REGULATION OF NEUROGENESIS IN THE ADULT MAMMALIAN BRAIN

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.) DER NATURWISSENSCHAFTLICHEN FAKULTÄT III - BIOLOGIE UND VORKLINISCHE MEDIZIN DER UNIVERSITÄT REGENSBURG

vorgelegt von

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2003

Promotionsgesuch eingereicht am: 18.02.2003

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It is only when we forget all our learning that we begin to know." – Thoreau

"If you have built castles in the air, your work need not be lost. That is where they should be. Now put the foundation under them." - Thoreau

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LIST OF ABBREVIATIONS

5,7-DHT	5,7-dihydroxytryptamine
5-HT	serotonin
5-HTP	5-hydroxytryptophan
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	brain-derived neurotrophic factor
BrdU	bromodeoxyuridine
CA1/CA3	Ammon's horn or *cornu ammonis* of the hippocampus
cAMP	cyclic adenosine monophosphate
CBF	cholinergic basal forebrain
ChAT	choline acetyltransferase
CNS	central nervous system
CPS	cryoprotectant solution
CY5	Indodicarbocyanine
DAPI	4,6-diamidino-2-phenylindole
DBH	dopamine beta-hydroxylase
DCX	doublecortin
DG	dentate gyrus
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
FGF-2	basic fibroblast growth factor
FITC	fluorescein isothiocyanate
Flk-1	fetal liver kinase-1
GABA	gamma-amino butyric acid
GCL	granule cell layer
hr	hour(s)
HVC	higher vocal center
ICV	intracerebroventricular
IGF-1	insulin-like growth factor-1
KA	kainate
LC	locus coeruleus
LV	lateral ventricle
MCL	mitral cell layer
min	minute
ML	molecular layer
NET	norepinephrine transporter
NeuN	neuronal nuclear antigen
NMDA	N-methyl-D-aspartate

NSE	neuronal specific enolase
NT-3	neurotrophin-3
OB	olfactory bulb
ON	over night
PB	phosphate buffer
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCPA	p-chlorophenylalanine
PFA	paraformaldehyde
PGL	periglomerular layer
PI	propidium iodide
PSA-NCAM	polysialylated neural cell adhesion molecule
Rb	retinoblastoma gene
RECA	rat endothelial cell antigen
RHOX	rhodamineX
RMS	rostral migratory stream
RT	room temperature
SGZ	subgranular zone
SVZ	subventricular zone
TBS	tris buffered saline
TGF-ß	tumor growth factor-ß
TH	tyrosine hydroxylase
TUC-4	TOAD (turned on after division)/Ulip/CRMP
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling
TX100	triton-X100
VEGF	vascular endothelial growth factor

INTRODUCTION

Generation of new neurons in the adult mammalian brain

The discovery that stem, or progenitor, cell populations produce neurons in the adult brain highlights the dynamics of a previously assumed "static" organ. During development, all organs are created from groups of proliferating cells, which have the potential to differentiate into those cell types that are needed for a functional organ. In adult tissues, these organ-specific stem cells are thought to play a major role in replacement of cells lost to physiological wear and injury[1-3]. The existence of neural progenitor cells in the adult central nervous system (CNS) is therefore seen as a paradox, since the brain has classically been thought of as a structure with very limited regenerative capacity. Nevertheless, in recent years, it became evident that the adult brain is generating certain neuronal populations through a process termed "adult neurogenesis".

As early as 1912, scientists have noted mitotic activity in the cells of the lateral ventricle of the adult rat[4]. And although Altman proposed the concept of persistent neurogenesis in the adult rodent brain as early as 1965[5], the neuroscience community was hesitant to recognize the importance of this observation until 1977 when Kaplan and Hinds were able to confirm, through the use of electron microscopy and ^{3}H-thymidine labeling, the neuronal fate of newly generated cells in the dentate gyrus (DG) and subventricular zone (SVZ)/olfactory bulb (OB)[6]. Through the use of better labeling methods for newborn cells, it is now generally accepted that the DG of the hippocampus and the OB are the two main areas of adult neurogenesis in the rodent.

The fact that new neurons are generated in the adult mammalian CNS has major implications for the study of the brain's regenerative capacity. (1) Neural plasticity is not restricted to axonal sprouting and synaptic remodeling. The possibility of addition or replacement of neurons could provide a fundamentally new approach to compensate for neuronal loss during aging and after injury or disease. (2) The existence of multipotent progenitor cells in the adult brain provides the possibility to study their functional behavior either *in vivo* or after isolation *in vitro*. This allows new insights into important concepts of progenitor cell biology, such as self-renewal, multipotentiality, lineage-

commitment and differentiation. Moreover, strategies using these cells as autologous sources for the therapy of neurodegenerative diseases can be developed. (3) Signals, which are necessary to generate neurons from undifferentiated cells, are present in certain regions of the adult brain. The special microenvironment that makes a region neurogenic provides molecular signals required for each step of the generation of new neurons. In order to make therapeutic use of neurogenesis it is important to know the sequence of events and signals that lead to continuous neuronal replacement. These signals could be used to induce neurogenesis of other neuronal populations that undergo neurodegeneration.

Subventricular Zone/Olfactory Bulb Neurogenesis

The newborn neurons that are generated in the OB originate from the subventricular zone (SVZ) of the lateral ventricle (LV). The SVZ is seen as a residual proliferative matrix left over from the embryonic neural tube, which harbors rapidly dividing neural progenitor cells. In the adult, the progeny of the proliferating SVZ cells migrate through the so-called “rostral migratory stream” (RMS) and proceed to the OB (Figure 1B). It is important to note that two neuronal phenotypes are generated in the OB, GABAergic granule cells (Figure 1D), which represent the majority (99%) of the new OB neurons, and 1% dopaminergic periglomerular interneurons (Figure 1E)[7-9].

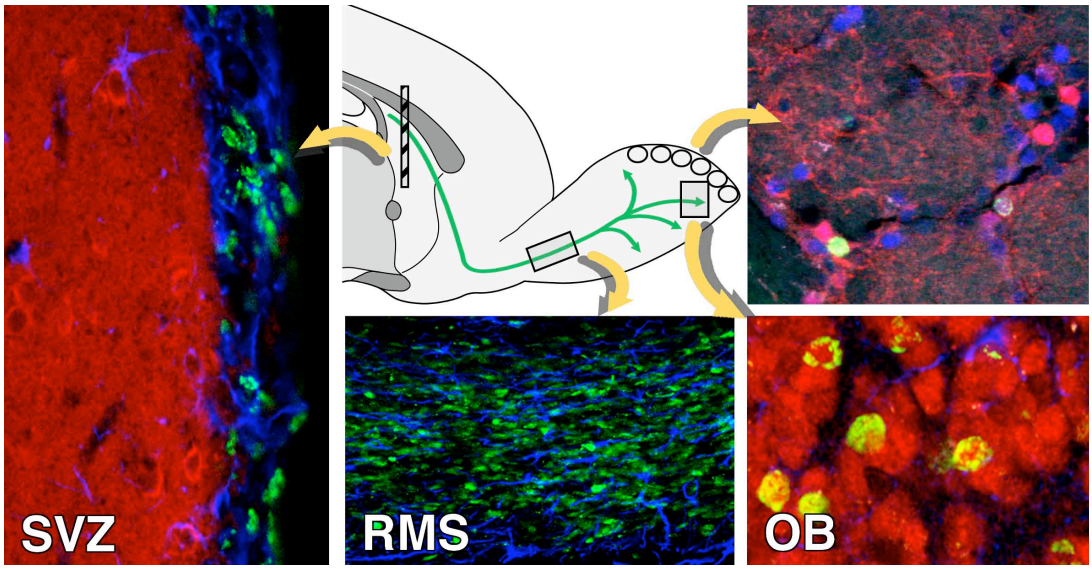


Figure 1
Neurogenesis in the adult olfactory bulb (OB) is initiated by proliferation in the subventricular zone (SVZ) of the lateral ventricle (left). The newly formed cells (green – BrdU labeling) migrate through the rostral migratory stream (RMS) and into the olfactory bulb, where they differentiate into two different neuronal types: 1 – periglomerular dopaminergic interneurons (yellow, upper right), and 2 – GABAergic granular neurons (yellow, lower right).

The olfactory granule cells are inhibitory interneurons that make their dendritic connections to the mitral cells (MCL) and to the middle tufted cells (see Figure 2 to illustrate the connectivity in the OB). The periglomerular neurons project their dendrites into the corresponding glomerulum and connect to the incoming olfactory axons from the sensory epithelium; however, their axons project to mitral cells that reach into the glomerular layer. Recently, it has been shown that these newly formed neurons are functionally integrated into the synaptic circuitry of the OB[10] and there are implications that olfactory interneurons may be important for maternal behavior, such as adaptive behaviors in mating and pregnancy[11]. In addition, data supporting the functional integration of newly generated neurons have shown that sensory deprivation, by plugging one nostril in the rat, downregulates neurogenesis in the ipsilateral GCL[12]. Once the nasal plug is removed, the level of neurogenesis returns to normal[13] indicating an involvement of neurogenesis in olfactory functioning.

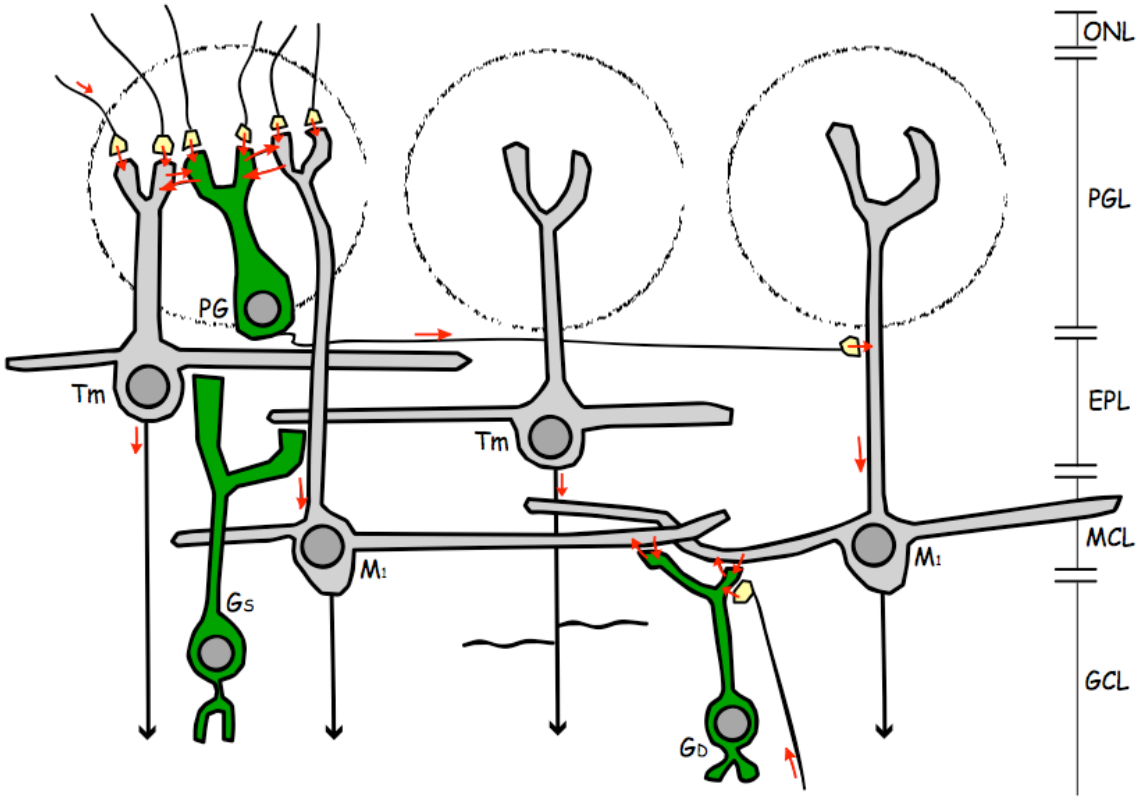


Figure 2
Depiction of olfactory bulb connectivity. Periglomerular (PG) neurons (green cell) reside in the periglomerular layer (PGL), make dendritic connections in the olfactory nerve layer (ONL) and project their axons to mitral cells (M_1) that reside in the mitral cell layer (MCL). Mitral tufted cells (T_m) reside in the external plexiform layer (EPL) and project their dendrites into glomeruli of the PGL, while their axons make connections with the granule cells (G_S/G_D) of the granule cell layer (GCL). The excitatory superficial and deep granule cells make their dendritic connections with the tufted mitral cells in the EPL and the mitral cells of the MCL, respectively (adapted from Shepherd and Greer[14]).

Hippocampal Neurogenesis

In the hippocampus, a structure involved in the processing and storage of new information, progenitor cells are born along the thin border between the granule cell layer (GCL) and the hilus, termed the subgranular zone (see Figure 3 for anatomical details). After cell division neuroblasts disperse along the subgranular zone and migrate a short distance into the GCL along the processes of existing neurons and radial glia like cells[15]. The newly formed cells take on the morphology and immunohistochemical properties of granule cells and express the granule cell marker Calbindin and neuronal markers, such as NeuN (a pan-neuronal marker) and NSE, and to a very small degree S100ß (a calcium binding protein and astrocyte-specific marker)[16-18].

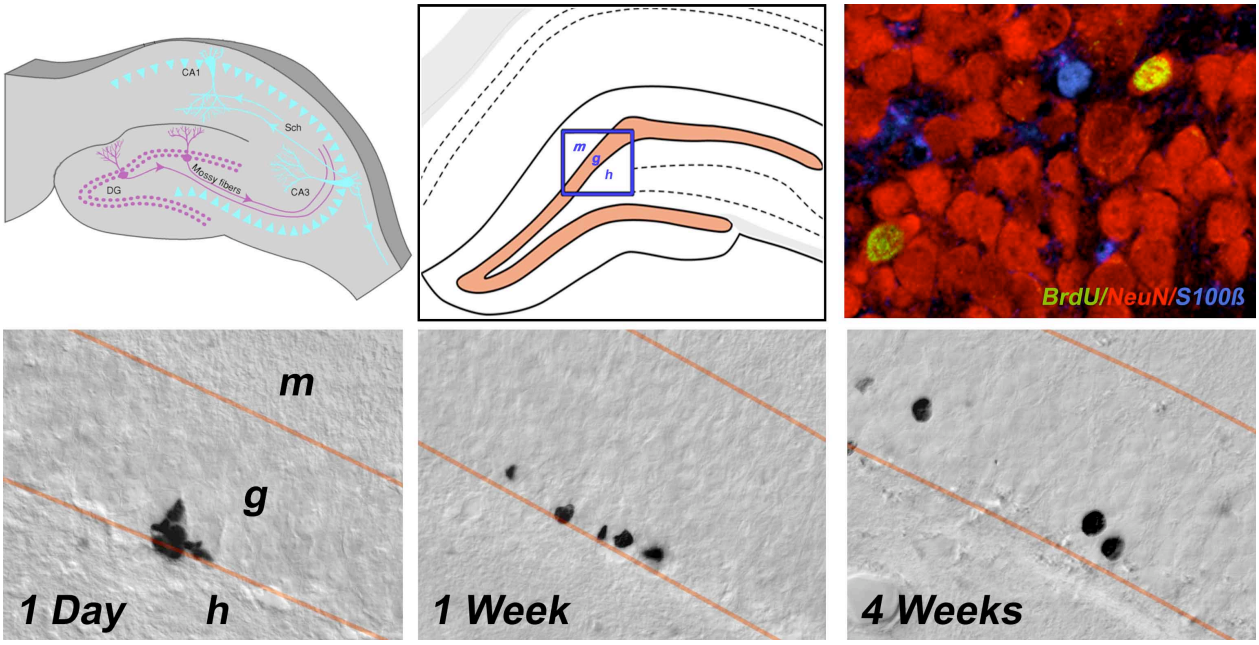


Figure 3
Neurogenesis in the adult hippocampus. Proliferation of progenitor cells is detected in the subgranular zone (border between granule cell layer, g and the hilar region, h) through BrdU labeling (1 day – bottom left). Notice the clumps of proliferating cells that can be up to 100 cells (bottom left), which within the first week after BrdU labeling (bottom middle) begin to redistribute along the subgranular zone and later (bottom right) migrate into the granule cell layer. In the following weeks the newly formed cells differentiate into granular neurons and begin to express neuronal markers, such as NeuN (red, top right) and Calbindin (picture modified from[19]).

These newly formed neurons, like all other granule cells of the DG, project their axons to the CA3 region, form synaptical connections and are not electrophysiologically discernible from the other granule cells [10, 20-23]. As the axons migrate through the GCL to make their connections within the hilus and CA3 region, they interact with projections within the SGZ and hilus of axons originating from the dorsal raphe (serotonergic) and

the locus coeruleus (noradrenergic). As the dendrites proceed through the molecular layer, they receive synaptic input from axons coming from the entorhinal cortex (glutamatergic) and the medial septum (cholinergic) via the perforant path and fimbria-fornix, respectively (Figure 4).

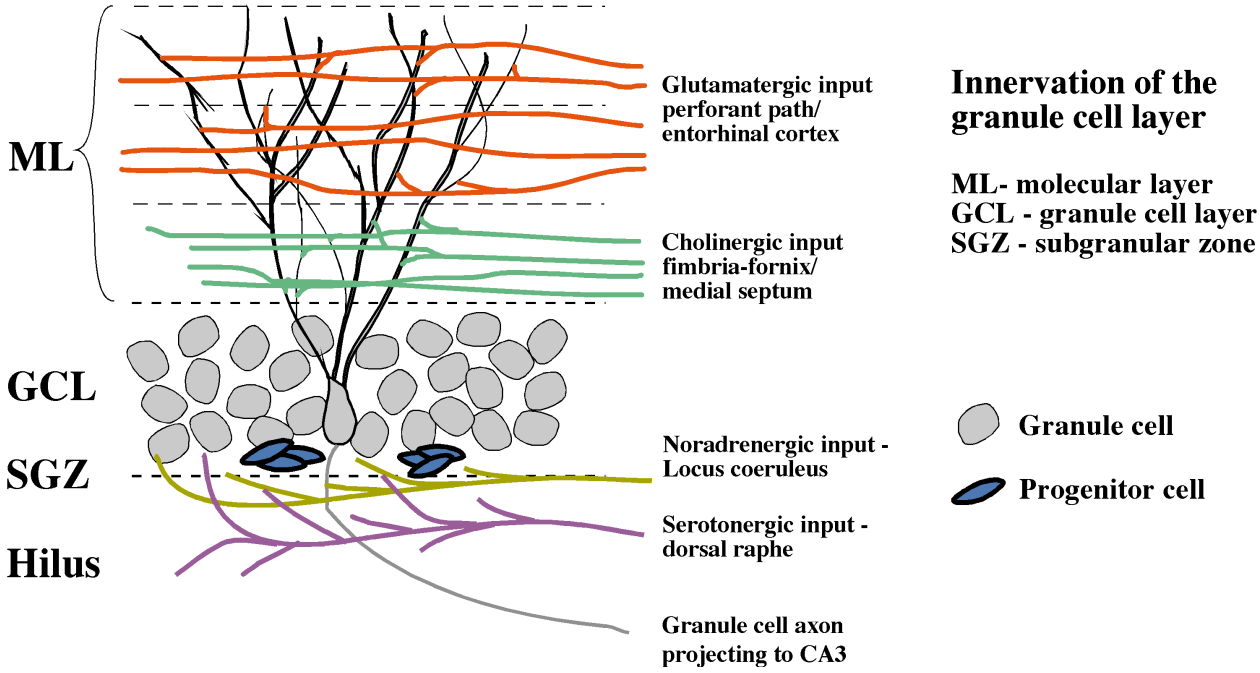


Figure 4

Depiction of neurotransmitter connectivity in the dentate gyrus. The granule cells residing the granule cells layer (GCL) project their axons to the CA3 regions and make their dendritic connections within the molecular layer (ML).

Studies using retrograde and anterograde labeling, as well as long-term potentiation have postulated the functional integration of adult generated granule cells into the hippocampal circuitry. Recently, electrophysiological and viral-labeling studies confirmed that adult born granule cells have functional synapses and are indistinguishable from their neighbors generated during early development[20-22, 24].

The capacity for the adult brain to generate new neurons within these neurogenic regions is immense. For example, it has been estimated, through BrdU labeling strategies, that several thousand new cells are born every day within the DG of the rat[25]. This number of new granule neurons generated each month represents 6% of the total population of the granule cells (between 1 and 2 million)[26]. These numbers, together with indications of physiological integration, would suggest that the newly formed neurons very likely play an important functional role in the hippocampus[27]. Within the

olfactory system neurogenesis occurs at a magnitude higher than in the hippocampus. Quantitatively, in rats it was estimated that at least 30,000 new neurons per day reach the OB and these are capable of forming functional connections and fully integrating into the existing circuitry (Figure 5).

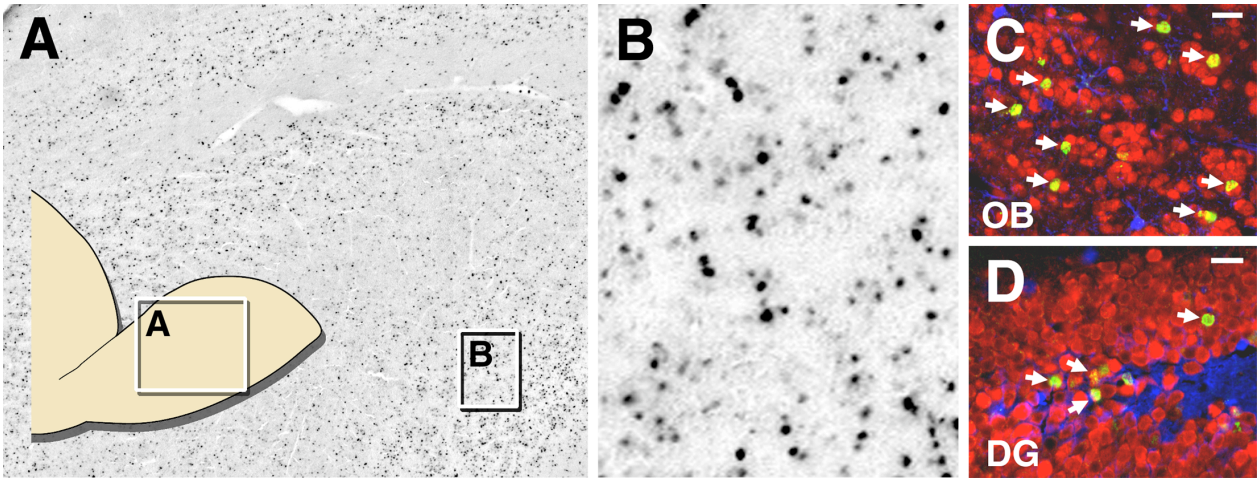


Figure 5

Neurogenesis in the adult olfactory bulb. Animals received BrdU injections on 12 consecutive days and were analyzed four weeks later. (A,B) A representative sagittal section reveals the high density of BrdU-positive cells in the olfactory bulb. All labeled cells seen in the olfactory bulb migrated from the SVZ, since the bulb is free of BrdU-positive cells immediately after labeling (data not shown). (B) Enlarged frame from (A). (C,D) Double labeling of BrdU-positive cells with neuronal and glial markers. Antibodies against NeuN (red) and S100ß (blue) and were combined with anti-BrdU (green) to detect neurogenesis in (C) the olfactory granule cell layer – OB and (D) the granule cell layer of the dentate gyrus – DG. Arrows point out cells that are double-positive for BrdU and NeuN and represent newborn neurons. For quantification of double-labeling see Table 1. Scale bars in (C,D) 20 µm. (Adapted from[28])

Developmental Aspects of Adult Neurogenesis

During development the SVZ forms adjacent to the ventricle zone (VZ) and is most prominent in the ganglionic eminences[29] (Figure 6). Later in embryonic development the SVZ generates cells for the diencephalon[30] and cortex[31, 32], as well as for the adjacent basal ganglia. The cell types and lineage-relationships of the embryonic SVZ are not fully understood; however, the location and proliferative activity of the adult SVZ suggest that it is derived from the embryonic SVZ of the lateral ganglionic eminence[33]. It has not yet been shown conclusively as to whether the progenitors in the embryonic SVZ are equivalent to those in the adult SVZ.

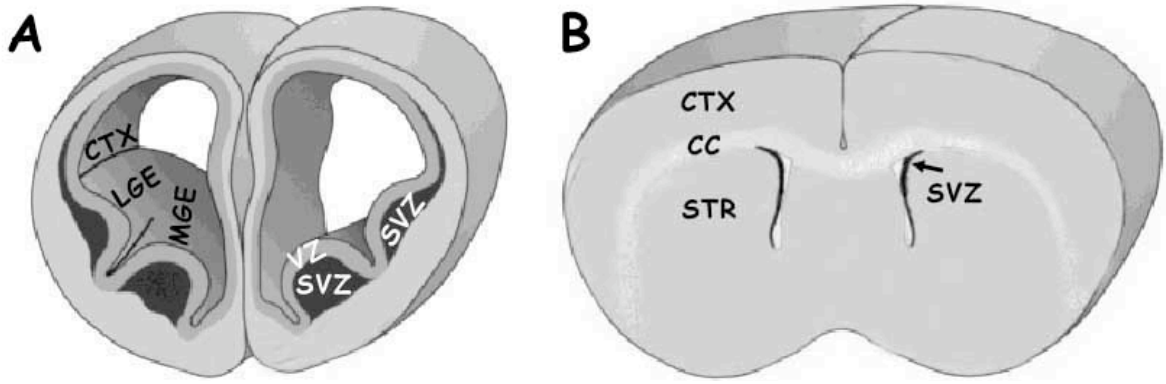


Figure 6
(A) Coronal section through the embryonic mouse forebrain at day 15 post conception. The proliferative ventricular zone (VZ) lines the lateral ventricles. A secondary proliferative region, the subventricular zone (SVZ), is prominent in lateral and medial ganglionic eminences (LGE and MGE) and transiently appears under the VZ of developing neocortex (CTX). (B) Coronal section through the adult mouse telecephalon. Embryonic VZ is transformed into a terminally differentiated ependymal layer lining brain ventricles. Proliferative SVZ remains adjacent to the ependymal layer of the lateral walls of the lateral ventricles. This region is thought to be derived from SVZ of lateral ganglionic eminence (A). CTX = neocortex; CC = corpus callosum; STR = striatum. (Modified after Garcia-Verdugo, et al.[33])

During hippocampal development, the granule cell is the neuronal cell type that is last to be born and in rodents, after birth, is generated from a secondary germinal matrix in the hilus region[34, 35] . It seems that this matrix is capable of sustaining the viability of undifferentiated cells, therefore creating the basis for continued neurogenesis in this region. In the adult, the secondary germinal matrix is refined to a small band along the border between the hilus and the GCL, termed the “subgranular zone”[35]. Here, the progeny of proliferating progenitors migrate locally to differentiate into neurons of the dentate GCL.

Neurogenesis in the adult brain appears to be a continuation of early postnatal neuronal production, as there is no onset of adult neurogenesis, but persisting neurogenesis that started during embryonic phases. In this sense, the development or continued maintenance of such structures as the OB or the DG never ends, since neurogenesis can be observed into very late stages of life[19], even in primates and humans[36, 37]; however, in both rodents and macaque monkeys, cell production in the DG has been shown to decline with age[19, 36]. Nevertheless, neurogenesis is still detectable in very old age, which opens the possibility of enhancing neurogenesis in the aged brain to study changes in the age-related decline in hippocampus-dependent learning[38].

Phylogenetic Aspects of Adult Neurogenesis

Species shown to have neurogenesis	
Invertebrates	Several specials of *Orthoptera* and *Coleoptera*, milkweed bug, cockroach, cricket, darkling beetle, lady bird, decapod crustaceans (crayfish, shore crab, lobster)
Non-mammalian vertebrates	Goldfish, zebrafish, gymnotiform fish, frog, lizard, turtle, canary, songbird, chickadee
Mammalian vertebrates	Mouse, rat, gerbil, guinea pig, squirrel, vole, pig
Humans and primates	Tree shrew, marmoset, macaque monkey, human

Invertebrates

In invertebrates, persistent neurogenesis has been shown to occur in the mushroom bodies of the insect brain, which are brain structures involved in learning and memory and considered as functional analogues of the hippocampus[39-42]. In decapod crustaceans, neurogenesis occurs among the different neuronal types of the central olfactory pathway throughout adult life, suggesting structural plasticity of the brain circuitry[43-45]. In addition, it has been shown that adult crickets, which are introduced into an enriched sensory and social environment, have an increased neuroblast proliferation and neuronal numbers in the mushroom bodies[46]. However, neurogenesis was not found in the adult brain of the honeybee[47], the fruitfly[48], the monarch butterfly[49] or the migratory locust[50].

It is not exactly certain why neurogenesis persists in some insect species and not in others. To use phylogenesis as a criterion is not sufficient, since adult locusts, which are phylogenetically close to crickets, do not maintain proliferating neuroblasts, whereas some species, which are evolutionarily farther removed, such as *Tenebrio* (meal-worm or darkling beetle) or *Harmonia* (lady bird), have adult neurogenic patterns similar to crickets.

Non-mammalian vertebrates

Adult neurogenesis has also been shown to occur in non-mammalian vertebrates, such as fish, frogs, lizards, turtles and birds[51-53]. Neuronal recruitment occurs widely throughout the adult songbird brain[54], but has been studied best in the telencephalic

high vocal center (HVC) and adjacent mediocaudal neostriatum[53, 55]. In both cases, the extent of ongoing neuronal recruitment is extraordinary: the adult canary HVC alone recruits over 1.4% of its neurons daily[56, 57]. Within this model, it has been established that neurogenesis varies dramatically with seasonal changes in song production[58, 59]. In chickadees, the learning behavior related to finding stored food is also correlated with seasonal adult neurogenesis [60].

Fish, in particular, have exceptional abilities to regenerate parts of their CNS after injury and experimental insult[61, 62]. As is seen in mammalian systems, neurogenesis in the adult teleost CNS appears to be restricted to specific proliferative zones. These regions in the adult forebrain have been reported in zebra fish[63, 64], goldfish[65], and gymnotiform fish[51].

In the adult frog, new cells are continuously generated in the preoptic recess ventricular zone. From here, they are recruited to the hypothalamic preoptic nucleus[66]. In both the lizard and turtle telecephalon, postnatal neurogenesis is also observed, with an intense production of neurons in the medial cortex of the lizard, which has homology with the hippocampal DG of mammals[67, 68]. The ependymal cell layer underlying the medial cortex retains its proliferative properties in adulthood and continues to produce new cells[69]. The lizard OB and nucleus sphericus are two other telencephalic regions that also undergo adult neurogenesis[70, 71].

Mammals

Discovered initially in rodents, adult neurogenesis has been demonstrated to occur in the brains of all vertebrate species investigated thus far[5, 36, 53, 67, 72-77], including humans[37], suggesting that it is both a primeval and a highly conserved process. Although early attempts to detect neurogenesis in adult primates were hampered by insensitive detection methods[78] or difficulties with the methods used, several reports now demonstrate that neurogenesis continues in the primate dentate[36, 73, 74] and possibly in other areas, such as the neocortex and the amygdala[79, 80]. In addition, it has been shown in the primate that the newly generated cells in the SVZ are capable of migrating a remarkably long distance (several centimeters) to reach the OB[81].

Work with terminally ill patients has recently confirmed that humans also generate new neurons. The proliferation marker bromodeoxyuridine (BrdU) was injected into

patients to monitor tumor cell proliferation. Some of these individuals subsequently died from their illness and the hippocampi were evaluated for the presence of BrdU-labeled neurons. Since BrdU was systemically administered, all dividing cells had been labeled and, indeed, newborn neurons were detected in the GCL of all individuals ranging up to more than 70 years of age[37].

In summary, adult neurogenesis is a common phenomenon among lower vertebrates, where continuing growth of neural structures as well as complete repair after neuronal damage can be observed. In mammals, the extent and regions of neurogenesis appear to be more restricted, although it is still detectable in the DG of the human brain. Nevertheless, in the invertebrate nervous system large species differences occur.

Fundamentals of Adult Neurogenesis

In order to experimentally modify adult neurogenesis, it is necessary to separate the mechanism into the crucial components. Neurogenesis is often thought of only in terms of progenitor proliferation; however it encompasses progenitor *proliferation* as well as *fate determination* (in the case of multipotent cells), *migration,* and *differentiation* into fully mature neurons. Moreover, as will be described in more detail below, the outcome of adult neurogenesis is also largely dependent on the capability of the immature neurons to *survive* and not succumb to *cell death*.

1) Proliferation
2) Migration
3) Differentiation
4) Survival

Accordingly, just because *proliferation* is increased or decreased under certain conditions, it does not necessarily mean that, as a consequence, the number of neurons has increased or decreased. The migration of these cells can also be influenced, thereby producing an effect in the target area or in the area that the proliferating cells migrated to. A change in the fate of a cell is also crucial when one thinks about regulating neurogenesis. If a pool of cells that were originally destined to become neurons is suddenly persuaded to become astrocytes (or vice versa), this can have

detrimental effects. Last, and certainly not least, the survival or proper elimination of these newly formed neurons is also crucial to the overall outcome.

With tens of thousands of neurons being born every day in the adult rodent brain, it becomes apparent that cells must also be lost to maintain the brain within the limited confines of the skull. Although it was shown in the 80's that the total number of granule cells in the hippocampus and OB continue to increase throughout the lifetime of the rat[16, 82], the numbers are nowhere near the numbers calculated from proliferative data (Figure 7). This has led to the assumption that an elimination process removes surplus cells.

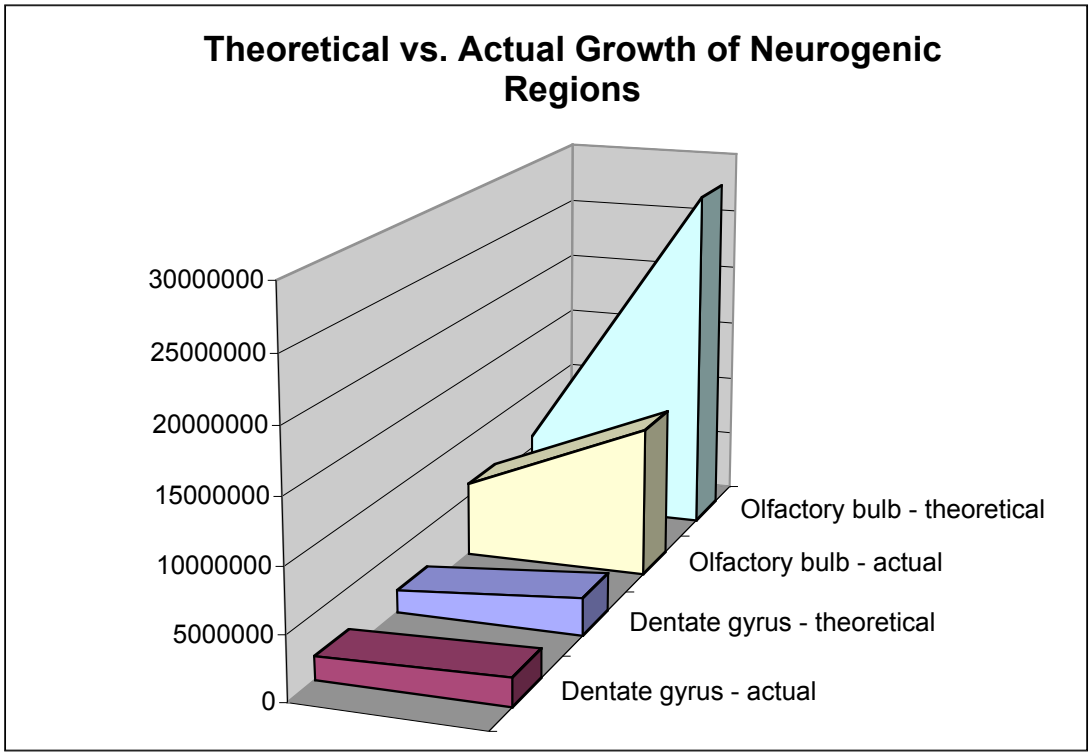


Figure 7
From Bayer and Kaplan studies it is known that the number of granule cells in the dentate gyrus and the OB continually increase over the first year of the rat's adult life (real growth[16, 83]). However, one can estimate from the proliferation data a much higher growth (calculated), which suggests a possible elimination mechanism through cell death that regulates the actual numbers.

Work done in collaboration with Manfred Biebl has shown programmed cell death in adult brain regions with known neurogenic activity[28]. Other reports have confirmed that neuronal progenitor cells are eliminated in the postnatal and adult SVZ and RMS[84-86]. Our stereological assessment, however, emphasized that the elimination of cells occurs in a caudal to rostral gradient of apoptotic cells, with an increasing number of

TUNEL-positive cells towards the OB. More importantly, our work reveals that areas of adult neurogenesis contain up to 100 times more apoptotic cells than non-neurogenic regions. Since about 80% of these cells were actually localized in the olfactory GCL, it was concluded that a large proportion of cells generated in the SVZ are eliminated after reaching their target area, the OB.

When analyzing neurogenesis and programmed cell death in the DG, a similar ratio of BrdU-positive to TUNEL-positive cells was detected in the GCL. The majority of dying cells was found at the border between the hilus and GCL, again suggesting that elimination of cells occurs primarily in the neurogenic region of the DG[87]. Taken together, these data indicate that programmed cell death has an important regulatory function by eliminating supernumerous cells from neurogenic regions and thus contributes to a self-renewal mechanism in the adult mammalian brain.

In order to understand whether the newly formed cells or the already existing neurons are eliminated, another study was performed in our lab in collaboration with Beate Winner[9]. Adult rats were injected with BrdU and a time-course analysis was carried out. At specific time points after BrdU injection, the rate of the loss of BrdU-positive cells in the OB was measured. It turns out that approximately 50% of newly formed BrdU-positive cells are eliminated within the first 3 mos. However, newly formed neurons that survive this elimination phase are detectable for the rest of the lifetime of the rat, or at least to the last time point measured (19 months) (Figure 8). From these results, it can be concluded that progenitor cells and young neurons are the ones being eliminated. This mechanism mirrors the embryonic brain development, where approximately 50% of the neurons are overproduced and eventually eliminated through programmed cell death[88].

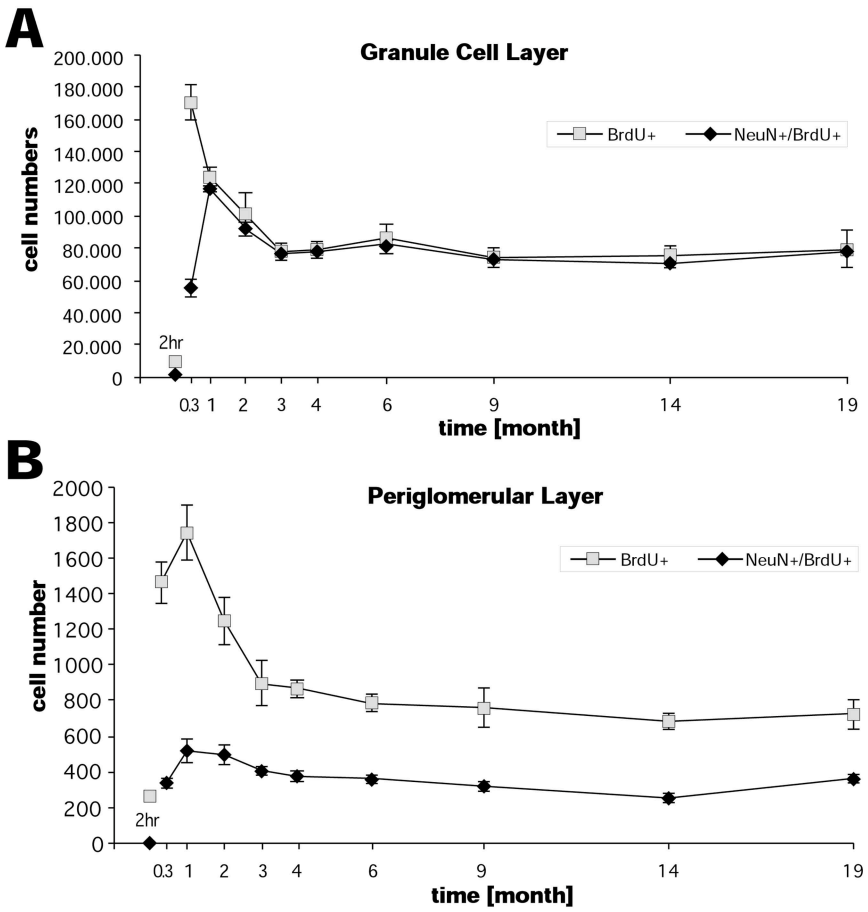


Figure 8

Time course analysis of the total number of newly generated cells and new neurons in the olfactory bulb granule cell and glomerular layer. The total number of BrdU immunopositive cells (squares) per region was determined over a period of 19 months after BrdU injection in (A) the granule cell layer and (B) the glomerular layer. The percentage of new neurons (rhombus) was determined by confocal microscopy as the number of BrdU-positive cells that co-expressed the neuronal marker NeuN. Neuronal cell numbers were determined by multiplying neuronal percentages with the total number of BrdU-positive cells. The data are presented as the mean number of BrdU-positive and BrdU/NeuN double positive cells per region ± S.E.M. for each time point post BrdU injection (n = 6 - 8 per group) (Adapted from Winner, et al[9].

Regulating Adult Neurogenesis: Which Stimuli Make a Difference?

The scope of this dissertation is to define more precisely the mechanisms and signals that influence adult neurogenesis in order to better understand why and how it occurs and to possibly make use of neurogenesis as a neuroregenerative tool. At the onset of this dissertation in 1998-1999, relatively little was known regarding the factors that control the generation of new neurons, however the knowledge has dramatically increased since then. The following chapter will mostly describe the relevant work that was published before 1999 and led to the design of the experimental studies described below. The *Discussion* section that follows the *Experiments* gives a more detailed overview of more recent developments in the field in relation to the findings of these experiments (see especially the comprehensive table of neurogenic factors in Appendix).

Systemic Influences:

Age

The very first neurogenesis studies showed that the production of new nerve cells didn't end in the early postnatal period, but was rather a continual process[89]. Since then, it has been shown that neurogenesis in the hippocampus decreases with age; although even in very old animals, there remains a low-level of neurogenesis[19, 38]. It could be shown that a decrease in the proliferation of neuronal progenitors was responsible for the decrease in neurogenesis, whereas the proliferation of other cells, such as astrocytes in neighboring regions, remained unchanged[19].

Environment (sensory stimulation)

"Enriched environment" paradigms, where animals are placed into housing conditions that are more similar to their natural surrounding, have been shown to increase neurogenesis[38, 90-92]. The enriched environment seems to be neuroprotective for young neurons that would otherwise be eliminated by apoptosis under standard laboratory conditions[93]. The "enriched" animals also showed improved motor skills and better performance in learning tasks[91]. Most importantly, the stimulatory effect on neurogenesis occurred at all ages, including senescence, even when the animals were

housed under enriched conditions for only a few weeks[38]. The unique capability of regulating the production of new neurons in a structure so closely associated with acquisition of new information suggests a possible function for newly generated neurons in certain types of learning.

In the OB, sensory deprivation attained through a “nasal plug” leads to a decrease in neurogenesis in the GCL[12]. However, when rats were challenged daily with different scents, it was shown that not only did neurogenesis increase in the OB, but the rats also became better at olfactory discrimination tasks, whereby the hippocampal neurogenesis and hippocampus-dependent learning remained unchanged[94]. In addition, olfactory synaptic circuitry seems to be important for adaptive behaviors during mating and pregnancy[11]. It appears that neurogenesis in the adult brain reacts specifically and regionally to different sensory modalities.

Activity (physical exercise)

Among the stimulatory factors within an enriched environment, voluntary physical activity appears to be a very strong activator for the proliferation of hippocampal progenitor cells[95, 96]. The sole introduction of a running wheel into a standard laboratory cage doubled hippocampal neurogenesis, suggesting that cerebrovascular parameters, such as blood flow, glucose uptake, and neovascularization could be mediators of this effect[97]. In fact, one study has shown that the exercise-induced increase in neurogenesis is dependent on the uptake of blood-borne insulin-like growth factor-1 (IGF-1)[98]. These results underline the truth in the ancient Latin proverb "Mens sana in corpore sano" (Roman poet Juvenal)- “a sound mind in a sound body”. Rehabilitation strategies for stroke or trauma patients emphasizing a multitude of sensory stimuli, motor tasks and complex training situations have long incorporated the concept of stimulatory effects from an enriched environment.

Stress

Environmental signals can also be detrimental to neurogenesis. Stressful experiences are known to activate the hypothalamic-pituitary adrenal (HPA) axis and increase levels of circulating adrenal steroids. Several different types of stressful experiences, such as exposure to predator odor, subordination stress, acute

psychosocial stress and resident-intruder stress, have been shown to inhibit granule cell production in the DG of rats, tree shrews and marmoset monkeys[72, 73, 99-103]. It is likely that the changes in granule cell genesis are the result of stress-induced activation of the HPA axis and ultimately elevations in glucocorticoid levels, since reducing corticosteroid levels in aged rats restores the rate of cell proliferation, resulting in an increased number of new granule neurons[104, 105]. But not only stressful experiences in *adulthood* influence proliferation and survival of neurons in the differentiated mammalian brain. Prenatal stress in rhesus monkeys and female rats has also been shown to induce a reduction in neurogenesis in the DG throughout life[106, 107].

Extracellular cues:

Hormones

Glucocorticoid hormones, such as cortisol and corticosterone secreted by the adrenal cortex, have been shown to inhibit the production of new granule neurons by suppressing the proliferation of granule cell precursors[104, 108], as well as the number of newly formed neurons in the DG[109]. On the other hand, removal of circulating glucocorticoids by adrenalectomy results in a clear increase in progenitors in the DG of young adult rats[104, 105, 108].

Growth Factors

Although some of these neuromodulatory signals trigger proliferation, the direct mitogenic stimulus to the progenitor cells appears to be mediated via growth factors. From *in vitro* data, it was already known that basic fibroblast growth factor (FGF-2) or epidermal growth factor (EGF) are required to keep neural progenitor cells in an undifferentiated and proliferative state[110, 111]. Therefore, it was consequential that these growth factors would be the first ones to be tested *in vivo* as well. Dentate precursor cells are known to express EGF receptors and direct infusion of the growth factor into the DG stimulates proliferation[112]. Chronic infusion of EGF and FGF-2 in the ventricular system of adult rats, however, triggered proliferation predominantly in the SVZ, but was nearly ineffective in stimulating proliferation in the subgranular zone[18]. In adult mice, the same effects were seen in the SVZ when EGF was infused into the LV[113]. Nevertheless, when using this route of administration, EGF induced a prominent

phenotypic shift leading to more astrocytes and fewer neurons in the OB and DG[18]. Via peripheral application, selective induction of neurogenesis has also been achieved using FGF-2[114] or IGF-1[115].

Interestingly, it has been shown that not only does neurogenesis in the DG decrease with age[19], but serum IGF-1 levels also decrease. When aged rats were infused with IGF-1 into the LV, neurogenesis levels were restored by almost 3-fold[116]. These data suggest that IGF-1 may be an important regulator of neurogenesis in the aging hippocampus and that an age-related decline in IGF-1-dependent neurogenesis could contribute to age-related cognitive changes.

Neurotransmitters

Recent evidence supports the view that neurotransmitter systems could influence the production of new granule neurons in the DG. The glutamate system is probably the most studied neurotransmitter system when it comes to neurotransmitters and their role in neurogenesis.

Lesioning of the entorhinal cortex, the major glutamatergic input into the hippocampus, has been another model to show glutamate's role on hippocampal neurogenesis. When the entorhinal cortex was unilaterally lesioned with ibotenic acid, thereby destroying cells through an excitotoxic mechanism, an increase in proliferation in the DG was observed[117]. This study suggests that glutamatergic input to the DG via the perforant path appears to suppress the proliferation of granule cell precursors. However, this study was lacking the phenotypical analysis of newborn cells, which leaves the question open as to whether the increased *proliferation* detected led to an actual increase in newly born *neurons*.

There have been several studies performed examining the role of glutamate receptors. It was observed that proliferation in the GCL was significantly decreased following treatment with NMDA[117]. In addition, NMDA is capable of blocking the increase in hippocampal proliferation after adrenalectomy[118]. Conversely, adult rats were also injected with glutamate antagonists[117-120] . All of these compounds have been shown to increase the number of proliferating cells in the DG, either in rat, gerbil or tree shrew[72, 120, 121]. Moreover, these compounds were also able to increase the number of new neurons in the DG[117, 119, 120].

OVERALL GOAL

Taking these findings together, several influences with high complexity such as environmental stimulation, physical exercise, stress and aging have an impact on neurogenesis. However, the underlying molecular basis for these regulatory mechanisms are not well understood. It was, therefore, the goal of this study to broaden the current knowledge especially by focusing on molecular signals that may regulate neurogenesis. This dissertation concentrates on the analysis of three separate classes of molecules:

(1) transcription factors
(2) neurotransmitters
(3) growth factors

Their possible function during adult neurogenesis was analyzed using three methodological approaches:

(1) knockout strategy
(2) intraventricular infusion
(3) neurochemical lesion

Thus, the aims were not only to characterize different molecular targets but also to compare different intervention strategies for adult neurogenesis.

SPECIFIC AIMS

In order to analyze neurogenesis, histological methods have been developed based on the use of a thymidine-analogue for labeling of dividing cells. However, there has been an ongoing debate over whether bromodeoxyuridine (BrdU) is an appropriate marker to be used, in combination with neuronal markers and confocal analysis, for the unequivocal detection of new neurons. It was unclear whether in BrdU-labeling approaches, BrdU is marking DNA repair as well. Clarifying this issue and providing a methodological basis for the subsequent experiments was crucial and lays the foundation for the subsequent experiment - **Experiment I**.

Many of the growth factors, which have been shown to upregulate neurogenesis, activate a chain of events through several signaling cascades that eventually lead to the

specific targeting of transcription factors. In **Experiment II**, I was interested in evaluating what the possible role of the transcription factor E2F1, a member of the E2F family of cell cycle control genes, could be in regulating adult neurogenesis. The primary aim was to study the effect of E2F1-deficiency on adult neural progenitor cells by performing stereological analyses on the progenitor proliferation in the DG and SVZ, and the resulting generation of new neurons. Finally, because E2F1 has been reported to be involved in cell death of postmitotic cells, I also wanted to determine the frequency of apoptotic cells in the regions of adult neurogenesis.

Experiments III & IV were aimed at identifying the role of neurotransmitter input on adult neurogenesis. In these experiments, the cholinergic and noradrenergic inputs into the hippocampus and OB were selectively lesioned through the use of transmitter-specific neurotoxins and it was our objective to investigate the effect of these selective denervations on neuronal differentiation and survival in the DG and OB.

Since it is known that an increase in neurogenesis is one of the responses to a diseased state such as hypoxia, I wanted to ask the question of whether or not vascular endothelial growth factor (VEGF), which is upregulated during hypoxia or ischemia, is capable of stimulating neurogenesis. In **Experiment V**, the effects of VEGF on proliferation and survival of neural progenitor cells in the DG of the hippocampus and the SVZ/OB system were investigated through the use of intracerebroventricular (ICV) injections.

As a continuation of Experiment I, I was interested in more precisely defining the course of events during the formation of neurons in the adult brain. **Experiment VI** aims at determining the time course of expression for several genes that are expressed during the neuronal maturation process. Knowing and understanding the specific expression for certain genes during neurogenesis could afford a broader range of markers for labeling newborn neurons. The specific goal was to analyze the expression of doublecortin (DCX) in the neurogenic regions of the adult brain, as well as to determine whether DCX expression changes with age.

OVERVIEW

Experiment I: Critical evaluation of BrdU labeling

Experiment II: Impaired adult neurogenesis in mice lacking the transcription factor E2F1

Experiment III: Cholinergic denervation in neurogenic regions of adult brain

Experiment IV: Noradrenergic denervation in neurogenic regions of adult brain

Experiment V: Vascular endothelial growth factor (VEGF) induces neurogenesis in the adult brain

Experiment VI: Transient expression of *doublecortin* during adult neurogenesis

EXPERIMENTS

This dissertation was designed to evaluate different influences on adult neurogenesis. Consequently, the experimental data are presented as individual studies, with separate introduction, result and discussion sections. At the end a General Discussion section will put the data of all experiments into a common perspective. Since the materials and methods used to detect and quantify neurogenesis are identical, they are appended at the end of the thesis; whereas, only the specific details of the design of each study are presented in the individual experiments. The first experiment represents an exception to this general structure since it was designed to critically evaluate the methodological basis for the following experiments. Therefore, in Experiment I, the data are presented in the form of a review, and directly combined with a literature overview.

Experiment I - Critical evaluation of BrdU labeling

(Cooper-Kuhn, C. M. and Kuhn, H. G., 2002, Developmental Brain Research 134:13-21)

Objective

Since the early sixties, *in vivo* observations have shown the generation of new neurons from dividing precursor cells. Nevertheless, these experiments suffered from critique, suggesting that the prevailing labeling method, which incorporates tagged thymidine analogs, such as ^{3}H-thymidine or BrdU, may not be detecting a proliferative event, but rather could mark DNA repair in postmitotic neurons. Even today many scientists are still skeptical, because the question of specificity for thymidine labeling has not been sufficiently answered. This first experiment aims at (1) presenting histological evidence of specificity of BrdU labeling for neural progenitor cell activity in the adult brain, (2) validating and comparing BrdU labeling with other histological methods, and (3) combining BrdU and labeling methods for apoptosis to argue against DNA repair being a major contribution of BrdU-positive cells.

Introduction to Experiment

As a prerequisite to studying progenitor cell activity *in vivo*, it is necessary to not only identify proliferating cells, but to also stably label their progeny. BrdU labeling is currently the prevailing method to study proliferation and neurogenesis *in vivo*[122].

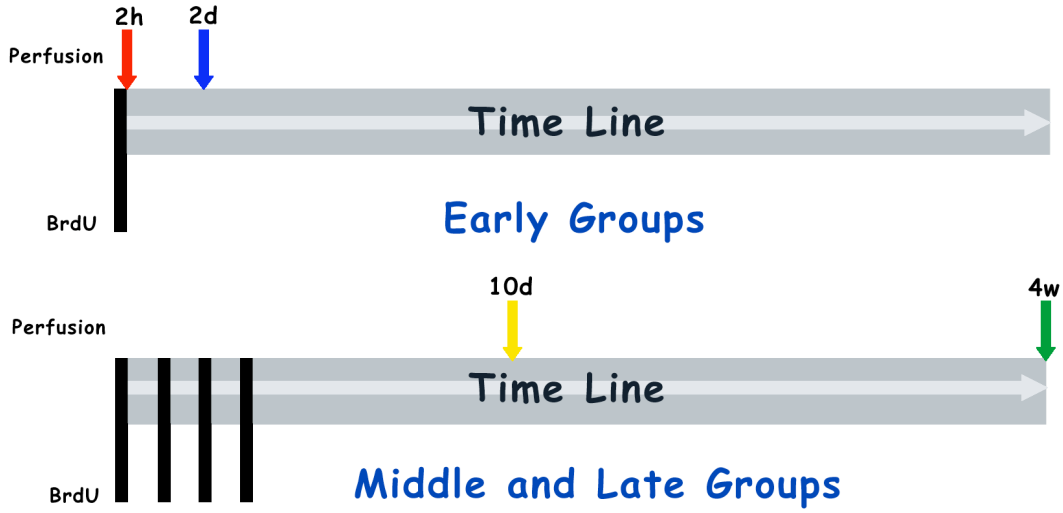
Systemically injected BrdU is incorporated as a thymidine analog into the DNA of all cells undergoing DNA synthesis, within the time that BrdU is present in the cellular thymidine pool (for review see[123-125]). BrdU integrates into the DNA during the DNA-synthesis phase of the cell cycle and is passed on to the daughter cells. Once incorporated into the DNA, BrdU can be detected immunohistologically in postmitotic cells for the remainder of their life, unless the cells undergo consecutive proliferation, which will exponentially dilute the BrdU signal in the nucleus[126]. If a cell becomes postmitotic, the permanent BrdU label enables one to study the fate of newborn cells by detecting it in combination with cellular markers for neuronal development, such as neuron-specific proteins. In a similar fashion, ^{3}H-thymidine autoradiography was previously used. After ^{3}H-thymidine labeling of dividing cells, silver grains are created in a photo emulsion that overlays the histological section. The histological evaluation is burdened by a time-consuming exposure followed by the matching of silver grain signals to the underlying cells. ^{3}H-thymidine was successfully applied to initially detect neurogenesis in adult brain tissue before other histological markers were available[5, 127, 128].

An alternative method to measure proliferative activity *in vivo* is the detection of mitotic markers, such as PCNA (proliferating cell nuclear antigen – a protein expressed in S and late G_1 phase) and Ki-67 (a protein expressed throughout the cell cycle). Although excellent markers for proliferating cells, these markers are not suitable for detecting the neuronal progeny of dividing cells, since by the time a cell expresses mature neuronal markers, it has already left the cell cycle. Retroviral labeling appears to be the only other alternative method for detecting neurogenesis, because for successful retroviral labeling, a cell must go through complete cell division. Retrovirally labeled cells will pass on the retroviral genome to their progeny and will continue to express a reporter gene even after neuronal maturation[129, 130].

Although widely used to detect *in vivo* neurogenesis, labeling with thymidine analogs is not free of methodological problems. In order to address whether BrdU might label cells undergoing DNA repair, as well as to demonstrate that BrdU-labeled cells undergo a gradual development from a dividing progenitor cell to a mature neuron, the co-localization of BrdU in a time series using markers for mitosis, progenitor cells, and immature and mature neurons was analyzed. Double-labeling data is also presented, showing cell division and apoptosis, in order to determine whether BrdU is detectable in

cells undergoing DNA repair in the final stages of apoptotic DNA degradation. Finally, we propose methodological standards for the detection and quantification of neurogenesis *in vivo*, whether new regions or new species are proposed.

Experimental Design



For specific details on Experimental Design, see Materials and Methods on page 106.

Results and Discussion

Time course analysis of BrdU-labeled cells

Until the *in vivo* generation of neurons from dividing progenitor cells can be followed directly through the use of time-lapse video microscopy, it is necessary to detect adult neurogenesis histologically via markers that are incorporated during cell division. BrdU is incorporated into all cells undergoing cell division; however, two additional sources of DNA incorporation are also possible: (a) mitochondrial DNA synthesis and (b) DNA repair. Mitochondrial labeling can be excluded by verifying that the BrdU signal is detectable within the nucleus, which can easily be accomplished with confocal microscopy. DNA repair is carried out by cellular enzyme complexes, which secure the genomic stability of cells (for review see[131, 132]). DNA repair often occurs in the CNS as an attempt to rescue postmitotic neurons after damage by irradiation or mutation-inducing chemicals, although a high percentage of these cells will eventually undergo cell death[133-135].

One attempt to determine specificity of BrdU labeling is to show the sequential co-expression of markers for neuronal development at multiple time points after BrdU labeling (see Figure 9 and Figure 10). It is difficult to argue that BrdU-labeled DNA repair in mature neurons, if BrdU is first detected in immature dividing cells and only several weeks later in mature neurons.

(1) The cell cycle marker Ki-67, as well as other cell cycle markers [136], are only detectable in BrdU-positive cells during the first days after labeling, when the cells are still in S-phase. Moreover, mitotic figures have previously been described to be a common phenomenon in brain regions with progenitor cell activity[137, 138].

(2) While the progenitor marker, Nestin, co-labeled with BrdU within the first two days after BrdU injection, markers for young neurons, ß-III-tubulin[139, 140] and DCX[141, 142], were only transiently detected between 2 and 10 days after BrdU application.

(3) Finally, the mature neuronal marker NeuN[143] was expressed after 10 days. Since co-labeling of NeuN with BrdU was not shown within the first 2 days after BrdU injection, it is unlikely that postmitotic neurons, which undergo DNA repair, are able to incorporate detectable amounts of BrdU. These data argue strongly for a gradual development of the BrdU-positive cells into neurons and are in disagreement with the hypothesis that DNA repair mechanisms in damaged postmitotic neurons could be the cause for BrdU signals in intact adult brain tissue.

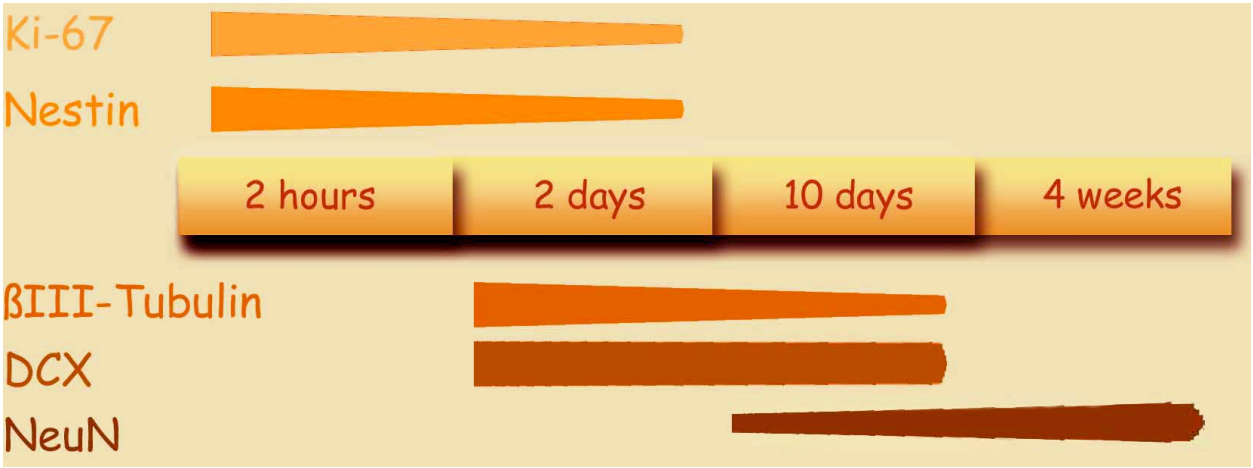


Figure 9
Depiction of antibody expression pattern in relation to BrdU labeling. Immediately after BrdU labeling, cells are positive for Ki-67 and Nestin and these expressions taper off after 2 days. By 2 days, expression of ß-III-Tubulin is detectable and begins to taper off by 10 days, whereby DCX expression remains strong through 10 days and is no longer detected at 4 weeks. NeuN isn't detectable until 10 days and remains strong throughout the life of a neuron.

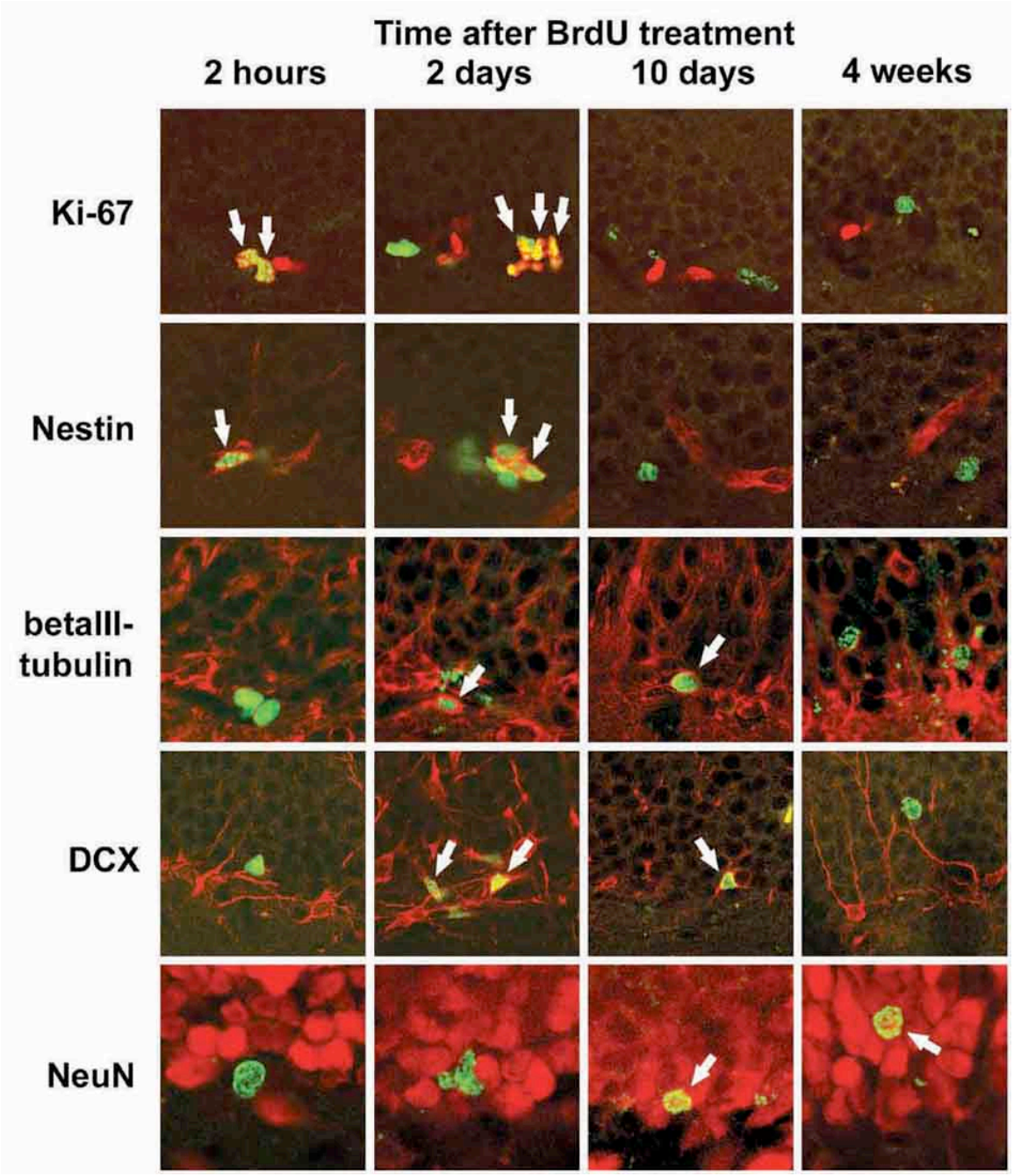


Figure 10
BrdU labeling in the adult dentate gyrus. At different time points after BrdU application (2 hrs, 2 days, 10 days and 4 weeks), co-labeling of BrdU with stage-specific markers reveals that newly generated cells in the granule cell layer of the dentate gyrus undergo neuronal development. At 2 hrs and 2 days after BrdU injection, most BrdU positive cells are still in cell cycle, as determined by Ki-67. At these time points, some of the BrdU positive cells express the neural progenitor marker, Nestin, as well. It is important to note that at later time points no co-labeling of BrdU-positive cells with Ki-67 or Nestin was observed. ß-III-tubulin and DCX, which represent markers for immature neurons, both co-label transiently with BrdU at 2 days and 10 days post BrdU application. Finally, NeuN, a marker for mature neurons, labels only BrdU-positive cells after 10 days and later, which confirms that BrdU is not initially (at 2 hrs) incorporated into mature neurons.

Electron microscopy

Ultrastructural identification of mitotic precursor cells of the DG and the LV can unequivocally distinguish between dividing cells and mature cells with DNA damage. When combined with BrdU or ^{3}H-thymidine labeling, electron microscopic reconstruction provides powerful evidence that labeled cells are initially immature, dividing cells, without signs of damage[89, 137]. At longer intervals after thymidine analog injection, the labeled cells were characterized as young neurons or, after several weeks, as mature neurons with synaptic connections[6, 16, 144]. The dividing cells in the SVZ appear to be more immature and free of synapse-like structures, whereas the hippocampal progenitor cells were described as “mitotic neuroblasts with synapses on their cell bodies and processes which resemble axons”[89]. Taken together, electron microscopy studies confirmed that ^{3}H- thymidine-positive cells undergo development from immature progenitors to mature neurons without signs of DNA damage - yet another argument against DNA repair as a major source of thymidine labeling.

Retroviral labeling

Detection of newborn neurons using a retroviral construct [130, 145, 146] provides several advantages compared to BrdU labeling. (1) The stable integration of the retroviral genome into the chromosomal DNA can only occur after nuclear membrane breakdown. This ensures that an infected cell will stably express viral genes only when it has undergone mitosis. Retroviral labeling, therefore, distinguishes between cell division and DNA repair. (2) Since the viral DNA is integrated into the host genome, it will be replicated and transmitted to every daughter cell. In comparison to incorporated thymidine analogs, which are diluted during every new cell division and eventually lost if a cell continually divides, retroviral labeling allows for the study of all progeny, regardless of the number of divisions. (3) The introduction of reporter genes such as ß-galactosidase[147] or green fluorescent protein[148], which freely distribute in the cytoplasm, make not only the detection of newborn cells possible, but also the study of the cell shape, including axonal and dendritic processes.

Retroviral labeling has been used to detect neurogenesis in the adult CNS[84, 149-151]. It serves as a strong argument for the validity of studies using BrdU to detect neurogenesis in the adult brain. Experiments with limited dilutions of retrovirus, which

statistically produce only single infections, have indicated that cells act as multipotent neural progenitor cells in the adult LV[152].

But several disadvantages make retroviral infection less suitable for *in vivo* applications: (1) Retrovirus particles have to be directly applied to the CNS region of interest, since the blood-brain-barrier is an impermeable obstacle for the virus particles. Stereotaxic injection of the virus, on the other hand, causes damage from the cannula tract. Thus, when using retroviruses, possible lesion-induced effects on neurogenesis have to be taken into account when studying progenitor cell activity in the intact brain. (2) Biosafety and technical issues during generation and use of retrovirus particles, such as helper virus-free preparations[153], as well as titer and stability of virus particles[154-156], make the method more technically demanding and costly than BrdU labeling. (3) Although all progeny of an infected cell will carry the retroviral genome, it is not guaranteed that the reporter gene will be continually expressed in all cell types, especially in the CNS, where shutdown of retroviral gene expression has been observed[157, 158]. Nevertheless, positive signals, such as GFP, from retrovirally labeled neurons are still the strongest argument for the occurrence of neurogenesis in the adult brain.

BrdU, DNA repair and cell death

BrdU is incorporated into all cells during DNA synthesis, including those that undergo DNA repair. Obviously, complete replication of the genome during cell division will incorporate BrdU to a much higher extent than limited DNA repair, but it is unclear whether the immunohistological BrdU signal can only be generated from cell division or from DNA repair as well.

An experimental approach to determine the origin of BrdU signals is the induction of DNA repair while BrdU is present. In response to ionizing radiation, cells immediately activate DNA repair pathways, which detect and correct DNA damage, including modified bases, sugar damage, strand breaks, and clustered DNA damage[134, 135, 159, 160]. Base excision and nucleotide excision repair involve integration of new nucleotides and, therefore, BrdU incorporation, whereas DNA strand breaks are corrected by DNA ligase[131, 132, 161]. Ionizing radiation has been shown to dose-dependently increase DNA damage followed by DNA repair, as well as cell death[162]. When the number of BrdU-positive cells is measured in the LV after increasing doses of irradiation, a dose-

dependent decline in proliferation and BrdU-labeling was found[136, 163, 164], whereas the number of apoptotic cells sharply increased[165, 166]. Although this experiment cannot disprove that a small fraction of residual BrdU positive cells may still have undergone DNA repair, it is unlikely that DNA repair represents a major source of BrdU labeling in the LV.

To directly demonstrate whether cells can incorporate significant amounts of BrdU during apoptosis, mice were injected with BrdU (50mg/kg) and perfused 2 hrs later. BrdU labeling was then combined with two independent markers for apoptosis: active caspase-3, which marks the final step in a protease cascade, and TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling), which reveals the resulting DNA degradation. Both apoptosis and cell division are found in the DG and the LV[28], but no BrdU-labeling was detected in cells positive for active caspase-3 or TUNEL (Figure 11). Although DNA repair was not directly assessed in these experiments, the data strongly argue that a standard dose of BrdU (50mg/kg bodyweight) injected into adult rodents is not sufficient for detecting DNA repair during apoptosis.

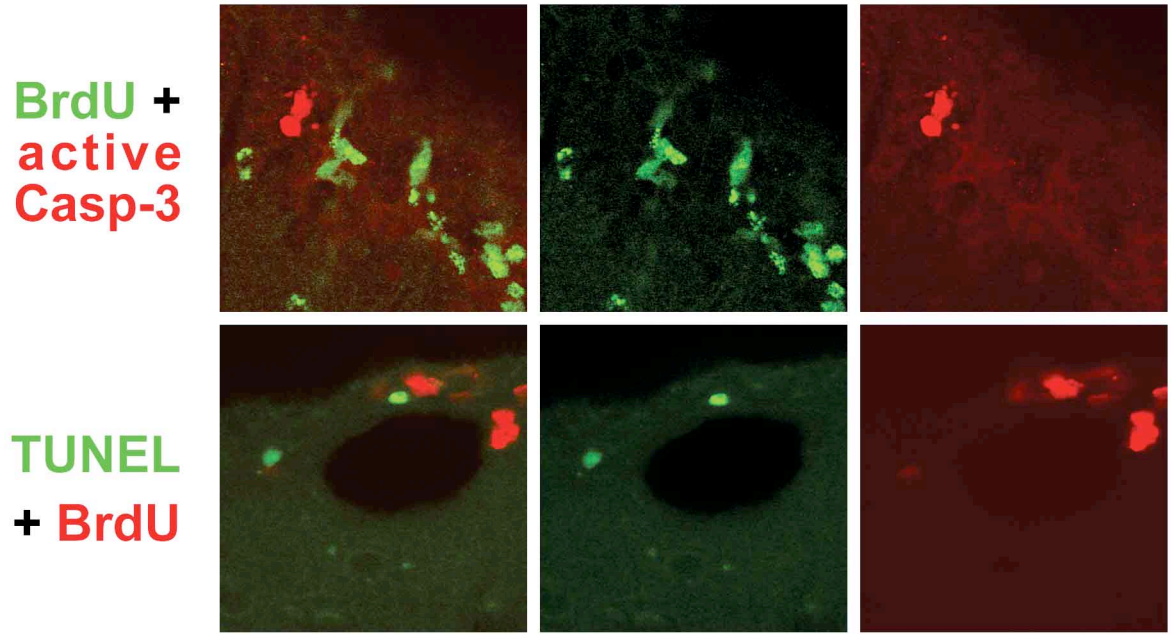


Figure 11

Proliferation and apoptosis in the adult brain. Both, cell division and apoptosis are occurring in the dentate gyrus and the ventricle wall; however, when BrdU incorporation was analyzed in the subventricular zone (SVZ) 2 hrs after systemic injection, no co-labeling with apoptotic markers, such as active Caspase-3 and TUNEL, was observed. This confirms that the standard dose of BrdU used is sufficient for labeling dividing cells. But BrdU it is not being incorporated into cells undergoing cell death, although cell death may be accompanied by DNA repair.

Requirements for detecting neurogenesis in new regions

Thus far, two regions of the adult brain have been unequivocally shown to continuously produce new neurons, the LV/OB system and the DG. Neurogenesis was verified in these regions with multiple methods, such as ^{3}H-thymidine, BrdU, retroviral labeling and electron microscopy, providing very strong arguments that adult neurogenesis is not an artifact. *In vitro* experiments, which showed the generation of neural progenitor cell cultures from other sites in the adult brain, including spinal cord, cerebral cortex and white matter tracts, suggest that progenitor cells are present throughout the brain and therefore, adult neurogenesis might be a more common phenomenon than currently assumed. Recently, first reports on spontaneous cortical neurogenesis in the primate[80] and lesion-induced cortical neurogenesis in the rat and mouse[141, 167] indicate that the current view of limited neurogenesis in the adult brain may have to be revised.

It is very likely that novel brain sites with adult neurogenesis, such as the cerebral cortex, will have rather limited amounts of newly generated cells. But in instances where a low number of labeled cells are detected, the chances are higher that a substantial quantity of these cells could be false positive. In order to increase the confidence that neurogenesis is a common phenomenon in several areas of the adult brain, the data from the present experiment advocate the notion of using multiple histological criteria, rather than relying exclusively on a single detection method.

Time course analysis. With the use of sequential markers for progenitor cells, neuroblasts and mature neurons, BrdU can be co-labeled at different time points after BrdU injection. This criterion assumes that each cell undergoes a sequence of steps from a dividing progenitor to a fully mature neuron. The detection of these steps with several immunohistochemical markers at multiple time points (Figure 10) would make a strong case for neurogenesis in a novel region. Moreover, in case the place of origin differs from the place of neuronal differentiation, it is of interest to find the migratory route of the immature cells, which again is best shown in a time course investigation.

3D-confocal analysis. Individual BrdU positive cells should be systematically analyzed by confocal microscopy in order to distinguish true co-labeling of BrdU with neuronal markers from false-positive cells. Considering the risk that BrdU-positive satellite glia cells, which can be very closely associated with cortical and striatal

neurons, can mimic co-labeling in standard immunofluorescence analysis, confocal analysis becomes an even more essential tool[18].

Retroviral labeling. To unequivocally confirm neurogenesis in new areas, BrdU labeling should be validated by a separate method as well. Due to the fact that retroviral vectors can only integrate into the genome during cell division, successful retroviral infection is currently the most convincing argument for the occurrence of adult neurogenesis.

Electron microscopy. Ultrastructural analysis is extremely helpful in determining the nature of labeled cells, in particular the cell type, as well as the differentiation state. Moreover, electron microscopy is relying on structural criteria, rather than on immunohistological markers, in order to determine cell types. Nevertheless, exhaustive and quantitative analysis of large areas such as the neocortex at the ultrastructural level are extremely time consuming.

Alternatives: Markers for cycling cells and young neurons

In some cases, systemic BrdU application and more invasive methods, such as intracranial retrovirus injections, are not the most desirable procedures: (1) For the study of neurogenesis in human subjects, ethical concerns will strictly limit the use of such labeling techniques, especially if intended for research purposes. And only in very rare cases, such as previous unrelated BrdU treatment for diagnostic reasons, is it possible to obtain labeled human brain samples from autopsies. This was the case for the pioneer study, which demonstrated ongoing neurogenesis in the hippocampus of adult humans[37]. (2) Studies, which directly analyze the effects of altered stress levels on test animals, will certainly have to take into account that invasive methods, such as repeated peripheral injections, not to mention surgery, will alter the levels of stress hormones, which, in turn, have been shown to regulate neurogenesis (for review see[101]). In these instances, it might be desirable to use, for example, the oral application route for BrdU via drinking water. Although this will allow for the detection of neurogenesis, the quantitative analysis of oral BrdU application is hindered by the inability to control the amount of BrdU uptake by individual experimental animals.

In experiments, where animals are studied in the wild, it may become necessary to completely exclude any interference by the experimenter prior to the histological analysis. In this case, it will be necessary to resort to cell cycle markers, such as Ki-67

or PCNA, and markers that are only transiently expressed in young neurons. Especially the markers for neuroblasts and young neurons, such as beta-III-tubulin[139, 140], DCX[141, 142], PSA-NCAM[168], Hu[169, 170], and TUC-4 (previously named TOAD-64[171]), could prove to be extremely helpful, if they are truly specific for earlier stages in neuronal development and are no longer expressed in fully mature neurons. It will be necessary to prove that these markers have the assumed cell type and stage specificity in the particular species of interest.

BrdU labeling: methodological considerations

BrdU is widely used for labeling newborn neurons *in vivo*, but several cautionary observations for this technique must be discussed. BrdU is incorporated into the DNA as a thymidine analog. But the size of the bromine atom can alter the DNA structurally, with implications for the integrity and functionality of the DNA. It is, therefore, necessary to reduce the concentration of injected BrdU to the minimal detectable dose.

Cytotoxicity. With the integration of a halogenated compound into the DNA, the stability of the DNA can be drastically altered, increasing the risk of sister chromatin exchange, mutations and DNA double-strand breaks[172, 173]. Given in higher doses during embryonic development, BrdU can lead to teratogenic malformations and behavioral changes[174-176]. Nevertheless, the use of systemic injections in adult rodents is most commonly applied (50mg/kg bodyweight). When using higher doses or more continuous labeling (e.g. via drinking water), one should take into account that mitotic cells are sensitive to prolonged BrdU exposure and may react with altered gene expression or cell death[177]. However, experimental data that are based on the comparison of BrdU labeling between control and experimental animals should lead to valid information.

Diffusion barriers. Thymidine analogs given systemically will distribute throughout the body and can readily cross the blood brain barrier in rodents. But ^{3}H-thymidine labeling was shown to be stronger after ICV, rather than intraperitoneal, injection. It is also unclear what concentration is reached in the brain and whether the uptake into the CNS differs between species. In the primate, it was assumed that the blood-brain-barrier is not permeable and initial studies used ICV injection to demonstrate adult neurogenesis[178]. However, in later experiments, including the first study in humans, intravenous BrdU application was equally successful. Since intraventricular injection of BrdU requires surgery, the systemic application is more commonly preferred. But when

studying neurogenesis under pathological conditions, such as trauma or stroke, the breakdown of the blood-brain-barrier after lesion may alter BrdU availability and may change quantitative data when compared to unlesioned controls. Under these conditions it is necessary to validate BrdU with independent markers for progenitor cell proliferation and neurogenesis, such as Ki-67, DCX or ß-III-tubulin.

Summary

The detection of *in vivo* neurogenesis requires a stable labeling technique for dividing cells and their progeny. BrdU is incorporated in dividing cells; however, under some circumstances and certain concentrations it can also label cells undergoing DNA synthesis, including DNA repair. Nevertheless, several experimental data argue against the concern that the concentration in which BrdU is commonly used may be sufficient to detect cells that undergo DNA repair. (1) Mitotic figures and cell cycle markers can be detected in regions of neural progenitor cell activity. (2) Over time, BrdU positive cells undergo development from dividing precursor cells to fully mature neurons, as demonstrated by immunofluorescence (Figure 9) and electron microscopy. (3) Studies using retroviral vectors, which integrate exclusively into dividing cells, confirmed the thymidine labeling studies of adult progenitor cell proliferation and neurogenesis. (4) Apoptotic cell death, which is accompanied by activation of DNA repair mechanisms, is not labeled by BrdU incorporation. (5) Irradiation drastically and immediately reduces the number of BrdU labeled cells *in vivo*, although DNA repair is increased after irradiation.

Taken together, these findings confirm that neurogenesis is not an artifact, but a prominent characteristic of the adult mammalian brain. Nevertheless, observation of neurogenesis in new species or new brain regions, as well as in disease models, requires careful histological analysis in order to avoid false-positive results.

Experiment II - Impaired adult neurogenesis in mice lacking the transcription factor E2F1

(Cooper-Kuhn et al., 2002, Molecular and Cellular Neuroscience 21:312-323)

Objective

During nervous system development the fate of neural progenitors - whether to undergo proliferation, differentiation or apoptosis - is controlled by various signals, such as growth factors. This experiment addressed the question of whether the transcription factor E2F1, which is targeted by several signaling cascades that are activated by growth factors, is involved in neurogenesis in the adult brain. Using a knockout strategy, the adult brains of E2F1$^{-/-}$ mice were analyzed, focusing on regions of adult neural progenitor cell proliferation, neurogenesis and cell death. Five separate analyses to address the effect of E2F1 deficiency on postnatal neurogenesis were performed: (1) Brain and body weights of E2F1-deficient and wildtype mice as neonates and as adults were compared to get an overall measure of alterations that may occur during postnatal maturation of the brain. (2) A neuroanatomical characterization of adult E2F1-deficient brains gives indications whether developmental brain defects are present that would compromise the analysis of adult neurogenesis. This analysis includes the stereological neuronal counts in the cerebellum, a brain structure predominantly generated postnatally, in comparison to the neocortex, whose neurons are generated entirely during embryonic stages. (3) To study the effect of E2F1-deficiency on adult neural progenitor cell proliferation *in vivo*, BrdU labeling and stereological analysis was employed to determine the number of cells in S-phase in the hippocampus and in the LV. (4) Consecutively, in the hippocampus and OB, the number of new neurons being generated from dividing progenitor cells 4 weeks after BrdU labeling was determined. (5) Finally, because E2F1 has been reported to be involved in cell death of postmitotic cells, the frequency of apoptotic cells in the regions of adult neurogenesis was also determined.

Introduction to Experiment

The E2F family of transcription factors has been implicated in controlling proliferation as well as cell death of embryonic cells, including neural progenitor cells *in vitro*[179]. Therefore, it was hypothesized that individual E2F family members may play an important role during generation and replacement of neurons in the adult brain. The E2F transcription factors consist of a family of 7 genes (Figure 12), and these transcription factors regulate the expression of genes whose products are important in cell proliferation, from the G1 to the S phase of mitosis. A functional E2F transcription factor consists of an E2F peptide heterodimerized with a DP1 or DP2 peptide. E2F-1, -2, and –3 are the closest related family members, all sharing the capacity to efficiently induce the S phase. E2F-4 and –5, in contrast, are weak inducers. Their activity is regulated through their association with so called "pocket proteins" (Rb, p107, and p130). The retinoblastoma gene (Rb) can inhibit the transcriptional activation capacity of E2F factors by masking their transcriptional activation domain – this converts them to transcriptional repressors[180-182]. During G1 phase, D-type cyclins modulate Rb by activating cyclin-dependent kinases, which in turn phosphorylate Rb. Once Rb is hyperphosphorylated (late G1), it releases transcriptionally active E2F factors (Figure 13).

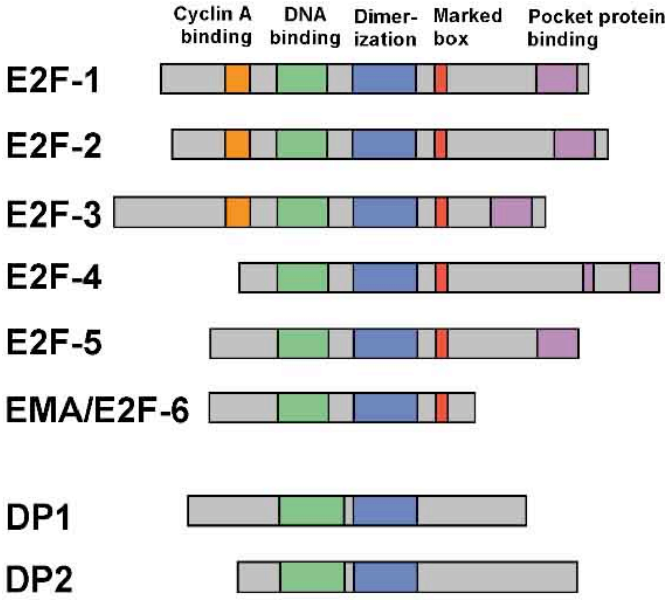


Figure 12
E2F family members and their dimer peptides (DP1/2).

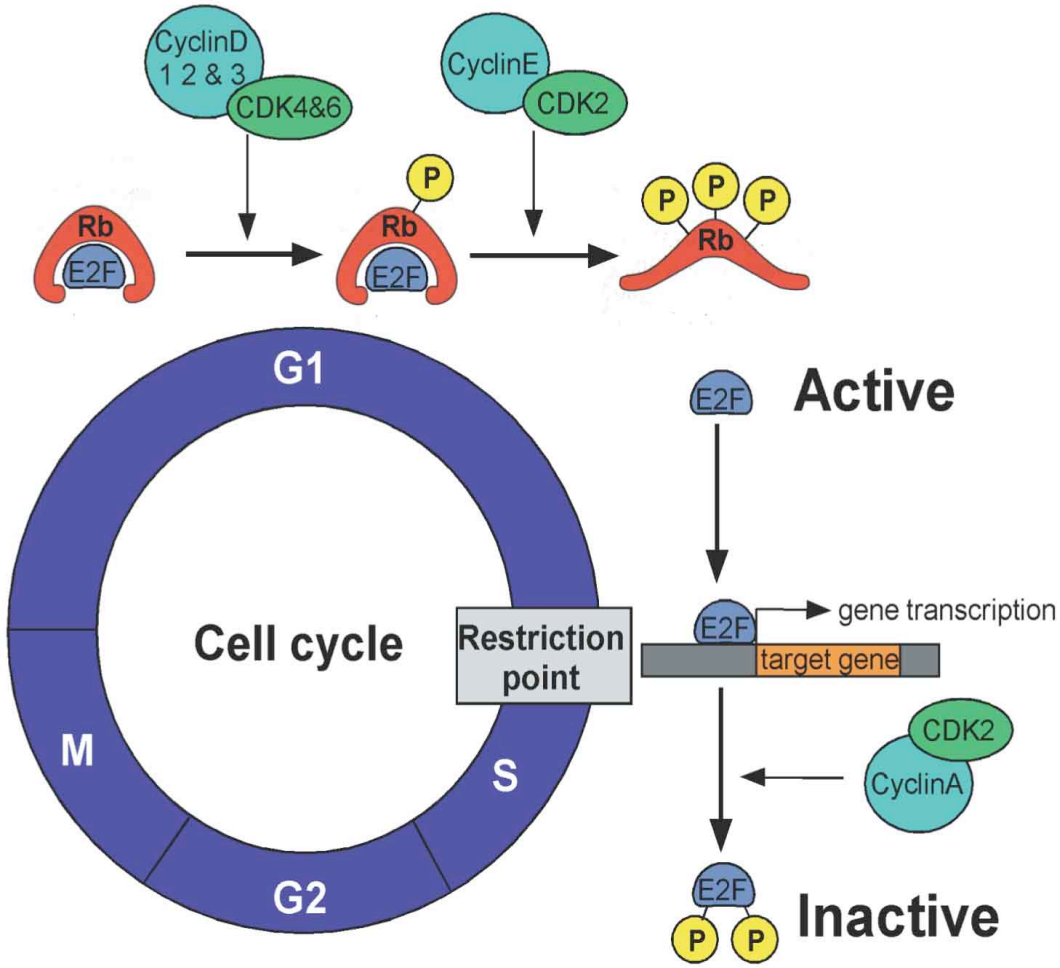
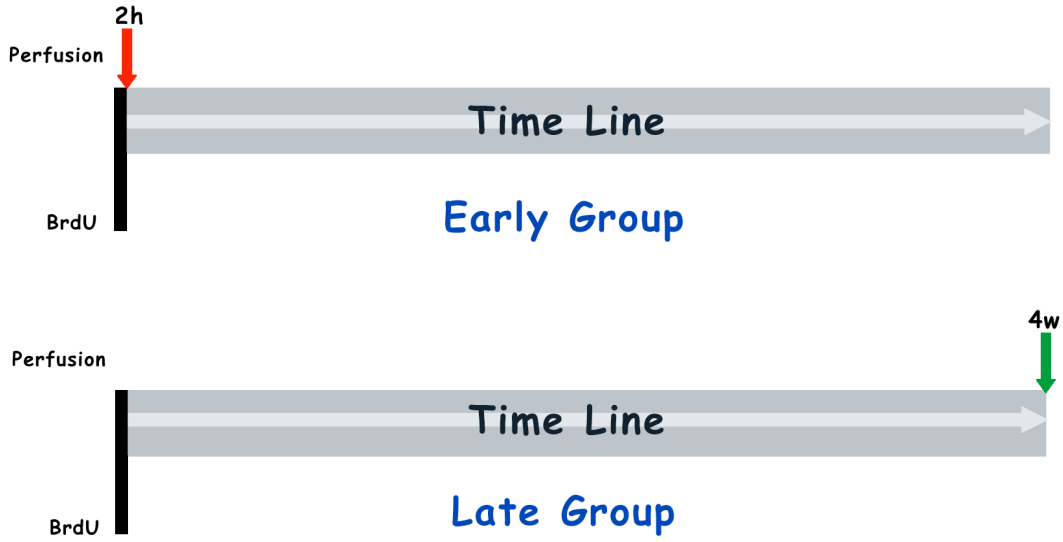


Figure 13
Depiction of cell cycle. During G1 phase, Rb becomes phosphorylated by cyclin kinases. Once Rb is hyperphosphorylated, its conformation changes, thus releasing active E2F, which is thereby able to regulate the expression of a number of genes whose products are required for S-phase entry. Free E2F is deactivated by Cyclin-A/Cdk2 during S-phase. Multiple mitogenic signaling pathways, as well as growth inhibitory signals, ultimately converge upon E2F at the G_1 phase checkpoint (with permission from M. Vroemen).

Within most cell types, the overexpression of E2F1 serves to induce proliferation[183-185]; however, it is also capable of inducing apoptosis in ceratin cell types, probably through a protective, apoptotic pathway that eliminates cells that have lost their cell cycle control[186]. In E2F1-deficient mice, organs like the testis suffer from atrophy, whereas a reduced apoptotic elimination of thymocytes during T-cell maturation leads to a postnatal hyperplasia of the thymus[185, 187]. Moreover, E2F1-deficient mice have a higher risk of developing intestinal tumors in later stages of life[185], leaving it open as to whether organs such as the brain are affected in one way or another.

Of the six E2F family members, E2F1 is most prominently expressed in the embryonic nervous system, with high abundance in regions of neural progenitor cell activity[188-191]. Nevertheless, mice with targeted deletion of E2F1 appeared normal in gross anatomy and behavior compared to wild type mice, probably due to functional compensation by other E2F family members[185, 187]. E2F1 continues to be expressed in the nervous system in postnatal periods while other E2F family members are downregulated at later stages of development[188-191]. Therefore, it was hypothesized that a possible effect of E2F1 deletion in the CNS could reveal a phenotype in postnatally and/or adult generated granule cell populations of the OB, hippocampus and cerebellum.

Experimental Design



For specific details on Experimental Design, see Materials and Methods on page 106.

RESULTS

Loss of E2F1 affects size of adult mice and brain weight.

As a basic, but crude measurement for quantitative changes in E2F1-deficient mice, the body weight and the wet brain weight of wildtype and E2F1-deficient animals at 4 days after birth (neonatal) and at 3 months of age (adult) was first compared. Table 1 shows that neither body weight nor brain weight differed between newborn wildtype and E2F1-deficient mice. However, in adulthood, the E2F1-deficient mice were slightly,

but significantly, smaller in body weight (20% reduction) and brain weight (10% reduction) compared to wildtype, which is in accordance with previous data on atrophy of several organs[185, 187].

Table 1
Neonatal and Adult Body and Brain Weights

	Wildtype	E2F1 deficient	*P* value
Neonatal (P4)			
Body weight	2.384 ± 0.054	2.504 ± 0.125	0.39
Brain weight	0.196 ± 0.004	0.206 ± 0.006	0.16
Adult (P90)			
Body weight	27.591 ± 1.087	22.083 ± 0.941	0.001**
Brain weight	0.475 ± 0.007	0.431 ± 0.005	0.001**

Neonatal and adult body and brain weights were determined on postnatal day 4 (P4) and day 90 (P90), respectively (*n* = 12 per group). Equal numbers of male and female mice were used. Weights (g) are presented as group means +/- SEM. Significance levels in the unpaired two-tailed *t*-test are given as *P* values.** Statistically significant difference between wildtype and E2F1-deficient mice at the 1% level.

Neuroanatomy of adult E2F1-deficient animals

The study of neural progenitor cell activity in adult mice with targeted mutations requires that the animals are viable and have no apparent abnormalities from embryonic brain development. E2F1-deficient mice have been reported to be fertile, viable, and without gross anatomical malformations[185, 187]. Since the brains of E2F1 deficient animals have not yet been systematically analyzed, the brain morphology of E2F1 deficient animals was compared with wildtype littermates. Analysis of multiple brain regions, such as neocortex, diencephalon, midbrain and cerebellum, on Nissl-stained sections revealed no missing or overtly altered cell groups (Figure 14A). All layers of the neocortex are present and appear to have identical organization and size (Figure 14C). To detect possible differences between prenatally and postnatally generated brain structures, neuronal cell numbers in the neocortex, whose neurons are generated exclusively during the embryonic phase, were compared with the postnatally generated GCL of the cerebellum. Stereological cell counts of Nissl-stained tissue sections revealed a small, but significant reduction in the number of cerebellar granule cells in E2F1-deficient mice (Figure 14B). In contrast, the neuronal numbers of the neocortex were not significantly decreased (Figure 14D). These data implicate that structural

changes, due to the lack of functional E2F1 protein, become more prominent in postnatal stages of life.

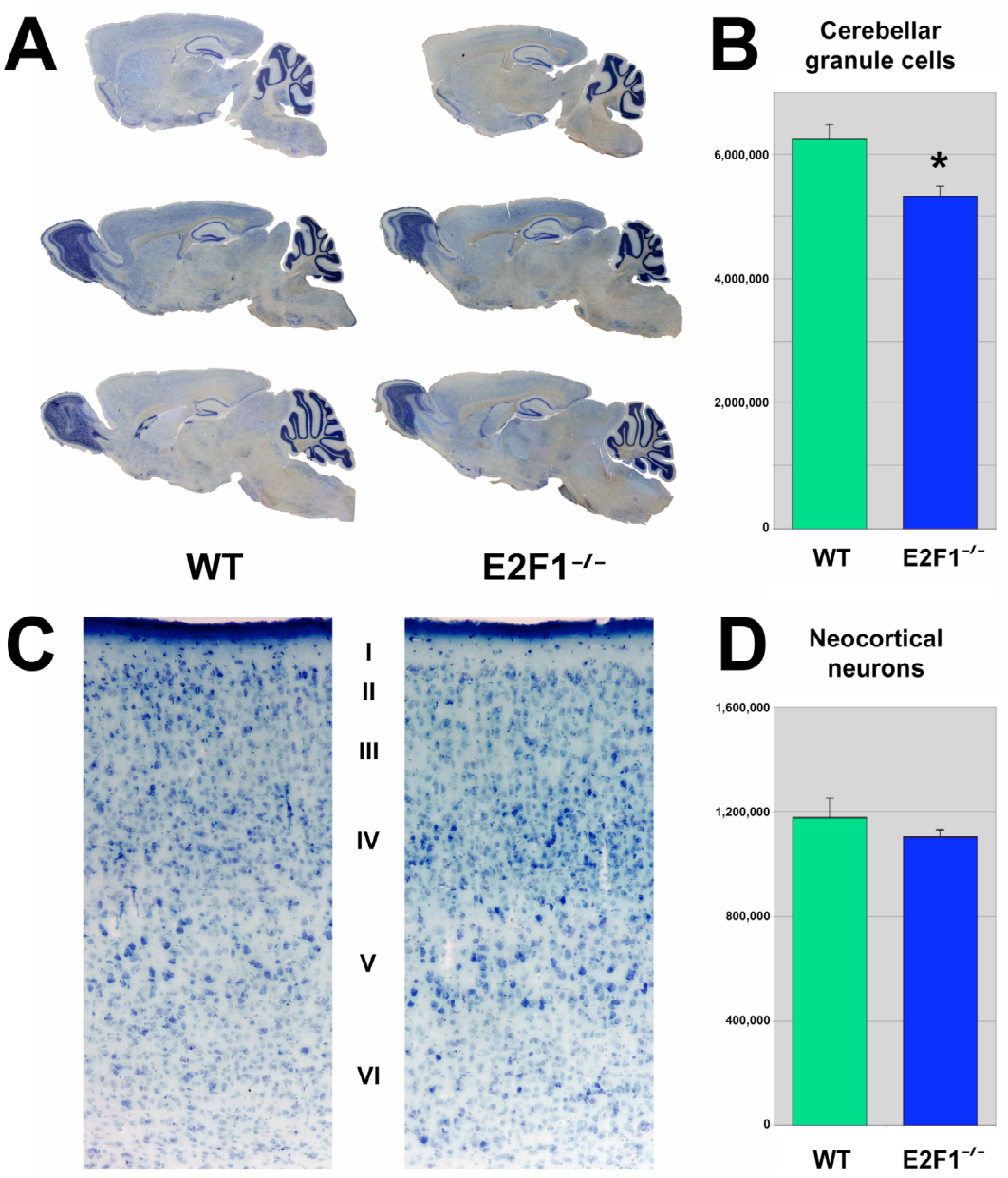


Figure 14
Neuroanatomical characterization of E2F1-deficient animals. (A, C) Anatomical overview of adult E2F1-deficient brains in comparison to wildtype as demonstrated on representative Nissl-stained parasagittal sections. The E2F1-/- brains appear to develop normally. The presence of multiple brain nuclei in the forebrain, thalamus, hypothalamus, ventral midbrain, and brain stem (details not shown) as well as the normal layering of the neocortex (C, primary motor cortex) were confirmed. (B, D) When neurons in the cerebellum and the neocortex were counted, a significant reduction in cerebellar granule cell numbers (B) of E2F1-deficient mice was observed, whereas cortical neuron numbers (D) were not altered. Neuronal cell counts are represented as group means +/- SEM (*n*=6 animals per group). *Statistically significant difference between wildtype (WT) and E2F1-deficient mice at the 5% level (Student's *t* test).

Loss of E2F1 leads to less proliferation in dentate gyrus and subventricular zone.

In order to determine the role of E2F1 in adult neurogenesis, neural progenitor cell proliferation in the DG and the LV wall was analyzed. Adult E2F1-deficient and wildtype littermates (2 months of age) received a single BrdU injection (50 mg/kg) 2 hrs before perfusion (n=5 animals per group) to restrict labeling to cells in the S–phase of the cell cycle. In the DG GCL, quantification of BrdU-labeled cells detected a significant lower level of cell proliferation in the absence of functional E2F1 (Figure 15). In the LV a similar reduction of cells in S-phase was observed (Figure 16).

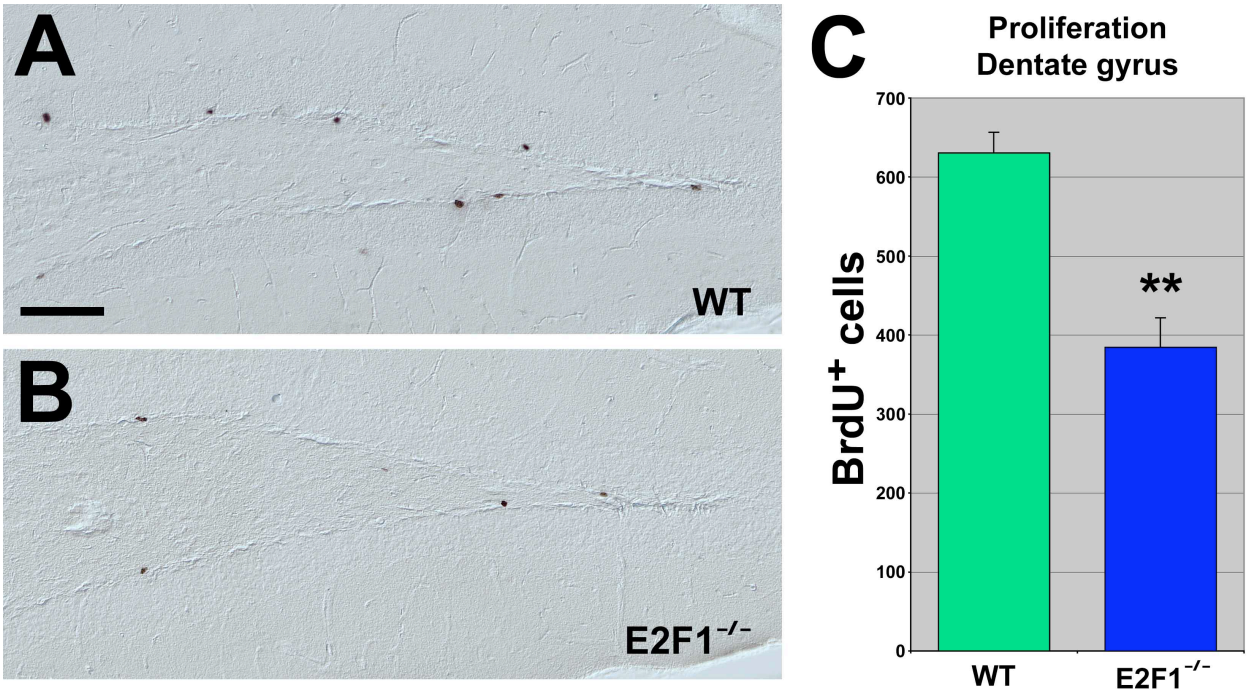


Figure 15
Proliferation in dentate gyrus visualized by BrdU labeling. (A–C) Cell division in the dentate gyrus granule cell layer of (A) wild-type and (B) E2F1-deficient mice. Note the typical location of newborn cells at the inner border of the granule cell layer. (C) Quantification. The dorsal hippocampus was analyzed on approx. 8 coronal sections per animal (*n* = 5 per group). The proliferative activity in the dentate gyrus is expressed as the total number of BrdU-positive cells per dentate gyrus granule cell layer. (D–F) **Statistically significant difference between wild type and E2F1-deficient mice at the 1% level, (Student's t test). Interference contrast optics. Scale bar in A is 100 µm.

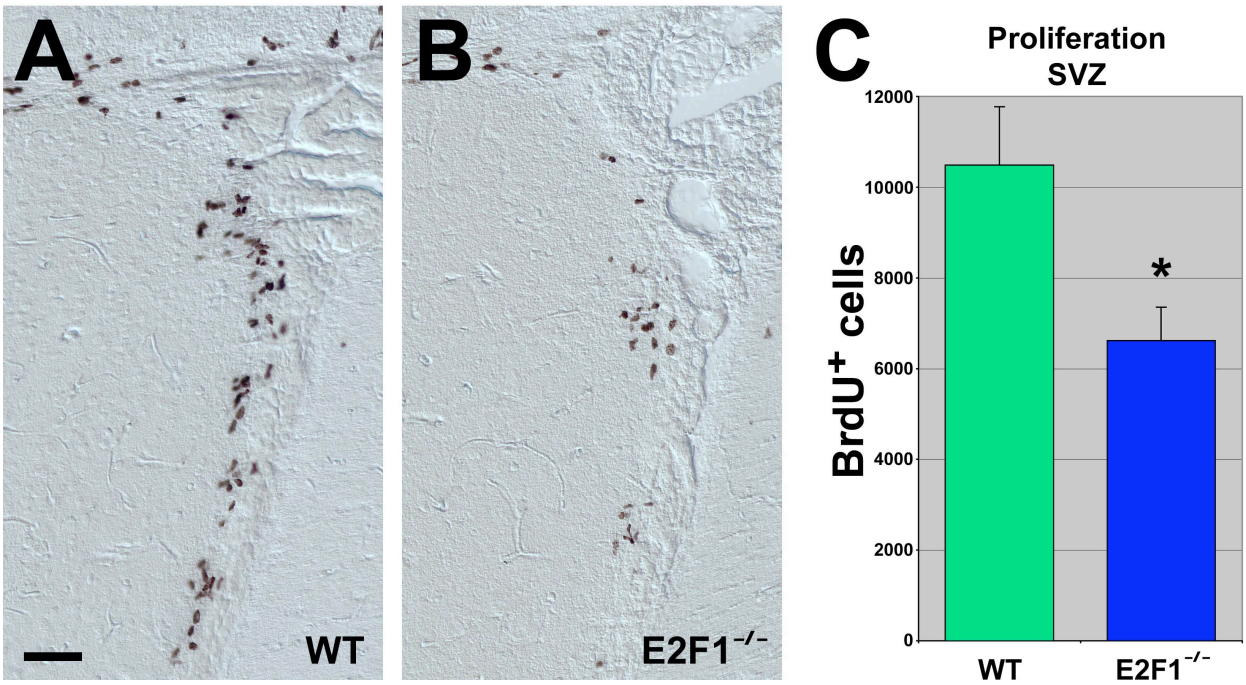


Figure 16
Proliferation in SVZ visualized by BrdU labeling. Cell division in the subventricular zone of (A) wild-type and (B) E2F1-deficient mice. (C) Stereological BrdU cell counts are represented as the total number of BrdU-positive cells per subventricular zone (n = 5 animals per group). *Statistically significant difference between wild type and E2F1-deficient mice at the 5% (Student's t test). Interference contrast optics. Scale bar in D is 50 µm.

Lower numbers of proliferating cells, as observed in the E2F1-deficient animals, could be due to (i) a lower proliferation rate of the existing neural precursor cells or (ii) size differences of the proliferative region, which may have restricted the amount of progenitors during development. These hypotheses were tested by determining the density of BrdU-positive cells in relation to the volume (volumetric density) and to the total number of cells (labeling index). In the DG GCL, 175 BrdU-positive cells/mm^3 represent 0.21% of the total cell population in wildtype animals, whereas, in E2F1-deficient mice, 109 BrdU-positive cells/mm^3 represent 0.13% of the total cell population ($p<0.01$). A significant difference is also observed in the SVZ with 330,000 BrdU-positive cells/mm^3 representing 34% of the total cell population in wildtype animals, whereas 181,000 BrdU-positive cells/mm^3 represent 16% of the total cell population in E2F1-deficient mice ($p<0.05$). These results illustrate that the reduced proliferation observed under E2F1 deficiency is independent from the volume and cell numbers of the respective proliferative areas.

Loss of E2F1 results in significant decrease of neurons in dentate gyrus and olfactory bulb.

In order to determine whether E2F1-dependent changes in proliferation affect adult neurogenesis, adult E2F1-deficient and wildtype mice were assessed 4 weeks after BrdU injections (Table 2). The numbers of newborn neurons as presented in Figure 17 were then calculated from the stereological count of BrdU-positive cells and multiplied with the percentage of neurons among the newborn cells as determined by confocal microscopy.

Table 2
BrdU Labeling in the Dentate Gyrus, Olfactory Bulb, and Lateral Ventricle Wall

Area	Wildtype	E2F1 deficient	*P* value
Estimated total number of BrdU-positive cells per structure			
Dentate gyrus—4 weeks after BrdU			
Granule cell layer	480.7 ± 52.7	242.6 ± 36.3	**0.003****
Molecular layer	60.7 ± 13.9	73.7 ± 8.9	0.45
Hilus	23.2 ± 5.6	25.7 ± 6.4	0.77
Olfactory bulb—4 weeks after BrdU			
Granule cell layer	38,971.9±1609.2	25,718.9 ± 1840.0	**<0.001****
Plexiform layer	1,972.9 ± 187.8	1,460.2 ± 197.2	0.08
Percentage neuronal (NeuN_) cells among newborn (BrdU_) cells			
Dentate gyrus—granule cell layer	73.0%	78.0%	
Olfactory bulb—granule cell layer	97.0%	93.2%	

The number of BrdU-positive cells for each area was estimated using immunohistochemistry and a systematic randomized sampling procedure (Williams and Rakic, 1988) similar to the optical disector (Gundersen *et al.,* 1988). Note the high numbers of cells being labeled by a single injection of BrdU. When combined with confocal analysis these numbers reveal a very prominent neurogenic activity in the adult mouse forebrain. Values are presented as group means +/- SEM. Significance levels in the unpaired two-tailed *t* test are given as *P* values. ** Statistically significant difference between wild type and E2F1-deficient mice at the 1% level.

In all brain regions with prominent adult neurogenesis - the GCLs of the DG and of the OB - a statistically significant reduction of BrdU-positive cells in E2F1-deficient mice at 4 weeks after BrdU labeling was found (Table 2). When ascertaining the relative proportion of neurons among the newborn cells, significantly less new neurons were generated in the adult CNS of E2F1-deficient mice. The number of newborn granule cells in E2F1-deficient mice was reduced to 53% of control levels in the DG and to about 63% in the OB (Figure 17). Although proliferation of other cell types, such as glial cells and endothelial cells, were not directly assessed, neighboring regions containing no

known neurogenesis, but rather glial and endothelial proliferation, e.g. the DG molecular layer, the hilus region and the olfactory plexiform region, revealed no significant changes in the number of BrdU-positive cells (Table 2).

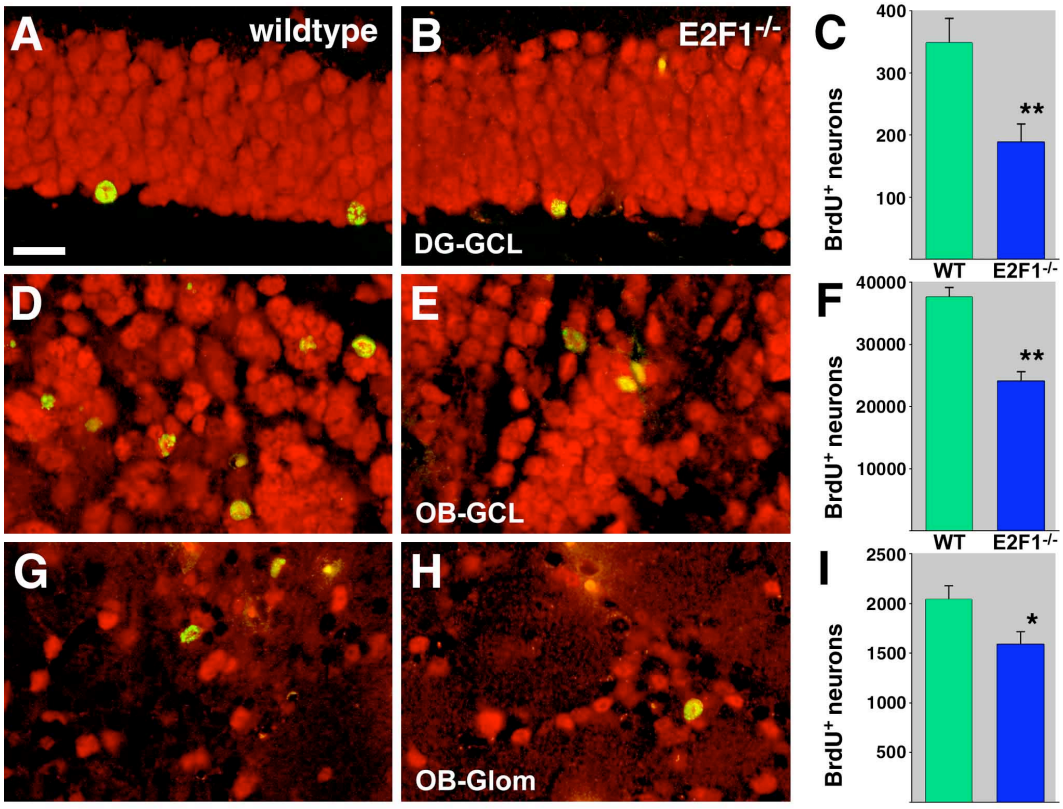


Figure 17
Adult neurogenesis in E2F1-deficient and wild-type animals. Decreased numbers of newly generated granule cells in the dentate gyrus (A–C), the olfactory bulb granule cell layer (D–F), and the olfactory bulb glomerular layer (G-I) of adult E2F1-deficient mice (n = 7) and wild-type littermates (n = 8). Confocal analysis was used to determine the percentage of neurons (NeuN-positive cells—red) among the population of newborn cells (BrdU-positive cells—green) at 4 weeks after BrdU labeling (double-labeling appears yellow). The total number of newly generated neurons was estimated by combining the stereologically assessed number of BrdU-positive cells with the percentage of neurons among the BrdU-positive cells (for details see Experimental Methods) and represented as group means +/- SEM. **Statistically significant difference between wild-type and E2F1-deficient mice at the 1% level (Student's *t* test). Scale bar in *A,* 20 µm.

Loss of E2F1 results in decreased cell death in dentate gyrus and olfactory bulb granule cell layers.

As previously shown for the rat, regions of neurogenesis in the adult mouse CNS harbor a high density of TUNEL-positive cells; therefore, TUNEL profiles were quantitatively evaluated in order to detect possible differences between E2F1-deficient and wildtype mice. Specificity of TUNEL labeling was controlled by the co-labeling of

apoptotic profiles with anti-active caspase-3 antibody (Figure 18). In E2F1-deficient animals the numbers of TUNEL-positive profiles in the GCL of the DG and OB were about 60-70% lower than in wildtype animals (Figure 19A and B). The LV wall revealed no significant changes in cell death (Figure 19C); however, a trend towards reduced apoptotic cells was detected.

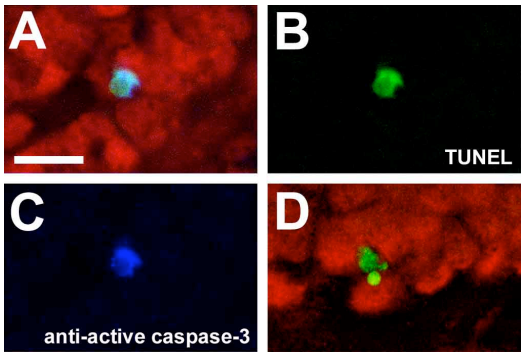


Figure 18
In order to detect apoptotic cells in tissue sections the TUNEL assay was performed (green signal in A, B, and D). Other hallmarks of apoptosis, like anti-active caspase-3 immunoreactivity (blue signal in A and C) or nuclear fragmentation (D) colabeled with TUNEL-positive cells, verify the specificity of TUNEL labeling. Red signal in A and D, anti-NeuN. Scale bar, 15 µm.

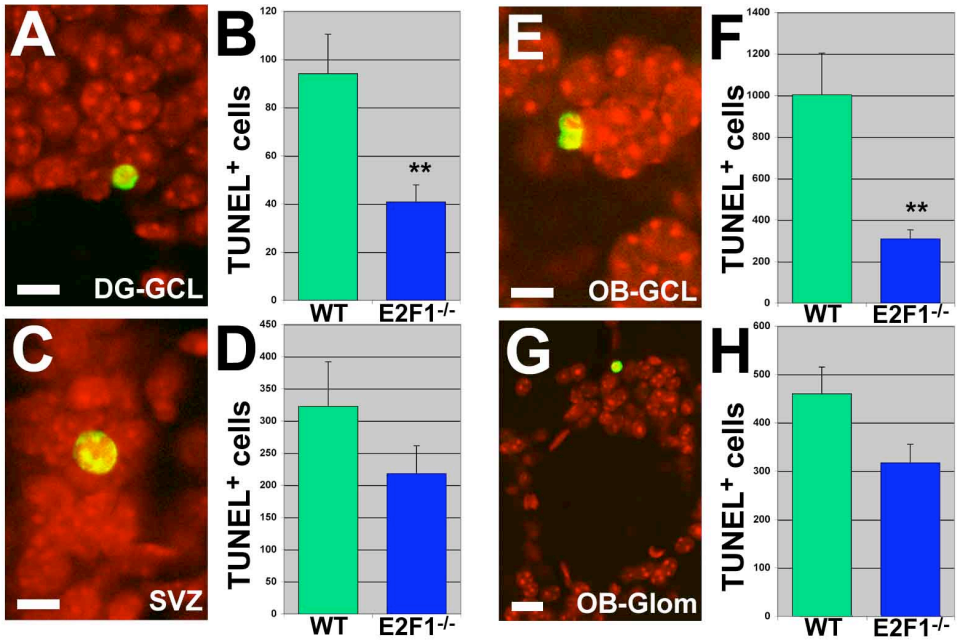


Figure 19
Apoptotic cells were consistently detected throughout the neurogenic regions of the adult brain. (A, C, E, G) Numerous TUNEL-positive cells (green) were identified in (A) the DG-GCL, (C) the SVZ, (E) the OB-GCL, and (G) the OB-PGL. (B, D, F, H) Quantification of TUNEL-positive cells in wildtype (n = 8) and E2F1-deficient mice (n = 7) revealed a significant reduction in the DG-GCL and OB-GCL in the knockout animals, whereas in the SVZ and OB-PGL no significant changes were detected (Student's t test). Red signal, nuclear counterstaining ToPro 3. Scale bars in A, C, E, and G 7.5 µm.

DISCUSSION

Functional significance of E2F1 in other animal models

Although E2F1-deficient mice are viable and fertile, the functional importance of E2F transcription factors for cell cycle progression becomes obvious in other animal models. In drosophila, mutation of the E2F1 homologue (dE2F) is early embryonic lethal[192]. Once the maternal contribution of gene products in the fly embryo has ended, a cessation of DNA synthesis is observed in virtually all cells of the embryo. In mammals, the functional redundancy of E2F family members becomes apparent when the effects of multiple deletions are studied. A recent report of fibroblasts defective in E2F1, E2F2 and E2F3 shows a complete abolition of S-phase entry, whereas single and double-mutant cells were not affected[193]. From these experiments it becomes apparent that the E2F transcription factors play an essential role in cell cycle progression, proliferation and development.

Cell death in regions of adult neurogenesis

Several members of the E2F family of transcription factors affect cell cycle progression, but E2F1 is the only known member to induce cell death as well[181, 186, 187, 194]. The mechanism of the pro-apoptotic E2F1 activity is not fully understood, but it is assumed that forcing proliferation-incompetent cells past the G1-S restriction point of the cell cycle may induce aberrant proliferation and subsequent apoptosis[195-197]. In the initial description of E2F1-deficient mice, one of the most prominent effects found was a hyperplasia of the thymus due to a defect in apoptotic elimination of thymocytes during T-cell maturation[187]. Other experiments demonstrated that overexpression of E2F1 in postmitotic neurons *in vitro* induces apoptosis[198, 199], whereas elimination of E2F1 function can prevent induction of apoptosis in neurons[198, 200, 201].

The effect of E2F1 deficiency on apoptosis suggests that the reduction in neurogenesis and cell numbers would be more drastic if apoptosis was not affected in parallel. As for the cerebellar granule cells, it can only be speculated as to how the neonatal generation of cerebellar granule cells *in vivo* is influenced by E2F1 deficiency, since only the total number of cerebellar neurons in adult animals is measured is this experiment (see Figure 14 on page 47). Compared to the regions of adult neurogenesis

the reduction in the number of cerebellar granule cells is rather small, perhaps due to an even stronger decrease in apoptotic elimination of granule cell progenitors. Along these lines, *in vitro* experiments have shown that E2F1 deficiency completely abolishes neuronal death in cerebellar granule cells induced by beta-amyloid[202].

In vitro overexpression of E2F1 leads to a strong upregulation of cell death activators[203]. The induction of E2F1 in postmitotic neurons activates DNA-synthesis and concomitant cell death[198, 199]. For the pathophysiology of Alzheimer's disease, it has become increasingly evident that neuronal cell death is accompanied by an upregulation of cell cycle activators, including E2F, and S-phase entry[204-207]. Under E2F1-deficiency, neurons were resistant to staurosporine-, large T-antigen and low K^+-induced neurotoxicity *in vitro*[198-201]. The ability of Myc to induce apoptosis in fibroblasts is also markedly reduced in cells deleted for E2F1[208]. These studies hint towards an active and direct role of E2F1 in the molecular regulation of apoptosis.

Postnatal phenotype of E2F1-deficiency

It has been hypothesized that a functional compensation for the lack of E2F1 is accomplished by other family members, leading to the assumption that no apparent phenotype is observed during embryonic brain development of E2F1-deficient mice[187]. In fact, five E2F family members are highly expressed in the developing brain and in-situ hybridization signals for E2F1, E2F2 and E2F5 are preferentially detected in the ventricular zone, where neural progenitors cells undergo rapid cell division[189, 190].

In E2F1-deficient animals the neurogenesis of granule cells in the OB, DG and cerebellum are affected; however, the number of neurons of the neocortex, which develops exclusively during the embryonic phase, is unchanged by E2F1 deficiency, probably due to compensation by other E2F family members[188-191]. But why are the other E2F family members downregulated postnatally? One explanation could lie in the decreased complexity of neurogenic activity in the postnatal brain compared to prenatal neural development. Postnatal and adult neurogenesis is mainly required for the production of large homogenous populations of interneurons. Therefore, from an evolutionary point of view a decline of redundancy between E2F family members in the postnatal CNS is probably more permissive than during embryonic brain development.

Adult neurogenesis is a cellular mechanism that encompasses rapid cell division of neural progenitors cells in the LV and hippocampus, followed by migration and neuronal and glial differentiation. With a cell cycle length of 14 hrs for progenitors in the LV[209] and 25 hrs for the DG[25] and the daily generation of several ten thousand neurons in adult rodent brain, a tight molecular regulation of cell cycle progression and cell death is required. The present study shows that E2F1 plays a significant role in the generation of granule cells in the postnatal and adult CNS by affecting proliferation and cell death. E2F1 deficiency reduces neuronal cell numbers, rather than distorts brain structures, suggesting a role in controlling the number of granule cells in the postnatal brain.

Experiments III & IV - Neurotransmitters and their position in the game of Adult Neurogenesis

Recently, several secreted molecules have been implied in the extrinsic regulation of cell proliferation within the developing telencephalon (for review see[210]). Among them, neurotransmitters are prominent candidates for transcellular signals that could influence the development of CNS cells, since they contribute to the extracellular milieu throughout the CNS maturation period[211-213]. Moreover, several studies strongly indicate the role of neurotransmitters as growth regulators for immature neurons[210, 214-221]. Of the neurotransmitters, the most is known about GABA and glutamate with regard to their influences on neural progenitor cells. It has been established that GABA exerts a variety of trophic influences through the stimulation of the $GABA_A$ receptor during neural development (for review see[218, 222]) and particularly, $GABA_A$ receptor activation can control the cell cycle kinetics in neuronal progenitors[217, 219]. Moreover, GABA can influence other essential aspects of neuronal maturation *in vitro* and *in vivo*, such as neuronal survival, growth cone pathfinding, and neuroblasts movement, including migration on radial glia[223-229]. In the case of glutamate, work has shown that glutamate, via the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate (AMPA/KA) receptor activation, could downregulate periventricular progenitor proliferation in rat embryonic cortical explants[219]. In the adult, several studies have shown that glutamate acts to block DG cell proliferation, although the exact mechanisms by which this occurs are currently unknown (for review see[210]).

Other neurotransmitters, such as serotonin, have also been shown to play an important role during development and progenitor proliferation. The early occurrence of serotonergic innervation in developing cortical pathways[230, 231] suggests a role for serotonin in brain circuit formation (for review see[232]) and synaptogenesis[233, 234]. Moreover, 5-HT (serotonin) is required for normal early brain maturation, since the depletion of 5-HT during this critical period leads to a decrease in synaptic density and impairs the cognitive capabilities in adult rats[234].

Two neurotransmitter systems were chosen in this study – acetylcholine, and noradrenaline - mainly because of their specific innervations into the DG and the OB. Although neurotransmitter systems are often studied pharmacologically by the use of

receptor agonists and antagonists, the current study used neurochemical lesions to study the effects on neurogenesis for two reasons. First, lesions chronically deplete a neurotransmitter system and therefore long term effects can be studied. Second, selective loss of specific classes of neurons is a hallmark of neuropsychiatric diseases, such as Alzheimer's disease. Here, the loss of cognitive function appears to be directly coupled to the loss of cholinergic neurons in the basal forebrain and it is therefore important to find out which long term consequences the loss of neurotransmitter has with regard to adult neurogenesis.

Prior to 1999 little was known about the possible effects that **acetylcholine** and **noradrenaline** could have on progenitor proliferation and neurogenesis in the adult brain. The next two experiments attempt to address the question as to whether these three neurotransmitters are able to regulate adult neurogenesis. In principle there are two strategies to study effects of neurotransmitter. Most often the pharmacological approach of stimulating and blocking neurotransmitter receptors is used, however since there is little knowledge about the receptor subtypes that are present in regions of neurogenesis, it was decided to use the neurochemical lesion strategy. Here, alteration in neurotransmitter concentration causes neurotoxin-induced cell death of neurons with specific neurotransmitter phenotypes and specific innervation into the hippocampus and the OB.

Experiment III – Cholinergic Denervation Lesion

Objective

This experiment aims at defining the role of cholinergic input into the neurogenic regions by using an immunotoxic lesion approach. The strategy is to selectively lesion the cholinergic neurons of the cholinergic basal forebrain (CBF), which project to the DG and the OB. The immunotoxin 192IgG-saporin was infused into the LV of adult rats, leading to specific neuronal cell death of cholinergic neurons of the basal forebrain. The specific goals of this experiment were to (1) determine the effects of cholinergic denervation on hippocampal and OB neurogenesis and (2) to analyze cell death in regions of neurogenesis after cholinergic lesions to those areas.

Introduction to Experiment

The cholinergic neurons of the forebrain (CBF), consisting of the medial septum, nuclei of the vertical and horizontal limbs of the diagonal band, and the nucleus basalis of Meynert (in human), have a widespread connection to the neocortex, the hippocampus, the OBs and the amygdala (Figure 20) and are involved in enhancing cortical responses to incoming sensory stimuli.

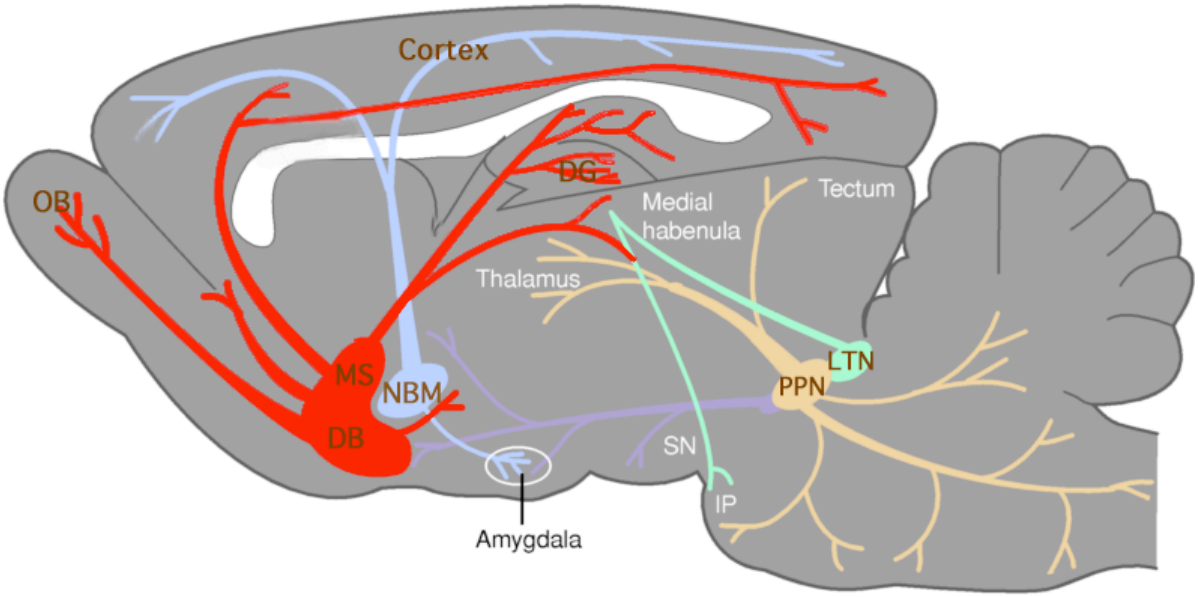


Figure 20
Sagittal section of an adult rat brain depicting the different cholinergic innervations. Of particular importance to this study is the basal cholinergic forebrain (CBF – red and light blue), which provides cholinergic innervation to the neocortex, hippocampus, olfactory bulb and amgdala. The CBF consists of the medial septum (MS), the vertical and horizontal limbs of the diagonal band of Broca (DB), the nucleus basalis of Meynert (NBM) and the substantia nigra (SN). The hippocampus receives its innervation from the MS and the vertical limb of the DB, whereas the OB receives its innervation from the horizontal limb of the DB. (Adapted from [235])

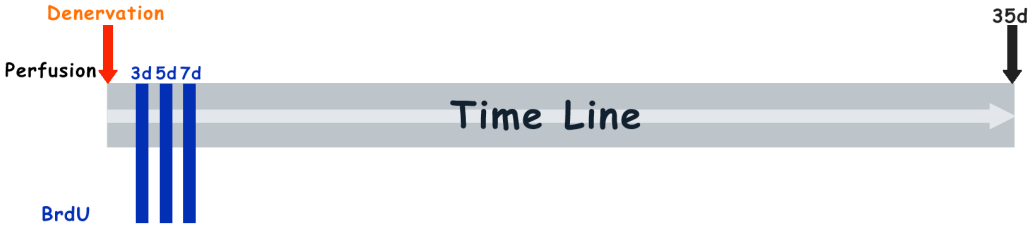
Cholinergic innervation into the OB has been shown to be quite extensive, with the highest amounts of cholinergic fibers present in the glomerular layer and deep external plexiform layer[236-239]. The main olfactory innervation of cholinergic fibers originate from the magnocellular basal forebrain nuclei - the substantia innominata and the horizontal limb of the diagonal band of Broca[240, 241] - and terminate in the PGL as well as onto the granule spines (for review see[14])

Within the DG, cholinergic innervation is especially in the inner 1/3 of the molecular layer directly adjacent to the GCL[242], where the cholinergic axons make synaptic contact with dendritic projections of the granule cells (see Figure 4 on page 14). The role of the

basal forebrain cholinergic system in learning and memory has held considerable interest since the discovery of cholinergic neurodegeneration in the basal forebrain in Alzheimer's disease. The hippocampus is well defined in the context of learning and spatial memory and the cholinergic system seems to be crucially connected to these functions. Lesions to the septo-hippocampus connection have been shown to impair spatial memory in rats[243] and acetylcholine has been shown to be not only necessary for learning and memory[244], but its presence within the neocortex is also sufficient to ameliorate learning deficits and restore memory following damage to the NBM[245].

The immunotoxin, 192IgG-saporin, consists of the monoclonal antibody 192IgG, which is coupled to saporin; a ribosome-inactivating toxin derived from the plant Saponaria officinalis (soap wort). The antibody component of 192-saporin is directed against rat p75, the low-affinity neurotrophin receptor. When injected intracerebrally, 192IgG-saporin binds to the surface of p75 bearing neurons and is internalized by endocytosis. Once in the cytoplasm, the saporin moiety escapes endosomes and enzymatically inactivates the large ribosomal subunit halting protein synthesis and leading ultimately to cell death[246]. Key to this experimental strategy is that the cholinergic neurons of the basal forebrain specifically express the neurotrophin receptor p75 and their development and survival depends on the supply of the nerve growth factor (NGF). When this immunotoxin is administered ICV, the result is widespread with a massive depletion of ChAT activity in the CBF projections and a substantial p75NGFr-immunoreactive neuronal loss[247-249], while sparing other neurotransmitter systems and non-cholinergic CBF neurons[250, 251]. It has been previously shown that a single injection of 192IgG-saporin results in a complete and selective loss of ChAT-immunoreactive neurons in the basal forebrain, long-lasting cortical cholinergic hypoactivity, and deficits in learning and memory tasks[248, 252-258].

Experimental Design



For specific details on Experimental Design, see Materials and Methods on page 107.

RESULTS

In order to evaluate the effect of cholinergic denervation on adult neurogenesis, adult Fischer rats were infused into the LV with 192IgG-saporin or PBS. Four weeks after the final BrdU injection, which is sufficient time for allowing the newborn cells to differentiate, the animals were perfused and tissues prepared for analysis. The DG of the hippocampus and the OB were analyzed for the amount of new cells generated. To characterize cell fate, BrdU labeling was combined with the neuronal marker, NeuN, which recognizes neuronal cell bodies and nuclei[143] and the astroglial marker, S100□, which labels astrocytic bodies[259].

ICV injections of 192IgG-saporin result in selective elimination of cholinergic neurons in medial septum.

Once the animals were sacrificed and perfused, the medial septum was analyzed in order to be certain that the lesion was accurate and complete. ChAT immunoreactivity demonstrates the extent to which the cholinergic forebrain was lesioned (Figure 21).

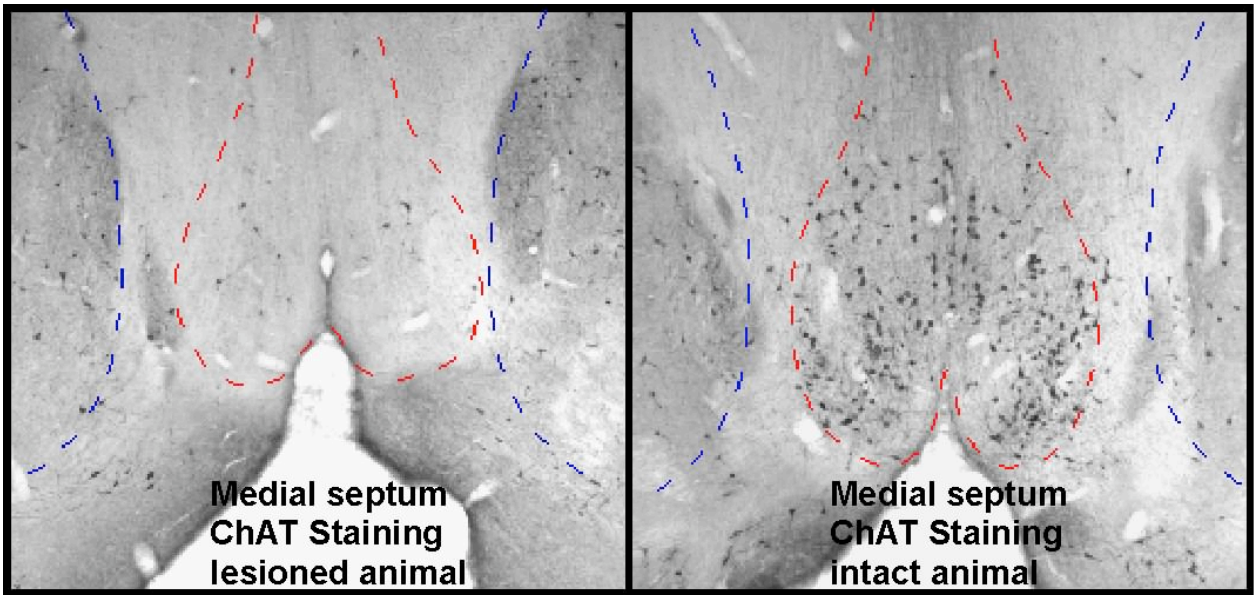


Figure 21
Extent of cholinergic denervation in medial septum. ChAT staining showing complete denervation of cholinergic neurons residing in medial septum (lesioned animal – left, control animal – right). Note the residual positively-stained ChAT neurons in the striatum (blue area) due to lack of p75 expression.

Decrease of Neurogenesis in Dentate Gyrus after Cholinergic Denervation

Four weeks after cholinergic denervation, BrdU-positive cells were counted in the GCL, the molecular layer and the hilus of the DG. Quantifications revealed a trend

towards less newly generated cells in the GCL and hilus of the 192IgG-saporin animals (Figure 22). But since the BrdU-positive cell population can consists of several different cell types, triple immunofluorescence and confocal laser scanning microscopy specifically determined the proportion of neurons among the new cells. The total number of BrdU-positive cells was multiplied by the percentage of neuronal (BrdU+/NeuN+) and non-neuronal cells (BrdU+/S100ß+ or BrdU+/NeuN-/S100□-). In the granule cells layer of the DG, there was a statistically significant 35% decrease in the number of new neurons ($p<.05$). On average, 1342 new neurons in the GCL of lesioned animals were detected; whereas, in the control animals 2064 newly born neurons were counted (Figure 23).

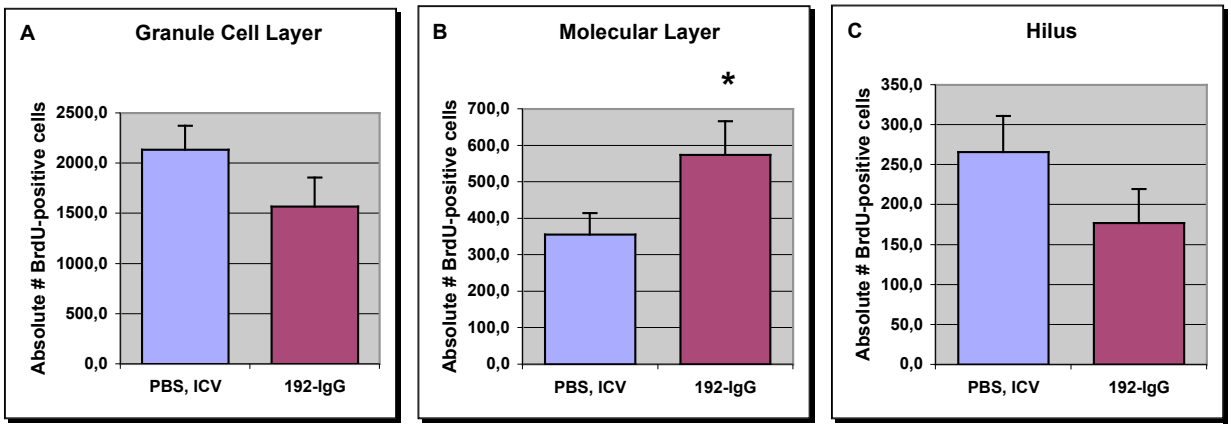


Figure 22
Bars represent total numbers of BrdU-positive cells within hippocampus of control animals (blue) and cholinergic lesioned animals (red). The hippocampus was divided into granule cell layer (A), molecular layer (B) and hilus (C). There was a trend towards less newly formed cells within the GCL and hilus; however, there was a significant increase in proliferation within the molecular layer (B).

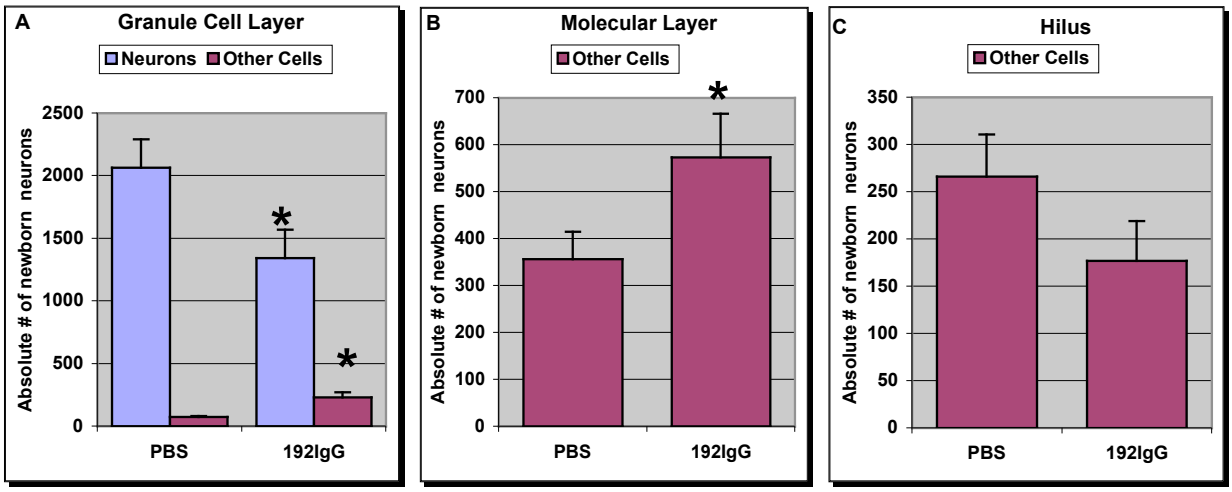


Figure 23
Graphs depicting the total number of newborn neuronal (blue) and non-neuronal (red) cells in the hippocampus. In the granule cell layer (A) there was a significant decrease in the number of newborn neurons after lesion, whereas the number of non-neuronal cells increased. In the molecular layer (B), there was a significant increase in the number of non-neuronal cells, whereas in the hilus (C) there was no significant difference. Newborn neurons were not observed in the molecular layer or hilus.

Cholinergic Denervation causes increase in Non-neuronal Cells in the Dentate Gyrus

The granule cells layer consists mainly of neurons but a few non-neuronal cells are generated as well. When the non-neuronal BrdU-positive cells in the GCL were quantified, a 205% increase was detected ($p<.05$, Figure 23A). Within the DG, the molecular layer and hilus were also analyzed and served as a control area, since these regions, under normal circumstances, do not generate new neurons in the adult. The molecular layer showed a significant increase in BrdU-labeling ($p<.05$, Figure 23B), whereas the hilus shows a tendency towards a decrease (Figure 23C). The molecular layer and GCL are directly perturbed by the loss of incoming axons, and these regions have previously been described to contain a high number of proliferating microglia after denervation[260].

Cholinergic Denervation causes an increase in Cell Death in the Dentate Gyrus

Compared to non-neurogenic regions, the DG and the OB contain more than 10-fold higher numbers of apoptotic cells. Therefore, the question of whether or not cholinergic denervation has an effect on cell death in the GCL was addressed. At a time point (five weeks after lesion) when the lesion is permanent, but the hippocampus is still denervated, apoptosis was quantified by TUNEL labeling. A 77% increase in TUNEL-

positive cells in the GCL was observed ($p<.05$) of the lesioned animals (Figure 24). TUNEL-positive cells were also quantified in the molecular layer and the hilus; but in contrast to the GCL, these regions had no significant changes in TUNEL labeling.

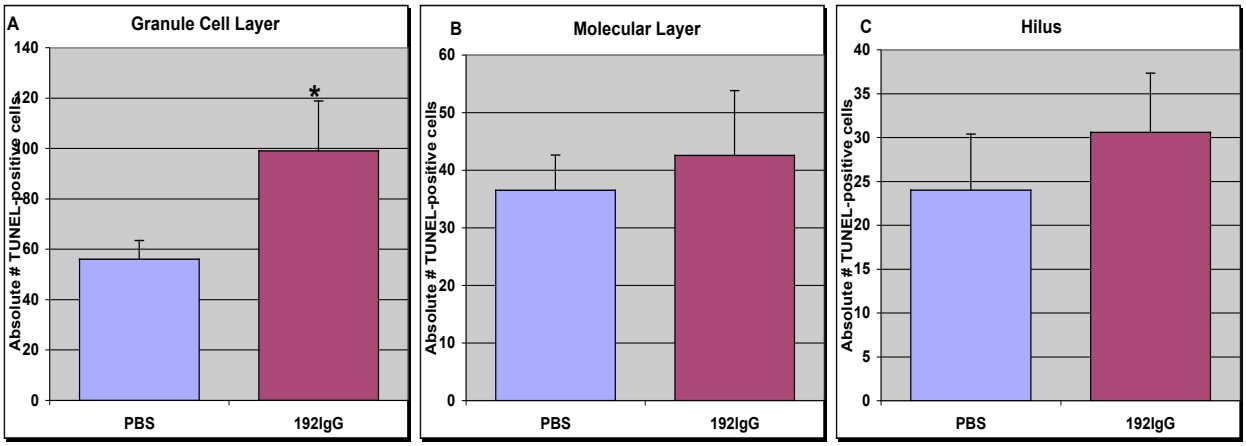


Figure 24
Graphs depicting cell death in the hippocampus after cholinergic denervation. In the granule cell layer (A) there is a significant increase in TUNEL-positive cells after lesion, whereas in the molecular layer (B) and hilus (C) there is no change.

Decrease of Neurogenesis in Olfactory Bulb after Cholinergic Denervation

Two neuronal cell types are generated in the adult OB, granule cells and periglomerular neurons (see on page). Therefore the analysis of the OB was subdivided into the granule cell layer (GCL) and the periglomerular layer (PGL) in order to assess whether these regions are affected differently by cholinergic denervation. Within the GCL, five weeks after the lesion, there was a 34% decrease in the number of newborn neurons ($p<.05$), along with a 35% decrease in new non-neuronal cells ($p<.05$) of the lesioned animals. A total of 77,265 new neurons were counted in the GCL of the PBS animals, whereas in the 192IgG-saporin animals, there was a total of 51,344 new neurons (Figure 25A).

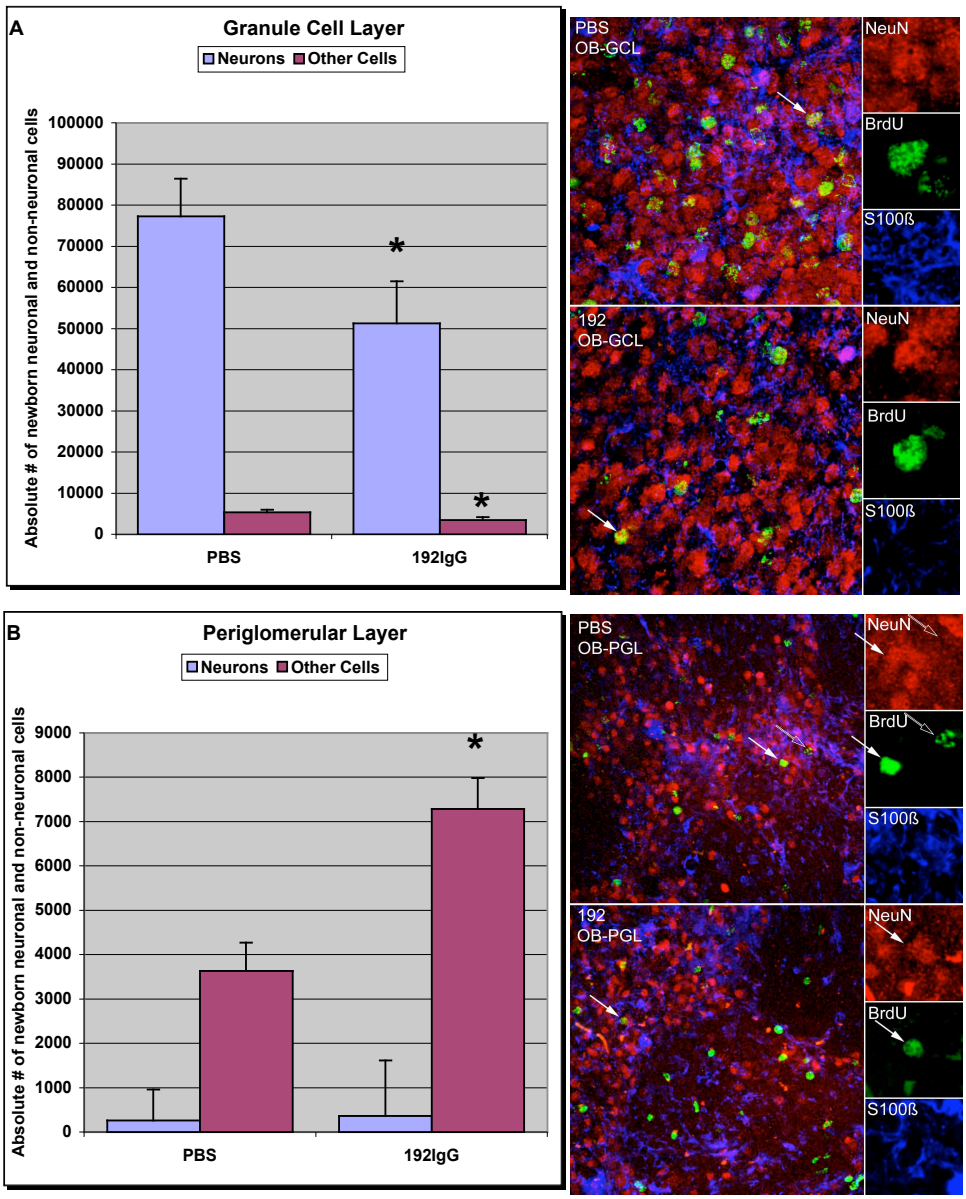


Figure 25
(A) Cholinergic denervation causes a decrease in the number of newborn neuronal (blue) and non-neuronal (red) cells within the granule cell layer of the olfactory bulb. (B) Cholinergic denervation causes an increase in the number of non-neuronal cells (red) within the periglomerular layer of the olfactory bulb, while leaving the newborn neuronal (blue) population unchanged.

In contrast to the GCL, the number of newborn neurons in the PGL was unchanged. However, there was a 100% increase ($p<.05$) in newborn non-neuronal cells in the PGL of lesioned animals (Figure 25B). This increase in non-neuronal cells may be due to the presence of microglia into this region after denervation, similar to the reported increase in microglial cells after denervation of the DG[260].

Cholinergic Denervation causes an increase in Cell Death in the Periglomerular Layer

As previously shown that in the adult rat, the highest number of TUNEL-positive cells in the brain are found within the OB, primarily within the GCL[28]. A 63% increase was detected in the number of TUNEL-positive cells ($p<.05$) in the PGL of the lesioned animals (Figure 26B) but a non-significant trend towards less apoptotic cells in the GCL. One would expect to see an increase in cell death in the GCL (Figure 26A), particularly since a significant decrease in the generation of new neurons was observed (Figure 25A); however, TUNEL-positive cells were quantified five weeks post-lesion, which is late enough after the lesion that one would no longer be quantifying the acute cell loss due to an acute denervation lesion.

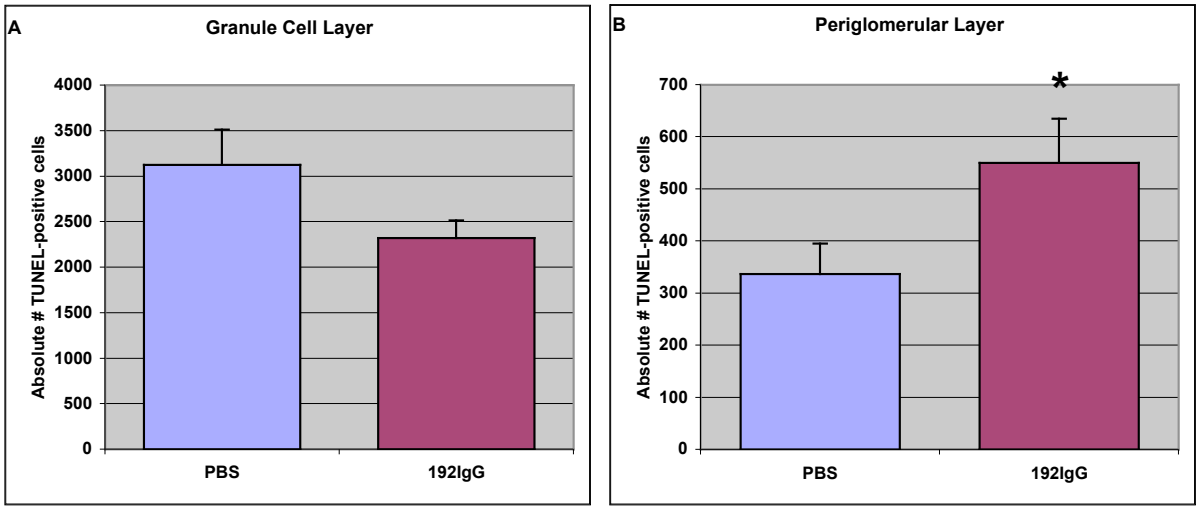


Figure 26
Cholinergic denervation has no effect on cell death in the granule cell layer of the olfactory bulb (top); however, cell death is increased within the periglomerular layer after lesion (bottom).

DISCUSSION

Lesion of the CBF system, generated by ICV injections of 192IgG-saporin, leads to less newly born neurons in regions of adult neurogenesis. In the hippocampus, a 35% decrease in the number of newly generated granule neurons was detected. Therefore, it can be proposed that the cholinergic system plays a stimulatory role for neurogenesis. However, it cannot be answered with this experiment whether or not acetylcholine acts directly on the neuronal progenitor cells in the adult brain and would need, for example, *in vitro* studies on isolated cells. In addition, the effects on neurogenesis after lesioning

the CBF could be due to either primary or secondary effects. Acetylcholine could act directly on the progenitor cells within the hippocampus and SVZ, or it could act indirectly through other neurotransmitters and their systems. These speculations, nevertheless, cannot be answered by this experimental approach.

The cholinergic system is known to be involved in information processing related to learning and memory. In particular, the loss of cholinergic function has been associated with cognitive decline during aging and in Alzheimer's disease. Although still correlative, several arguments support the view that hippocampal neurogenesis might be involved in similar cognitive functions. (1) Enriched environment increases neurogenesis and spatial learning in the Morris water maze, a hippocampus dependent task[91]. (2) Learning situations can increase neurogenesis[261, 262]. (3) Hippocampal neurogenesis decreases with aging[19, 263]. (4) Restoring neurogenesis in aged animals ameliorates symptoms of age-dependent cognitive decline[38, 104, 116, 264]. Although it is too early to connect neurogenesis to disease progression in neurological disorders such as Alzheimer's disease, it is intriguing to propose that the loss of neuronal elements may not only be based on cell death of mature neurons but on the decreased production of new neurons as well. Based on this theory, neurodegeneration could be redefined in certain cases as an imbalance in production vs. elimination, rather than the inability to sustain the survival of a fixed number of neurons. This is still a provocative hypothesis, however it may open completely new avenues to find novel therapeutic strategies for neurodegenerative diseases.

Besides reduced neurogenesis in the DG, a similar decrease in neurogenesis was observed after cholinergic denervation in the GCL of the OB. Even though the function of adult olfactory neurogenesis has not yet been determined, it can be assumed from several studies that neurogenesis is involved in olfactory information processing, such as olfactory discrimination learning[94, 265, 266, 11]. In this regard it is also relevant to mention that cholinergic input to the OB has been shown to be directly involved in local neuronal plasticity related to olfactory learning[267-269]. As a clinical perspective, numerous reports demonstrated the connection between olfactory deficits and neurodegenerative diseases. For example, functional testing of Alzheimer's patients reveals significant impairment in olfactory function (for reviews, see[270-273]). Moreover, aged rats have impairments in olfactory reversal learning[274], which could have a possible correlation

with the fact that neurogenesis decreases with age in the rat OB[275]. Taking these observations together with the current experiment, it is possible that one of the functions of the cholinergic input into the OB is to regulate the extent of neuronal production. It is however too early to conclude that neurogenesis is involved in the olfactory pathology of certain neurodegenerative diseases.

Experiment IV – Noradrenergic Denervation Lesion

Objective

This experiment aims at defining the role of noradrenergic input into the neurogenic regions by using a chemical lesion approach. The strategy is to selectively lesion the noradrenergic neurons of the locus coeruleus (LC), which project to the dentate gyrus and the olfactory bulb. The immunotoxin DBH-saporin was infused into the lateral ventricle of adult rats, leading to specific neuronal cell death of noradrenergic neurons of the LC. The specific goals of this experiment were to (1) determine the effects of noradrenergic denervation on hippocampal and olfactory bulb neurogenesis and (2) to analyze cell death in regions of neurogenesis after noradrenergic lesions to those areas.

DBH-saporin, which consists of a monoclonal antibody to Dopamine beta-hydroxylase coupled by a disulfide bond to saporin (a ribosome inactivating protein), has been shown to be selectively toxic to peripheral noradrenergic sympathetic neurons in rats after systemic[276]and ICV injection. Dopaminergic neurons of the substantia nigra and ventral tegmental area and serotonergic neurons of the raphe, all monoaminergic neurons that do not express DBH, survived all anti-DBH-saporin doses[277].

Introduction to Experiment

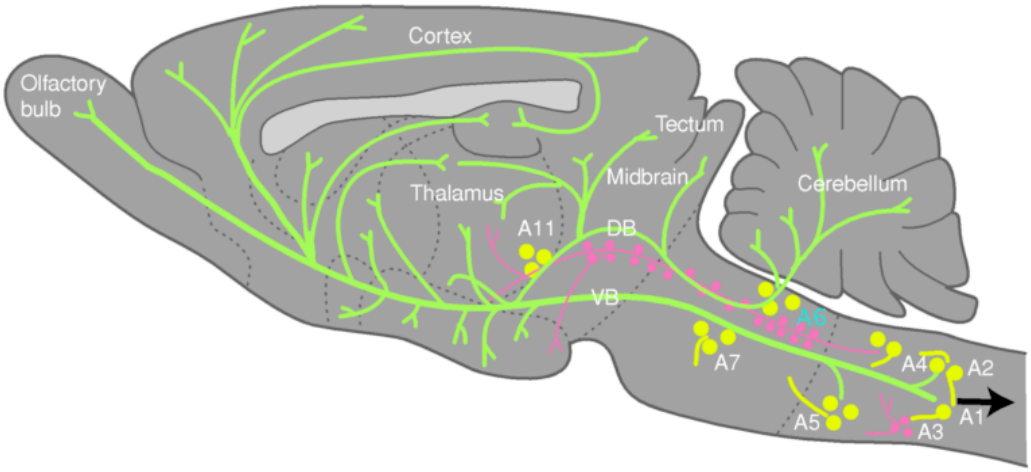


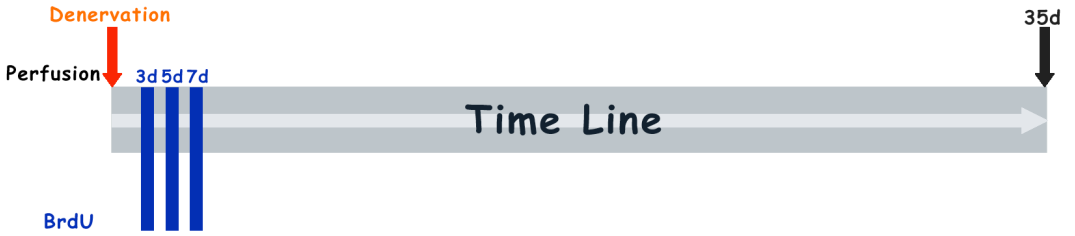
Figure 27
Depiction of noradrenergic innervation in the adult rat brain. The locus coeruleus (A6 – in blue) projects to the cortex, cerebellum, hippocampus, olfactory bulb, and a small portion to the spinal cord. Of particular interest to this study are the noradrenergic neurons projecting from the LC to the hippocampus and olfactory bulb. (Adapted from [235])

The majority of noradrenergic neurons reside in the locus coeruleus of the pons. These neurons project throughout the cortex, cerebellum and spinal cord (Figure 27). A large portion of the adrenergic neurons reside in the medulla and project to the spinal cord, particularly to the sympathetic preganglionic column, and some terminate in the hypothalamus, as well as a small portion ending up in the locus coeruleus. There is also a group of neurons that reside in the nucleus of the solitary tract.

Most interestingly for this study, projections from the locus coeruleus innervate the hippocampus specifically along the subgranular zone (between hilus and gcl), the region of progenitor proliferation and neurogenesis. At least 40% of all LC neurons project to the olfactory bulb; the innervation is almost 10 times greater than to any part of the cerebral cortex[278]. Noradrenergic input into the olfactory bulb innervates the granule cell layer, mitral layers and plexiform layers; however, little innervation into the glomerular layer has been reported[279]. In spite of this innervation pattern, no data from pharmacological or lesion studies on noradrenaline in relation to adult neurogenesis have been reported thus far.

In the developing brain, noradrenaline may play a role by modulating synaptic plasticity during critical periods of circuit formation[280, 281]. In the olfactory bulb, noradrenaline input from the locus coeruleus (LC) appears to be necessary for the newborn rat to form a learned odor preference[282].

Experimental Design



For specific details on Experimental Design, see Materials and Methods on page 107.

RESULTS

In order to evaluate the effect of noradrenergic denervation on adult neurogenesis, adult Fischer rats were infused into the lateral ventricle with DBH-saporin or PBS. Four wks after the final BrdU injection, which is sufficient time for allowing the

newborn cells to differentiate, the animals were perfused and tissues prepared for analysis. The dentate gyrus of the hippocampus and the olfactory bulb were analyzed for the amount of new cells generated. To characterize cell fate, BrdU labeling was combined with the neuronal marker NeuN and the astroglial marker S100□.

Decrease of Neurogenesis in Dentate Gyrus after Noradrenergic Denervation

Four weeks after noradrenergic denervation, BrdU-positive cells were counted in the GCL of the dentate gyrus. Quantifications revealed a significant reduction in the number of newly generated cells in the GCL of the DBH-saporin animals compared to sham-lesioned animals (PBS injection) (Figure 28). Triple immunofluorescence and confocal laser scanning microscopy determined the relative amount of neurons vs. other cell types among the new cells. The total number of BrdU-positive cells was multiplied by the percentage of neuronal (BrdU+/NeuN+) and non-neuronal cells (BrdU+/S100ß+ or BrdU+/NeuN-/S100□-). Since the percentage of neurons among the new cells did not differ between DBH-saporin and PBS animals, the production of newly generated neurons was significantly reduced (Figure 29). On average, 1341 new neurons were detected in the granule cell layer of lesioned animals, whereas, in the control animals, 2064 newly born neurons were counted.

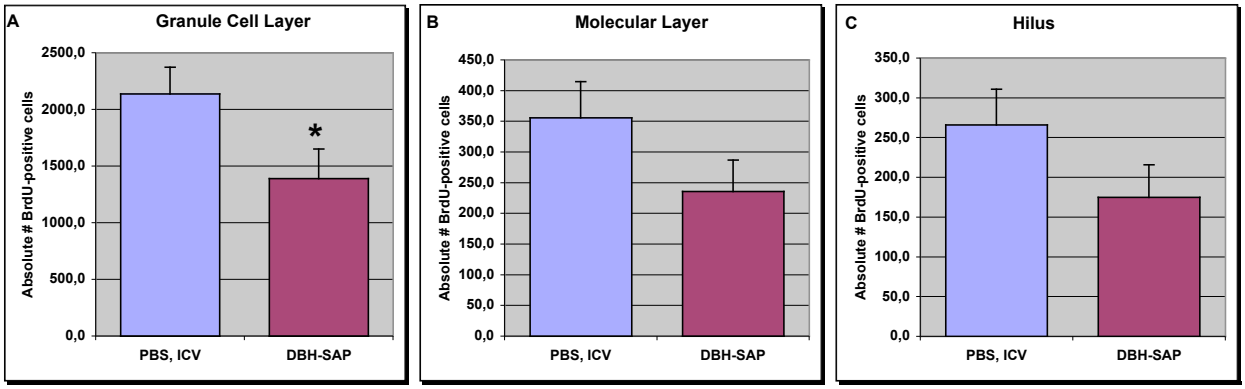


Figure 28
Bars represent total numbers of BrdU-positive cells within hippocampus of control animals (blue) and noradrenergic lesioned animals (red). The hippocampus was divided into granule cell layer (A), molecular layer (B) and hilus (C). Within the GCL, there was a significant decrease in the proliferation of progenitor cells due to noradrenergic denervation; however, within the molecular layer and hilus, there were no significant changes.

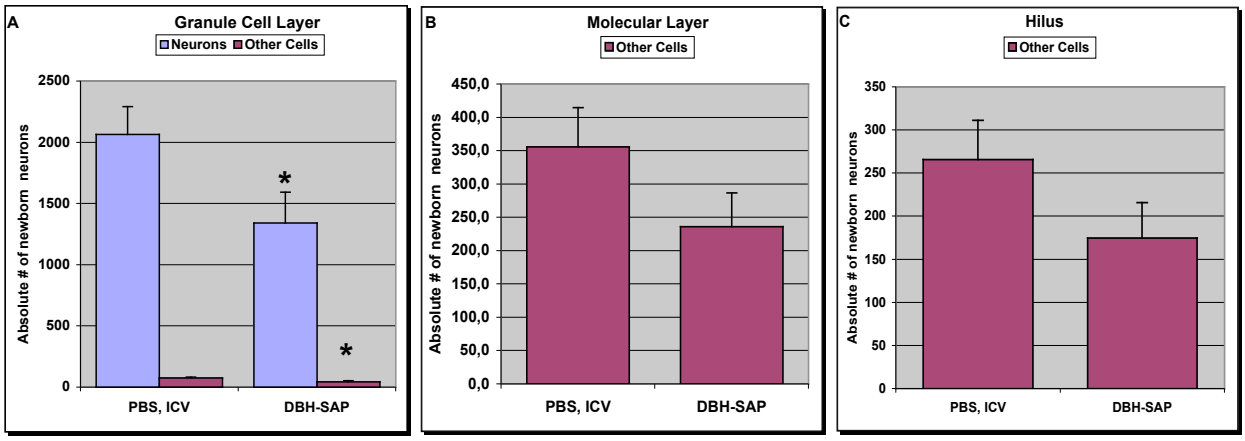


Figure 29
Graphs depicting the total number of newborn neuronal (blue) and non-neuronal (red) cells in the hippocampus. In the granule cell layer (A) there was a significant decrease in the number of newborn neurons and non-neuronal cells. In the molecular layer (B) and the hilus (C) there was no significant difference. Newborn neurons were not observed in the molecular layer or hilus.

Noradrenergic Denervation causes decrease in Non-neuronal Cells in the Dentate Gyrus

The GCL consists mainly of neurons; however, a few non-neuronal cells are generated as well. When the non-neuronal BrdU-positive cells in the GCL were quantified, a 40% decrease was detected ($p<.015$, Figure 29A). Within the DG, the molecular layer and hilus were also analyzed and served as a control areas, since these regions, under normal circumstances, do not generate new neurons in the adult. The molecular layer and the hilus show no alteration in the number of BrdU-positive cells (Figure 29B & C).

Cell Death in the Dentate Gyrus is not altered by Noradrenergic Denervation

Cell death is a prominent regulatory mechanism in neurogenic regions in the adult brain. Therefore, the question of whether or not noradrenergic denervation has an effect on cell death in the GCL was addressed by quantifying TUNEL labeling. No changes in the number of TUNEL-positive cells were observed in the GCL of the lesioned animals (Figure 30). Apoptotic cells were also quantified in the molecular layer and the hilus and similarly, these regions had no significant changes in TUNEL labeling.

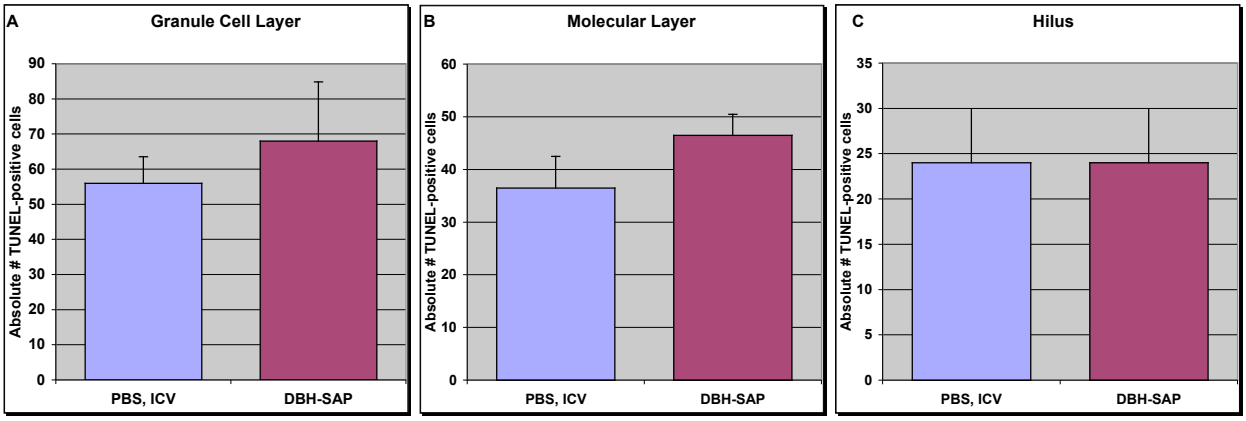


Figure 30

Graphs depicting cell death in the hippocampus after noradrenergic denervation. No changes in the granule cell layer (A), the molecular layer (B) or hilus (C) were observed.

Increased Periglomerular Neurogenesis in Olfactory Bulb after Noradrenergic Denervation

Two neuronal cell types are generated in the adult olfactory bulb, granule cells and periglomerular neurons (see Figure 1 on page 11). Therefore the analysis of the olfactory bulb was subdivided into the granule cell layer (GCL) and the periglomerular layer (PGL) in order to assess whether these regions are affected differently by noradrenergic denervation. Within the GCL, five weeks after the lesion, there was no significant alteration in the number of newly generated neurons or new non-neuronal cells (Figure 31A).

In contrast to the GCL, the number of newborn neurons in the PGL was significantly increased (108%, $p<0.01$), whereas the number of non-neuronal cells was not altered (Figure 31B).

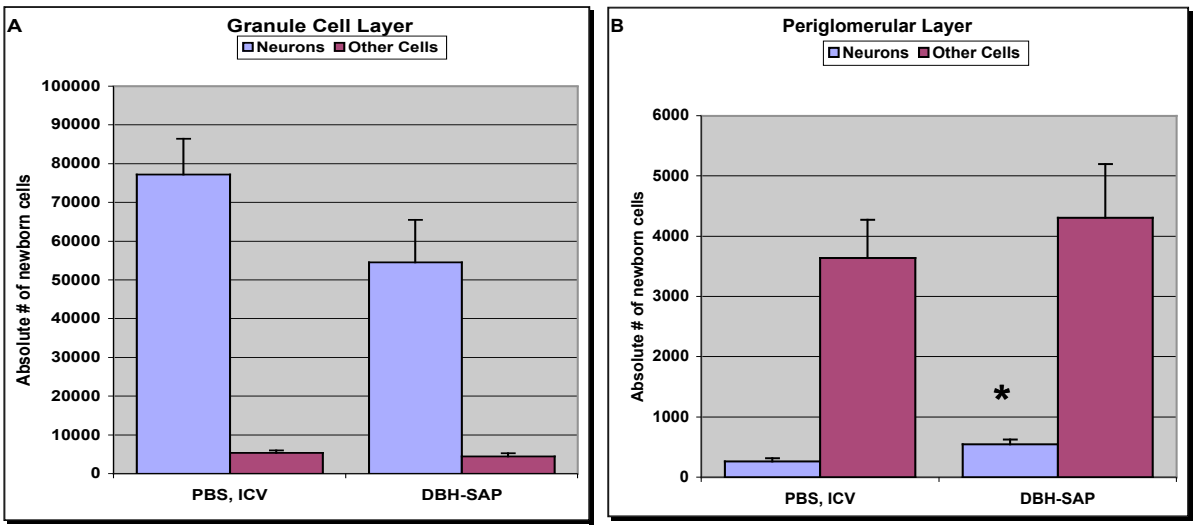


Figure 31
(A) Noradrenergic denervation causes no alteration in the number of newborn neuronal (blue) or non-neuronal (red) cells within the granule cell layer of the olfactory bulb. **(B)** Noradrenergic denervation causes an increase in the number of neuronal cells (blue) within the periglomerular layer of the olfactory bulb, while leaving the newborn non-neuronal (red) population unchanged.

Noradrenergic Denervation causes a Decrease in Cell Death in the Olfactory Granule Cell Layer

As previously shown that in the adult rat, the highest number of TUNEL-positive cells in the brain are found within the olfactory bulb, primarily within the GCL[28]. A 42% decrease was detected in the number of TUNEL-positive cells ($p<.05$) in the GCL of the lesioned animals (Figure 32A), whereas the rate of apoptosis in the PGL was not altered (Figure 32B).

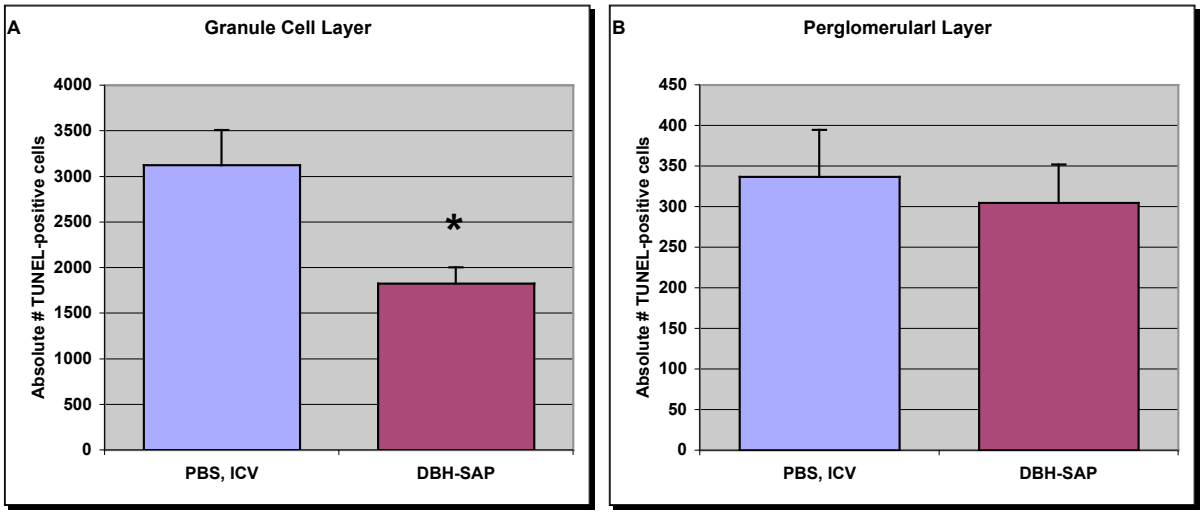


Figure 32
Noradrenergic denervation has no effect on cell death in the periglomerular layer of the olfactory bulb (A); however, cell death is decreased within the granule cell layer after lesion (B).

DISCUSSION

The major noradrenergic innervation into the dentate gyrus occurs along the border between the GCL and the hilus, the so-called subgranular zone. This innervation pattern corresponds with the reduction in neurogenesis that was observed after noradrenergic denervation. Lesion of the noradrenergic neurons residing in the locus ceruleus with DBH-SAP leads to a 35% decrease in hippocampal neurogenesis. Therefore, it can be assumed that normal noradrenergic functioning may play a stimulatory role in hippocampal neurogenesis. However, as with the cholinergic lesions, it cannot be determined through this experimental design whether norepinephrine acts directly on neuronal progenitors or whether the effects are due to indirect pathways.

In addition to the loss of neurons within the dentate gyrus, there was also a decreased generation of non-neuronal cells, such as astrocytes, within the GCL. Since several studies have indicated that the subgranular zone harbors multipotent progenitor cells, it is possible that the loss of noradrenergic input affects this cell population, subsequently leading to an overall reduced cytogenesis of neurons and glia. However, one cannot rule out the possibility that separate effects of noradrenergic deficiency on individual lineage-restricted progenitor populations occurred.

In the olfactory bulb, there is a strong noradrenergic projection to the GCL, mitral layers and plexiform layers from the LC, and this innervation is almost 10 times greater than in the cerebral cortex[278]. However, there were no significant changes in the number of newly born neurons observed within the OB-GCL. Conversely, there is very little innervation into the periglomerular layer[279], whereas, lesions to the LC resulted in an 108% increase in the number of newly born neurons within the PGL. At this point there is not enough data to speculate on the reasons for the differential effects of noradrenergic denervation on olfactory neurogenesis.

A functional role of norepinephrine in neuronal development has been postulated. For example, norepinephrine is critical in the development of early olfactory preferences in infant rats[283], as well as in reproductive and maternal behaviors[284]. Moreover, the classical view is that released norepinephrine from mature sympathetic neurons and noradrenergic neurons of the LC is re-uptaken by the norepinephrine transporter (NET). But NET is also expressed in the young embryo in a wide range of neuronal and non-neuronal tissues suggesting that NET has additional functions during embryonic

development (for review see [285]) In the locus ceruleus, NET expression is stimulated by growth factors such as FGF-2, NT-3 and TGF-ß, and subsequently NET regulates differentiation of noradrenergic neurons in the peripheral nervous system and by promoting expression of tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH). In a neuroblastoma cell line, NE induced cell survival, while proliferation was inhibited[286]. Most importantly for this study, desipramine, a tricyclic antidepressant that blocks norepinephrine reuptake, has recently been reported to induce neurogenesis in the DG, mainly through a cell survival mechanism that involves activation of cAMP signaling (for review see [287]). However, similar to the data presented here, the SVZ and OB system were unaffected. It is therefore likely that one of the functions of norepinephrine is specifically regulating the birth of new granular neurons from progenitor cells in the subgranular zone of the hippocampus.

The reduction of newly born PGL neurons could be due to a chain reaction, since the mitral cells within the GCL make their dendritic connections with the dendrites of the PGL neurons. In theory, since the PGL neurons are the first-line connections to the olfactory nerve layer and are the “first responders” within the OB to olfactory stimulation, it could be possible that when norepinephrine innervation is missing within the GCL, the PGL neurons are the ones to suffer, because they may not be able to make their proper axonal connections.

Experiment V – Vascular endothelial growth factor induces neurogenesis in the adult brain

(In submission and in collaboration with Anne Schänzer, Till Acker, Heike Beck, Karl-H. Plate and H. Georg Kuhn.)

Objective

Previous studies have demonstrated that a multitude of factors, including hypoxia/ischemia, are strong activators of neurogenesis in the adult mammalian CNS. Hypoxia, the final condition of several neurological diseases, is a critical stimulus for neurogenesis, since reduced oxygen levels can promote survival, proliferation and differentiation of neuronal progenitor cells *in vitro*[288]. However, the underlying mechanisms of neurogenesis and its up-regulation by hypoxia are not fully understood. Vascular endothelial growth factor (VEGF), a key regulator of developmental, hypoxia-induced, and tumor-induced angiogenesis, is rapidly up-regulated in response to hypoxia[289-291]. The goals of this experiment were to determine whether VEGF is involved in the regulation of adult neurogenesis by analyzing neurogenesis in the hippocampus and OB after infusion of recombinant VEGF into the LV of adult rats.

Introduction to Experiment

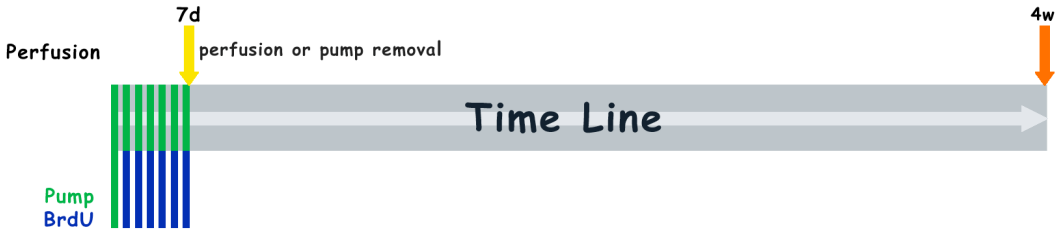
It is speculated that apart from its well-characterized effects on the vascular system, recent studies suggest that VEGF may act directly on neuronal cells. In the adult brain, VEGF is expressed in neurons and glial cells and under normal conditions, highest mRNA levels were found in the OB and DG[291], the two regions of adult neurogenesis. Under hypoxia, cortical neurons and microglial cells express elevated levels of VEGF, while activation of its receptor Flk-1 (fetal liver kinase-1, otherwise known as VEGFR-2) is increased on neuronal cells[292, 293]. VEGF is capable of protecting cells isolated from the central and peripheral nervous system from apoptosis, as well as promoting cell survival *in vitro*[294-296]. Mice, in which the hypoxia response element has been deleted from the VEGF promoter, fail to up-regulate VEGF under hypoxia and develop motor neuron degeneration in the spinal cord[297]. These data suggest a role for VEGF as a neurotrophic factor for mature neurons. However, Flk-1 expression has been

described on progenitor cells from the fetal retinal and cortex[298, 299] as well as on hematopoietic stem cells[300], suggesting that VEGF may also be involved in the regulation of neural progenitors. The hypothesis was, therefore, that VEGF-signaling might play a role in the generation of new neurons from neural progenitor cells in the adult brain.

Studies from our laboratory, in collaboration with Prof. Plate in Frankfurt, have shown that through the use of a specific antibody against VEGFR-2, a strong and uniform staining of the ependymal cell layer throughout the LV is revealed. Ependymal cells lining the lateral and medial side of the LV, demonstrated an intense, cell membrane-specific staining. In addition, VEGFR-2 expression could be observed in endothelial cells of medium-sized vessels as well as in neuronal cells, although at lower levels. Ependymal cells did not express other VEGF receptors, such as VEGFR-1 and neuropilin-1; however, neuropilin-1 expression was clearly detectable in cells residing in the SVZ and the RMS.

The choroid plexus plays an important role in supporting neuronal function by secreting growth factors such as FGF-2, IGF-1, and TGF-ß into the cerebrospinal fluid[301]. High-levels of VEGF mRNA and protein expression were observed in epithelial cells of the choroid plexus when analyzed by *in situ* hybridization and immunohistochemistry. In contrast, little or no VEGF mRNA could be detected in ependymal cells. Previous reports on VEGF expression in the choroids plexus suggested a local autocrine function in CSF production by altering the permeability of the blood-liquor-barrier[301]; however, using ELISA, VEGF levels in the cerebrospinal fluid of adult rats suggested an additional paracrine function for the brain. VEGF protein was also detectable at moderate levels preferentially on the luminal side of the ependymal layer; however, little or no VEGF mRNA could be observed in ependymal cells, suggesting that VEGF is not produced by ependymal cells but rather bound to the receptor. Although other cell types in the CNS, like neurons and astrocytes, are able to produce VEGF during development or under stress conditions, such as hypoxia, our data suggest that VEGF secreted into the cerebrospinal fluid by the choroid plexus epithelium could be one of the primary sources of VEGF for Flk-1 expressing cells in the LV wall of the intact adult brain.

Experimental Design



For specific details on Experimental Design, see Materials and Methods on page 107.

RESULTS AND DISCUSSION

VEGF stimulates olfactory neurogenesis.

The presence of Flk-1 in the LV and the production and release of VEGF from the choroids plexus prompted us to examine whether VEGF infusion *in vivo* could lead to a higher number of new neurons generated in the SVZ/OB system. (For detailed materials and methods, please see Methods Section). Immediately after seven days of growth factor infusion, no significant difference in the number of BrdU-positive cells in the LV wall could be detected between VEGF-infused (19573 +/- 1987 BrdU-positive cells, mean +/- standard error) and aCSF-infused animals (25334 +/- 2195, P>0.075, *t*-test). This implies that VEGF had little or no influence on the proliferative activity of neural progenitor cells in the adult LV wall. At 4 weeks post-infusion, however, a highly significant difference between the two groups was observed. Namely, VEGF-infused rats had 80% more BrdU-labeled cells in the OB (aCSF: 36249 +/- 3312 vs. VEGF: 58630 +/- 4094, P<0.0005, *t*-test).

Triple labeling for BrdU, NeuN and S100□, in combination with confocal microscopy, was performed in order to determine the differentiated phenotype of the newly generated cells (Figure 33). The relative proportion of neurons among the newborn cells in the OB did not differ between the groups: 93% of the BrdU-positive cells were NeuN-positive in aCSF-controls compared to 96% in VEGF-infused rats, indicating a largely unchanged neuronal differentiation. On average, 56,000 +/- 3800 new neurons per OB were generated in VEGF treated animals, compared with 34,000 +/- 3080 new neurons in aCSF controls (P<0.0001, *t*-test) – more than a 60% increase -

leading to the conclusion that VEGF is a potent stimulator of neurogenesis in the adult brain.

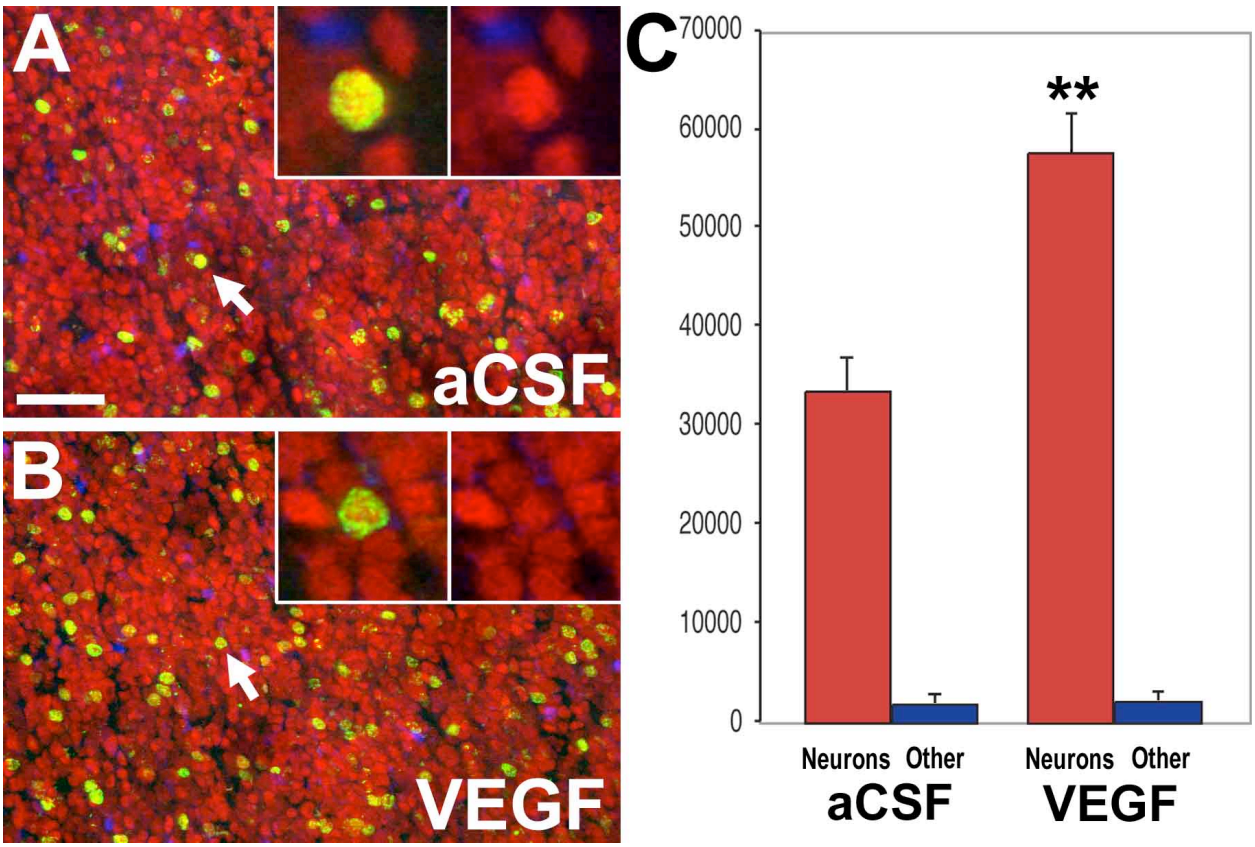


Figure 33
Increased neurogenesis in the olfactory bulb of (B) adult VEGF-infused animals compared to (A) aCSF-infused controls. Confocal analysis was used to determine the percentage of neurons (NeuN-positive cells - red) among the population of BrdU-positive cells (green) 4 weeks after growth factor infusion/BrdU labeling (double-labeling appears yellow). C, The total number of newly generated neurons was estimated by combining the stereologically assessed number of BrdU-positive cells with the percentage of neurons among the BrdU-positive cells and is represented as group means +/- standard error. (**) indicates statistically significant differences at the 1% level (*t*-test). Scale bar in A, 50 µm.

VEGF stimulates hippocampal neurogenesis.

VEGFR-2 has previously been localized within the proliferating clusters of hippocampal progenitor cells[302]. The next step in the experiments was then to determine a possible effect of VEGF on hippocampal neurogenesis. Immediately after infusion, proliferation of hippocampal progenitor cells was not affected by VEGF (aCSF: 2235 +/- 266 BrdU-positive cells vs. VEGF: 1944 +/- 185, P>0.38, *t*-test). However, as depicted in Figure 34, VEGF-infusion stimulated the generation of approximately 60% new neurons (aCSF: 1024 +/- 97 BrdU/NeuN-positive cells vs. VEGF: 1687 +/- 129, P< 0.001, *t*-test). Since similar data in the DG and in the LV/OB system was observed, it

was concluded that VEGF infusion promotes adult neurogenesis by exerting a survival effect on neuronal progenitor cells.

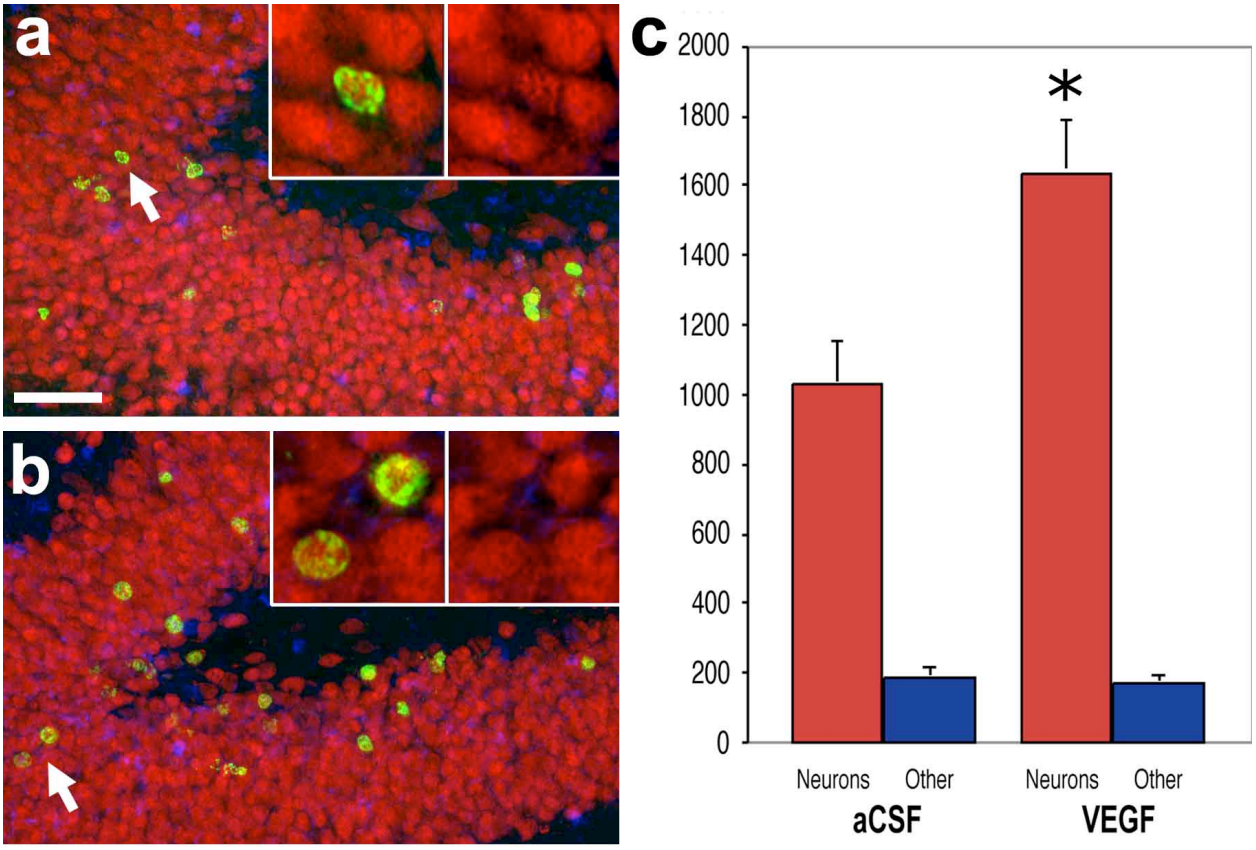


Figure 34
Increased neurogenesis in the dentate gyrus of (B) adult VEGF-infused animals compared to (A) aCSF-infused controls. Confocal analysis was used to determine the percentage of neurons (NeuN-positive cells - red) among the population of BrdU-positive cells (green) 4 weeks after growth factor infusion/BrdU labeling (double-labeling appears yellow). C, The total number of newly generated neurons was estimated by combining the stereologically assessed number of BrdU-positive cells with the percentage of neurons among the BrdU-positive cells and is represented as group means +/- standard error. (**) indicates statistically significant differences at the 1% level (*t*-test). Scale bar in A, 50 µm.

VEGF decreases cell death in SVZ/OB and hippocampus

Increased neurogenesis in the absence of significant changes in neural progenitor proliferation under VEGF stimulation suggests that VEGF has a survival-promoting effect on progenitor cells and immature neurons in the adult brain. Our group has previously shown in the adult rat brain that programmed cell death is specifically localized to the areas of neurogenesis and counterbalances the proliferative activity of neural progenitor cells in the LV wall[9, 28]. When apoptotic cell death was analyzed in the LV wall/OB system of VEGF-treated animals, TUNEL labeling in the LV/OB system of VEGF-treated animals revealed that cell death was significantly decreased immediately

after infusion, whereas 4 weeks after VEGF infusion no significant difference was observed. Within the hippocampal DG, the number of apoptotic cells was also significantly reduced by VEGF to about 50% of the control level (aCSF: 69.5 +/- 9.9 TUNEL-positive cells vs. VEGF: 35.0 +/- 6.3, P<0.005, *t*-test). Therefore, the increased neurogenesis under VEGF is very likely due to decreased apoptotic elimination of progenitor cells and immature neurons. These data support the hypothesis that programmed cell death is an important mechanism for the regulation of adult neurogenesis and that VEGF plays an active role in preventing apoptotic cell death.

VEGF does not stimulate proliferation of glial cells.

Since VEGF is known to stimulate proliferation of other cell types, such as endothelial cells, the non-neuronal fraction of newborn cells in the OB was evaluated, however no differences were observed (aCSF: 2396 +/- 188 cells vs. VEGF: 2478 +/- 156, P>0.8, *t*-test, see also Figure 33C). Similarly, the number of non-neuronal BrdU-positive cells in the DG GCL were not altered (aCSF: 209 +/- 44 cells vs. VEGF: 182 +/- 34, p>0.6, *t*-test). Analysis of regions, such as the molecular layer of the DG where neurogenesis does not occur but rather genesis of endothelial and glial cells, also revealed no changes in the number of BrdU-positive cells (aCSF: 682 +/- 75 cells vs. VEGF: 686 +/- 124, P<0.49, *t*-test), suggesting a specific effect of VEGF on the neuronal progenitor populations in the adult brain. Thus, VEGF did not induce endothelial cell proliferation in the brain.

VEGF does not induce neovascularization of the ventricle wall or the dentate gyrus.

These data demonstrate an effect of VEGF on neurogenesis in the adult brain, however VEGF is most prominently known for its effects on the vascular system. In addition, vascular VEGF mediates the testosterone-induced stimulation of neurogenesis in adult songbirds by release of BDNF (brain-derived neurotrophic factor) from the vasculature[303]. In order to verify whether ICV infusion of VEGF into the rat brain exerted its effect on neurogenesis indirectly through signaling from vascular cells, it was determined whether endothelial proliferation or vascular permeability were altered. At the dosage used, VEGF did not induce neovascularization adjacent to the LV or in the

DG as determined by quantitative immunohistochemistry against the rat endothelial cell marker, RECA. Moreover, when the dividing non-neuronal cells in the neurogenic regions were analyzed, which consisted mostly of glial and endothelial cells[302], no differences between VEGF and aCSF-treated animals were observed. Secondly, Evans blue dye was injected intravenously into a separate set of animals on the last day of intraventricular VEGF infusion and no leakage in the blood-brain-barrier of VEGF-treated animals was detected compared to controls. Moreover, a change in brain volume was not observed, which would imply enhanced vascular permeability. These observations, together with expression patterns of VEGF and Flk-1 strongly suggest that VEGF affects neurogenesis rather directly than indirectly via the vascular system.

A close interaction of the vascular system and neurogenic activity in the adult brain has recently been suggested[302, 303], however an indirect effect of VEGF by increasing vascular permeability was proposed. The fact that VEGF can regulate neurogenesis in the adult nervous system, and that neural progenitor cells directly respond to VEGF via activation of the VEGF receptor Flk-1, adds an unexpected novel dimension to angiogenic/neurogenic interactions. Moreover, the production and release of VEGF from the choroids plexus indicates a possible new route of controlling neurogenic activity in the brain.

Experiment VI – Transient expression of doublecortin during adult neurogenesis

(Accepted by J. Comp. Neurol. in collaboration with Jason Brown, Sebastien Coulliard-Despres, Jürgen Winkler, Ludwig Aigner, H. Georg Kuhn.)

Objective

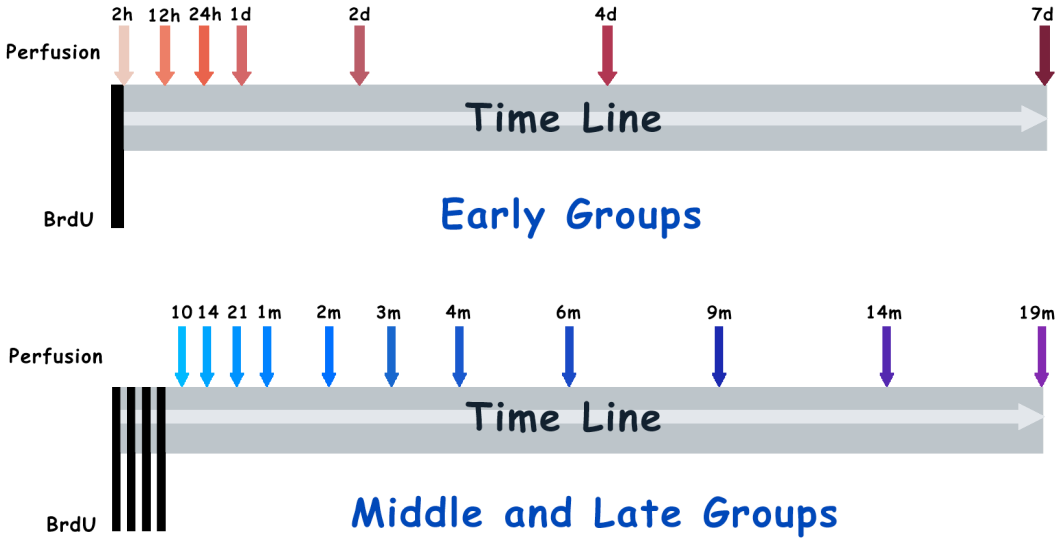
During the development of the CNS, the microtubule binding protein doublecortin (DCX) is associated with migration of neuroblasts. Besides this developmental role, expression of DCX remains high within certain areas of the adult mammalian brain. These areas, mainly the DG and the LV wall in conjunction with the RMS and OB, also retain the capacity to generate new neurons into adulthood. Adult neurogenesis is typically detected by incorporation of bromodeoxyuridine (BrdU) into dividing cells and co-labeling of BrdU-positive cells with markers for mature neurons. In order to elucidate whether DCX could act as an indicator for adult neurogenesis and possible replace the use of BrdU, the temporal expression pattern of DCX in neurogenic regions of the adult brain was investigated. The purpose of this study was 1) to elucidate the spatial-temporal pattern of DCX expression within the neurogenic regions of the adult rat CNS, (2) to determine the onset of DCX expression in newly generated precursor cells, (3) to ascertain the duration of the DCX expression during the neuronal maturation process, and (4) since a decline in adult hippocampal neurogenesis is known to occur in aged animals [19], changes in DCX expression with aging was also investigated.

Introduction to Experiment

In order to detect neurogenesis, techniques such as incorporation of thymidine-analogs or retroviral labeling have been used to track dividing cells and their cell fate within the CNS. However, these methods have their limitations in the detection of neurogenesis as described in Experiment I. The difficulties associated with these methods call for new, specific and quantifiable indicators of neurogenesis. Expression of such a marker should ideally be induced in the neuronal progenitor population in order to detect the new neurons while being generated, and later downregulated in mature neurons.

DCX could be such a candidate marker for adult neurogenesis. First detected at embryonic day 10.5, DCX is expressed at high levels in the developing rat CNS and in migrating neurons and is required for proper neuronal migration.[304-306]. Central to this study, DCX expression is retained within the areas of continuing neurogenesis in the adult brain[307]. The morphology of DCX-expressing cells is consistent with that of migrating neuroblasts. Moreover, many of these cells were co-labeled with PSA-NCAM, an antigen also present on migrating neuroblasts[308]. The *Dcx* gene was originally described in the context of human migration disorders[304, 309, 310]. In addition, mutant alleles of *Dcx* provoke a migratory impairment of neural progenitor cells and lead to lissencephaly or to a subcortical bandheterotopia, or so-called "double cortex syndrome"[304, 309] (for review see[311]).

Experimental Design



For specific details on Experimental Design, see Materials and Methods on page 108.

RESULTS

Doublecortin expression during adult hippocampal neurogenesis

Time course analysis. The pattern of DCX expression within newly generated cells was examined by immunofluorescence labeling of DCX and BrdU in the neurogenic regions of the adult rat brain at different time points after BrdU

administration. At the earliest time point analyzed, i.e. 2 hours post-BrdU administration, 60% of the newborn cells co-labeled for DCX and BrdU (Figure 35 and 36A).

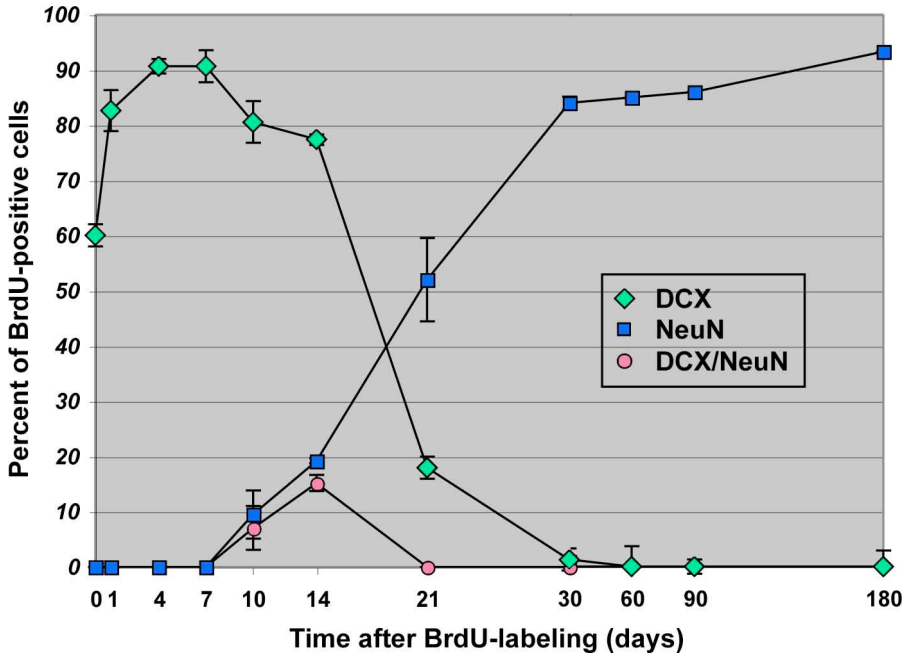


Figure 35
Quantification of BrdU-positive cells expressing DCX, NeuN, or both in the dentate gyrus. Note that the peak of BrdU/DCX labeling is around 4-7 days after BrdU labeling. BrdU/NeuN double labeling begins to increase after DCX expression has decreased. The time of BrdU/DCX/NeuN co-labeling coincides with DCX expression decreasing and NeuN expression increasing.

The fact that the majority of BrdU-positive cells were expressing DCX at the earliest time point strongly suggested that proliferating cells were already expressing DCX. To further substantiate this observation, frequent co-labeling of DCX with Ki67 was also demonstrated in the hippocampus and LV wall (Figure 37). Between the seventh and tenth day post-labeling, the percentage of BrdU-positive cells expressing DCX further increased to more than 90% (Figure 36C/D). Thereafter, DCX expression was rapidly downregulated. It was observed in only 2% of the BrdU-positive cells by one month and became undetectable by two months after labeling (Figure 36F).

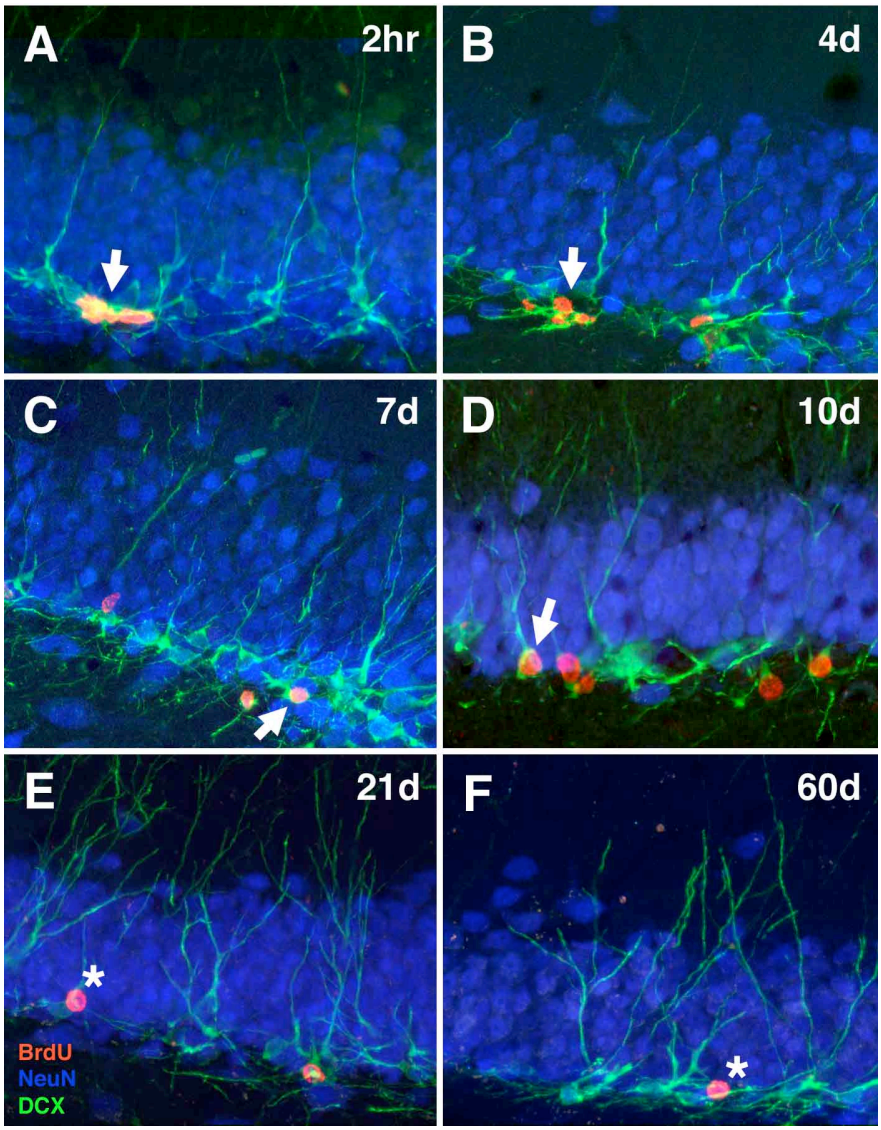


Figure 36
BrdU time course showing expression pattern of DCX and NeuN in the dentate gyrus. At 2 hrs after BrdU labeling, there are clumps of cells that are DCX-positive. By 7 to 10 days, the BrdU/DCX-positive cells have spread out along the subgranular layer and the cells begin to take on a different morphology. By 21 days after BrdU, the majority of the cells are no longer expressing DCX, but rather NeuN (starred cells in E and F)

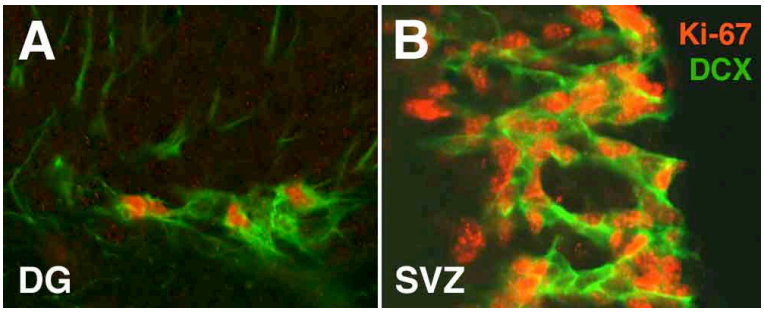


Figure 37
Co-labeling of DCX (green) with Ki67 (red) along the subgranular zone of the dentate gyrus (A) and subventricular zone of the lateral ventricle wall (B).

Towards the final stage of neuronal differentiation, the newborn cells begin to express proteins typically present in mature neurons such as the nuclear neuronal marker NeuN, neuronal-specific enolase (NSE), or calbindin[17-19]. For that reason, NeuN was used to determine when newly generated neuronal precursors become mature and to what extent their expression overlaps with the expression of DCX. BrdU-labeled cells immunoreactive for NeuN were first detected in the hippocampus at 10 days after BrdU injection (Figure 35). The majority of the NeuN positive cells co-expressed DCX between Day 10 and 14, thereafter NeuN/DCX co-labeling was not detectable anymore. The percentage of BrdU-positive cells expressing NeuN increased to about 80% one month after labeling and increased further to more than 90% at later time points analyzed (Figure 35 and Figure 36F). Adjacent non-neurogenic regions, the hilus and molecular layers of the DG, were analyzed as control areas. As expected, the co-localization of BrdU-positive cell bodies with DCX or NeuN was not detected within these regions (data not shown).

Morphology of DCX expressing cells. It is interesting to note that the morphology of DCX-expressing cells changed as neuroblasts matured (Figure 38). Two types of cellular morphologies were observed. Within the first days after BrdU-labeling, DCX-positive cells formed a cluster in the subgranular zone adjacent to the inner margin of the GCL. Some of these cells were without defined processes, whereas others resembled neuroblasts with processes oriented parallel to GCL (Figure 38A). Later, at about 10 days after BrdU labeling, DCX-positive cells were integrated into the GCL and displayed processes spanning the entire layer and further into the molecular layer (Figure 38B).

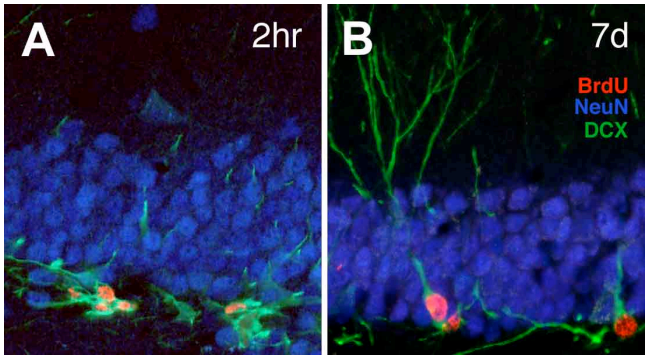


Figure 38
Morphology of DCX-positive cells changes over time. Two hours after BrdU-labeling, DCX co-labeled cells reside in clumps and assume a horizontal orientation (A). By 7 days, the cells have a more neuronal-like morphology, with dendritic processes orientating vertically into the molecular layer (B).

Doublecortin expression in the aging dentate gyrus.

An age-dependent decrease of neurogenesis within the GCL of the DG has previously been reported[19]. Consequently, the analysis of DCX expression was performed as a function of age. The highest incidence of DCX immunoreactivity was observed in the younger animals examined, i.e. 2 month old rats. The total amount of cells expressing DCX notably decreased by 11 months of age, and very few DCX positive cells were detectable in 21 month old rats (Figure 39). The reduction of DCX-expressing cells in the DG is consistent with and correlates with the reported age-dependent decrease in neurogenesis.

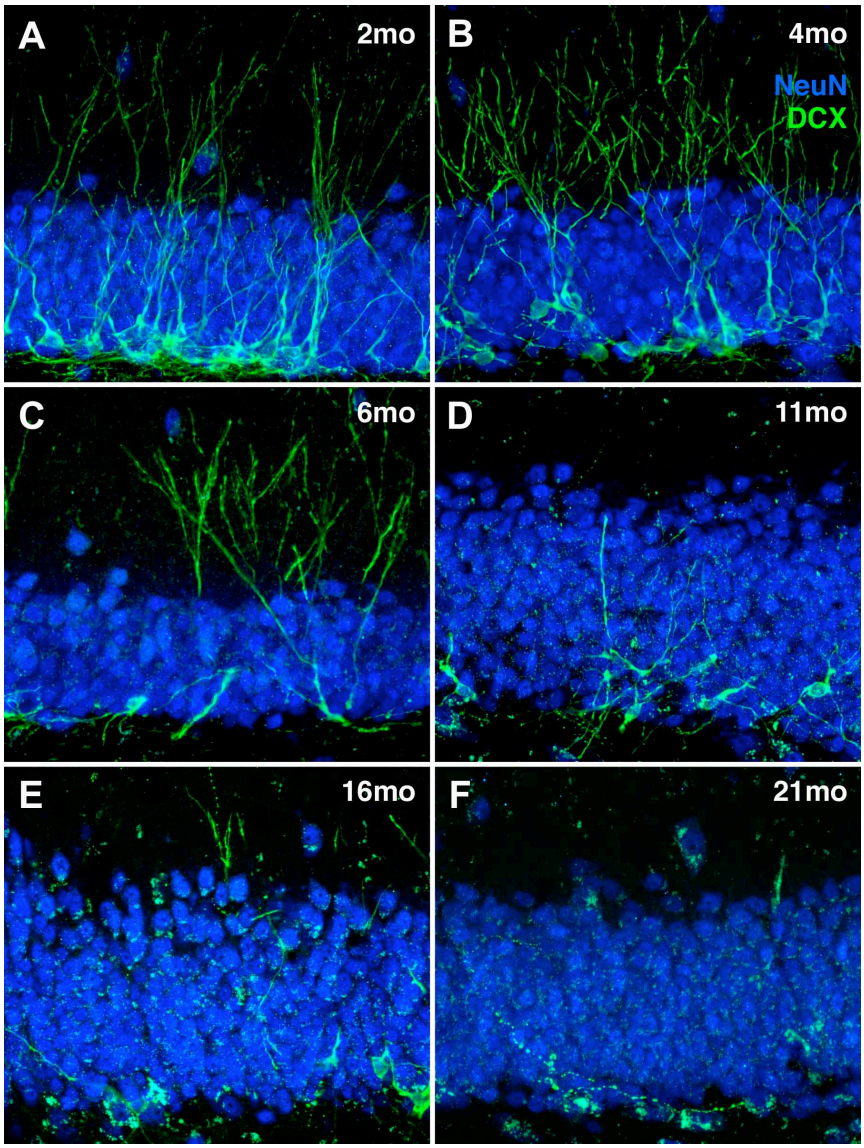


Figure 39
DCX expression in the aging dentate gyrus. The strongest labeling of DCX occurs in the younger animals (A and B), whereby over time DCX expression decreases and becomes almost non-existent in aged animals (F).

Doublecortin expression in the SVZ/OB system

Subventricular zone/rostral migratory stream.

DCX is already strongly expressed in the SVZ of the LV, where neuronal commitment already occurs, (Figure 40). Similar to the hippocampus, numerous cells in the SVZ were double-labeled for DCX and the proliferation marker Ki-67, indicating frequent cell division of neuroblasts (Figure 37). The morphology of the DCX-expressing cells in the SVZ is mostly bipolar with short processes (Figure 40A). The DCX-positive cells are organized in chain-like structures, which has been previously been described for migratory neuroblasts[312]. In the RMS, the DCX-expressing neuroblasts have a more elongated morphology consistent with cells migrating towards the OB (Figure 40B).

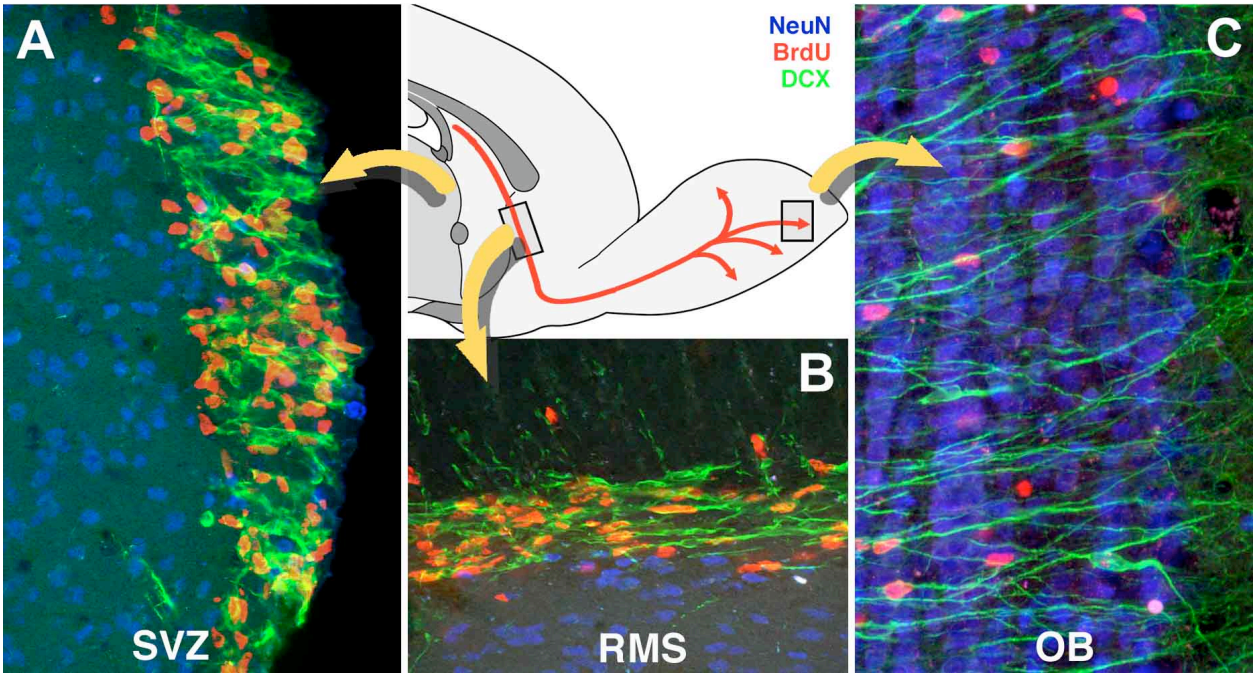


Figure 40
DCX expression and morphology in the subventricular zone, rostral migratory stream and olfactory bulb. In the SVZ, DCX-positive cells (green) have a neuroblasts-like morphology and form chain-like structures (A). Once they have moved into the RMS, they assume a more elongated morphology (B) within the olfactory bulb, they cells adopt a more complex morphology, similar to granular neurons of the OB (C).

Olfactory bulb.

Finally, in the OB the DCX-expressing cells adopt a more complex morphology, similar to the one observed for the cell integrating into the cell granular layer of the hippocampus (Figure 40C). Consequently, BrdU-labeled cells coming from the SVZ were first detected in the OB four days after BrdU injection. This interval reflects the time

required for neuroblasts to migrate from the SVZ, where they arise and incorporate BrdU, to the OB. At four days after labeling, only 2% of the BrdU-labeled cells in the OB were found to express DCX (Figure 41). At this time point, the percentage is relatively low since most to the BrdU-labeled cells observed in the OB result from *in situ* cell division of non-neuronal cells. As the bulk of newly generated neuroblasts from the SVZ reach the OB, this percentage increased rapidly. Ten days after labeling, approximately 70% of the BrdU-positive cells in the OB were expressing DCX (Figure 41 and Figure 42D). As in the hippocampus, the BrdU-positive cells expressing DCX within the OB decreased to undetectable levels by 1 month after labeling and remained undetectable throughout the subsequent time points (Figure 41 and Figure 42F). The induction of NeuN expression in newly generated neuroblasts integrating in the OB also appeared to have a kinetic similar to what is observed in the hippocampus. The co-localization of BrdU with NeuN was first detected at 14 days after BrdU injection. At this time, 24% of the BrdU-labeled cells expressed NeuN (Figure 41). The percentage of BrdU-labeled cells expressing NeuN increased to nearly 90% by 1 month and remained at this level in the later time points analyzed. Interestingly, in the SVZ/OB system, the co-expression of NeuN and DCX within neuroblasts was virtually absent. The reasons for this are still unclear and need to be definitively answered, but it could be explained by a different transient expression than in the DG. It could be possible that the olfactory granule neurons go through a differentiation phase where they no longer express DCX and haven't yet begun to express NeuN.

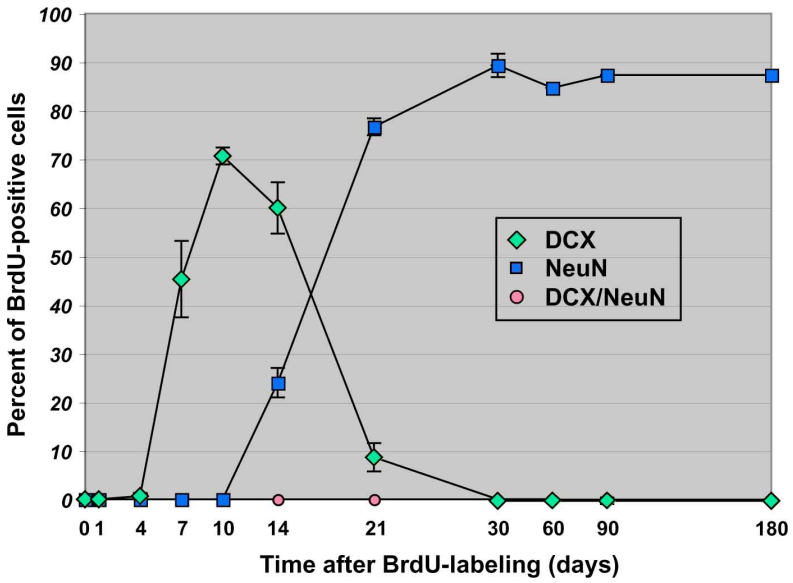


Figure 41
Quantification of BrdU-positive cells expressing DCX, NeuN, or both. Note that the peak of BrdU/DCX labeling is around 10 days. This time point is later in the OB than in the dentate gyrus, since the cells need more time to migrate to the OB granule cell layer. BrdU/NeuN double labeling begins to increase after DCX expression has decreased. Note that co-labeling of DCX and NeuN is virtually non-existent.

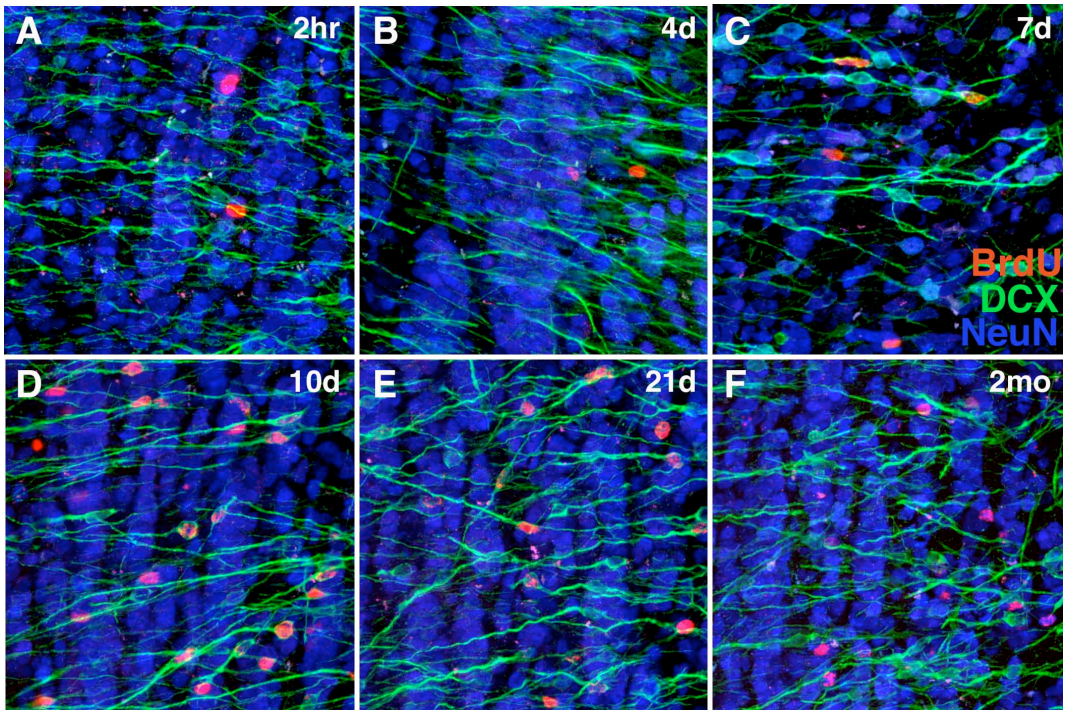


Figure 42
BrdU time course showing expression pattern of DCX and NeuN in the granule cell layer of the olfactory bulb. By 7 to 10 days after BrdU, there are DCX co-labeled cells detectable (C and D); however, by 21 days and 2 mos, the majority of BrdU-positive cells have become NeuN-positive as well (E and F).

Doublecortin expression in the aging olfactory bulb.

As with the hippocampus, OB neurogenesis has been shown to decrease with age[19]. And as with the hippocampus, a decrease in DCX expression in the aging rat was also observed. The highest incidence of DCX immunoreactivity was observed in younger animals, with the total amount of expression notably decreasing by 16 months of age (Figure 43A-E). By 21 months, DCX-positive cells were barely detectable (Figure 43F). The reduction of DCX-expressing cells in the OB GCL is consistent with and substantiates the reported age-dependent neurogenesis decrease.

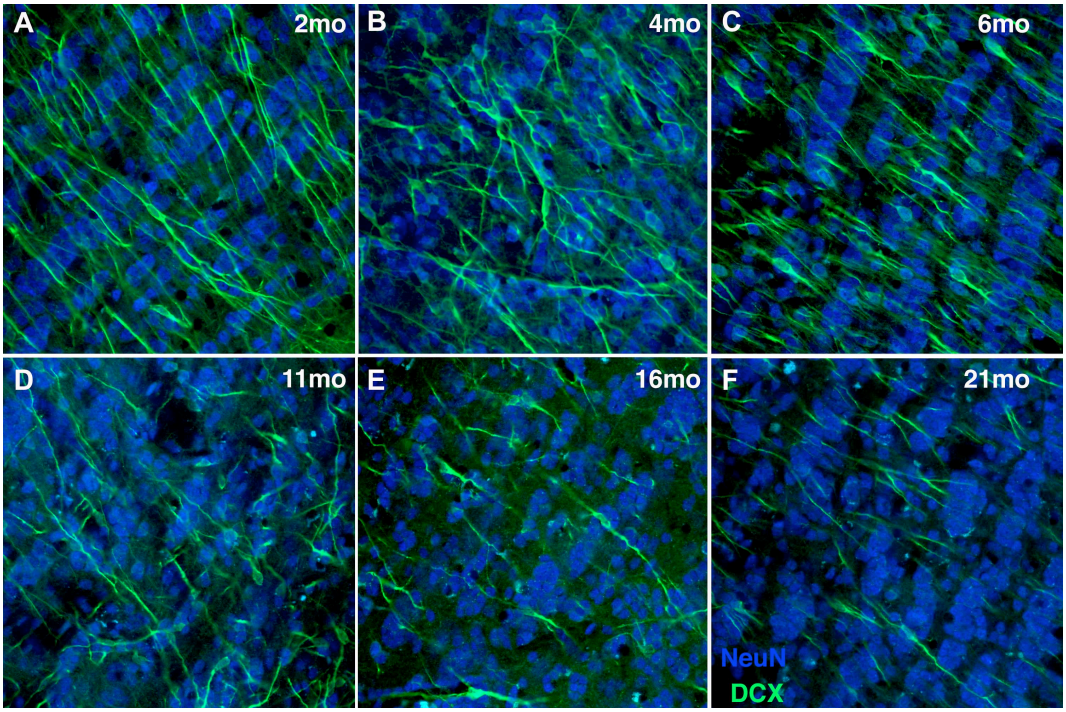


Figure 43
DCX expression (green) in the olfactory bulb granule cell layer decreases with age. DCX immunoreactivity is strong in the young adult rat (A-C); however, with increasing age, expression notably decreases (D and E) and is barely detected in the aged rat (F).

DISCUSSION

Expression of the nervous system specific protein DCX is associated with neuronal migration during development of the nervous system and in the adult[304-306]. DCX is expressed at very high levels in the prenatal brain and can be detected within most of the cells in the embryonic intermediate zone, as well as in the cortical plate, but not in proliferative regions, like the embryonic ventricular zone[305, 306]. Within these regions, DCX is expressed by migrating neuronal precursors, both during radial and tangential

migration[305, 306, 310]. In the adult mammalian CNS, DCX expression is very low. However, in some regions including the two neurogenic areas of the adult brain, the SVZ/RMS/OB axis and the DG of the hippocampal formation, DCX expressing cells are observed[299, 307]. In addition, DCX expression is up-regulated by conditions that induce or promote adult neurogenesis and localizes in migrating neuronal precursors[141, 304].

Analysis of DCX in newly generated cells showed that DCX is transiently expressed in proliferating progenitor cells and newly generated neuroblasts. When the newly generated cells begin expressing mature neuronal markers, DCX immunoreactivity is sharply decreased below the level of detection and remained undetectable thereafter. The transient expression pattern of DCX in neuronally committed progenitor cells/neuroblasts indicates that DCX is a suitable marker for adult neurogenesis and may provide an alternative to BrdU labeling. This assumption is further substantiated by our observation that the amount of cells expressing DCX is decreased with age, which coincides with the previously reported reduction of neurogenesis in the aging DG.

DCX expression in adult neural precursor cells?

Due to the continuous generation of new neurons in the adult subgranular zone of the hippocampus and in the SVZ of the LV/RMS/OB axis, active neural progenitor cells are postulated in these regions. The identity of neural progenitor cells is still under debate. Detailed electron microscopy studies by Alvarez-Buylla and coworkers, however, characterized the different cell types in the SVZ, and according to the current hypothesis, neural progenitor cells of the adult SVZ are slowly dividing cells, most likely specialized glial cells[137, 313] giving rise to fast dividing precursor cells that enter the RMS and migrate towards the OB. DCX was found to be expressed in the majority of BrdU positive cells in the hippocampus 2h after BrdU labeling and was frequently co-labeled with the proliferation marker, Ki-67, in the hippocampus and SVZ. It is, therefore, an intriguing idea that DCX might be expressed in neural progenitor cells. Neither the BrdU labeling paradigm used in this study, nor the Ki-67 staining can discriminate between the two possibilities. In some cells of the adult brain, DCX is found to be co-expressed with nestin, a marker for immature progenitor cells[307]. Similarly, DCX co-localizes with neuronal precursors migrating along radial glial cells in the developing cortex and with

TuJI-expressing cells in the adult rodent RMS[306]. Therefore, the present data support DCX as a marker for neuronal precursor cells. Interestingly, there is apparently a difference of DCX expression in proliferative regions of the embryonic and the adult brain. DCX is not detected in the embryonic ventricular zone during cortical development[306], but it is expressed in proliferating cells of the adult hippocampus and SVZ. This could mean that fetal and adult neural progenitor cells have different expression patterns and properties.

DCX expression in mature neurons?

DCX is not exclusively expressed in immature neurons, but also found intermittently in neurons with differentiated morphology within non-neurogenic regions, including cortex, striatum and corpus callosum[307]. In addition, DCX labels some hippocampal mossy fibers into the CA3 region[307]. In this experiment, DCX expression is observed in 2% of BrdU labeled cells in the hippocampal DG one month after BrdU labeling. It is not unlikely, that these cells have already acquired a mature morphology and grown their axon towards CA3, but still express DCX. The characteristic features of mature neurons that express DCX are not known. It might be, that these comprise neurons associated with higher levels of structural plasticity. At least in the cortex and in hippocampal mossy fibers, structural plasticity and sprouting is a well-documented phenomenon. Structural plasticity, like axonal sprouting and dendritic rearrangement, and neuronal precursor cell migration are regulated by the same underlying cellular mechanisms resulting in re-modeling of the neuronal cytoskeleton. Since DCX is a microtubule-associated protein that binds, bundles and stabilizes microtubules, DCX could be integrated into various neuronal cytoskeleton-depending scenarios including migration of neuronal precursor cells, nuclear translocation, and axonal and dendritic maturation. LIS1, a protein functional similar to DCX and associated with neuronal migration, has been shown to regulate neuroblast proliferation, nuclear translocation and positioning, dendritic elaboration and axonal transport in drosophila[314, 315]. Similar activities for DCX, depending on the cellular background in which it is expressed, could be postulated.

DCX, a transient marker for neuronal committed precursor cells.

DCX was originally described to be associated specifically with migrating neurons during development and in the adult; however, the present data support DCX as a transient marker for neuronal committed precursor cells. As the number of BrdU-labeled cells that express DCX starts to decline, labeled cells expressing NeuN can be detected. One could argue that DCX-expressing cells have a short half-life and that these NeuN-positive cells originate from a different population of BrdU-labeled cells that required a period of time to mature. Two aspects, however, argue against this hypothesis. Firstly, 90% of the BrdU-labeled cells in the hippocampus expressed DCX ten days after BrdU incorporation. Although the number of BrdU-labeled cells diminishes with time, only about 25% of the BrdU are lost between the tenth days and the first month after BrdU administration[9]. Therefore, the death of some BrdU-labeled cells is clearly insufficient to explain the loss of DCX-expressing cells with time. Secondly, as the number of DCX-expressing cells declines, the number of NeuN-expressing cells, in contrast, increases among the BrdU-labeled cells. During this period of transition, BrdU-positive cells that co-expressed DCX concomitantly with NeuN were observed. This demonstrated that the populations of DCX- and NeuN-expressing cells originate from the same precursors, but are at different stages of neuronal maturation. The low frequency of co-expression of these two neuronal proteins within the same cell reveals the short period of overlapping expression.

In the hippocampus, where migration is only a minor event, DCX is expressed in neuronal precursors for the same amount of time (up to one month) as it is expressed in neuronal precursors in the SVZ/OB system, where neuronal precursors migrate long distances. In the hippocampus, neuronal precursors migrate only a very short distance from the subgranular zone into the granular layer of the DG. The more striking event is a rearrangement of cellular processes, the elaboration of dendritic and axonal processes, and a general change of cell morphology towards a mature neuron. Similar events occur during structural plasticity of mature neurons, where synaptic refinement and collateral sprouting play major roles. The onset of neuronal maturity of neuronal precursor cells in the SVZ/OB and hippocampus, using NeuN as a marker, is roughly the same, i.e. about three to four weeks.

In this experiment, DCX is specifically expressed and only detected in neuroblasts within the first month after their emergence. This observation was made in two independent sites of adult neurogenesis, the hippocampal formation and the SVZ/OB system. The temporal expression pattern and specificity of DCX within the adult neurogenic regions therefore suggest that DCX can be a useful marker for neurogenesis. Recent studies have begun to employ DCX as a marker for new neurons in the classical neurogenic regions, but also in the cortex[141], striatum[316] and the CA region of the hippocampus[317].

CONCLUDING DISCUSSION

The present dissertation aimed at finding molecular signals that define the microenvironment of neural progenitor cells and that may regulate neurogenesis in the adult brain. The focus was on the analysis of three separate classes of molecules: *transcription factors, growth factors, and neurotransmitters* and their possible function during adult neurogenesis. In general it can be concluded that altering the availability of these factors can modulate neurogenesis.

Transcription factors were chosen, because most extracellular signals, such as growth factors, neurotrophic factors and cytokines, act through extracellular receptors which signal to the nucleus to change gene transcription. A key transcription factor for the control of proliferation is E2F, which is essential in promoting the transition from G1 to the S-phase of the cell cycle. This experiment was able to show that E2F1, the most prominently expressed E2F family member in the brain, is involved in postnatal and adult neurogenesis, because E2F1 deficient mice had significantly reduced neuronal progenitor proliferation. The use of a knockout strategy to analyze adult neurogenesis was only possible, because other E2F family members apparently compensate for the lack of E2F1 during embryonic brain development. The involvement of a key transcription factor for cell cycle control in adult neurogenesis is not a surprise, since all new neurons are generated from proliferating progenitor cells; however it was important to find out (i) whether the most prominently expressed E2F family member (E2F1) is involved and (ii) whether decreased proliferation is automatically followed by reduced neurogenesis. This was the case; however, the effect on neurogenesis would have been even greater had E2F1 deficient animals not displayed a reduced apoptotic elimination as well, suggesting that downregulation of apoptosis may have played a role in downplaying the effects of decreased proliferation.

With respect to other cell cycle regulators, it is important to note that p27 is a signaling molecule that acts upstream from E2F by inhibiting cell cycle progression. A recent study, using p27 knockout mice, has shown that these mice have an increased pool of neural progenitor cells and a higher number of neurons being generated in the

adult brain[318]. Therefore, p27 knockout data on neurogenesis are in agreement with the E2F data presented here.

Neurotransmitters are some of the most prominent molecules that define the extracellular milieu of neurons in the brain. Although their roles for communication between mature neurons are mainly studied, these molecules are also effective in controlling cell fate of immature cells (for review see[210]). The general conclusion of Experiments III & IV is that neurotransmitters can alter the microenvironment of neural progenitor cells, whether directly or indirectly, and that these changes lead to significant alterations in neurogenesis. In principle, the data suggest that acetylcholine and noradrenaline are stimulatory to adult hippocampal neurogenesis, since neurotoxin lesions specific to these neurotransmitter systems lead to a reduced number of new neurons and serotonin is inhibitory, since an increase in neurogenesis is observed after neurotoxin lesion to this system.

The cholinergic lesion data are the most interesting since elimination of neurons in the CBF produces not only less neurons but is also known for producing memory impairment. In addition, neurodegeneration of the cholinergic neurons in the basal forebrain is one of the hallmarks of the pathology in Alzheimer's disease patients. Whether reduced neurogenesis may contribute to the pathology and/or symptoms of neurodegenerative diseases, such as Alzheimer's, cannot be determined from these first correlative evidences, but will need further investigations. It is, however, an intriguing concept that altered levels of neurogenesis could be directly involved in neurodegenerative disorders.

Growth factors are, by definition, molecules that stimulate proliferation of certain cell populations. For that reason, growth factors appear to be an ideal target to stimulate the proliferative activity of neural progenitor cells. Previous studies from our laboratory, however, have shown that FGF-2 and EGF, two potent *in vitro* stimulators of neural progenitor cell proliferation, have quite different effects *in vivo*[18]. Whereas FGF-2 increased neurogenesis in the OB after intraventricular infusion, EGF reduced neurogenesis and concomitantly increased gliogenesis, probably due to altering the fate of the multipotent neural progenitor cells. Experiment V involved the testing of **VEGF**, a growth factor for a variety of cells, and especially vascular endothelial cells. Again a

different result was observed than expected: contrary to other growth factors, VEGF did not increase the proliferation of progenitor cells *in vivo*, but rather the selective survival of neuronally-committed progenitor cells, resulting in a strong increase in neurogenesis in the OB and DG. This trophic effect was confirmed by the fact that apoptotic cell death was reduced in the neurogenic regions after VEGF treatment. Therefore, it is possible to conclude that in this system VEGF functions not as a growth factor in the strict sense, but rather as a trophic or survival factor. And in this regard, VEGF acts very similar to BDNF, which also has been shown to induce neurogenesis in the adult rodent brain[319-321].

Neurogenesis: A balance of proliferation and apoptosis

The findings from this dissertation highlight the important fact that adult neurogenesis depends not only on progenitor proliferation but also on apoptotic elimination. The number of new neurons being generated at a certain time point therefore depends not only on the number dividing cells but also on the balance between cell production and cell elimination. Adult neurogenesis mimics some of the processes that occur during embryonic development, where approximately half of the neurons produced are eliminated in order for the regions of the brain to ultimately function properly[322]. This shows the close relationship between cell birth and cell death and the intricacies that must be involved. But, the important point to understand, and which nature illustrates to us in such elegance, is that this balance exists throughout development and even further into adulthood and that it works. In order to experimentally control neurogenesis, the goal is not to just increase the proliferation of progenitor cells, because if the balance gets out of control, then an unwanted tumor could be the possible result. This balance is regulated by a number of factors, genes and global stimuli, and a large body of recently published work helps to understand the elements that are responsible (see Table in Appendix). The experiments described in this dissertation also show the variability of this system and how genes, growth factors and neurotransmitters can regulate this balance in very different ways (Table 3).

Table 3

	Method of Intervention	Neurogenesis	Cell Death in Neurogenic Regions
E2F1 knockouts	Knockout strategy	↓	↓
Cholinergic denervation	Neurochemical Lesion	↓	↑
Noradrenergic denervation	Neurochemical Lesion	↓ (DG) ↑ (OB-PGL)	–
VEGF infusion	ICV infusion	↑	↓

Comparison of the different experimental strategies:

Three different intervention strategies were applied in this dissertation to analyze adult neurogenesis. A **knockout strategy**, such as employed to study the role of E2F1, enables one to study the influence of endogenous genes and this strategy is an essential tool for understanding the physiological role of a specific gene. Nevertheless, inducible knockouts would probably be a better approach, because the gene's functions during the embryonic phase would remain untouched. In the case of E2F1, it seems that the other gene family members supplant the loss of E2F1 during development, since gross anatomical analysis of these animals yielded no malformations. All the same, the removal of a gene that is essential to the development of the animal or that doesn't have another gene to substitute its function could greatly interfere with the analysis or understanding of its role in adult neurogenesis.

Neurochemical lesions involve the removal of endogenous neurotransmitters and help to define the local chemical environment of progenitor cells. The advantages of using a neurotoxin (vs. pharmacological agents) are the specificity and selectivity for a certain cell population. In the case of 192IgG-saporin, it has been elegantly shown that this immunotoxin specifically and selectively eliminates p75-immunoreactive neurons in the cholinergic forebrain and allows one to study the effects of this particular denervation. On the other hand, neurotransmitter denervations also cause a multitude of other side effects, i.e. learning and memory deficits, mood disorders, depression, etc.,

and one must consider whether or not the effects that one sees on adult neurogenesis are secondary to the other changes that occur in a lesioned animal.

The utilization of **intraventricular infusions** enables one to study the effects of exogenous compounds on adult neurogenesis. This application has the advantage that the LV is close to the proliferative region, namely that SVZ and it also eliminates the problems encountered with the blood-brain barrier. However, surgery on an animal, anesthetics and cannula tracts can all have repercussions on progenitor cells and should be considered when planning these sorts of experiments. Previous pre-clinical testing of ICV growth factor infusion has shown that substantial side effects can occur in proximity to the CSF space, even down to the spinal cord level[323]. In addition, depending on what molecule is being injected, the molecule could have "sticky" characteristics and not make it farther than the subependymal lining of the LV. In Experiment V, since VEGF is endogenously produced in the choroids plexus, it was unlikely that this growth factor would have these problems.

Disease-related Models and the Clinical Perspective

The adult CNS is classically known as a structure with very limited regenerative capacity. In this respect the existence of neural progenitor cells in the brain is seen as a paradox, since multipotent cells in other organs are generally in charge of cellular replacement after physiological or disease-related degeneration, whereas in the adult brain spontaneous structural recovery is usually not observed. But some of the compensatory reactions to injury, which were previously thought to represent synaptic or functional plasticity, could very well have their origin in a limited neuronal replacement.

Traumatic brain lesion

Traumatic brain injury is capable of causing an increased progenitor population in the DG[324,325] and SVZ[325], as well as an increase in the number of newborn neurons in the DG[326]. It is, however, interesting to note that cortical contusions had the opposite effect – namely, there was a decrease in the number of newborn neurons in the DG[327]. Brain injury induced by traumatic lesions can cause a transient increase in the number of progenitor cells of the LV[328-330], but these studies were not able to demonstrate any neuronal contribution of progenitor cells to the lesion site.

Epilepsy/Seizure

When epileptic activity is induced in animal models a prominent induction of neurogenesis is observed in the DG[21, 331-335]; however, the newborn neurons formed aberrant connections and did not extend neurites along the mossy fiber pathway to the CA3 region[21]. This increase in neurogenesis is observed regardless of the mechanism for the induction of seizures (e.g. chemical or electrical stimulation). When protective strategies, such as "environmental enrichment" are employed in combination with seizure induction, the intervention proved to be effective in preventing seizures and neuronal cell death[93]. Nevertheless not all epilepsy-related structural changes are linked to altered neurogenesis, since progenitor proliferation after seizure was inhibited by irradiation, whereas the synaptic remodeling of the mossy fiber pathway was not altered[163]. Recently, it has been shown that the neurogenic effects of seizures are not only affecting the rate of progenitor proliferation in the hippocampus, but also in the LV, where prolonged seizures up-regulated the rate of SVZ progenitor proliferation[336].

Hypoxia/Ischemia

Focal and global ischemia have been shown to be potent in inducing neurogenesis in the DG of the rat and gerbil and the LV[142, 337-341]. Ischemic neuronal damage often involves excitotoxicity, characterized by over stimulation of glutamate receptors and Ca2+ influx. This knowledge has led to further exploration of the role of glutamate receptors in the context of brain ischemia. It has been observed that of NMDA blockers systemically injected before and directly following global ischemia completely blocked the increase in neurogenesis otherwise seen with ischemia[121, 339].

The two very promising studies have recently reported that, after ischemia, neurogenesis is inducible in brain regions which are normally not generated in the adult: in the striatum and in the CA1 region of the hippocampus. The first study by Lindvall and colleagues observed after focal ischemia of the striatum that cells from the LV migrate laterally into the striatum and form new neurons of the type that is most severely affected, the medium spiny interneurons[316]. The second study by Nakafuku and colleagues showed that after global ischemia up to 50% of the damaged CA1 pyramidal neurons can be replaced[317]. This regenerative neurogenesis was only observed after a combination of FGF-2 and EGF was infused into the LV. With an extraordinary broad set

of methods the study demonstrated that cells divide in the alveus region of the LV, migrate to the CA1 region, form appropriate synapses and become electrophysiologicallly active. Moreover, behavioral defects of the ischemia were compensated only in the EGF/FGF-2 infused animals. Together with data on possible neurogenesis in the cortex[79, 80, 141], these studies demonstrate that the potential for neurogenesis in other brain areas was previously underestimated.

Taken together, the previous chapters suggest that signals are present throughout the mammalian brain, which allow limited neuronal self-renewal to occur. Whether this limited self-renewal contributes to some of the functional plasticity and spontaneous recovery that can be observed after brain damage, needs to be determined. This fundamental observation of spontaneous neuronal repair could change our view of the brain's regenerative capacity; but then again, without giving us the immediate ability to regenerate large or complex brain areas.

Although the field of adult neurogenesis is not more than 10 years old, a growing number of factors have been shown to regulate progenitor proliferation and/or neurogenesis *in vivo*. The factors most pertinent to the current study were discussed above; however, it would go beyond the scope of this study to discuss all regulatory factors. For a brief summary of which major influences change neurogenesis, see Table 4 below. For a comprehensive overview of the individual experiments and citations, see Table I in the Appendix.

Table 4

Signals/Situations influencing Proliferation and/or Adult Neurogenesis	
Up-regulate	**Down-regulate**
Gonadal steroids	Glucocorticoids/Stress
Growth factors	Glutamate
Neurotrophic factors	Cell cycle inhibitors
Cell cycle activators	Aging
Cell death inhibitors	Irradiation
Dietary restriction	Drugs of abuse (cocain, nicotine, amphetamine, cannabinoids)
Enriched environment	Sensory deprivation
Physical activity	Serotonin
Epilepsy	Vitamin E
Ischemia and brain injury	
Antidepressants	

Outlook

During development neural progenitors are generated in germinal matrices, such as the ventricular zone. As embryonic and fetal development near the final stages, these matrices gradually form the SVZ of the LV and the subgranular zone of the DG. These zones are ultimately the regions that are responsible for the production of new neurons in the adult brain. Why do they keep proliferating new neurons for these regions, whereas other areas of the brain grind to a halt? What makes the cells in these regions so special? Is it the environment that's affording this phenomenon to take place? Or could it be a combination of both? The current experiments have attempted to contribute answers to these stimulating questions. Especially in the case of the neurotransmitter lesions it was possible to show that by altering the input into these neurogenic regions, thereby changing the microenvironment for these cells, the neural progenitors responded in varying ways – either by producing more new neurons or less. Whereas, in a different scenario, changing particular genes and thereby altering the

program of cell cycle (E2F1 knockout strategy) showed that by specifically altering a process within the progenitor cell itself results in a decrease in neurogenesis.

In order to make use of this phenomenon of neurogenesis as a possible therapeutic tool, it will be essential to understand the biological and physiological significance of new neurons in the adult brain. Further studies will need to be performed in order to show that more neurons lead to a better functioning brain. And once the stimulus for producing new neurons is understood, then it will be essential to be able to control the amount of proliferation. The thought of one day being able to heal devastating diseases, such as Parkinson's, Alzheimer's and even ischemia, provides hope for a better future.

MATERIALS AND METHODS

Animals, Injections, and Tissue Preparation

Experiment I - Critical evaluation of BrdU labeling

Female Wistar rats (Harlan, Sulzfeld, Germany) received either a single intraperitoneal injection of BrdU (50mg/kg) or four consecutive daily injections of BrdU (50mg/kg/day) at the age of 8 weeks. Animals with a single injection were perfused 2 hours or 2 days later. During the 2 hr time interval, only cells in S-phase are labeled by BrdU incorporation. Animals with multiple BrdU injections were perfused 10 days or 4 weeks later. The later time interval is sufficient to allow newly generated cells to undergo full neuronal differentiation. The animals were perfused transcardially with 4% PFA/PB. The brains were removed, post-fixed ON in 4% PFA/PB, and transferred to 30% sucrose/PB. The brains were sagitally divided and each hemisphere was cut at 40µm on a sliding microtome in dry ice and stored at -20ºC in CPS.

Experiment II - Impaired adult neurogenesis in mice lacking the transcription factor E2F1

E2F1 mutant mice were previously generated by inserting a PGK-Neo cassette into exon 3 and 4[187] leading to a deletion of the DNA binding and heterodimer regions. To reduce the influence of flanking regions from 129/SVJ ES cell construct, the knockout strain was backcrossed at least 10 times with the C57/BL6 mouse strain. For histological analysis, E2F1-deficient animals and wildtype C57/BL6 mice were generated as littermates from heterozygote breedings.

In order to determine the proliferative cell populations in the CNS, 10-12 week old E2F1-deficient and wildtype mice were injected with BrdU (50mg/kg) and killed 2 hours later (n=5 for each group). In a second experiment in order to determine the number of newly generated neurons, animals were treated with a single intraperitoneal injection of BrdU (50mg/kg) at the age of 8 weeks and perfused 4 weeks later (n=8 for each, E2F1-deficient and wildtype). The animals were deeply anesthetized with a cocktail of ketamine, acepromazine and xylazine and perfused transcardially with 4% PFA. The brains were removed, post-fixed ON in 4% PFA/PB, and transferred to 30% sucrose/PB.

The brains were sagitally cut at 40-□m on a sliding microtome in dry ice and stored at -20°C in CPS.

Experiment III - Cholinergic Denervation Lesion

250g male Fischer-344 albino rats (n=13; Harlan Sprague-Dawley, Sulzfeld, Germany) were anesthetized by an intramuscular injection consisting of 62.5-mg/kg ketamine (Ketanest), 3.175-mg/kg xylazine (Rompun), and 0.625-mg/kg of acepromazine maleate dissolved in 0.9% sterile saline. Rats were mounted in a small animal stereotaxic apparatus (David Kopf, Tujunga, CA) with bregma and lambda in the same horizontal plane. A stainless steel cannula (10µl Hamilton syringe) was implanted in the LV (AP +8 mm, ML -1.5 mm from the center of the interaural line in flat skull position, DV +5.8 mm). 192IgG-saporin (5µl, 0.7 µg/ml, MAB390, dissolved in PBS) or PBS (5µl) was injected at a flow rate of 1µl/min, resulting in a delivery of 3.5µg of toxin into the LV. On days 3, 5 and 7 after infusion, animals received intraperitoneal injections of bromodeoxyuridine (BrdU, 50mg/kg, Sigma, St. Louis, MO). Four weeks after the final BrdU injection, the animals were deeply anesthetized and intracardially perfused with 4% PFA. Brains were removed, post-fixed ON in 4% PFA/PB and transferred to 30% sucrose/PB. The OBs were transected at the fornix level and cut sagitally in 40µm sections on a sliding microtome in dry ice and stored at -20ºC in CPS. The rest of the brain was coronally cut at 40µm.

Experiment IV – Noradrenergic Denervation Lesion

Animals and tissue were treated the same as in Experiment III (n=12), with the exception that 6 rats received 7.5µg (5µl) of mouse anti-DBH-SAP (MAB394) into the same LV coordinates and the control group received PBS. Injection flow rate was also 1µl/min.

Experiment V - Vascular endothelial growth factor induces neurogenesis in the adult brain

Stainless steel cannulas, which were connected to osmotic minipumps (Model 2001, Alza) were implanted into two month old male Fischer-344 rats (n=36) according to a previously established protocol [18]. The animals received recombinant mouse VEGF-A_{165} or aCSF, artificial cerebrospinal fluid, (n=18 each) at a flow rate of 1 µl/hr, resulting

in a delivery of 2.4ng growth factor/day for 7 days. During the last 6 days of the pump period, animals received daily intraperitoneal injections of BrdU (50 mg/kg). After 7 days of ICV infusion, 8 animals per group were intracardially perfused with 4% PFA. The remaining animals had the pumps removed and were perfused after an additional four-week period without growth factor infusion. Adult male rats were infused for 1 week with recombinant mouse $VEGF_{165}$ dissolved in artificial cerebrospinal fluid (aCSF) or aCSF alone. During the infusion period, the animals received daily single intraperitoneal injections of BrdU (50 mg/kg). In order to determine the number of cells that were generated from dividing progenitor cells, 6 animals per group were perfused on the last day of growth factor infusion. Four weeks after the infusion, the remaining rats (n=8 per group) were perfused and this time point served to determine the survival rate and neuronal differentiation of the newly generated cells. The animals were deeply anesthetized with anesthetic cocktail and perfused transcardially with 4% PFA. The brains were removed, post-fixed ON in 4% PFA/PB, and transferred to 30% sucrose/PB. The brains were sagitally cut at 40-μm on a sliding microtome in dry ice and stored at -20°C in CPS.

Experiment VI - Transient expression of doublecortin during adult neurogenesis

Female Wistar rats (Charles River-Wiga, Sulzfeld, Germany) were kept in normal light dark cycle (12 hour light/12 hour dark) and had free access to food and water. Animals received at 2 months of age intraperitoneal injections of BrdU (50 mg/kg). Animals perfused at 2 hours, 12 hours, 24 hours, 1, 2, 4 and 7 days after BrdU treatment received only a single BrdU injection. The animals that were sacrificed at 10, 14, and 21 days, 1, 2, 3, 4, 6, 9, 14, and 19 months after BrdU injection received daily injections on 4 consecutive days. Each time point consisted of 3 animals. At perfusion time point, the animals were deeply anesthetized with a cocktail of ketamine, acepromazine and xylazine and perfused transcardially with 4% PFA. The brains were removed, post-fixed ON in 4% PFA/PB, and transferred to 30% sucrose/PB. The brains were sagitally cut at 40-μm on a sliding microtome in dry ice and stored at -20°C in CPS.

Histological Protocols

BrdU-DAB Protocol

Day 1

- Rinse 3x for 10 min in TBS
- 0.6% H_2O_2/TBS for 30 min
- Rinse 2x in TBS
- 50% formamide/2XSSC for 2hr at 65^{o}C, shaking
- Rinse in 2XSSC for 5 min
- 2N HCl for 30 min at 37^{o}C *Shake plate immediately after addition to avoid tissue clumping.*
- 0.1M borate buffer pH8.5 for 10 min at RT
- Rinse 4x5 min in TBS
- Block: 3% donkey serum/0.1% TX100/TBS for 30 min
- Primary antibody in 3% donkey serum/0.1% TX100/TBS ON at 5°C

Day 2

- Rinse 2x10 min in TBS
- Secondary antibody in 3% donkey serum/0.1% TX100/TBS for 1 hr

  **make ABP solution to allow mixture to equilibrate for at least 30 min*
- Rinse 3x10 min in TBS
- Avidin-Biotin-Peroxidase (10µl/ml of A and B) for 1 hr
- Rinse 2x10 min in TBS
- DAB solution: 5-10 min

  1 ml TBS
  12.5 µl DAB (20 mg/ml)
  0.3 µl H_2O_2 (30%)
  16.67□l of 8% $NiCl_2$
- Rinse 4x in tap water
- Rinse in TBS
- Mount
- Dehydrate through alcohol series, ending with NeoClear
- Coverslip with NeoMount

BrdU Triple Immunofluoroscence Protocol

Day 1

- Rinse 3x for 10 min in TBS
- 50% Formamide/2xSSC for 2hr at 65°C, shaking
- Rinse in 2XSSC for 5 min
- 2N HCl for 30 min at 37°C *Shake plate immediately after addition to avoid tissue clumping.*
- 0.1M borate buffer pH8.5 for 10 min at RT
- Rinse 4x5 min in TBS
- Block: 3% donkey serum/0.1% TX100/TBS for 30 min
- Primary antibody in 3% donkey serum/0.1% TX100/TBS ON at 5°C
  e.g. rat anti-BrdU + mouse anti-NeuN + rabbit anti-S100

Day 2 (following secondary antibody, all steps in dark)

- Rinse 2x10 min in TBS
- Secondary antibody in 3% donkey serum/0.1% TX100/TBS for 2 hr, RT, shaking
  donkey anti-rat–FITC 1:1000
  donkey anti-mouse-RHOX 1:1000
  donkey anti-rabbit-CY5 1:1000
- Rinse 5x15 min in TBS
- Mount and coverslip

Immunofluoroscence Protocol

Day 1

- Rinse 3x for 10 min in TBS
- Block: 3% donkey serum/0.1% TX100/TBS for 30 min
- Primary antibody in 3% donkey serum/0.1% TX100/TBS ON at 5°C
  e.g. rat anti-BrdU + mouse anti-NeuN + rabbit anti-S100b

Day 2 (following secondary antibody, all steps in dark)

- Rinse 2x10 min in TBS
- Secondary antibody in 3% donkey serum/0.1% TX100/TBS for 2 hr, RT, shaking
  donkey anti-rat–FITC 1:1000
  donkey anti-mouse-RHOX 1:1000
  donkey anti-rabbit-CY5 1:1000
- Rinse 5x15 min in TBS
- Mount and coverslip

Standard TUNEL Protocol

- Rinse sections in TBS or PBS
- Rinse in H_2O
- 50%, 70%, 96%, 100% isopropanol – 1 min each
- 100%, 96%, 70%, 50% isopropanol – 1 min each
- Rinse in H_2O
- Rinse 3x in PBS – 5 min

All remaining steps in dark

- Equilibration Buffer (50µl/section) – 1 min, RT
- TdT Solution (50µl/section) – 1 hr, 37°C (70µl reaction buffer + 30µl TdT Enzyme)
- Stop Buffer – 10 min, RT (1:34, Stop concentrate:H_2O)
- Rinse 3x in PBS – 5 min
- Secondary antibody: 1 hr, RT, shaking (sheep anti-Dig–FITC 1:40 in PBS)
- Rinse 3x in PBS – 5 min

Can now do one of the following:

1. Mount and coverslip
2. Mount, follow with nuclear staining:
   - propidium iodide 1:1000 (PBS), 5 min at RT in dark
   - rinse 3x in PBS – 5 min
   - coverslip
3. Block and continue with another fluorescent staining

TUNEL + Immunofluorescence Protocol

Day 1

- Standard TUNEL protocol to final rinse step
- Block: 3% donkey serum/TBS for 30 min
- Primary antibodies in 3% donkey serum/TBS ON at 5°C
  e.g. mouse anti-NeuN + rabbit anti-s100

Day 2

- Rinse 2x10 min in TBS
- Secondary antibody in 3% donkey serum/TBS for 2 hr at RT, shaking in the dark.
  For example:

donkey anti-sheep-FITC *1:1000 (for Tunel)*
donkey anti-mouse-RHOX *1:1000 (for NeuN)*
donkey anti-rabbit-CY5 *1:1000 (for S100ß)*

- Rinse 5x for 15 min in TBS (minimum), or ON at 5°C (no shaking!)
- Mount and coverslip

TUNEL + BrdU Protocol

Day 1

- Standard TUNEL protocol to final rinse step
- Fix: 4% paraformaldehyde in 0.1M PO_4 for 20 min at RT
- Rinse 3x in PBS – 5 min
- 2N HCl for 30 min at 37°C *Shake plate immediately after addition to avoid tissue clumping.*
- 0.1M borate buffer pH8.5 for 10 min at RT (brings pH back to neutral)
- Rinse 4x in TBS for 5 min (the more the better)
- Block: 3% donkey serum/TBS for 30 min
- Primary antibodies in 3% donkey serum/TBS ON at 5°C
  e.g. rat anti-BrdU + mouse anti-NeuN
  best results if primary antibodies incubate 2x ON (36 hr)

Day 2

- Rinse 3x for 5 min in TBS
- Secondary antibody in 3% donkey serum/TBS for 2 hr at RT, shaking in the dark.
  For example:
  donkey anti-sheep-FITC 1:1000 (for Tunel)
  donkey anti-rat-RHOX 1:1000 (for BrdU)
  donkey anti-mouse-CY5 1:1000 (for NeuN)
- Rinse 5x for 15 min in TBS (minimum), or ON at 5°C (no shaking!)
- Mount and coverslip

Nissl Staining

- Mount sections on gelatin-coated slides and let dry thoroughly
- De-fat in equal parts of chloroform/2-ETOH (100%) for 5 min
- Rehydrate as follows:

100% 2-propanol	until clear (20 dips), repeat
95% 2-propanol	until clear (20 dips), repeat
70% 2-propanol	until clear (20 dips)

50% 2-propanol	until clear (20 dips)

- Rinse in deionized water 2X
- Stain in 1.5% cresylviolett 2 min **Note**: heat to 60 degrees and filter before use.
- Rinse in deionized water 2X
- Rinse in tap water for 10 min
- Differentiate in 70% ETOH/0.5% acetic acid for a few seconds
- Rinse in tap water

If staining is too light, go back to Step 4 and repeat staining

- Dehydrate as follows:

70% 2-propanol	until clear (20 dips)
95% 2-propanol	until clear (20 dips), repeat 2X
100% 2-propanol	until clear (20 dips), repeat 2X
NeoClear	until clear (20 dips), repeat

- Coverslip with NeoMount.

DAPI/Propidium iodide Staining

- 1mg/ml stock solution
- Incubate sections/slides in 1:5000 solution for 10 min
- Wash 3X
- Coverslip

Antibodies

Antibody	Company	Dilution used
Primary Antibodies:		
Goat anti-ChAT	Chemicon, Temecula, CA	1:100
Goat anti-doublecortin C-18	Santa Cruz, Santa Cruz, CA	1:500
Mouse anti-ß-III Tubulin	Promega, Madison, WI	1:500
Mouse anti-Nestin	BD PharMingen, San Diego, CA	1:1000
Mouse anti-NeuN	Chemicon, Temecula, CA	1:500
Rabbit anti-active Caspase-3	R&D Systems, Minneapolis, MN	1:1000
Rabbit anti-Ki-67	Jackson ImmunoResearch, West Grove, PA	1:500
Rabbit anti-Ki-67	Novacastra Laboratories Ltd., Newcastle Upon Tyne, UK	1:500
Rabbit anti-S100ß	Swant, Bellinzona, Switzerland	1:2500
Rat anti-BrdU	Biozol, Eching, Germany	1:500
Rat anti-BrdU	Accurate, Westbury, USA	1:500
Secondary Antibodies:		
Donkey anti-goat-biotin	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-goat-FITC	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-goat-rhodamineX	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-mouse-biotin	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-mouse-CY5	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-mouse-rhodamineX	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-mouse Alexa 488	Molecular Probes, Eugene, OR	1:300
Donkey anti-rabbit-CY5	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-rabbit-FITC	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-rabbit-rhodamineX	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-rabbit Alexa 488	Molecular Probes, Eugene, OR	1:300
Donkey anti-rat-biotin	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-rat-CY5	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-rat-FITC	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-rat rhodamineX	Jackson ImmunoResearch, West Grove, PA	1:300
Donkey anti-rat Alexa 568	Molecular Probes, Eugene, OR	1:300
Donkey anti-sheep-FITC	Jackson ImmunoResearch, West Grove, PA	1:300

DNA Purification

Tails clippings from the mice were collected and processed with the DNeasy Tissue Kit (QIAGEN) according to the manufacturer's protocol.

PCR

To determine the genotype of the breedings, PCR analysis using 3 Primers (E2F1 5': GGA TAT GAT TCT TGG ACT TCT TGG (50µM), E2F1 3': CTA AAT CTG ACC ACC AAA CGC (100µM), PGK-Neo 5' CAA GTG CCA GCG GGG CTG CTA AAG (50µM) and the following PCR parameters were used: (1) 94°C 3 min, (2) 94°C 1 min, (3) 57°C 1 min, (4) 72°C 1 min, (5) repeat step (2)-(4) 30 times, (6) 72°C 10 min.

Quantities used:

12.5µl Master Mix

2µl E2F1-5' primer

4µl E2F1-3' primer

2µl E2F1-pgk primer

1µl DNA

3.5µl H_2O

Stereology

For quantification, a systematic, random counting procedure, similar to the optical disector[342] was used as described by Williams and Rakic[343]. To determine the number of Nissl- or DAPI-stained cells as well as BrdU-positive or TUNEL-positive cells in individual brain regions, separate series of every 6th section (240 µm interval) of the left hemisphere from each brain were analyzed. The volume of each structure was determined by tracing the areas using a semi-automatic stereology system (StereoInvestigator, MicroBrightField, Colchester, VT). Positive cells, which intersected the uppermost focal plane (exclusion plane) and the lateral exclusion boundaries of the counting frames, were not counted. As BrdU-labeled cells and TUNEL-labeled cells are comparatively rare in the DG, no counting frames were used, but rather all cells in the DG were counted on each section under exclusion of the uppermost focal plane. In the case of Experiment II (E2F), in order to calculate total cell counts in specific brain areas, sections were counterstained with cresylviolet for Nissl-staining (Cerebellum and cortex)

or with the fluorescent nuclear marker 4',6-diamidino-2-phenylindole (DAPI, DG and SVZ).

To examine defined areas from free-floating sagittal sections several structures were not analyzed in their entirety, but rather between selected anatomical landmarks for reasons of reliability and reproducibility. *Olfactory bulb* (Figure 44B). For stereological quantification the GCL, which continually incorporates new neurons, was distinguished from the plexiform layer, which served as a control region. *Lateral ventricle wall* (Figure 44C). All sections, where the LV formed an open cavity, were used for analysis (between figure 82 and 89[344]). The *rat* stereotaxic atlas by Paxinos and Watson[344] was used as a reference for sagittal sections, because sagittal sections are not available in the corresponding *mouse* atlas[345]. *Dentate gyrus* (Figure 44C). Cells were counted in the molecular layer, the GCL and the hilus. A series of every 6th section was selected, starting where the *dorsal* DG separated from its *ventral* part (between figure 88 and the midsagittal section[344]).

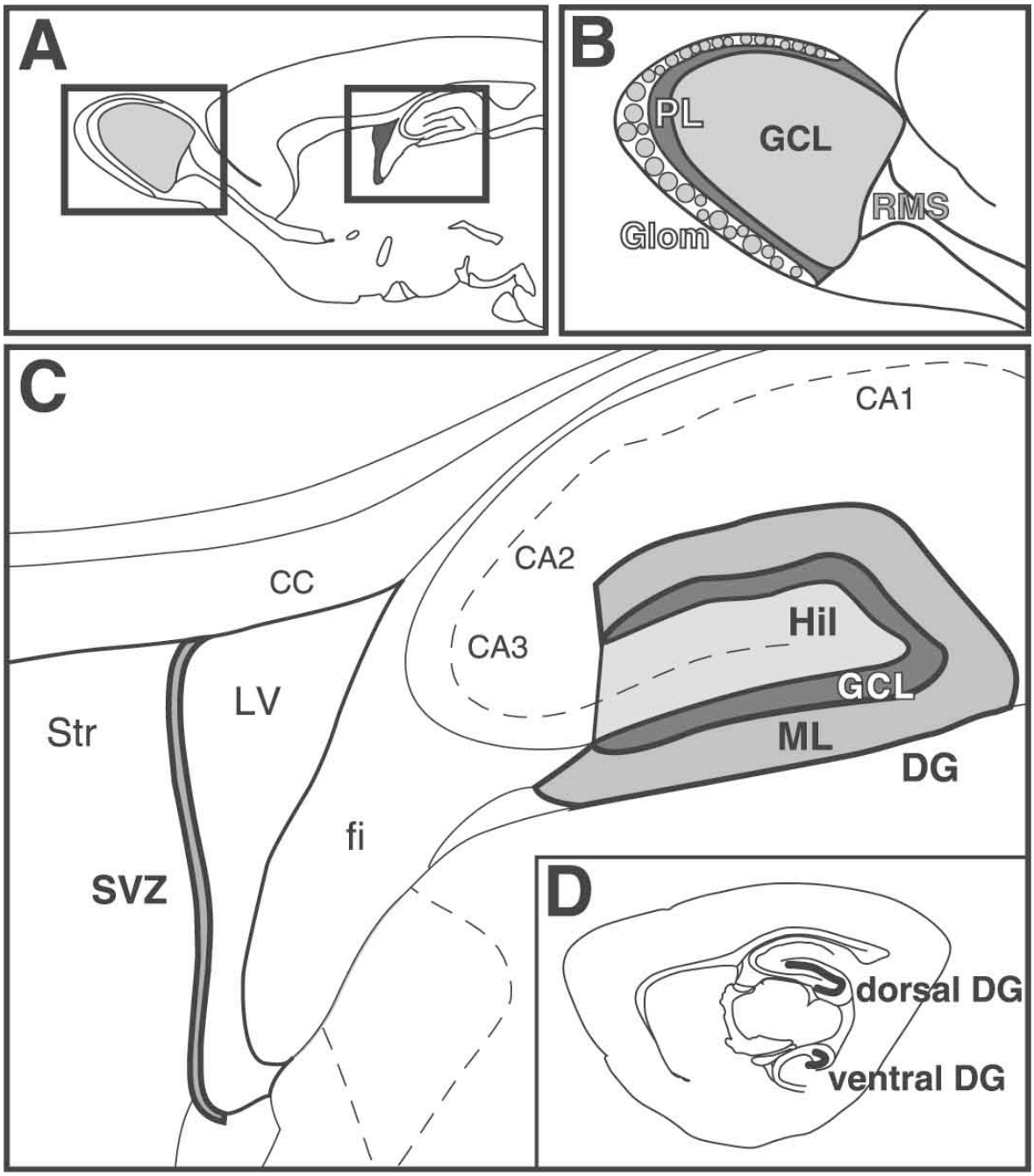


Figure 44

Analysis of neurogenic regions. (A) Sagittal view of the mouse brain (modified, with permission of the publisher, from Paxinos and Watson, 1998[344]) illustrates the anatomical sites of adult neurogenesis: the olfactory bulb, the lateral ventricle wall, and the dentate gyrus. (B) For analysis, the olfactory bulb was differentiated into separate layers, the granule cell layer (GCL) and the plexiform layer (PL), which was defined as the area between the GCL and the glomerular layer (Glom). RMS—rostral migratory stream. (C) The lateral ventricle wall (LV) adjacent to the striatum (Str) contains the proliferative subventricular zone (SVZ). The ventricle wall was analyzed (thick lines) from the ventral tip of the ventricle to the point where the corpus callosum (CC) forms the roof of the ventricle. The dentate gyrus was separated into the

granule cell layer (GCL), molecular layer (ML), and hilus region (Hil). CA1, CA2, CA3—cornu ammonis regions 1–3; fi—fimbria fornix. (D) In order to obtain clearly defined areas for these three subregions, the dorsal dentate gyrus (DG) was analyzed on all sections where it was separated from its ventral counterpart.

Adaptations for specific experiments

Experiment II - Impaired adult neurogenesis in mice lacking the transcription factor E2F1

Cerebellum and neocortex. Nissl-stained series of every 6th sagittal sections were used to determine number of neurons in the cerebellum and neocortex. The cerebellum was analyzed on sections that included the lateral or the medial cerebellar nucleus as a landmark (between figures 80 and 86[344]). A counting frame size of 20x20 µm and a grid of 750x750 µm to space the count frames systematically across the cerebellum resulted in the detection of more than 700 granule cells per animal. To determine the volume and the number of neurons in the neocortex, sections were selected that included the capsula interna as an external landmark[344]. On these sections the neocortex was analyzed in its entire depth (layer I-VI) and length (from the rhinal fissue to the occipital pole). In order to estimate the total number of cortical neurons a counting frame size of 30x30 µm and a grid of 300x1200µm was used, leading to the detection of more than 500 neurons per animal. In order to determine the fraction of BrdU-positive cells among the total population of cells, DAPI-positive cells were stereologically counted in the GCL of the DG (counting frame size of 20x20 µm and a grid of 120x120µm) and the SVZ (counting frame size of 30x30 µm and a grid of 100x100µm). The resulting cell number was used to calculate the percentage of BrdU-positive cells relative to the total number of cells.

Experiment IV – Cholinergic Denervation Lesion

Although the lesion was bilateral, since the toxin was administered through the LV, the ipsilateral side was nevertheless used for analysis. To determine the number of BrdU-positive or TUNEL-positive cells in the OB, separate series of every 4th section (160-µm interval) were analyzed. A counting frame of 40 x 40-µm and a grid size of 350x350 µm were used for analysis of BrdU. TUNEL-positive cells in the OB were counted exhaustively.

Experiment V - Vascular endothelial growth factor induces neurogenesis in the adult brain

For the LV, counting frame and sampling grid sizes of 20x20 µm and 50x100 µm and for the OB 60x60 µm and 300x300 µm were used.

Microscopy

Fluorescent signals were detected using a confocal scanning laser microscope (Leica TCS-NT, Leica Lasertechnik, Heidelberg, Germany) and a 40xPL APO oil objective (1.25 NA). To avoid cross detection of FITC signals in the rhodamine channel and Rhodamine-X detection in the CY-5 channel, the fluorescent images were sequentially recorded for each channel with only the corresponding single wavelength laser line (488 nm, 568 nm or 647 nm) activated using acusto-optical tunable filters. In order to exclude false-positive signals from overlying cells, pinhole settings were used that corresponded to an optical thickness of less than 2 µm. Randomly selected BrdU-positive cells were analyzed in their entire z-axis in order to exclude false double-labeling due to an overlay of signals from different cells[18]. A minimum of 50 BrdU-positive cells per region of interest were examined for co-labeling in each animal and time point.

Neuronal differentiation

To determine the percentage of differentiation of newborn cells, a series of every 6th section was stained for double immunofluorescence using antibodies against BrdU, NeuN, S100ß, etc. For each brain and each neurogenic region, 50 BrdU-positive cells were randomly selected and analyzed for double labeling with BrdU. The resulting percentages for each group were multiplied with the stereologically estimated numbers of BrdU-positive cells in order to estimate the number of newly generated neurons, astrocoytes, etc.

Statistical analysis

The data are expressed as mean values +/- standard error of mean (S.E.M.) Statistical analysis was performed using the unpaired, two-sided t-test comparison (Student's t-test) (Statview, SAS Institute, Cary, NC). Significant differences were

assumed at a level of $p < 0.05$. The data are expressed as mean values +/- standard error of mean.

Chemicals

ABC Kit (avidin-biotin-peroxidase complex; Vectastain Elite, Vector Laboratories, Burlingame, CA, USA)

1M Acetic Acid

6ml Glacial Acetic acid
QS to 100ml

1% Agarose Gel, 100ml

1g agarose
6µl 10mg/ml ethidium bromide

Anesthesia Cocktail:

62.5mg/kg ketamine (Ketanest)
3.175mg/kg xylazine (Rompun)
0.625mg/kg acepromazine maleate
dissolve in 0.9% sterile saline

Apoptag In Situ Cell Death Detection Kit (Intergene, Purchase, NY)

Borate Buffer (0.5M Stock) ph 8.5

15.45g boric acid
450ml H_20
5ml 10N NaOH (to bring pH to 8.5)
QS to 500ml

10X Blue Juice (10ml)

5ml Glycerin
2ml 100mM EDTA, pH 8.0
25mg 0.25% bromophenoblue
25mg 0.25% xylenecyanol
3ml H_2O

BrdU

Dissolve in sterile NaCl
Mouse – 10mg/ml
Rat – 20mg/ml

CPS – Cryoprotection Solution (1 liter), store at RT

250ml Glycerin
250ml Ethylene glycol
500ml 0.1M PO4

Cresyl Violet (Nissl Staining)

1.5g Cresyl Violet
98ml H_2O

1ml 1M CH_3COOH
1ml 1M Sodium acetate

aCSF (artificial CSF), pH 7.2

8.65g NaCl
0.22g KCl
0.21g $CaCl_2 \cdot 2H_2O$
0.16g $MgCl_2 \cdot 6H_2O$
0.21g Na_2HPO_4
0.03g $NaH_2PO_4 \cdot H_2O$
QS to 1 liter with H_2O, sterile filter

DAB

20mg/ml in H_2O

DAPI (4',6-diamidino-2-phenylindole)

DNA Molecular Weight Marker VIII (Roche Diagnostics, Mannheim, Germany)

Dneasy Tissue Kit (QIAGEN, Hilden, Germany)

Formamide Deionisation

4 large spoonfuls of Resin in 1 liter of Formamide; stir 1 hour. Filter and freeze.

HCL 5N

[HCl] = 11.6M
43ml concentrated HCl
57ml H_20

Master Mix for PCR (QIAGEN, Hilden, Germany)

4% Paraformaldehyde (1liter)

40g paraformaldehyde
0.5l H_2O
1ml 10N NaOH, stir until clear
Filter into larger vessel
Filter 0.5l 0.2M PO_4 into same vessel

10X PBS, pH 7.4 – Ca^{2+}, Mg^{2+}-free (Dulbecco's)

40g NaCl
1g 1KCl
7.2g $Na_2HPO_4 \cdot 2H_2O$ (or 5.4g Na_2HPO_4 water free)
1g KH_2PO_4 (water free)
QS to 500ml with H_2O

Phosphate buffer (PO_4)

0.2M PO_4 from water-free Natriumphosphate:

1 Liter dH_2O

5.52g Sodium-dihydrogen-phosphate (NaH_2PO_4 water free)
21.9g Disodium-hydrogen-phosphate (Na_2HPO_4 water free)
OR

0.2M PO_4 from water-based Natriumphosphate:

1 Liter dH_2O
6.35g Sodium-dihydrogen-phosphate monohydrate ($NaH_2PO_4 \times H_2O$)
41.35g Disodium-hydrogen-phosphate heptahydrate ($Na_2HPO_4 \times 7\ H_2O$)

0.1M Phosphate Buffered Saline (PBS)

500ml 0.2M PO_4
500ml dH_2O
9g Sodium chloride

Propidium iodide

ProLong (Molecular Probes, Eugene, Oregon, USA)

30% Saccharose (500ml)

400ml 0.1M PO_4
150g Saccharose
store at +5°C

1M Sodium Acetate

82.03g
QS to 1 liter

20X SSC, pH 7.0

3.0M NaCl 86.65g
0.3M NaCi x $2H_20$ 44.1g
QS to 500ml

ToPro-3 (1:2500; Molecular Probes, Eugene, OR)

Tris Buffered Saline (TBS)

870ml dH_2O
100ml 1M Tris-HCl pH7.5
30ml 5M NaCl

10X TBS (1l), pH 2.4-2.5, store at +5°C

855ml dH_2O
132.2g Trizma HCl
19.4g Trizma Base
90g NaCl

TBS++

TBS buffer

0.1% Triton-X100

3% donkey serum

TdT-Enzyme (from Apoptag Kit)

TUNEL Dilution Buffer (Roche Diagnostics, Mannheim, Germany)

TUNEL-Reaction Buffer (from Apoptag Kit)

APPENDIX

Table I

Interventions influencing Proliferation in Dentate gyrus	
Up-regulate	**Down-regulate**
Adrenalectomy[104, 105, 108]	Prenatal stress[106, 107]
NMDA blockers[117-120]	Predator-odor exposure stress, rats[103]
Ovarian steroids - estrogen[346-348]	Acute psychosocial stress, tree shrew[72, 102]
HB-EGF, ICV[349]	Resident-intruder stress, monkeys[73]
Lithium[350]	Alpha-tocopherol[351]
Fetal raphe grafts into hippocampus after 5,7-DHT lesion[214]	Glucocorticoids - corticosterone[104, 108]
Entorhinal cortex leions[117]	Ovariectomy[348]
Haloperidol, gerbils[352]	NMDA[117]
Focal ischemia, rat[142, 340, 353]	5,7-DHT lesion into medial and dorsal raphe[214]
Transient focal ischemia, gerbil[354, 355]	Acute methamphetamine[356, 357]
Global ischemia, gerbil[358]	NMDA blockers following focal ischemia, rat[121, 353]
Cerebral ischemia[349]	Electromagnetic exposure, gerbil[359]
Kainic acid[333]	Irradiation[163, 360]
Caspase inhibitors following status epilepticus[361]	Stretozotocin-induced diabetes[362]
Traumatic brain injury[324, 325]	CREB-/- transgenic mice[287]
mCD24-/- transgenic mice[363]	
Strain variations[364, 365]	

Interventions influencing Neurogenesis in Dentate gyrus	
Up-regulate	**Down-regulate**
Enriched environment[60, 90-92]	Corticosterone[109]
Enriched environment in aged animals[38, 264]	Mineralocorticoid receptor knockout mice[366]
Running[95, 96]	PCPA[348, 367]
Dietary restriction[368, 369]	Nicotine[370]
Dehydroepiandrosterone, subcutaneous[109]	Cortical contusion[327]
FGF-2, subcutaneous[114]	Irradiation[371]
IGF-1, peripheral[115]	Presenilin-1 knockout mice had no effect with enriched environment[372]
IGF-1, ICV in aged animals[116]	IL-6, transgene[373]
VEGF[299]	E2F1-knockout mice[374]
Chronic antidepressant treatment[375]	
Transient global ischemia, gerbil/rat[337, 338]	
Seizures[21, 332-335]	
Electro-convulsive shock seizures[376, 377]	
Traumatic brain injury[326]	
Mossy fiber stimulation, LTP[378]	
Rolipram, inhibitor of cAMP breakdown[287]	
Overexpression of wildtype presenilin-1[379]	

Interventions influencing Proliferation in SVZ	
Up-regulate	**Down-regulate**
Estrogen, prarie vole[380]	TGF-□ knockout mice[381]
HB-EGF, ICV[349]	Irradiation[136]
Focal ischemia, rat[142])	Erythropoietin, ICV[382]
Prolonged seizures[336]	Amyloid beta-peptide, ICV[383]
Atypical neuroleptics[384]	
Traumatic brain injury[325]	
OB ablation[385, 386]	
N-acetyl cystein[387]	
Anti-erythropoietin antibody, ICV[382]	
Infusion of ectodomain of either EphB2 or ephrin-B2[388]	
$p27^{Kip1}$ knockout mice[318]	
mCD24 knockout mice[363]	

Interventions influencing Neurogenesis in Olfactory Bulb	
Up-regulate	**Down-regulate**
Enriched odor exposure[94]	EGF, ICV[18]
FGF-2, ICV[18]	Nasal plug[12]
FGF-2, subcutaneous[114]	ZnSO4 intranasal perfusion[389]
BDNF, ICV[320]	Anti-erythropoietin antibody, ICV[382]
Adenoviral BDNF overexpression of SVZ cells[319]	E2F1 knockout mice[374]
Erythropoietin, ICV[382]	

Curriculum vitae

Christiana Marie Cooper-Kuhn

Degrees:

Bachelor of Science in Microbiology
Colorado State University, Colorado USA, 1992

Masters of Science in Microbiology
Colorado State University, Colorado USA, 1995
Title: Toxoplasma gondi-specific IgM, IgG, and IgA responses in the serum, saliva, and duodenal secretions of cats following oral inoculation with an oocyst-forming field strain.

Education and Work Experience

August 1988-May 1989 Union College
Lincoln, NE 68506

August 1989-Dec. 1992 Colorado State University
Fort Collins, CO 80523
Bachelor of Science in Microbiology

Research Associate for the Department of Clinical Sciences, Colorado State University. Laboratory Director, Michael R. Lappin, DVM, PhD. My primary responsibilities consisted of laboratory services and research utilizing immunodiagnostic tests. I used immunofluorescent antibody assay, ELISA, and agglutination tests for the diagnosis of *T. gondii*, Rocky Mountain Spotted Fever, and Lyme disease. I assisted in several research projects studying *T. gondii* in felines, resulting in two coauthor publications.

August 1993-June 1995 Colorado State University
Fort Collins, CO 80523
Masters of Science in Microbiology

Graduate Research Associate in the Department of Microbiology, Colorado State University. Principle advisor, Michael R. Lappin, DVM, PhD. My thesis focused on the intestinal immune response of cats to *Toxoplasma gondii* through which I accumulated extensive experience with protein electrophroessis, Western blot immunoassay, purification of oocysts from fecal material, purification of tachyzoites from murine peritoneal fluid, purification of bradyzoites from murine brain tissue,

and cell culture techniques. I have extensive experience with procedures in cats and mice, including anesthesia, duodenal endoscopy, aqueous paracentesis, and jugular venipuncture. My work during this period led to four coauthor publications.

Sept. 1995-Sept. 1996 Institute for Hygiene and Microbiology
University of Würzburg
Würzburg, Germany

Graduate Research Associate for the Institute for Hygiene and Microbiology. Principle Advisor, Dr. med Uwe Groß. My project focused on identifying new bradyzoite-specific genes of *Toxoplasma gondii*. While continuing to use my knowledge of cell culture and animal handling, I also learned techniques involving molecular biology, including PCR, cloning, subcloning, subtractive hybridization, plasmid preparation/purification, cDNA, RNA, and genomic DNA purification.

July 1998-present Department of Neurology
University of Regensburg
Regensburg, Germany
PhD in Neuroscience
Title: Regulation of neurogenesis in the adult mammalian brain.

PhD Student at the University of Regensburg, Klinik und Poliklinik für Neurologie, Germany. Principle Advisor, Prof. Dr. med. Jürgen Winkler und Prof. Dr. rer. nat. Stephan Schneuwly. My dissertation focuses on cellular and molecular influences on neurogenesis in the adult rodent. I have accumulated knowledge on the mechanisms of proliferation and differentiation and have been involved in trying to understand the fate of neural stem cells. I have gained experience in the following techniques: immunohistochemical staining, confocal microscopy, stereological analysis, in situ hybridization, microtome and cryostat tissue sectioning, rat and mouse perfusions and the removal of brains for histology, setting up a mouse breeding colony for single and double knockouts and performing genomic DNA isolations and PCR for genotyping, as well as extensive experience in performing surgeries on rats, involving the administration of immunotoxins into the lateral ventricle, as well as into the parenchyma.

Literature Lists:

Original Publications:

1. Lappin MR, Cayatte S, Powell CC, Gigliotti A, **Cooper C**, Roberts SM (1993) *Detection of Toxoplasma gondii antigen-containing immune complexes in the serum of cats.* Am J Vet Res 54:415-419.
2. Chavkin MJ, Lappin MR, Powell CC, **Cooper CM**, Munana KR, Howard LH (1994) *Toxoplasma gondii-specific antibodies in the aqueous humor of cats with toxoplasmosis.* Am J Vet Res 55:1244-1249.
3. Burney DP, Lappin MR, **Cooper C**, Spilker MM (1995) *Detection of Toxoplasma gondii-specific IgA in the serum of cats.* Am J Vet Res 56:769-773.
4. Cannizzo KL, Lappin MR, **Cooper CM**, Dubey JP (1996) *Toxoplasma gondii antigen recognition by serum immunoglobulins M, G, and A of queens and their neonatally infected kittens.* Am J Vet Res 57:1327-1330.
5. Lappin MR, Chavkin MJ, Munana KR, **Cooper CM** (1996) *Feline ocular and cerebrospinal fluid Toxoplasma gondii-specific humoral immune responses following specific and nonspecific immune stimulation.* Vet Immunol Immunopathol 55:23-31.
6. Lappin MR, Ungar B, Brown-Hahn B, **Cooper CM**, Spilker M, Thrall MA, Hill SL, Cheney J, Taton-Allen G (1997) *Enzyme-linked immunosorbent assay for the detection of Cryptosporidium parvum IgG in the serum of cats.* J Parasitol 83:957-960.
7. Biebl, M., **C. M. Cooper**, J. Winkler and H. G. Kuhn (2000). *Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain.* Neurosci Lett 291(1): 17-20.
8. Kohl Z, Kuhn HG, **Cooper-Kuhn CM**, Winkler J, Aigner L, Kempermann G (2002). *Preweaning enrichment has no lasting effects on adult hippocampal neurogenesis in four-month old mice*. Genes, Brain, Behav. 1:46-54.
9. **Cooper-Kuhn CM**, Kuhn HG (2002). *Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain.* Brain Res Dev Brain Res Mar31;134(1-2):13-21.
10. **Cooper-Kuhn CM,** Vroemen M, Brown J, Ye H, Thompson MA, Winkler J, Kuhn HG (2002). *Impaired adult neurogenesis in mice lacking the transcription factor E2F1.* Mol Cell Neurosci Oct;21(2):312-23.
11. Winner B, **Cooper-Kuhn CM,** Aigner R, Winkler J, Kuhn HG (2002). *Long-term survival and cell death of newly generated neurons in the adult rat olfactory bulb.* Eur J Neurosci Nov;16(9):1681-9.

Submitted Manuscripts:

1. Jason Brown, **Christiana M. Cooper-Kuhn**, Sebastien Coulliard-Despres, Jürgen Winkler, Ludwig Aigner, H. Georg Kuhn. *Transient expression of doublecortin (DCX) in immature neurons of the adult dentate gyrus and olfactory bulb.* (J Comp Neurol, accepted for publication)
2. Anne Schänzer, **Christiana M. Cooper-Kuhn**, Till Acker, Heike Beck, Karl-H. Plate and H. Georg Kuhn. *In vivo activation of the vascular endothelial growth factor receptor flk-1 leads to increased neurogenesis in the adult rodent brain.* (Nature, in review)
3. Jason Brown, **Christiana M. Cooper-Kuhn**, Gerd Kempermann, Henriette van Praag, Jürgen Winkler, Fred H. Gage and H. Georg Kuhn. *Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis.* (Eur. J. Neurosci.)

Manuscripts in Preparation:

1. Jochen Klucken, **Christiana M. Cooper-Kuhn**, Jixiang Xu, Jürgen Winkler, Moses Chao and H. Georg Kuhn. Elevated stem cell proliferation and neurogenesis in adult p27kip deficient mice.

Scientific Meetings:

1. **Cooper CM**, Ramirez G, Kempermann G, Gage FH, Winkler J, Kuhn HG (1998) *Growth factors induce proliferation and differentiation of neural progenitors in the adult brain.* Eur. J. Neurosci. 10(Suppl.10):226.
2. Kuhn HG, **Cooper CM**, Kempermann G, Gage FH, Winkler J (1998) *Growth factor effects on proliferation and differentiation of neural progenitors in adult rat brain.* Soc Neurosci. Abstr. 24:784.
3. Kohl Z, Kuhn HG, **Cooper C**, Winkler J, Aigner L, Kempermann G (2000) *No lasting effects of preweaning enrichment on size of the dentate gyrus and adult hippocampal neurogenesis.* Soc. Neurosci. Abstr. 26:54.
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Workshops

1. Student at the **Route28 Summits in Neurobiology** “Therapeutic Potentials of Neural Stem Cells in the Adult CNS”, August 1999, Port Ludlow, WA, USA.
2. Organizer of the **Route 28 Summits in Neurobiology** “The Id of Stem Cells: Redefining stem cell idenetity and plasticity for CNS repair”, August 2001, Port Ludlow, WA, USA.
3. Organizer of the **Route 28 Summits in Neurobiology** “(Stem) cell based therapeutic approaches for Parkinson’s disease”, August 2002, Frauenchiemsee, Germany.

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ACKNOWLEDGEMENTS

When I started in Jürgen Winkler's and Georg Kuhn's laboratory, I never dreamed that it would amount to this. I am deeply indebted to Prof. Dr. med. Jürgen Winkler for his encouragement, guidance, mentoring, and research support throughout these studies. I also appreciate his patience and tolerance through my numerous mishaps. The work accomplished in this dissertation is a testimony to the foresight and perseverance of these two great scientists and role models.

My gratitude goes out to the chair of our department, Prof. Dr. med. Ulrich Bogdahn, for being so supportive of the research and for creating a friendly and positive work environment. I am fortunate to have the opportunity to work with a group of energetic people in the Neuroregeneration Lab. I appreciate all their friendships and their collective encouragement to finish this dissertation. I especially want to thank Ludwig Aigner, Robert Aigner, Manfred Biebl, Sebastian Coulliard-Despres, Maren Engelhardt, Claudia Karl, Kathrin Pfeifer, Sonja Plötz, Johanna Tebbing, Maurice Vroemen, Frank-Peter Wachs, Norbert Weidner, Daniel Wilhelm, Beate Winner, Petra Wise, and my collaborators, Jason Brown and Anne Schänzer.

I'd like to express my appreciation to Prof. Stephan Schneuwly for his willingness to be my advisor in the Department of Biology and for the time and energy that he has provided.

To Gerd Kempermann, Phil Horner and Theo Palmer I am forever grateful for giving me the opportunity to expand my horizons and to help with the organization of Route28. Thank you for treating me as your equal.

Without my family and friends, my successes in life would amount to nothing. I am grateful to my parent's never-ending belief in me and their unconditional love and for providing me with the opportunities in my life that have led me to where I am. To my friends and other "family" - Kerri Anthony, Matthew Cooper, Tricia Johnson, Alexandra Siedhoff, Melissa Brewer, Anne Weidner, Sabine Heiming and Nicole Bücheler – your friendships have been the most powerful source of inspiration and encouragement.

This dissertation has been a journey on a long and winding road, which I never could have surmounted without the love, support and encouragement from my friend, colleague and husband, Georg. Thanks for traveling with me.

And to my daughter, Hannah Riley, I dedicate this piece of work. Without you I would have finished quicker, but through you I've gained a whole new meaning to life, which makes me even more appreciative of this achievement.

"Is it possible after all, that in spite of bricks and shaven faces, this world we live in is brimmed with wonders, and I and all mankind, beneath our garbs of commonplaceness, conceal enigmas that the stars themselves and perhaps the highest seraphim cannot resolve?" – Herman Melville