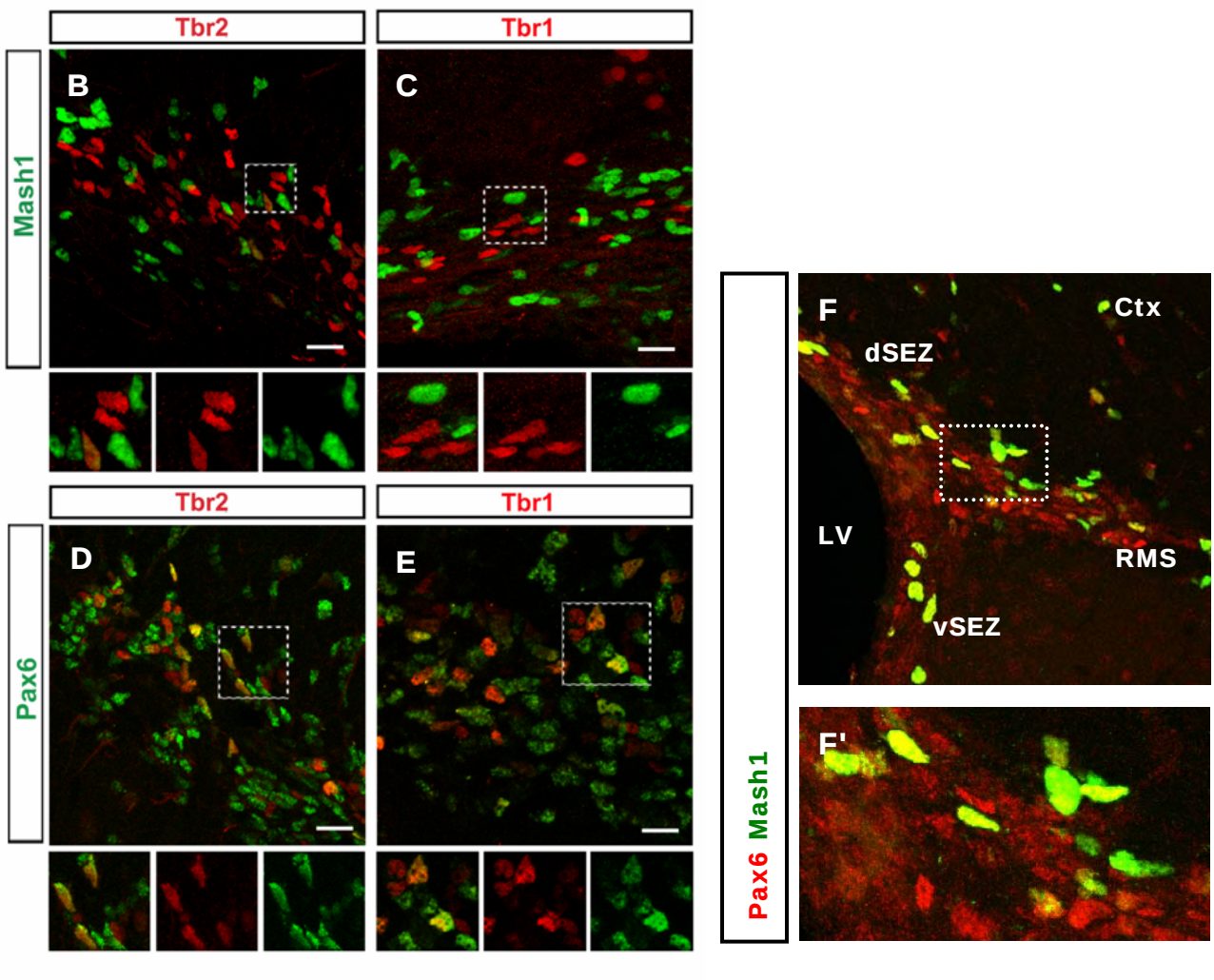


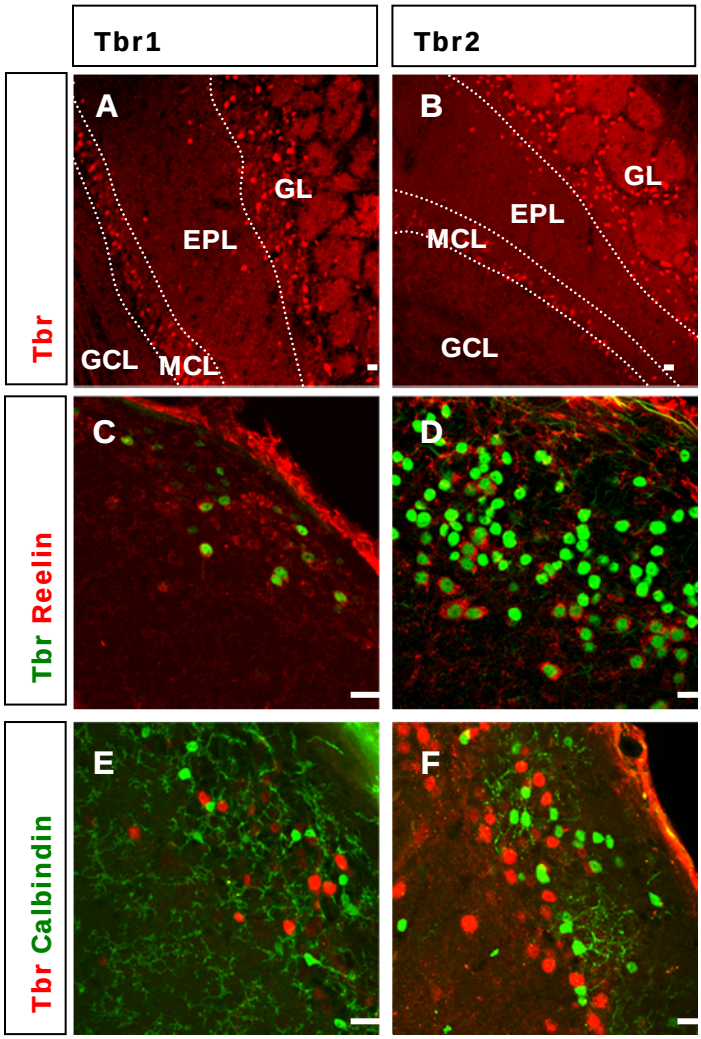
Fig. 47: Pax6 and Mash1 expression in regard to Tbr transcription factors.
(A) The Dlx transcription factor family (pan-Dlx, green) does not colocalize with Tbr1 (red) as shown by immunohistochemistry.
(B, C) Mash1 colocalizes in wildtype mice only with Tbr2, but not with Tbr1.
(D, E) Fluorescent micrograph depicting immunohistochemistry for Pax6 (green) and Tbr2 (red) and Tbr1 (red). Both transcription factors colocalize substantially with Pax6. Boxed areas are shown in higher maginifactions.
(F) Pax6 and Mash1 are colocalized in dorsal and ventral regions of the adult subependymal zone. Boxed area is shown in higher magnification in (F').
Scale bars 20µm. dSEZ = dorsal subependymal zone, vSEZ = ventral subependymal zone, RMS = rostral migratory stream, Ctx = Cortex, LV = lateral ventricle

4.2.4 Adult generation of glutamatergic neurons in the olfactory bulb

To elucidate the neuronal fate of the Tbr expressing neuroblasts, I examined by immunohistochemistry if periglomerular neuronal markers would colocalize with Tbr proteins in the glomerular layer. However, most of the common markers for periglomerular neurons such as calbindin, calretinin and tyrosine hydroxylase did not colocalize with Tbr1+ or Tbr2+ cells (Fig. **48**). In addition none of the Tbr expressing cells expressed GFP in the GAD67::GFP mouse line, indicating that they are not

GABAergic. Notably, some of the Tbr-positive cells express Reelin (I. Hack et al., 2002) (Fig. **48** C, D).

To test if the Tbr1 or Tbr2 expressing cells in the glomerular layer of the olfactory bulb are the progeny of Tbr progenitors generated in the adult subependymal zone or if these cells are generated during embryogenesis we gave BrdU for three weeks into the drinking water followed by a BrdU free period of three weeks. None of the Tbr-expressing cells colocalized with BrdU demonstrating that Tbr expressing cells in the glomerular layer are not generated during adulthood but during embryonic neurogenesis instead. Consequently we hypothesized that the progeny of adult Tbr expressing cells down-regulate Tbr transcription factors upon entrance in the olfactory bulb and that there are glutamatergic interneurons present without Tbr transcription factors. Consistently we could not detect Tbr proteins in the granule layer of the olfactory bulb as all granule neurons are GABAergic.



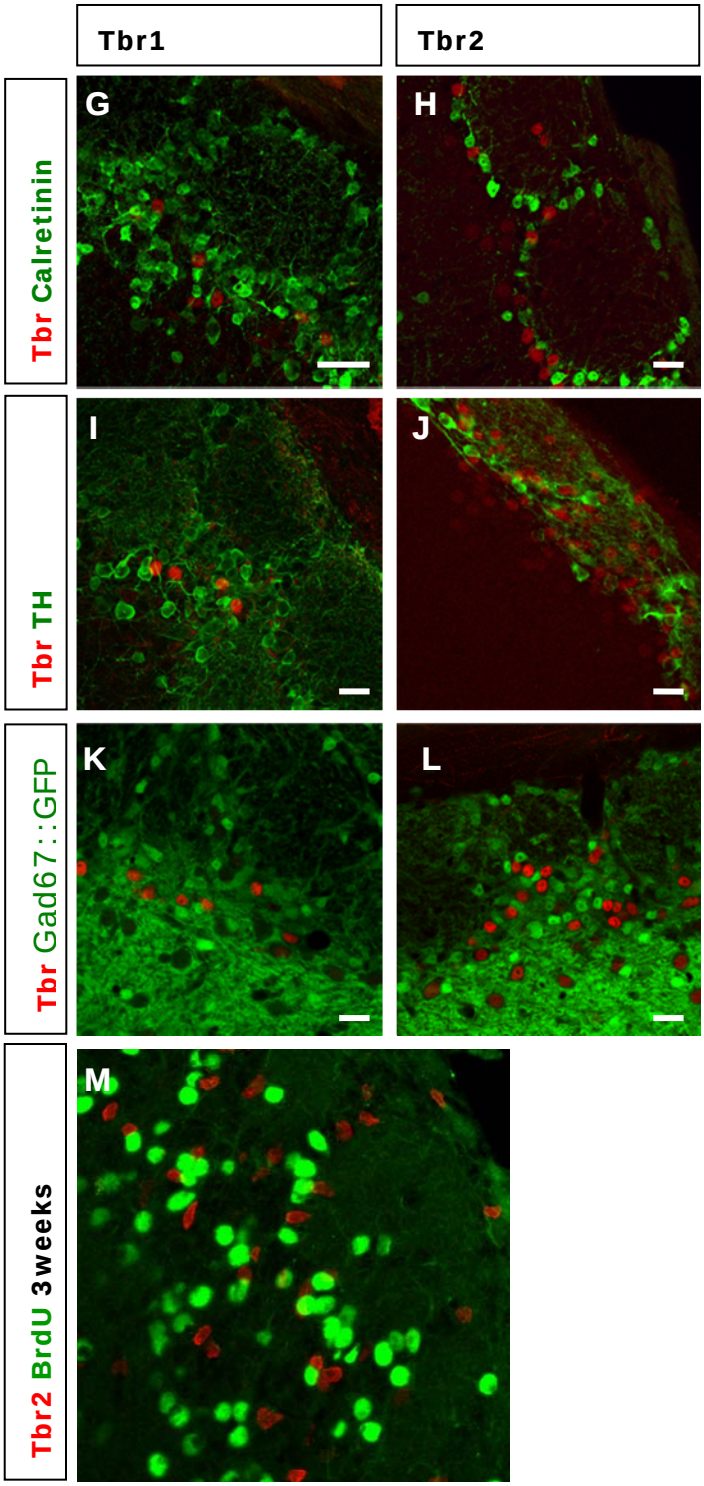
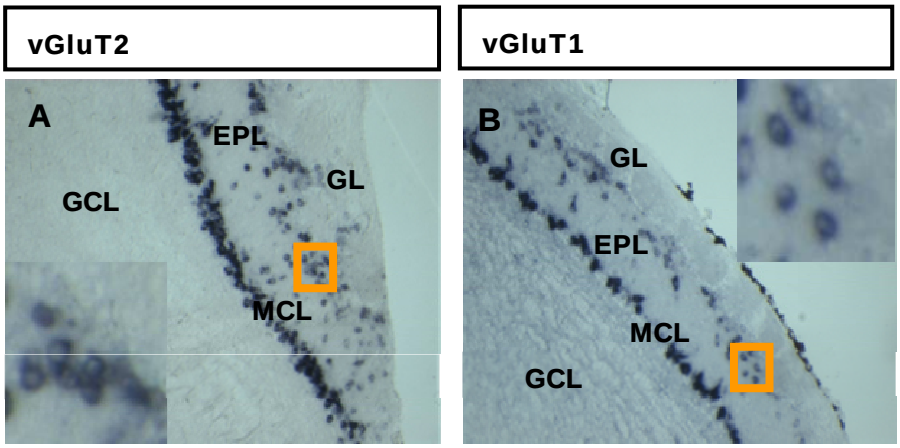


Fig. 48: Tbr1 and Tbr2 cells in the olfactory bulb are GAD67, calbindin, calretinin and tyrosine hydroxylase negative.

(A, B) Tbr1 and Tbr2 are expressed in the adult olfactory bulb in the mitral cell layer and glomerular layer. Notably, no Tbr-positive cells were observed in the granule cell layer. (C, D) Some of the Tbr+ cells expressed Reelin, but most other common markers did not colabel Tbr-positive cells. Immunohistochemistry for Tbr (red) and (E, F) Calbindin, (G, H) Calretinin and (I, J) Tyrosine Hydroxylase (TH) did not result in colocalisation.
(K, L) In addition Tbr-positive cells are negative for the GABA synthesizing enzyme GAD67 in the olfactory bulb of Gad67::GFP mouse line.
(M) Tbr2 (red) and BrdU (green) do not colocalize in the glomerular layer of the olfactory bulb.
MCL = mitral cell layer, EPL = external plexiform layer, GL = glomerular layer; Scalebar = 20 µm.

Given the down-regulation of Tbr transcription factors upon arrival into the olfactory bulb and the hypothesis that Tbr expressing progenitors of the dorsal subependymal zone would generate glutamatergic progeny, we performed in-situ hybridization for the vesicular glutamate transporter 1 or 2 (vGluT1 or vGluT2) to test the glutamatergic identity of adult BrdU labelled cells in the olfactory bulb. Indeed, we detected intense vGluT1 and vGluT2 mRNA signals in numerous cells located in the mitral cell layer (G. Gheusi et al., 2000), the external plexiform layer (EPL) and the glomerular layer (GL), but not in the granule cell layer (Fig. **49** A, B).

Notably, mitral cells expressed both transporters vGluT1 and vGluT2. Tbr expressing cells in the olfactory bulb are mostly glutamatergic interneurons and expressed either vGluT1 or vGluT2 (C - G). Notably, a substantial proportion of vGluT1 or vGluT2 mRNA containing cells did not colocalize with Tbr protein. In general, the vGluT1 and vGluT2 mRNA expressing cells showed a great heterogeneity and several classes of interneurons are present: (i) Tbr1 and Tbr2 positive cells without mRNA for vGluT1 or vGluT2; (ii) vGluT1 or vGluT2 mRNA positive cells that did not colocalize with Tbr proteins; (iii) neurons that expressed both, Tbr proteins or vGluT mRNA.



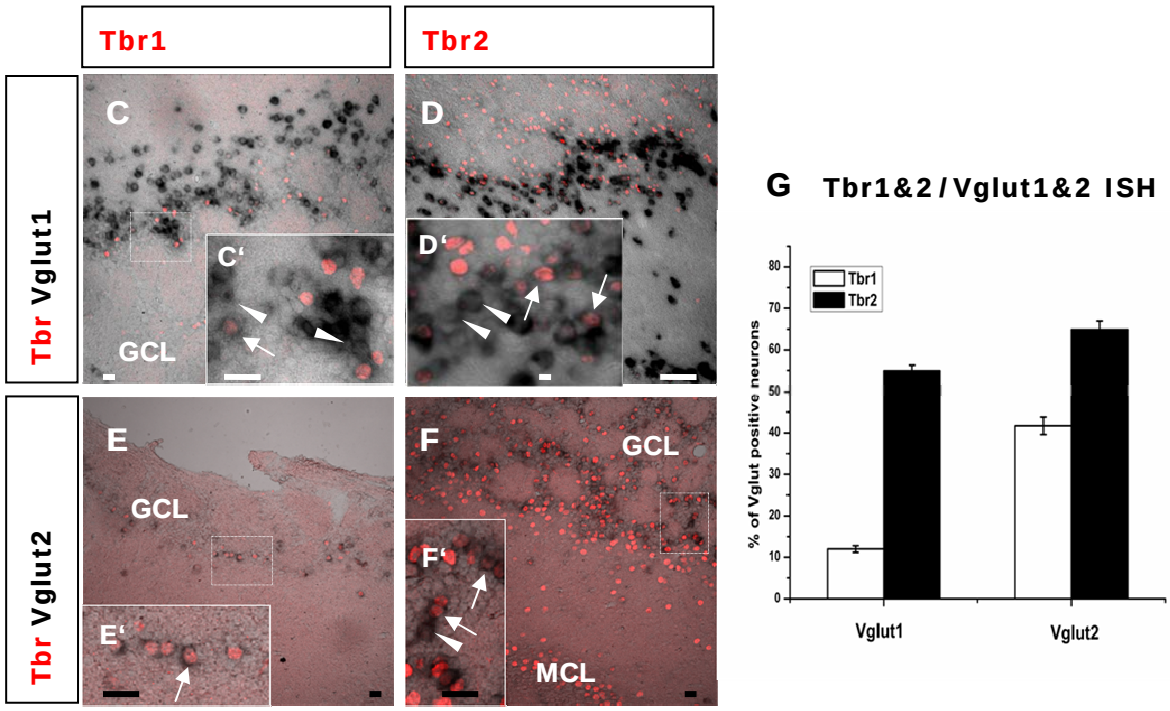


Fig. 49: Glutamatergic neurons in the olfactory bulb express partially Tbr1 and Tbr2.
(A, B) In situ hybridization for vGluT2 and vGluT1 in the adult olfactory bulb shows mRNA expression in the mitral cell layer (MCL), external plexiform layer (EPL) and glomerular layer (GL). Insets show higher maginifactions of the gomerular layer.
In-situ hybridization for vGluT1 (C, D) and vGluT2 (E, F) counterstained with immunohistochemistry for Tbr1 (red, C, E) and Tbr2 (red, D, F). Notably, not all vGluT+ cells are Tbr+. Arrows indicate double-positive cells for vGluT and Tbr, whereas arrowheads indicate only vGluT+ cells. Insets show higher magnifications.
GCL = granule cell layer, MCL = mitral cell layer; Scalebars = 20 µm

We further tested the generation of glutamatergic neurons that had down-regulated Tbr transcription factors in the adult olfactory bulb by combining BrdU-labelling with in-situ hybridization for vGluT1 or vGluT2 (Fig. **50**). BrdU was given for three weeks in the drinking water followed by three weeks chase with BrdU free water to allow sufficient time for neurotransmitter differentitation. Consistent with previous reports a substantial proportion of the BrdU-positive cells differentiate into periglomerular neurons immunoreactive for tyrosine hydroxylase, calbindin or calretinin (K. Kosaka et al., 1995; B. Winner et al., 2002; Z. J. Allen, 2nd et al., 2007a; P. Panzanelli et al., 2007; S. Parrish-Aungst et al., 2007). The GABAergic phenotype of periglomerular neurons was also confirmed in GAD67::GFP mice where more than 80 % of the BrdU+ cells colocalized with GAD67-GFP (Fig. **50** A - E) (P. Panzanelli et al., 2007). Strikingly a small proportion of BrdU-positive cells (2 ± 0.5%, n(cells)=1632) located in the glomerular layer were found to coexpress vGluT2 mRNA (Fig. **50** E, F, G).

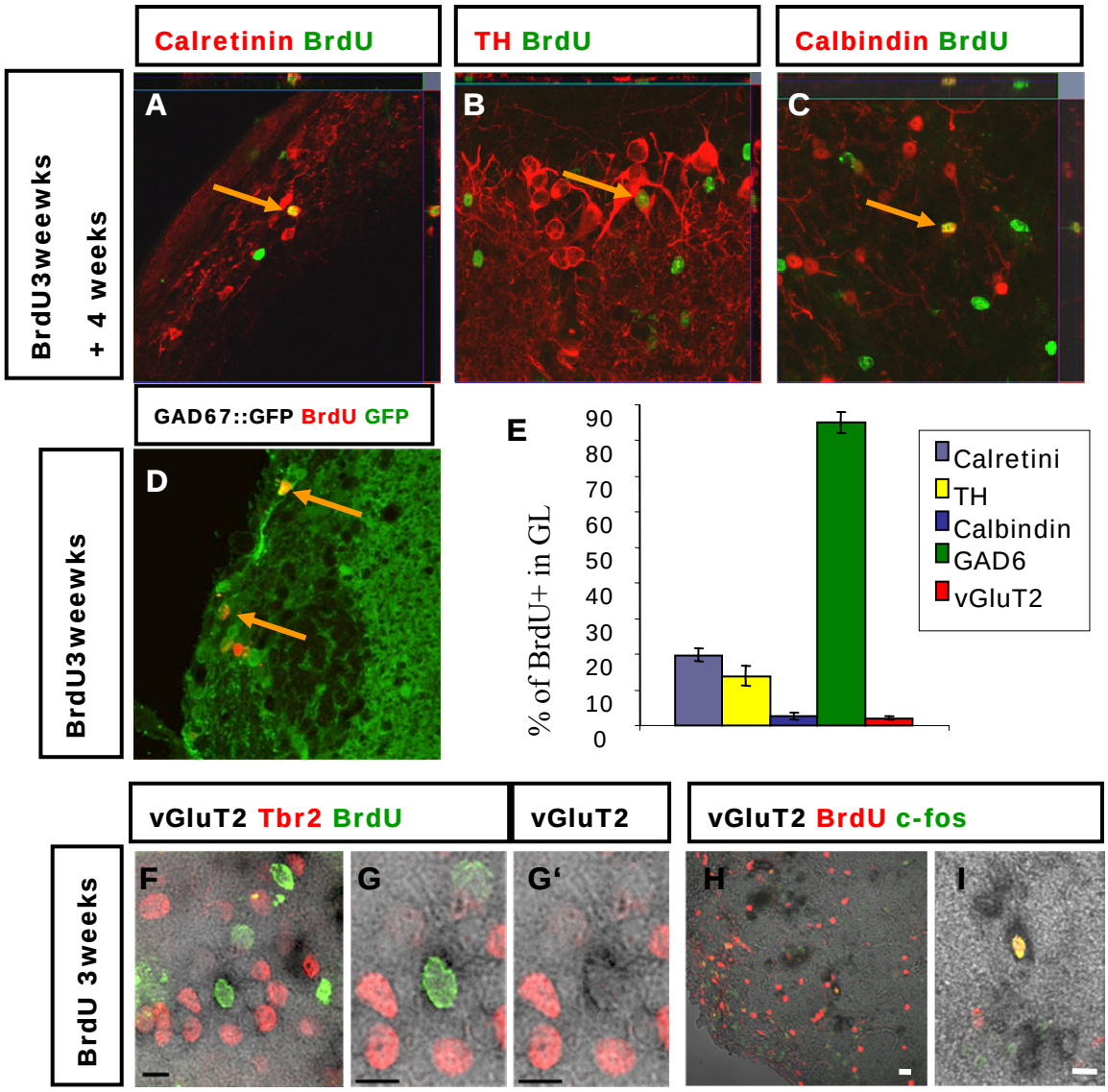


Fig. 50: Newly generated glutamatergic neurons defina a subpopulation of BrdU-positive periglomerular neurons and integrate functionally in the olfactory bulb.

For fate mapping analysis BrdU was given for three weeks into the drinking water followed by a chase of four weeks without BrdU to allow sufficient time for neurotransmitter differentitation. Established periglomerular neuron markers are expressed at significant frequencies: Immunohistochemistry for BrdU (green) and Calretinin (red) (A), Tyrosine Hydroxylase (TH) (red) (B), and Calbindin (red) (C). Orange arrows indicate double-positive cells for BrdU and corresponding periglomerular neuron marker.
(D) The majority of BrdU+ cells (red) is of GABAergic identity as demonstrated with immunohistochemistry for GFP (green) in GAD67::GFP mice.
(E) Quantification of marker expression of BrdU+ cells amongst the periglomerular neurons. Notably, the majority of adult born periglomerular neurons adopt a GABAergic transmitter phenotype.
(F, G) In-situ hybridization for vGluT2 and immunohistochemistry for BrdU (green) resulted in double-positive cells in the glomerular layer of the adult olfactory bulb. Notably these BrdU+/vGluT2+ cells are negative for Tbr2. Higher magnication is shown in (G, G').
(H, I) Funtional integration of glutamatergic periglomerular neurons as shown by c-fos immunohistochemistry (green), BrdU (red) and vGluT2 ISH.
(J – J") Four weeks after adult recombination in GLAST::CreERT2 mice crossed with the RosC reporter line colocalization of vGluT2 and YFP was observed. Boxed area is shown in higher magnification in (K – K")

Notably, no double-positive cells were observed shortly after BrdU-labelling suggesting that these BrdU cells are not dying cells. None of the vGluT1 expressing neurons (n(cells)=1236) could be labelled by BrdU, suggesting that only the vGluT2, but not the vGluT1 expressing subset of cells are adult generated. Moreover, no vGluT1 or vGluT2 expressing cells in the external plexiform layer had incorporated BrdU, indicating that these interneurons are not generated in the adult olfactory bulb. Finally, we examined whether adult-generated vGluT2-positive (vGluT2+) cells become functionally incorporated into neuronal circuits by staining for the immediate early gene c-fos known to be regulated by neuronal activity (S. S. Magavi et al., 2005; M. P. Leussis and S. C. Heinrichs, 2007). Indeed, c-fos immunoreactivity was detected in a substantial fraction of cells positive for both BrdU and vGluT2 (Fig. **50** H, I; experiment performed by Jovica Ninkovic) suggesting that these glutamatergic neurons generated 3 - 6 weeks earlier become functionally active by this time. Moreover, we observed the long-term survival of vGluT2 expressing neurons until two months after BrdU-labelling, suggesting that at least some of them are stably incorporated into the adult olfactory bulb network.

4.2.5 Adult generated neurons form functional glutamatergic synapses

While co-expression of vGluT2 and c-fos are in BrdU-positive cells within the olfactory bulb is a strong indicator for functional glutamatergic neurons being generated in the adult, we further aimed to ensure the establishment of functional glutamatergic synapses by adult generated neurons using electrophysiology. Towards this end, we took advantage of the culture system of primary subependymal progenitors (for details see 6.9.2 Non-expanded adult progenitor cultures). This culture system allows maintenance of isolated subependymal zone progenitors in vitro without EGF and FGF2 in defined medium. Under these conditions cultured progenitors give rise predominantly to neuronal progeny. First, we isolated the whole wall including lateral and ventral regions. While seven days after plating the majority of cells express members of the Dlx gene family, in agreement with their GABAergic fate, a small percentage (1.5 %; n(cells)=574) expressed Tbr2 consistent with their low abundance in the subependymal zone in vivo (Fig. **51**). Interestingly, Tbr2-positive cells appeared as small clusters of 2 - 4 cells, consistent with a clonal origin of these cells.

To examine whether Tbr2 expressing cells may be enriched in cultures prepared from the dorsal wall of the lateral ventricle we separated the upper dorsal part from the lower ventral part of the ventricle during culture preparation. Seven days after plating, I quantified the number of Tbr2-positive cells per DAPI stained nuclei and found a striking regionalization. Compared to the mixed culture where I found 1.5 % Tbr2 expressing cells virtually no Tbr2-positive cells were detectable in cultures derived from the ventral part of the ventricular wall. However, in the cultures of cells from the dorsal subependymal zone four times more Tbr2-positive cells were present (Fig. **51** D). This data demonstrates the enrichment of dorsal wall cultures with Tbr2 expressing progenitor cells and that these progenitors are derived from the dorsal subependymal zone. To test if Tbr2-positive cells are indeed generated in vitro we transduced the dorsal and ventral cultures two to four hours after plating with retroviral vectors encoding only GFP. Indeed we found some GFP transduced Tbr2 expressing clones seven days after plating in dorsal wall derived cultures. Additional immunohistochemistry for GFP, Tbr2 and ß-III-Tubulin demonstrated the neuronal idendity of these progenitors and their generation in vitro (Fig. **51** E).

Further in line with the presence of Tbr2-positive progenitors in these cultures we found a small proportion of cells that exhibited vGluT immunoreactivity which became concentrated in puncta after several weeks in vitro suggesting the formation of functional glutamatergic synapses (Fig. **51**). To determine the presence of functional glutamatergic synapses unambiguously we performed perforated patch recordings (B. Berninger et al., 2007).

Fig. **51** shows a representative example of an adult subependymal zone derived neuron that exhibited a CNQX sensitive autaptic response (electrophysiology performed by Benedikt Berninger). This data demonstrate that adult subependymal zone cells can generate glutamatergic neurons that form functional synapses in vitro.

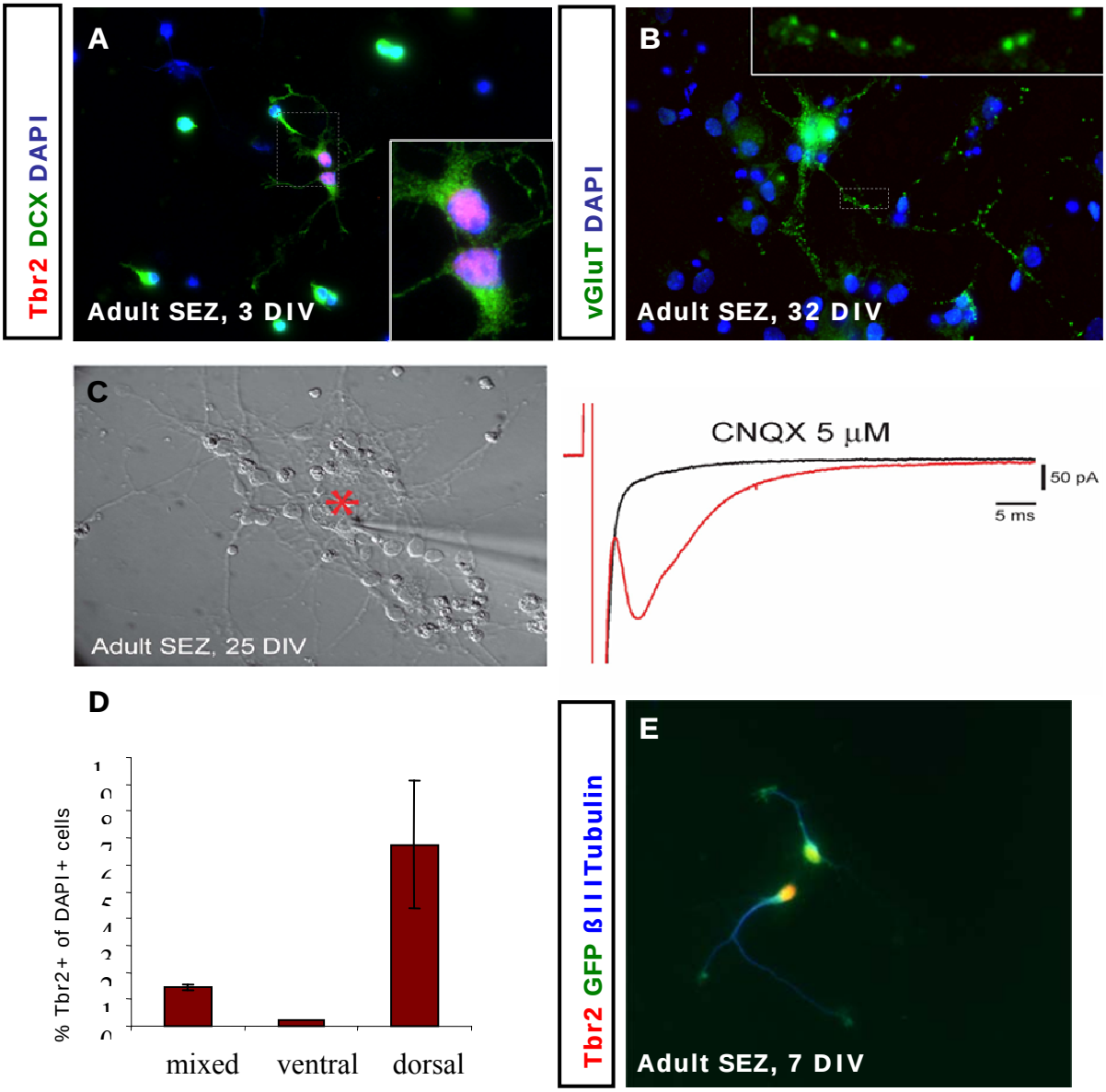


Fig. 51: Cultured adult subependymal zone stem and progenitor cells give rise to a small proportion of glutamatergic neurons in vitro.

(A) Micrograph depicts two subependymal zone neuroblasts expressing Doublecortin (DCX) (green) and Tbr2 (red) after 3 days in vitro (3 DIV); DAPI in blue. The insert shows a high magnification view of the Tbr2+ nuclei.

(B) Micrograph depicts two neurons derived from primary cultured progenitors expressing vGluT (green) immunoreactivity 32 DIV. The insert shows the punctuate pattern of vGluT immunoreactivity suggestive of synaptic localization.

(C) Left: micrograph shows an adult subependymal zone derived cultured glutamatergic neuron; right: stimulation of the neuron evoked an autaptic response that was blocked by the AMPA/kainite receptor antagonist CNQX revealing its glutamatergic nature. Electrophysiology performed by Benedikt Berninger.

(D) Quantification of Tbr2+ cells per DAPI+ nuclei in mixed, ventral and dorsal ventricular wall cultures. Mixed cultures contained 1.5 % Tbr2+ progenitors. Whereas ventral wall culture preparation hardly contained Tbr2+ cells, dorsal wall culture preparation were enriched of Tbr2+ progenitors and contained four times more Tbr2+ cells.

(E) Tbr2+ cells are generated in vitro demonstrated by retroviral transduction two hours after plating. Immunohistochemistry for GFP (green), Tbr2 (red) and ßIIITubulin (blue) after seven days in vitro demonstrated the proliferation of progenitors and generation of this cell type in vitro.

4.2.6 Tbr2-expressing progenitors migrate towards sites of neocortical injury

One of the implications of this discovery of endogenous progenitors for glutamatergic neurons in the adult subependymal zone is the possibility of their recruitment towards sites of neocortical injury where they may replace glutamatergic neurons. We therefore employed an injury model which has previously been shown to elicit remarkable repair of cortical neurons from endogenous sources of progenitors which either migrated from the adult subependymal zone towards the lesion site or alternatively, were activated locally (S. S. Magavi et al., 2000; J. Chen et al., 2004).

In this lesion model rhodamine labelled latex beads (Lumaflour) were coupled with the phototoxic chemical chlorine e_6 (see Material & Methods for detailed protocol). Following cortical injections the coupled latex beads are retrogradely transported and label only callosal projection neurons in the contralateral cortex (S. S. Magavi et al., 2000). Subsequent laser illumination of the contralateral hemisphere allows activation of chlorine e_6 followed by a release of oxygen radicals which kills exclusively chlorine e_6 beads containing neurons.

4.2.6.1 Chlorine e_6 coupled latex beads on embryonic day E 14 cultures

In order to test the functionality of chlorine e_6 coupled beads in neurons, I prepared cortical embryonic day E 14 cultures. I added 2 µl coupled latex beads suspension to each well onto embryonic day 14 cortical cultures at three days in vitro. Laser illumination was performed 7 days after latex beads addition and all cultures were fixed two days after laser illumination. As control no beads were added, neither laser illumination was performed. A second control consisted of laser illumination without the addition of coupled latex beads. No difference could be observed between the controls demonstrating that the laser illumination alone or the addition of coupled latex beads suspension did not induce neuronal cell death (Fig. **52** A, B). Only cells in wells containing chloreine e_6 geads died following laser illumination (Fig. **52** C, D)

Following laser illumination for 10 minutes per well of a 24-well plate at lower (0.1 W) and higher (0.5 W) laser dosis, cultures were severly affected. Hardly any cell with neuronal morphology could be observed after illumination for 10 minutes with 0.5 W suggesting that a lot of cells die quickly upon coupled latex beads uptake and high energy dosis of laser leading to chlorine e_6 activation. Also after a smaller energy dose (illumination for 10 minutes with 0.1 W) cells with neuronal morphology were less frequently observed in these cultures.

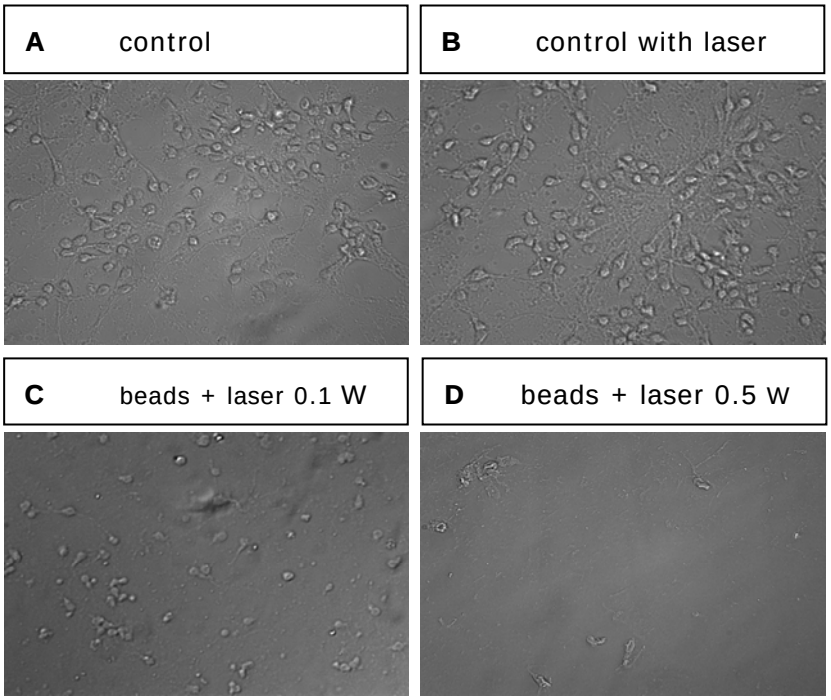


Fig. 52: Laser test in vitro leads to induction of cell death in embryonic day E14 cultures.
After 3 days in vitro coupled latex particles were added to half of the embryonic day E 14 cultures. All cultures were fixed two days after laser illumination.
(A) In the control no laser illumination and no latex particles were added. (B) In the second control laser illumination was performed without the addition of latex particles. Notably, no difference was observed between control experiments suggesting that the laser itself does not exhibit toxic effects in vitro.
(C, D) Cultures were severly affected after 10 minutes illumination with laser under both conditions with 0.1 W (C) and 0.5 W (D).

4.2.6.2 Transport of rhodamine latex beads and laser illumination

Next I tested the retrograde transport of rhodamine latex beads (without coupling to chlorine e_6) in vivo. Latex beads were injected into the cortex of adult mice. At least three up to eight injections were performed using a glass capillary at a maximal depth of 1.0 mm at the approximate level of bregma. To allow sufficient transport, animals were sacrificed seven days after beads injection. Rhodamine latex beads suspension remained close to the injection site. Immunohistochemistry for the mature neuronal marker NeuN (green) demonstrated that on the contralateral hemisphere demonstrated that the beads (red) have been transported retrogradely and are localised in the soma of contralateral projection neurons (Fig. **53**).

Following injection of coupled chlorine e_6 latex beads, I observed the same transport properties like in pure latex beads suspension. Following laser illumination, the neuronal marker NeuN (green) is down-regulated in the region containing neurons that had taken up and retrogradely transported the chlorine e_6 beads (Fig. **54**) and

neuroblasts were observed in the cortical area close to the subependymal zone presumably invading the cortex from this neurogenic niche (Fig. **54**).

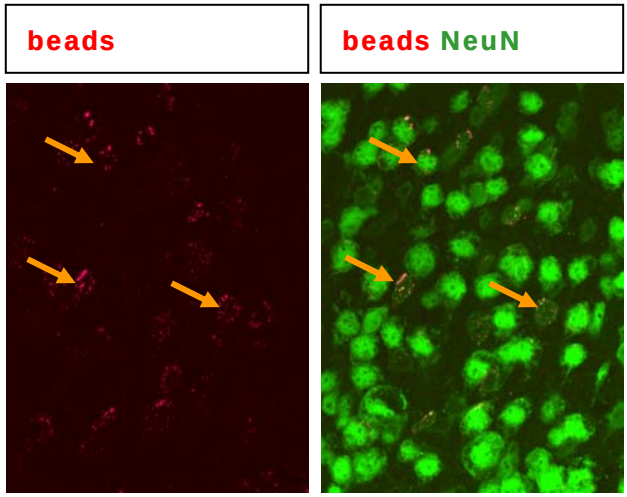


Fig. 53: Transport of rhodamine latex beads to the contralateral hemisphere.
Immunohistochemistry for NeuN (green) showed that latex particles are located in the soma of neurons in the contralateral hemisphere.Orange arrows indicate neurons containing latex beads.

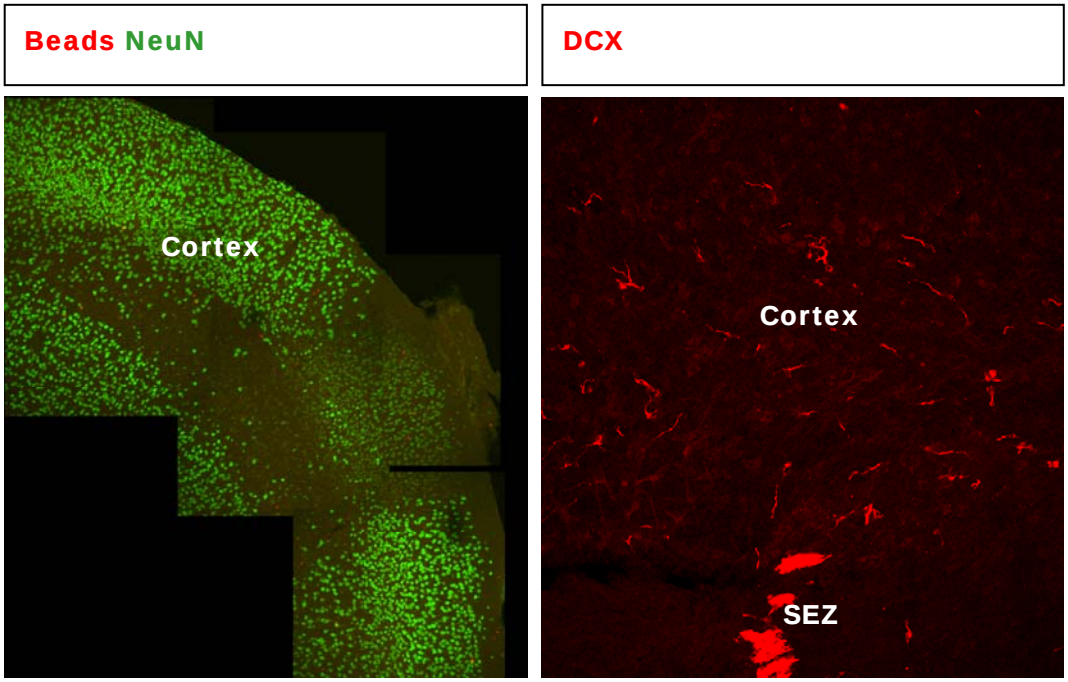


Fig. 54: Consequences of laser illumination in vivo
After an energy dose of 10 minutes and 10 mW the neuronal marker NeuN (green) is down-regulated suggestive of a neuronal loss upon laser illumination. Close to the subependymal zone (SEZ) neuroblasts (DCX, red) invaded the cortex presumably migrating from this neurogenic niche to the lesion site.

4.2.6.3 Tbr2-expressing progenitors migrate towards sites of neocortical injury

Given that Tbr2 immunoreactive progenitors are generated from dorsal regions of the subependymal zone and this neocortical injury paradigm is close to precisely these subependymal regions, we investigated if Tbr2-positive progenitors could be detected in the injured neocortex. Indeed, one week after laser treatment, clusters of Tbr2+/DCX+ neuroblasts were found in the corpus callosum (Fig. **56**) and some Tbr2+/DCX+ cells had also entered the cortical grey matter (Fig. **56**), while such invasion was never observed in control mice that did not undergo latex beads injection or laser illumination (Fig. **55**).

Thus, our data suggest that Tbr2-expressing progenitors in the adult dorsal subependymal zone may serve as an endogenous source of progenitors that can be recruited to the cerebral cortex upon injury.

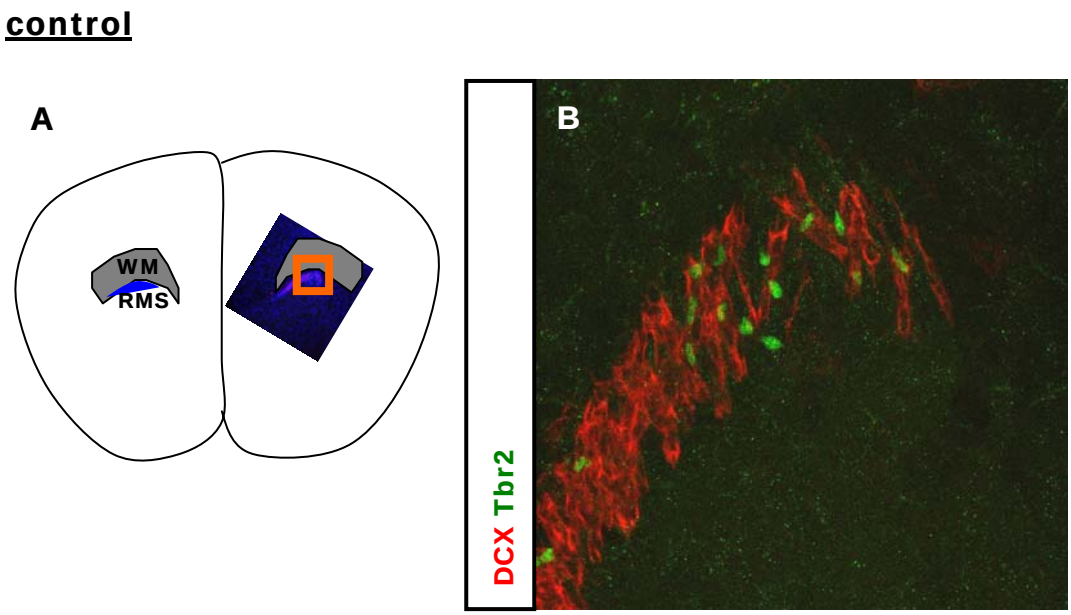


Fig. 55: Tbr2-positive cells are present in the rostral migratory stream in control mice.
(A) Schematic drawing depicting a coronal forebrain section. The white matter is indicated as grey fields underneath migrate neuroblasts towards the olfactory bulb (rostral migratory stream, blue). Orange boxed field is shown at higher magnification in (B).
(B) Immunostaining for Tbr2 (green) and DCX (red) demonstrates that Tbr2 expressing cells partially express DCX and are present in dorsal regions of the subependymal zone and rostral migratory stream.

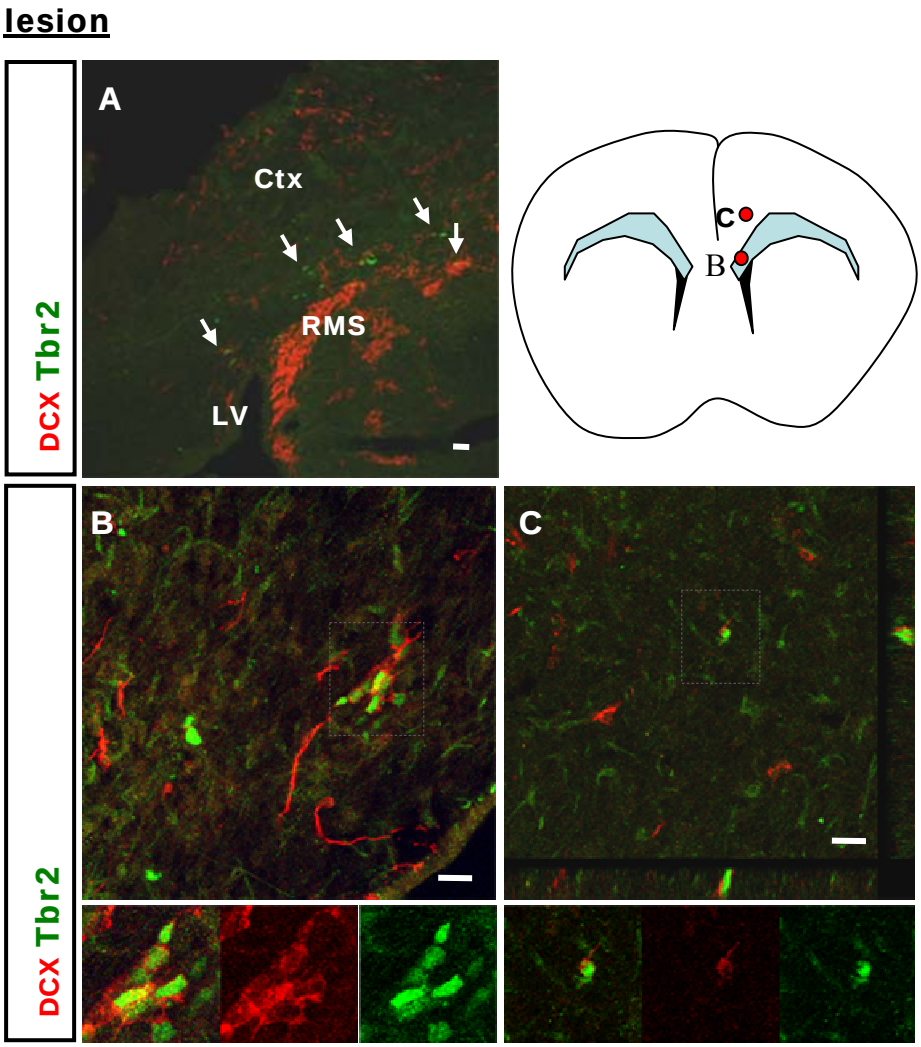


Fig. 56: Tbr2-positive cells migrate towards the lesioned cerebral cortex and express DCX.

(A) Overview of the lesioned cortex and rostral migratory stream one week after ChlorineE induced lesion of callosal projection neurons. Tbr2 expressing cells are present in dorsal regions of the subependymal zone and rostral migratory stream (arrows) and are sometimes clustered (B).
Notably, Tbr2-positive neuroblasts (C) are able to enter the cortical grey matter and may provide a source of endogenous repair.

5 Discussion

5.1 Summary

In this study I give evidence for the functional regionalization of the adult subependymal zone and the molecular mechanisms implementing the regionalization. In the ventral wall of the subependymal zone which is derived from the ganglionic eminences neuroblasts are specified by the transcription factor Dlx2. In contrast, the dorsal wall of the subependymal zone expresses transcription factors linked to cortical glutamatergic neurogenesis.

Dlx1 & Dlx2 are present in the majority of neuroblasts in the rostral migratory stream and in virtually all neuroblasts in the ventral subependymal zone and additionally, in the majority of ventral transit-amplifying progenitors. Notably, besides Dlx and BrdU double-positive transit-amplifying progenitors, I found Dlx-negative transit-amplifying progenitors some expressing Olig2 (Fig. **57**).

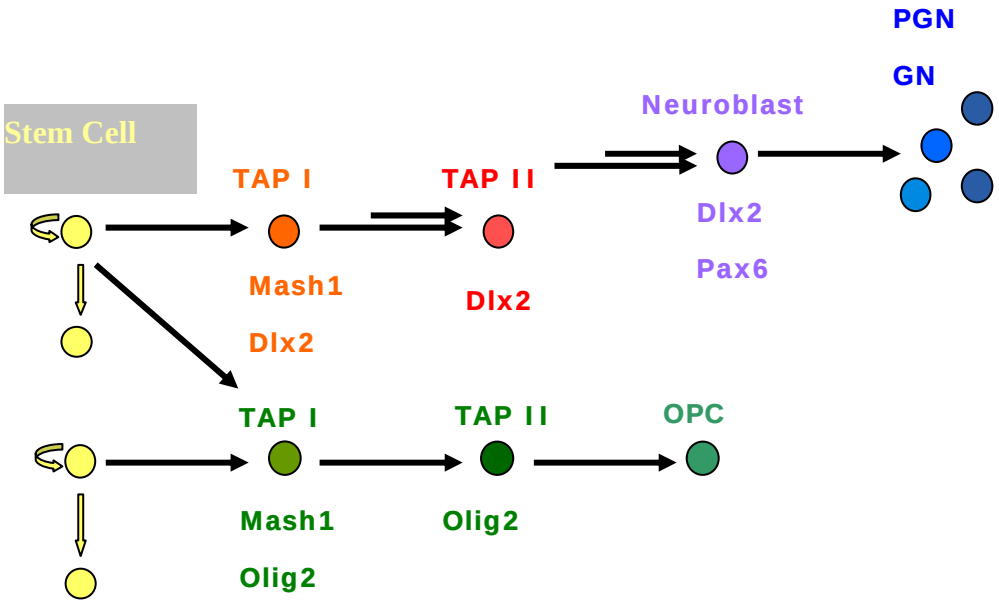


Fig. 57: Schematic lineage diagram of the adult ventral subependymal zone

The simplified diagram shows the lineage progression of adult neural stem cells (NSCs) of the adult subependymal zone. At least two distinct types of transit-amplifying progenitors (TAPs) are generated either from different or the same neural stem cells. Oligodendrocyte precursors (OPCs) of the white matter tract are generated from TAPs that express transcription factors Olig2 and Mash1. In contrast, transit-amplifying progenitors destined for generating neuronal progeny are positive for transcription factors of the Dlx-gene family and Mash1. Upon maturation neuroblasts express further transcription factors, such as Pax6, Dlx, Sp8 and Er81. TAP = transit-amplifying progenitor; PGN = periglomerular neuron; GN = granule neuron; OPC = oligodendroglial precursor cell

Given that over-expression of Olig2 in the adult subependymal zone promotes generation of oligodendrocytes (M. A. Hack et al., 2005) it is likely that this progenitor pool gives rise to oligodendrocytes. Thus, at least two populations in the ventral subependymalzone generate neuronal and oligodendroglial progeny. Notably, the transcription factor Mash1 is present in both lineages (Fig. **19** C, Fig. **31**).

Dominant-negative Dlx2 constructs demonstrate that repression of Dlx-mediated target gene activation leads to a block in neurogenesis and converts cells into the astroglial lineage. However, a small proportion of neuroblasts reached the olfactory bulb following Dlx2-Engrailed transduction suggesting that these cells were not affected and might be originated from the dorsal subependymal zone. Besides the generic role for Dlx2 in adult ventral neurogenesis Dlx2 plays a crucial role in periglomerular neuron specification. Cell-autonomous manipulations by injections of retroviral vectors demonstrate that Dlx2 potently promotes the specification of neuroblasts and regulates neuronal subtype specification favouring a dopaminergic periglomerular neuron fate. Notably, the latter effect occurs by a cooperation of Dlx2 and Pax6 at the expense of the calretinin-positive periglomerular neuron fate. Pax6 functions not only in specification of dopaminergic neurons but also plays a neurogenic role in the adult subependymal zone. We demonstrate that Pax6 is expressed highly in dorsal regions and low in ventral regions of the subependymal zone.

Here I described the adult generation of a novel population of glutamatergic olfactory neurons most likely arising from the dorsal wall of the subependymal zone. I demonstrated the presence of Tbr2 and Tbr1 expressing cells in the dorsal subependymal zone by immunohistochemistry. Furthermore I provided evidence that Tbr1 and Tbr2 positive cells are generated in the adult subependymal zone by BrdU labelling, retroviral injections into the rostral migratory stream, and in vivo genetic fate mapping. Consistent with the transcription factor sequence in cortical development (Pax6 → Ngn2 → Tbr2 → Tbr1) I demonstrate colocalization of Tbr proteins with Pax6 and that Tbr1 is followed by Tbr2. Furthermore we detected proliferating Neurogenin2-GFP expressing cells in Neurogenin2$^{+/GFP}$ mice (J. Seibt et al., 2003) that colocalized with Tbr2 protein in the rostral migratory stream (collaboration with Olivier Raineteau; experiments performed by Eleanor Helps). Notably, this lineage is distinct from Dlx expressing progenitors arising in ventral regions of the subependymal zone. We followed the fate of Tbr-GFP expressing progenitors by fate mapping analysis in Tbr2$^{BAC-GFP}$ mice (G. S. Kwon and A. K. Hadjantonakis, 2007) (collaboration with

Robert Hevner; experiments performed by Rebecca Hodge). Notably, we found BrdU+/Tbr2-GFP+ cells that had down-regulated Tbr2 protein located in the glomerular layer of the olfactory bulb. Furthermore, we demonstrate the generation of adult born vGluT2 expressing cells on mRNA and protein level in the olfactory bulb and give evidence for their functional integration with c-fos staining. Consistently, in subependymal zone cultures, we observed generation of Tbr2 progenitors and a small number of glutamatergic neurons that form functional glutamatergic synapses.

Thus, the high degree of specificity in the neuronal subtypes generated in the adult olfactory bulb is due to an unsuspected diversity of subependymal zone progenitors that are defined by their combinatorial expression of different transcriptional cues. These specific transcriptional codes are reminiscent of the patterning during development.

5.2 Function of Dlx2 in adult ventral-lateral neurogenesis

In order to examine the function of Dlx2 in the adult subependymal zone we constructed retroviral vectors for over-expression and dominant-negative approaches. Dlx2 over-expression in subependymal zone progenitors resulted in a higher proportion of DCX-positive neuroblasts amongst the transduced cells, due to an increase in neuronal specification, but not proliferation. Moreover, a larger proportion of Dlx2 transduced cells reached the olfactory bulb, due to an increase in migration velocity. Accordingly, Dlx2-Engrailed transduction causes a severe decline of neuroblasts both in vivo and in vitro. Dlx2-Engrailed primarily suppresses those genes that would normally be activated by Dlx2, but possibly affects also targets of Dlx1. As little is known about the targets of these transcription factors or the proteins with which they interact to regulate their targets, we cannot exclude that Dlx2-Engrailed may also act on Dlx1 regulated genes. The possible compensation for the loss of Dlx2 by Dlx1 as observed in mice with targeted deletion of these genes individually (M. Qiu et al., 1995; S. A. Anderson et al., 1997a) prompted us to take this approach rather than a protein knock-down that may require elimination of both Dlx1 and Dlx2. Our results demonstrate that Dlx2 and/or Dlx1 activated target genes are essential for progression towards a neuronal lineage and for initiation or maintenance of a high proliferation rate. Indeed, the latter role fits well with the early expression of Dlx2 in transit-amplifying progenitors (F. Doetsch et al., 2002).

In the light of my data on the neurogenic function of Dlx2 in adult subependymal zone progenitors, the expression of Dlx transcription factors in a subset of transit-amplifying

progenitors (Colak et al., 2008) suggests that the neurogenic lineage becomes already determined at the transit amplifying progenitor stage. The existence of at least two subtypes of transit-amplifying progenitors in the ventral subependymal zone, characterized by the mutual exclusive expression of Olig2 and Dlx1 & Dlx2 under physiological conditions, suggests that these cells belong to distinct lineages. In contrast to the observations in the adult subependymal zone, substantial coexpression of Dlx1 & Dlx2 and Olig2 occurs in the embryonic ventral telencephalon where a common oligodendrocytes / interneuron precursor has been identified (G. Miyoshi et al., 2007; M. A. Petryniak et al., 2007). During embryonic development, deletion of Dlx1 & 2 results in a fate switch from neuro- to oligodendrogenesis (M. Kohwi et al., 2007b). Therefore, we examined the transcription factor Olig2, a key determinant of oligodendrocyte fate in development (Q. R. Lu et al., 2002; D. H. Rowitch, 2004) and adulthood (M. A. Hack et al., 2005; B. Menn et al., 2006; D. Colak et al., 2008). Here specification towards the neuronal lineage appears to occur via a gradual Dlx1 & Dlx2-mediated down-regulation of Olig2, while the lack of co-expression of these transcription factors in the adult subependymal zone suggests that other mechanisms are involved in the suppression of Olig2. Indeed we found that in the adult subependymal zone, BMP–mediated signalling – which is conspicuously absent in the developing ventral telencephalon (T. Shimogori et al., 2004; M. Fernandes et al., 2007) - is required for Olig2 suppression (D. Colak et al., 2008). Thus, besides similarities, there are important differences in the precise molecular mechanisms involved in neuronal versus oligodendroglial fate specification in the developing and adult telencephalon. Indeed, many adult subependymal zone progenitors adopt an astroglial identity following expression of Dlx2-Engrailed, while most cells deficient of Dlx1 and Dlx2 in the developing telencephalon revert to an oligodendroglial fate (M. A. Petryniak et al., 2007). Intriguingly, adult neural stem cells are of astroglial identity (F. Doetsch et al., 1999b) and we observed a certain proportion of GFAP-positive, Dlx2-Engrailed transduced cells incorporating the DNA analogue BrdU, suggesting that some progenitors, when inhibited to progress towards neurogenesis, revert to an astrocytic fate what might include a neural stem cell fate. This possibility could be tested in a neurosphere forming assay upon injection of Dlx2-Engrailed into the subependymal zone. The transduced Dlx2-Engrailed stem cells could be isolated, cultured, and could be identified by GFP that they inherit to their daughter cells within a sphere.

5.3 Transcriptional code for GABAergic versus glutamatergic periglomerular neuron specification

5.3.1 Specification of GABAergic periglomerular neurons from ventral – lateral Dlx transcription factors expressing progenitors

Besides its role in generic neurogenesis, Dlx2 also exerts an important function in neuronal subtype specification, promoting the acquisition of a periglomerular neuron identity, in particular of the dopaminergic subtype. Notably, these effects of Dlx2 are virtually identical to those of Pax6 (M. A. Hack et al., 2005). Like in the case of Dlx2, over-expression of Pax6 results in increased neurogenesis, while interfering with Pax6 function inhibits neurogenesis (M. A. Hack et al., 2005). Moreover, Pax6 promotes the subtype specification of dopaminergic periglomerular neurons (M. A. Hack et al., 2005; M. Kohwi et al., 2005). These findings are suggestive of a cooperation of these transcription factors. While we found no evidence for either of these factors regulating expression of the other in adult subependymal zone derived cells, co-immunoprecipitation of Pax6 and Dlx proteins demonstrated their physical interaction. More strikingly, in the absence of Pax6 protein Dlx2 no longer promotes the acquisition of a periglomerular neuron fate demonstrating that Pax6 and Dlx2 are each required for the specification of tyrosine hydroxylase expressing periglomerular neurons. Cells double-positive for Dlx2 / Pax6 increase from roughly 10 % in the subependymal zone to 20 % in the rostral migratory stream suggesting that some of the tyrosine hydroxylase positive cells are already generated in the subependymal zone. The proportion of double-positive cells is even higher considering only the neuroblast population reaching about 40 %. This higher percentage of double-positive cells within the rostral migratory stream is consistent with our previous findings that the rostral migratory stream contains most of the stem / progenitors giving rise to periglomerular neurons including dopaminergic neurons in mice (M. A. Hack et al., 2005; M. Alonso et al., 2008) and in rat (Mendoza-Torreblanca et al., 2008). However, the proportion of periglomerular neurons resulting from rostral migratory stream injections is only 10 % after three weeks and even smaller for the proportion of tyrosine hydroxylase positive neurons amongst these. This could be explained by a large fraction of the newly arriving neurons undergoing cells death, or by Pax6 and Dlx2 cooperating on a broader fate specification that is then further refined by extrinsic signals within the olfactory bulb.

Thus, the present and previous studies suggest the following transcriptional code for the specification of GABAergic periglomerular neurons: (i) Calretinin+ periglomerular neurons lack Pax6 and Dlx, but express Sp8 (Z. J. Allen, 2nd et al., 2007a), (ii) calbindin+ periglomerular neurons contain Dlx and Meis2, (Z. J. Allen, 2nd et al., 2007b), but not Pax6, and (iii) dopaminergic periglomerular neurons contain both Dlx and Pax6 (and Meis2 and Er81, Allen et al., 2007). Our functional analysis highlights the necessity for Pax6 and Dlx co-expression and collaborative function for the specification of dopaminergic periglomerular neurons. In the absence of the function of Pax6, periglomerular neurons fail to differentiate along the dopaminergic lineage, but rather assume a calretinin+ fate (hence the periglomerular neurons subtype lacking Pax6 and Dlx2).

The above mentioned transcriptional code would suggest that over-expression of Dlx2 in the absence of Pax6 would lead to a calbindin+ fate. However, Dlx2 over-expression could not rescue or convert the fate of transduced cells into calbindin+ periglomerular neurons in the absence of Pax6, consistent with the absence of endougenous Pax6 in this population. Notably, the transcription factor Meis2 is required for a calbindin+ fate. If Meis2 expression would be inhibited by the presence of Pax6, Cre-mediated loss of Pax6 would lead to upregulation of Meis2 and a calbindin+ fate upon over-expression of Dlx2. As this is not the case, Pax6 presence at early maturation stages might be required for Meis2 expression. Additionally, over-expression of Dlx2 might not be able to induce Meis2 presence and hence could not convert the transduced cells into a calbindin+ fate. This would suggest that Meis2 might be regulated by Pax6, but not Dlx2, expression. Alternatively, additional unkown factors besides Dlx2 and Meis2 might be required to assess a calbindin+ periglomerular neuron fate.

Notably, calretinin+ neuron specification requires Sp8 (Z. J. Allen, 2nd et al., 2007a) and in the absence of Sp8 Pax6 is up-regulated, suggesting that the balance between Pax6 / Dlx2 and Sp8, respectively, regulates the proportion of periglomerular neurons with distinct identities. Conversely, over-expression of either Pax6 or Dlx2 promotes dopaminergic at the expense of calretinin+ periglomerular neurons. This indicates that the respective increase in calretinin+ periglomerular neurons is not merely a ‘passive’ consequence of the loss of the dopaminergic phenotype, but rather that Dlx2 and Pax6 actively inhibit calretinin+ subtype specification.

These observations also bear relevance in regard to the diverse origin of periglomerular neuron subtypes at distinct positions and the proposition that distinct stem cell pools are

fate-restricted towards the generation of specific neuronal subtypes (M. A. Hack et al., 2005; W. Kelsch et al., 2007; F. T. Merkle et al., 2007). Our studies indicate a substantial degree of plasticity: forced expression of either Pax6 or Dlx2 in the rostral migratory stream can convert progeny towards a tyrosine hydroxylase positive periglomerular neuron fate both at the expense of calretinin+ periglomerular neurons and granule cells indicating that independent of their respective origin the derivatives of distinct stem cell pools have the competence to interpret the transcriptional cues and adopt different neuronal subtypes. Given that the majority of adult subependymal zone stem cells are derived from the ganglionic eminences (K. M. Young et al., 2007), the fate restriction of their progeny may be acquired already at the embryonic stage and inherited to the adult offspring. Alternatively, adult subependymal zone stem cells may be exposed to local domains of niche factors such as BMP and Sonic hedgehog (V. Palma et al., 2005; D. Colak et al., 2008) thereby creating distinct progenitor domains with different fate restrictions.

5.3.1.1 Function of dopaminergic periglomerular neurons in the adult olfactory bulb

Periglomerular neurons synapse on the incoming olfactory sensory neurons and on mitral cells. The vast majority of periglomerular cells are of GABAergic identity, nevertheless they exhibit a great heterogeneity. Some of the glutamic acid decarboxylase (GAD) immunoreactive periglomerular neurons release in addition to their neurotransmitter GABA also dopamine and express tyrosine hyrdroxylase (B. J. Maher and G. L. Westbrook, 2008). Interestingly, upon odour deprivation or naris occlusion the levels of tyrosine hydroxylase expression decrease dramatically and recover after functional inputs are restored (H. Baker et al., 1984; E. Weruaga et al., 2000; J. G. Brinon et al., 2001). This suggests that the expression of tyrosine hydroxylase in periglomerular neurons depends on peripheral stimulation (H. Baker et al., 1983; H. Baker, 1990). Notably, early neonatal unilateral anosmic rats showed reduced size of the deprived olfactory bulb and a loss of neurons in the granule cell layer and glomerular cell layer (P. C. Brunjes, 1994). This indicates that the day of naris occlusion plays a crucial role in the results, which is consistent with the postnatal generation of some olfactory neurons (P. C. Brunjes, 1994).

Furthermore, axonal projections of periglomerular neurons are generally short and their size is rather small compared to dopaminergic neurons of the substantia nigra. Dopaminergic periglomerular neurons synapse on olfactory sensory neurons and belong

to the type I glomerular neurons (K. Kosaka et al., 1997) and act in concert with calbindin+ and calretinin+ periglomerular neurons. Dopamine has been shown to inhibit synaptic transmission from the olfactory nerves by acting presynaptically on dopamine (D2) receptors (A. Y. Hsia et al., 1999; D. A. Berkowicz and P. Q. Trombley, 2000; M. Ennis et al., 2001; G. Shepherd, 2004) and postsynaptically on $GABA_A$ receptors (I. Brunig et al., 1999; G. Shepherd, 2004). Dopamine receptor activation in the olfactory bulb causes a significant depression of synaptic transmission at the first relay between olfactory receptor neurons and mitral cells (A. Y. Hsia et al., 1999). Thus dopamine plays an important role in olfactory bulb processing and acts directly on input control.

The generation of dopaminergic tyrosine hydroxlase positive periglomerular neurons is of particular interest in light of diseases where dopaminergic neurons degenerate, e. g. Parkinson's disease (S. B. Dunnett et al., 2001; J. Lotharius and P. Brundin, 2002). Transplantation or migration of endogenous progenitors that give rise to dopaminergic neurons and integrate synaptically would have the potential to ease these diseases. Several embryonic brain cell transplantation studies in Parkinson's disease have been performed with various results (S. B. Dunnett et al., 2001; I. Mendez et al., 2008). Additionally, it is of particular importance to understand the molecular cues leading to a dopaminergic phenotype in adult neurogenesis, since these cells might serve as a potential source for endogenous repair.

5.3.2 Specification of glutamatergic periglomerular neurons from dorsal Tbr1 & Tbr2 expressing progenitors

Thus far, the adult subependymal zone had been viewed as a region giving rise nearly exclusively to GABAergic olfactory interneurons. Here we describe a novel type of olfactory interneuron that has a glutamatergic phenotype. The transcription factors involved in the generation of glutamatergic neurons is conserved in cortical, cerebellar and hippocampal neurogenesis and follows the sequence Pax6 → Ngn2 → Tbr2 → Tbr1.

5.3.2.1 Transriptional code of adult-born glutamatergic olfactory neurons

Our discovery of generation of glutamatergic olfactory neurons not only further support the concept of subependymal regionalization, but also suggests the persistence of several Pax6 regulated lineages in the adult subependymal zone. While Pax6 is co-

expressed and functions together with Dlx2 in the adult subependymal zone to generate dopaminergic olfactory interneurons (M. A. Hack et al., 2005), Neurogenin2 or Tbr2 never colocalized with Dlx transcription factors. However, they also overlapped with Pax6, supporting the existence of two different lineages arising from Pax6-positive progenitors. This much smaller population up-regulates Neurogenin2 and Tbr2 at the neuroblast stage, followed by Tbr1. Notably, the dorsal subependymal zone that contains the Neurogenin2 expressing progenitors has been shown to originate from the dorsal telencephalon during embryogenesis (K. M. Young et al., 2007), the region that also expresses Neurogenin2 during development (G. Gradwohl et al., 1996; L. Sommer et al., 1996; C. Fode et al., 2000). These data therefore suggest that patterning and regionalization persists at least partially from development into the adult neurogenic zones.

As observed for Pax6, also Mash1 expressing progenitors seem to proceed along two different lineages. Mash1 is clearly involved in the generation of GABAergic granule cells and periglomerular neurons, at least at postnatal stages (C. M. Parras et al., 2004). Notably, both Mash1 as well as Pax6 are already expressed in transit-amplifying progenitors, i.e. prior to the neuroblast fate (C. M. Parras et al., 2004). Neurogenin2 and Tbr1 appear later in the lineage, exclusively at the neuroblast stage. While some of the cells expressing these two transcription factors still divide and incorporate BrdU, Tbr1 expressing cells no longer divide and hence represent postmitotic DCX-positive neuroblasts migrating towards the olfactory bulb. Indeed, this sequence of transcription factors has been shown to be a direct functional cascade of transcriptional regulation during cortical development: (i) Pax6 directly regulates Neurogenin2 (R. Scardigli et al., 2003), (ii) and Neurogenin2 regulates Tbr2 and Tbr1 (C. Fode et al., 2000; C. Schuurmans et al., 2004; B. Berninger et al., 2007; P. Mattar et al., 2008). In both cases, the loss of Pax6 or Neurogenin2 leads to an at least partial diversion from the generation of glutamatergic to GABAergic neurons (C. Schuurmans et al., 2004; V. Nikoletopoulou et al., 2007; D. D. O'Leary et al., 2007). Moreover, over-expression of Neurogenin2 in adult derived neurosphere cells is sufficient to up-regulate Tbr1 and direct virtually all neurons towards a functional glutamatergic identity (B. Berninger et al., 2007). Additionally, we performed short-term fate mapping analysis using $Tbr2^{BAC\text{-}GFP}$ in order to follow the GFP-positive progeny derived from Tbr2 expressing progenitors (collaboration with Robert Hevner) (D. Geschwind, 2004; G. S. Kwon and A. K. Hadjantonakis, 2007). We observed that adult Tbr2 expressing progenitors found

in the rostral migratory stream have already largely down-regulated the Tbr2 protein when they arrive in the olfactory bulb, while some still express Tbr1. However, several weeks after BrdU incorporation, most of the BrdU labelled vGluT2-positive neurons did no longer contain Tbr1, suggesting that Tbr1 becomes also down-regulated.

In summary, this molecular code therefore strongly implicates the Neurogenin2-positive and Tbr2-positive progenitors as candidates for the generation of the vGluT2 expressing glutamatergic olfactory neurons of the glomerular layer. Taken together with the observation that progenitors located in the dorso-rostral subependymal zone also contribute to the generation of some non-GABAergic, calretinin+ interneurons (M. Kohwi et al., 2005; K. Kosaka and T. Kosaka, 2007; S. Parrish-Aungst et al., 2007; K. M. Young et al., 2007) these findings further support the regional differences between dorsal and ventral regions of the adult subependymal zone with the later generating GABAergic neurons while the former generate additional neurons, including the glutamatergic subtype.

5.3.2.2 Functional integration and electrophysiology of glutamatergic olfactory neurons

Here we provide two independent sets of evidence that a subset of adult subependymal zone stem or progenitor cells can indeed generate glutamatergic neurons. Firstly, we found that some adult birth-dated BrdU labelled cells in the glomerular layer express vGluT2, a transporter for glutamate into synaptic vesicles. Secondly, in primary adult subependymal zone cultures we could detect vGluT immunoreactive neurons which exhibited the characteristic punctuate pattern suggestive of the formation of functional synapses. Additionally we also observed functional glutamatergic transmission by electrophysiology. I also provide evidence that these Tbr2-positive progenitors are indeed generated in these cultures by retroviral transduction shortly after preparation.

The physiological maturation and integration of glutamatergic olfactory cells is supported by the expression of the immediate-early gene c-fos, a member of a family of transcription factors that are rapidly regulated by neuronal activity (S. S. Magavi et al., 2005). Detection of c-fos has previously been used to demonstrate integration of newborn neurons in the adult dentate gyrus (S. Jessberger et al., 2007) and the olfactory bulb (K. M. Guthrie et al., 1993; S. S. Magavi et al., 2005). In the olfactory bulb, c-fos and other immediate-early genes are specifically expressed in neurons and reflect with fidelity the pattern of neuronal activity (S. S. Magavi et al., 2005). Neuronal activity in

the olfactory bulb measured by electrophysiological as well as optical imaging techniques produces a similar pattern as immediate early gene expression in the olfactory bulb (D. A. Wilson and M. Leon, 1988; M. Meister and T. Bonhoeffer, 2001; S. S. Magavi et al., 2005). The expression of c-fos in the BrdU labelled vGluT2-positive olfactory neurons therefore strongly supports a functional integration of these newborn glutamatergic neurons into the neuronal network several weeks after their birth. Consistent with their glutamatergic identity these cells do not express GABAergic neuron markers. Thus, we conclude that vGluT2 expressing neurons are truly glutamatergic neurons rather than exhibiting a mixed transmitter phenotype like dopaminergic - GABAergic periglomerular neurons.

5.3.2.3 Generation of specific glutamatergic subtypes at embryonic and adult stages

Glutamatergic neurons in the olfactory bulb comprise several sets of projection neurons, amongst them are mitral and tufted cells, and several classes of glutamatergic interneurons in the external plexiform layer and glomerular layer, such as short axon cells and external tufted cells or tufted interneurons (K. Kosaka and T. Kosaka, 2007; S. Parrish-Aungst et al., 2007; S. Saino-Saito et al., 2007). Several members of the T-box transcription factor family, such as Tbr1, Tbr2 and Tbx21 (R. F. Hevner et al., 2001), are expressed in olfactory bulb projection neurons and interneurons in the external plexiform layer and glomerular layer (A. Bulfone et al., 1998; Z. J. Allen, 2nd et al., 2007a). Our analysis of Tbr1 and Tbr2 in combination with the expression of the vGluT1 & vGluT2 revealed a surprising heterogeneity. A significant proportion of vGluT1 and / or vGluT2 expressing cells in the glomerular layer do not express Tbr1 or Tbr2, while other vGluT1 or vGluT2 positive cells express these factors. This is not due to their recent birthdate, as already three weeks after BrdU incorporation vGluT2 expressing neurons have down-regulated Tbr1 or Tbr2. In contrast other olfactory neurons born during embryonic neurogenesis, e. g. mitral cells, maintain these transcription factors. Notably, periglomerular neurons that maintain Tbr1 or Tbr2 expression are both generated during embryonic neurogenesis what can be demonstrated by BrdU labelling at embryonic day E14 (experiments performed by Jovica Ninkovic). Given that vGluT2-positive but not vGluT1-positive olfactory neurons are adult generated we further tested if the embryonically generated vGluT2 or vGluT1 positive population would remain constant over time or decrease with age. We injected BrdU at embryonic day E 14 and sacrificed animals at postnatal day P16 or

P60. Consistent with the notion that vGluT2 expressing neurons generated in the embryo become replaced by adult generated ones, the proportion of vGluT2-positive cells labelled with BrdU at E14 declined whereas the vGluT1 expressing population remained constant between P16 and P60, supporting the view that the vGluT1-positive population is entirely established at embryogenesis and not altered by adult neurogenesis (experiment performed by Jovica Ninkovic). Thus, some olfactory neurons differ in their expression of either vGluT1 or vGluT2. Notably, mitral cells express both vGluT1 and vGluT2. Amongst these different types only the vGlut2-expressing neurons (not co-expressing Tbr1 or 2) located in the glomerular layer, but not those in the external plexiform layer, were generated in the adult subependymal zone, while the generation of most other subtypes of glutamatergic neurons in the olfactory bulb is restricted to embryonic development. This specificity has also been observed for the GABAergic interneurons with only some of them continuing to be newly generated life-long while other subtypes are exclusively generated at embryonic stages (D. C. Lagace et al., 2007; J. Ninkovic et al., 2007; R. Batista-Brito et al., 2008; I. Imayoshi et al., 2008).

5.3.2.4 Function of glutamatergic periglomerular cells in the adult olfactory bulb

An exciting question is the functional role of this specific glutamatergic interneuron population in the adult olfactory bulb. It has been previously shown that vGluT2 expression is particularly high in the glomerular layer and besides being present in olfactory nerve endings it can be also detected in juxtaglomerular neurons such as external tufted neurons (M. M. Gabellec et al., 2007). Thus, the fact that the newly added glutamatergic neurons become selectively incorporated into the glomerular layer is consistent with their specific choice of vGluT2 expression. The intriguing specificity of adult generation of vGluT2 expressing neurons in the glomerular layer prompts the suggestion that they perform a specific role in the glomerular neuronal network, the first synaptic station of the incoming afferents. Interestingly, synapses using vGluT2 for loading synaptic vesicles generally appear to exhibit a higher release probability compared to those expressing vGluT1 (G. J. Murphy and J. S. Isaacson, 2003). Since periglomerular neurons send their axons into the neighbouring glomeruli one function might be to activate nearby glomeruli. For this reason, the neuronal network in the glomerular layer is not only composed of inhibiting neurons, but also contains excitatory glutamatergic periglomerular neurons. Hence, the newly discovered

generation of glutamatergic interneurons in the glomeruli might enhance the complex view about the connectivity between neighbouring glomeruli and processing of incoming signals from the olfactory sensory neurons. The selective replacement of the vGluT2 expressing subtype might suggest that they change the network over time and that these glomeruli change their pattern of activation over time.

Periglomerular neurons are particularly affected by adult neurogenesis and are constantly turned over (J. Ninkovic et al., 2007). Furthermore, we could not determine so far if the newly generated glutamatergic olfactory neurons belong to type 1 or type 2 periglomerular neurons. Whereas type 1 periglomerular neurons contact olfactory sensory neurons, type 2 establishes synapses mostly with mitral or external tufted cells. Notably, some type 2 calretinin+ but GABA immunonegative neurons have been identified in the mouse olfactory bulb (K. Kosaka and T. Kosaka, 2007). Amongst the periglomerular neurons generated over a period of nine months in adult mice, the dopaminergic-GABAergic subpopulation dominates, followed by a prominent contribution of calretinin+ neurons (J. Ninkovic et al., 2007). However, about 5 % of adult generated periglomerular neurons were marker-negative for calbindin, calretinin, GABA, and tyrosine hydroxylase (J. Ninkovic et al., 2007) and hence may correspond to the newly generated vGluT2 expressing population described here.

Additionally, adult generated neurons respond in a different manner to odour stimuli than embryonic generated neurons. Newly generated neurons are most responsive to novel odours soon after their synaptic integration into the olfactory network. Moreover, their learning/habituation paradigms are different, with the adult generated neurons rather enhancing their activity upon repetitive stimulation while the embryonic generated neurons decrease their response (S. S. Magavi et al., 2005).

These considerations further highlight the distinct contribution that each of these neuronal subtypes makes to the neuronal network, not only due to the specific generation of subpopulation of interneuron during adult neurogenesis but also due to their different turn-over and functional activity.

5.4 Regionalization of the adult subependymal zone and neuronal subtype specification

Recent evidence indicates that the subependymal zone in adult mice is regionalized. Stem cells of the rostral migratory stream produce a substantial number of periglomerular dopaminergic neurons (M. A. Hack et al., 2005; M. Alonso et al., 2008;

J. G. Mendoza-Torreblanca et al., 2008); Cre-mediated fate mapping with reporter lines demonstrate that the adult subependymal zone contains stem cells derived from multiple regions of the embryonic neuroepithelium (medial and lateral ganglionic eminence, and cortex) and generate distinct subtypes (calbindin, calretinin, and tyrosine hydroxylase) of olfactory interneurons in different proportions (M. Kohwi et al., 2007a; K. M. Young et al., 2007); especially Emx1-Cre lines demonstrated the participation of the dorsal wall of the lateral ventricle that generates tyrosine hydroxylase and calretinin expressing periglomerular neurons, however, no calbindin-positive cells derived from Emx1-Cre could be observed (K. M. Young et al., 2007); superficial and deep granule neurons are generated from two different pools of neural stem cells residing in rostral and caudal regions of the subpependymal zone (W. Kelsch et al., 2007); and a detailed analysis of the progeny of neural stem cells along the whole ventricular rostro-caudal axis demonstrated that stem cells in different locations give rise to distinct subpopulations of olfactory interneurons (F. T. Merkle et al., 2007). Consistent with the idea of regionalization, neural stem cells produce the specific progeny characteristic for their region of origin even when they were heterotopically grafted in other areas of the subependymal zone or grown in culture (W. Kelsch et al., 2007; F. T. Merkle et al., 2007). These data suggest that adult neural stem cells are restricted in their potential and are only able to generate specific types of neurons (H. Simon et al., 1995). Taken together, these reports show the regionalized generation of distinct olfactory interneuron populations produced from fate restricted neural stem cell pools. However, the subependymal zone was so far still believed to generate nearly exclusively GABAergic interneurons with a small population of Calretinin-positive periglomerular neurons that were GABA-immunonegative (K. Kosaka and T. Kosaka, 2007; P. Panzanelli et al., 2007; S. Parrish-Aungst et al., 2007).

Notably, when we cultured the dorsal and ventral wall separately, I observed an enrichment of Tbr2-positive progenitors only in the dorsal wall derived cultures. Thus, Tbr2 expressing progenitors are derived from dorsal neural stem cells. Furthermore, this excludes the alternative that Tbr2-positive progenitors are generated in ventral regions and up-regulate Tbr2 during migration to dorsal-rostral regions of the subependymal zone. Thus, our in vitro and in vivo data demonstrate the adult subependymal zone as a novel source for adult generated glutamatergic olfactory neurons.

In my studies I now identified a Dlx-dependent lineage arising from ventral regions of the adult subependymal zone which expresses Dlx and Pax6 and a novel dorsal subependymal progenitor pool that undergoes the same transcription factor sequence that characterizes the specification of many glutamatergic subtypes throughout the brain, e. g. cortical and cerebellar neurogenesis (R. F. Hevner et al., 2006): Pax6 → Neurogenin2 → Tbr2 → Tbr1. Pax6 is coexpressed in most Neurogenin2 or Tbr2 expressing cells and Pax6 is also expressed in some Dlx-positive cells in the subependymal zone. Pax6 and Tbr2 expressing cells partially colocalize with Mash1, a transcription factor present in progenitor cells, whereas hardly any Tbr1 expressing cell was positive for Mash1. Additionally, after a short BrdU pulse, I observed only Tbr2/BrdU double-positive cells. However, three days after the BrdU pulse we found Tbr1/BrdU+ cells suggesting that these are the immediate progeny of Tbr2-expressing progenitors.

My findings highlight that neural stem cells rather than being plastic and homogenous, produce only a specific type of neuron according to their developmental origin. My data are consistent with previous reports which suggest that the dorsal wall of the subependymal zone generate olfactory interneurons and contributes to adult olfactory neurogenesis (R. E. Ventura and J. E. Goldman, 2007; K. M. Young et al., 2007; S. Willaime-Morawek and D. van der Kooy, 2008). However none of these studies reported that neural stem cells of the dorsal wall would generate glutamatergic progeny.

5.4.1 The role of Pax6 and Mash1 in neural lineages arising from the subependymal zone

According to the developmental origin of dorsal subependymal neural stem cells we discovered Tbr1 & Tbr2, and Neurogenin2 expressing cells in this neurogenic niche. We demonstrated immunofluorescence for Tbr1 and Tbr2, and secondly took advantage of $Tbr2^{BAC\text{-}GFP}$ mice for fate mapping analysis of Tbr2-expressing cells. We could also detect Neurogenin2 protein and GFP in $Ngn2^{+/GFP}$ mice, and most of these Ngn2-GFP expressing cells colocalized with Tbr2 or Tbr1 protein (collaboration with Olivier Raineteau). Notably, Tbr1 and Tbr2 both colocalized with Pax6 in the dorsal supbependymal zone and rostral migratory stream. This data not only support the concept of regionalization, but also suggest the persistence of an unkown progenitor pool. Furthermore, this lineage is derived from a Pax6 expressing progenitor state. Notably, Pax6 and Mash1 colocalize and Mash1 is also present at early progenitor

stages. Interestingly, also ventral derived lineages in the adult subependymal zone are regulated by Pax6 and are furthermore defined by the presence of Dlx1 & Dlx2. Interestingly, during embryonic development Pax6 and Dlx are also shared in a lineage (G. S. Mastick and G. L. Andrews, 2001). In contrast Pax6-positive progenitors arising from dorsal regions express sequentially Neurogenin2, Tbr2, and Tbr1 (Fig. **58**). Moreover, Pax6 is crucial for adult neurogenesis since loss-off-function experiments demonstrated a block into the neuronal lineage progression (M. A. Hack et al., 2004; M. A. Hack et al., 2005). During embryonic neurogenesis Pax6 is present in radial glial cells and governs the generation of cortical neurons whereas it is present only at low levels in the lateral ganglionic eminence (M. Gotz et al., 1998; N. Heins et al., 2002; P. Malatesta et al., 2003; T. T. Kroll and D. D. O'Leary, 2005). The expression of Pax6 laregely restricted to dorsal regions in the adult subependymal zone reflects its embryonic expression and supports the regionalization of the adult neurogenic niche. However, the direct interaction of Dlx transcription factors and Pax6 in the ventral subependymal zone and rostral migratory stream exhibits a difference to the embryonic telencephalon where Pax6 is exclusively expressed in dorsal regions (M. Gotz et al., 1998; N. Heins et al., 2002; C. Englund et al., 2005). This developmental regionalization is further maintained by the expression of Dlx transcription factor that is predominantly present in ventral regions of the adult subependymal zone. According to their developmental regionalization Dlx transcription factors never colocalized with Tbr1 (Fig. **42**). This data suggest that there are at least two different lineages arising from Pax6 expressing progenitors, a larger defined by Pax6 and Dlx expression and a smaller lineage defined by the subsequent appearance of the transcription factor Pax6, Neurogenin2 and Tbr2 (Fig. **58**).

Pax6-positive cells in the subependymal zone can be specified towards a specific neuronal lineage at the transit-amplifying progenitor state, or neural stem cells are determined to generate specific progeny depending on their location. My data suggest that neural stem cells in different regions generate specific lineages, one expressing Pax6 and/or Dlx in ventral regions and one determined by the presence of Pax6/Tbr1 & Tbr2 in dorsal regions.

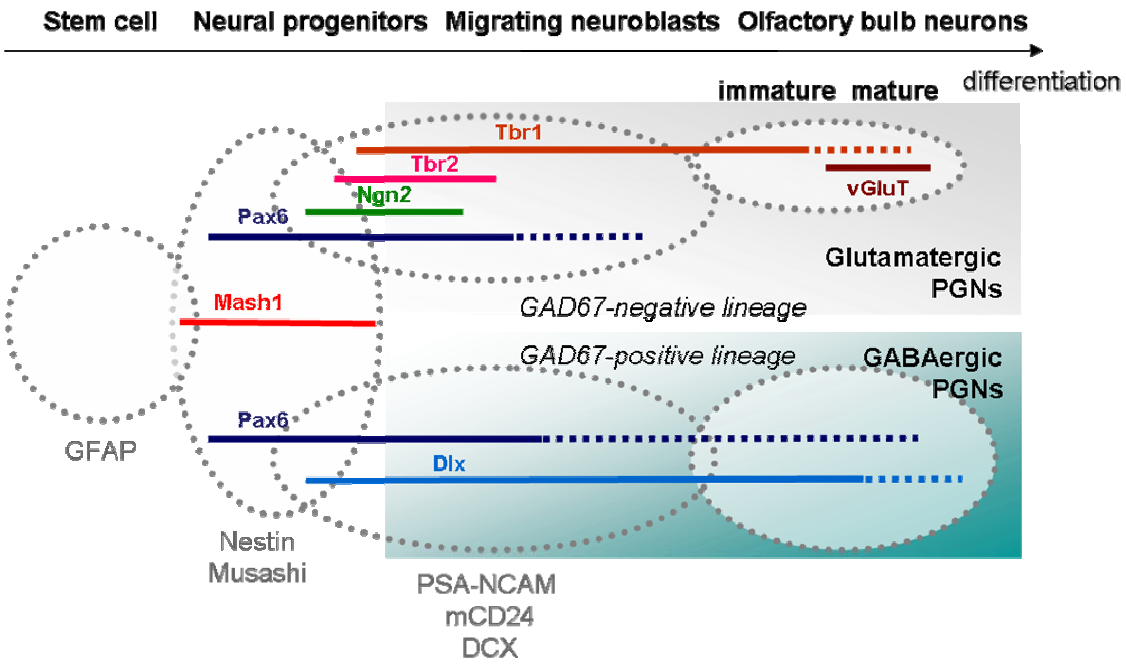


Fig. 58: Summary of the transcription factor sequence in a subset of neuroblasts derived from the rostral-dorsal subependymal zone.

At least two separate lineages can be distinguished from progenitors expressing Mash1+ and Pax6+: (i) the majority expressing transcription factors of the Dlx gene family that are linked with the generation of GABAergic olfactory interneurons and are GAD67-positive. Following maturation this lineage forms GABAergic periglomerular neurons. (ii) a subpopulation express Neurogenin2, followed by the T-domain transcription factors Tbr1 and Tbr2 that are GAD67-negative. This subset of periglomerular neurons is directed to a glutamatergic fate and express vGluT upon maturation in the olfactory bulb. PGN = periglomerular neuron

Interestingly, the bHLH transcription factor Mash1 is coexpressed with Pax6 in transi-amplifying progenitors giving rise to neuronal lineages. The ventrally arising lineage expresses besides Pax6 and Dlx additionally Mash1, whereas the dorsal Pax6 derived lineage would co-expess Mash1 at early stages, and later on Neurogenin2 and Tbr2. This suggests that Pax6 / Mash1-positive transit-amplifying precursors can give rise to both GABA and glutamatergic lineages. This may further imply that Pax6 / Mash1-expressing transit-amplifying progenitors may be able to generate different sets of neurons, comprising both GABAergic and glutamatergic neurons. Alternatively these two transcription factors may be shared between different lineages - like in development (H. Toresson et al., 2000) - that are distinct right from the start (Fig. **58**), as suggested recently (F. T. Merkle et al., 2007).

In the ventral embryonic telencephalon Mash1 in combination with Dlx1 & Dlx2 regulate Notch signaling (K. Yun et al., 2002). Whereas Mash1 is necessary to maintain Notch signaling, Dlx transcription factors down-regulate Notch signaling and promote lineage progression. Mash1 and Dlx1 & Dlx2 are both expressed in transit-amplifying

progenitors in the subependymal zone. As soon as these progenitors progress into the neuronal lineage (DCX+), Mash1 is down-regulated and we hardly detected any Mash1+/Dlx+/DCX+ neuroblast. Consistently Mash1 expression is less frequent upon retroviral over-expression of Dlx2 in the adult subependymal zone which promotes neuroblast formation. This is consistent with the down-regulation of Mash1 upon maturation and maintenance of Dlx2 in ventral embryonic progenitors (K. Yun et al., 2002). Furthermore Notch signaling regulates stem cell numbers of the subependymal zone (T. O. Alexson et al., 2006; A. Androutsellis-Theotokis et al., 2006; J. J. Breunig et al., 2007) and Mash1 might be necessary to keep Notch signaling active in progenitors of the adult subependymal zone.

Besides the neuronal lineages, Mash1 protein is also present in Olig2-positive transit-amplifying precursors giving rise to oligodendrocytes (C. M. Parras et al., 2004; M. A. Hack et al., 2005). In Mash1 mutants both neurons and oligodendrocytes in subependymal zone derived neurosphere are decreased in number (C. M. Parras et al., 2004). Thus Mash1 specifies also non-neuronal cell types arising from the subependymal zone and may exert a more generic function which is required at a similar step in neuronal and oligodendrocyte lineages.

5.5 Neurogenic potential in vitro – plasticity of progenitors

5.5.1 In vitro models for studying the behaviour of adult neural stem cells

In order to study neural stem cells in vitro, we used the neurosphere assay in which cultured cells are expanded in the presence of growth factors, namely epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2). Under these non-adherent conditions a small number of cells form neurospheres which can be differentiated in neurons, oligodendrocytes, and astrocytes (B. A. Reynolds and S. Weiss, 1992; M. A. Hack et al., 2004; B. Berninger et al., 2007; A. Chojnacki and S. Weiss, 2008). However, neurospheres are distinct in their behaviour compared to the in vivo situation: (i) neurospheres differentiate upon plating predominantly into GFAP+ astrocytes and give rise to only a small number of neurons probably due to the treatment with EGF and FGF2 (M. B. Luskin, 1993; F. Doetsch et al., 2002); and (ii) they down-regulate transcription factors like Dlx which are linked to a neuronal fate and instead up-regulate Olig2 (M. A. Hack et al., 2004).

Therefore we established a new culture system that mimicks the in vivo situation better than the neurosphere assay. Instead of expansion in non-adherent conditions and treatment with growth factors, we directly plated the cells after preparation. Retroviral transduction demonstrated that these cells generate predominantly neurons, and only few astrocytes and oligodencrocytes. Furthermore, retroviral clonal analysis demonstrated that only few cells underwent asymmetrical cell divisions and gave rise to large clones. More often we observed small pure neuronal clones and only few pure non-neuronal clones.

Taken together, primary non-expanded subependymal cultures may provide an alternative model for studying the cell biological processes like cell cycle and symmetric or asymmetric cell divisions as compared to the classical neurosphere assay. Furthermore, this model may allow important insights into the molecular mechanisms underlying cell fate specification.

5.5.2 Neurogenic potential of Dlx2 in adult subependymal zone derived cultures

We used both the neurosphere assay and primary subependymal zone cultures to test the effects of Dlx2 and Dlx2-Engrailed overexpression. However, since neurosphere derived cultures do not express endogenously Dlx transcription factors, we over-expressed Dlx2-Engrailed in direct plated subependymal zone progenitors. Notably, we observed the same ablation of neurogenesis like in vivo. We observed an increase in the number of GFAP-positive astrocytes following Dlx2-Engrailed transduction compared to the control. Concomitantly less Dlx2-Engrailed transduced cells adopted a neuronal fate compared to the control transduced cells. Gain-off-function experiments with Dlx2 in adult subependymal zone derived neurospheres which hardly express Dlx transcription factors endogenously demonstrated the neurogenic potential of Dlx2 retroviral transduction. Notably, even in non-expanded primary subependymal zone progenitors, retroviral over-expression of Dlx2 further promoted the generation of neuroblasts compared to the control. However, when I performed the same experiments in neurosphere cells using lentiviral vectors that also transduce non-dividing cells I observed only a small neurogenic effect. The number of ß-III-Tubulin+ cells was increased mostly at the expense of marker negative cells, comprising oligodendrocytes and progenitors. This might suggest that oligodendrocyte precursors are easier to convert towards a neuronal fate than neurosphere derived GFAP-positive astrocytes. Consistently their number is not altered upon lentiviral over-expression of Dlx2.

Furthermore, this might also suggest that the differentitation state of a cell is of particular importance. Because retroviral vectors transduce exclusively dividing cells, the infected cells are during transduction in a less differentiated state and might be easier to convert into a neuronal fate.

Also other transcription factors present in the adult subependymal zone exhibit a neurogenic potential, like Pax6 or Neurogenin2. The functional relevance of Neurogenin2 expression in the specification of glutamatergic neurons from adult subependymal zone cells may be suggested by the fact that over-expression of Neurogenin2 in adult derived neurosphere cells is sufficient to up-regulate Tbr1 and directs virtually all neurons towards a functional glutamatergic identity (B. Berninger et al., 2007). Interestingly, adult neurosphere cells spontaneously generate glutamatergic neurons at a low rate providing additional evidence for the notion that the adult subependymal zone harbours stem cells that give rise to glutamatergic neurons. Furthermore, retroviral transduction of Neurogenin2 in postnatal cortical astrocytes cultures exhibited a substantial increase of neurons in these cultures.

The neurogenic effect of Dlx2 in neurosphere cells supports the conclusion that Dlx2 is sufficient to instruct progenitors to acquire a neuronal, GABAergic fate. However, in more challenging conditions like transduction of non-dividing neurosphere derived cells the potential of Dlx2 is restricted. Interestingly, Neurogenin2 exhibited the same neurogenic potential in neurosphere derived cultures but also could convert a substantial portion of postnatal astrocytes into a neuronal fate.

5.6 Hope for endogenous brain repair

The combinatorial transcription factors codes of adult subependymal zone progenitors located at distinct positions to specify distinct neuronal phenotypes bears interesting implications for repair. If progenitors or even stem cells are restricted to a specific neuronal fate (F. T. Merkle et al., 2007), this may imply that specific subsets can serve for repair of only specific types of neurons. Thus, the regionalization of the subependymal zone and the discovery of a novel source of progenitors generating glutamatergic neurons in the adult may provide a reservoir for cortical repair of such neurons, whereas ventral regions would be more suitable for striatal injuries.

Several lesion models demonstrated the possibility to recruit new neurons from these two regions although to low extents (S. S. Magavi et al., 2000; A. Arvidsson et al., 2002; H. Nakatomi et al., 2002; J. Chen et al., 2004; T. Collin et al., 2005; T.

Yamashita et al., 2006). The potential of these regions to contribute to endogenous repair may depend on the range of neuronal subtypes they can produce or alternatively on the plasticity of progenitors to adopt to a new environment. While earlier transplantation studies had proposed a broad potential of neural stem cells in regard to the generation of different neuronal subtypes (F. H. Gage, 2000), this view has recently been challenged by the demonstration of the existence of distinct stem cells with restricted potential regarding subtype specification (F. T. Merkle et al., 2007).

The endogenous repair of glutamatergic neurons has been demonstrated in different lesion models (S. S. Magavi et al., 2000; H. Nakatomi et al., 2002; J. Chen et al., 2004) with neurons apparently originating from distinct sources of progenitors. While repair of glutamatergic projections neurons in the hippocampus originates from a periventricular region overlying the hippocampus (H. Nakatomi et al., 2002), the repair of neocortical layer 2, 3 or layer 6 neurons after chlorine e_6 induced neocortical cell death seems to originate in the subependymal zone at rather rostral positions or alternatively local progenitors residing in the neocortex are activated locally (S. S. Magavi et al., 2000). To test the recruitment of Tbr-positive progenitors I established this lesion model for specific callosal cortical projection neurons. Indeed, using the latter model we could demonstrate the emergence of Tbr2+ neuroblasts from the dorso-rostral subependymal zone into the neocortex. This is precisely the location where Tbr2-positive progenitors reside also in the intact brain, suggesting that neuronal cell death in the neocortex is able to recruit these progenitors for repair. While these progenitors normally generate glutamatergic interneurons of the glomerular layer, at least some of them can seemingly be diverted towards a different glutamatergic fate, such as the cortical projection neurons (S. S. Magavi et al., 2000; J. Chen et al., 2004). Interestingly, the transcription factor code that we observed in the glutamatergic progenitors of the adult subependymal zone is identical with the generation of glutamatergic cortical pyramidal neurons in neocortical development with Pax6 preceeding Neurogenin2 followed by Tbr2 and Tbr1 (R. F. Hevner et al., 2006). However, glutamatergic olfactory neurons apparently down-regulate Tbr1 upon arrival and integration into the olfactory network, whereas deep layer cortical neurons maintain Tbr1 expression. This implies that it may take only a few molecular changes to redirect these progenitors towards specific subtypes of glutamatergic neurons.

Notably, injury of striatal projection neurons also prompts the recruitment of neuronal progenitors to the generation of DARPP32+, medium spiny GABAergic neurons (A.

Arvidsson et al., 2002), a subtype that is normally not generated in the adult forebrain. Interestingly, these striatal projection neurons seem to be replaced from progenitors streaming out of the ventral subependymal zone, the same region namely the lateral ventricular wall where these neurons originate during development (H. Toresson et al., 2000). Indeed, this is the region of the adult subependymal zone that continues to express Dlx transcription factors (M. H. Porteus et al., 1994; F. Doetsch et al., 2002), while Pax6+ (M. A. Hack et al., 2005), and even more Neurogenin2+ and Tbr2+ progenitors are restricted to the dorsal subependymal zone in adulthood, reminiscent of their patterning during development (N. Bertrand et al., 2002).

Taken together, these data suggest that adult neuronal progenitors are specified towards the generation of GABAergic and glutamatergic neurons in a region-specific manner, providing distinct reservoirs of progenitor pools for repair of GABAergic and glutamatergic neurons respectively. Within their respective transmitter fate these progenitors can apparently be endogenously reprogrammed towards the generation of other GABAergic neurons, such as the medium spiny neurons (A. Arvidsson et al., 2002) or glutamatergic projection neurons (S. S. Magavi et al., 2000) upon injury. Understanding the molecular code of these distinct subtypes of adult progenitors is therefore a major step towards utilizing these neurons for repair of specific types of neurons. Indeed, the recent discovery of molecular fate determinants of subsets of cortical projection neurons (B. J. Molyneaux et al., 2007) may allow directing this novel source of adult progenitors for glutamatergic neurons much more efficiently towards the repair of cortical projection neurons.

Thus, the combinatorial code of different transcription factors activated in adult subependymal progenitors located at specific positions allows the generation of highly specific subtypes of both GABAergic as well as glutamatergic neurons in the adult subependymal neurogenic niche and shows that this regional specificity has profound implications for repair.

6 Material & Methods

6.1 Standard Solutions

Para-Formaldehyde (PFA) 4% (1 l):

PFA was diluted with autoclaved Millipore water from a frozen (-20°C) 20% PFA stock in 50 ml aliquots prepared according to the following protocol:

67 g Na_2HPO_4 x $2H_2O$ were dissolved in 800 ml water thereby heated up to 60°C (temperature control!). 200 g PFA (Sigma) was added and stirred for around 5 minutes, until the cloudy solution was homogenous. Around 18 g of NaOH pellets were added until complete dissolvent of PFA. The pH was adjusted to 7.4 with 37% HCl.

Phosphate buffered Saline (PBS) (0.15 M):

400 g NaCl (1.37 mol/l), 10g KCl (0.02 mol/l), 10 g K_2HPO_4 (0.015 mol/l), 58.75 g Na_2HPO_4 x 2 H_2O (0.066 mol/l) were dissolved in 5 liter millipore water and autoclaved in 1l-bottles. This 10x PBS stock was diluted 1:10 with autoclaved Millipore water to around 0.15 M 1x PBS.

Storing solution for vibratome sections (100 ml):

30 ml Glycerol, 30 ml Ethylenglycol, 30 ml autoclaved H_2O, and 10 ml phosphate buffer 10x [0.25 M, pH 7.4: $NaH_2PO_4xH_20$ 6.5 g, NaOH 1.5 g up to 40 ml H_20]. The storing solution was stored in the fridge and can also be sterile filtered to avoid contamination.

Lysis buffer for tail biopsies (10 ml):

1 ml 1 M Tris HCL pH 8.5, 100 µl 0.5 M EDTA, 200µl 10% SDS, 2 ml 1 M NaCl, 6.6 ml H_2O (autoclaved) are stored together at RT in one solution. Before lysis 100 µl Proteinase K (10 mg/ml) was added freshly.

Tris 10 mM for DNA (1 l):

1,211 g Trisbase (M=121.1 g/mol) dissolved in 1 liter Millipore water. pH was adjusted to 8.0 with HCl.

Electrophoresis buffer (1 l) for Western Blot:

30.3 g Trizma, 144 g Glycine, and 10 g SDS are dissolved in 1 liter of water. pH was adjusted to 8.45.

Sample buffer for Western Blot:

2 ml 1 M Tris-HCl pH 8.5, 8 ml 20% SDS, 5 ml Glycerol, 1.6 ml ß-Mercaptoethanol, 3.4 ml H_2O, 50 mg Bromphenolblue. Store aliquots at -20°C.

TBST for Western Blot:

100 ml TBS 10x [1 l: 12.1 g TRIS-Base and 87.8 g NaCl are dissolved in 900 ml autoclaved water and pH is adjusted with 5 M HCl to pH 8.0. Autoclave.], 10 ml 20% Tween [200 ml H_2O and 50 ml Tween 20], and 900 ml H_2O are mixed.

6.2 Animals

The following mouse lines were used for the experiments. All animal procedures were performed in accordance to the policies of the use of animals and humans in Neuroscience Research, revised and approved by the Society of Neuroscience and the state of Bavaria under license number 55.2-1-54-2531-23/04 and license number 55.2-1-54-2531-144/07.

mice (22 − 25 g) were used as wildtype animals at the age of 2 – 3 months. No difference was made between genders and both females as well as males were used for injections and histology.

mice contain the Pax6 gene flanked by loxP sites (R. Ashery-Padan et al., 2000); the two loxP sites are located before exon 4 and at an intron between exon 6 and 7, the region encoding the amino terminus of Pax6, including the initiatior methionine and most of the paired domain (Fig. **37**). Mice heterozygous for this genotype were crossed with mice homozygous for this genotype; for injections these animals were used at the age between 2 − 3 months.

mice (T. Mori et al., 2006) express the fusion protein of Cre recombinase with the mutated estrogen receptor (ERT2) in the Glast locus (knock-in). When induced with tamoxifen (see also tamoxifen induction) the CreER fusion protein translocates to the nucleus and cuts out DNA pieces located between two loxP sites. This mouse line was crossed with the (A. Novak et al., 2000) wich contains a Stop signal flanked by loxP sites followed by a GFP. Upon Tamoxifen induction and Cre shuttling to the nucleus GFP expression is turned on under the pCAGGS promoter.This mouse line was used to monitor the progeny of Glast-expressing astrocytes from the subependymal zone. The progeny could be monitored by GFP immunohistochemistry; the Glast::CreERT2 mouse line was crossed with the Z/EG reporter line on a Pax6 $^{flox/flox}$ and Pax6 $^{flox/wt}$ background. To increase the number of triple-transgenic animals the following crossing

was performed: Pax6 $^{flox/flox}$ Glast::CreERT2 $^{wt/+}$ Z/EG mice with Pax6 $^{flox/wt}$ Glast::CreERT2 $^{wt/+}$ Z/EG animals. Mice that were Glast::CreERT2 $^{+/+}$ were only used for matings, but not for experiments.

mice (N. Tamamaki et al., 2003) where GFP expression is driven by the expression of the GABA synthesising enzyme GAD (Glutamic Acid Decarboxylase) and therefore the vast majority of the GABAergic neurons in the adult brain are detectable via GFP staining. These mice were kept on a Black6 background and crossed on this background.

Beside GAD67::GFP mice, we used also **GAD65-GFP transgenic** mice. In this line, a 6.5 kb segment of the GAD65 gene that includes 5.5 kb of the 5'-upstream region, the first two exons and a portion of the third exon and the introns in between drives the expression of GFP (G. Lopez-Bendito et al., 2004). The expression of GFP in the olfactory bulb was similar to GAD67::GFP mice (data not shown).

mice were used in the study of Tbr transcription factors in adult neurogenesis. These animals mimick the expression of Tbr2 (G. S. Kwon and A. K. Hadjantonakis, 2007) by a bacterial-artificial-chromosome (BAC). The BAC transgene was obtained from the GENSAT consortium (D. Geschwind, 2004). The BAC covers around 225 kb of mouse genomic DNA, containing sequences spanning about 186 kb upstream and about 18 kb downstream of the Eomes (Tbr2) locus. Enhanced green fluorescent protein (EGFP) and a polyA sequence were inserted directly upstream of the Eomes coding region preserving gene structure while providing a readout of promoter activity. This mouse strain was kept on the original pure FVBN background. Tbr2$^{BAC-GFP}$ mice were used to visualize Tbr2 protein in the adult dorsal wall of the subependymal zone, rostral migratory stream, and olfactory bulb and short term fate mapping of Tbr2-positive cells.

6.2.1 Genotyping

To maintain colonies and experimental mice, numbered ear clips were put alternating the left and right ear: even numbers in the right ear, uneven numbers left ear. Numbers ranged from 0001 – 9999 and tail biopsies of less than 5 mm length were taken. The tails were transferred to 1.5 ml reaction tubes and incubated in 0.5 ml lysis buffer (see standard solutions) over night at 55°C (or at least for 3 hours) in a tail shaker at 55x10 rpm (Uniequip, Vortemp 56EVC). After lysis hairs and tissue residues were re-moved by centrifugation at 10,000 rpm for approximately 5 minutes. The supernatant was

transferred to a new 1.5 ml tube and DNA was precipitated by adding 0.5 ml Isopropanol, followed by another centrifugation step (max. speed, 10 minutes). The supernatant was removed from the pellet and tubes were dried upside down for 1 hour at room temperature, or alternatively for 30 min at 37°C until the DNA was completely dry and the pellet was transparent. The dry DNA was dissolved in 150 µl 10 mM Tris Buffer pH 8, followed by 1 − 2 hours shaking at 55°C.

The DNA was kept at 4°C until PCRs were performed. In general 1 − 2 µl DNA and 10 mM dNTPs were used in 30 µl reaction tubes. The final concentrations of primers for PCR were 10 pM in the reaction mix.

6.2.1.1 Pax6 floxed mice

The genotyping protocol for the $Pax6^{flox/flox}$ and $Pax6^{flox/wt}$ mice was adapted from Ashery-Padan et al., 2000.

Primer sequences:

Pax1: 5'- GCG GTT GAG TAG CTC AAT TCT A - 3'

Pax2: 5'- AGT GGC TTG GAC TCC TCA AGA -3'

Pax3: 5'- CGT GTG CCC CAG CTT CCG GT - 3'

Reaction mix: 2.5µl 10x buffer, 2.0µl $MgCl_2$, 1.0µl dNTPs, 1µl Primer 1, 2 and 3, 0.3µl Taq Polymerase, 14.2µl H_2O, 2µl DNA.

Cycling conditions (PCR program):

	94°C	2 min
30 x	94°C	50 sec
	58°C	50 sec
	72°C	50 sec
	72°C	10 min

Band size: WT 300 bp, Pax6 floxed allel 400 bp

6.2.1.2 Glast::CreERT2 mice

The genotyping protocol of the Glast::CreERT2 mice was adapted from Mori et al., 2006.

Primer sequences:

Glast F8 (forward): GAG GCA CTT GGC TAG GCT CTG AGG A

Glast R3 (reverse): GAG GAG ATC CTG ACC GAT CAG TTG G

CER 1 (CreERT2 specific primer): GGT GTA CGG TCA GTA AAT TGG ACA T

Reaction mix: 3.0 µl 10x buffer, 3.0µl $MgCl_2$, 6µl Q-Solution (Qiagen), 0.6µl dNTPs, 1µl each Primer, 1.0µl Taq Polymerase, 12.4µl H_2O, 1µl DNA.

Cycling conditions (PCR program):

	94°C	2 min
35 x	94°C	20 sec
	55°C	20 sec
	72°C	30 sec
	72°C	5 min

Band size: WT 700 bp, Glast::CreERT2 recombinant 400 bp

6.2.1.3 Z/EG reporter, GAD67::GFP, and GAD65-GFP mice

The genotyping protocol of the GAD67::GFP, GAD65-GFP, and the Z/EG reporter line is the same as the primers are designed for detection of DNA encoding for GFP.

Primer sequences:

eGFP-F2: CTA CGG CAA GCT GAC CCT GAA GTT C

eGFP-R2: GCC GAT GGG GGT GTT CTG CTG GTA G

Reaction mix: 2.5 µl 10x buffer, 1.0µl $MgCl_2$, 0.5µl dNTPs, 1µl each Primer, 0.5µl Taq Polymerase, 1.0µl Glycerol, 1.0µl DMSO, 15.5µl H_2O, 1µl DNA.

Cycling conditions (PCR program):

	94°C	2 min
35 x	94°C	20 sec
	55°C	20 sec
	72°C	30 sec
	72°C	5 min

Band size: WT no band, transgenic 400 bp

6.2.1.4 Tbr $^{BAC\text{-}GFP}$ mice

Genotyping of the $Tbr^{BAC\text{-}GFP}$ mice was adapted according to the protocol from the mutant mouse regional resource center, UC DAVIS (D. Geschwind, 2004; G. S. Kwon and A. K. Hadjantonakis, 2007).

Primer sequences:

11151 F1: 5'- CCG TCT GCG ATT CGC TAA A - 3'

GFP R2: 5'- TAG CGG CTG AAG CAC TGC A - 3'

Reaction mix: 2.5 µl 10x buffer (with 15mM $MgCl_2$), 0.5µl dNTPs, 0.5µl each Primer, 0.5µl Taq Polymerase, 19.5µl H_2O, 1µl DNA.

Cycling conditions (PCR program):

	95°C	1 min
35 x	94°C	20 sec
	55°C	30 sec
	72°C	30 sec
	72°C	5 min

Band size: WT no band, transgenic 300 bp

6.2.2 Stereotactic injections

Microsurgery for viral injections into different regions of the adult murine brain was performed according to the following protocol.

Anaesthesia:

For viral injections mice were deeply anaesthetised with around 100 µl up to 180µl (depending on the body weight) using the following mix: 1.0 ml Ketamin 10%, (injected at approximately 100 mg/kg body weight; cp-pharma, Burgdorf, Germany), 0.25 ml 2% Xylazinhydrochlorid (injected at 5 mg/kg body weight; trade name Rompun, Bayer, Leverkusen, Germany) and 2.5 ml 0.9 % NaCl solution (Braun, Germany). For injection of the anaesthesia insulin needles (U-100, 1 ml, BD Micro Fine, PZN: 324870) were the most suitable device because of their very short and thin cannula. After injection of the aneaesthesia mice were checked for pain reactions, i.e. pinching the tail or toes. Otherwise 20 – 50 µl anaesthetics were additionally injected.

Injection:

Mice were fixed in the stereotactic apparatus (Stoelting) and eyes were kept wet using 0.9% NaCl solution or Bepanthen Augen- und Nasensalbe. The fur on top of the head was disinfected with 70% EtOH and a small midline incision was performed. The skull was dried with a Sugi (Kettenbach GmbH, REF 31603) and bregma was searched by pressing gently on the skull with forceps. The digital display of the stereotactic apparatus was set to zero after an empty glass capillary (Kwik-Fil Borosilicate glass capillaries, WPI, 1B150F-4) was set onto bregma and a very small dot with a pen was put on the skull under the glass capillary to find the zero point for the following injections easier. The capillary was set to the coordinates and a dot was put on the skull at the position of the coordinates. A drill (Foredom) was used to open the skull cautiously at the position of the dots and I took care that the meninges stayed intact.

Coordinates were re-checked, using again the empty glass capillary. A new capillary was inserted containing now viral suspension and set again onto bregma. The digital display was adjusted to zero again and 0.5 – 1 µl viral suspension was injected at the coordinates very slowly (5 – 10 min) using an air system (WPI, picopump, PV 820; connected to a Jun-Air compressor). A pulse generator (pulse/delay generator PDG 204) gave a pulse every 5 or 10 sec and pulses were given at the lowest possible pressure and pulse length. After finishing the injection the capillary was retracted 2 minutes up to 5 minutes. The skin covering the skull was sewed by filaments (Ethicon Vicryl, 4-0, SH-1 plus, 21.8 mm 1/2c, 70 cm filament) with at least two stitches. For recovery from anaesthesia mice were put in an airing cupboard at 37°C and checked every 5 – 10 minutes.

Coordinates (relative to Bregma):

SEZ: 0.7 (anterioposterior), 1.2 (mediolateral) and 2.0 - 1.6 (dorsoventral)

RMS: 2.55 (anterioposterior), 0.82 (mediolateral), 2.9 - 3.0 (dorsoventral)

Lateral Ventricle: 0.0 (anterioposterior), 0.8 (mediolateral), -2.0 (dorsoventral)

Dentate Gyrus: -2.0 (anterioposterior), 1.6 (mediolateral), -1.9 (dorsoventral)

6.3 Virus – vector construction and production

Retroviral vectors have been used in most experiments in the present study and only in very few experiments lentiviral vectors. Therefore, I focus mostly on retroviral vectors in the technical part, since the preparation of the virus and the DNA is basically the same.

6.3.1 Viral vectors designed in this study

Two retroviral backbones have been used in this study: pMXIG and CAG-DsRed. The inserts into the pMXIG vector does not contain any internal promoter but its inserts are driven directly from the viral LTRs. Transduced pMXIG cells could be easily monitored by GFP immunostaining as this vector contains an IRES-GFP cassette.It shows therefore very strong transcription of inserted viral DNA and has an early onset of protein expression, i. e. GFP could be detected 24 hours after transduction. However, LTR sequences are often silenced and I noted a decrease in reporter positive cells 2 – 3 weeks after transduction.

The contains besides the LTR an internal chicken ß-actin promoter. As this vector is also a self-inactivating retroviral vector (SIN-vector) the LTR is inserted further upstream into the genome and silencing occurs to a lower extent compared to the pMXIG retroviral vector. This issue became more relevant in survival times over 3 weeks. Furthermore, the CAG-driven vector contains an IRES-DsRED cassette and was used in combination with IRES-GFP vectors for double transductions. Transduced cells could be monitored by immunostaining for RFP. Both vectors contain furthermore an Ampicillin resistence.

Dlx2 viral vectors:

The complete cDNA of Dlx2 in the pCAX expression plasmid (kind gift of G. Mastick, for details see also (G. L. Andrews et al., 2003)) was removed by use of EcoRI and XhoI and inserted into the multiple cloning site of pMXIG using again the EcoRI and XhoI site (T. Nosaka et al., 1999; M. A. Hack et al., 2005; D. Colak et al., 2008). The multiple cloning site of the pMXIG vector is located between the upstream LTR and the IRES sequence. Dlx2 cDNA was also inserted into a lentiviral vector encoding for IRES-GFP and a ubiquitin promoter. This vector was only used in neurospheres experiments to transduce also non-dividing cells.

The Dlx2 sequence was also inserted into the CAG-driven retroviral vector with IRES DsRed. The Dlx2 insert was amplified by PCR using the same primers as for the lentiviral vector but flanked by basepairs recognized by the enzymes Sfi and PmeI.

Primer sequences for Dlx2 for insertion into the **lentiviral** vector:

mDlx2f GAGGATCCACCATGACTGGAGTCTTTGACAGTC

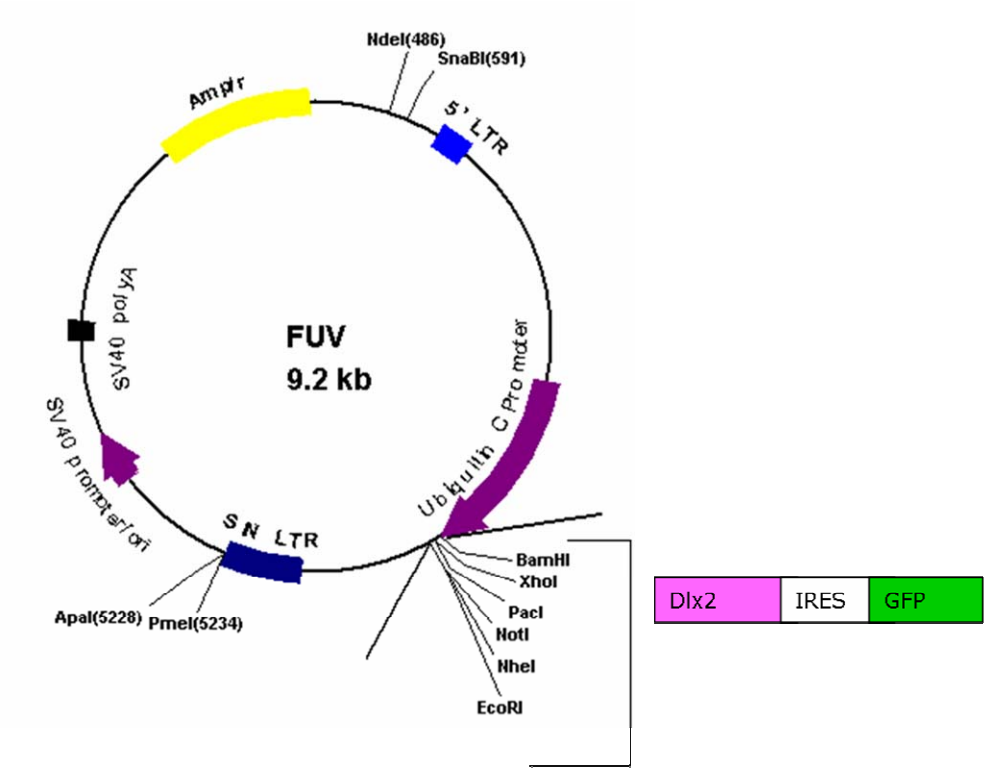
mDlx2r GTCCACTCGAGGTTAGAAAATC

The amplified fragment contains full length Dlx2 cDNA and is 1011 basepairs long.

PCR Reaction mix: 15 µl 10x buffer, 1.5 µl $MgSO_4$, 2.25 µl dNTPs, 2.25 µl each Primer, 1.5 µl Pfx Polymerase, 39.75 µl H_2O, 1 µl pure vector DNA. PCR was tested with 10 % Enhancer, 5 % Enhancer and no enhancer. The best results were obtained with 10 % Enhancer provided with the Polymerase.

Cycling conditions (PCR program):

	94°C	5 min
30 x	94°C	15 sec
	63°C	30 sec
	68°C	70 sec
	72°C	5 min



Dlx2-Engrailed retroviral vector:

The Dlx2-Engrailed fusion-construct was made by subcloning the homeodomain coding region of mouse Dlx2 (pCAX-Dlx2) and the Engrailed Repressor Domain (pSlax13-EnR; kindly provided by M. Kengaku) by PCR. The amplified fragments were then digested with XhoI and XbaI (for Dlx2 homeodomain) and with BamHI and XhoI (for Engrailed Repressor domain) and ligated into pCDNA 3.1. The fusion protein was digested with BamHI and XbaI and inserted into the retroviral vector pMXIG.

Dlx2 homeodomain:

mDlx2HDr GCTCTAGATTATATCTCGCCGCTTTTCCACATC

mDlx2HDf ATCTCGAGAAGGAAGACCTTGAGCCTGAAA

Primers amplify the cDNA of the homeodomain of Dlx2 and is 246 basepairs long.

PCR reaction mix: 5µl 10 x buffer, 0.5 µl $MgSO_4$, 0.75 µl dNTPs, 0.75 µl each Primer, 0.5 µl Pfx Polymerase, 13.25 µl H_2O, 0.5 µl pure vector DNA.

Cycling conditions (PCR program):

	94°C	5 min
30 x	94°C	15 sec
	61°C	30 sec
	68°C	30 sec
	72°C	5 min

Drosophila Engrailed:

drEngf GAGGATCCACCATGGCCCTGGAGGATCGCTGCAG

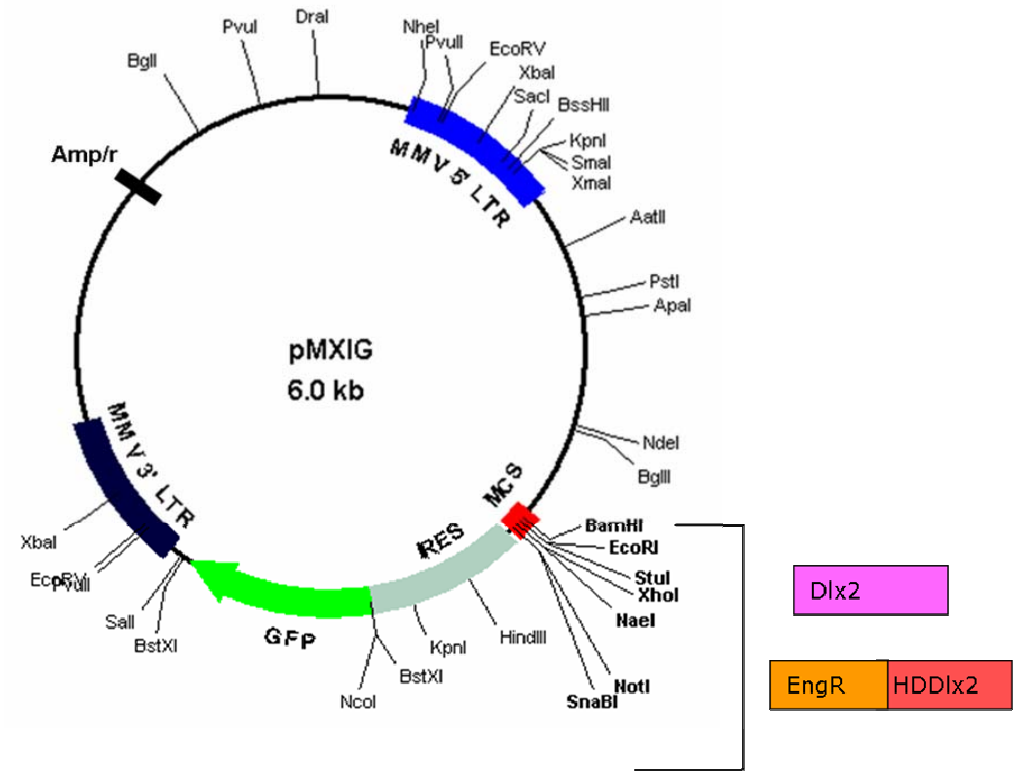
drEngr ATCTCGAGGTTCAGGTCCTCCTCGGAGAT

The amplified fragment contains drosophila Engrailed and is 930 basepairs long.

PCR Reaction mix: 15 µl 10 x buffer, 1.5 µl $MgSO_4$, 2.25 µl dNTPs, 2.25 µl each Primer, 1.5 µl Pfx Polymerase, 39.75 µl H_2O, 0.5 µl pure vector DNA. The mastermix was divided into three parts and 10 % enhancer, 5 % enhancer or no enhancer was added. Best results were obtained with 10 % enhancer.

Cycling conditions (PCR program):

	94°C	5 min
30 x	94°C	15 sec
	60°C	30 sec
	68°C	65 sec
	72°C	5 min



6.3.2 DNA preparation for virus production (CsCl-Gradient)

This method provides very pure DNA and contains only supercoiled plasmid DNA while nicked (relaxed circled) DNA is separated. A 250 ml bacterial culture was used.

Harvest:

Notably, care was taken that the culture was not overgrown. Optimum was around 90% confluency. Spin down bacteria at 5000 x g for 20 minutes and drain off supernatant, re-suspend the pellet in 10 ml Qiagen buffer1 with RNAse. After complete re-suspension, 10 ml Qiagen buffer 2 for lysis was added, tube shaked gently and incubated for exactly 5 minutes. 10 ml of cold Qiagen buffer 3 (kept on ice) was addd, and mixed well. The mixture was filtered through a folded paper filter pre-wetted with clean water into a 50 ml tube (volume around 25 ml, some rest may stay in the filter). DNA was precipitated by adding 20 ml Isopropanol to the filtrate (1:1), mixed, and followed by a centrifugation step at 8000 x g for 1 hour at 4°C. Supernatant was drained off, the white pellet was washed carefully with 70% EtOH and spun again for 10 minutes. After draining off EtOH the wet pellet was dissolved in 15 ml TE pH 8.0. The pH of the solution should be around pH 8 (otherwise adjust pH).

Phenol extraction (may be omitted):

The Pour DNA solution was poured into a Phase Lock Heavy 50 ml tube, 7.5 ml of phenol equilibrated with TE and 7.5 ml Chloroform was added and shook well. Then the solution was spun at 1500 x g at RT for 20 min in a swing out rotor. The upper, aequeous phase was poured into a new 50 ml tube and 1.5 ml of 3 M Sodium Acetate pH 5.2 and 15 ml Isopropanol was added for DNA precipitation, mixed well and centrifuged at 8000 x g for 1 hour at 4°C. The supernatant was drained off and the pellet was washed with 70 % EtOH and spun again for 10 minutes. Rests of EtOH were removed, the DNA pellet was air-dried until transparency. Dry pellets may be stored at -20°C.

CsCl-gradient preparation and ultracentrifugation:

The dry pellet was dissolved completely in 8.0 ml TE. The DNA solution (8.0 ml) was added to 8.80 g CsCl in a 50 ml tube and the salt was dissolved completely (solution will get cold). 800µl of saturated EtBr was added and the solution was mixed until a fine precipitate was formed. Samples were warmed up to 37°C for 15 minutes in the water bath and centrifuged at full speed in a swing out rotor for 10 minutes so that the precipitate was clinged to the surface and the wall of the Falcontube. (Phenolextraction

may be omitted but then more precipitate will be formed!). The supernatant was filled into an 11.2 ml OptiSeal tube (Beckmann) until the meniscus touched the basis of the tube's neck. Tubes were balanced precisely to 0.0 g for the following ultracentrifugation step: 65.000 rpm for 5 hours and 30 minutes at 20°C at maximum acceleration and slow brake settings. Start of the centrifuge was delayed so that the spin ended at the next working day. After centrifugation finished visible DNA bands were immediately extracted.

Extraction of the EtBr from the rotor tube:

For harvest of the DNA band, a UV lamp in vertical direction and a dark room are required, as well as skin protection (i. e. wear lab coats, nitril gloves and face protection).

When the centrifuge stopped, tubes were removed from the rotor as soon as possible, and the tube was fixed in front of the UV-lamp in a holder. The tube's shoulder was pierced with a canula (around 22 G) for ventilation of the tube when DNA-band was withdrawed. The tube was put in a fitting clamp, so that the band was visible halfway down the tube. Now the second needle (around 22 G) was put into the tube shortly under the DNA band. The UV-lamp was used to check the height of the DNA band (upper band: nicked DNA, lower band: super-coiled DNA). The needle was connected to a 2 ml syringe and as much DNA was sucked as possible by moving the syringe up and down. Content of the syringe was transferred into a 15 ml tube. DNA can be stored at that stage at room temperature overnight under light protection.

Removal of EtBr from the DNA:

An equal volume of n-Butanol saturated with TE was added to each of the DNA + EtBr solutions, shook well and spun briefly in a swing out rotor and upper organic phase was removed. The extraction was repeated until lower aqueous phase was completely colourless (around 5 – 6 times). Then an equal volume of Diethyl-Ether was added, solution was mixed and upper, organic phase was removed to remove rests of n-Butanol from the water phase. The Ether will remove itself during the next steps due to room temperature. DNA solutions were transferred into 50 ml tubes and diluted with 2 volumes of sterile TE. 1/10 new volume of 3 M Sodium-Acetate pH 5.2 and another 2 volumes of cold 100 % EtOH (stored at -20°C) were added, the solution was mixed and incubated on ice for 1 – 2 hours or overnight at 4°C, followed by a centrifugation step 10000 x g for 1 hour at 4°C. Supernatant was removed and washed with 10 ml cold 100 % EtOH, spun again at 10000 x g for 10 minutes and again rests of EtOH were

removed. The pellet was air-dried until transparency. DNA was dissolved under a tissue culture flow in 500 µl of sterile 10 mM Tris-HCl pH 8. Vigorous shaking was avoided! DNA solution was transferred into a 1.5 ml reaction tube, eventually followed by another spin at full speed for 5 minutes to further remove insoluble particles. Average yield: around 1 mg of pure DNA.

6.3.3 Retroviral preparations

The retrovirus preparation with GPG293 cells requires only the addition of the retroviral expression plasmid, as all other viral genes are integrated in the genome of these cells (W. S. Pear et al., 1993; J. K. Yee et al., 1994).

The lentiviral production follows basically the same protocol. The cell line used for lentiviral preparation does not contain any integrated viral DNA; therefore three plasmids have to transfected.

Growing GPG293 cells:

GPG293 cells were used for preparation of all retroviruses. These cells were kept in culture and passaged up to twenty times maximum until a new vial of frozen cells was defrosted.

Cells were kept in DMEM/high glucose/Glutamax (Gibco) containing 10% FCS (Invitrogen, heat inactivated at 56°C for 30 min), 5ml/500ml Non-Essential Amino Acids (NEAA, 100 x, Gibco), 5ml/500ml Sodium-Pyruvate (100 x, Gibco) and under a triple selection of antibiotics (basic medium):

1 mg/ml penicillin/streptomycin (Gibco, general antibiotic for tissue culture, no specific function or repression in GPG293 cells)

1 mg/ml tetracycline (Sigma, repression of the VSVG production)

2 mg/ml puromycin (Sigma, selection for the integrated VSVG gene and Tet-repressor that regulates VSVG expression)

0.3 mg/ml G418 (=Geneticin, Gibco, selection for integrated MMLV genome (gag-pol)).

The medium above was stored for maximum 6 weeks in the fridge to avoid decay of the antibiotics. Cells were passaged two times per week using diluted 0.25% Trypsin (1:5 with PBS) and splitted at a ratio of 1:3 to 1:5.

Retroviral preparation:

GPG293 cells were expanded in 75cm^2 flasks to yield a sufficiently lare number of cells for viral packaging. The first step of viral production is to seed the cells in 10-cm culture dishes in basic medium without Tetracycline (but all other antibiotics), whereby VSVG expression is induced. At least three 10-cm dishes were used for one viral vector batch fitting into one ultracentrifuge tube (Beckmann). Otherwise 6 plates were used for supernatant for two centrifugatial tubes. The next day after splitting (confluency 80-90%), cells were washed with Opti-MEM (Gibco) containing 10% FCS (6ml per 10cm-dish) to remove any antibiotics. Two washing steps were performed to ensure absence of antibiotics, followed by an incubation step of around one hour. Transfection of the GPG cells was performed with the following mix (W. S. Pear et al., 1993; J. K. Yee et al., 1994; M. A. Hack et al., 2004; D. Colak et al., 2008): (for 6 plates) 2 x 50ml tubes with 9 ml Opti-MEM (1.5 ml for each 10-cm dish in each tube), with 360 µl Lipofectamine 2000 (Invitrogen, 60 µl per 10-cm dish) in one tube, mix. 150 µg of pure DNA (24 µg DNA per 10-cm dish) was added to the second tube and mixed. Solutions were allowed to settle for 5 minutes at room temperature and checked if the Lipofectamin gets slightly cloudy. The content of the Lipofectamin tube was added to the DNA containing solution, mixed and incubated 30 minutes until Lipofectamin-DNA complexes are formed. Then 3 ml of transfection mix was added in a dropwise manner per plate. Transfection medium was replaced the next morning by packaging medium (DMEM/high glucose/Glutamax, 10% FCS, NEAA, Pyruvate) (10 - 12 ml of packaging medium per 10-cm dish).

The first medium collection (harvest) was performed 48 hours after transfection and a second harvest was done three days following transfection. All centrifuge tubes, screw caps and rotor buckets were sterilized in pure 100% EtOH. Culture medium was collected in falcon tubes and transfected GPG cells were fed with packaging medium. The supernatant was filtered through a pre-wetted low-protein binding 0.45 µm low-protein binding PVDF filter (Millipore) followed by an ultracentrifugation step at 50000 x g for 90 minutes at 4°C (Beckmann, SW40Ti rotor). Supernatant was removed carefully with a Pasteur pipette connected to a vacuum pump. The transparent pellet was soaked for at least 3 hours on ice or over night in a TBS-5 buffer [to around 300ml ultrapure water add: 20 ml 1 M Tris-HCl, pH 7.8, 10.4 ml 5 M NaCl, 4 ml 1 M KCl, 2 ml 1 M $MgCl_2$, fill up to 400 ml with ultrapure water and store at 4°C] and re-

suspended carefully with a 200 µl-pipette. Aliquots of 5 µl or 10 µl were stored at -80°C.

Determination of retroviral titres:

Viruses were used for injections when titres were above 10^6 viral particles per µl. Dissociated embryonic day E14 – E15 cells from cortex or striatum were used for determination of titres. Transduction was performed 2 hours after plating and at least 6 wells transduced with the same retroviral batch were prepared with the following dilution series: 1 µl of virus was pipetted in the first well and 1 µl was pre-diluted into 400 µl medium. 200 µl (0.5 µl virus in total), 100 µl (0.25 µl virus in total), 20 µl (0.05 µl virus in total), 10 µl (0.025 µl virus in total) and 1 µl (0.0025 µl virus in total) of virus containing medium was put into according wells. Cells were cultured for 2 – 3 days in 24-well plates on PDL coated cover-slips to allow expression of the transgene. After fixation for 15 minutes at room temperature in 4 % PFA, cells were processed for GFP immunohistochemistry. Colonies were counted. In most cases the 1 µl (0.0025 µl) or 10 µl (0.025 µl) transduction of the diluted retrovirus with medium allowed to count single colonies and thereby to assess the viral titre.

6.3.4 Principles of viral targeting of adult neural stem cells

Tissue specific deletion and over-expression of candidate genes can be performed elegantly by the Cre-loxP system where Cre is driven by a cell type specific promoter (i. e. GlastCreERT2) and cuts out a candidate gene flanked by loxP sites. However, most cells of one type are affected.

Alternatively, adult neural stem cells can be manipulated in vivo by injected viral vectors. Advantageously, the manipulated cells stay in a normal, healthy environment and non cell-autonomous effects maybe avoided. The number of transduced cells can be varied by varying the titre and thereby cell autonomous effects can be studied upon low titre injection. Gain- and loss-off-function experiments can be performed in wildtype animals by the use of dominant-negative as well as over-expression constructs of the candidate gene. However, mice have to undergo a surgery during injection and a very small lesion due to insertion of the glass capillary cannot be avoided. In addition, beforehand it is important to study the transduction pattern: distinct viruses show specific transduction pattern of cells and depending on which cells should be manipulated one should consider the transduction pattern. Here, we compared two

species: Retrovirus versus Lentivirus (both VSVG pseudotyped) (for details see chapter 6.3.4.2 Comparison of Retrovirus versus Lentivirus).

Moreover, viral vectors can also be modified with cell type specific promoters. Consequently, only cells which switch on this promoter will activate the virally inserted gene, e. g. the synapsin promoter for neurons, and the GFAP promoter for astrocytes. However, this approach is not feasible when cells change their fate upon differentiation: i. e. the human GFAP promoter is only active in astrocytes, but during generation of TAPs and transition into neuroblast this promoter is switched off and GFP can only be followed for a short time.

Viral vectors can also be altered by different envelope proteins. Here, I tested the LCMV (lympho chorio meningitis virus) glycoprotein. I observed upon cortical injections a predominant transduction of GFAP-positive astrocytes (Fig. **59**). Injection into the adult subependymal zone exhibited neuroblasts transduction after 2 - 3 days post injection. Therefore this virus could not be used for specific targeting of adult neural stem cells as it also targets neuroblasts. Finally, the location of the injection site can be altered. Neuroblasts can be transduced rather late upon maturation in the rostral migratory stream or early in the lineage directly in the subependymal zone. Notably, GFAP+ neural stem cells extend processes between the ependymal cells into the ventricular wall. This process extension into the ventricle was taken as an advantage to transduce cells by ventricular injections.

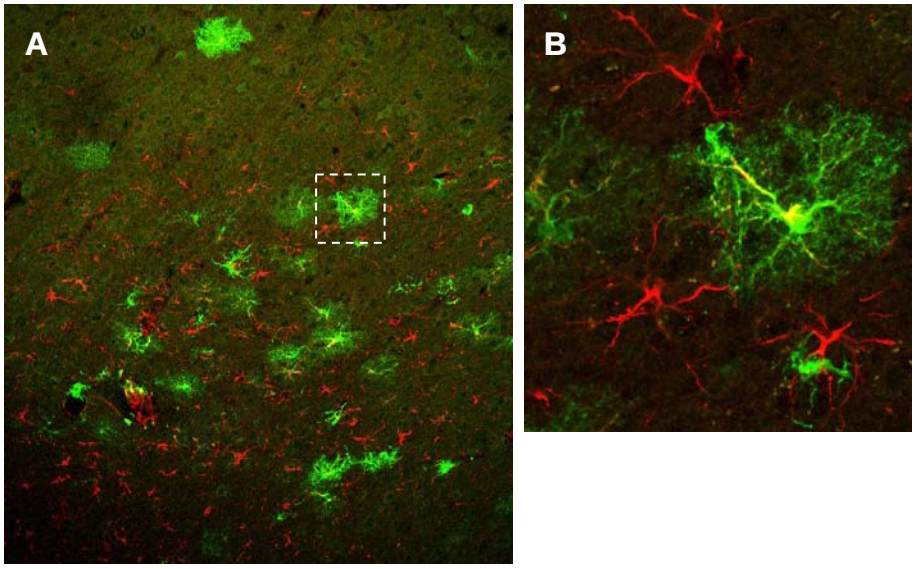


Fig. 59: Cortical injection of LCMV pseudotyped lentivral vectors.

(A) 7 days post injection into the cortex most of the transduced cells exhibits an astroglial identity as demonstrated in immunohistochemistry for GFP (green) and GFAP (red).
(B) Boxed area is shown in higher magnification in (B). Note the activation of GFAP around the injection site due to the lesion from the capillary injection.

6.3.4.1 Injection into the lateral ventricle

As slow dividing neural stem cells extend processes into the ventricle, ventricular injections have been performed. To hit the processes of neural stem cells, viral vectors were very slowly infused into one ventricle over a time of at least 10 minutes (1-2 µl). This experiment was done in young (3 – 4 weeks old C57Bl/6 mice) and adult mice (more than 8 weeks). Retroviral injections gave in both cases very poor transduction rates due to the slow division of stem cells. As this type of virus transduces only dividing cells, no ependymal cells were transduced and hardly any neuroblast could be observed in the olfactory bulb. In contrast, Lentiviruses transduced a lot of ependymal cells, both in adult and young mice but the number of transduced GFAP+ astrocytes decreased dramatically with age of the animals (Fig. **60**).

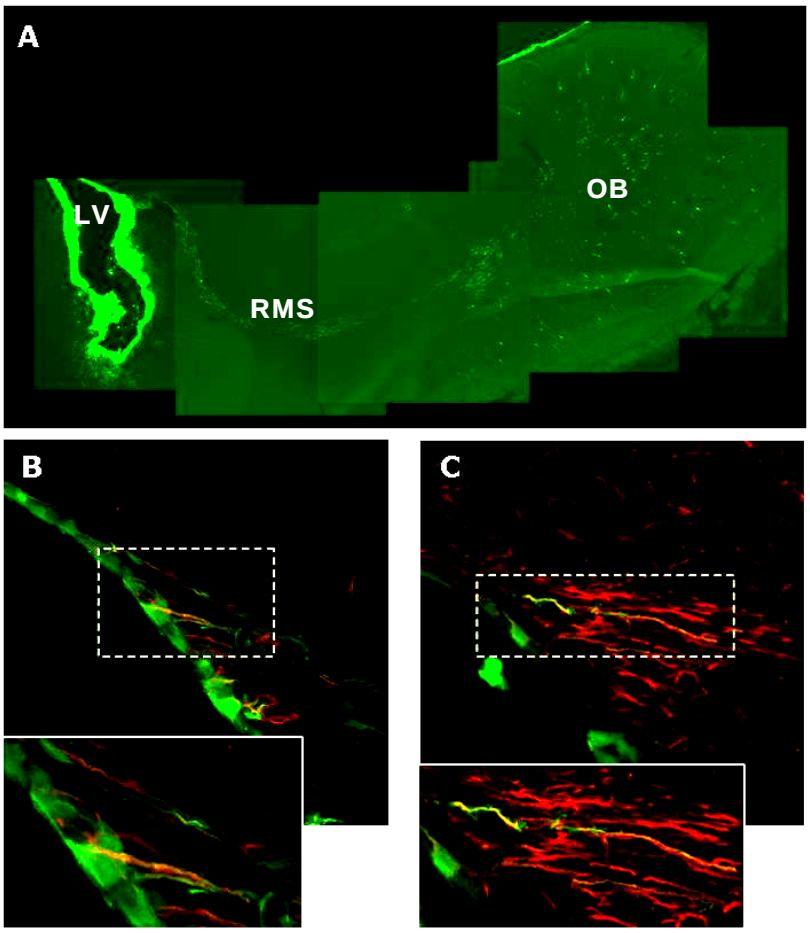


Fig. 60: Ventricular injection of lentiviral vectors into 3 weeks old mice
(A) Overview of the ventricle, rostral migratory stream and olfactory bulb. Endogenous GFP expression is enhanced by immunohistochemistry for GFP (green). Mice survived for one week after injection.
(B, C) Two examples of transduced neural stem cells: Immunohistochemistry for GFP (green) and GFAP (red). Note the transduced green and red fibres. These cells must have contacted the ventricle as they are virally transduced and are therefore considered as neural stem cells. Insets show higher magnifications of the boxed areas. LV = lateral ventricle, RMS = rostral migratory stream, OB = olfactory bulb.

As neural stem cells are amongst the GFAP expressing astrocytes neuroblasts could be observed in the olfactory bulb only in young animals. Neural stem cells could be transduced until the age of four weeks. In older animals the number of transduced stem cells decreased dramatically. This technique maybe applied for the manipulation of ependymal cells in adult animals, but not for the manipulation of astrocytes and their progeny. Higher viral titres and injection volume may be increased to have better transduction rates in adult mice. However, as the total volume of the first and second ventricle in the mouse is around 12 µl, injections volumes are limited. We therefore injected directly into the subependymal zone in the present studies.

6.3.4.2 Comparison of Retrovirus versus Lentivirus

Only Lentiviruses have an integrase complex and are therefore able to cross the nuclear membrane and insert their genome into non-dividing cells. As a consequence transduction occurs randomly at the injection site in every cell type. Retroviruses lack this complex, although they are also crossing the cell membrane as well and enter the cytoplasm. If the cell is not dividing within a certain time window, no transduction will occur (L. Naldini, 1998).

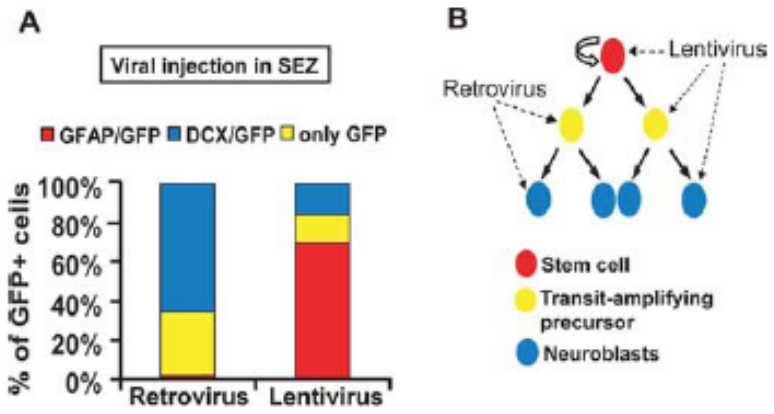


Fig. 61: Comparison of transduced cells in the SEZ after retro- and lentiviral injection.
The transduced cells were identified by GFP immunohistochemistry counterstained for the neuroblasts specific antigen DCX and the astrocytes specific anitgen GFAP. Cells that were negative for both GFAP and DCX were considered to be TAPs or oligodendrocyte precursors. Note the strong increasein the number of transduced GFAP+ cells upon lentiviral transduction.

For comparison of Retro- and Lentiviruses GFP encoding vectors were injected into the subependymal zone and brains were analyzed two days later. Quantification of the transduced cells was performed by immunohistochemistry for GFP and two markers: GFAP for astrocytes and DCX for neuroblasts (Fig. **61**).

Adult neural stem cells are slow dividing, in contrast to their progeny. Transit-amplifying progenitors and some neuroblasts are fast dividing. Retroviral vectors therefore incorporate mostly into these transit-amplifying progenitors and neuroblasts, whereas lentiviral vectors transduce mostly GFAP-positive neural stem cells.

6.4 Time-lapse videoimaging

Sagittal sections (250 µm) from virally injected adult (2-3 months old) mouse forebrain were prepared and maintained at 32°C. Slices were continually superfused (2 ml/min) with artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 26 $NaHCO_3$, 10 glucose, 3 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$ and 1.25 Na_2HPO_4 (bubbled with 95 % O_2/5 % CO_2; pH = 7.4). For time-lapse videoimaging of cell migration images (at least 6-10 z-sections, with abouth 10µm interval) were acquired every 15 sec for at least 1 hour with a BX61WI (Olympus) up-right microscope equipped with CCD camera (CoolSnap HQ2) and DG-4 Xenon light source (Sutter Instruments). Multiple z-steps acquisition in our time-lapse experiments of cell migration allowed us to follow the same cell in different z-planes.

6.5 Histology and BrdU administration

For immunohistochemistry, animals were deeply anaesthetized using 5 % Chloralhydrate (0.15 ml/10 g bodyweight) and transcardially perfused first with a pre-flush of PBS followed by 4 % PFA in PBS. Brains were postfixed in 4 % PFA overnight and the following day cyroprotected with 30% sucrose solution in PBS and after brains had sank down in the sucrose solution they were cut at 20 µm thickness at the cryostate. For long term storage cryosections were frozen at -20°C. Alternatively, and later the preferred method, vibratome sections were cut at 60-µm thickness. After perfusion and postfixation in 4 % PFA overnight (this step can be omitted) brains were washed once in PBS, embedded in 4 % agarose in PBS and cut at the same day. For long term storage of vibratome sections 1ml of storing solution (see standard solutions) and at least 5 sections per 1.5 ml tube were stored at -20°C. Sections can be stored for at least 2 years.

For detection of proliferating cells, the DNA base analogue 5-Bromo-2′-deoxy-Uridine (BrdU, Sigma) was injected intraperitoneally (50 mg/kg body weight) one to two hours before perfusion to label fast proliferating cells (short pulse). BrdU was dissolved at a

concentration of 5 mg/ml in sterile 0.9 % NaCl solution (Braun). Complete dissolvement could only be reached by 2 hours at 37°C. Aliquots were stored at -20°C.

BrdU was given into the drinking water at a concentration of 1mg/ml (sometimes 1 % sucrose was added for sweetening the water) and stirred for at least 1 hour at room temperature. 50 ml of BrdU-water was sufficient for 2 - 3 mice per cage for 2 - 3 days and was exchanged two times per week. BrdU drinking water was stored in the fridge for several weeks.

6.6 Immunohistochemistry

Primary antibodies (for details see table) were incubated with specimen overnight at 4°C in 0.1 M PBS containing 0.5 % Triton-X-100 (Sigma) and 10 % normal goat serum (Gibco) or 0.5 % Triton-X-100 containing 2 % Bovine Serum Albumin (Sigma).For detection of BrdU in proliferating cells, sections were incubated in 2 M HCl for 1 hour at room temperature, followed by one incubation step with borate buffer (0.1 M, 10 min, pH 8.5 – 8.6) for 10 minutes for neutralisation of the pH. Afterwards sections were rinsed once in PBS and incubated in the primary α-BrdU-antibody in 0.5 % Triton-X-100 containing either 10 % NGS or 2 % Bovine Serum Albumin. Following incubation in the primary antibody, specimen were washed in PBS 1 – 3 times and incubated in solutions containing subclass specific secondary antibodies conjugated to Alexa 488 (1:1000, Invitrogen), Cy2, Cy3, Cy5 (1:1000, Dianova) or biotin (1:200, Vector Laboratories). For stainings of coverslips from the in vitro experiments secondary antibodies conjugated to FITC and TRITC were preferred (less background). After several washes in PBS specimen were placed by the use of brushes on glass slides and dried shortly (in case of the vibratome sections) and embedded with Aqua Polymount (Polysciences, 18606).

Some weakly expressed antigens were detected by high sensitivity tyramide signal amplification (Perkin Elmer) that allowed amplification of the signal and simultaneous detection of twoproteins by two antibodies generated in the same animal.. After incubation of specimen in the primary and biotinylated secondary antibody, specimen were washed again and incubated with a horseradish-peroxidase coupled to Streptavidin. The colour (green or red) was precipitated on the specimen in an amplification buffer for 4 min at a dilution of 1:75. This kit was also used for fluorescent in-situ hybridization in combination with a horse-radish peroxidase coupled anit-Digoxigenin antibody (Roche).

Antigen	Full name of antigen	company	Pretreatment	species	dilution
α-activated Caspase		Promega	Tyramide Kit	rabbit	1:100
α-APC	Adenomatous polyposis coli	Calbiochem		mouse	1:200
α-BrdU	5-Bromo-2′-deoxy Uridine	Abcam Biozol	2M HCl, 0.1M Borate buffer	rat	1:400 1:100
α-Calbindin	Calcium binding protein	Sigma		mouse IgG1	1:2000
α-Calretinin	Calcium binding protein	Chemicon		mouse IgG1	1:2000
α-c-Fos		Calbiochem			1:2000
α-Ctip2	Pyramidal neurons in cortex layer V	abcam		rat	1:500
α-Cux1	Pyramidal neurons in cortex layer II and III	Santa Cruz		rabbit	1:100
α-DCX	Doublecortin	Chemicon Abcam		guineapig rabbit	1:2000 1:2000
α-Dlx	Distal-Less; homeobox TF	Gift of J. Kohtz	2 days ON	rabbit	1:750
α-Dlx2	Distal-Less 2; homeobox TF	Chemicon	Tyramide Kit	rabbit	1:200
α-GAD65	Glutamic acid decarboxylase 65	Chemicon		mouse IgG2a	1:1000
α-GAD65/67	Glutamic acid decarboxylase 65/67	Sigma		rabbit	1:500
α-GFP	Green fluorescent protein	Invitrogen Aves Lab		Rabbit chick	1:500 1:500
α-Mash1	Mammalian achate schute Homolog 2; bHLH TF	Gift of D. Anderson	Tyramide Kit	mouse IgG1	1:200
α-NeuN	Neuronal nuclei	Chemicon		mouse IgG1	1:100
α-NG2	Chrondoitinsulphate glycoprotein	Chemicon		rabbit	1:200
α-O4	Oligodendrocyte progenitor marker	Gift of J. Price	No Triton	mouse IgM	1:50
α-Olig2	bHLH TF;	Chemicon		rabbit	1:1000
α-Pax6	Paired domain TF	Developmental Studies Hybridoma Bank Chemicon		mouse IgG1 rabbit	1:500 1:500
α-RFP	Red fluorescent protein	Chemicon Rockland		rabbit	1:500
α-Tbr1	T-box TF 1	Chemicon		rabbit	1:1000
α-Tbr2	T-box TF2	Chemicon		rabbit	1:1000
α-TH	Tyrosine Hydroxylase	Chemicon		mouse IgG2a	1:1000
α-ß-IIITubulin	Neuronal marker	Sigma		mouse IgG2b	1:500
α-vGluT1	vesicular glutamate transporter 1	Synaptic Systems		rabbit	1:2000
α-vGluT2	vesicular glutamate transporter 2	Synaptic Systems		rabbit	1:2000

6.7 Quantitative analysis and statistics

Stainings were analyzed at an Olympus FV1000 or Leica SPE laser-scanning confocal microscope with optical sections of maximum 0.5 – 2 µm intervals. Virally transduced cells were identified by GFP-immunoreactivity and colocalization with cell type specific antigens was quantified either in single optical sections of confocal pictures or for each GFP-positive cell at high magnification at the epifluorescence Olympus microscope (BX61). Between 5 and 10 sections per animal or between 2 and 5 coverslips per in vitro experiment were counted until comparable numbers of transduced GFP-positive cells per animals or experiments were reached. Quantifications of immunohistochemistry in wildtype animals and the number of single- or double-positive cells were assessed with single optical sections using the Olympus Fluoview program. The total number of cells counted in all (injected) animals or experiments is indicated in the text or figure legends. One (injected) animal or experiment represents one mean value (n) and standard deviations were calculated between animals or experiments (unless indicated otherwise). All error bars are presented as standard errors of the mean (± SEM). Group comparisons were made with the unpaired t-test and P-values smaller than 0.05 were considered significant, P-values smaller than 0.001 were considered highly significant. Significant changes are indicated with the following symbols on top of the corresponding diagram bars (*, #, °, +). In addition, for group comparisons between three groups (control, Dlx2 and Dlx2-Eng) ANOVA tests were performed to test for the significance.

The formula for the **standard deviation** of the mean is calculated by:

$$\sigma(r) = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - r)^2}.$$

N = number of samples

X_i = value of the sample

r = mean of all samples

The **standard deviation of the mean** was calculated by the following formula:

$$\sigma_{\mathrm{mean}} = \frac{\sigma}{\sqrt{N}}$$

N = number of samples used to sample the mean.

The statistical significance for two groups was calculated by the **t-Test** by the use of statistical softwares. P-values smaller than 0.05 were considered significant, P-values smaller than 0.001 were considered highly significant.

$$t = \frac{\bar{x} - \bar{y}}{\sqrt{(s_x^2 + s_y^2)/n}}$$

S^2_n = sample variance of x or y calculated by the formula $s_n^2 = \frac{1}{n}\sum_{i=1}^{n}(x_i - \overline{x})^2$

$\bar{x}$ = mean value of all samples of x

$\bar{y}$ = mean value of all samples of y

6.8 *In situ* hybridization

In situ hybridization was carried out on cryostate sections of 20µm thickness or vibratome sections of 60 µm thickness whith the latter giving better results probably due to the thickness that shows stronger signal and better penetration of the *in situ* probe from both sides. In general the in vitro transcription and the *in situ* hybridization were carried out under semi-sterile conditions using sterile pipet tips and gloves.

An *in situ* hybridization can be divided into two main steps: (i) generating the anti-sense RNA specific for the according RNA in the tissue by in vitro transcription (described in chapter 6.8.1 In vitro transcription); (ii) hybridization of the RNA probe and its detection in the section (described in 6.8.2 In situ hybridization). In general the *in situ* hybridizations were developed in a stain visible in normal light. However, in special cases a fluorescent in situ hybridization protocol was applied (see chapter 6.8.3).

6.8.1 In vitro transcription: generation of digoxigenin labelled probes

The cDNA containing ISH plasmid was linearized by use of an enzyme at the end cDNA probe insert. Therefore, the RNA polymerase falls off the DNA strand at a defined point after transcribing the according cDNA into RNA.

Linearization of 1 µg DNA was performed with 1 µl of the according enzyme and 2 µl 10 x buffer in 20 µl final volume. After digestion DNA was purified by use of a column (Qiagen) or Phenol extraction (described above).

Transcription was performed in an 1.5 ml reaction tube by using 1 µg DNA, 2 µl digoxigenin labelled dNTPs (Roche), 4 µl 5 x buffer (Stratagene), 1 µl RNAse inhibitor

(Roche) and 1 µl of the according RNA Polymerase (T3, T7, Sp6) (Stratagene). The mixture was kept for 1 - 2 hour at 37°C and RNA was cleaned over a RNA binding column (Qiagen). 1 µl of the resulting RNA probe was examined on a gel for RNA presence. All probes were then tested on sections. As a rule of thumb 2 µl per 100 µl hybridization buffer was used first until the optimal concentrations were determined.

6.8.2 In situ hybridization

For vibratome sections the hybridization was performed in a sterile Eppendorf tube and the following steps in 24- or 6-well plates. For cryostate sections the hybridization was carried out in chambers containing chamber solution (1 x SSC containing 50 % Formamide) to avoid drying of the sections overnight. The amount of hybridization buffer was calculated per slide or sections: For a cryostate section 150 µl hybridization buffer was used or alternatively 500 µl for 3 - 4 vibratome sections in one reaction tube. After adding the probe to the hybrization buffer the mixture was heated up to 74°C for 4 minutes to separate RNA strands and mixed again. Vibratome sections were added to the tube containing the hybrdisation solution or the buffer was put onto the slides with the cryostate sections and sterile glass slides (shortly breamed) were carefully put on top and the chamber solution was added into the chamber. Hybrization was carried out in an oven or water bath at 65°C overnight.

Hybridization buffer	10 ml
Formamid	5 ml
20 x SSC	1.5 ml
Deinhardt's solution 50x	200 µl
50 % Dextran sulfate	2 ml
t-RNA	500 µl
H_2O	Up to 10 ml

The next day the washing solution was pre-warmed in a water bath to 65°C. Cryostate sections were moved to a sterile washing chamber, vibratome sections were moved into a 6-well plate containing washing solution. Sections were incubated twice for 30 minutes at 65°C in washing solution followed by two washing steps in 1 x MABT for 30 minutes at room termperature.

Washing solution	50 ml
20 x SSC	2.5 ml
10 % Tween	0.5 ml
Formamide	25 ml
H_2O	22 ml

MABT 5x	2 l
Maleic acid (500 mM end concentration)	116.08 g
NaCl (750 mM end concentration)	87.7 g
10% Tween 20	20 ml
H_2O	1980 ml
Adjust pH to 7.5	~ 70 g NaOH

Blocking solution was prepared. For vibratome sections 500 µl per probe was use in a 24-well plate and for cryostate sections 500 µl per slide was used. The blocking solution was added drop wise onto the slides that were put back into the chamber and covered by parafilm to avoid drying. Vibratome sections were transferred from a 6-well to a 24-well plate and sections were incubated for 1 hour at room temperature. The coupled to alkaline phosphatase (Fab Fragments, Roche, 1:2000) was diluted in blocking solution meanwhile. The blocking solution on the slides was discarded and replaced by antibody containg solution and covered again by parafilm. The vibratome sections were transferred to the next well containing the antibody solution. Sections were incubated at 4°C over night.

Blocking solution	10 ml
MABT 5 x	2 ml
Bovine Serum	2 ml
10 % Blocking Reagent	2 ml
H_2O	4 ml

The next day, sections were washed 4 – 5 times in 1 x MABT buffer and twice in freshly prepared AP staining buffer. NBT and BCIP (3.5 µl per ml AP staining buffer) (Roche) were added to the AP buffer and put onto the sections until the desired staining intensity was reached. Reaction was stopped by washing the section in autoclaved water. Sections were processed for immunohistochemistry as described above or mounted and coversliped.

AP staining buffer	50 ml
1 M Tris pH 9.5	5 ml
10 % Tween 20	0.5 ml
5 M NaCl	1 ml
1 M $MgCl_2$	2.5 ml
H_2O	Up to 50 ml

6.8.3 Fluorescent *in situ* hybridization

Fluorescent ISHs were performed for combination with immunohistochemistry and colocalisation at the confocal level. The tyramide Kit (Perkin Elmer) was applied in combination with anti-Digoxigenin antibody coupled with horse-radish-peroxidase (anit-DIG-POD, Roche, 11 207 733 910).

Prehybridization

- Cut sections at 75 µm (ISH immediately after cutting is best, storage at 4°C in 4% PFA is possible)
- Remove sections for use and wash in PBS (make with DEPC water) 2 X 5 min
- Wash briefly (30 seconds) in DEPC water
- Incubate for 5 min in 2.3 % sodium metaperiodate (dissolve in DEPC water)
- Wash briefly (30 seconds) in DEPC water
- Wash in 100 mM Tris pH 7.5 for 5 min
- Incubate in 1 % sodium borohydrate (dissolved in 100 mM Tris pH 7.5) for 10 min
- Wash briefly in PBS
- Wash 2 X 5 min in PBS
- Incubate 5 min in 4% PFA
- Wash 2 X 5 min in PBS
- Incubate in pre-hybridization solution for 1 hour at 55°C
- Incubate in hybridization mix with specific probes overnight at 55°C

Postbridization

- Wash 2 X 30 min in 2X SSC, 50 % formamide at 55°C
- Wash 5 min in 2 X SSC at 55°C
- Wash 30 min in 0.5 X SSC, 60°C

- Wash 30 min in 0.1 X SSC, 60°C
- Wash 2 X 5 min in Buffer 1
- Block for 30 min at room temperature in TNB blocking buffer
- For DIG labeled probes, incubate overnight at 4°C with anti-digoxigenin-HRP (anti-DIG-POD) diluted 1:100 - 1:250 in TNB buffer
- Wash 3 X 5 min in TNT buffer
- Incubate in amplification diluent solution (provided with the Kit) for 10 min
- Wash 3 x 5 min in TNT buffer
- For fluorescence visualization, incubate in Streptavidin conjugated fluorophore (Texas Red or Rhodamine; NEL721) diluted 1:100 – 1:500 in TNB buffer
- Wash 6 X 10 min in TNT buffer or overnight
- Mount and coverslip

Reagents

NTE:

NaCl 5 M, 50 ml

Tris pH 8 1M, 5 ml

EDTA 0.5M pH8, 1 ml

Water to 500 ml final volume

Hybridization Mix (10 ml):

50 x Dendhardt's solution 200 µl

t-RNA 500 µl

50 % Dextran sulfate 2 ml

Formamide 5 ml

SSC 20 X 1.5 ml

Water 800 µl

TNB Buffer:

0.1 M Tris, pH 7.5

Prehybridization Solution:

25 ml Formamide

7.5 ml SSC 20X

Water to 50 ml

Buffer 1 (10 X, 1 l):

60.55 g Trisbase (FW= 121.1g/mol)

87.66 g NaCl (FW = 58.44 g/mol)

Volume to 1 L, pH 7.5

TNT Wash Buffer:

0.1 M Tris, pH 7.5

0.15 M NaCl

0.3% Triton X-100

0.15 M NaCl

0.5 % (wt/vol) blocking reagent (TSA kit, Perkin Elmer)

Stir for 1-2 hr at 60°C until fully dissolved. Filter and store at <20 °C. Solution should be milky white in colour with no particulates.

6.9 Tissue culture

Different approaches have been used in this study: SEZ-derived neurospheres as stem cell expansion in vitro with EGF / FGF2; primary subependymal zone culures without EGF / FGF2. E13 or E14 cultures for viral tittering and specificity test of the Dlx2-Engrailed construct.

6.9.1 Neurosphere cultures

For culturing neurosphere cells from the adult subependyma we followed the protocol described by Johansson et al. (1999). 5 – 6 C57Bl/6 mice were used for one preparation. Mice were killed in a CO_2 containing chamber; decapitated and whole brains were isolated and kept in HBSS containing 10 mM Hepes until dissection. Brains were cut at the midline between the two hemispheres and in the middle. The medial wall of the ventricle was removed and the lateral wall was isolated by cutting beneath the wall with scissors. These tissue pieces were kept in HBSS/Hepes. At the end of the preparation 3.4 mg Trypsin (Sigma, T9201) and 3.5 mg Hyaluronidase (Sigma, H3884) was weighed in a 15ml-Falcon tube. Aliquots of weighed Trypsin/Hyaluronidase were kept at -20°C.

Solutions 1 – 3 are thawn and stored on ice during the tissue preparation:

Solution 1 [5ml per prep.]	**Solution 2** [10ml per prep]
HBSS 10x (Life Tech) [50ml]	HBSS 10x (Life Tech) [25ml]
D-Glucose (Sigma, Stock: 300mg/ml) [9ml]	Sucrose (Sigma) [154g]
HEPES (Life Tech, 1M) [7.5ml]	H_2O [up to 500ml]
H_2O (autoclaved, Millipore) [up to 500ml]	

Solution 3 [19ml per prep.]
BSA (Sigma) [20g]
HEPES (Life Tech, 1M) [10ml]
EBSS (Gibco, 1X) [up to 500ml]

The dissociation medium was freshly prepared by adding 5 ml of Solution 1 to the enzymes in the Falcontube and filter sterilized. Tissue was disscociated for 15 min at 37°C, gently triturated with a 5 ml pipette for 5 times up-and-down and again dissociated for 15 min at 37°C. At the end of the maximal incubation time of 30 minutes there were still some pieces of tissue visible.

Tissue was mixed with 5 ml ice cold Solution 3 and cells were passed through a 70 µm-strainer (Falcon, 2350) to remove bigger pieces of tissue, followed by a centrifugation step at 1300 rpm for 5 minutes in a Falcontube. The supernatant was removed and cells were resuspended in 10 ml ice cold Solution 2 and centrifugated at 2000 rpm for 10 minutes. This centrifugation step is similar to a sucrose gradient and gets rid of dead cells which are very small in size. The supernatant was again removed and cells were resuspended in 2 ml ice cold Solution 3. A new 15 ml tube was filled with 12 ml ice cold Solution 3 and 2 ml of the cell suspension was added on top of the new tube, followed by another centrifugation step at 1500 rpm for 7 minutes. The supernatant was removed and re-suspended in 6 ml Neurosphere media in a small cell culture flask.

Neurosphere media	50 ml
DMEM/F12 + Glutamax	47 ml
B27 supplement	1 ml
Penicillin/Streptomycin (100x)	0.5 ml
HEPES (1M)	0.4 ml
EGF2 (20 µg/ml)	1µl/ml; add fresh
FGF (10 µg/ml)	1µl/ml; add fresh

Cells were cultured in the presence of 20 ng/ml EGF and 10 ng/ml FGF2 (added every second day) under non-adherent conditions to allow for the formation of neurospheres. Neurosphere cells were passaged three times. For passaging the neurosphere containing medium was collected in a 15 ml tube and centrifuged at 800 rpm for 5 minutes. Most of the supernatant was removed spearing 500 µl in which cells were then dissociated mechanically. Cells were counted after the first split two little flasks and after the second one, big flasks were used.

For improvement of the neurosphere growth conditioned medium of previous culture, sterile filtered medium was used until plating of the neurospheres on poly-D-lysine coated cover slips at a density of 120,000 cells per well (24 well plates) in medium containing FGF2. At that stage cells were transduced with pseudotyped retroviruses (see also (M. A. Hack et al., 2004)). The next day the medium was replaced by Neurobasal supplemented with B27 without any addition of growth factors.

6.9.2 Non-expanded adult progenitor cultures

This culture system was newly established in the laboratory in order to better mimic the in vivo. Neurospheres are grown under the addition of growth factors like EGF and FGF2, and up-regulate transcription factors that are not present in neural stem cells under physiological conditions, for example Olig2 (M. A. Hack et al., 2004). In contrast subependymal zone primary cultures grown without EGF / FGF2 express transcription factors that are down-regulated in neurospheres like transcription factors of the Dlx family (Fig. **24**).

Dissociation of the subependymal zone was performed as described above (for details see chapter: 6.9.1 Neurosphere cultures). Six mice were prepared and the subependymal cells were then plated on six cover-slips coated with poly-D-lysine without any addition of EGF and FGF2 in DMEM/F12+Glutamax supplemented medium (neurosphere medium) and transduced by viral vectors 2 - 4 hours later. The next day the medium was filled up to 1 ml and one day later half of the medium was replaced by fresh medium. After 7 days in culture, cells were fixed with 4 % PFA in PBS for 15 minutes at room temperature and processed for antibody staining. Alternatively, after 4 weeks in culture, electrophysiology was performed and cells were fixed afterwards in PFA 4 % and processed for immunohistochemistry.

6.9.3 E13 embryonic cultures

The day of the vaginal plug was considered as embryonic day E 0. The timed pregnant females were killed and embryos were removed by a caesarean section and the whole uterus was isolated. Embryos were decapitated, the brain was isolated from the skull, the hemispheres separated, and the meninges removed. The telencephali were dissected and put into HBSS/HEPES into a 15ml-Tube, followed by a centrifugation step of 5 minutes at 1000 rpm. The supernatant was removed off and 2 – 3 ml of DMEM/PS/FCS was added. Cortices were dissociated mechanically by the use of a

fire-polished Pasteur pipette coated with DMEM/PS/FCS. The cell suspension was centrifuged again at 1000 rpm for 5 minutes and suspended in 1 – 3 ml DMEM/PS/FCS and counted in a Neubauer chamber. Cells were plated at a density of 300,000 cells per well in a volume of 500µl medium. Viral transduction was performed between 2 and 3 hours after plating and the next day the same volume of differentiation medium (DMEM-Glutamax/PS/B27) was added to reduce the serum concentration. The same step was done on the day 4 and 6 in vitro. Cells were fixed on the seventh day in vitro and processed for immunohistochemistry.

6.10 Co-Immunoprecipitation and Western Blot

For one Co-Immunoprecitpitation of Dlx and Pax6 at least 6 olfactory bulbs, 4 cortices and 12 subepenymal zones were used. Tissue was shock-frozen in liquid nitrogen or on dry ice directly after preparation and may be stored at -80°C. However, best results were obtained when the tissue's lysis was performed on the same day of preparation. All steps are performed on ice!

Buffer B (high salt): 2.0 ml of low salt Buffer A plus 160 µl 5 M NaCl solution in water.

Buffer BP (binding buffer): mixture of Buffer A and Buffer B (3:1.8). Isotonic NaCl concentration.

Buffer A (low salt)	5 ml
Hepes (1M)	50 µl
KCl (1M)	50 µl
H_2O	4.9 ml
Proteinase Inhibitor Cocktail - Leupeptin (1mg/ml) - Aprotinin (500µg/ml) - Pepstatin (1mg/ml in MeOH) - Pefablock (100mg/ml)	 10 µl 10 µl 20 µl 20 µl

Total lysate:

Tissue was defrosted on ice and 500 µl Buffer B was added and and the tissue was chopped into pieces with a blue pipette tip, followed by pottering to completely break tissue pieces. Then the liquid was put back into a 1.5 ml reaction tube on ice and incubated for 5 minutes to allow swelling of the cells. Then the cells were sonifiered three times every 5 minutes (10 % amplitude), and Igepal 10 % was added to a final concentration of 1 % in each sample. Samples were then incubated on ice for

45 minutes. Thereafter, samples were centrifuged for 5 minutes at 10.000 rpm at 4°C and the supernatant was carefully removed (= total lysate). The pellet contains DNA, nuclear membrane and not soluble particles. As the samples are until now in high salt the salt concentration has to be reduced to 0.9 % NaCl (isotonic). Thereafter 500 µl total lysate received 800 µl buffer A; this mixture contains now all soluble proteins and 145 mM NaCl.

Preclearing:

ProteinG agarose beads (Immunoprecipitation Kit, Roche, 11 719 386 001) were washed. per sample 70 µl 50 % proteinG-agarose, spun down very shortly, supernatant was removed and 200 µl Buffer BP was added. In total 2 – 3 washes were performed. After the last washing step, as much of the supernatant was removed as possible. The extract was added to the washed beads and incubate for 30 minutes up to one hour in a rolling wheel shaker that also turns the Eppendorf tubes upside down. For removal of the proteinG agarose beads that bound the unspecific proteins, these were shortly centrifuged shortly and the supernatant was transfered to a new 1.5 ml reaction tube. These samples are now clean for the following immunoprecipitation. Keep 20 µl of the supernatant for Western Blot Gel (= input control).

Co-Immunoprecipitation:

3 – 5 µl specific antibody (Pax6 mouse IgG1, Developmental Studies Hybridoma Bank, 2.2 mg/ml; purified by Dr. D. Schulte) per sample was added and the extract was incubated for 1 – 2 hours at 4°C in a rolling wheel shaker. ProteinG-agarose beads were washed meanwhile as described above: 50 – 100 µl 50 % proteinG-agarose beads per sample, depending on the amount of tissue and antibody. Extract containing the antibody was added to the beads and incubated overnight on the rolling wheel shaker at 4°C. The next day samples were spun shortly (1 minute at 10.000 rpm at 4°C) and supernatant was transferred to a new tube. The extract contained all proteins that are not precipitated under the used conditions (may be analyzed by Western Blot). ProteinG agarose beads contain now the precipitated protein complexes. The beads were washed at least 4 (!) times with 600 µl Buffer BP and the last time with 1 ml 1 x PBS (+ inhibitors). 20 µl aliquots from the first and the last washing step for Western Blot Gel (= washing controls) were kept on ice or -20°C.

As much supernatant was removed from the beads as possible from the last washing step and sample buffer was added (see standard solutions) and heated up to 95°C for 5 minutes. Probes were placed immediately on ice. Proteins can now be stored at -20°C or immediately processed for Western Blot.

Western Blot gel:

Samples were loaded on SDS-PAGE gels.

Collecting Gel 4 %, 7.5 ml: 4.575 ml clean H_2O, 0.975 ml acryl amide (Biorad), 1.875 ml gel buffer pH 6.8 (0.5 M Tris pH 6.8), 37.5 µl 20 % SDS, 37.5 µl APS, 7.5 µl TEMED (Biorad).

Separation Gel 12 %, 15 ml (2 gels with 1.5 mm thickness): 5.1 ml clean H_2O, 6 ml 30 % acryl amide, 3.75 ml gel buffer pH 8.8 (1.5 M Tris pH 8.8), 75 µl 20% SDS, 75 µl 10 % APS, 7.5 µl TEMED.

The Western Blot Gels run under the following conditions: 1 h 80 V, 2 h 120 V. Gels were equilibrated for 30 minutes in Transfer buffer (200 ml MetOH, 100 ml electrophoresis buffer (see standard solutions), and 700 ml H_20).

Blot:

Membranes (PVDF, Biorad) were prewetted with MetOH, which was replaced stepwise by transfer buffer. Blot was performed in semi-dry conditions (Panther, Platin): 320 mA per gel / 15 V limited, 45 minutes. Immediately after the end of the blot PVDF membranes were washed three times for 5 minutes in 1 x TBST (see standard solutions).

Detection:

Membranes were prepared for antibody staining by blocking in 1 x TBST containing 5 % milk powder (Biorad) for 1 hour at room temperature. The primary antibody Dlx2 (1:1000 in TBST containing 5 % milk powder) was applied overnight at 4°C followed by another washing step the next day for three times 15 minutes in 1 x TBST. The secondary HRP labelled antibody (anti-rabbit 1:10.000 in TBST containing 5 % milk powder) was applied for two hours at room temperature. Afterwards membranes were washed again three times in 1 x TBST for at least 30 minutes. Detection of proteins on the membranes was done in a dark chamber using an ECL-Plus detection Kit (Amersham). The Kit was prepared (2 ml Solution A; 50 µl Solution B) and the film

(Amersham photo paper) was developed with different length of illumination (between 1 minute and 30 minutes depending on the strength of the light signal).

6.11 Chlorine e_6 induced lesion model of cortical projection neurons

This lesion model is described in (J. D. Macklis and R. Madison, 1985; R. D. Madison and J. D. Macklis, 1993; V. L. Sheen and J. D. Macklis, 1994, 1995; S. S. Magavi et al., 2000; J. Chen et al., 2004) and was established according to the published protocols. The principle of photoacitvated cell death includes injection of chlorine e_6 coupled latex beads into the cerebral neocortex, retrograde transport through callosal projection neurons into the contralateral hemisphere, and illumination with a red laser (wavelength 633 nm). This leads to the release of oxygen radicals from chlorine e_6 and consequently to neuronal death.

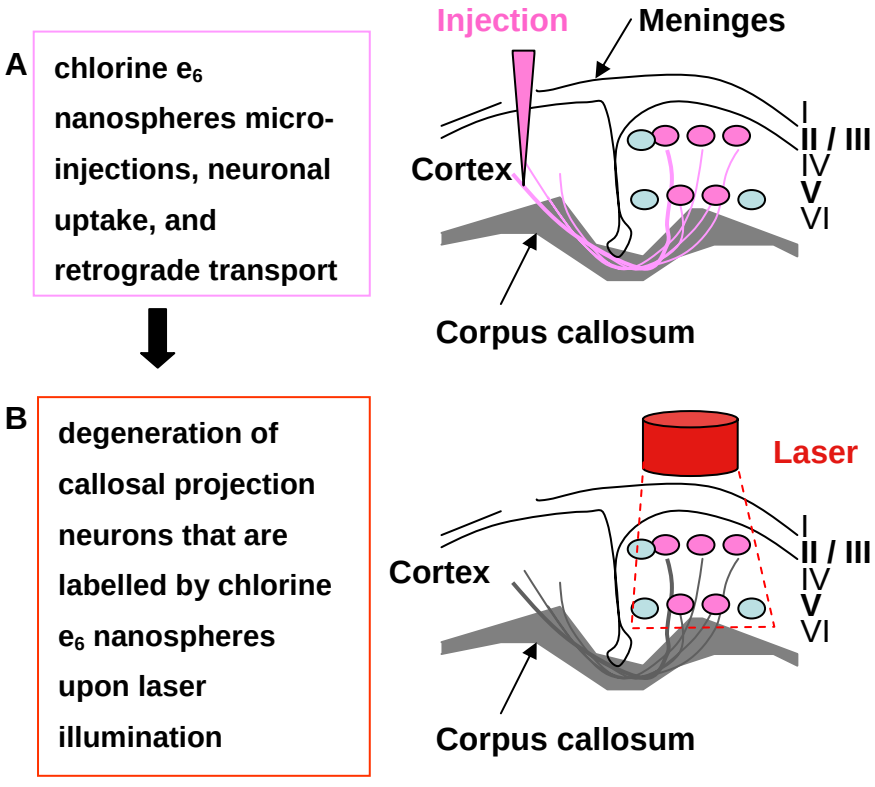


Fig. 62: Principle of targeted neuronal cell death of callosal projection neurons (adapted from (V. L. Sheen and J. D. Macklis, 1995)).

(A) Latexbeads were coated with chlorine e_6 and re-suspended in a small volume. The nanospheres were injected into the ipsilateral side of the cortex several times. Nanospheres are taken up by axons of cortical projection neurons and transported through the corpus callosum to the contralateral hemisphere as depicted by pink cell bodies. Callosal projection neurons are present especially in layer II, III, and V of the cortex.

(B) The contralateral hemisphere was illuminated with a red laser (wave length 633 nm) as depicted in the lower scheme. Upon wave-length specific illumination chlorine e_6 produces singlet oxygen radicals that lead to activation of apoptotic pathways and cell death of callosal projection neurons in layer II, III, and V of the cortex.

Microsphere coating with chlorine e_6 and injection:

1.79 mg Chlorine e_6 (Frontier) was dissolved in 3 ml 0.01 M phosphate buffer [diluted from an autoclaved 0.1 M stock solution with autoclaved Millipore water; 0.1 M stock: 1.76 g of NaH_2PO_4 x 2 H_2O and 6.9 g of Na_2HPO_4 x 2 H_2O (Merck) were dissolved in 0.5 l millipore water, pH was adjusted to 7.4 and autoclaved]. 5 mg of 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (M = 191.7 g/mol, MP Biomedicals) was added to the chlorine e_6 solution and the mixture was kept for 30 minutes on ice (= activation of Chlorine e_6).

25 µl of rhodamine latex microspheres (latexbeads, Lumaflour, USA) were diluted in 150 µl 0.01 M phosphate buffer and 1.5 ml of the activated Chlorine e_6 solution was added into the tube and shook for 1 hour at RT. The reaction was stopped by the addition of 0.1 M glycine buffer [0.1 M glycine buffer pH 8; glycine (Sigma)]. Coated beads were centrifuged in an ultracentrifuge at 100,000 g (32,000 rpm; 30 – 40 minutes, SW40Ti) and washed at least twice until the supernatant was completely clear with 0.01 M phosphate buffer. After the last washing step as much of the supernatantas possible was removed an the pellet was resuspended and resuspended in 50 µl volume 0.01 M phosphate buffer (normally no additional buffer was added any more).

Injection of the coated beads was done as described above. Around 200 nl coated latexbeads were injected into the cortex at least five times at the level of bregma and laser illumination was performed 5 – 7 days later on the contralateral hemisphere. After removal of the skull bone (window of 2 mm diameter) illumination was performed for variable length of time [1 – 5 minutes] and at variable intensities [1 mW – 50 mW].

7 Appendix

7.1 Abbreviations

APS	Ammonium persulfate
bHLH	basic Helix-Loop-Helix
BrdU	5-Bromo-2′-deoxy-Uridine
cDNA	complementary DNA
CTX	Cortex
DAPI	4’-6-Diamidino-2-phenylindol
DCX	Doublecortin
DIV	days in vitro
Dlx2Eng	Dlx2 homeodomain cDNA fused to the engrailed repressor domain
DNA	Desoxyribonucleic acid
dNTPs	Deoxy-nucleotide-triphposphates
E	embryonic day
EGF	epidermal growth factor
eng	engrailed repressor domain
EPL	external plexiform layer
EtBr	Ethidium Bromide
EtOH	Ethanol
FGF	fibroblast growth factor
Fig.	Figure
GABA	γ-amino-butyric acid
GAD	Glutamic Acid Decarboxylase
GE	ganglionic eminence
GFAP	Glial fibrillary acidic protein
GFP	green fluorescent protein
GL	glomerular layer
GN	granule neuron
HC	Hippocampus
HRP	Horse radish peroxidase
IRES	internal ribosome entry site
ISH	In situ hybridization
LTR	long terminal repeat
LV	lateral ventricle
MCL	mitral cell layer
MetOH	Methanol
mRNA	messenger ribonucleid acid
n	number of samples
NE	neuroepithelium
OB	Olfactory Bulb
ON	overnight
P	postnatal day
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PFA	paraformaldehyde
PGN	periglomerular neuron
RFP	red fluorescent protein
RMS	rostral migratory stream
RNA	ribonucleic acid
rpm	rounds per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS – polyacrylamid gel electrophoresis
SEM	standard error of the mean
SEZ	subependymal zone
STDEV	standard deviation
SVZ	Subventricular zone
TAP	transit-amplifying progenitor
TEMED	N,N,N',N'-tetramethylethylenediamine
TF	transcription factor
TH	Tyrosine Hydroxylase
TSA	tyramid signal amplification
UTR	untranslated region
VZ	Ventricular zone
WM	White matter
WT	wildtype

7.2 List of figures

8 References

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9 Curriculum vitae

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