

**PSYCHOSOCIAL INFLUENCES ON THE  
DYNAMICS OF ADULT HIPPOCAMPAL  
NEUROGENESIS**

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Disputation am: 27.01.2012

*Für meine geliebte Familie*

*Mit euch erforsche ich die Welt jeden Tag ein Stückchen mehr!*



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## 1. Introduction

*„Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers the nerve paths are something fixed, ended, and immutable: everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.“ (Ramón y Cajal, 1913).*

The „Father of neuroscience“ (DeFelipe, 2002), Santiago Ramón y Cajal, investigated the degenerative and regenerative capacities of the central nervous system at the beginning of the 20<sup>th</sup> century. Although his new and innovative science was ground-breaking some of his conclusions deserved to be reconsidered.

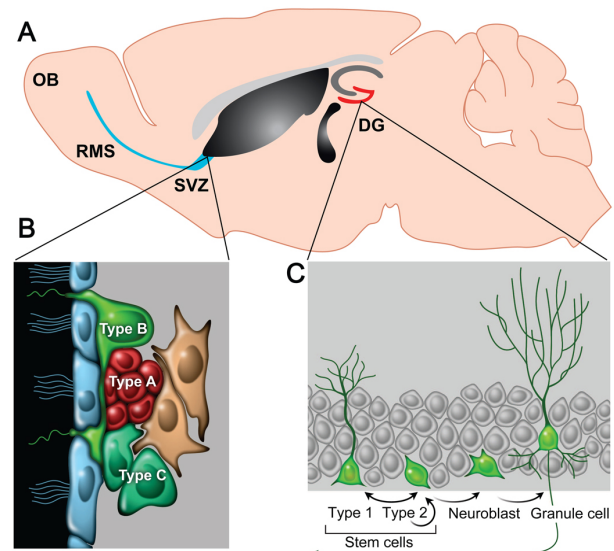
Only half a century ago new methods enabled the discovery of adult neurogenesis by Joseph Altman (Altman, 1962). Namely, he showed that new neurons are generated not only in the embryonic and early postnatal brain, but also in the adult rodent and cat brain (Altman and Das, 1965, 1967, Altman, 1969). It took another decade until Michael Kaplan could reproduce these first findings on adult neurogenesis under usage of electron microscopy, followed by others (Kaplan and Hinds, 1977, Stahnisch and Nitsch, 2002, Colucci-D'Amato et al., 2006). Since then several regions of the brain have been shown to generate new neurons in the postnatal and adult period in higher vertebrates including birds (Goldman and Nottebohm, 1983, Doetsch and Scharff, 2001), reptiles (Lopez-Garcia et al., 1990), fish (Zupanc and Sirbulescu, 2011), rodents (Altman and Das, 1965, Kaplan and Hinds, 1977), primates (Gould et al., 1999b, Gould et al., 1999c, Gould, 2007) and humans (Eriksson et al., 1998, Knoth et al., 2010).

In the healthy mammalian brain under physiological conditions active neurogenesis seems restricted to the subventricular zone (SVZ) (Altman and Das, 1966, Altman, 1969) and the subgranular zone (SGZ) of the dentate gyrus (Altman and Das, 1965, Kaplan and Bell, 1984) in vivo as shown in FIGURE 1-1 (Mu et al., 2010).

The dentate gyrus (DG) is one important part of the hippocampus, receiving input from the enthorinal cortex via the perforant path and connecting via the Mossy fibers to CA3. The hippocampus itself is located in the medial temporal lobe, participating in important

functions via connections to Prefrontal Cortex, Nucleus accumbens, Hypothalamus and Amygdala. The hippocampal structure is critically important for the formation of semantic memory (Squire et al., 2004), but also participates in regulation of stress response via the HPA axis (Herman et al., 1989) and several other brain tasks.

**FIGURE 1-1:** Neurogenic niches in the adult rodent brain (A) Adult Neural stem cells are primarily located in two germinal zones of the brain: the SVZ of the lateral ventricles and the SGZ of the hippocampal dentate gyrus (DG). (C) In the adult SGZ, a population of GFAP<sup>+</sup>, Nestin<sup>+</sup>, SOX2<sup>+</sup> radial cells (Type-1 cells) gives rise to actively proliferating, mainly GFAP<sup>-</sup>, Nestin<sup>+</sup>, SOX2<sup>+</sup>, nonradial neural stem cells (type 2a cells) that generate both GFAP<sup>+</sup> astrocytes as well as neuroblasts (type-2b and -3 cells). Neuroblasts then migrate into the granule cell layer and mature into neurons. (Adapted from (Mu et al., 2010))



### 1.1. Adult Hippocampal Neurogenesis (ahNG)

In adult mammals one focus of neurogenesis research is the SGZ of the dentate gyrus in the Hippocampus (Kaplan and Hinds, 1977, Eriksson et al., 1998). In this zone granule cell neurons are generated throughout life from a population of stem and progenitor cells (Altman and Das, 1965, Kaplan and Hinds, 1977, Cameron et al., 1993, Eriksson, 2003). This process is usually divided into four distinct processes. During *proliferation* stem and progenitor cells divide rapidly, followed by a passage through distinct *differentiation* stages. Within a first selection process most newborn cells undergo apoptosis (Biebl et al., 2000, Kuhn et al., 2005), while few migrate and reach afterwards the *survival* stage. Last but not least the new granule cell neurons undergo *maturation /functional integration* into the hippocampal network (van Praag et al., 2002, Schmidt-Hieber et al., 2004, Hattiangady and Shetty, 2008, Kempermann, 2011b). As displayed in FIGURE 1-2 this different features of adult hippocampal neurogenesis (ahNG) also affect different cell types (FIGURE 1-2). Typically proliferation studies investigate effects on stem and

## Introduction - Adult Hippocampal Neurogenesis (ahNG)

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progenitor cells in the adult dentate gyrus, while survival studies focus on immature and mature granule cells. Differentiation focuses on the whole process from stem cell towards functionally integrated neuron.

Of all neurogenic regions in the mammalian brain more studies focused on the hippocampus than on any other area (668 hits at 'Pubmed' for "*adult neurogenesis*" AND *hippocampus* and 253 hits for "*adult neurogenesis*" AND "*subventricular zone*" at 7<sup>th</sup> of December 2011 (<http://www.ncbi.nlm.nih.gov/pubmed>)). This special interest in ahNG derives from its major role in important cognitive functions such as learning, memory consolidation and spatial navigation (Barnea and Nottebohm, 1996, Gould et al., 1999a, Feng et al., 2001, Shors et al., 2001, Leuner et al., 2004, Sahay et al., 2011). Also the cellular adaption within the dentate gyrus seems quite huge compared to other brain regions. Every month 250.000 new neurons are incorporated into the rodent dentate gyrus summing up to 6% of its total cell number (Cameron and McKay, 2001). Although it has been claimed that there is no real turnover, new neurons are constantly added and the elimination process does not depend on the age of the neurons (Crespo et al., 1986). The constant cellular changes caused by adult neurogenesis is one of the reasons for major structural alterations in different neurological and psychiatric disease, for example epilepsy (Parent et al., 1997, Gong et al., 2007, Parent, 2007), Alzheimer's disease (Rodriguez and Verkhratsky, 2011) Parkinson's disease (Arias-Carrion et al., 2007, Geraerts et al., 2007), depression (Jacobs et al., 2000b, Malberg et al., 2000, Kempermann, 2002, Drew and Hen, 2007, Perera et al., 2011, Snyder et al., 2011) and others (for review see (Steiner et al., 2006b, Winner et al., 2011)). Adjacent to the involvement of adult hippocampal neurogenesis in several diseases is this process generally assumed to be driven by endogenous factors produced during neurodegeneration (Yoneyama et al., 2011).

The role of the hippocampus for healthy brain function seems strongly correlated to the function of adult neurogenesis and is mainly associated with either a reduction or an increase in ahNG (Samuels and Hen, 2011). Most importantly, ahNG is strongly involved in learning and memory consolidation (Gould et al., 1999a, Shors et al., 2001, Jessberger et al., 2009, Appleby et al., 2011a). Beside many known functional implications latest studies also emphasize a role of adult neurogenesis in social, especially reproductive behavior in mammals (Stranahan et al., 2006, Mak et al., 2007, Snyder et al., 2009, Mak and Weiss, 2010, Glasper et al., 2011, Snyder et al., 2011).

Social behavior not only allows survival in a complex structured society, but is also necessary for individual health as described later on. In the context of ahNG, social interactions rely on flexible adaptations to hierarchical structures, memory and learning of complex behavioral rules. To understand the interdependency of ahNG, social features and medical implications, deep comprehension of the differentiation process from stem cell to neuron is essential.

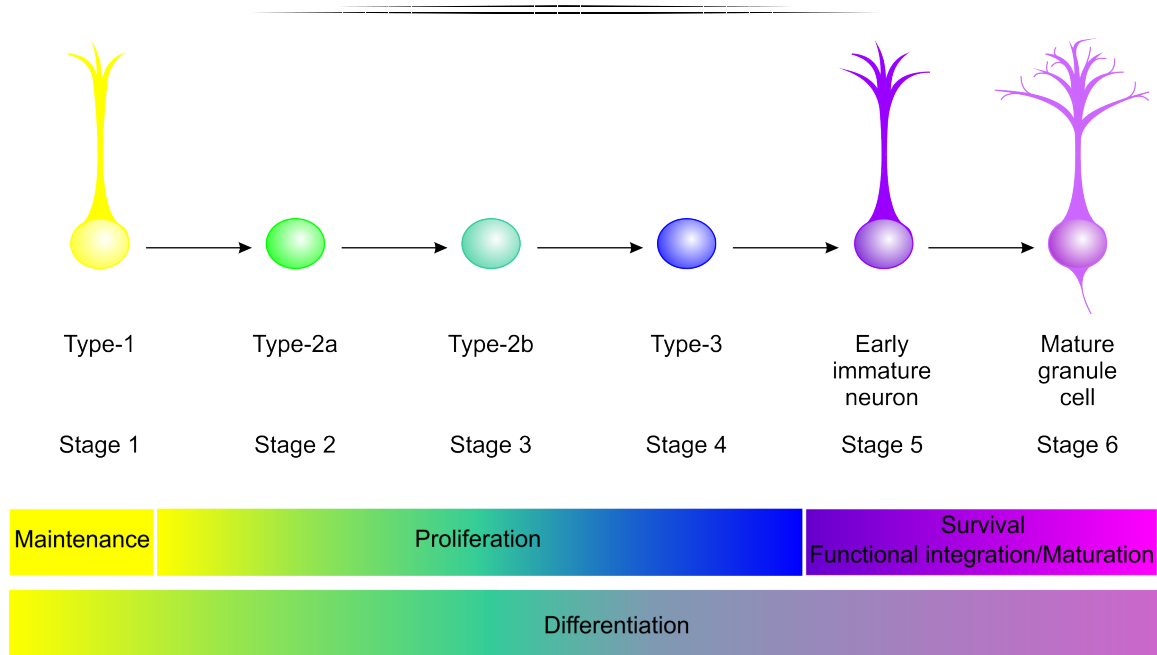
### ***1.2. The different phases of ahNG***

To understand the function and mechanisms of ahNG, detailed information about its dynamics, composition and architecture are required (Seri et al., 2004).

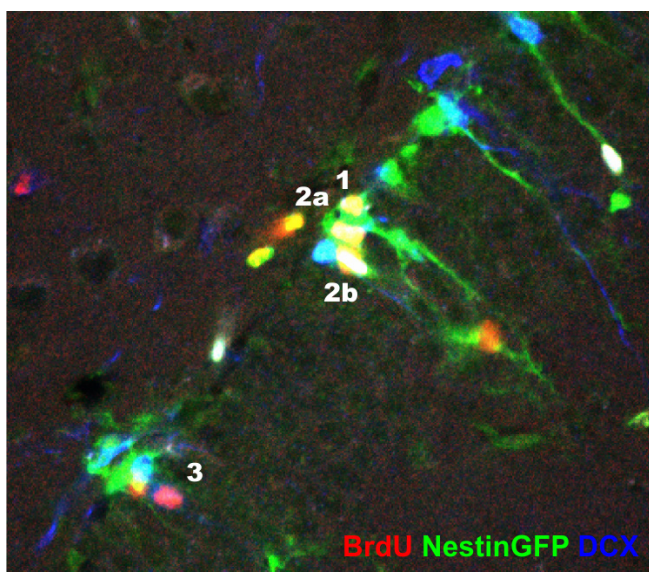
Besides methodological convergence on either proliferation or survival paradigms it is possible to distinguish cells within specific phases of differentiation from each other, even though the complete process has not been understood, yet. In general dividing radial glia like stem cells (type-1 cells) give rise to precursor/stem cells (type-2a cells), which proliferate in the SGZ and give rise to daughter cells (type-2b cells). These neuroblasts (type-2b and -3) migrate a short distance within the granule cell layer (Seki and Arai, 1993, Kuhn et al., 1996). Afterwards they start to differentiate through distinct stages into granule cells (Kempermann et al., 2003, Plumpe et al., 2006) and project an axon to the Cornu Ammonis region 3 (CA3) of the hippocampus (Markakis and Gage, 1999). Four to seven weeks after birth, these newly generated neurons are integrated into the hippocampal circuitry (Jessberger and Kempermann, 2003). Thus, the granule cells evolved within the process of adult neurogenesis become functional (Song et al., 2002, van Praag et al., 2002).

Nomenclature of the different cell types involved in ahNG strongly varies (TABLE 1-B). In my thesis I thus stay with the terminology used in previous publications from Kempermann et al. (e.g. 2004). Therefore, new neurons originate from type-1 cells, pass through the mitotic type-2 and type-3 cell stages, until they express postmitotic markers and characteristics (FIGURE 1-2 and 1-3, TABLE-1-A). Previous models of ahNG assume, that the various differentiation stages precede linear after each other, causing a flexible hippocampal network, which can adapt to the needs of the system, e.g. for manners of memory formation (Kempermann et al., 2004, Appleby et al., 2011b).

## Introduction - The different phases of ahNG



**FIGURE 1-2:** Existing model of differentiation in ahNG. Six stages of neuronal development in the adult hippocampus can be readily identified on the basis of morphology, proliferative ability, and expression of markers (TABLE 1-A). The different cell types participating in ahNG all possess distinct features (TABLE 1-B). Previously assumed neuronal development originates from the putative stem cell (type-1 cell; stage 1). Neuronal development then progresses over three stages of putative transiently amplifying progenitor cells (type-2a, type-2b and type-3 cells; stages 2–4), followed by a transient early postmitotic period, characterized by Calretinin expression (stage 5). Image combines figures from (Kempermann et al., 2004, Kempermann, 2011b).



**FIGURE 1-3:** Overview of cells within the different phases of differentiation.

Type-1 cells (1) are thought to be the origin of ahNG. They give rise to type-2a cells (2a) located in the subgranular zone. Their daughter cells, type-2b cells express not only Nestin and SOX2, but also migration factor DCX. Type-3 cells lack NestinGFP. They grow first processes through the granular layer of the dentate gyrus.



**TABLE 1-A:** Overview of the markers used to identify cells within the different phases of ahNG.

Crosses indicate expression within the specific cell type and key references are given for each marker. Type-1 cells can be identified by astrocytic marker GFAP (GFAP stands for Glial fibrillary acidic protein), NestinGFP and / or SOX2, while type-2a cells typically only express SOX2 and NestinGFP, although it has been reported that they rarely also express GFAP. Type-2b cells are usually identified by SOX2 / NestinGFP and doublecortin (DCX) expression. Migration factor DCX also labels type-3 cells and immature neurons. The latter additionally express Calretinin (CR) and Neuronal Nuclei clone A60 (NeuN). Mature granule cells lack CR, but express besides NeuN also Calbindin (not used in this study) as soon as they are functionally integrated.

Marker	type-1	type-2a	type-2b	type-3	Early Immature Neurons	Mature Granule Cells	Reference
NestinGFP	+	+	+				(Yamaguchi et al., 2000, Kronenberg et al., 2003)
GFAP	+	+/-					(Seri et al., 2001, Filippov et al., 2003, Steiner et al., 2004)
SOX2	+	+	(+)				(Steiner et al., 2006a, Lugert et al., 2010)
DCX			+	+	+		(Brown et al., 2003b, Rao and Shetty, 2004, Couillard-Despres et al., 2005, Plumpe et al., 2006, Suh et al., 2007)
CR					+		(Brandt et al., 2003)
NeuN					+	+	(Kempermann et al., 2003, Kempermann et al., 2004)

### 1.2.1. Type-1 cells: Neural stem cells



Type-1  
Stage 1

In the past “astrocytes” of the dentate gyrus were assumed as origin only for gliogenesis and, thus, as gliogenic progenitor cells (Kaplan and Bell, 1984, Cameron et al., 1993, Palmer et al., 2000). In 2001 and 2004 Seri and colleagues showed that glial fibrillary acid protein (GFAP) - positive astrocytes can also give rise to hippocampal granule cell neurons (Seri et al., 2001, Seri et al., 2004). Further studies confirmed the necessity of GFAP-positive type-1 cells as stem cells in the process of adult neurogenesis in various brain areas both in vivo and in vitro (Garcia et al., 2004). Studies, using neural stem cells isolated from adult brains of men and rodents, point to importance of these cell type for adult neurogenesis (Palmer et al., 1999, Uchida et al., 2000, Imura et al., 2006). Since in vitro

experiments yield some methodological problems (e.g. the culture conditions themselves influence stem and precursor cell identity) I will refer mostly to in vivo studies further on.

Since then dentate gyrus adult neurogenesis is thought to originate from neural stem cells. These type-1 cells, named also radial astrocytes or quiescent neural progenitors, show a characteristic morphology: a triangle-shaped soma, long and strong apical processes, spanning the entire granular cell layer. Additionally, the neural stem cells possess astrocytic properties (Seri et al., 2001, Filippov et al., 2003, Kronenberg et al., 2003, Kempermann et al., 2004, Seri et al., 2004, Encinas et al., 2006).

Type-1 cells are usually identified by their unique morphology and specific marker expression. Besides GFAP and other markers, they also express Nestin and SOX2 (Kempermann et al., 2004, Steiner et al., 2006a, Encinas and Enikolopov, 2008). Nestin is a class VI intermediate filament protein expressed in the developing central nervous system (CNS) in early embryonic neuroepithelial stem cells (Lendahl et al., 1990). This protein has been widely used as a marker for stem / progenitor cells, glioma cells, and tumor endothelial cells in the mammalian CNS. On the other hand sex determining region-Y-box 2 protein (SOX2) is involved in the regulation of embryonic development, determination of cell fate and used as a typical marker in embryonic stem cells and since recently also for precursor cells in postnatal and adult neurogenesis. The SOX gene family is strongly involved in transcription and plays a dominant role in neural stem cell

activity. SOX2 regulates the maintenance of proliferation in NSC by repressing GFAP transcription (Cavallaro et al., 2008, Mu et al., 2010).

Since type-1 cells and astrocytes share some common properties, it is essential to clearly differentiate between these two cell types. Besides morphological differences also markers can be used for identification. Morphologically the main difference lies within the position of the process. In astrocytes located in the subgranular zone the processes mainly proceed parallel, horizontally towards the granular zone, while the process of type-1 cells pervades the granular zone and runs radial. By means of marker expression, both cell types can be labeled with GFAP in the SGZ of mice, whereas only postmitotic astrocytes express S100 $\beta$  (Steiner et al., 2004).


Some dispute within the scientific community focuses on the potency of type-1 cells. Some researchers claim these cells to be only progenitor cells, other assume them to be stem cells (Palmer et al., 1999, Seaberg and van der Kooy, 2002, Bonaguidi et al., 2011). By definition, adult (somatic) stem cells must be undifferentiated, possess the ability to self-renew and to differentiate into two or more different tissue-specific cell types of one or more lineages. Hence, they are at least multipotent (National Institute of Health, Stem Cell Information (<http://stemcells.nih.gov/>)). Neural stem cells fulfill most of these requirements: they are able to self-renew and differentiate into neurons, astrocytes and oligodendrocytes (Gage, 2000). Stem cells are supposed to leave the quiescent G0 state and undergo asymmetric division to maintain the stem cell pool. On the other hand progenitor cells are lineage restricted, and show only limited capability to differentiate and self-renew (Kempermann et al., 2004).

Researchers have shown that stem cells in the subventricular zone (SVZ) can divide asymmetrically (Alvarez-Buylla et al., 2001), but other studies also imply asymmetric division for type-1 cells in the SGZ of the dentate gyrus (Kronenberg et al., 2003). Another study suggested that this asymmetric mode of division could be shifted towards symmetric divisions by treatment with the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Encinas et al., 2006). In this case, asymmetrical division means a division giving rise to two different cell types: one stem cell and one either neuronal or glial progenitor cell. Symmetric divisions, in contrast, generate two equal cells.

Dividing type-1 cells are quite rare within the adult hippocampus compared to other progenitor cells. Only 2% of all mitotic cells labeled with 5-Bromo-2'-deoxyuridine

(BrdU) are type-1 cells (Kronenberg, 2003). Although this could imply a minor role within ahNG, results of previous studies indicate an important value of this differentiation stage towards neurogenesis. With a strong reduction of type-1 cells correlates a strong reduction of the whole ahNG leading to fewer granule cell neurons within the dentate gyrus (Hellstrom et al., 2009). The reasons why only little is known about type-1 cells include too few tools to directly study this differentiation stage (Eisch and Mandyam, 2007, Eisch et al., 2008). In vitro analyses display one possibility to overcome the problem of few samples, but they are usually quite artificial compared to in vivo experiments and, thus, may give results, which not represent nature. By using transgenic mice the resolution of the type-1 cells can be enhanced, thus, newer publications focus more intensively on this first differentiation stage (Encinas et al., 2006, Encinas and Enikolopov, 2008, Bonaguidi et al., 2011, Encinas et al., 2011, Namba et al., 2011). On the one hand, it has been reported this year, that type-1 cells divide symmetrically into astrocytes and therefore disappear with increasing age (Encinas et al., 2011) on the other hand have other groups observed dividing type-1 cells in vitro and in vivo which divide asymmetrically (Bonaguidi et al., 2011, Namba et al., 2011). Additionally, first studies investigate possible influences on the proliferative behavior of type-1 cells. A recent study by Walter et al., for example, found an increase in type-1 cells after stroke (Walter et al., 2010). Still the role of type-1 cells in ahNG is not completely understood and the picture of type-1 cell characteristics remains diffuse. Summing up, based on data available so far, type-1 cells are the stem cells of the adult dentate gyrus that give rise to type-2a cells, the next stage within differentiation, but as all other features of type-1 cells, this is still under exploration and possible redifferentiation from type-2a to type-1 as described by Suh et al. needs to be considered (Suh et al., 2007).

### 1.2.2. Type-2a cells: Amplifying progenitors


 The neuronal progenitor cells in general lack the radial-glia-like process as well as the astrocytic features of type-1 cells. These type-2 cells are located in the adult SGZ and express Nestin and SOX2, just as type-1 cells, but they neither feature a process nor express GFAP (Filippov et al., 2003). Filippov et al. (2003) subdivided NestinGFP expressing type-2 cells electrophysiologically into two subclasses, while Kronenberg et al. showed histologically the existence of type-2a and 2b cells (Kronenberg et al., 2003).

Type-2a  
Stage 2

Type-2 cells that are co-labeled with Doublecortin (DCX) are classified as type-2b cells where SOX2 expression goes down, whereas type-2a cells lack DCX. Since then type-2a cells are assumed to give rise to type-2b cells. Also, Suh, et al. found different subtypes among SOX2-positive cells (Suh et al., 2007). Type-2 cells have the potential to self-renew and generate both neurons and astrocytes (Steiner et al., 2006a). Roughly 89% of these SOX2-positive cells differentiate into neurons, while approximately 7% became astrocytes (Suh et al., 2007). These features shared with type-1 cells indicate a possible lineage relationship between type-1 and type-2 cells (Mu et al., 2010). Since the total number of SOX2-positive cells remains constant, only the ratio of BrdU labeled SOX2-positive cells increases, it is assumed, that these cells also divide asymmetrically similar to type-1 cells (Suh et al., 2007).

Transient amplifying precursor cells (type-2a cells) arise from type-1 cells. These type-2a cells are believed to be one of the cell types highly regulated within the process of ahNG. It has for example been shown, that the population of SOX2-positive cells proliferates strongly with increased exercise leading to an increased population of DCX-positive type-2b and type-3 cells as well as to more granule cell neurons (Kronenberg et al., 2003, Steiner et al., 2008). Also other factors such as environment, but also genetic and molecular influences, for example neurotransmitter, e.g. gamma-Amino butyric acid (GABA), highly affect the proliferation rate of type-2a cells besides other differentiation stages (Cameron et al., 1995, Brezun and Daszuta, 1999, Wang et al., 2005, Kempermann et al., 2006, Masuda et al., 2011).

### 1.2.3. Type-2b cells: Neuroblasts-1

 In general type-2 cell stages can be clearly differentiated from type-1 cells, not only in the mean of markers, but also by morphology and electrophysiology (Filippov et al., 2003). These cells are identified by a small soma and an irregularly shaped nucleus. Additionally, type-2 cells have plump, short processes which are orientated parallel to the subgranular zone (Keiner et al., 2010). Type-2b cells particularly express Nestin, SOX2 and DCX, besides other markers (Kempermann et al., 2004, Encinas and Enikolopov, 2008). DCX is widely used as a marker for migrating neurons. This factor undergoes a cycle of dephosphorylation and phosphorylation that regulates its binding to microtubules in growing neuritis, migrating neuroblasts and neurons (Francis et al., 1999,

Type-2b  
Stage 3

Gleeson et al., 1999, Coquelle et al., 2006). Most type-2b cells are still located within the subgranular zone, but the expression of migration factor DCX implies first migrating behavior. Also type-2b cells are the first ones to express Prox1, a strong sign for neurogenic differentiation (Steiner et al., 2008). Thus, type-2b cells are progenitor cells with lineage determination. That means SOX2 positive type-2b cells only proliferate to give rise to new neurons, but are not origins of gliogenesis anymore (Wegner and Stolt, 2005, Suh et al., 2007). Prox1 seems, additionally, to enforce granule cell maturation during development, besides the maintenance of neuronal progenitors in adult neurogenesis (Lavado et al., 2010). The same study also found first indications for enhanced apoptosis in absence of Prox1, but even more important, the absence of type-2b cells caused by depletion of Prox1 led to a strong reduction in the number of type-1 cells.

The type-2b differentiation stadium is particularly vulnerable to different kinds of stimuli. Steiner and colleagues (2008) for example described the effects of short-term exercise, enrichment (ENR) and kainic acid (KA) on this differentiation stage. They found all stimuli effective on this stage of ahNG even after a short period of 24 hours (Steiner et al., 2008). Also a previous study indicated an effect on the transition towards type-3 cells by seizures, but only type-2a and type-3 cells were analyzed in detail (Jessberger et al., 2005).

### 1.2.4. Type-3 cells: Neuroblasts-2



Type-3  
Stage 4

“Neuroblast-like” Type-3 cells have lost Nestin, but are still DCX-positive (Kronenberg et al., 2003). They can belong to two different cell types. Either they are proliferative type-3 cells, which arise from type-2b cells, or they are already early postmitotic immature granule cells. Type-3 cells are migratory progenitor cells with lineage determination. Therefore they give only rise to immature neurons. The proliferative activity is significantly increased along with the expression of DCX compared to other differentiation stages, especially in aging individuals. While the number of all cell types decreases with age stays the number of type-3 cells stable (Walter et al., 2011). Thus, it is not surprising that this cell stage can even be modulated by short term environmental ENR, but not by only 24 hours of exercise (Steiner et al., 2008). Plümpe et al. demonstrated a high variability in type-3 cell morphology from horizontal to diverse forms of radial processes (Plümpe et al., 2006).

### 1.2.5. Early immature neurons



Early  
immature  
neuron  
Stage 5

The mitotic expansion phase of neuronal development including type-1 to type-3 cells (differentiation stages 1-4, FIGURE 1-2) is followed by a postmitotic differentiation and maturation phase (Brandt et al., 2003). Early immature neurons arise from type-3 cells and project an axon to the CA3 region of the hippocampus (Markakis and Gage, 1999). These cells are already postmitotic, neuronal, but still need to be fully integrated into the hippocampal network. They still express migration factor DCX within the early phase, but additionally also express neuronal marker NeuN (Neuronal Nuclei).

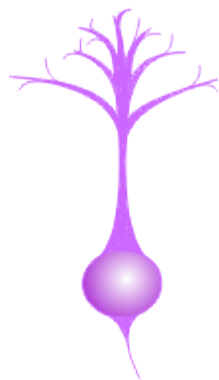
Furthermore these cells can be identified by transient expression of the calcium-binding protein Calretinin (Brandt et al., 2003). Immature granule cells have left the cell cycle and can therefore only further differentiate into mature granule cells (Kempermann et al., 2004).

The survival of new neurons is determined during the first 3 weeks after their birth (Kempermann et al., 2003) and is strongly regulated by surrounding neuronal network activity (Tashiro et al., 2006, Keiner et al., 2010) Neurons that have survived this period and further differentiate into mature granule cells are completely incorporated into the hippocampal network (Dayer et al., 2003).

As mentioned above, the number of precursor cells in ahNG is expanded in an activity-dependent manner (Kronenberg et al., 2003). Cells from this expanded pool differentiate into early immature neurons, shown by Calretinin expression and advancing extension of dendrites and axons (Brandt et al., 2003). Of these early immature neurons only a subset becomes functionally integrated and persist long-term (Kempermann et al., 2004). Thus, the early postmitotic phase of adult neurogenesis seems to be of particular interest in the context of regulation of neuronal development. At this stage most of the quantitative regulation occurs (Garcia et al., 2004) via distinct factors leading to apoptosis (Kuhn et al., 2005, Lepousez and Lledo, 2011). It has also been hypothesized, that most of the activity-dependent regulation of cell survival occurs at or strongly affects this stage (Kempermann et al., 1997b, Nilsson et al., 1999, Brandt et al., 2003, Kronenberg et al., 2003, Plumpe et al., 2006). The absolute number of Calretinin expressing new neurons can be influenced by stimuli like environmental enrichment (ENR), physical activity

(RUN) and kainic acid-induced hippocampal seizures (KA) assuming a prolongation of the stimulation on proliferation towards survival stages (Brandt et al., 2003). Also molecular factors such as neurotrophic tyrosine kinase receptor type 2 (TrkB) strongly influence the rate of this differentiation stage, but it is not clear yet, what exactly causes this effect (Bergami et al., 2008). We do know, nevertheless, that several neurotrophic factors, like brain derived neurotrophic factor (BDNF), act via bonding to and activation of this receptor. Interestingly, this extracellular signaling factor is included in antidepressant action, enhancing survival rates of newborn neurons (D'Sa and Duman, 2002).

### 1.2.6. Mature granule cells



Mature  
granule  
cell  
Stage 6

Four to seven weeks after birth, new neurons are integrated into the hippocampal circuitry. Thus, these newly generated glutamatergic granule cells become fully functional (Song et al., 2002, van Praag et al., 2002, Jessberger and Kempermann, 2003). This means, they are electrically active, fire action potentials in response to synaptic input, form synapses and release neurotransmitters in response to action potentials (Song et al., 2002). These mature granule cells express another calcium-binding protein: Calbindin, rather than Calretinin, and can also be identified by their NeuN expression (Kempermann et al., 2004). As soon as NeuN-positive cells become functional they project not only dendritic processes into the molecular layer, but also

an axon into the CA3 region of the hippocampus and finally establish synapses (Stanfield and Trice, 1988, Markakis and Gage, 1999, Hastings and Gould, 2003, Toni et al., 2008). At this differentiation stage also some regulation occurs determining their survival or apoptosis. Regulation of survival of the newborn neurons is activity-dependent underlying strong genetic control (Kempermann et al., 1997a, b). Although NeuN is quite a good marker for granule cell neurons, not all cells positive for NeuN also become functionally integrated. To really measure maturity in the way of functional integration one needs to use Calbindin. Nevertheless, to ease the complexity of the differentiation within the postmitotic stages NeuN positive cells, lacking DCX expression are called later on premature granule cells.



**TABLE 1-B:** Overview over the key features of the different cell types participating in ahNG.

	<b>Classification</b>	<b>Mitotic features</b>	<b>Potency</b>	<b>Differentiation stage</b>	<b>Alternative names</b>
<b>Type-1</b>	Putative stem cell	Unlimited / Limited self-renewal	Multi-potent, origin for neurogenesis and gliogenesis	<b>1</b>	Quiescent neuro - progenitor, radial astrocyte (rA), quiescent neural stem cell (qNSC), B-cell
<b>Type-2a</b>	Progenitor cell	Limited self-renewal	Multi-potent	<b>2</b>	Amplifying neuro – progenitor (ANP), active neural stem cells (aNSC), IPC
<b>Type-2b</b>	Progenitor cell	Limited self-renewal	lineage determined	<b>3</b>	Neuroblast-1 (NB), D1-cell
<b>Type-3</b>	Progenitor cell	Limited self-renewal	lineage determined	<b>4</b>	Neuroblast-2 (NB), D2 and D3-cell
<b>Early Immature Neurons</b>	Neuron	Post- mitotic		<b>5</b>	Immature granule cell / neuron
<b>Mature Granule Cells</b>	Neuron	Post- mitotic		<b>6</b>	Granule cell, NGN, G-cell
<b>References</b>	(Kempermann et al., 2004)	(Kempermann et al., 2004, Encinas et al., 2011)	(Kempermann et al., 2004)	(Kempermann et al., 2004)	(Seri et al., 2001, Kempermann et al., 2004, Seri et al., 2004, Encinas and Enikolopov, 2008, Ma et al., 2009, Bonaguidi et al., 2011, Encinas et al., 2011)

### ***1.3. Influences on ahNG***

#### **1.3.1. Non-pathological effects**

AhNG is regulated by inputs from the environment and presumably allows a plastic adaptation and fine-tuning of hippocampal circuits to the functional needs across the lifespan. Proliferation and survival of new neurons can be modulated by many factors such as stress (Gould et al., 1997), environmental enrichment (ENR) (Kempermann et al., 1997b), physical activity as running (RUN) (van Praag et al., 1999a, van Praag et al., 1999b), social housing / isolation (Stranahan et al., 2006) and distinct learning tasks (Gould et al., 1999a, Dobrossy et al., 2003, Leuner et al., 2004, Hairston et al., 2005). An experiment coupling the spacing effect of learning with adult neurogenesis has shown strong effect, that learning over time not only increases memory, but also survival of new neurons in the adult dentate gyrus (Sisti et al., 2007). Also several further studies gave strong evidence on the correlation between ahNG and memory consolidation. Most impressively it was shown that depletion of newly generated neurons also leads to depletion of newly acquired memories (Arruda-Carvalho et al., 2011).

All of the above mentioned influences on ahNG either vary proliferation or survival or both. Exercise is a typically example of a stimulus mainly enhancing proliferation, while ENR mainly increases the survival of new neurons. A major question in the field of neurogenesis focuses on the possibility to couple effects. A recent paper by Fabel and colleagues for example shows nicely an additive effects of RUN and environmental enrichment (ENR) on adult neurogenesis (Fabel et al., 2009). In order to be able to completely understand, control and regulate adult neurogenesis in the context of medical therapies (see below), one needs to consider reciprocal effects in far more detail.

#### **1.3.2. Pathological effects**

Pathological conditions like seizure (Parent et al., 1997, Gray and Sundstrom, 1998), depression (Jacobs et al., 2000a, Malberg et al., 2000, Kempermann, 2002, Czeh and Lucassen, 2007), Alzheimer's disease (Jin et al., 2004), following alcohol addiction (Nixon and Crews, 2002, Crews and Nixon, 2003, Nixon, 2006) opiate abuse (Eisch et al., 2000) and cerebral ischemia (Liu et al., 1998, Tanaka et al., 2004, Lichtenwalner and Parent, 2006, Qiu et al., 2007) also have a strong impact on adult neurogenesis both in the

dentate gyrus and the subventricular zone. An overview for the impact of the different factors on hippocampal adult neurogenesis can be seen in **TABLE 1-C**.

**TABLE 1-C:** *Some major factors influencing ahNG*

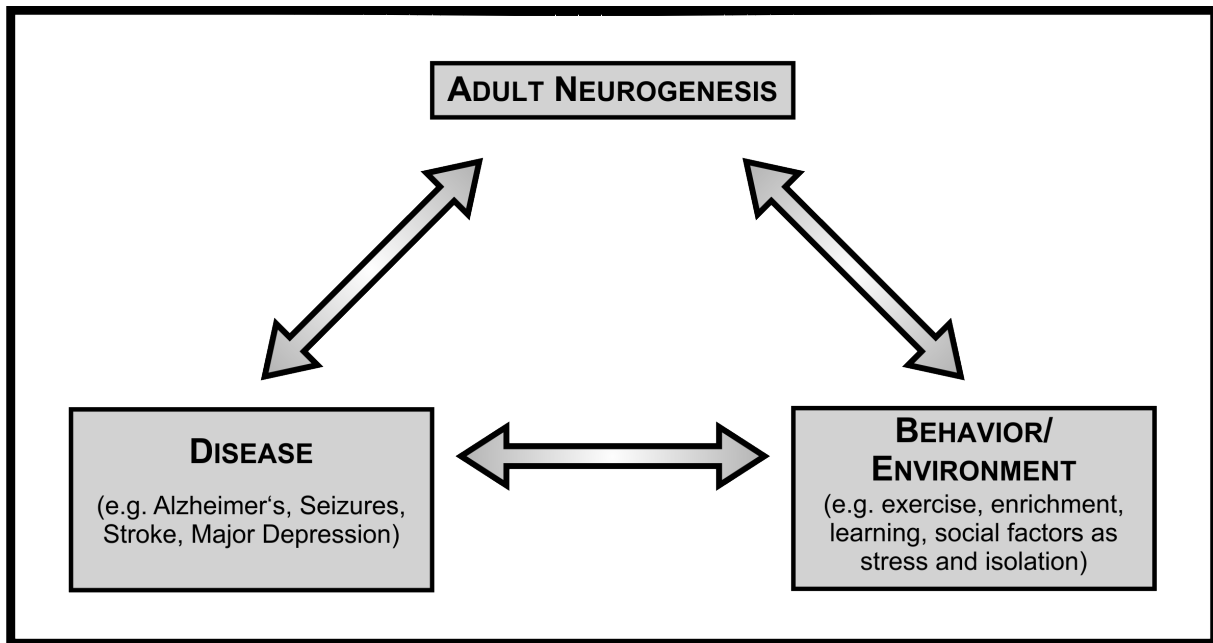
Factor	Proliferation	Survival	Reference
Genetic Background	Yes	Yes	(Kempermann et al., 1997a, Kempermann and Gage, 2002)
Glucocorticoids	Decrease	Decrease	(Cameron and Gould, 1994, Wong and Herbert, 2004)
Stress	Decrease	Contradictory results	(Gould and Tanapat, 1999, Czeh et al., 2001, Mirescu and Gould, 2006)
Depression	Further studies needed	Further studies needed	(Jacobs et al., 2000a, Kempermann and Kronenberg, 2003)
Antidepressants	Increase	Increase	(Duman et al., 2001, D'Sa and Duman, 2002, Encinas et al., 2006)
Social hierarchical structures	No change	Increase (dominant animal)	(Kozorovitskiy and Gould, 2004)
Stroke / Ischemia	Increase	Increase	(Liu et al., 1998, Lichtenwalner and Parent, 2006)
Epilepsy	Increase	Increase	(Parent et al., 1997, Kempermann, 2006)
Isolation	No exact data on isolation alone	No exact data on isolation alone	(Stranahan et al., 2006)

Introduction - Influences on ahNG

Factor	Proliferation	Survival	Reference
Running (RUN)	Increase	Increase	(van Praag et al., 1999a, van Praag et al., 1999b, Holmes et al., 2004)
Enriched Environment (ENR)	No change	Increase	(Kempermann et al., 1997b)
RUN followed by ENR	Not analyzed (n.a.)	Increase	(Wolf, 2007)
Learning over time	n.a.	Increase	(Sisti et al., 2007)
Learning	n.a.	Increase	(Gould et al., 1999a, Leuner et al., 2004)
Water maze learning	Decrease	Depends on time point of generation of progenitor cells	(Leuner et al., 2004, Leuner et al., 2006a, Leuner et al., 2006b, Aztiria et al., 2007, Drapeau et al., 2007)
Associative learning	n.a.	Increase	(Leuner et al., 2004, Leuner et al., 2006a, Leuner et al., 2006b)
Fear learning	Decrease	No change	(Pham et al., 2005)
Sleep deprivation	Decrease followed by over compensation	n.a.	(Mirescu et al., 2006)
Aging	Decrease	Decrease	(Kuhn et al., 1996, Klempin and Kempermann, 2007)

### **1.3.3. Correlation between ahNG, disease and behavior**

Behavioral and environmental factors influencing adult neurogenesis also alternate the differentiation process as well as proliferation and survival and functional integration in pathological condition. This indicates a reciprocal effect between diseases, adult neurogenesis, including ahNG, and social / behavioral factors (FIGURE 1-4). To mention some examples of this effect, Komitova showed in 2005, that enriched environment (ENR) increases adult neurogenesis in the subventricular zone (SVZ) after lesions (Komitova et al., 2005), whereas Crews indicated a year earlier, that the inhibition of proliferation by ethanol can be reversed with exercise (Crews et al., 2004). Especially regarding major depression, several new animal models were developed within the last years and examined in the context of ahNG (Fernando and Robbins, 2011). It is known since some time, that patients suffering from major depression own a smaller hippocampus with strongly reduced neurogenesis (Bremner et al., 2000, Videbech and Ravnkilde, 2004, Malykhin et al., 2010). Furthermore even a change in the White / Grey matter distribution seems connected to this psychiatric disease (Abe et al., 2010). Also a correlation between antidepressants and increased adult neurogenesis often accompanied by increased cognitive performance was shown by different research groups (Banar and Duman, 2007, Perera et al., 2011, Surget et al., 2011). Thus, the focus on adult neurogenesis in the context of neurological and psychiatric diseases has been promising.



*FIGURE 1-4: Hypothesized interaction between the different factors: adult Neurogenesis, disease and behavior / environment. The correlation between these different factors has been shown in multiple publications, but it has not been proven yet, that adult neurogenesis has a direct effect on the correlation between disease and behavior. Critical voices claim adult neurogenesis as only a mediator variable, just indirectly affecting this correlation, if not even affecting it at all.*

### 1.3.4. Increased physical activity



External stimuli, such as exercise, also strongly influence rates of ahNG. Mice love exercise; given the chance they spend up to 89% of the dark phase of the day within the running wheel. On average they even run 6.8 km per 24 hours (Kavanau, 1966, Allen et al., 2001). Regarding ahNG running (RUN) has been shown to strongly increase proliferation in various studies. RUN mainly influences type-2a cells, thus, stage 2 of differentiation, causing an enhanced population of type-2b, type-3 and postmitotic cells (Kronenberg et al., 2003, Steiner et al., 2008). The ratio of apoptosis seems to be independent on exercise, thus, the increased proliferation also leads to more new neurons after several weeks, due to an increased pool at the start. In regard to neurological and psychiatric disease exercise, including RUN, strongly optimizes regeneration, for example by increasing oxygen supply towards the whole body, especially the brain. In the

context of moderate and major depression exercise has even been shown to be quite affective as complementary treatment in humans (de la Cerda et al., 2011, Nahas and Sheikh, 2011, Schuch et al., 2011). This way even treatment resistant depression can be improved (Mota-Pereira et al., 2011).

Often exercise is named as one feature of environmental enrichment. Thus, modern studies exclude running wheels from enrichment cages. Still the larger size of the cage gives the animals more space to move, maybe not necessarily increasing their exercise, but their possibility to withdraw from stressors. In respect to ahNG exercise and enrichment cause different changes in ahNG. While RUN mainly affects proliferation, enrichment mainly causes an increased survival rate (Kempermann et al., 1997b, van Praag et al., 1999b). This indicates two different mechanisms (Kronenberg et al., 2003, Fabel et al., 2009).

### **1.3.5. Environmental Enrichment (ENR)**

“Enriched environments are “the complex combination of social and inanimate stimulation” (Rosenzweig and Bennett, 1996) and consist of a large group of animals living in a big cage with exchangeable tunnels, bridges, and other toys. This set up is usually compared to the rather Spartan conditions of regular laboratory housing.” (Kempermann et al., 2010). Thus, the classical concept of enrichment fosters general activity (more space), social interaction (large group sizes), learning (complex special representation) and introduces increased novelty.

Several studies performed within the last two decades proved ENR to be a strong inducer of hippocampal neurogenesis (Kempermann et al., 1997b, 1998b, Kempermann et al., 2002, Brown et al., 2003a, Kronenberg et al., 2003, Hattori et al., 2007, Fabel et al., 2009). This classic set up for enrichment mainly increases survival of newborn neurons to an extraordinary extent, without significantly changing proliferation rates, although an increase in proliferation has been found for the 129/SvJ mouse strain suggesting a strong influence of inheritance (Kempermann et al., 1998a). Cells originated during classic enrichment have even been shown to be involved in memory consolidation (Bruehl-Jungerman et al., 2005). We know that the classic ENR studies have this effect, but we do not know which aspect of enrichment is the key feature! Does only the aggregation of all aspects of this housing condition cause the change in adult neurogenesis, or are single aspects (e.g. social aspects) sufficient to induce the same increase in survival of new

neurons? It could even be possible that the enhancement of ahNG caused by one aspect gets diminished by another one.

Stress, as one example for an effect influencing enrichment possibly, decreases proliferation and survival. Mice housed in an enrichment cage have plenty options of hiding from aggressors and avoiding open fields. Besides the effect of classical enrichment on ahNG it can also ameliorate emotional disturbances induced by psychological stress (Fox et al., 2006, Schloesser et al., 2010). Environmental enrichment endows stress resilience, abating the animal's emotional reactivity to protect it from the consequences of uncontrollable stress exposure (Mohammed et al., 1993, Chapillon et al., 1999, Larsson et al., 2002, Pollak et al., 2008).

Up to now, only few studies focused on the correlation between enrichment, behavior and ahNG. A recent study by Schloesser et al. (2010) explored the effect of enrichment on mice displaying submissive behavioral traits developed during chronic social stress within the social defeat paradigm. They found that enrichment is highly effective in rescuing the behavioral traits in mice with functional adult neurogenesis. Transgenic mice lacking ahNG could not be behaviorally adapted by enrichment indicating functional significance of adult neurogenesis for beneficial behavioral adaptations (Schloesser et al., 2010).

### 1.3.6. Social Behavior



Mice are very social animals; similar to humans they react strongly towards isolation, but also to any kind of aggressive behavior (Vanderlip, 2001). Also both wild living mice as well as laboratory individuals develop complex social structures (Lathe, 2004).

Social behavior can be divided into reproductive behavior and non reproductive behavior. Several studies exploring the behavior of mice in diverse social contexts exist. Some studies also investigate a correlation between abnormal behavior and neurological / psychiatric diseases (Henry, 1982, Malatynska and Knapp, 2005, Matsumoto et al., 2005, Ricceri et al., 2007, Costa-Pinto et al., 2009, Silverman et al., 2010, Fischer and Hammerschmidt, 2011, Karelina and DeVries, 2011, Stuller et al., 2011), but only very few studies focus



on a connection towards ahNG. Social behavior always focuses on the interaction between individuals on the one hand or the selective refusal of interaction, by isolation or restriction of social groups on the other hand. Distinct forms of interaction between mice can be observed: first of all reproductive behavior and any behavior correlated to it, secondly behavior among animals of the same gender, thirdly breeding behavior. All of these kinds of behavior include distinct behavioral patterns including any kind of communication. For mice communication on both the “verbal” and nonverbal level are very important. Sounds produced by mice in distinct situations trigger behavior (Portfors, 2007). For examples do male mice “sing” towards females to convince them to mate with quite high success (Holy and Guo, 2005, Hammerschmidt et al., 2009). Also the connection between mothers and their offspring relies to huge amounts on communication, by sound production as well as nonverbal communication in the broadest sense. Isolated pups call for their mothers in the range of ultrasounds, causing them to retrieve the “lost child” to the nest (D'Amato et al., 2005). In the context of non-reproductive behavior a phenomenon called social buffering has been well described in several species, besides humans. In general it states company reduces stress and allows better recovery from aversive experiences (Kikusui et al., 2006).

Since several years various studies showed a correlation between social impacts and adult neurogenesis within the subventricular zone (SVZ). From the SVZ in the lateral wall of the lateral ventricle stem cells migrate with the rostral migratory stream (RMS) to the olfactory bulb (OB) (Altman and Das, 1966, Altman, 1969). There they differentiate into interneurons and integrate into the granule cell layer and the periglomerular region to modulate the output of projection neurons (Kageyama et al., 2011). For social behavior in mice olfactory input seems quite important, thus, it does not surprise, that most studies in this context focus on SVZ adult neurogenesis. Several sensory stimulations modulate adult neurogenesis in both hippocampus and the SVZ – OB neurogenic system. Odorant cues display an important and best studied item in rodents in this context (Kageyama et al., 2011). Also the dentate gyrus, with its connections towards the amygdala and other important brain regions for social behavior is in this context of high relevance, but much less studied.

Only few studies focused on the impact of social factors on adult neurogenesis within the mammalian hippocampus. It seems that social housing has positive effects on ahNG in

mice over isolation, but a clear effect was only found for an attenuation of the decrease caused by stress (Stranahan et al., 2006). Furthermore, Stranahan also displayed a decreasing effect of RUN under isolation compared to an increase found for group housing. This effect was verified also in 2009 (Leasure and Decker, 2009). Additionally, rats not only show reduced survival rates for ahNG, when kept in isolation, but this decrease is rescued by subsequent group housing (Lu et al., 2003). A recent publication by Dranovsky et al. was even able to demonstrate different effects of isolation on the distinct differentiation stages, while the number of DCX-positive cells (type-2a, -3, immature granule cells) gets strongly diminished is the number of type-1 cells even enhanced guiding to the assumption, that isolation keeps stem cells from differentiation along the neural lineage (Dranovsky et al., 2011).

In the context of medicine social isolation has been shown to cause negative effects. Isolation triggers depressive-like behavior, aggression, hormonal levels and even reversal learning (Uyeno, 1966, Sayegh et al., 1990, Han et al., 2011, Jahng et al., 2011).

A study by Kozorovitskiy and Gould focused on the effect of social hierarchy on ahNG and found higher numbers of BrdU-positive cells in dominant animals than subordinate and controls (Kozorovitskiy and Gould, 2004). Exposure to psychosocial stress by introducing another individual was shown to decrease ahNG in tree shrews (Gould et al., 1997). Stress also seems to influence adult neurogenesis in dependency on increased aggressive behavior. Dominant animals with a short latency to attack intruders have higher ahNG, additionally they react with no decrease of the neurogenesis rate compared to the males with long latency to attack (Veenema et al., 2007). Furthermore, decrease of the survival of newborn neurons caused by chronic psychosocial stress was accompanied by changes in behavior towards a submissive behavior. This behavioral change can be rescued by enrichment, but only in dependency on intact ahNG (Schloesser et al., 2010).

A recent study also found a positive effect of mating behavior on ahNG in mice (Corona et al., 2011). Also indirect evidence leads to this assumption. For instance, Gloria Mak found a correlation between female preferences for odors of dominant males over subordinate animals and adult ones. Furthermore, this study also claimed a correlation of pheromones and proliferation (Mak et al., 2007). Then again exposure to other individuals in voles only caused a short term effect decreasing ahNG after interaction

with the same gender, whereas no long term effect was found after three weeks (Fowler et al., 2002).

Possibly the large increase in ahNG caused by environmental enrichment is partly, maybe even mainly, caused by the fact, that these mice are housed in much bigger groups, usually the group sizes differ by at least a factor of two, sometime enrichment cages even house three times the number of animals per regular standard cage.

### **1.3.7. Depression**

Major Depression is under the fourth leading contributor to the global burden of disease with further increase in the amount of disability adjusted life years (DALY). Within the next year this psychiatric disease is expected to rise towards the second most threatening illness with respect to DALYs (WHO, [http://www.who.int/mental\\_health/management/depression/definition/en/](http://www.who.int/mental_health/management/depression/definition/en/)). Thus, it is most important to understand major depression in order to be able to find the best treatment possible.

Stress displays a major risk factor for development of major depression (Caspi et al., 2003, Risch et al., 2009). Furthermore, it has been shown in several studies that stress of any kind, including psychosocial stress, decreases the proliferation rate of progenitor cells within the adult hippocampus (Mirescu and Gould, 2006). Also with respect to survival of newborn neurons some studies report a reduction (Czeh et al., 2002, Dagate et al., 2011). Additionally, investigations focusing on ahNG, behavior and antidepressant effect showed a correlation. It has even been stated, that intact adult neurogenesis is absolutely required for antidepressant action, e.g. after stress exposure (Santarelli et al., 2003, Surget et al., 2008, Perera et al., 2011). Also in animal models of depression a recent study indicates the necessity of the production of new hippocampal neurons for amelioration of stress-induced behavioral changes by antidepressants (Schloesser et al., 2010). Surprisingly, it was even found that stress coping itself can stimulate ahNG (Lyons et al., 2010). Glucocorticoids, such as corticosterone (CORT), are released in response to stressful experiences and dysregulation of glucocorticoids has been shown to lead to cognitive impairment and depressive phenotypes (Mirescu and Gould, 2006, Snyder et al., 2011). The partial ablation of glucocorticoid receptors (GR) in mice leads to a valuable mouse model of depression (Ridder et al., 2005). Furthermore a very recent study was able to show the necessity of intact adult neurogenesis for normal stress response with respect to both endocrinal and behavioral factors (Snyder et al., 2011).

Thus, depression is one example for a disease linking social behavior and adult neurogenesis.

### ***1.4. Function of ahNG***

The mammalian brain, including human and mouse brain, is highly plastic. Adaptations occur all live long from minor structural changes caused by adaptations in synaptic plasticity, via cellular plasticity towards changes of whole brain structures. AhNG dynamically responds to many extrinsic and intrinsic stimuli and may be important for cognition, behavior, pathophysiology, brain repair and responses to therapies.

#### **1.4.1. Behavior**

Adult mammalian neurogenesis in the dentate gyrus is highly correlated to emotional behavior, control of the HPA axis and memory function besides others (Kempermann, 2008, Snyder et al., 2011). In respect to mating behavior few studies have hypothesized a correlation between hippocampal neurogenesis and successful reproduction (Mak et al., 2007, Leuner et al., 2010, Mak and Weiss, 2010). Although these results are hard to interpret, some correlation still seem to exist, as it has been shown for the subventricular zone (for review see (Lau et al., 2011). Additionally, Oxytocin, a hormone strongly released in mothers during delivery and breast feeding, increases proliferation in the adult dentate gyrus and survival when administered for several days (Leuner et al., 2011). Furthermore this hormone even increases cognitive performance (Tomizawa et al., 2003). Also other cycle dependent hormones correlate with either decrease or increase in ahNG. Additionally, other endocrine factors influence adult neurogenesis regulating anxiety and mood (Dranovsky and Hen, 2006, Ageta et al., 2008, Bergami et al., 2008).

Even though the scientific community just started to unravel this interconnection it seems highly plausible, that survival relevant behavior, on individual as well as species level, is influenced by evolutionary preserved neurogenesis in mammals (for review see (Lau et al., 2011).

#### **1.4.2. Learning and Memory**

In mammals the hippocampus is strongly related to episodic and spatial reference memory. Several studies have reasoned a functional relevance of adult neurogenesis within the hippocampal dentate gyrus for learning and memory. While Fordyce and

Wehner (1993) linked RUN and improved spatial learning performance has Van Praag et al. (1999a) observed that mice living with a RUN wheel showed an increase in adult neurogenesis, improved learning in the Morris water maze, and enhanced long-term potentiation (LTP) at perforant path-dentate gyrus cell synapses. Lambert et al. showed a positive effect of exercise on working memory, while cognitive stimulation did not cause any significant effect (2005). Reduction in newly born hippocampal neurons in adult rats leads to impairment in hippocampal-dependent conditioning tasks in rats, whereas hippocampal-independent tasks are not affected (Shors et al., 2001). Consequently, a correlation between adult neurogenesis, learning and LTP exists. Further subsequent studies evaluated the role of adult born neurons in this manner. Specifically, contextual fear conditioning, spatial learning and novel object recognition are known to be dependent on intact adult neurogenesis, although few studies could not reproduce these effects (for review see (Deng et al., 2010). Nevertheless, the majority of publications indicates a more or less complex connection between ahNG and different forms of learning (Gould et al., 1999a, Dobrossy et al., 2003, Leuner et al., 2004, Snyder et al., 2005, Sisti et al., 2007). To further uncover the impact of neurogenesis did Garthe et al. study mice in a water maze learning task with hidden platform. They discovered the necessity of intact adult neurogenesis for complex search strategies, confirming theoretical assumptions of metric spatial representation in the hippocampus in the context of memory (Garthe et al., 2009). Memory, as well as learning, is crucial for survival and adaptation to environments and social structures. In the past new neurons were assumed to replace old granule cells within the hippocampus in empirical studies and mathematical models (Becker, 2005, Chambers and Conroy, 2007), but recent studies showed, that new granule cell neurons are added to the hippocampal network, rather than replacing older granule cell neurons (Aimone et al., 2009, Appleby and Wiskott, 2009). Theoretical attempts to investigate the function of ahNG recently hypothesized a role of new neurons in avoidance of catastrophic interference (Wiskott et al. 2006). An increase in the number of granule cells avoids maximal, therefore unspecific, connectivity of single cells and increases storage capacity while decreasing signal superposition (Wiskott, 2006) A further study by the same group introduces a theoretical model of ahNG, which indicates a permanent pool of progenitor cells for fast adaptations to new environments. Thus, a low permanent neurogenesis rate is present in the adult brain, which rapidly increases with need. Furthermore, they confirm the best adaptation in case of an additive effect, instead

of replacement by new neurons (Appleby et al., 2011a). In line with this mathematical approach is the neurogenic reserve theory, which states the necessity of ahNG for lifelong adaptations to new environments and new situations (Kempermann, 2008).

Tashiro et al. also found a strong influence of time on the survival and network integration of new neurons in the adult hippocampus. Particularly effective was environmental stimulation three weeks after the administration of BrdU, accordingly three weeks after cell division (Tashiro, 2007). Keeping in mind, that ahNG takes between one and four weeks from stem cell towards maturation of new granule cells and even longer for complete functional integration, this means mathematical models need to be adapted in respect to time course.

### **1.4.3. Medicine**

Several studies confirm a correlation between neurological / psychiatric diseases and ahNG. Thus, stroke (Yagita et al., 2001, Zhu et al., 2004, Wang et al., 2011a), inflammation (Vallieres et al., 2002, Jakubs et al., 2008, Wolf et al., 2009) and Depression (Sahay and Hen, 2007) represent just some examples of medical implications of ahNG. This field of research is not only very broad, but often also quite contradictory. As part of this work only depression is investigated to exemplary show a correlation between adult neurogenesis, behavior and disease. Thus, no further details are given. Nevertheless, several good reviews are described focusing on this theme trying to combine the available data into new models of neurogenesis. In case the interested reader may want to know further detail, the following reviews are recommended: (Eisch et al., 2008, Kempermann et al., 2008, Lazarov and Marr, 2010, Gil-Mohapel et al., 2011)

Overall, the biological data so far is insufficient to determine either the specific function of new hippocampal neurons or how these new neurons are related to synaptic plasticity and hippocampal memory formation. In the scientific community, function of ahNG seems of increasing interest also linking the development of new neurons to advantages in species survival. Nevertheless, the focus shifts more and more towards a general point of view linking genetics, molecular, cellular and behavioral information.

### ***1.5. Research goals and questions***

The discovery, that neurons are continuously generated in the adult hippocampus opened a new field of neuroscientific research, also with respect to the development of therapeutic strategies. The dynamic generation of new granule cells from multipotent stem cells can be regulated by various factors that act on different steps of ahNG. While some impacts only alter the proliferation rates of early progenitors, cause others a strong effect on functional integration of immature neurons. Some features of this process have been investigated, but major questions regarding the exact time course of lineage progression and detailed succession remain open. Thus, it is important to further investigate the differentiation of stem cells along the neurogenic lineage.

In the first part of my thesis I characterize and describe ahNG in greater detail. This is of general interest for further investigations, and the time course of differentiation can give a clearer picture of the underlying mechanisms. How and how often granule cells are generated during adult neurogenesis, differentiate and become integrated into the preexisting hippocampal network is only partially understood.

Proliferation of neural stem / progenitor cells can be detected with the S-phase marker BrdU. Here, I analyze the temporal progression of newly generated cells through all known differentiation stages (Type-1 till (pre-)mature granule cells) at several time points ranging from two hours post injection (p.i.) towards 21 days p.i. This enabled me to perform quantitative and qualitative analyzes of the different stages of neurogenesis.

To date, no single empirical study is available showing all differentiation stages in dentate gyrus neurogenesis at once. Thus, too few data have been generated for a more general model. The aim of the present pseudo-longitudinal study was designed to overcome this problem. More mice, time points and cells were studied than in any previous study. The data obtained in this work will help to clarify the dynamics behind ahNG. To be able to design appropriate studies for further investigations, a theoretical model of adult hippocampal neuronal development will be a powerful tool. This new model shows the kinetics of neurogenesis and will be verified by means of mathematical modeling in the near future.

Research on the adult dentate gyrus gives new perspectives on cell genesis and deepens our understanding of brain functions. The regulation of this process depends on manifold

## Introduction - Research goals and questions

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stimuli / suppressors. In the second part of my thesis, I focus on the influence of behavioral and social interaction, as well as depression state on sequential developmental steps. The aim was to connect the dynamics of neural stem cell biology in the adult dentate gyrus with environmental mechanisms. Social interaction has first priority in the context of human survival and evolution, thus, it is necessary to understand implications of structural brain adaptations on social behavior.

It is known for several years, that both exercise and environmental enrichment have a strong effect on ahNG in mice and other species, but only very limited studies were carried out to analyze the impact of social behavior and / or social enrichment on ahNG in mammals. None on these focused on the changes in dynamics of ahNG. In order to solve some remaining mysteries in this field of research we need to determine why environmental factors have such a huge influence. I here validate a few environmental factors such as group dynamics and auditory stimuli, their influence on cell proliferation, neuronal survival and differentiation. In addition, the effect of major depression as social influenced disease in combination with stress was determined. This states also a change in dynamics of ahNG by accumulation of stressors and rare conditions.



## **2. Results**

### ***2.1. Dynamics of neuronal development in the adult dentate gyrus***

In the first part of my thesis I further determine the differentiation process of adult neurogenesis in detail. Special focus lies on the obtainment of huge data sets by means of high subject number and exact analyzes of all BrdU positive cells within the dentate gyrus by optimal histological methodology. First results regarding the exact differentiation process of new granule cell neurons within the adult dentate gyrus were obtained during my Master's thesis. For the first part of my PhD thesis I revised and expanded this study to achieve a deeper comprehension of the process of differentiation during ahNG. For a better understanding I exhibit part of the results from my Master's thesis in the following section concerning absolute cell counts of BrdU-, DCX-, or CR-positive cells stained with 3,3'-Diaminobenzidine (DAB). To distinguish between previous results and preliminary results of my PhD thesis all novel information is shown with Grey bars or highlighted in Grey in tables and figures. All displayed phenotypic analyses were performed as part of my PhD thesis.

All mice received three intraperitoneal (i.p.) injections of BrdU every six hours on day 0 (see methods). The data collection for this project started 2 hours after the first BrdU injection due to the bioavailability of BrdU of approximately 0.5 - 2 hours (Cameron and McKay, 2001). That enables us to obtain a "snapshot" of all cells that are marked during S-phase at the beginning of the experiment. This first analysis indicates the distribution of cells in specific stages of differentiation, which are labeled with BrdU immediately. The next data set was collected 14 hours after the first BrdU injection (which is two hours from the third BrdU administration), i.e. when BrdU will just be eliminated from the body. Thus, this additional group of animals will serve as a "snapshot" of all stained cells after the third injection. With this additional data, the model of the differentiation process can be further optimized and our understanding deepened.

#### **2.1.1. Quantitative analyses**

##### ***2.1.1.1. Absolute number of BrdU-positive cells***

Groups of animals were perfused at distinct time points after the first BrdU injection to determine the differentiation process of mitotic cells over time. The observed time frame

## Results - Dynamics of neuronal development in the adult dentate gyrus

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includes ten observed time points spanning from 2 hours till 21 days. During my Master's thesis I obtained the absolute number of BrdU-positive cells labeled with DAB. Within the first two days after the first injection the number of BrdU-positive cells rises strongly until it reaches its peak. After day two the cell number slowly decreases to a brief stagnation stage between five days and seven days of differentiation. Between day seven and day ten the total number of BrdU-positive cells decreased rapidly again, followed by a moderate decrease between ten days and three weeks, where the lowest number of BrdU-positive cells is detected (FIGURE 2-1). Detailed information about the absolute cell numbers +/- standard error of the mean (SEM) is given in TABLE 2-A. Analysis of variance (ANOVA) revealed significant differences across groups ( $F(12,61) = 49.12$ ;  $p < 1.0 * 10^{-15}$ ). Fisher's post-hoc test disclosed significant differences between most groups at a  $\alpha$ -level of 0.05 (TABLE 5-1, see Appendix).

Within my Master's Thesis I had already stained slices from the same animals for BrdU, GFP and DCX to analyze early mitotic differentiation. Only the BrdU counts were also incorporated into the present study.

The absolute number of BrdU-positive cells was counted for several different staining (TABLE 2-A). Besides its assessment under the light microscope as performed during my Master's thesis absolute BrdU cell counts were investigated in addition by means of two different fluorescence staining under the confocal microscope: a) BrdU, GFP, DCX staining or b) BrdU, DCX, NeuN staining (compared to the BrdU-DAB values) (FIGURE 2-1, TABLE 2-A). Moreover, I calculated a merged data set from my three different cell counts to control for the maximum in time and the gross time course (FIGURE 2-1D).

## Results - Dynamics of neuronal development in the adult dentate gyrus

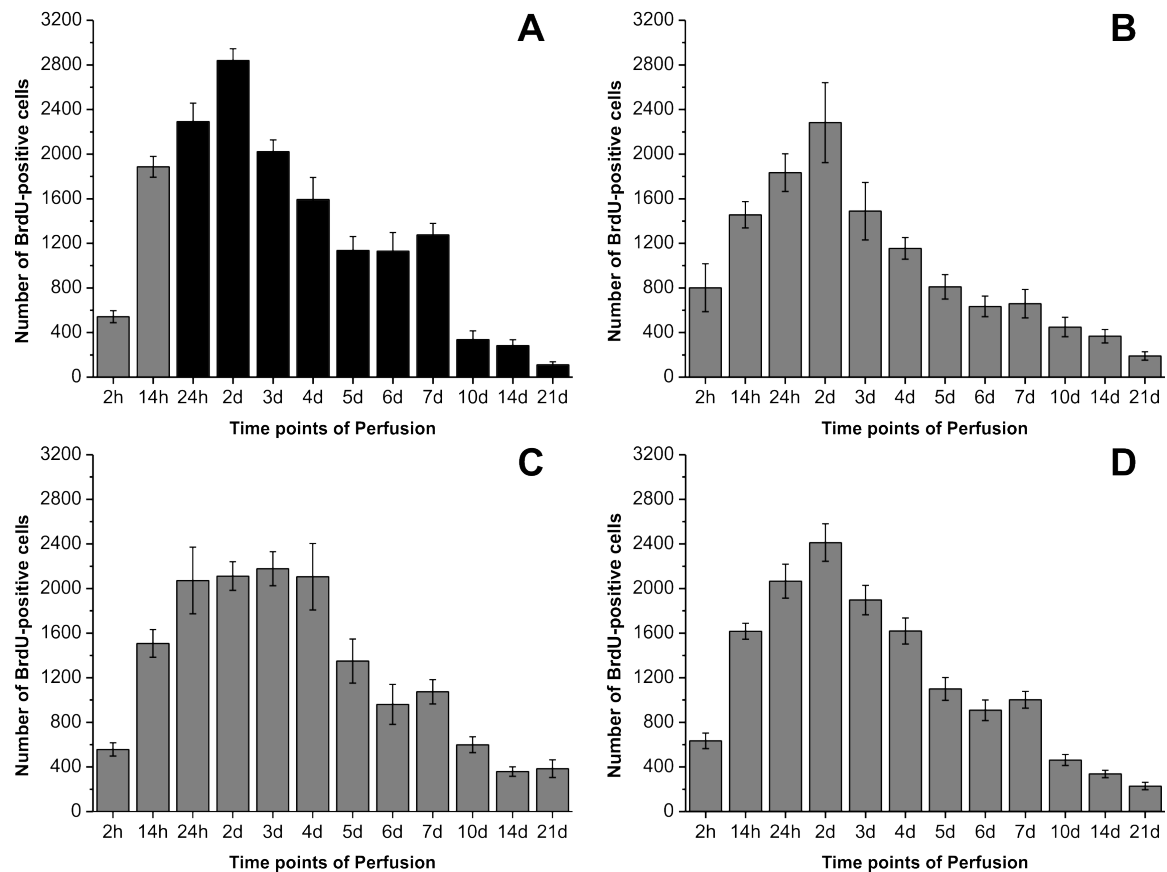
*TABLE 2-A Comparison of the mean  $\pm$  SEM for the absolute number of BrdU- positive cells obtained with different methods*

	BrdU DAB staining	BrdU / NestinGFP / DCX staining	BrdU / DCX / NeuN staining
2h	543 $\pm$ 133	802 $\pm$ 527	557 $\pm$ 147
14h	1887 $\pm$ 228	1456 $\pm$ 288	1508 $\pm$ 304
1d	2292,5 $\pm$ 406	1835 $\pm$ 414	2072 $\pm$ 733
2d	2841 $\pm$ 256	2283 $\pm$ 877	2112 $\pm$ 313
3d	2023,7 $\pm$ 275	1488,9 $\pm$ 683	2178 $\pm$ 403
4d	1594,5 $\pm$ 482	1155 $\pm$ 237	2106 $\pm$ 730
5d	1137,9 $\pm$ 329	810,9 $\pm$ 290	1349,1 $\pm$ 524
6d	1129,5 $\pm$ 412	635 $\pm$ 227	961 $\pm$ 441
7d	1275,5 $\pm$ 254	659 $\pm$ 313	1074 $\pm$ 266
10d	337,5 $\pm$ 192	450 $\pm$ 212	599 $\pm$ 175
14d	284,5 $\pm$ 127	368 $\pm$ 148	359 $\pm$ 104
21d	111 $\pm$ 66	191 $\pm$ 92	384 $\pm$ 193

Comparable time courses as well as roughly similar numbers are found for all three different staining, but the standard deviation (SD) is smaller for the DAB staining than for the confocal staining. Mean SD for all different time points investigated reaches a value of 262.67 for the cells observed under the light microscope with the DAB staining, while the mean overall standard deviation lies at 358.58 for the first confocal staining and at 360.83 for the second confocal staining. This indicates a higher quality and better resolution of BrdU-positive cells under the light microscope. Although FIGURE 2-1C only displays a plateau between 24 hours and 4 days the peak at day two after the first injection is preserved as well as the plateau between day five and seven in the merged calculation (FIGURE 2-1D). These differences in precise numbers are caused by the inevitable selection of different slices for different staining. This may also be the cause for different numbers within the literature combined with a later peak at roughly three

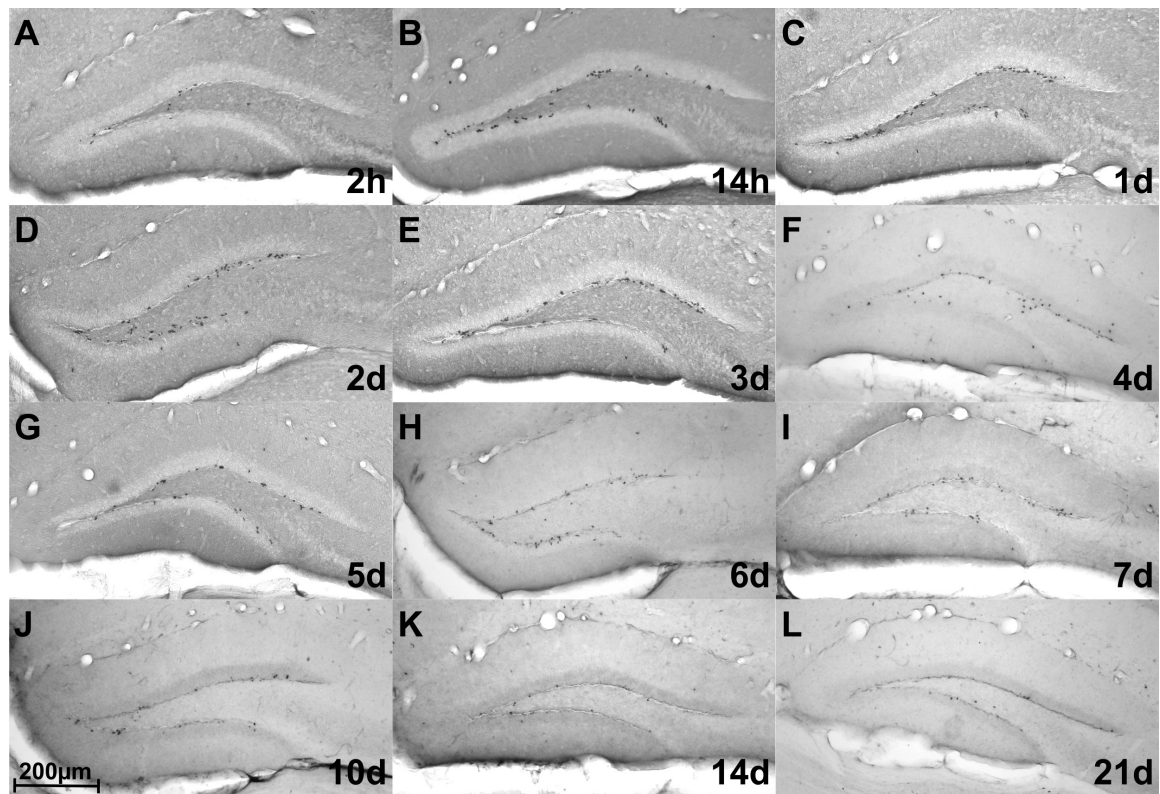
## Results - Dynamics of neuronal development in the adult dentate gyrus

days after BrdU injection (TABLE 3-A, Discussion). Although DAB analyses are more precise regarding the threshold for visibility of the BrdU labeling, confocal analyses reveal quite good quality data, as well. For BrdU-DAB analyses as well as for confocal analyses differences in BrdU cell counts across groups are highly significant ( $p < 1.0 \times 10^{-15}$ ). Results of Fisher's post-hoc test for both confocal analyzes are also displayed in TABLE 5-A (see Appendix).



**FIGURE 2-1:** Absolute counts of cells labeled positively for BrdU (A) assessed with BrdU DAB staining ( $p < 0.0001$ ), Black bars indicate data collected during my Master's thesis, while Grey bars display new information (B) assessed with fluorescence triple staining for BrdU, GFP, DCX ( $p < 0.0001$ ) and (C) assessed with fluorescence triple staining for BrdU, DCX, NeuN ( $p < 0.0001$ ). (D) Mean number of BrdU-positive cells for all three different staining ( $P < 0.0001$ ). Error bars display Standard error of the mean (SEM).

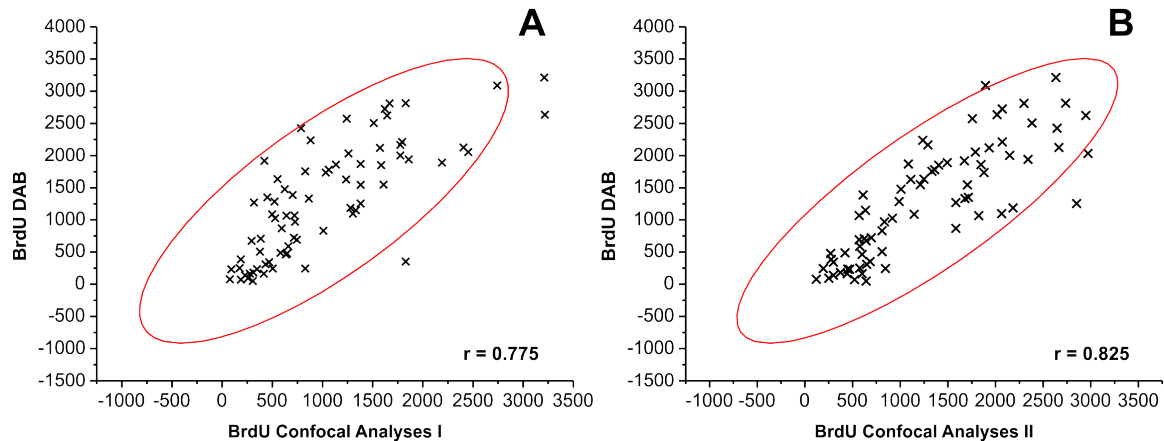
To further illustrate the differences in absolute BrdU numbers at the light microscope representative photographs are shown (FIGURE 2-2). Differences between early time points and late time points are easily visible.



**FIGURE 2-2:** Photographs taken with the light microscope to further illustrate the differences in absolute numbers of BrdU DAB positive cells dependent on the time after BrdU administration. (A) 2 hours after BrdU injection only few BrdU-positive cells can be detected (B) 14 hours after the first and 2 hours after the last BrdU injection several positive cells are visible under the microscope. These cells display our starting population (C) 1 day after the first BrdU administration several cells were visualized and (D) 2 days after BrdU the highest number of BrdU-positive cells was visible. At (E) day 3 and (F) day 4 the number of positively detected cells decreases until a first plateau is reached lasting for (G) day 5 (H) day 6 and (I) day 7. At day 10 post injection the number of positive cells has rapidly decreased again (J). At (K) day 14 and (L) day 21 only few cells are still detectable.

To assure the quality of my data, I determined the correlation coefficient between the different staining methods and microscopical analyses (FIGURE 2-3). The correlation coefficient indicates good quality of the data obtained with the different methods. The counts determined in the DAB staining highly correlated with both the first fluorescence staining ( $r = 0.775$ ,  $p = 4.44089 \times 10^{-16}$ ) as well as the second fluorescence staining ( $r = 0.825$ ;  $p = 0$ ).

## Results - Dynamics of neuronal development in the adult dentate gyrus

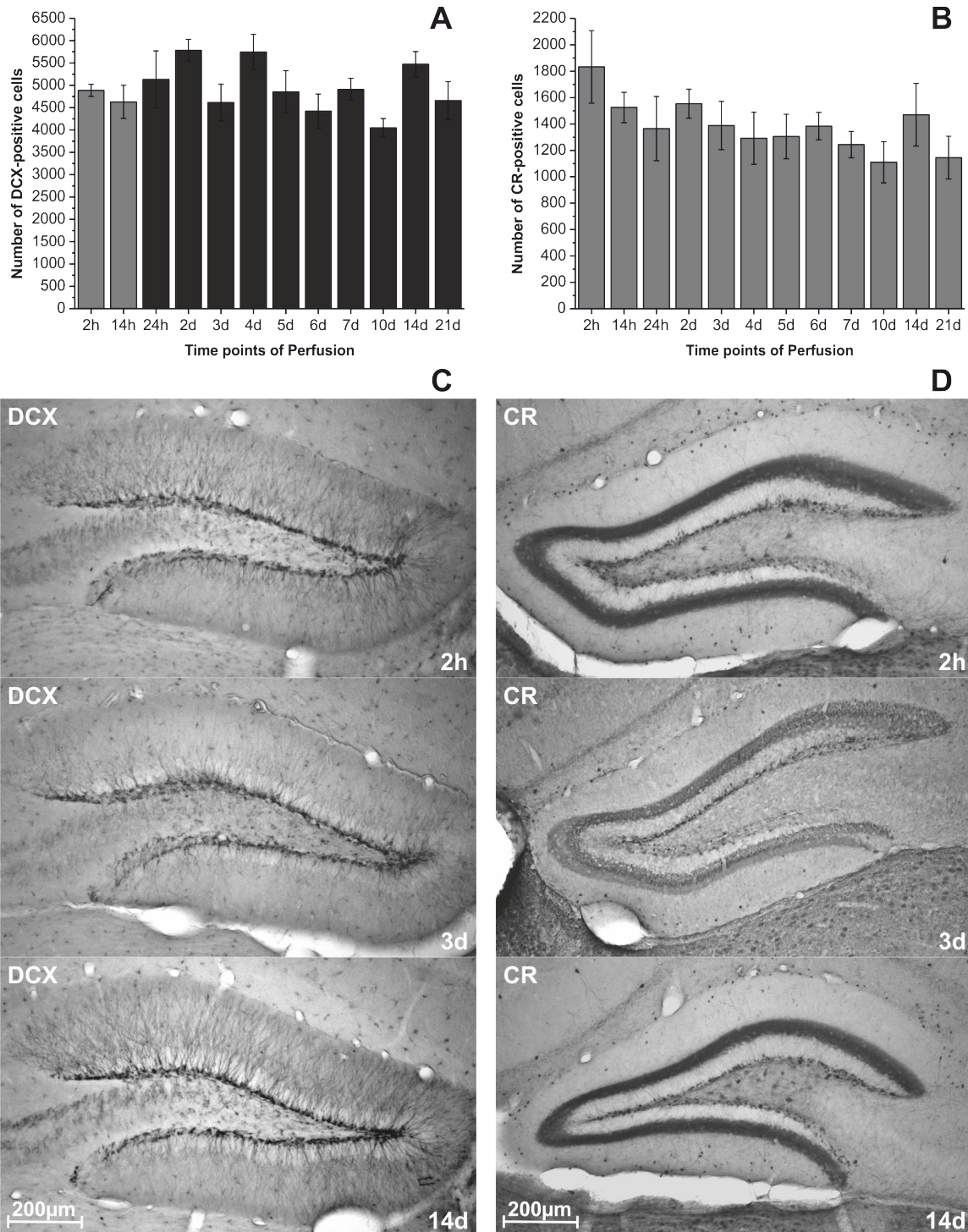


**FIGURE 2-3:** Correlation of the cell counts in different staining techniques (A) Correlation between DAB and the first fluorescence staining revealed a correlation coefficient of  $r = 0.775$  and  $p < 0.0001$  (B) Correlation between DAB and the second fluorescence staining revealed a correlation coefficient of  $r = 0.825$  and  $p < 0.0001$ .

### 2.1.1.2. Absolute number of Doublecortin-positive and Calretinin-positive cells

In addition to absolute BrdU cell counts, I obtained data regarding the absolute number of Doublecortin (DCX)-positive cells. DCX is a microtubule-associated protein, expressed transiently in the course of adult neurogenesis and is associated with structural plasticity and migration. In the hippocampus DCX identifies migrating progenitor cells transferring from the subgranular zone into the granular zone (type-2b / 3 cells). It is also widely used to label immature granule cells that are not yet fully integrated into the hippocampal network. Analyses of the absolute number of DCX-positive cells over time revealed no significant differences between groups ( $F(12,61) = 1.68583$ ,  $p = 0.09237$ ) (FIGURE 2-4). Furthermore, I also analyzed the absolute number of Calretinin (CR)-positive cells within the dentate gyrus, independently of BrdU. CR, a calcium-binding protein, displays a widely used transient, but early, postmitotic marker to label immature granule cells in the adult dentate gyrus, that are not yet fully integrated into the hippocampal network. The absolute number of these type of cells did not reveal any significant differences across time ( $F(12, 61) = 1.046$ ,  $p = 0.42044$ ) (FIGURE 2-4). In the literature it is described that aging leads to a significant decrease of this cell population over time. Three weeks seem to be a too narrow time frame to monitor this effect on early postmitotic neuronal cells.

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**FIGURE 2-4:** Absolute number of DCX-positive and CR-positive cells in the dentate gyrus. Both DCX-positive cells as well as CR-positive cells are expressed with almost equal numbers over the examined period of three weeks. A and B illustrate the absolute values with  $p = 0.09$  for DCX and  $p = 0.42$  for CR. The light microscope images in C and D further illustrate the similar amounts of cells within the DG at 2 hours, 3 days and 14 days post injection for DCX (C) and CR (D).

## Results - Dynamics of neuronal development in the adult dentate gyrus

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Immunohistochemical investigations regarding CR-positive cells and DCX-positive cells yielded similar results across all experimental groups (TABLE 2-B). Therefore, I conclude that the absolute number of type-2, type-3 (DCX-positive) cells and the absolute number of early postmitotic cells (CR-positive) stays relatively constant within the DG over time disregarding age as diminishing factor (FIGURE 2-4). Since the mice were housed under standard laboratory conditions these results appear to be reasonable. No additional neurogenesis-stimulating factor was applied, which could have influenced the number of the different mitotic cell stages. Also, the group of animals perfused after just 24 hours showed no increase or decrease in the absolute number of type-1, type-2 or DCX-positive cells. At the 24 hours time point the last BrdU injection was just 12 hours ago.

An issue of concern is that the numbers might be confounded by the stress of the injections. It was shown that stress has a strong influence on neurogenesis (Gould et al., 1997, Mirescu and Gould, 2006, Warner-Schmidt and Duman, 2006). Since we found no differences in the absolute cell numbers after 24 hours, carefully performed BrdU injections as such do not seem to have any effect on these precursor cell pools. Therefore, the injections were either not stressful at all, or the acute stress was too short to produce a detectable effect. From past publications it is known that ~48% of all DCX positive cells are type-2b cells (type D1) and the rest ~52% are type-3 cells (type D2 and D3, see TABLE 1-B) (Seri et al., 2004). Based on this information we can calculate the absolute number of type-2b type-3 cells respectively at each time point within the SGZ (TABLE 2-B). This information is used later on to calculate the mitotic rate of both: type-2b and type-3+ cells.



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**TABLE 2-B:** Total number of DCX-positive and CR-positive cells.

All counted CR-positive and DCX-positive cells for each time point plus / minus standard deviation are displayed, rounded to the full number. Additionally, the total number of type-2b and type-3 cells was calculated under the assumption that 48% of all DCX-positive cells are type-2b cells and the rest are type-3 and early immature neurons (type-3+ cells) (based on (Seri et al., 2004)).

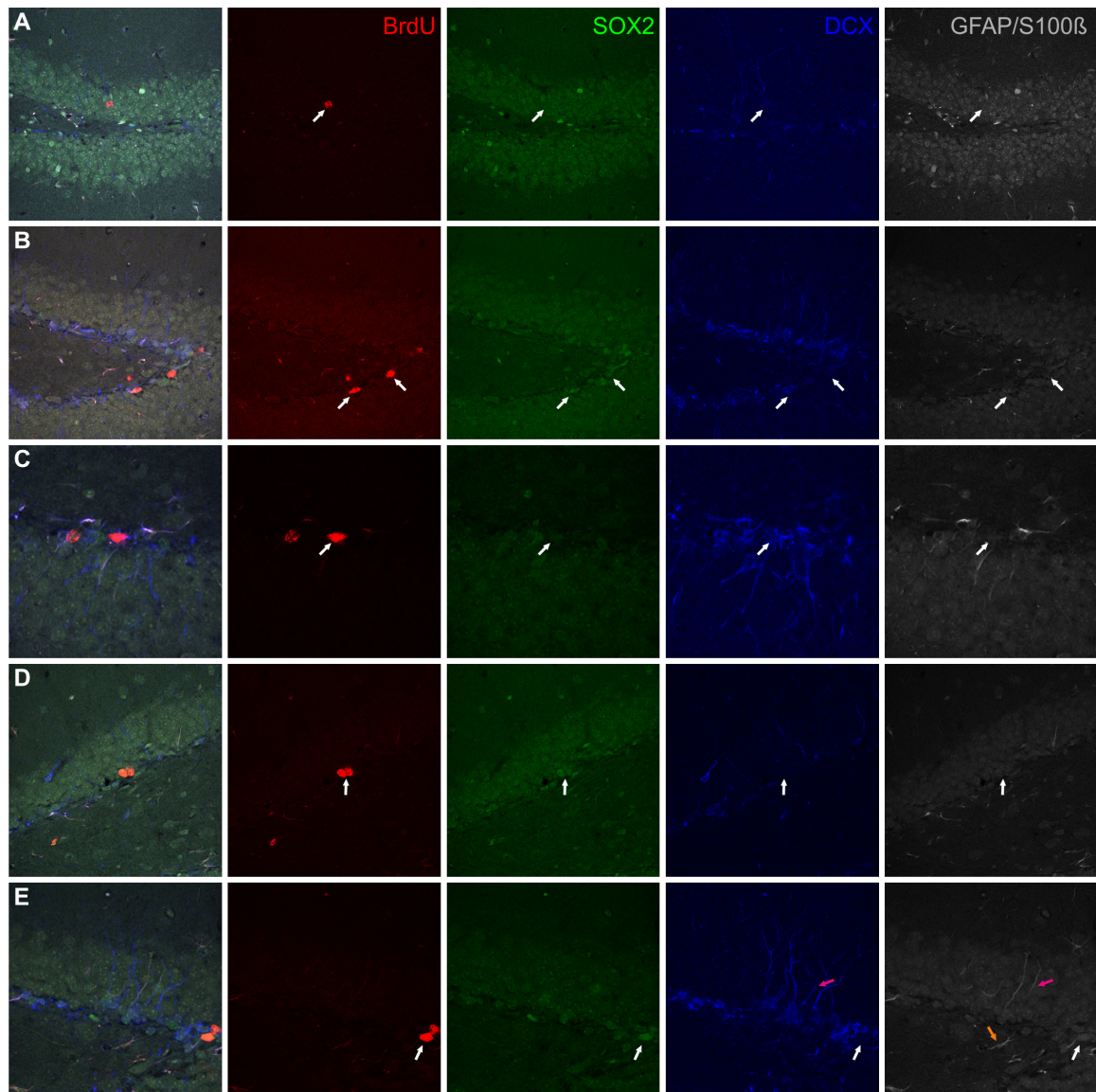
	Total number of CR-positive cells (immature neurons)	Total number of DCX-positive cells	Calculated number of total type-2b cells	Calculated number of total type-3+ cells
2h	1833 ± 674	4887 ± 330	2346	2541
14h	1525 ± 284	4629 ± 908	2222	2407
1d	1365 ± 595	5132 ± 1561	2463	2669
2d	1554 ± 270	5786 ± 601	2772	3014
3d	1389 ± 484	4616 ± 1094	2216	2400
4d	1292 ± 485	5746 ± 971	2758	2988
5d	1305 ± 46	4854 ± 1255	2330	2524
6d	1384 ± 254	4422 ± 935	2123	2299
7d	1244 ± 244	4910 ± 605	2357	2553
10d	1110 ± 383	4049 ± 505	1944	2105
14d	1470 ± 581	5472 ± 691	2627	2845
21d	145 ± 398	4659 ± 1041	2236	2423

### **2.1.2. Phenotypic analyses**

The data sets I collected by means of different staining protocols are based on different absolute BrdU values caused by slight differences in the distribution of BrdU between slices (FIGURE 2-1). Since I wish to be able to compare the values, it is necessary to bring them on a common basis, thereby level out the differences in BrdU distribution. As a conclusion, all values are processed mathematically and correlated to the BrdU-DAB cell counts.

#### ***2.1.2.1. Analyses of early mitotic differentiation stages***

To allow better analyses of the early differentiation phase, immunofluorescence was used to label proliferating cells with BrdU and classify them according to the different stages in ahNG (see FIGURE 1-2, Introduction). For the first four stages of differentiation slices were stained with BrdU (Red), GFAP / S100 $\beta$  (Grey), SOX2 (Green), and DCX (Blue) (FIGURE 2-5). Cells positive for BrdU, GFAP / S100 $\beta$  and SOX2, but negative for DCX and showing radial process were identified as type-1 cells. Type-2a cells expressed BrdU and SOX2, but lacked GFAP / S100 $\beta$  and DCX, while type-2b cells additionally expressed DCX. Type-3 cells expressed BrdU and DCX, but neither SOX2, nor GFAP / S100 $\beta$  (TABLE 1-A, Introduction). All cells positively stained for BrdU, SOX2 and GFAP / S100 $\beta$ , but showing horizontal processes were identified as astrocytes. The pool of cells only positive for BrdU but not any other stained marker contains postmitotic cell stages as well as oligodendrocytes.

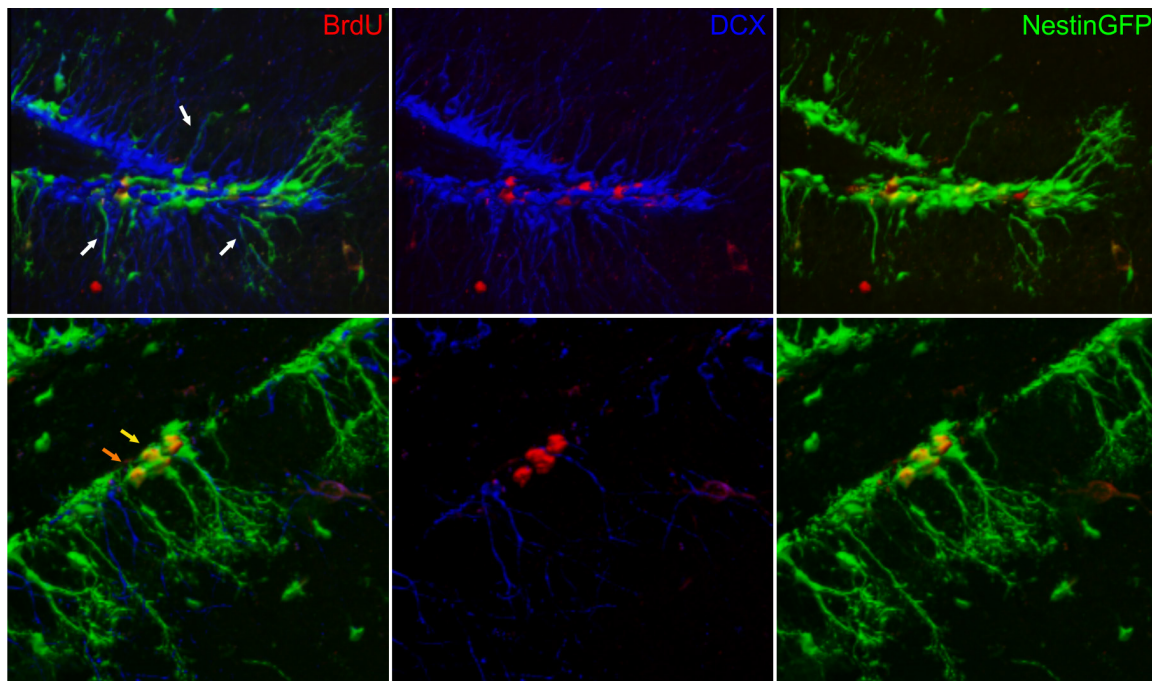


**FIGURE 2-5:** Confocal analyzes of the mitotic stages of differentiation. As markers we used BrdU (Red), SOX2 (Green), DCX (Blue) and GFAP / S100 $\beta$  (Grey). Arrows are used to demonstrate various cell types (A) cell only positive for BrdU (B) left cell is positive for both SOX2 and DCX, such identified as type-2b cell, while the right cell only expresses SOX2 (type-2a cell) (C) type-3 cell expressing only DCX, winding her process around a GFAP-positive process of a type-1 cell (D) Closely co localized type-2a cells (E) White arrows indicate a SOX2 / S100 $\beta$  positive astrocyte with a process oriented into the direction of the hilus. The orange arrow additionally shows processes of another astrocyte, while the pink arrow indicates a type-1 process co localized to a type-3 process.

*Morphological observations*

Analyses of fluorescence staining indicate a specific location of type-1 cells in correlation to cells further passed through the differentiation process. In the studied mice type-1 cells are mainly collocated with either type-2a cells or type-3 cells. In case of adjacent type-2a cells it looks like these two cells recently passed through mitosis and division (FIGURE 2-6).

In case of a type-1 cell, that is directly adjoining a type-3 cell, the two processes are closely located and seem to wind around each other (FIGURE 2-6), maybe indicating signaling between these two different cell types further regulating ahNG.



**FIGURE 2-6:** Type-1, type-2a, type-2b and type-3+ cells are located within the subgranular zone of the dentate gyrus. First row: Processes of type-1 and type-3 cells often entangle each other (exemplary indicated by White arrows). Second row: type-1 cells are often part of clusters. Within these clusters type-1 (yellow arrow) and type-2a cells (orange arrow) are located close to each other.

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### 2.1.2.2. *Distribution of mitotic cell stages over time*

Within a period of 21 days after the initial BrdU administration, the proportion of cells within the different stages of differentiation changed quite rapidly and drastically. Initially, different types of mitotic cells were labeled with BrdU. Depending on the further expression of GFAP, SOX2 and DCX they were classified according to the description in the introductory part.

Overall significance for the changes among the different cell types varies strongly. While the changes in the relative proportion of type-1 cells are non-significant ( $p = 0.99961$ ) all other changes show strongly significant effects of time. For type-2a cells, astrocytes and unidentified cells changes are as strong as  $p < 1.0 * 10^{-15}$ . Type-2b cells contingent changes with  $p = 1.05414 * 10^{-10}$  and type-3+ cells with  $1.01676 * 10^{-10}$ .

After the first injection of BrdU  $2.38 \pm 2.57\%$  of all analyzed cells express BrdU within their cell body and GFAP within the nicely extended radial process. Thus, these cells are identified as type-1 cells. A rather huge proportion of type-2a cells ( $50.02 \pm 6.69\%$ ) dominates the population, while the rest consists of  $22.32 \pm 3.86\%$  type-2b and  $19.01 \pm 5.24\%$  type-3 cells. Neither postmitotic nor astrocytic cells could be found and the fraction of unidentified cells was rather low with  $6.26 \pm 3.14\%$ . (FIGURE 2-7, 2 h)

Two hours after the last BrdU administration (14 hours time point) the distribution of cells within the defined cell stages has already changed due to the higher proportion of cells labeled, but also due to the ongoing differentiation during the 12 hours since the first observed time point. Besides a negligible small fraction of non-identified cells ( $4.33 \pm 1.74\%$ ), also the proportion of type-1 cells has not changed significantly ( $2.47 \pm 2.57\%$ ). The contingent of type 2a-cells dropped significantly ( $p = 1.206 * 10^{-5}$ ) from more than 50% to only  $37.35 \pm 5.45\%$ . On the other hand, the proportion of type-2b cells increased towards a maximum of  $31.52 \pm 6.24\%$  with  $p = 0.02498$ . Also the relative proportion of type-3+ cells increased non-significantly towards  $24.32 \pm 9.65\%$  ( $p = 0.14247$ ). No astrocytes were found earlier than 2 days after initial BrdU administration. (FIGURE 2-7, 14h)

On day one, 24 hours, after the first BrdU injection the proportion of type-1 cells remains constant, as also during the rest of the investigated time ( $1.71 \pm 1.14\%$ ). Type-2a ( $28.77 \pm 8.49\%$ ) and type-2b ( $28.53 \pm 5.41\%$ ) cells decrease in their number. With respect to the

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previous time point only changes for type-2a cells are significant ( $p = 0.00202$ ), while the p-value for type-2b only reaches  $p = 0.45839$ . The fraction of type-3+ cells and of cells with unknown cell type further increases to  $29.63 \pm 9.87\%$  ( $p = 0.14249$ ) and  $11.35 \pm 4.29\%$ , respectively ( $p = 0.06967$ ) (FIGURE 2-7, 1d).

Two days post injection (p.i.) the absolute number of BrdU peaks while the distribution of cells within the different stages changes further towards later mitotic stages. Additionally, first astrocytes were observed within the tissue ( $0.72 \pm 0.64\%$ ). Type-1 cells in general stagnate around 2%, at this time point they account for  $2.04 \pm 1.37\%$  of all cells analyzed, whereas type-2a cells drop further towards  $22.66 \pm 2.71\%$ . The change of cells of this cell type between one day p.i. and two days p.i. is significant with  $p = 0.025$ . Again changes within type-2b cells are non-significant, although a further decrease can be observed ( $21.48 \pm 11.20\%$ ,  $p = 0.0831$ ). Also the changes in the proportion of type-3+ cells ( $33.02 \pm 7.22\%$ ) are non-significant ( $p = 0.34723$ ), while the increase in the fraction of cells of unknown type towards  $20.07 \pm 4.57\%$  gets significant with  $p = 0.02519$ . (FIGURE 2-7, 2d)

On day three no significant changes at all occur although the tendency observed on the previous time point continues for each cell type. The proportions observed are  $1.65 \pm 1.35\%$  for type-1,  $18.72 \pm 3.79\%$  for type-2a,  $17.43 \pm 7.59\%$  for type-2b,  $36.01 \pm 4.47\%$  for type-3+ cells,  $2.14 \pm 1.96\%$  for astrocytes and  $20.07 \pm 4.57\%$  for all others.

During day four a maximum proportion of type-3 cells was observed, while all type-2 cells further decreased in their rate and both astrocytes and other cells further increased. Type-1 cells maintained stable with  $1.44 \pm 1.58\%$ . Changes for type -2a cells ( $11.65 \pm 3.92\%$ ) get significant with  $p = 0.00764$  as do changes for type-3+ cells ( $43.43 \pm 5.85\%$ ) with  $p = 0.03511$ . Only the decrease in the proportion of type-2b cells ( $10.31 \pm 2.82$ ) remained non-significant ( $p = 0.06951$ ) as did the increase in other cells ( $28.14 \pm 3.82$ ) and astrocytes ( $5.04 \pm 2.24\%$ ). (FIGURE 2-7, 4d)

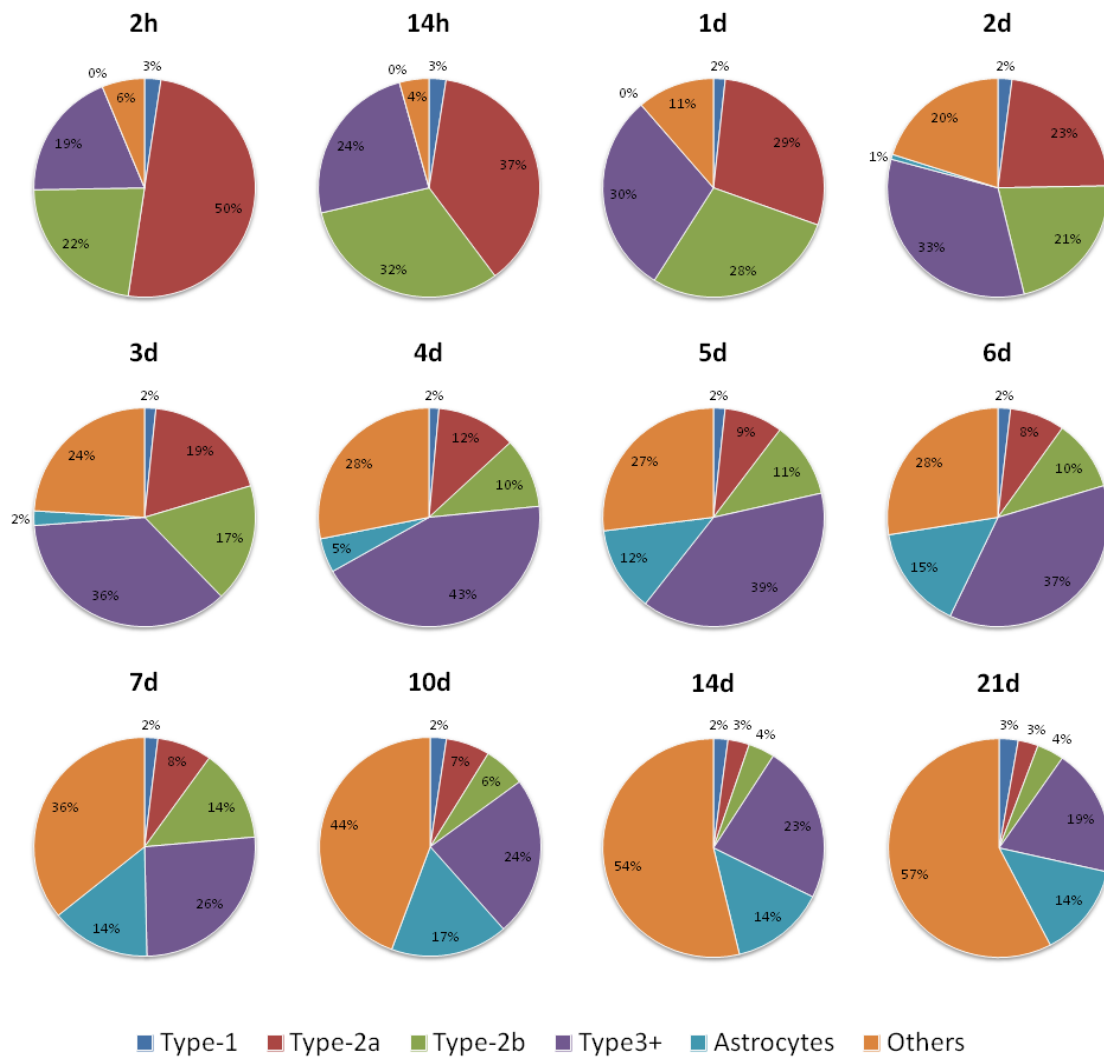
The absolute number of BrdU-positive cells remains rather stable between five days and seven days p.i. with respect to the DAB staining and also considering the absolute values obtained under the confocal for the BrdU-DCX-NeuN staining and the BrdU-GFP-DCX staining (FIGURE 2-1). With respect to the distribution of mitotic differentiation cell stages increase type-1 cells slowly, but non-significantly, from  $1.169 \pm 2.58\%$  over  $1.83 \pm$

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1.75% towards  $1.94 \pm 2.19\%$ . Additionally, type-2a cells also remain rather stable with proportions ranging from  $8.59 \pm 4.56\%$  at day five towards  $8.02 \pm 2.73\%$  at day seven and  $8.06 \pm 2.27\%$  at day six. Seven days p.i. the fraction of type-2b cells comes to a local maximum with  $13.62 \pm 9.17\%$  although differences towards day five ( $11.18 \pm 7.64\%$ ) and day six ( $10.45 \pm 7.93\%$ ) are quite small and highly insignificant. Regarding type-3+ cells, a rather huge drop in its proportion occurs at day seven ( $26.09 \pm 2.23\%$ ,  $p = 0.00389$ ), while changes between day four and six are also non-significant with values of  $39.12 \pm 6.39\%$  at day five and  $36.81 \pm 4.20\%$  at day six. On the contrary, the increase in the proportion of astrocytes between day four and five ( $12.45 \pm 2.96\%$ ) seems highly significant with  $p = 2.6761 * 10^{-4}$ , while differences between day five, six ( $15.31 \pm 5.25\%$ ) and seven ( $14.61 \pm 3.63\%$ ) are non-significant. For the not further identified population of BrdU-positive cells a further increase occurs between day five ( $26.97 \pm 8.63\%$ ), six ( $27.54 \pm 9.77\%$ ) and seven ( $35.73 \pm 9.18\%$ ), with a significant change towards the last time point of  $p = 0.03523$ . (FIGURE 2-7, 5d, 6d and 7d).

At the later time points the survival of cells gets much more prominent, thus the mitotic stages in ahNG decrease in importance and also in their proportion. On the other hand the value of postmitotic cells increases, which are included in the pool of unidentified cells within this staining. More precisely, type-1 cells again remain rather stable with a small non-significant increase from seven to ten days ( $2.37 \pm 2.70\%$ ) and 14 ( $2.11 \pm 2.49\%$ ) to 21 days ( $2.78 \pm 6.80\%$ ). Also the further decrease in the proportion of type-2a, type-2b and type-3+ cells as well as for astrocytes, are non-significant for the later time points. Concretely the values range from  $6.43 \pm 2.17\%$  at day ten to  $3.14 \pm 3.67\%$  at day 14 and  $2.95 \pm 4.63\%$  at day 21 for type-2a cell populations. Type-2b cells vary from  $6.13 \pm 5.64$  at day ten to  $3.87 \pm 3.85\%$  at day 14 and  $3.97 \pm 6.85\%$  at day 21 p.i. A decrease towards  $23.56 \pm 3.95\%$  at day ten,  $23.22 \pm 5.46\%$  at day 14 and  $18.81 \pm 5.48\%$  was observed for type-3+ cells. Astrocytes, on the other hand, peak at ten days p.i. with  $17.21 \pm 7.05\%$ . Afterwards, a decrease towards  $13.89 \pm 3.80\%$  at day 14 and  $13.83 \pm 4.67\%$  at the last observed time point occurs. For the population of unidentified BrdU-positive cells a further significant increase occurs between day seven and ten ( $44.31 \pm 5.88\%$ ) with  $p = 0.02751$  as well as between ten and 14 ( $53.78 \pm 8.49\%$ ) with  $p = 0.0154$ . Only the change towards 21 days p.i. ( $57.67 \pm 5.67\%$ ) is non-significant. (FIGURE 2-7, 10d, 14d, 21d)





**FIGURE 2-7:** Relative distribution of cells within each differentiation stage over time. While type-1 cell remain stable in their proportion, all other cell types vary highly. Type-2a cells reach their maximum within the first two hours, while type-2b cells peak 14 hours after BrdU injection. The highest proportion of type-3 cells was found four days after BrdU administration. In contrast, the amount of cells not further identified (others) increases during the whole observed period of time, indicating an increase in postmitotic cells.

### 2.1.2.3. Development of mitotic cell types over time in absolute numbers

To get a better impression on the real numbers of cells within each developmental stage, it is also important to focus on the absolute numbers in addition to the relative distribution. Therefore, the percentages obtained during confocal analyses were multiplied with the absolute numbers achieved from the DAB staining for BrdU-positive cells. In comparison to the relative amounts of the distinct cell types analyzed before, this



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time the focus laid more on the individual cell types changing their absolute numbers over time.

### *Type-1 cells*

With respect to type-1 cells an overall slightly significant change was observed with  $p = 0.00143$ . Fisher's post-hoc test also revealed significant changes between some time points. A huge significant increase ( $p = 0,01515$ ) between the two hour time point after one BrdU injection and the 14 hour time point, 2 hours after the third injection, from  $13.36 \pm 15.78$  cells per dentate gyrus towards  $47.62 \pm 38.36$  cells can be noticed (FIGURE 2-8 A). Some non-significant fluctuations occurred between the following time points with  $40.27 \pm 27.90$  cells at 24 hours,  $58.24 \pm 39.73$  type-1 cells at two days p.i. Only the decrease towards three days p.i. ( $30.9 \pm 21.81$  cells/DG) becomes slightly significant with  $p = 0.04265$ . All other changes in the number of type-1 cells are not significant in relation to the previous time point. In total numbers the calculations revealed  $19.78 \pm 18.86$  type-1 cells per DG at four days p.i.,  $18.82 \pm 27.99$  cells/DG at five days,  $15.35 \pm 13.56$  at six days and  $24.68 \pm 26.95$  one week p.i. For the time points observed later, lower numbers of type-1 cells were obtained as expected keeping in mind the relative stable proportion of type-1 cells with roughly 2% and the decrease in BrdU-positive cells. At day ten p.i.  $5.69 \pm 6.75$  cells/DG were received as result of the calculations. 14 days ( $7.35 \pm 8.11$  cells/DG) and 21 days p.i. ( $2.17 \pm 5.31$  cells/DG) similar numbers of type-1 cells were found. (FIGURE 2-8 A)

### *Type-2a cells*

Changes between different time points with respect to type-2a cells are highly significant with  $p < 1.0 * 10^{-15}$ . A first huge increase from two hours p.i. ( $274.30 \pm 90.55$  type-2a cells/DG) to 14 hours p.i. ( $706.22 \pm 150.59$ ) was expected. A slight decrease from 14 hours to two days ( $639.68 \pm 51.93$  cells/DG) with  $679.86 \pm 277.06$  cells/DG at one day after the initial injection of BrdU was followed by a steep drop between 2 days, 3 days ( $382.47 \pm 108.93$  cells/DG) and 4 days ( $180.71 \pm 63.81$  cells/DG). The absolute numbers of type-2a cells stagnates between five days and seven days post injection with  $94.47 \pm 47.38$  cells/DG, followed by a steep drop towards the later time points. At ten days post injection only  $21.55 \pm 15.55$  cells are located within the subgranular zone of the dentate gyrus. At 14 days p.i. the number further decreases to  $7.99 \pm 7.84$  and reaches  $3.01 \pm 4.8$

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type-2a cells after 21 days. (FIGURE 2-8 B). Regarding significances, over all p-values are at  $p < 1.0 * 10^{-15}$ . The increase from 2 h to 14h ( $p = 1.16467 * 10^{-9}$ ), as well as the decreases between two days and three days ( $p = 4.03518 * 10^{-5}$ ), and three days and four days ( $p = 9.59164 * 10^{-4}$ ) are significant. All other transitions have p-values bigger than 0.05.

### *Type-2b cells*

While type-2a cells peak after 14 hours p.i., type-2b cells increase between the first two observed time points to a plateau between 14 hours and 2d post injection, while the calculated absolute number decreases by nearly 50% afterwards. In concrete terms the values are  $119.81 \pm 33.01$  at two hours,  $587.86 \pm 104.74$  at 14 hours,  $660.47 \pm 198.72$  at 24 hours,  $613.35 \pm 351.05$  at 2days,  $353.55 \pm 165.3$  at 3 days,  $164.7 \pm 68.1$  at four days and  $124.32 \pm 85.9$  type-2b cells per DG after five days p.i. Between day five and six ( $112.63 \pm 76.66$ ) nearly no change was observed, while surprisingly an increase occurs towards the next observed time point ( $187.68 \pm 165.71$ ). The absolute number of type-2b cells drops towards the later time points. At day ten p.i. only  $17.42 \pm 17.81$  are left within the dentate gyrus, while at day 14 ( $12.88 \pm 18.46$ ) and 21 ( $5.33 \pm 8.27$ ) even less cells are positive for both SOX2 and DCX (FIGURE 2-8 C). Overall significance for type-2b cells reaches  $3.77476 * 10^{-15}$ . Changes between two hours and 14 hours ( $p = 4.27906 * 10^{-7}$ ), two days and three days ( $p = 0.00184$ ), three days and four days ( $p = 0.0211$ ), as well as between seven days and ten days ( $p = 0.04402$ ) are significant.

In order to make an assumption about what percentage of all type-2b cells and all type-3+ cells are mitotic, absolute numbers of these cell types in mitosis (obtained from confocal analyses at two and 14 hours p.i.) were multiplied with the total population of type-2b or type-3+ cells, respectively (TABLE 2-B). For the type-2b-positive cells these values demonstrate that 5.1% of all cells in this differentiation stage undergo mitosis after one injection of BrdU and 26.5% after three BrdU administrations.

### *Type-3+ cells*

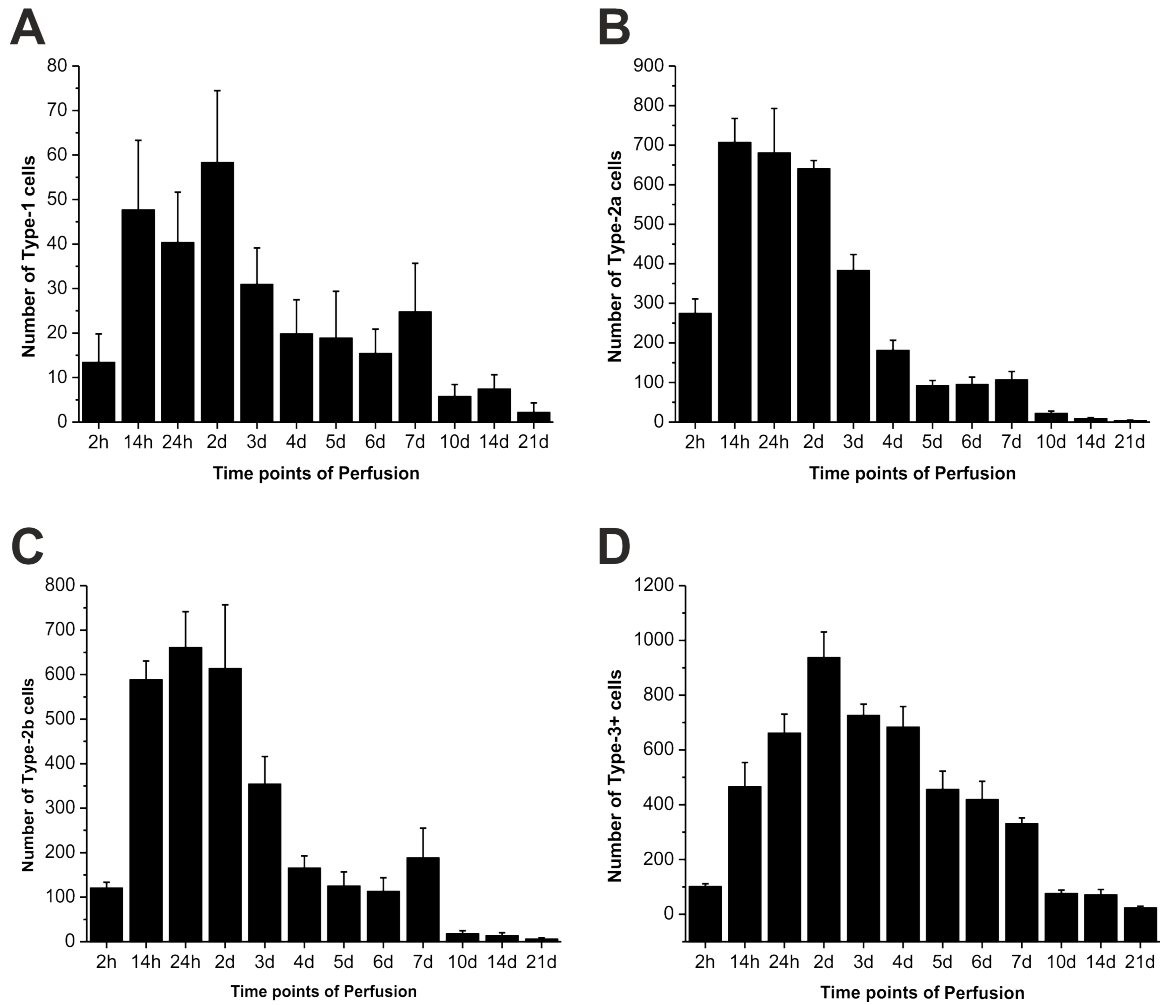
Type-3 cells including early postmitotic cells (type-3+ cells) also change highly in their number, even with high significance ( $p < 1.0 * 10^{-15}$ ). A stable increase of cells can be monitored during the first examinations until the absolute number of type-3+ cells peaks

## Results - Dynamics of neuronal development in the adult dentate gyrus

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at two days p.i. The numbers obtained during multiplication of the relative proportions with the absolute number of BrdU-positive cells result in  $100.83 \pm 26.61$  cells at two hours,  $464.6 \pm 219.55$  at 14 hours,  $660.45 \pm 172.63$  at 24 hours, and  $936.5 \pm 230.94$  cells at day two p.i. per dentate gyrus. All these transitions are significant with  $p = 4.1664 * 10^{-5}$  (2 h, 14h),  $p = 0.02065$  (14h, 24h),  $p = 0.00139$  (24h, 2d),  $p = 0.01005$  (2d, 3d). A significant decrease towards day three after the initial BrdU injection ( $725.49 \pm 110.83$ ) as well as between day four ( $682.50 \pm 185.38$ ) and day five ( $455.43 \pm 177.82$ ) occurs ( $p = 0.0058$ ). The absolute number of type-3+ cells slightly decreases between day five, six ( $417.90 \pm 165.04$ ) and seven, but all these transitions are non-significant. Only the drop of roughly 75% between day seven ( $329.96 \pm 54.37$ ) and ten ( $75.49 \pm 48.61$ ) reaches a p-value  $< 0.05$  of exactly  $p = 0.00303$ . At day ten p.i.  $70.42 \pm 48.61$  cells are still estimated to be located within the SGZ, which drops to  $22.33 \pm 16.43$  cells/DG at day 21 p.i. (FIGURE 2-8 D).

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**FIGURE 2-8:** absolute number of cells within each differentiation stage in dependency on time calculated from the relative numbers and the absolute BrdU values. (A) The number of type-1 cells varies between the different time points, with different phases, forming slightly fluctuating plateaus ( $p = 0.00143$ ) (B) The number of type-2a cells within the SGZ decreases after the first 14 hours ( $p < 1.0 * 10^{-15}$ ) (C) 24 hours after BrdU the maximum number of type-2b cells was observed ( $p = 3.77476 * 10^{-15}$ ). (D) The number of type-3+ cells within the SGZ peaks at two days p.i. ( $p = 1.064 * 10^{-10}$ )

From the above displayed absolute number of type-3+ cells (TABLE 2-B), the mitotic rate was calculated. Here I demonstrate that 4% of all type-3+ cells are undergoing division after the first BrdU injection, while 19.3% of all these cells express BrdU after 14 hours and three injections.

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### *Astrocytes and other cells*

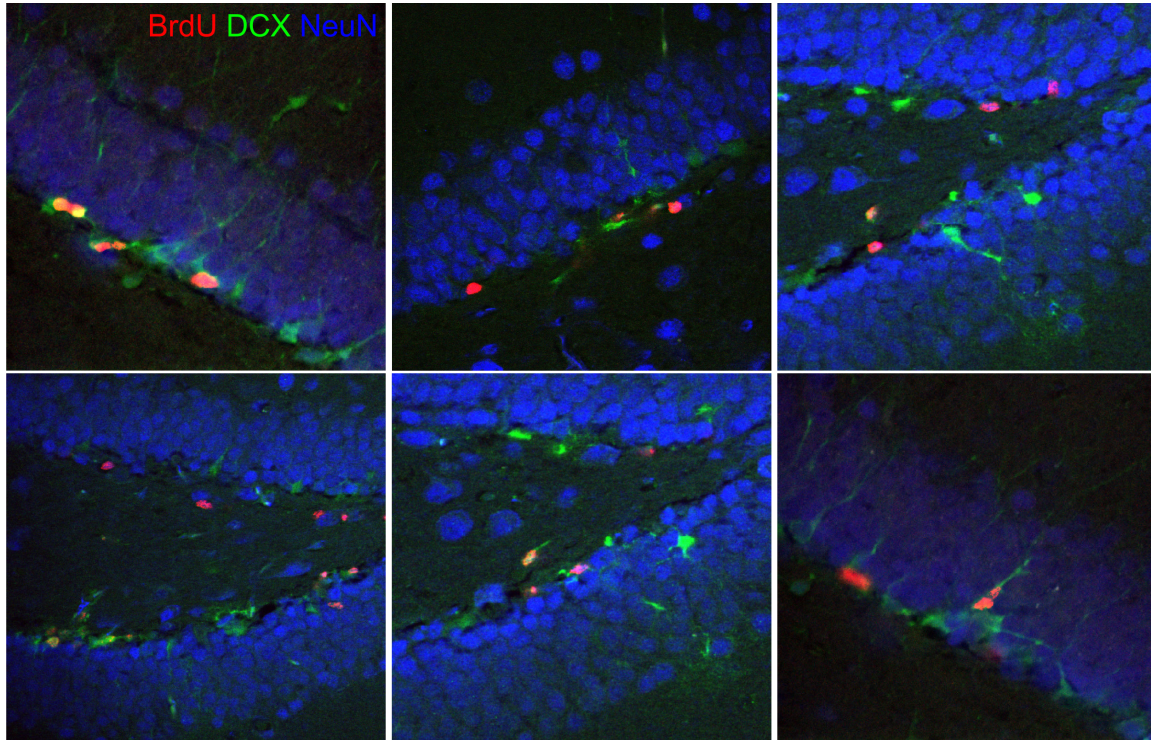
To complete the rough image of the absolute values of cells occurring during these early ahNG stages also astrocytes and cells which are BrdU-positive, but could not be included into one of the previously phenotyped groups, must be summed up. With respect to astrocytes overall changes reach significance with  $p = 2.48024 * 10^{-13}$ , while changes in the absolute value of not further phenotyped cells reaches significance with  $p = 2.53686 * 10^{-13}$ . For the cell population of unknown phenotype (called “other cells” further on) we found a strong increase during the first few time points, roughly followed by stagnation phase between day two and seven after the initial injection. Within the later time points the number of other cells drops again. Concretely this mixed population of diverse cells starts at  $34.69 \pm 18.56$  cells at 2 hours, reaches  $80.71 \pm 31.85$  at 14 hours, afterwards increases to  $252.45 \pm 66.80$  cells at day one to arrive at a number of  $572.77 \pm 165.35$  cells/DG after two days. The values between day three and seven fluctuate between 304 and 486 cells/DG until they finally drop again towards  $151.61 \pm 96.57$  (10d),  $148.22 \pm 57.19$  (14d) and  $62.85 \pm 36.71$  (21d) other cells per DG.

Regarding the astrocytes no cells can be found during the first few hours after injection. First clearly identifiable astrocytes were visible after two days ( $20.46 \pm 17.55$ ). Their number constantly increases till day seven after the initial administration of BrdU. Only the increase between four days and five days was found to be slightly significant with  $p = 0.02391$ . After the maximum value of  $183.53 \pm 42.72$  cells/DG, a sharp decrease of the absolute values occurs to  $65.74 \pm 62.28$  (10d),  $37.63 \pm 15.31$  (14d) and  $15.31 \pm 10.35$  cells per DG at 21 days post injection with a significant difference between day seven and ten ( $p = 1.69936 * 10^{-5}$ ).

#### **2.1.2.4. *Analyzes of the late mitotic and early postmitotic differentiation stages***

To ascertain the kinetics of the late mitotic and early postmitotic cells further analyses were performed. Sections of each brain were co-labeled with BrdU (Red), DCX / GFP (Green) and NeuN (Blue). Since NestinGFP mice were used we stained both DCX and GFP in Green. GFP and NeuN are known to have no overlap (Filippov et al., 2003, Kronenberg et al., 2003). Therefore, all triple-labeled cells are early immature neurons, already postmitotic, but not yet fully integrated into the hippocampal network. Also all NeuN- / BrdU-positive cells are clearly postmitotic, even a step ahead in their differentiation process. All cells which are only positive for DCX / GFP are still mitotic

cells in stage 1 to 4, thus type-1-3 cells (TABLE 1-B, FIGURE 1-2, introduction). From the results, I obtained thus far, it is possible to calculate the number of type-3 and postmitotic DCX-positive cells for each time point, as well as any potential postmitotic cell that has already down regulated DCX.



**FIGURE 2-9:** Exemplary photos of slices stained for BrdU, DCX and NeuN. Cells are counted according to their marker expression. BrdU / DCX-positive cells are assumed to be type-1-3 cells, BrdU- / DCX- / NeuN-positive cells are counted as early immature neurons and BrdU / NeuN-positive cells as premature granule cells.

#### **2.1.2.5. Distribution of mitotic and postmitotic differentiation stages over time**

Relative distribution indicates a major proportion of cells within early differentiation stages such as type-2a, type-2b and type-3 cells. From our previous analyzes we know that the proportion of type-1 cells varies only roughly around 2% and seems quite stable. Thus, in the following analyzes of the later time points they will be neglected.

With progressing time after cell division the percentage distribution changes towards higher proportions of postmitotic cells. These postmitotic cells express both NeuN and DCX in the early immature phase and only NeuN as soon as they reach the premature differentiation stage (FIGURE 2-10). Overall significances were obtained for all analyzed

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cell types. Cells in the mitotic stages 1 to 4 of ahNG start from a maximal proportion of cells at two hours after the first BrdU injection ( $p < 1.0 * 10^{-15}$ ). In contrast, early immature neurons reach a plateau with strong fluctuations between day three and six p.i. ( $p = 8.22419 * 10^{-11}$ ) and premature granule cells even reach their maximal proportion at the latest observed time point, 21 days after the initial BrdU administration with  $p = 4.77015 * 10^{-11}$  (FIGURE 2-10).

Cells within the mitotic stages of ahNG are positively stained for BrdU and GFP / DCX. The relative proportion of these cells strongly depends on the time point after perfusion (FIGURE 2-7, see above). Also the fraction of both immature and premature granule cells varies highly within the first three weeks after the initial administration of BrdU. Within the first two hours mitotic cells mainly contribute to the total number of BrdU labeled cells with  $78.82 \pm 5.02\%$ , while no postmitotic cells were detected. 14 hours p.i. the first NeuN-positive cells were found within the dentate gyrus still a major fraction remained mitotic ( $67.94 \pm 5.69\%$ ), but  $7.69 \pm 2.14\%$  expressed both markers migration factor DCX and neuronal marker NeuN. Additionally,  $2.8 \pm 0.94\%$  of all cells even expressed only BrdU and NeuN indicating a premature cell status. The decrease among mitotic cells gets significant with  $p = 0.03731$ , while the changes for both postmitotic differentiation stages remains non-significant.

The fraction of postmitotic cells strongly increases with time. One day p.i. only  $57.94 \pm 7.2\%$  of all BrdU-positive cells are still mitotic while  $12.79 \pm 4.86\%$  are immature and  $6.73 \pm 1.98\%$  are premature. Although the day one time point does not reach statistical significance for any of the above cell types the trend continues. Two days after the initial dose of BrdU only  $39.38 \pm 6.77\%$  are mitotic ( $p = 6.53922 * 10^{-4}$ ), while 1 / 5 of all cells are found to belong to the population of immature cells ( $21.28 \pm 3.35\%$ ,  $p > 0.06$ ) and  $16.97 \pm 3.46\%$  are premature granule cells ( $p > 0.12$ ). Over the next few time points changes are relatively minor in the distribution of the different cell types. Mitotic cells reach  $25.93 \pm 2.93\%$  (3d p.i.),  $25.83 \pm 9.25\%$  (4d),  $23.39 \pm 10.19\%$  (5d) and  $21.05 \pm 6.07\%$  (6d) during this period, followed by an increase towards  $30.31 \pm 10.06\%$  one week after the BrdU administration. With respect to the immature neurons values between  $24.93 \pm 5.91\%$  (4d) and  $35.47 \pm 2.31\%$  (6d) are reached with additionally  $32.53 \pm 5.59\%$  (3d),  $29.61 \pm 9.21\%$  (5d) and  $29.71 \pm 4.93\%$  (7d) on the other observed time points. The premature granule cells on the other hand fluctuate roughly around 24% with  $20.44 \pm 7.62\%$  at three days p.i.,  $25.92 \pm 8.08\%$  four days p.i.,  $26.48 \pm 5.23\%$  five days p.i.,  $23.6 \pm$

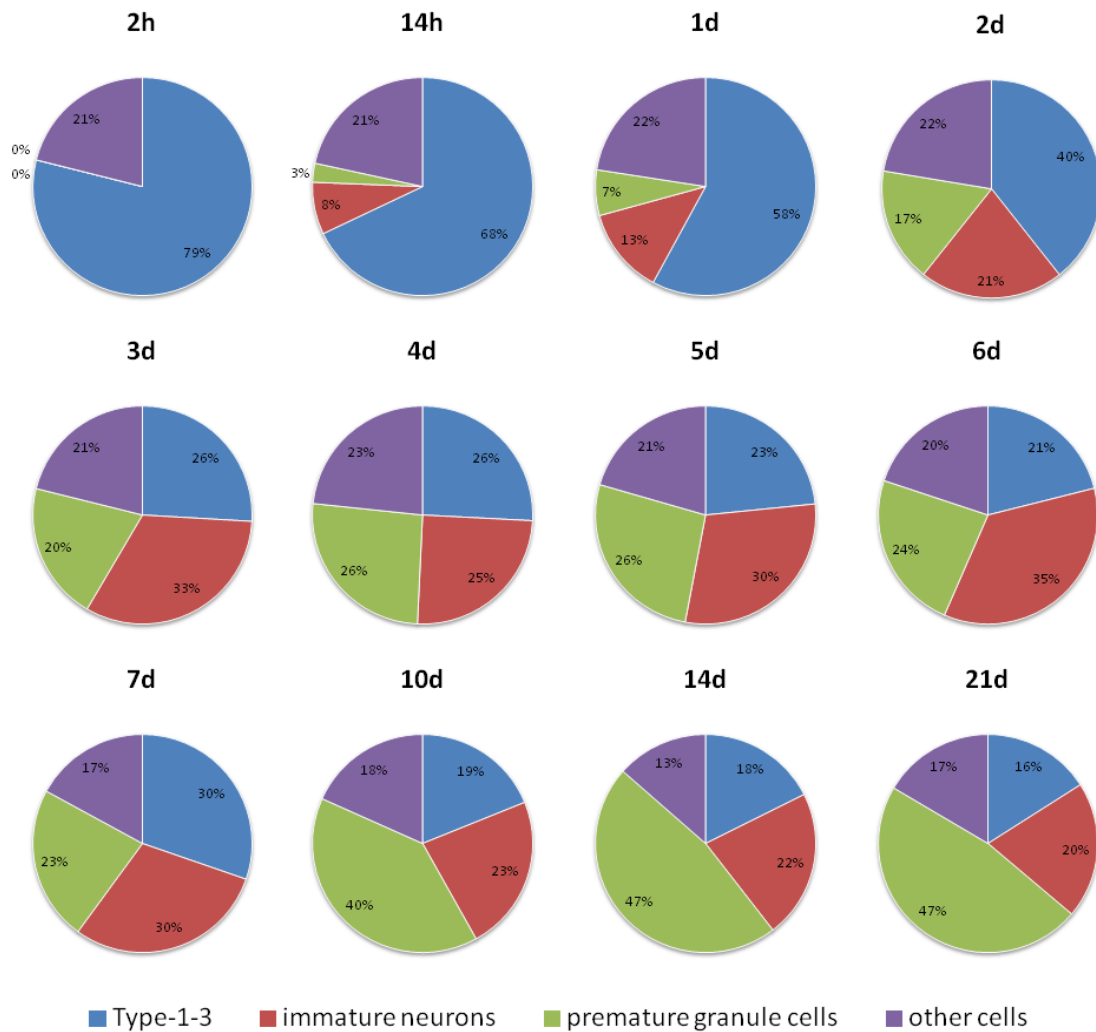
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4.86% six days p.i. and  $22.93 \pm 7.95\%$  seven days after the initial administration of BrdU. All changes occurring between day three and seven are non-significant towards the previous time point in Fisher's post-hoc analyzes, except for day three versus two for immature neurons ( $p = 0.01236$ ) and mitotic cells ( $p = 0.00891$ ). Also the changes observed during the latest time points are non-significant for early immature neurons, but changes between seven and ten days are slightly significant for both mitotic cells ( $p = 0.03057$ ) and premature granule cells ( $p = 0.0136$ ). The values between day ten and 21 fluctuate around 17% for the cells in differentiation stages one till four. Among the immature neurons a trend towards an increase was observed, but p-values did not reach  $p < 0.05$ . Moreover, proportions of premature granule cells are further increasing. Speaking of concrete values, mitotic cells reach  $18.87 \pm 7.19\%$  (10d),  $17.63 \pm 17.36\%$  (14d) and  $15.95 \pm 11.32\%$  (21d), while immature neurons are observed with proportions of  $23.01 \pm 3.66\%$  (10d),  $21.89 \pm 18.67\%$  (14d) and  $20.25 \pm 12.55\%$  (21d). Premature granule cells increase towards  $39.82 \pm 7.67\%$  at ten days p.i. and finally reach  $47.31 \pm 22.47$  at 21 days p.i. with an intermediate measurement of  $46.96 \pm 28.44\%$  at 14 days (FIGURE 2-10).



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**FIGURE 2-10:** Relative distribution of cells within the different phases of adult neurogenesis separated for time after perfusion with special focus upon postmitotic cells.

### 2.1.2.6. Development of the distinct postmitotic cell types in absolute numbers

Besides relative numbers of cells within each stage of differentiation I also obtained the absolute number of cells (FIGURE 2-11). Overall significance was obtained for all analyzed cell types. Cells within the mitotic stages 1 to 4 of ahNG reach a maximum at 24hours after the first BrdU injection ( $p = 3.21965 * 10^{-15}$ ). In contrast, early immature neurons peak much later at three days post injection ( $p < 1.0 * 10^{-15}$ ) and premature granule cells even show a time delay of four days after BrdU until they reach their maximum with  $p = 1.55553 * 10^{-12}$  (FIGURE 2-11).

### *Type-1-3 cells*

Overall changes between the different time points regarding mitotic stages are highly significant, but Fisher's post-hoc test only reveals some significant changes towards the previous observed time point. Starting at two hours post injection I found on average  $450 \pm 122$  cells positive for both BrdU and DCX, but negative for NeuN. This number significantly increases to  $1028 \pm 240$  cells at the 14 hours time point ( $p = 2.66617 * 10^{-5}$ ). A further increase till the next 24 hours time point revealed absolute cell counts of  $1227 \pm 522$  mitotic cells. The significant drop between day one and two led to  $822 \pm 120$  cells ( $p = 0.0023$ ). Only small changes between this time point and the next two observations were found with  $570 \pm 149$  cells after three days ( $p = 0.04428$ ) and  $564 \pm 293$  cells after four days. The difference between three and four days p.i. is not-significant. The same holds true for all following time points, although a trend toward reduction in the number of mitotic cells in dependency to time was detectable. Concretely, five days p.i.  $345 \pm 247$  cells were found in this category followed by  $219 \pm 163$  (6d),  $327 \pm 135$  (7d),  $118 \pm 69$ (10d),  $62 \pm 67$  (14d) and  $71 \pm 59$  (21d) cells (FIGURE 2-11 A).

### *Early immature neurons*

In contrast to mitotic cells, early immature neurons express not only BrdU and DCX, but also NeuN, thus, they were triple-labeled. First postmitotic immature cells were found 14 hours p.i. The relative proportion of these cells rapidly increases starting from  $115 \pm 39$  cells at 14 hours, over  $245 \pm 78$  cells at day one,  $455 \pm 128$  cells at day two to  $697 \pm 112$  cells at day three post injection. Overall significance is striking high with  $p < 1.0 * 10^{-15}$  and Fisher's post-hoc test also indicates significant changes between day one and two p.i. ( $p = 0.00389$ ) and two days and three days p.i. ( $p = 6.65284 * 10^{-4}$ ). At day four absolute numbers of immature neurons ( $544 \pm 247$ ) decreases significantly with  $p = 0.02699$ , followed by a stagnation period with  $377 \pm 160$  cells at day five,  $344 \pm 169$  cells at day six and  $326 \pm 127$  cells at day seven p.i. After a further decrease between day seven and ten ( $135 \pm 33$  cells/DG) and day ten and 14 ( $69 \pm 42$  cells/DG) a second stagnation period ends with  $88 \pm 63$  cells at 21d after the initial BrdU administration (FIGURE 2-11 B). Besides the above mentioned p-values, significant changes also occur between day four and five ( $p = 0.01616$ ) and day seven and ten ( $p = 0.00828$ ).

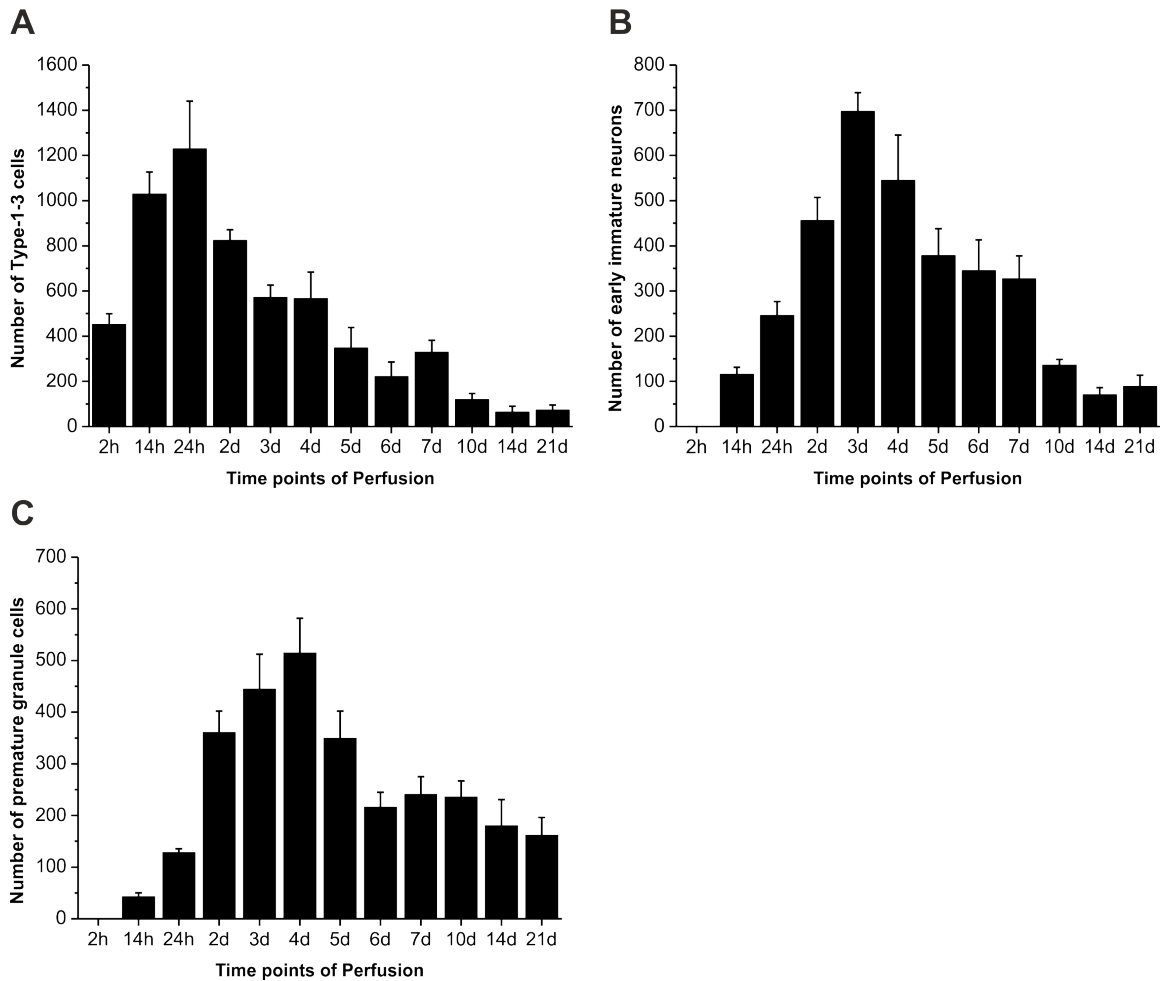
## Results - Dynamics of neuronal development in the adult dentate gyrus

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### *Premature granule cells*

At the first examined time point no granule cells expressing BrdU and NeuN, but lacking GFP / DCX were found. With increasing time the proportion of these granule cells also increased rapidly and significantly ( $p = 1.55553 * 10^{-12}$ ). At 14 hours after BrdU administration the first granule cells lacking DCX expression could be observed, adding up to  $43 \pm 18$  cells/DG. A strong but non-significant increase continues during the next observed time points with a significant transition between day one and two ( $p = 3.15622 * 10^{-4}$ ). In absolute numbers  $128 \pm 19$  cells were found 1 day p.i.,  $361 \pm 100$  at day two,  $445 \pm 178$  at day three and  $515 \pm 164$  cells at day four p.i. A significant decrease occurs afterwards, also continuing further on.  $350 \pm 138$  cells were found five days after the initial BrdU administration ( $p = 0.00664$ ) and  $216 \pm 70$  premature granule cells at the observed day six time point ( $p = 0.02654$ ). The later time points are characterized by a low fluctuation in absolute cell numbers with  $241 \pm 83$  (7d),  $236 \pm 76$  (10d),  $180 \pm 125$  (14d) and  $162 \pm 84$  (21d) cells per dentate gyrus.

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**FIGURE 2-11:** Absolute number of cells within distinct differentiation stages in dependency of time. (A) The number of mitotic cells within the SGZ rises during the first 24 hours after BrdU injection ( $p = 3.21965 * 10^{-15}$ ) (B) 14 hours after BrdU injection the first NeuN-positive cells can be detected. Immature neurons, positively labeled for DCX and NeuN, reach their peak at day 3 ( $p = 3.331 * 10^{-16}$ ) (C) 4 days after BrdU the maximum number of premature granule cells was observed ( $p = 1.55553 * 10^{-12}$ ).

### 2.1.2.7. Calculated absolute numbers of cells at each differentiation stage

To be able to compare the number of cells at each differentiation stage for both mitotic and postmitotic stages I obtained relative numbers and multiplied them with the absolute number of BrdU-positive cells obtained by means of BrdU DAB cell counts. This procedure enabled me to normalize all data with the common basis and thereby detach it from the different absolute numbers obtained with different staining. The new set of data however lacks some accuracy, but allows comparison across different data collection sets. Changes within the data remained minor with preservation of the major developmental characteristics for each analyzed cell type over time (FIGURE 2-11 and 2-12).

## Results - Dynamics of neuronal development in the adult dentate gyrus

### *Type-1-3 cells*

The absolute number of mitotic cells, calculated from the relative distribution and BrdU DAB cell counts changes significantly with  $p < 1.0 * 10^{-15}$ . An increase from  $426 \pm 99$  cells/DG at two hours to  $1290 \pm 247$  cells at 14 hours and to a maximum of  $1346 \pm 366$  cells at one day p.i. was observed, which is similar to the results obtained with all stained cells counted. Here, only the transition from two hours to 14 hours was significant ( $p = 2.67041 * 10^{-12}$ ). Afterwards, a huge drop towards day two post injection with only  $1113 \pm 167$  cells/DG ( $p = 0.02277$ ) was obtained from the calculated data, while a rather slow decrease was indicated between day three p.i. ( $525 \pm 95$ ) and six ( $237 \pm 118$ ). The reduction in absolute cell numbers from day two to three is significant with  $p = 7.00019 * 10^{-8}$ , while all other described transitions were non-significant. Additionally, the slight increase between day six and seven ( $396 \pm 201$ ) was non-significant. The only other transition with  $p < 0.05$  occurred to be the decrease between day seven and ten ( $57 \pm 19$  cells/DG;  $p = 0.00115$ ). The number of cells at the later time points seemed rather stable with  $59 \pm 85$  cells two weeks p.i. and  $19 \pm 19$  cells three weeks p.i., just a tendency towards a small decrease could be indicated between to two latest observed time points (FIGURE 2-12).

### *Early immature neurons*

The postmitotic cells peaked much later as the mitotic cells. For the early immature neurons a maximum was found three days after the initial administration of BrdU, but the transition between day two p.i. and three was non-significant. Thus, one could also assume an earlier peak between these observed time points. Overall, changes were significant with  $p < 1.0 * 10^{-15}$ , but Fisher's post-hoc test revealed high variations in p-values between the different observed time points. The first postmitotic, NeuN-positive cells were found 14 hours p.i. with only low numbers of  $145 \pm 44$  early immature neurons. A few hours later, one day after the initial dose of BrdU this number increased to  $281 \pm 78$  cells/DG ( $p = 0.01336$ ). A further, even stronger increase occurred at day two p.i., where  $606 \pm 120$  cells were obtained as result of the calculations with a significant change towards the previous time point of  $p = 7.48415 * 10^{-8}$ . At the observed three day time point even  $655 \pm 132$  cells were assumed to be located within the DG. A rather strong decrease ( $p = 1.29387 * 10^{-6}$ ) was followed by a stable phase between four days and seven days post injection. In concrete numbers  $380 \pm 87$  (4d),  $321 \pm 94$  (5d),  $408 \pm$

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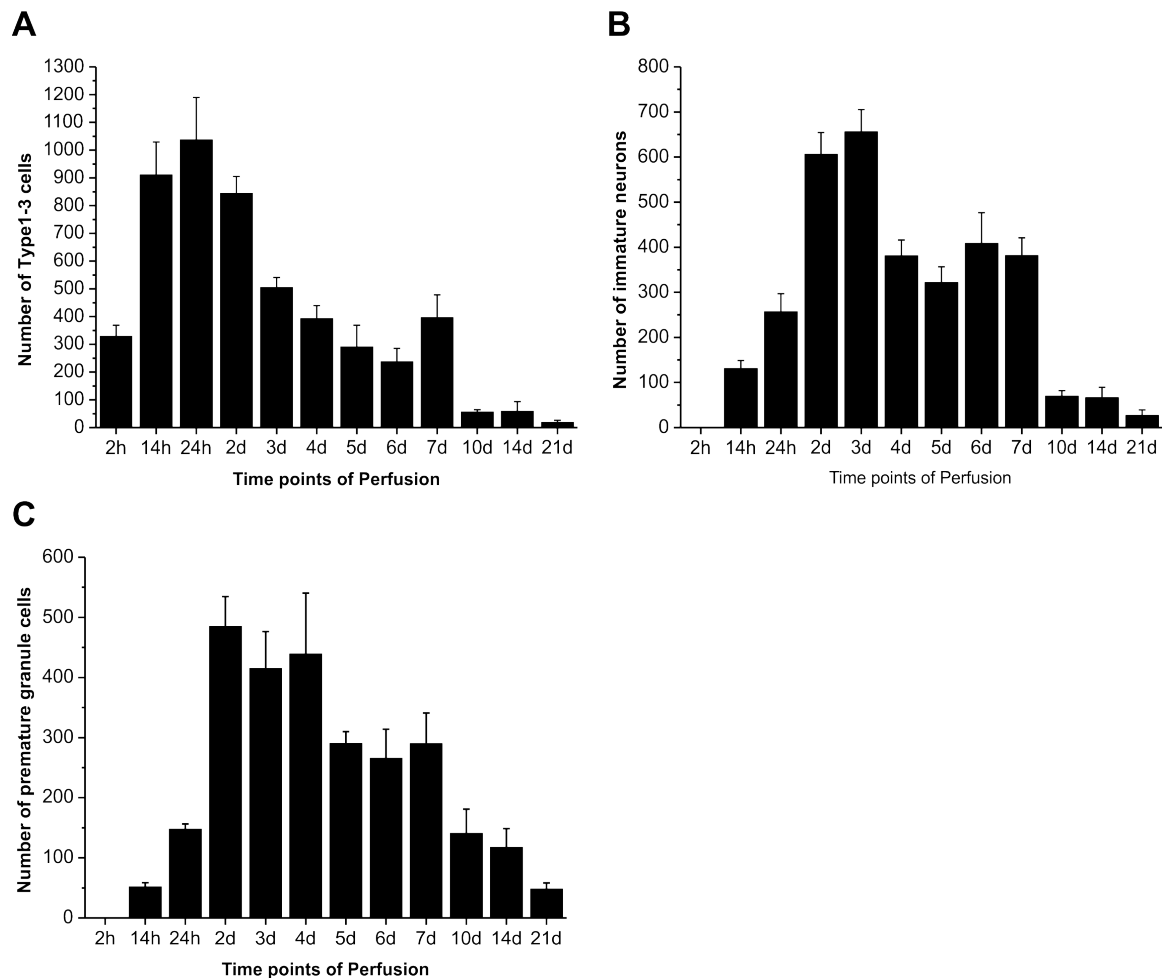
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168 (6d),  $381 \pm 99$  (7d) cells were obtained as result of the estimations. A second plateau is observed between ten days and 14 days with  $76 \pm 40$  and  $66 \pm 58$  cells/DG, respectively. Only 21 days p.i. less cells were estimated with  $27 \pm 30$  (FIGURE 2-12). Regarding the results of Fisher's post-hoc test only the decrease between seven and ten days was significant ( $p = 3.28671 * 10^{-7}$ ).

### *Premature granule cells*

Concerning the premature granule cells overall, a strong increase between the first observed time points, followed by a stagnation phase between day two and four was estimated. In concrete numbers, first  $51 \pm 17$  BrdU- and NeuN-positive cells were detected at 14 hours p.i., whereas one day p.i.  $148 \pm 20$  and two days p.i.  $485 \pm 121$  cells were gained as result of the multiplication of the BrdU numbers with the relative proportion of these cells.

A second quite stable phase with low fluctuations between 5d ( $291 \pm 52$ ), 6d ( $266 \pm 118$ ) and 7d ( $290 \pm 124$  cells/DG) was followed by a slow decrease towards the last observed time point at 21 days post injection.  $141 \pm 99$  (10d),  $118 \pm 76$  (14d) and  $48 \pm 25$  (21d) are estimated for these time points. ANOVA revealed significance with  $p = 2.1676 * 10^{-12}$ . Fisher's post-hoc test further showed significant changes towards the previous observed time point for two days ( $p = 2.43829 * 10^{-6}$ ), five days ( $p = 0.02057$ ) as well as ten days ( $p = 0.02473$ ).



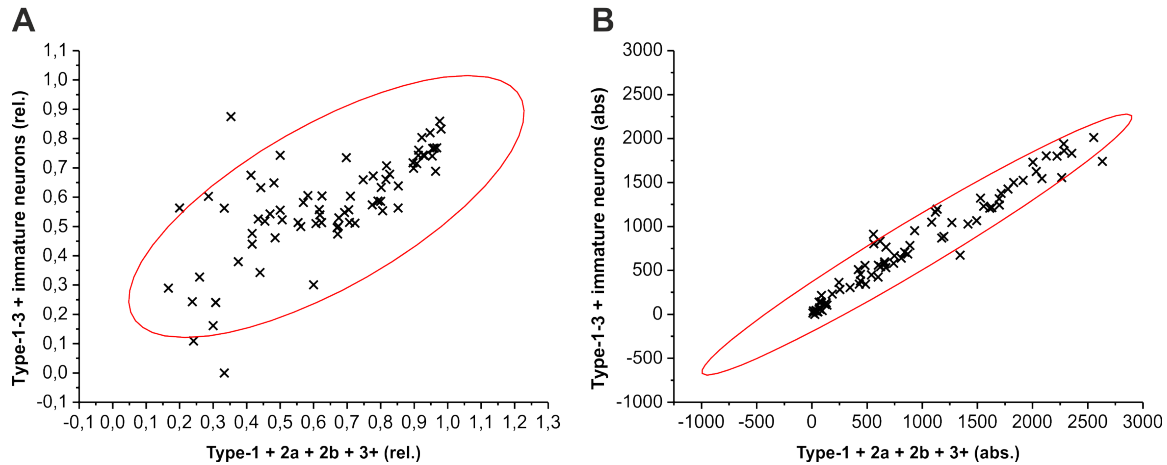
**FIGURE 2-12:** Absolute number of cells within distinct developmental stages for the second fluorescent staining (staining II) calculated from the relative proportion of cells within each stage times the absolute number of BrdU-positive cells obtained via DAB staining. (A) Cells within the mitotic stages of ahNG reach their maximum already after 24hours ( $p < 1.0 * 10^{-15}$ ), while postmitotic cells peak roughly at 3 days post BrdU injection ((B) Early immature neurons with  $p < 1.0 * 10^{-15}$  and (C) premature granule cells with  $p = 2.1676 * 10^{-12}$ )

#### 2.1.2.8. Combination of mitotic and postmitotic differentiation stages

To obtain an overview of the entire differentiation process, data obtained by means of two confocal staining were mathematically combined. To ensure similar data sets regression between the relative proportions and the estimated absolute numbers of mitotic and early immature granule cells were calculated from the two different data sets. By summing up type-1, 2a, 2b and 3+ cells from the one fluorescent staining and also adding type-1-3 cells and early immature neurons from the other fluorescent staining I received information about the same population of cells. These values are highly correlated to each

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other, as they should be, with  $r = 0.71495$ ,  $p = 8.35998 \cdot 10^{-13}$  (Pearson correlation) for the relative proportion of mitotic and early immature neurons and  $r = 0.9757$ ,  $p < 1.0 \cdot 10^{-15}$  for the estimated absolute number of cells (FIGURE 2-13).



**FIGURE 2-13:** Correlation of the population of all mitotic differentiation stages in combination with the early immature neuron for two different fluorescent analyses. The x-axis displays values obtained from the staining for mitotic differentiation stages, while the y-axis displays values estimated from the analyses of postmitotic differentiation stages. (A) Proportions of the same cell populations obtained in different analyses highly correlates with  $r = 0.71495$ . (B) Estimated absolute numbers of cells of the same population highly correlates with  $r = 0.9757$ .

To mathematically combine the two different data sets and calculate the number of type-3 cells one could think of three different possibilities:

Firstly, we can start the combination from the staining for the mitotic differentiation cell stages. It is possible to assume, that all other cells counted during the analyses of the mitotic stages of differentiation are either oligodendrocytes, endothelia or postmitotic cells. Since we have no information about the population of the oligodendrocytes and endothelial cells, it would be necessary to estimate the size of that population. For simplification we subtract the number of premature granule cells from the number of the unknown population and receive a rest, which would need to be oligodendrocytes, endothelial cells and maybe not yet identified other cell products of neurogenesis / gliogenesis / angiogenesis deriving from type-1, type-2a cell stages.

Additionally, the population of the type-3+ cells contains type-3 cells and early immature neurons, thus, we need to subtract the number of the immature neurons to receive the true population of type-3 cells (Method A). This procedure results in  $101 \pm 27$  cells at 2 h, 320



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$\pm 209$  cells at 14h,  $380 \pm 172$  cells at 1d,  $331 \pm 159$  cells at 2d,  $70 \pm 117$  cells at 3d,  $302 \pm 183$  cells at 4d,  $134 \pm 180$  cells at 5d,  $10 \pm 56$  cells at 6d,  $-51 \pm 70$  cells at 7d,  $-1 \pm 14$  cells at 10d,  $5 \pm 46$  cells at 14d and  $-4 \pm 18$  type-3 cells at 21 days p.i. (FIGURE 2-14).

Now, we combine all new numbers of cells within each developmental stage. As result we receive the same numbers of type-1, type-2a and type-2b cells, early immature neurons and premature granule cells as described above, but detailed percentages for the type-3 cells and all other non-neurogenic cells, excluding astrocytes. ANOVA reveals significant changes for the relative proportion of type-3 cells obtained from this calculation with  $p = 1.79892 * 10^{-5}$ . Two hours p.i.  $19.01 \pm 5.24\%$  of all BrdU labeled cells are classified as type-3 cells. Moreover, 14 hours and 24 hours after the initial BrdU administration proportions stay similar with  $16.63 \pm 9.74\%$  (14h) and  $16.84 \pm 8.05\%$  (24h). Two and three days p.i. we find a huge decrease to  $11.74 \pm 5.55\%$  (2d) and  $3.48 \pm 5.83\%$  (3d). Although Fisher's post-hoc test shows p-values bigger than 0.1 for both changes towards the previous time points, this seems quite surprising. The next time point shows values close to day one and two again of  $18.5 \pm 7.95\%$  (4d) with even a significant increase towards three days p.i. ( $p = 0.00531$ ). All other changes in the fraction of type-3 cells are non-significant in Fisher's post-hoc test, but a strong trend towards fewer cells within this differentiation stage seems detectable and plausible. Concretely calculations reveal  $9.51 \pm 12.56\%$  at day five,  $1.34 \pm 4.97\%$  at day six,  $-3.63 \pm 5.63\%$  at day seven,  $0.54 \pm 3.76\%$  at day ten,  $1.32 \pm 18.47\%$  at day 14 and  $-1.44 \pm 12.6\%$  at day 21 post injection. The relative proportion of all not further classified cells varies from 0.49% to 12.8% insignificantly with a small trend to increase with progressing time ( $p = 0.54688$ ).

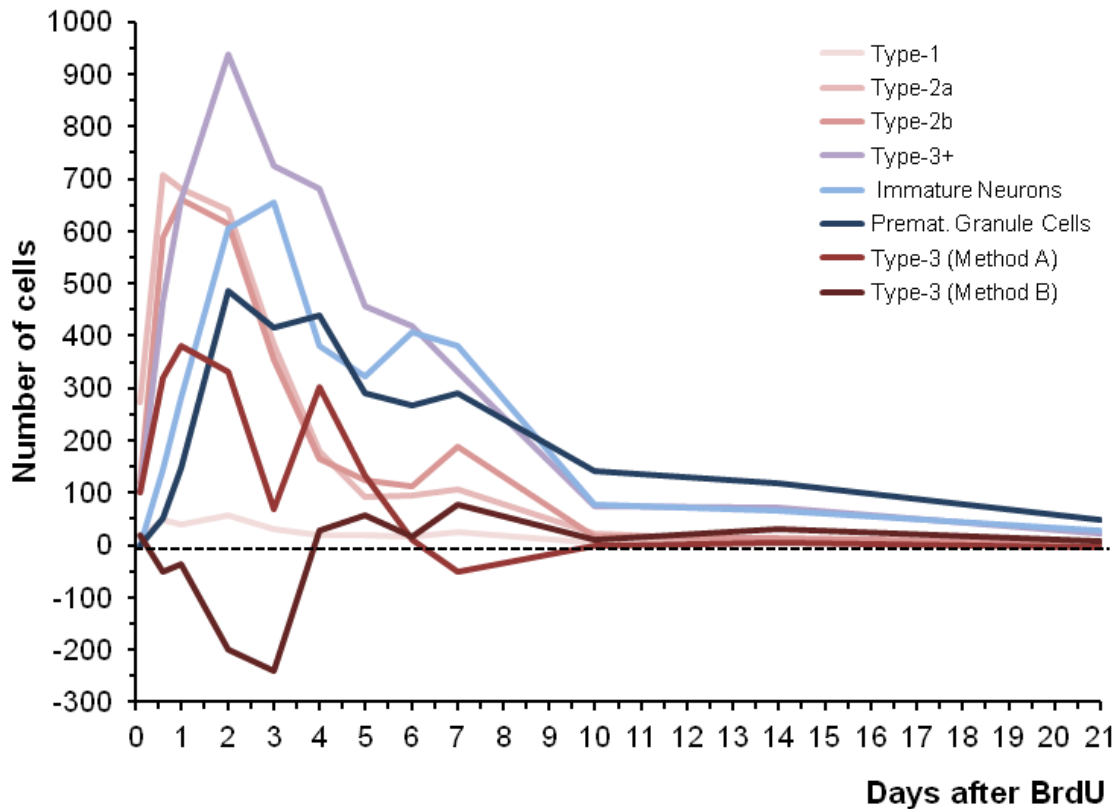
Secondly, it is possible to combine the two data sets starting from the analyses of the postmitotic differentiation stages. We can assume that the unknown population of the analyses of the postmitotic cell stages contains all astrocytes and oligodendrocytes / endothelia / other cells. Thus, we would subtract the number of astrocytes to receive the population of the not identified rest.

Furthermore, the population of the type-1-3 cells in combination with the early immature neurons should contain all type-1, type-2a, type-2b and type-3 cells. Thus, one possibility would be to subtract the number of all type-1, type-2a and type-2b cells to receive the number of type-3 cells (Method B). This way we also get detailed information about the type-3 cells and the unclassified cells. For this calculation the number of type-3 cells

varies non-significant with values between -6.81% and 8.51% and with absolute numbers between -242 und 78.

Thirdly, another way to combine both data sets is by starting from the calculated absolute number. Both are based on the BrdU DAB cell counts, thus, the variations due to different slices and different antibody combinations are ruled out as good as possible. Taken these absolute numbers we can calculate a relative distribution of all cell types, but we need to disregard the population of unclassified cells within each staining to prevent overestimation of the newborn glia cells and endothelia. Moreover, immature neurons are included in both staining, thus, this method would lead to an overestimation of immature neurons and no exact information about type-3 cells could be obtained. An overview on the estimated number of cells within the distinct differentiation stages are displayed in figure 2-14.

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**FIGURE 2-14:** Estimated absolute number of cells within each differentiation stage, as described above. Mitotic cell stages are displayed with Red lines, while postmitotic neuronal differentiation stages are illustrated with Blue lines. The baseline at zero is displayed with a dotted line. Type-3 cells are calculated by two different procedures (Method A / B, see text), thus, different values are obtained. Although negative values are not realistic, the trend in the time course is similar for both estimations.

For the relative distribution of cells of the different analyzed cell types, combination of both data sets revealed a new impression on ahNG, more detailed than illustrated by any other study before. Since I retrieved strongly different values for type-3 cells for each calculation method, I rely on the data set, which seems most plausible further on. Method A resulted in less negative values than method B, therefore the first set of data is processed.

Concretely, we find quite stable numbers of type-1 cells with low non-significant fluctuations between 1.55 and 3.78% ( $p = 0.994$ ). For type-2a cells ANOVA revealed significant changes ( $F(11,62) = 49.4078$ ,  $p < 1.0 \cdot 10^{-15}$ ) with a maximum number at the first observed time point. Two hours p.i.  $53.36 \pm 7.03\%$  of cells express SOX2 and lack both, a GFAP+ radial process and DCX. 14 hours p.i.  $37.93 \pm 5.5\%$  still belong to the

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population of type-2a cells with decreasing tendencies with increasing time. 14 days p.i. only  $4.2 \pm 4.14\%$  of all neurogenic cells were still type-2a cells. This ratio stayed the same towards the last time point 21 days p.i. (FIGURE 2-15). Fisher's post-hoc test indicates significant drops between 2 h and 14h ( $p = 3.76886 * 10^{-6}$ ), 14h and 24h ( $30.1 \pm 8.71\%$ ;  $p = 0.0124$ ), 24h and 2d ( $23.67 \pm 3.49\%$ ;  $p = 0.03845$ ), 3d ( $20.18 \pm 5.13\%$ ) and 4d ( $12.7 \pm 4.73\%$ ;  $p = 0.01311$ ) and 7d ( $10.867 \pm 2.91\%$ ) and 14d ( $p = 0.03198$ ).

Type-2b cells also vary strongly in their proportion during the observed time interval. ANOVA revealed significant changes with  $F(11,62) = 8.48664$  and  $p = 6.86092 * 10^{-9}$ . Fisher's post-hoc test displayed p-values above 0.05 for the differences in the proportion of type-2b cells between two hours p.i. and 24 hours p.i., but a tendency towards an initial increase between the first two time points followed by a slight decrease seems indicated. In numbers two hours after the initial BrdU administration we find  $23.74 \pm 3.42\%$  type-2b cells, at 14 hours  $32.04 \pm 6.43\%$  and at 24 hours  $29.87 \pm 5.29\%$ . A strong, but non-significant decrease towards the next observed time point with  $21.87 \pm 10.26\%$  type-2b cells ( $p = 0.06991$ ) is followed by a stagnation phase between four days p.i. and six days p.i. and a slight increase towards seven days p.i. The only significant change occurs between seven days with  $18.12 \pm 10.4\%$  type-2b cells and ten days with  $7.29 \pm 6.77\%$  type-2b cells ( $p = 0.01517$ ). All other transitions from one time point to the subsequent time point are non significant according to Fisher's post-hoc test.

The proportion of type-3 cells has two local maxima. The first peak occurs after two hours with  $20.35 \pm 6.01\%$  followed by a plateau until 2d p.i. ( $12.51 \pm 6.44\%$ ) and a steep drop towards three days ( $3.4 \pm 6.37\%$ ). A sharp increase results in the second maximum at 4d p.i. ( $19.78 \pm 8.09\%$ ). An additional decrease ends 6d p.i. in a rather stable proportion in the range of  $-0.06$  to  $+1.32\%$  type-3 cells. (ANOVA surrendered  $F(11,62) = 3.15734$ ,  $p = 0.00192$ ). Fisher's post-hoc test only indicates significant changes between 3d and 4d ( $p = 0.04023$ ).

With respect to the neuronal stages of differentiation we find a strong increase between the first time points. 14 hours p.i.  $7.82 \pm 2.18\%$  of all neurogenic cells are early immature neurons, six days after the initial BrdU administration we find a maximum proportion of cells within this differentiation stage of  $45.03 \pm 9.17\%$ , followed by a slight decrease with  $31.76 \pm 21.53\%$  early immature neurons 21 days p.i. ANOVA revealed, that these

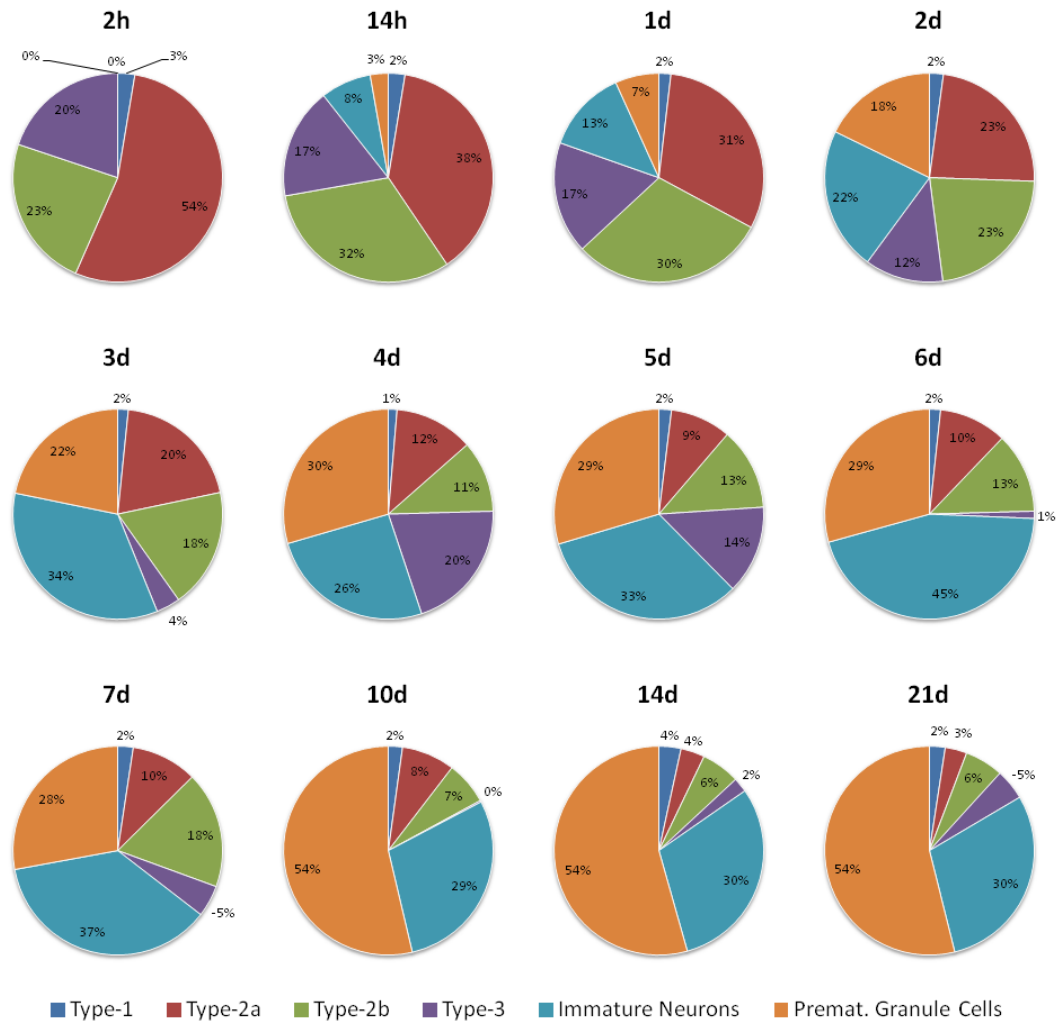
## Results - Dynamics of neuronal development in the adult dentate gyrus

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changes are significant with  $F(11,62) = 4.585224$  and  $p = 4.5192 * 10^{-5}$ , but Fisher's post-hoc test results in  $p > 0.05$  for all transitions in respect to the previous time point.

Premature granule cell neurons on the other hand increase in their proportion during the whole observed period of time. We finally end up with more than 60% ( $61.12 \pm 12.17\%$ ) of granule cells after three weeks indicating a relatively fast differentiation process. This increase in the fraction of neurons is highly significant according to analyses of variances (ANOVA,  $F(11,62) = 24.59241$ ,  $p < 1.0 * 10^{-15}$ ). Fisher's post-hoc test showed significant changes between seven days and ten days ( $p = 0.00119$ ) only. For further details regarding the distribution of cell types in dependency on the observed time point see FIGURE 2-15.

## Results - Dynamics of neuronal development in the adult dentate gyrus



**FIGURE 2-15:** Distribution of the different cell types in dependency on time, calculated from the two different data sets, described above. A nice shift from mitotic stages at the early time points to postmitotic stages at later times was confirmed (see text).

**2.2. Influences of group size on ahNG**

In this study we returned to previous analyses of the enrichment effect on ahNG. As described in the introduction enrichment consists of several features, such as activity, novelty, learning and social interaction. Usually control groups are housed among three to five animals per standard cage, while enriched animals are housed in groups of 10 to 15 animals. Additionally, the size of the cages allows voluntary distance to other individuals diminishing stress caused by agonistic behavior. In this study we used standard housing conditions with one exception: the cage measured always one meter by one meter. We analyzed net hippocampal neurogenesis in mice after being housed for seven days in the enlarged cages in the differently sized groups and 24 hours after BrdU injection. Thus, in this study we focused on the influence of social group effect on the proliferative features of ahNG. For detailed illustration of the experimental paradigm see FIGURE 4-1 (Material and Methods).

**TABLE 2-C:** Major outcomes of the statistical analyses of our different experimental groups.

*The close relation between mean and median stands for the good quality of our result, displaying high probability for a representation of the whole population. Moreover, the range between minimum and maximum shows relatively few outliers.*

	Mean	Median	Minimum	Maximum	Standard deviation (SD)	Standard error of the mean (SEM)
2 Animals per Cage	1972.5	1935	1752	2268	219.05	109.53
5 Animals per Cage	2359.2	2208	2034	2778	343.35	153.55
10 Animals per Cage	2818.8	2835	2262	3396	436.46	138.02
15 Animals per Cage	2999.4	3051	2424	3618	393.75	124.51

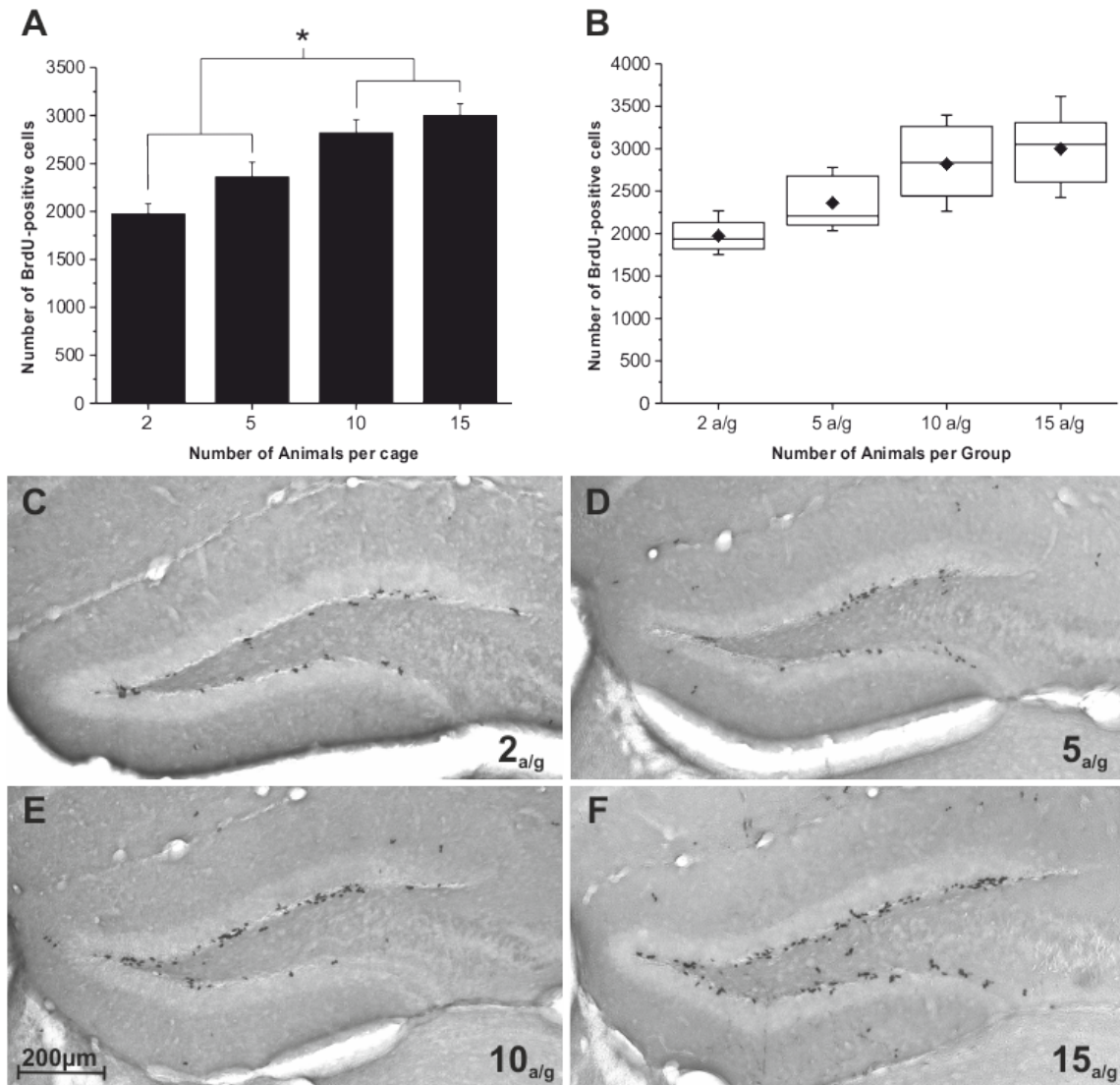
## Results - Influences of group size on ahNG

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Statistical values relevant for the analyses of the absolute BrdU cell counts are displayed in TABLE 2-C. Two-way ANOVA revealed that BrdU cell counts were significantly enhanced with growing group size. Fisher's post-hoc test further showed significant differences between two animals per cage and 10 animals per cage ( $p = 0.001$ ), two animals and 15 animals per cage ( $p = 0.039$ ), five animals and 10 animals per cage ( $p = 0.0001$ ) and five animals and 15 animals per cage ( $p = 0.006$ ). Neither could we find significant differences between 2 and 5 animals per group ( $p = 0.15$ ) nor between 10 and 15 animals per group ( $p = 0.31$ ), although the tendency of the results indicated more BrdU positive cells for the bigger group sizes (FIGURE 2-16). The correlation coefficient between number of animals per cage and number of BrdU-positive cells account to 0.68134 with  $p = 4.7222 * 10^{-5}$  (using Pearson's correlation). Linear regression reveals a gradient of 74.15375, y-axes intersection at 1961.4651,  $R^2 = 0.44439$ ,  $F(1,27) = 23.39484$  and  $p = 4.72228 * 10^{-5}$ .



## Results - Influences of group size on ahNG

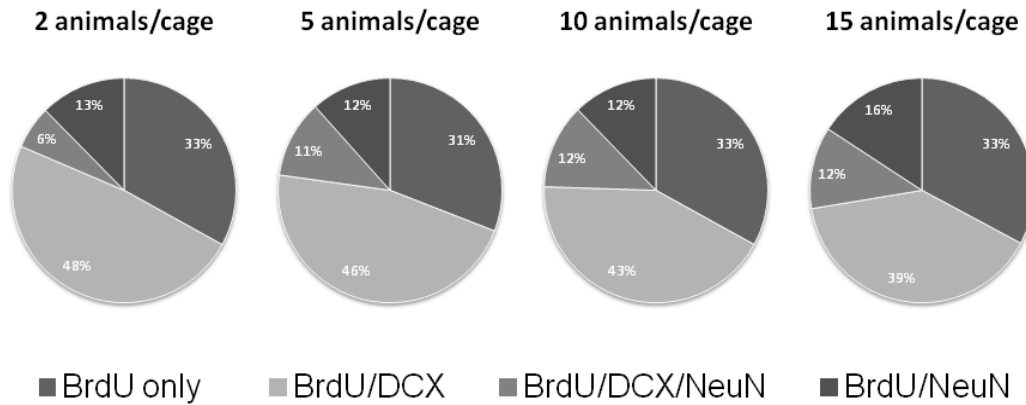


**FIGURE 2-16:** The absolute number of BrdU-positive cells highly depends on social conditions such as animals per group (a / g). (A) Adult neurogenesis is positively related to group size with significant differences between 2 and 10 a / c ( $p = 0.001$ ), 2 and 15 a / c ( $p < 0.0001$ ), 5 and 10 a / g ( $p = 0.039$ ) and 5 and 15 a / g ( $p = 0.006$ ). 2 and 5 a / g ( $p = 0.148$ ) as well as 10 and 15 a / g ( $p = 0.305$ ) are not significantly different. (B) Box plots for each group displaying the mean (diamond) the median (line), the interquartile range (box) and whiskers towards the minimum and maximum values (C-F) Light microscopically taken pictures further illustrating the differences in cell counts among groups.

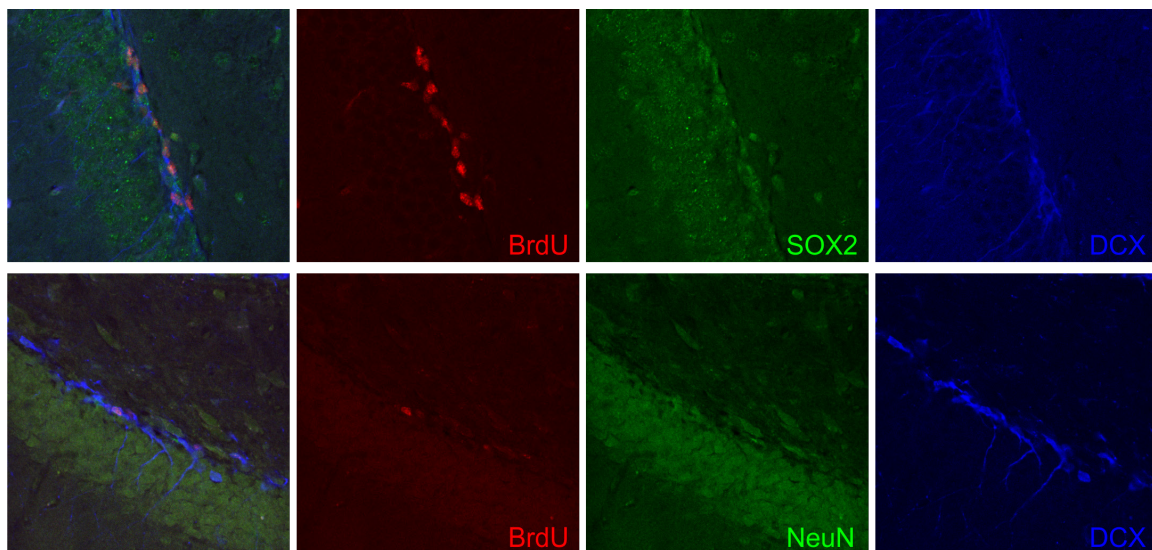
Phenotypical analyses revealed no significant differences correlated to the housing paradigm. We always found between 39% and 45% BrdU and DCX positive cells in the subgranular zone of the dentate gyrus. 6% to 12% of all BrdU-positive cells expressed both markers DCX and NeuN. Between 11% and 15% of the BrdU-positive cells also

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expressed NeuN, but not DCX. The rest of this cell population was only positive for BrdU (33% - 38%) (FIGURE 2-17, 2-18).



**FIGURE 2-17:** Phenotypical analyses revealed no significant differences between the different differentiation stages in ahNG.



**FIGURE 2-18:** Exemplary photos of cells analyzed under the confocal microscope with two different staining arrangements. First row: staining for BrdU, SOX2 and DCX, second row: staining for BrdU, NeuN and DCX

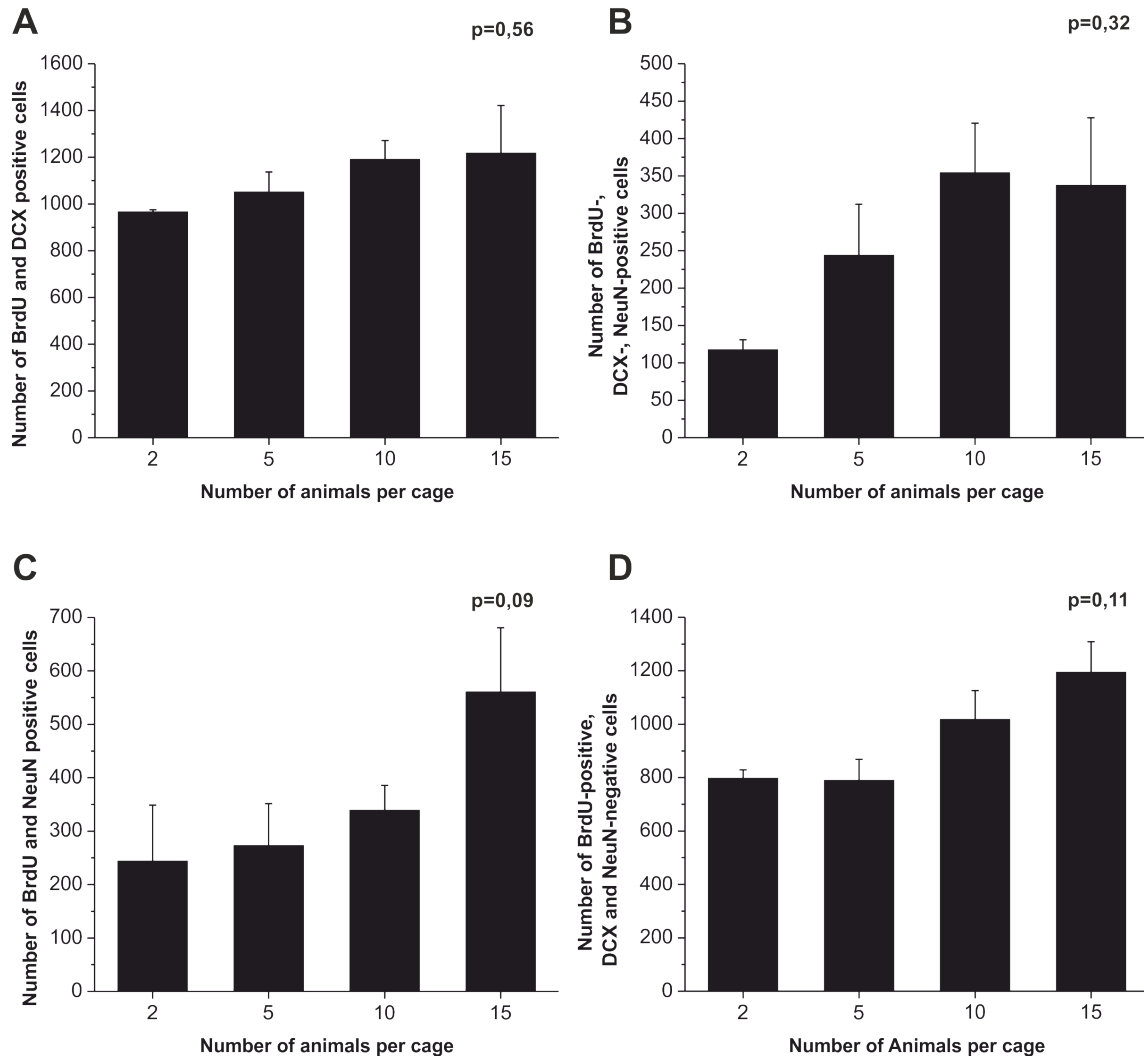
To get a better impression of the absolute number of cells of each type within the dentate gyrus we multiplied the relative proportion with the absolute number of BrdU positive cells. Results are displayed in FIGURE 2-19. Concretely, no significant changes were found in for the distinct cell types. Although tendencies seem plausible, with p-values

## Results - Influences of group size on ahNG

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above 0.09 it would be highly speculative to interpret any further information into the data set. Thus, I performed linear regression to obtain further information about the influence of group size on each cell type. This analyzes resulted in different strong positive correlations for each cell type. Concretely, we find a linear slope of  $y = 7.57104 * x + 801.2915$  with  $R^2 = -0.01763$ ,  $F(1,23) = 0.58427$  and  $p = 0.45241$  for the all BrdU-positive cells, negative for DCX and NeuN. For type-2b and type-3 cells (BrdU- and DCX-positive) a linear slope of  $y = 5.43641 * x + 1116.28146$ ,  $R^2 = -0.03531$ ,  $F(1,23) = 0.1814$  and  $p = 0.76412$  is revealed. Additionally, the linear regression for early immature neurons does not reach significance with  $p = 0.23933$  ( $y = 9.01898 * x + 205.64163$ ;  $R^2 = 0.01877$ ;  $F(1,23) = 1.4592$ ) as well as for premature granule cells ( $y = 14.72754 * x + 220.79583$ ;  $R^2 = 0.08482$ ;  $F(1,23) = 3.22436$ ;  $p = 0.0857$ ).

## Results - Influences of group size on ahNG



**FIGURE 2-19:** Although a tendency towards more new neurons as well as more cells in the transient stage between mitotic progenitors (DCX positive) and postmitotic neurons (NeuN positive) exists (B, C), no significant differences could be found at an  $\alpha$ -Level of 0.05. Furthermore, the number of progenitor cells as well as all none further specified cells varied non-significantly (A, D).

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### 2.3. *Influences of auditory stimuli on ahNG*

AhNG originates from precursor cells in the adult dentate gyrus and results in new granule cell neurons. Regulation of this process is related to and dependent on inputs from the external environment. Several studies showed a connection between adult neurogenesis and both ENRs and negative social cues, like stress and isolation. Positive social cues such as communication sounds would also represent a strong environmental input, but has not yet been studied in this context in mammals. In fact, there are few studies of direct effects of acoustic stimulation on adult neurogenesis, besides a controversial one suggesting modulation by music. We, thus, investigated whether patterned auditory input such as communication sounds might influence adult neurogenesis.

C57BL/6 mice undergo severe progressive sensorineural hearing loss with increasing age (presbycusis) independent of the gender of the animals (Hunter and Willott, 1987). In young adulthood, roughly at an age of 4-5 month, the hearing loss starts with mild high frequency impairment. Lower frequency impairment usually follows within the second half of life (12-13 months of age) (Hunter and Willott, 1987). To ensure the audibility of the auditory stimuli for our experimental subjects, as well as to account for attenuation effects we measured the auditory brainstem response (ABR) within samples of C57BL/6 mice. For the Measurement of the ABR following equation was applied:

$$\text{Attenuation (dB)} = 10 * \log_{10} \left( \frac{\text{Input Intensity (W}_1)}{\text{Output intensity (W}_2)} \right)$$

Results of the ABR analyses are displayed in TABLE 2-C.

Our stimuli are in the range from 4 kHz up to 80 kHz with peaks between 5 and 20 kHz for all stimuli besides pup calls, which peaked at 65 kHz. In general audibility highly depends on the volume. On the one hand the mice should not be stressed by too high sound intensity, whereas on the other hand stimuli had to be loud enough for the animals to be heard. Therefore, we decided to expose the animals to the diverse stimuli at  $70 \pm 10$ dB SPL.

## Results - Influences of auditory stimuli on ahNG

**TABLE 2-C:** Results of the Measurement of the ABR in C57BL/6 mice.

Animal	Stimulus threshold (dB attenuated)			
	Clicks	7kHz	32kHz	65kHz
1	65	40	30	10
2	60	50	45	5
3	65	45	40	15
4	65	45	35	10
5	60	45	35	5
6	60	40	25	5
7	65	40	45	10
8	60	35	35	0
9	65	35	20	0
10	60	40	25	10
Average hearing threshold	62.5	41.5	33.5	7.0

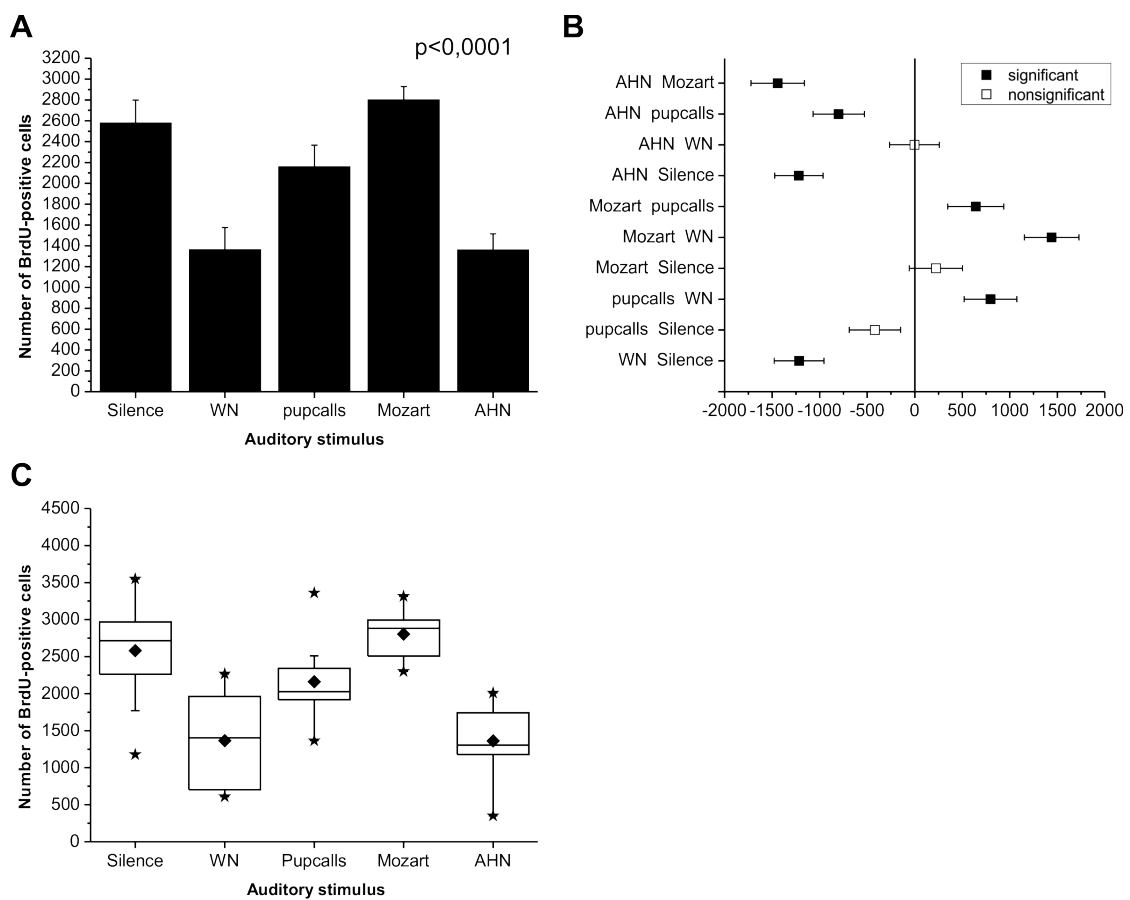
Influences of the distinct auditory stimuli on proliferation were assessed after three days of stimulation for two hours / day and 24 hours survival after BrdU injection (see methods, FIGURE 4-1). Net neurogenesis was analyzed seven days after a single injection of BrdU. In the course of this period mice were exposed to the auditory stimulus for 2 hours / day (see methods, FIGURE 4-1). In this particular paradigm BrdU counts reflect early survival rates of newly generated cells.

### **2.3.1. Influences of auditory stimulation on proliferation**

Three days of auditory stimulation already has a main impact on the absolute number of BrdU-positive cells. Within the control group, Animal House Noise (AHN),  $1362 \pm 481$  cells are labeled with BrdU. Similar numbers are found for White Noise ( $1364 \pm 637$ ), but more BrdU-positive cells are located within the subgranular zone of the dentate gyrus for all other stimuli as silence ( $2581 \pm 693$ ), Pup calls ( $2162 \pm 580$ ) and Mozart' music ( $2803 \pm 330$ ). Analyses of the absolute number of BrdU-positive cells for the proliferation paradigm revealed significant changes among groups with  $p = 1.62415 * 10^{-6}$  (FIGURE

## Results - Influences of auditory stimuli on ahNG

2-20 A). Fisher's post-hoc test further indicates significant differences among the following groups: Silence vs. White Noise ( $p = 3.77871 * 10^{-5}$ ), Silence vs. AHN ( $p = 2.45798 * 10^{-5}$ ), White Noise (WN) vs. Pup calls ( $p = 0.00641$ ), White Noise vs. Mozart ( $p = 1.20504 * 10^{-5}$ ), pup calls vs. Mozart music ( $p = 0.03572$ ), pup calls vs. Animal House Noise ( $p = 0.00519$ ) and Mozart vs. Animal House Noise ( $p = 8.17339 * 10^{-6}$ ) (FIGURE 2-20 B). Thus, with respect to our control, the AHN group, all stimuli, but White Noise (WN), produce a significant increase in cell proliferation in the adult dentate gyrus 24 hours after BrdU administration.



**FIGURE 2-20:** Illustration of the absolute number of BrdU-positive cells in dependency on the auditory stimulus (A) The histogram indicates the absolute number of BrdU-positive cells for Silence, White Noise (WN), pup calls, Mozart's Music and Animal House Noise (AHN) (B) Graph displays the results of Fisher's post-hoc test. Filled squares indicate significant differences, while White squares are non-significant (C) Box plots give detailed information on each data set with the stars displaying maxima and minima, diamonds the mean, boxes the inter quartile range and lines within the boxes the median.

## Results - Influences of auditory stimuli on ahNG

To further analyze the specific influence of auditory stimuli on the differentiation process, cells were co-labeled for BrdU, SOX2 and DCX. The distribution of cells within the distinct stages of ahNG varied in dependency on the specific auditory stimulus (FIGURE 2-21). For the fluctuations of the population of type-2a cells ANOVA revealed  $p = 4.21316 * 10^{-5}$ . The biggest fraction of type-2a cells was found under exposure to Mozart ( $71.12 \pm 7.63\%$ ). In addition, White Noise and pup calls caused high proportions of this cell type ( $56.26 \pm 12.21\%$  and  $51.98 \pm 4.82\%$ ), while silence and Animal House Noise only revealed moderate percentages ( $42.98 \pm 16.34\%$  and  $32.43 \pm 16.72\%$ ). Fisher's post-hoc test implied significant differences between Silence and Mozart ( $p = 1.59449 * 10^{-4}$ ), White Noise and Mozart ( $p = 0.04093$ ), White Noise and AHN ( $p = 0.00129$ ), Pup calls and Mozart ( $p = 0.01281$ ), Pup calls and AHN ( $p = 0.00918$ ) and Mozart and AHN ( $p = 2.38649 * 10^{-6}$ ).

With respect to type-2b cells overall p-values reached significance with  $p = 0.00613$ , while post-hoc analyses only showed p-values smaller than 0.05 for Silence vs. Pup calls ( $p = 0.012849$ ) and both Pup calls as well as Mozart vs. Animal House Noise ( $p = 7.93499 * 10^{-4}$ ,  $p = 0.00494$ ). Under exposure to pup calls only  $0.44 \pm 1.09\%$  are phenotyped as type-2b cells, and Mozart's music induced  $3.08 \pm 5.72\%$  type-2b cells. Higher proportions were found for White Noise ( $7.18 \pm 6.6\%$ ), Silence ( $8.78 \pm 6.43\%$ ) and Animal House Noise ( $12.45 \pm 7.15\%$ ).

The proportions of type-3+ cells fluctuated between  $2.38 \pm 2.47\%$  (Mozart) and  $29.91 \pm 15.58\%$  (AHN). The proportion of these cells under the exposure to pup calls seems also quite low with  $4.57 \pm 3.31\%$ , while silence and White Noise produced moderate quantities ( $12.22 \pm 5.48\%$  and  $15.91 \pm 7.95\%$ ). These variations are highly significant (ANOVA,  $p = 5.34095 * 10^{-6}$ ). Further post-hoc analyses indicated significant differences among all groups, except Silence vs. White Noise or Pup calls, respectively, and Pup calls vs. Mozart's music.

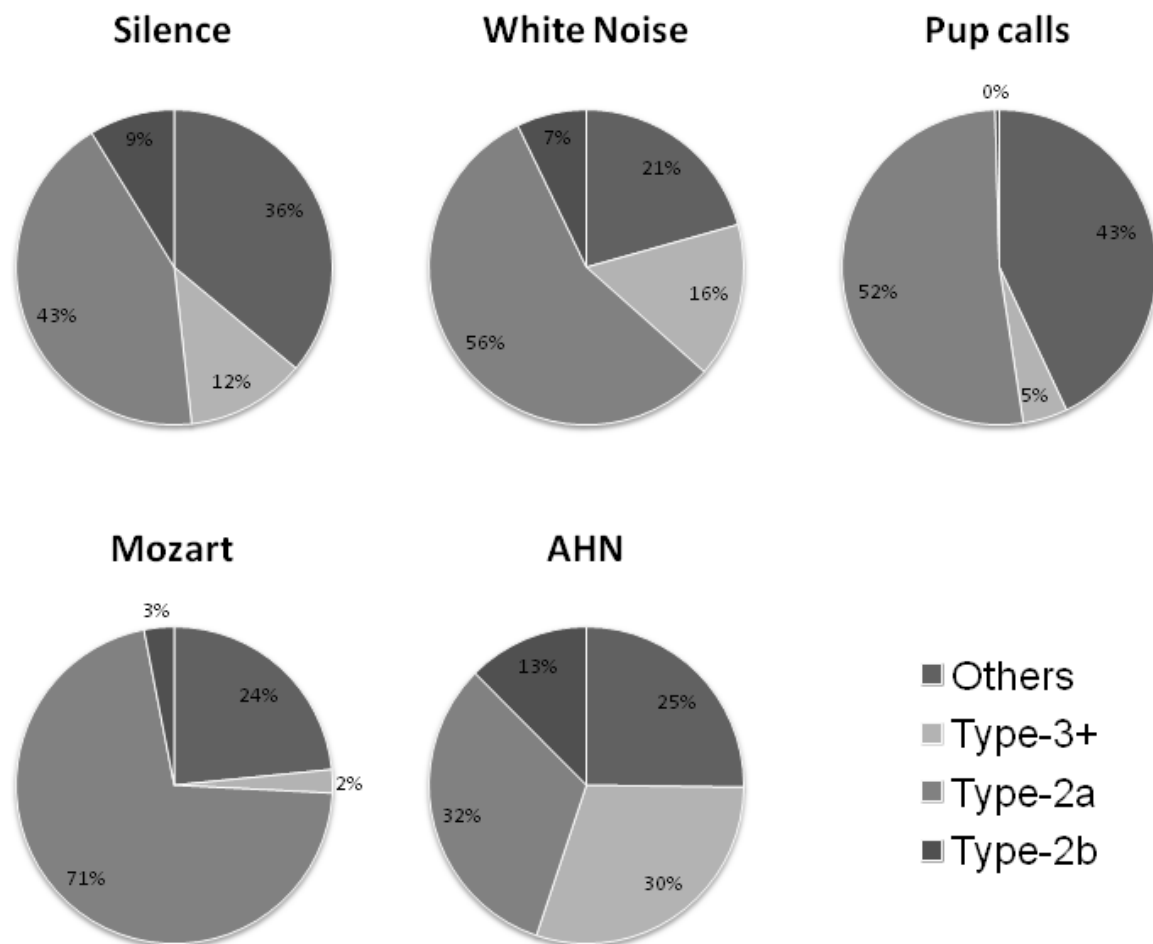
For the fluctuations of the population of cells of unknown cell type ANOVA revealed  $p = 0.00261$ . Fisher's post-hoc test further implied significant differences between Silence with  $36.02 \pm 16.64\%$  and White Noise with  $29.64 \pm 8.23\%$  ( $p = 0.00853$ ), Silence and Mozart with  $23.42 \pm 11.53\%$  ( $p = 0.02827$ ), Silence and Animal House Noise with  $25.21 \pm 5.74\%$  ( $p = 0.04931$ ), White Noise and Pup calls with  $43.01 \pm 5.39\%$  ( $p = 8.24 * 10^{-4}$ ),



## Results - Influences of auditory stimuli on ahNG

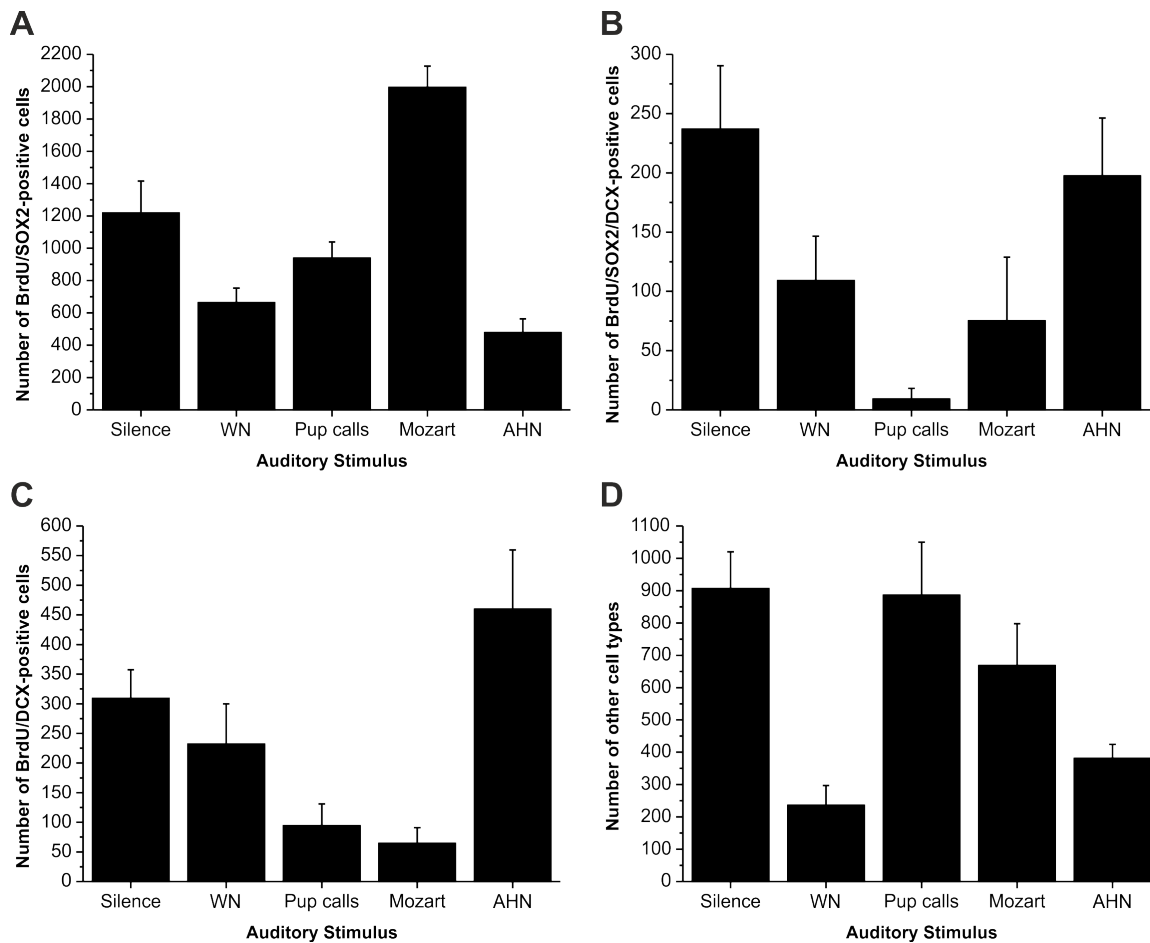
Pup calls and Mozart ( $p = 0.00283$ ) and Pup calls and Animal House Noise ( $p = 0.00485$ ).

With respect to the absolute number of cells within each differentiation stage we multiplied the relative proportion with the absolute number of BrdU-positive cells within the proliferation paradigm. Again ANOVA reveals significant p-values for all analyzed cell types. Concretely we obtained  $p = 2.96812 * 10^{-7}$  for type-2a cells,  $p = 0.03133$  for type-2b cells,  $p = 0.00153$  for type-3+ cells and  $p = 1.92178 * 10^{-4}$  for all other cell types (FIGURE 2-22).



**FIGURE 2-21:** Distribution of specific cell types in dependency on the auditory stimulus. Changes for all analyzed phenotypes are significant with  $p = 4.21 * 10^{-5}$  for type-2a,  $p = 0.006$  for type-2b,  $p = 5.34 * 10^{-6}$  for type-3+ and  $p = 0.003$  for all other cells types.

## Results - Influences of auditory stimuli on ahNG



**FIGURE 2-22:** Absolute number of cells within each differentiation stage highly depends on the acoustical environment. (A) Type-2a cells increase significantly with exposure to Mozart and silence compared to Animal House Noise with  $p < 0.0005$  (B) Type-2b cells are suppressed by pup calls while all other auditory stimulus have no significant impact. (C) The number of type-3+ cells decreases for all stimuli, but silence (D) all other cellular phenotypes are either not effected, or significantly increased (silence and pup calls).

Fisher's post-hoc Analyses also disclose significant differences in comparison to our control group (Animal House Noise) for each cell type. For type-2a cells we find a strong, significant increase for silence towards  $1218 \pm 595$  cells per DG ( $p = 4.66269 * 10^{-4}$ ) and Mozart's music ( $1996 \pm 346$  cells/DG;  $p = 2.2383 * 10^{-8}$ ). The differences in absolute cell number between Animal House Noise ( $478 \pm 240$ ) and White Noise ( $663 \pm 223$ ), respectively pup calls ( $939 \pm 198$ ) are non-significant (FIGURE 2-22 A).

In comparison we find type-2b cells strongly suppressed by Pup calls ( $p = 0.02665$ ), while all other stimuli do not change the number of these cells significantly in comparison to Animal House Noise, although the absolute numbers seem strongly

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variable. For Animal House Noise we find  $197 \pm 137$ , for Silence  $237 \pm 160$ , for White Noise  $109 \pm 92$  and for Mozart  $75 \pm 142$ , but for pup calls only  $9 \pm 18$  cells per dentate gyrus (FIGURE 2-22 B).

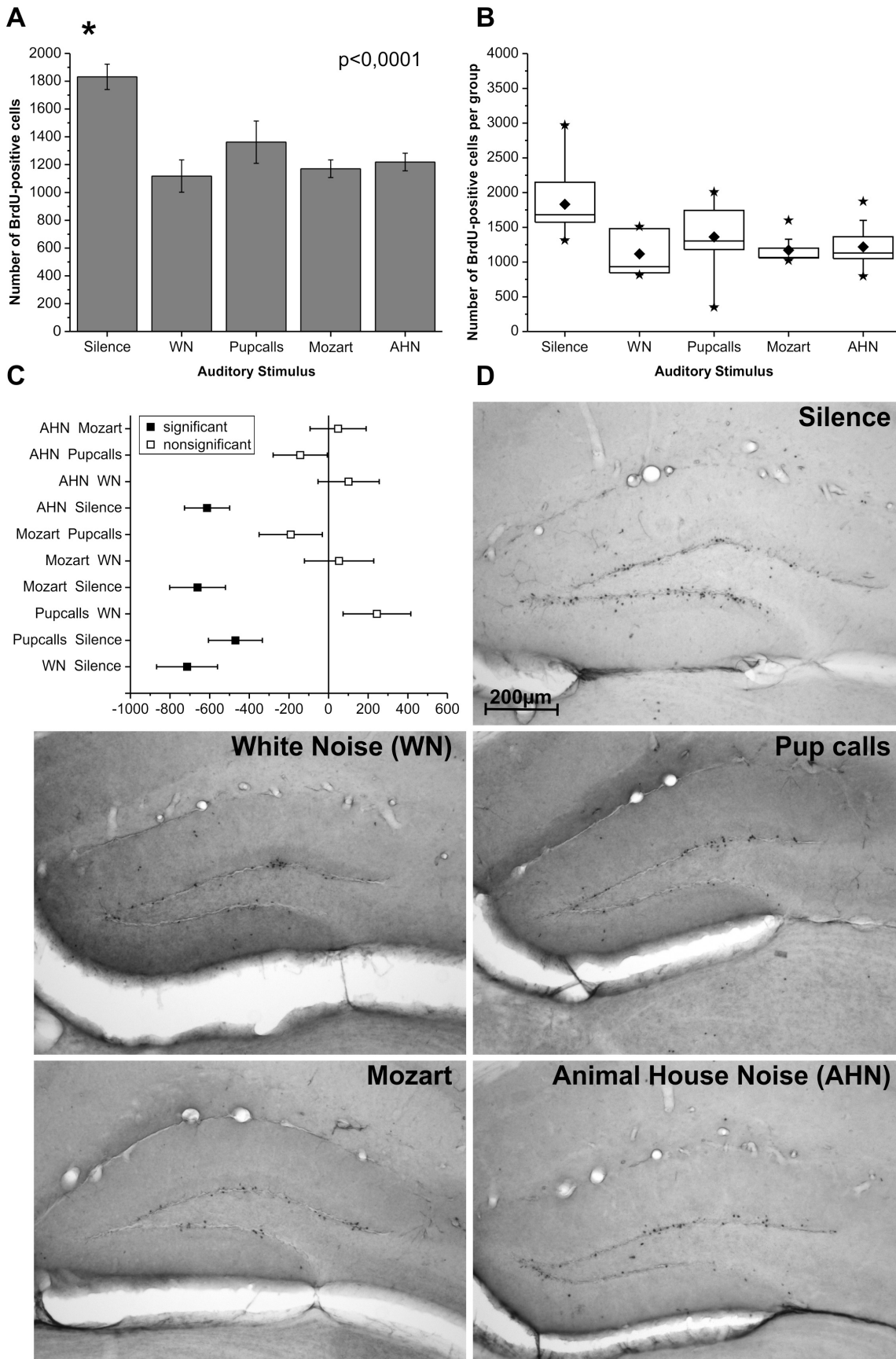
In contrast, the number of type-3+ cells is decreased by all auditory stimuli, but silence ( $309 \pm 144$  cells/DG) with respect to Animal House Noise ( $460 \pm 282$  cells/DG). White Noise ( $p = 0.02392$ ) has the lowest effect with  $232 \pm 166$  cells/DG, Pup calls ( $p = 0.00213$ ) already decreased the number of these cells by 1 / 5th to  $94 \pm 74$  and Mozart even reduced this cell type to 14%, or  $64 \pm 71$  cells/DG ( $p = 1.68156 * 10^{-4}$ ) (FIGURE 2-22 C).

All other cell types, including different glia cells and early postmitotic cells are increased with respect to Animal House Noise, but only the increase for silence and pup calls revealed significant changes ( $p = 4.58717 * 10^{-4}$ ,  $p = 0.00539$ ). I observed  $907 \pm 340$  cells/DG with silence,  $236 \pm 149$  with White Noise,  $886 \pm 329$  with pup calls,  $668 \pm 344$  with Mozart and  $381 \pm 122$  cells neither expressing SOX2 nor DCX with Animal House Noise. (FIGURE 2-22 D)

### 2.3.2. Influences of auditory stimuli on survival

Analyses of the absolute number of BrdU-positive cells within the survival paradigm revealed a high increase caused by silence, lack of any auditory stimulus, except the ones produced by the group of five female mice themselves. ANOVA revealed significant differences with  $p = 0.01534$  (FIGURE 2-23 A and B). Fisher's Post Hoc test further indicated significant differences among the following groups: Silence and White Noise ( $p = 0.00851$ ), Silence and Mozart ( $p = 0.01043$ ), and Silence and Animal House Noise ( $p = 0.00542$ ). All other differences among groups have p-values bigger than 0.1 (FIGURE 2-23 C). In absolute cell counts  $1832 \pm 396$  BrdU-positive cells were found per dentate gyrus in mice exposed to silence, while all other stimuli only lead to cell numbers between  $1118 \pm 308$  (White Noise) and  $1362 \pm 481$  (pup calls).

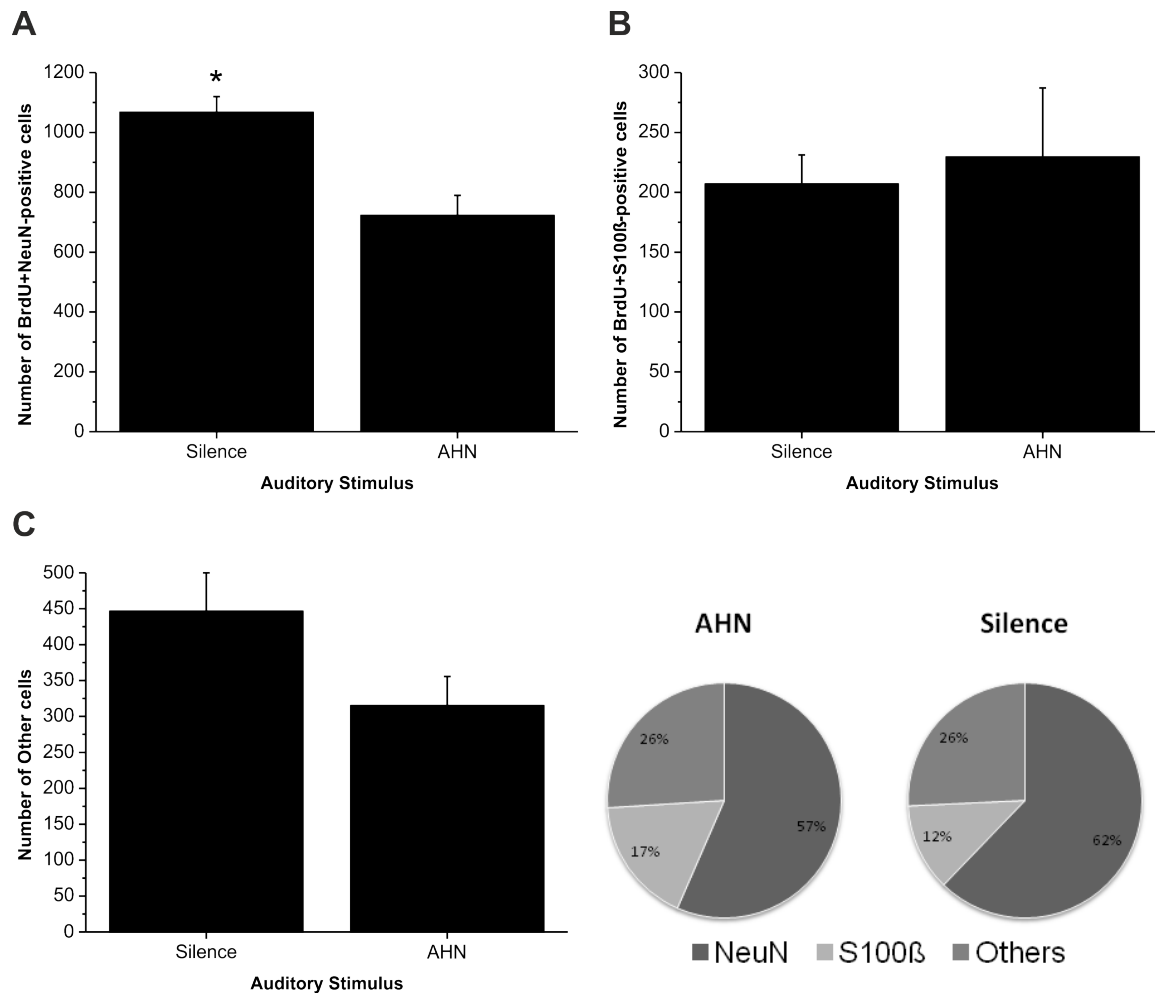
# Results - Influences of auditory stimuli on ahNG



**FIGURE 2-23:** *Survival of newborn cells is rapidly increased by lack of auditory stimuli (A) ANOVA revealed a significant increase of BrdU-positive cells by silence ( $p = 1.413 * 10^{-6}$ ) (B) Box plot illustrating the mean (diamond), media, minimum, maximum and the interquartile range for each group (C) Fisher's LSD reveals significant differences between silence and all other groups with  $p = 1.962 * 10^{-5}$  for White Noise.  $p = 0.001$  for Pup calls.  $p = 1.621 * 10^{-5}$  for Mozart Music and  $p = 1.464 * 10^{-6}$  for Animal House Noise. The other groups did not reveal any significant differences towards each other ( $p > 0.159$ ) (D) Photos illustrating the differences in BrdU cell counts among groups.*

In further investigations the focus lied upon the differentiation of cells in dependency on the auditory stimulus. Since the major effect was found for Silence, comparisons between the group exposed to silence versus the control group exposed to Animal House Noise was drawn. For the changes in relative proportions of the different groups no significant differences were revealed via ANOVA although the p-value for the neuronal differentiation was close to our significant level with  $p = 0.05733$ . A shift from gliogenesis towards neurogenesis seems indicated (FIGURE 2-24 D). With respect to the absolute values of cells found either positive for neuronal marker NeuN or astrocytic marker S100 $\beta$  we found only significant changes for BrdU / NeuN-positive neurons with  $p = 7.60631 * 10^{-4}$ . Astrocytes and other cells only reached  $p = 0.71401$  or  $p = 0.0721$ , respectively (FIGURE 2-24).

## Results - Influences of auditory stimuli on ahNG

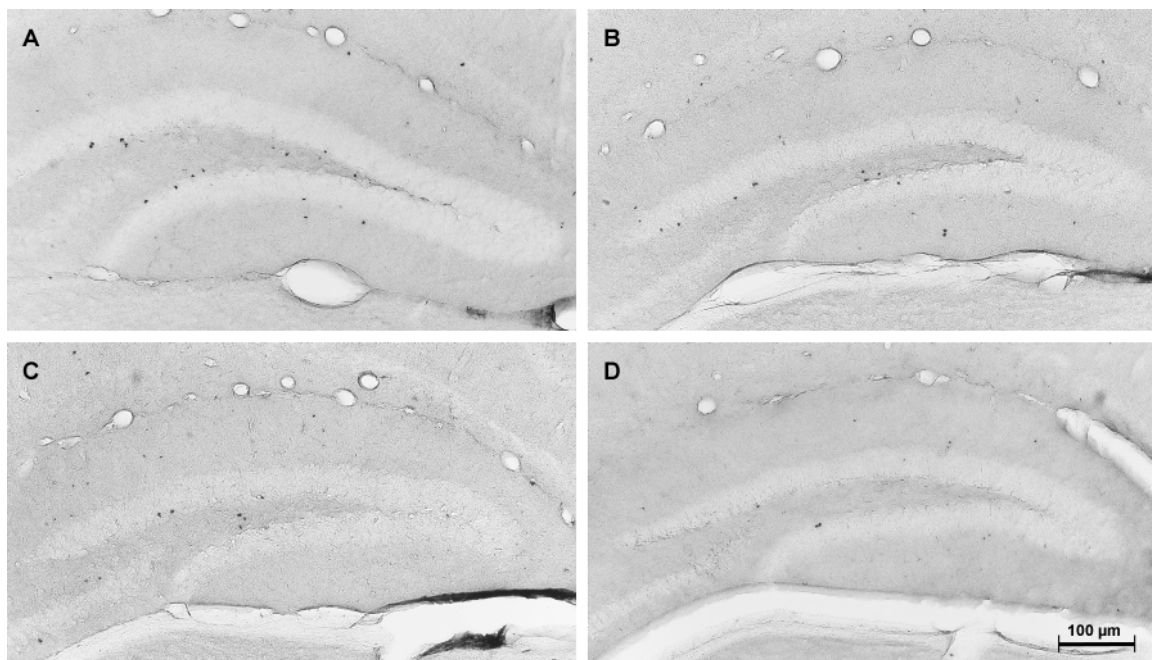


**FIGURE 2-24:** Results of phenotypization (A) Number of new neurons found within the granule zone of the dentate gyrus after seven days of stimulation. A strong increase was detected for the silence paradigm ( $p < 0.0001$ ). (B, C) No significant differences in the absolute number of neither astrocytes nor other cells were detected. (D) Relative distribution of cells found positive for BrdU and NeuN or S100 $\beta$ . A trend towards an increase of neurons and decrease of astrocytes with stable or increasing proportion of other cells seems plausible, although changes are non-significant.

#### 2.4. *Hippocampal neurogenesis in the GR<sup>+/-</sup> genetic mouse model of depression*

Within this part of this dissertation a genetic mouse model of depression was explored in the context of ahNG. Depression is one of the major medical threads within industrial countries highly related to the challenges of our environment and social live.

Between nine and ten animals per group received one week daily BrdU injections. Four weeks later both the absolute number of BrdU within the dentate gyrus was observed as well as the distribution of cells which underwent either neurogenesis or gliogenesis. Here, BrdU counts focus on the survival of newly generated cells. The experimental set-up is illustrated in FIGURE 4-1 (see Methods). Briefly, we used a two times two design with genotype (GR<sup>+/-</sup> mice versus GR<sup>+/+</sup> littermate controls) as examination factor on the one hand and seven days of standard housing or one hour of restraint stress daily, directly after intraperitoneal injection of BrdU. (Kronenberg, Kirste et al. 2010).

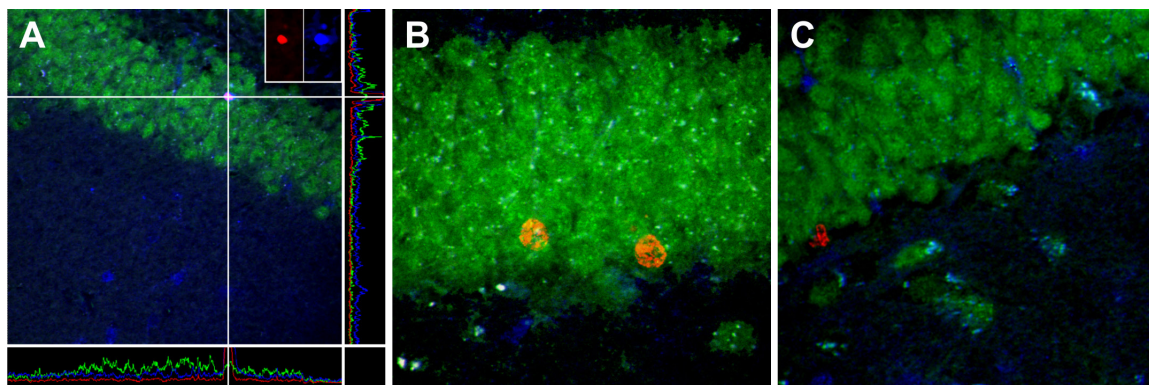


**FIGURE 2-25:** *BrdU<sup>+</sup> cells in the dentate gyrus are reduced as an effect of GR<sup>+/-</sup> genotype and as an effect of stress. The four images show BrdU-stained cells (DAB) in representative hippocampal sections of animals from the respective experimental groups: (A) GR<sup>+/+</sup> mice in the control condition, (B) GR<sup>+/+</sup> mice in the stress condition, (C) GR<sup>+/-</sup> mice in the control condition, (D) GR<sup>+/-</sup> mice in the stress condition. (Taken from Kronenberg, Kirste et al. 2010)*

## Results - Hippocampal neurogenesis in the GR<sup>+/-</sup> genetic mouse model of depression

Two-way ANOVA revealed that both stress and GR<sup>+/-</sup> genotype reduces the absolute number of BrdU-positive cells significantly (FIGURE 2-25 and 2-26a). Mean numbers of cells varied among groups with  $751 \pm 65$  cells/DG in the control condition with wildtype animals,  $671 \pm 137$  cells/DG for stressed wildtype mice,  $708 \pm 81$  cells/DG in GR<sup>+/-</sup> mice housed under standard conditions and  $569 \pm 114$  cells/DG for genetic altered mice exposed to the stress condition. The differences between genotypes revealed significance with  $F(1,34) = 4.60953$  and  $p = 0.03901$ , while the differences between housing conditions (restraint stress vs. standard) revealed significance with  $F(1,34) = 10.39734$  and  $p = 0.00276$ .

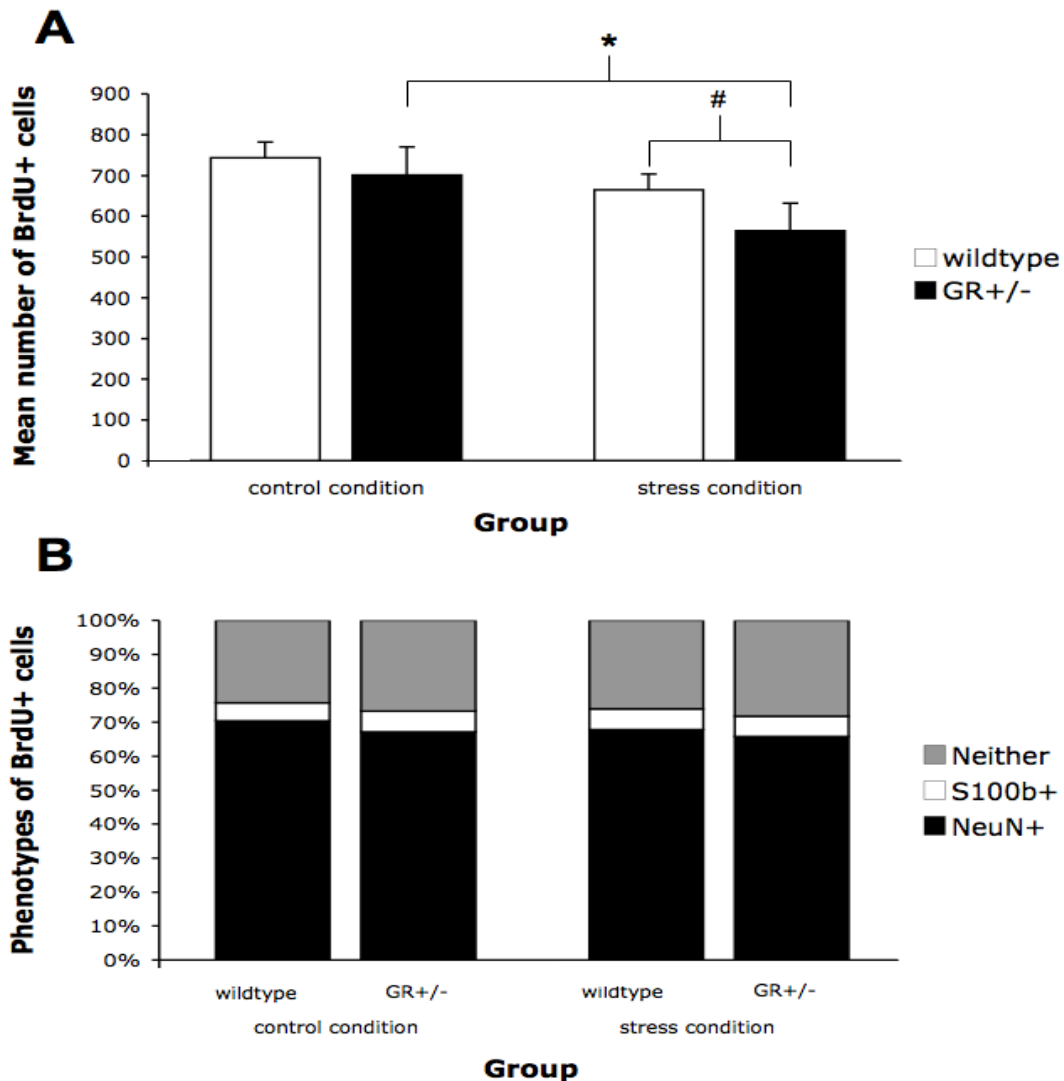
BrdU-labeled cells were subjected to further phenotypic analysis. The majority of cells showed co-labeling with neuronal marker NeuN, but also a constant number of cells expressing astrocytic marker S-100 $\beta$  was found (see also FIGURE 2-26 and 2-27B). No further significant effect of either experimental condition or genotype was found on neurogenesis or gliogenesis. (Kronenberg, Kirste et al. 2010).



**FIGURE 2-26.** Phenotypic analysis (A) Confocal image of a newly generated astrocyte in the granule cell layer expressing mature astrocytic marker S100 $\beta$  (Blue) and BrdU (Red). Emission intensities measured along the lines drawn in the larger image are shown in the bottom and right panels. Insets show the cell investigated in single channels with separate wave-lengths. (B) Newly generated neuron labeled with BrdU (Red) and expressing NeuN-immunoreactivity (Green). (C) BrdU-positive cell (Red) expressing neither neuronal marker NeuN (Green) nor astrocytic marker S100 $\beta$  (Blue). (Modified from Kronenberg, Kirste et al. 2010)



Results - Hippocampal neurogenesis in the GR+/- genetic mouse model of depression



**FIGURE 2-27.** Quantitative assessment of net neurogenesis (A). The number of BrdU+ cells was analyzed 4 weeks after a seven-day series of once-daily BrdU injections. Two-way ANOVA yielded a significant effect of factor ‘treatment condition’ (control versus stress condition:  $F_{1,33}=10.2$ ,  $p = 0.003$ , Fisher’s Post-hoc:  $p = 0.003$ ) and a significant effect of factor genotype ( $F_{1,33}=4.5$ ,  $p = 0.04$ , post hoc:  $p = 0.03$ ) on BrdU cell counts with no significant interaction between factors (genotype  $\times$  treatment interaction:  $F_{1,33}=0.8$ ,  $p = 0.39$ ). (B). Phenotypic analysis of BrdU+ cells did not reveal differences in neuronal (NeuN) or astrocytic (S100 $\beta$ ) marker expression between experimental groups. (Taken from Kronenberg, Kirste et al. 2010)

### **3. Discussion**

The time course through lineage progression of neural stem/progenitor cells in the adult dentate gyrus, e.g., cell proliferation, differentiation, maturation and integration are of important functional relevance (Aimone et al., 2011, Inokuchi, 2011). These processes have been proven to not only correlate strongly with hippocampal function, but additionally correlated to severe disease, such as major depression (for review see Eisch et al., 2008, Lucassen et al., 2010, Yan et al., 2011). In order to study the psychosocial influence on the dynamics of adult neurogenesis I focus on the differentiation from neural stem cells in the adult dentate gyrus to mature granule cell neurons, first. In this framework, details of the impact of defined stages of neuronal development are exposed with focus on the mitotic cell types. Furthermore, a first insight into the connection between social factors and ahNG is revealed in correlation to group composition. Additionally, I focus on distinct auditory stimuli as a first start to investigate the influence of communication on mammalian neurogenesis. Finally, a murine depression model is used to give a small impression of the dependency between social factors, disease and ahNG.

#### ***3.1. Detailed quantitative analyses of the differentiation process***

In all studies, researchers try to minimize the number of animals to the needs of their project at hand. Thus, the statistical power of the studies was only determined for the immediate question at hand (and thus too low for further assumptions). Previous studies on adult neurogenesis in mammals only focused on sections of the differentiation process. This leads to a lack of knowledge and following no complete picture of this important biological phenomenon is available, so far. In order to overcome this problem I used more mice, investigated more time points and analyzed more cells, than any previous study. This enabled me to perform a broad quantitative and qualitative analysis of the different stages of neurogenesis. I determined the relative and absolute numbers of cells at each developmental stage in order to generate as much data as possible for future mathematical modeling.

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### **3.1.1. Dynamics of absolute number of proliferative cells is mainly independent of BrdU dosage**

#### ***3.1.1.1. No correlation between time point of maximal BrdU cell number and BrdU dosage exists***

To investigate details of the neural differentiation in the adult murine dentate gyrus, I collected quantitative BrdU cell counts during a period of three weeks. As displayed in FIGURE 2-1, I found a strong increase for the first time points followed by a maximum at day two p.i. A first decline between day two and five ended in a plateau with rather constant cell counts. Seven days p.i. the decline continued and finally, only very few BrdU-positive cells at the late time points were observed.

TABLE 3-A displays the major outcomes of comparable studies by the mean of methods. Studies addressing either proliferation or differentiation typically inject BrdU only once. A standard dosage often used is 50 mg/kg, but few studies also use much higher concentrations. While some researchers prefer a higher dose to label all cells in S-phase, others argue less BrdU is also less harmful and does not affect the differentiation process by activating apoptosis (Taupin, 2007). Burns and Kuan stated that 50 mg/kg bodyweight of BrdU is sufficient to label most cells in S-phase (Burns and Kuan, 2005). In the contrary, Mandyam et al. found an dose dependent increase in the number of labeled cells with a ceiling effect for BrdU dosages higher than 150 mg/kg and claim an independence of dosage from bioavailability (Mandyam et al., 2007). Unfortunately, they did not address cell death in their study, thus it is impossible to know if the increased number of BrdU-positive cells (compared to 25, 50 and 100 mg/kg) affects apoptosis rates, as BrdU labels also cells with DNA synthesis, other than caused by S-phase (Taupin, 2007). However, in order to induce as few apoptosis as possible and try not to negatively influence differentiation by BrdU (which is well known as a traditional chemotherapeutic drug (Bagshaw et al., 1967, Russo et al., 1984, Greenberg et al., 1988)), we decided to use the lower concentration.

Although studies applying similar methods in the same strain of mice (mainly C57BL/6) should produce the similar results, variations in BrdU cell counts across groups are quite high (for references see TABLE 3-A). While studies performed in our laboratory (Brandt et al., Steiner et al., Garcia et al.) with 1x BrdU (50 mg/kg) typically resulted in a peak at three days p.i. and approximately 550 cells/DG, the group of Eisch (Mandyam et al.),

using a higher dose of 1x BrdU (150mg/kg) found a peak after 15h with roughly 7000 cells/DG. The laboratory of Enikolopov (Sierra et al., 2010, Encinas et al., 2011) also found an early peak at day 2 after administrating of 1xBrdU (250 mg/kg) with 4500 or 6000 cells/DG, respectively. Thus, no clear link between the BrdU dosage and the time point of the peak can be found. However, it seems that a rather small dosage as used by our group (Steiner et al., Brandt et al., Garcia et al.) leads to a smaller total number of cells/DG, while a dosage of 150 mg/kg and above leads to higher total numbers. This finding seems to be true, independent on the number or time point of injection.

### ***3.1.1.2. Differences in absolute BrdU cell number is caused by relabeling***

In respect to the total number of BrdU-positive cells found at the maximum, I observed between 5- and 6-fold the number of cells, in contrast to previous studies performed in our research group with single injection, but only half of the number found by experiments with similar BrdU dosage. Thus, we can reason, that my protocol induces both labeling of additional cells, hitting S-phase in cells at each injection time point, that were not labeled before, and also relabeling of the same cell, marked during previous BrdU administration. We know that BrdU bioavailability accounts to maximal 2h (Hayes and Nowakowski, 2002), but most probably to only 15 minutes (Mandyam, 2007). This indicates, that we do not have a cumulative BrdU concentration between the injections. Since I only found half of the number of cells described for the 150 mg/kg BrdU pulse, I know the difference in BrdU cell numbers is caused only by relabeling. Thus, in the attempts to identify CCL for individual cell types, we will take this distort by 50% into consideration.

### ***3.1.1.3. Increased BrdU dosages shift the peak of proliferative cells to a ~12-24h earlier time points by more efficient labeling of cells in late S-phase, but does not affect cell dynamics***

Since studies of Brandt et al., Steiner et al., and Garcia et al. did not include time points between day one and three p.i. it is impossible to say whether or not the concentration of BrdU changes the dynamics of neurogenesis by means of BrdU measures. Nevertheless, this could be indicated by comparison of 1d and 3d in the previous studies. Previous studies with 50 mg/kg BrdU pulse injection always show less cells at 1d than at 3d, thus, a peak before 2d seems unlikely (Garcia et al., 2004, Steiner et al., 2004).

## Discussion - Detailed quantitative analyses of the differentiation process

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Hayes and Nowakowski, however, administered BrdU i.p. every 4 h for either 12 or 24 hours (12 and 24 hours saturation paradigms (SSM)). For the 12 hour SSM (3x 50mg/kg BrdU) they found an increase in the absolute number of BrdU-positive cells within the first 36 hours followed by a subsequent decrease. For the 24 hours paradigm similar results revealed a peak after 48 two days. From these results a cell cycle length (CCL) of BrdU-labeled cells within the dentate gyrus of 12 – 14 hours was calculated (Hayes and Nowakowski, 2002). Keeping in mind, that BrdU concentration alone may affect dynamics of adult neurogenesis, these values could have a bias.

As mentioned, the BrdU peak in my experiments was found at day two. This is very similar to the dynamics described by Hayes and Nowakowski in the 24h SSM, as well as to those of Sierra et al. and Encinas et al. If one compares the total dosage of BrdU within 24 hours, we find 150 mg/kg for my study and Encinas study. Hayes and Nowakowski, as well as Sierra et al., administered 250 or 300 mg/kg, respectively. Again, only the dosage, but not the injection protocol seems to slightly affect the time course of adult neurogenesis. One possible explanation could lie in the better rate of identification caused by increased dosages. Especially cells at the end of S-phase only provide few DNA sections for BrdU incorporation, while cells at the beginning of S-phase will more likely incorporate BrdU over the total DNA length. Thus, the increase in cell detectability, caused by higher BrdU dosages, may mainly lead to higher proportion of cells at the end of S-phase during BrdU bioavailability. Thus, a wrong impression of faster proliferation emerges.

The time points of the peaks of BrdU-positive cells are displayed in TABLE 3-A in dependency on the BrdU application protocol. Comparison between the studies with 50mg/kg BrdU by our group and the studies with  $\geq 150$ mg/kg, we find a shift of the peak for approximately 24h. Keeping in mind that all studies with 50mg/kg did not received data for day two pi., we find a shift of  $\leq 24$  hours. The data presented by Hayes and Nowakowski (2002) shows more detailed a shift of 12h.

Thus, my main finding is, that increased BrdU dosages (either administered as pulse labeling at once, or in several injections within a short time frame) leads to an slightly advanced peak, but no changed dynamics of neurogenesis. For a deeper understanding of this phenomenon it will be necessary to further investigate cellular processes, which are altered by BrdU. These include proportion of DNA with BrdU incorporation in

## Discussion - Detailed quantitative analyses of the differentiation process

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dependency on S-phase stage, major DNA repair mechanisms, partially apoptosis and maybe also others. Taking into account the rather low total number of BrdU cells for dosages of 50 mg/kg and below, it is suggested that for future investigations the dose of 100 – 150 mg/kg should not be exceeded.

TABLE 3-A: Comparison of absolute numbers of BrdU-positive cells with previously described studies

Author, Reference	BrdU administration	Time points analyzed	BrdU peak	No. of BrdU+ cells at peak per DG (mean $\pm$ SE where possible)
Kirste, I	3x BrdU (50 mg/kg) every six hours	2h, 14h, 24h, 2d, 3d, 4d, 5d, 6d, 7d, 10d, 14d, 21d	2d	2841 $\pm$ 105 (DAB staining) 2283 $\pm$ 877 (fluorescence)
(Hayes and Nowakowski, 2002)	3x BrdU each 50 mg/kg (12 h SSM) 6x BrdU (24h SSM)	0.5h, 4.5h, 8.5h, 12.5h, 16.5h, 20.5h, 24.5h, 36h, 2d, 2.5d, 3d, 4d, 8d, 9d, 10d	36h (12h SSM) 2d (24h SSM)	~ 6000 ~ 7000
(Brandt et al., 2003)	1x BrdU (50 mg/kg)	4h, 24h, 3d, 7d, 2.5w, 4w	3d	~550
(Steiner et al., 2004)	1x BrdU (50 mg/kg)	4h, 24h, 3d, 7d, 28d	3d	531.8 $\pm$ 29.3
(Garcia et al., 2004)	1x BrdU (50 mg/kg)	4h, 24h, 3d, 7d, 4w	3d	~650
(Mandyam et al., 2007)	1x BrdU (150 mg/kg)	1 / 4h, 2 h, 8h, 15h, 24h, 2d, 4d, 10d, 30d	15h	~7000
(Sierra et al., 2010)	1x BrdU (250 mg/kg)	2h, 12 h, 24h, 2d, 3d, 4d, 8d, 11d, 15d, 18d, 22d, 32d	2d	~6000
(Encinas et al., 2011)	1x BrdU (150 mg/kg)	2h, 12h, 24h, 2d, 3d, 5d, 7d, 10d, 15d, 30d	2d	~4500

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### 3.1.2. Proliferation rates of late mitotic differentiation stages

To study the differentiation process in more detail, not only the quantity of BrdU-positive cells, but also of DCX- and CR-positive cells was assessed in dependency on time. Neither did absolute cell counts of late mitotic cell stages (DCX-positive Type-2b, Type-3 cells and early immature neurons), vary with time (independently of S-phase measures), nor did I find significant changes among CR-positive immature neurons (FIGURE 2-4). Nevertheless, it was possible to calculate the proliferation rate from this observations, phenotypic analyzes and previously published data, revealing 4% of all Type-3+ DCX-positive cells in S-phase after 1x BrdU and 19.3% after 3x BrdU administration 6 hours apart. Since Type-3+ cells include mitotic Type-3 cells and postmitotic immature neurons I reason, that Type-3 cells have this proliferation rates.

In addition, we find 5.1% of all Type-2b cells in division after one times BrdU and 26.5% after three injections. To estimate this numbers, I used data from confocal analysis of cells expressing both, BrdU and DCX. Thus, they depend on the used dosage and application protocol. My findings of the proliferation rates can be interpreted in two different ways: either a single dose of BrdU (50 mg/kg) is not sufficient to label all cells in S-phase as stated by Mandyam (Mandyam et al., 2007), or the division rate of these cells is quite fast. As I will display cell cycle length for Type-2b and 3 cells later one, this question will be answered, but a mixtures of both effects is most probable.

We know, that repeated administration of BrdU leads to higher detected cell numbers. Hayes and Nowakowski assumed a S-phase length between of 7.5 hours. Thus, some cells are labeled at the beginning and the end of S-phase by our protocol. Therefore, we have a mixture of cumulative- and re-labeling increasing both visibility and number of labeled cells. Under the assumption that relabeling accounts to 0 and all increase in cell number is caused by differentiation and cumulative labeling, the minimum proliferation rate has to be  $26.5\% / 3$  (corresponding to the number of injections) and  $19.3\% / 3$ , respectively: at least 8.83% of all Type-2b cells and 6.43% of all Type-3 cells are in S-phase. In conclusion we must have a proliferative rate between 8.8% and 26.5% for Type-2b cells, and between 6.4% and 19.3% for Type-3 cells.

Up to date, no data exists on proliferation rates especially of type-2b and type-3 cells, Encinas et al. estimated this value for type-1 and type-2a cells. They found a proliferation



rate of only 1% for type-1 cells and 10 – 20% for type-2a cells (ANPs) (Encinas et al., 2011).

Although my findings fit to the rates given for type-2a cells by Encinas et al. (2011), they need to be evaluated critically. Proliferation rates obtained from the total population of BrdU-positive cells are vague estimations describing only a pool of very diverse cells. Thus, it can only provide only very little information. To specifically address proliferation rates of distinct differentiation stages displays a major step to increase knowledge, but further research is needed.

To estimate proliferation rates for each differentiation stage individually, is a new and innovative idea and displays the right way to unravel remaining mysteries of differentiation. Future investigations will narrow down the rates further with slightly altered methods.

### **3.1.3. Cell cycle length (CCL) varies for different mitotic cell types**

#### ***3.1.3.1. Estimated CCL for BrdU-positive cells***

For the Type-3 cells I estimated a 5-fold increase in 12 hours. Under the assumption, that the total increase would be caused by cell division only, we can apply math to calculate the number of divisions (X) and the CCL. Here, N(14h) displays the number of cells found at the 14h time point, while N(2h) is the number of cells at 2h p.i.

$$X = \left[ \frac{\lg(N(14h))}{\lg 2} - \frac{\lg(N(2h))}{\lg 2} \right]$$

$$CCL = (14h - 2h) / \left[ \frac{\lg(N(14h))}{\lg 2} - \frac{\lg(N(2h))}{\lg 2} \right]$$

With the absolute values for BrdU-positive cells (TABLE 2-A) I calculated the CCL and found a length of 6.68 hours. Since I know that relabeling roughly affects 50% of my population (see 3.1.1.2.), I have to double the estimated CCL. Thus, I claim an average CCL for all BrdU% cells of 13.36 hours.

Up to date, only two other studies estimated the CCL in ahNG. Although both used slightly different methods, they come to similar results and state a CCL for all BrdU-positive cells of 12-14h, and 14h, respectively (Hayes and Nowakowski, 2002, (Mandyam et al., 2007). With my data, I cannot state any information about distribution of cell cycle phases among this total CCL. Thus, in this respect we need to rely on previously published data for future modeling. Hayes and Nowakowski (2002) identified an S-phase length of 7.6h, which will be considered later on.

### ***3.1.3.2. BrdU-positive cells can arrest form cell cycle***

In order to understand the dynamics of neuronal development in the adult hippocampus it is necessary to have detailed information about CCL for stem-, progenitor-, and precursor cells within the dentate gyrus, individually. Up to date only values for the complete BrdU cell population are available. As described in section 3.1.1., the dynamics of neurogenesis is independent of the total BrdU dosage, but higher concentrations lead to a shift of the maximal cell number observed towards earlier time points, caused through more efficient label of cells at the end of S-phase. This difference in time typically accounts to 12h – 24h (Hayes and Nowakowski, 2002). Furthermore, higher dosages of BrdU cause increased cell numbers with a ceiling effect is reached for BrdU with dosages of more than 150 mg/kg (Mandyam et al., 2007). Therefore, Hayes and Nowakowski labeled the maximal number of mitotic cells after three BrdU injections (12h SSM). The small shift of the peak between the 24h SSM and the 12h SSM, thus has to be caused by additional cells labeled, which newly enter S-phase, without having been in S-phase at previous injection times. This fact indicates, that cells not always continue within cell cycle, but that CCL must vary. Otherwise, Hayes and Nowakowski would have labeled the same cells with ever injection, causing not a second peak followed by a decrease, but a stable plateau. If this would hold true, the only effect would be the prevention of dilution of BrdU from the cells.

Since CCL for average BrdU-labeled cells is 13.36h / 14h in mice (Mandyam et al., 2007), a continued differentiation process without  $G_0$  - resting stage would lead to three divisions within roughly 42 hours, and in consequence to 3.4 divisions after 2 days (a theoretical 10.5-fold (!) increase in cell number). In my results I find a maximal number of BrdU-positive cells at this time point with roughly a 5.2-fold increase compared to the initial 2h time point. Thus, I maximal find 1.7 divisions within two days. As laid out in

chapter 3.1.2., we have cumulative labeling as well as partially double-labeling affecting 50% of the cohort. The number of cells labeled initially with the first BrdU thus, has not in total divided three times in a row. In conclusion, this means, BrdU+ cells have an intermediate G<sub>0</sub> state, where they rest before entering the next cell cycle, at least some times if not always.

### **3.1.3.3. *Different cell types leave the cell cycle and enter G<sub>0</sub>***

The variance in CCL found for 12h and 24h SSM can be caused by two means: either cells enter a G<sub>0</sub> resting state (quiescent cells), or the labeled cell pool, consists of cell types with individual CCL, or both. Type-1 cells have been stated as quiescent, but the number of this population with only 2% of all BrdU-positive cells is too low, to account for the variance. In addition, the bigger population of horizontal stem cells (Type-2a cells) have been found in quiescent state, but this has only been related to aging, yet (Lugert et al., 2010). My data reveals, that this cell type accounts for 54% of all BrdU-positive cells after a single injection. Thus, it is very likely, that the quiescence of this differentiation stage causes the variance in Hayes and Nowakowski's study. Nevertheless, I also found 23% of Type-2b and 20% of Type-3 cells after single BrdU administration (FIGURE 2-15, 2h). Quiescent phases in these cell types have never been described, yet, nevertheless this does not mean they cannot enter G<sub>0</sub>. Keeping in mind, that these cells sum up to nearly 50% of all BrdU-positive cells it is possible they also leave cell cycle. Mathematical modeling will have to prove this claim in near future.

### **3.1.4. Decrease in cell numbers after the initial peak is not linear**

The values obtained in my experiment show an abrupt drop in the total number of BrdU-positive cells between day seven and day ten. The results for day five till day seven are not significantly different from each other as well as day 10 till day 21 (TABLE 2-A, FIGURE 2-1).

Only few studies analyzed time points beyond three days after BrdU administration in such density, as I did. Although Hayes and Nowakowski analyzed animals between 0.5 hours and 5 weeks after the first injection of BrdU, for the SSM protocol, no results are given for time points beyond day 10. Furthermore, time points between 4d and 8d are not observed. Since the variance for the late time points (9d (216h) and 10d (240h)) was quite high in that study it is difficult to draw firm conclusions about the late time points. An

## Discussion - Detailed quantitative analyses of the differentiation process

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exponential decline as stated by Hayes and Nowakowski (2002) for the points after day two could be possible. However, Mandyam et al found, that within clusters a second peak occurs after four days (Mandyam et al., 2007). Mandyam found a first peak after 15 hours. Nevertheless, it could be that my findings with an initial peak at 2d and a second drop after seven days fit the differentiation scheme found in clusters, but with some time delay. This delay maybe caused by different injection protocols. While Mandyam et al. gave only a single BrdU pulse, I used three injections every 6 hours. Thus, my latest labeled cells start their differentiation with a delay of 12 hours.

A drop between day seven and day ten could make sense biologically. It can be explained by different reasons: Firstly, increased cell death rate after day seven caused by regulation of the neuronal integration could cause the effect. This seems unlikely, as we did not stimulated regulation of ahNG in any way, but rather used only standard conditions with as few handling as possible. Secondly, dilution of the BrdU label below the detection level could cause a sudden drop in BrdU cell counts. Hayes and Nowakowski (2002) assumed attenuation below detection level of the BrdU label after three divisions. Dayer et al. showed that in vivo BrdU is diluted after four divisions already and cannot be visualized anymore (Dayer et al., 2003). Between day seven and ten most cells labeled at the beginning of the investigation could have passed through the third division and, thus, have passed detectability. We used in our protocol a cumulative approach with three injections of BrdU. Thus, not all cells detected during differentiation necessarily started at the same time point with this process. Also we label a wide variety of different cell types with distinct dynamical properties (e.g. proliferation rates, see section 3.1.2.). This heterogeneous group of cells very unlikely shares identical dynamics for all members, thus a community effect causing a sudden decrease by a fourth division should not be the cause (for further details on distinct features of each cell types see chapter 3.2.).

Thirdly, Mandyam et al. tried to explain the appearance of the second peak with a switch of whole clusters from expansion phase towards migration and differentiation phase (2007). This approach is highly attractive, since cells within clusters are assumed to communicate with each other (see also Sections 2.1.2.1., Paragraph “Morphological examinations” and 3.2.1.2.). Up to date, the basic understanding of regulation and stimulation of Type-1 cell is rudimentary. Nevertheless, a whole section will address the question which dynamics Type-1 cells follow, and if this process can be influenced by any factor (see Section 3.2.1.).

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### **3.1.5. Average CCL may vary for different cell types**

We know, that the average CCL of BrdU-labeled cells in mice adds up to 14 hours, with 6-7.6 hours of S-phase, 4.5 hours of G<sub>2</sub>/M phase and 1.9 hours of G<sub>1</sub> phase (Hayes and Nowakowski, 2002, Mandyam et al., 2007). Although it is possible, that the assumptions underlying the mathematical model from Hayes and Nowakowski (2002) are too unspecified and therefore not completely correct (as implied in section 3.1.4.1.), Mandyam et al. (2007) were able to verify a cycle length of 14h for this merged cell population. Still the individual CCL for the distinct differentiation stages needs to be explored.

As mentioned above, I found a drop in BrdU-positive cells between day seven and ten. Similar, Mandyam et al. (2007) found the same effect a bit earlier in cell cluster, consisting of cells in various differentiation stages. One possible explanation for an inconsistent drop of the BrdU-positive cell number would be a different CCL in dependency on the cellular microenvironment even within cell types a possible stimulus would thus force cells of one cluster to leave proliferation stages (type-2) towards migration stages (type-3, early immature neurons) at once. This would explain the sudden leave from proliferation stage towards migration stage within clusters and reveals one possibility for the second peak.

In summary, the length of the cell cycle, estimated for all cells undergoing S-phase, displays an average value for all mitotic cell stages. Since it has been shown, that a subpopulation, if not all, progenitors in the adult brain divide infrequent from early development onward (Mathews et al., 2010), we can assume a bias caused by average division rates of the total BrdU-positive cell population. In consequence, CCL for individual differentiation stages and cell types needs to be investigated in near future.

Nevertheless, I found promising indications of distinct dynamics for separate cell types, as well as of a quiescent stage for more differentiation stages than type-1.

### **3.2. *Detailed phenotypic analyses of ahNG***

The phenotypic analysis of the BrdU-positive cells produced both relative amounts and absolute numbers of each cell type at each time point. A similar analysis was performed previously (Kronenberg et al., 2003), but several constraints reduced the possible gain of information on ahNG. Firstly, low efficiency of labeling a cohort of proliferative cells

was caused by the experimental protocol (1x 50 mg/kg BrdU). Secondly, results of that study were limited by the restricted temporal resolution (too few time points) and the comparatively low number of cells phenotyped (50 BrdU-positive cells per animal). Analyzing too few cells makes the analysis prone to cluster effects if a group of cells is found together. Poor temporal resolution can cause wrong assumptions (e.g. missing the real peak at day two). Low sample size again produces huge standard error as well as slightly wrong impressions caused by involuntary pre-selection.

In this work the number of time points was increased and all BrdU-positive cells were phenotyped exhaustively, respectively more than 200 cells/DG, in a one-in-six series spanning the entire dentate gyrus. Thus, I was able to gain detailed information on the differentiation process with respect to the individual cell types involved.

### 3.2.1. Type-1 cells



Type-1  
Stage 1

During the first differentiation stage Type-1 cells are located within the subgranular zone of the adult dentate gyrus of mammals. These neural stem cells are the origin of ahNG and thus give rise to progenitor cells, as hypothesized in previous models of the differentiation process (Kempermann et al., 2004). Fortunately some newer studies focus on this differentiation stage, solving some research questions, but also raising new questions (Bonaguidi et al., 2011, Encinas et al., 2011, Kempermann, 2011a). We are far from understanding the dynamics of this cell type, but some new conclusions will be drawn from my data and available knowledge, resulting in a hypothesis introducing Type-1 cells as safeguard.

#### 3.2.1.1. *The pool of BrdU-positive cells contains very limited numbers of Type-1 cells under standard conditions with varying CCLs*

In the adult dentate gyrus only few BrdU-positive type-1 cells can be found. I applied three times 50 mg/kg BrdU, adding up to a dosage of 150 mg/kg within 12 hours. This experimental protocol resulted in roughly 2% type-1 cells, with regard to total number of BrdU-positive cells (FIGURE 2-7). The result verifies previous findings by Kronenberg et al. (Kronenberg et al., 2003).

The absolute number of proliferating Type-1 cells increased significantly with the number of BrdU injections, implicating a benefit in detectability by relabeling. Concretely, I

found a 3.5-fold increase between 2h and 14h (FIGURE 2-8). Since proliferation rates are so low for type-1 cells, I can assume, that double labeling mainly occurs, while cumulative labeling should be rather seldom. Thus, this strong increase must be mainly caused by cell division. As mentioned above CCL is calculated from the given formula. With  $N(2h) = 13.36$  and  $N(14h) = 47.62$  I find a cycle length of 6.5 hours. Since I do not have any detailed information on whether double-labeling varies between cell types, I also assume an effect on 50% of the population as described for the total number of BrdU-positive cells above. Thus, we end up with a CCL of 13 hours, slightly less, than the average one.

The estimation of the CCL for Type-1 cells, only holds true under the assumption of symmetric division of Type-1 cells with the generation of two daughter type-1 cells. This self-renewal has nearly never been observed, yet. Only one very recent publication shows a possible symmetric self-renewal under standard conditions (Bonaguidi., et al. 2011). Thus, we can assume, that the CCL length needs to be estimated for different kinds of divisions.

### **3.2.1.2. *Type-1 cells are mainly quiescent, but can divide in different ways including self-renewal, neurogenesis, and gliogenesis***

As mentioned above, Type-1 cells have never been actually seen to undergo symmetric self-renewal. In my study, I see a 50% reduction in the number of type-1 cells after two days, as well as after seven days. Assuming a symmetric division causing two identical, not Type-1 cells (either Type-2 or astrocyte), CCL would average out at two days for the first division and for the second division another 120 hours. This huge variation in cycle length seems quite implausible. Therefore, we either need to reason a  $G_0$  state after the first division, or a more complex division paradigm, including also self-renewal and/or asymmetric division.

The morphological findings (see below 3.2.1.4.) indicate a possible asymmetric division of type-1 cells as described in earlier publications as well, but it does not automatically exclude the probability of symmetric division. Bonaguidi et al. even displays in his publication symmetric type-1 division yielding two type-1 cells (self renewal). Since type-1 cells are often very closely located, it is hard to be totally sure of cell division and not simple close position caused by other means. Nevertheless, recent in vitro

experiments support the possibility of the coexistence of symmetric and asymmetric type-1 cell division. Namba et al. found in a time lapse study in cultured hippocampal slices from early postnatal mGFAP-eGFP Tg mice different forms of dividing type-1 cells. 45% of all dividing GFAP-positive cells self-renewed, thus, they divided symmetrically into dentate gyrus stem cells. Another 45% also divided symmetrically, but into two neural progenitor cells. Only 10% were found to divide asymmetrically into one stem cell and one neural progenitor cell (Namba et al., 2011). Although this study was performed in early postnatal brains in vitro and the process could strongly vary in adult ones, it still proves the possibility of complex and variable division behavior of GFAP-positive neural stem cells.

### ***3.2.1.3. Type-1 cells can either be active or quiescent***

After day two the observed population of stem cells decreased strongly, but mostly insignificant (FIGURE 2-8 A). This data could mean a saturation of labeled type-1 cells after one BrdU administration, causing a common division start for all Type-1 cells at 2 hours. For CCL this would mean a value of more than 14h, maybe including  $G_0$ . In respect to an intermediate  $G_0$  state, a recent publication by Encinas and colleagues three asymmetric neurogenic divisions of type-1 cells, followed by one symmetric gliogenic division leading to the emergence from the stem cell pool (Encinas et al., 2011). Additionally, the S-phase length of the cell cycle of type-1 cells is suggested in this publication to amount to 7.8 hours. Mathematical modeling would need to test these values for the obtained data. Theoretically, it could be possible, that this theory fits the experimental results. During the three injections type-1 cells in S-phase were labeled unaware of the question, if the cells were hit during their first, second or third division. Therefore, we cannot confirm this cell cycle model without doubt.

### ***3.2.1.4. Type-1 cells communicate with cells in later differentiation stages***

In this study, phenotypical analyses indicate a specific location of type-1 cells in correlation to cells further passed through the differentiation process. Type-1 cells are mainly collocated with either type-2a cells or type-3+ cells (DCX+). In case of adjacent type-2a cells a recent mitosis and division seems highly probable, with some cells even sharing cytoplasm. These findings have also been recently reported by Bonaguidi et al. (Bonaguidi et al., 2011). They identified cells directly after mitosis and detected type-1 and type-2a cells sharing soma. Additionally, these identified gliogenic cell division



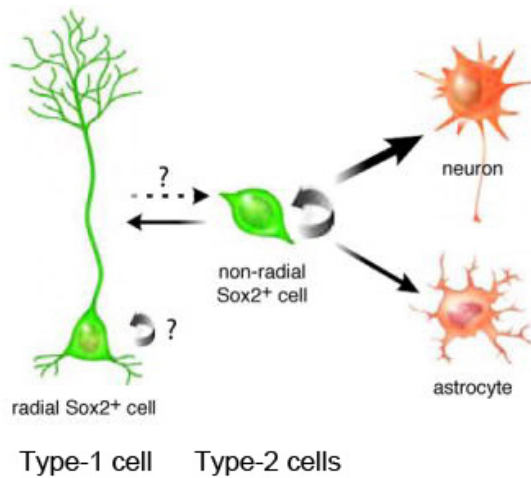
consisting of one type-1 cell and an adjacent GFAP<sup>+</sup> astrocyte, which cannot be confirmed in this study. In contrast to Bonaguidi et al., I furthermore found type-1 and type-3 cell processes winding around each other (FIGURE 2-6).

It is known, that Type-2a cells are the first ones during in vivo differentiation, which receive GABA input via interneurons, but although Type-2b and Type-3 cells receive stimulation (Deisseroth and Malenka, 2005, Tozuka et al., 2005, Wang et al., 2005). This way they receive information neural stem cells will not recognize immediately. Additionally, Type-2b cells start to build out first connections via processes leading to a first interconnection with the hippocampal network of type-3 cells, they transfer from GABAergic input only towards glutamatergic stimulation. Thus, type-3+ cells could possess the role to filter network information and only transfer relevant ones to type-1 cells.

Nevertheless, my findings are in line with a recent publication showing reduced number of type-1 cells caused by depletion of Prox1-positive type-2b and type-3 cells (Lavado et al., 2010). Thus, type-1 could be assumed to reappear without input / connections to type-2b/3 cells. Maybe, this displays also a signal for type-1 cells to leave the neurogenic niche via symmetric gliogenic division as described by Encinas et al. (2011).

### ***3.2.1.5. Type-1 cells can arise from type-2a cell division (redifferentiation)***

A mixture of asymmetric and symmetric division as well as partial redifferentiation from type-2 into type-1 could be possible. This is consistent with recent literature indicating redifferentiation from type-2 cells into type-1 cells (FIGURE 3-2). Suh et al., for example, followed the differentiation of SOX2 positive cells (Suh et al., 2007). He claimed that 9.8% of all SOX2 / BrdU positive cells become GFAP positive after an interval of 28 days. This could indicate both a possible gliogenesis as well as redifferentiation of type-2a cells into type-1 cells.



**FIGURE 3-2** Contribution of SOX2<sup>+</sup> NSCs (type-1 cells) to adult neurogenesis:

Non-radial SOX2<sup>+</sup> NSCs (type-2a cells) have potentials to generate identical cells and give rise to downstream neural cell types, suggesting that type-2a cells retain the self-renewing and multipotent NSCs. Type-1 cells preferentially gave rise to neurons, presumably due to the influence of stronger neurogenic niche. Type-2a cells also have the potential to give rise to Type-1 cells, suggesting that the equilibrium between radial and non-radial SOX2<sup>+</sup> NSCs (type-1 and Type-2a) may have a significant role in the homeostatic control of adult neurogenesis (Adapted and modified from (Suh et al., 2007).

### 3.2.1.6. Are type-1 cells the origin of ahNG?

Still the question remains if type-1 cells really are the origin of neurogenesis or if they display a differentiation stage, cells get into to be relatively immune to environmental influences, as backup for adult neurogenesis? Under the assumption, that type-1 cells give rise to type-2a cells, a decrease in the absolute number of type-1 cells as observed in this study seems very plausible. On the other hand the existence of Type-1 cells involved in gliogenesis and neurogenesis does not mean this cell type exclusively participates in both lineages. In Chapter 3.2.2. I will present Type-2a cells as origin for neurogenesis and gliogenesis.

In previous publications Nestin-positive cells were often divided into quiescent radial glia like stem cells (type-1 cells) and active stem / progenitor cells. The latter are often not identically characterized. Mostly Nestin is used as marker, seldom GFAP, sometimes morphology. Thus, it is not completely sure, if these activated stem cells are a subpopulation of type-1 or type-2 cells, maybe even type-2a cells (for review see (Wang et al., 2011b). Encinas et al. (2011) found further evidence for the quiescence of Type-1 cells, with identifying only 1% of them positively labeled for BrdU (1x BrdU with a dose of 150 mg/kg). On the other hand he identified 10% of all ANP cells (Type-2 cells) in S-phase with the same protocol. Nevertheless, the question whether type-1 cells or type-2

cells are the origin of ahNG, or if an intermediate cell type-1 takes over this role needs to be further investigated.

### ***3.2.1.7. Type-1 cells are affected by diverse stimuli***

In consideration of the results regarding type-1 cells, it must be considered, that they are more proliferative than assumed before. Type-1 cells have been discussed controversially in respect to stimulation by diverse factors. They did not change in number when the animal was exposed to a neurogenic stimulus (Kronenberg et al., 2003, Battista et al., 2006). Regarding the fact, that these studies phenotyped a very limited number of cells (<100), it could be possible that these cells still react, but below detection level. Other studies have found Type-1 cells responding to extrinsic stimuli, such as seizures, KA; cortical infarct and exercise (Huttmann et al., 2003, Kunze et al., 2006, Steiner et al., 2008, Lugert et al., 2010). But further research will reveal the importance of stimulation of type-1 cells in more detail.

Although, anti-mitotic treatment affects all stages of ahNG, but the GFAP-positive ones (type-1 cells), we do not know whether or not this cell stage is partially special in ahNG since it needs to fulfill a unique task (Seri et al., 2004). In the next section I therefore hypothesize type-1 cells as safeguards of the system. It seems like type-1 cells still remain some kind of mystery which still needs to be solved in future studies.

### ***3.2.1.7. Type-1 as safeguard for ahNG***

In this chapter 3.2.1 I argue, that Type-1 cells are very seldom proliferative, thus, they mainly stay in quiescent G<sub>0</sub> cell cycle stage (3.2.1.1.). Furthermore, I show a strong morphological connection between Type-1 and Type-2 cells, as well as Type-1 and Type-3 cells. This may imply a strong communication between these stages. Thus, Type-2a cells with first GABA input and Type-3 cells with some first dendritic connections are hypothesized to provide Type-1 cells with information about the microenvironment. By means of previously described data (Suh et al., 2007) I show a possible maintenance of the stem cell pool via redifferentiation from Type-2a cells, guiding to the question whether Type-1 cells really are the origin of ahNG. Furthermore, I showed, that Type-1 cells, as all other differentiation stages can be influenced by diverse stimuli, but stay unaffected by antimitotic treatment (Seri et al., 2004). Furthermore, a high amount of these cells remains quiescent (data not shown, based on NestinGFP analyses as part of my

Master's thesis). Quiescent stages are much less vulnerable to damage. This could imply a safety state.

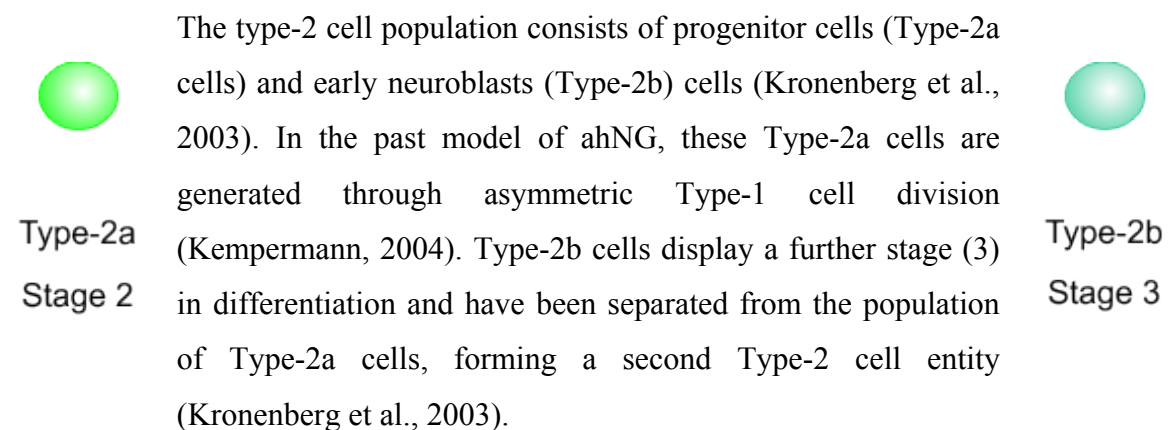
Based on this total information I thus hypothesize, that Type-1 cells are not necessarily involved in the differentiation process under normal condition, but they gain importance in case of damage to hippocampal tissue and/or later proliferation stages, this damage gets indicated by extrinsic factors, but also via the connections, or missing connections to Type-2a and Type-3 cells. Thus, Type-1 cells function as safeguards for ahNG.

In line with this theory did Kempermann state:

“Could it be that, in the absence of appropriate stimuli, the stem cells are indeed predestined for disposal but that this fate can be overcome by unleashing the potential that exists in individual precursor cells? This is a testable hypothesis. How plastic is adult neurogenesis?” (Kempermann, 2011).

Whether or not one of these theories, or a combination of both, holds true, future will reveal.

### 3.2.2. Type-2 cells



#### 3.2.2.1. *Type-2a have a CCL of 17.6 hours, which is probably strongly overestimated*

In respect to cell cycle we find a 2.57 fold increase between 2 h and 14h, caused firstly by additional BrdU administration and secondary by division of cells labeled after the first injection (FIGURE 2-8). No other increase was observed further on, thus, type-2a cells very likely divide only once followed by a quiescent phase. Contradictory results have been described regarding ANPs (type-2) cells recently. Encinas et al. found 2.45 divisions in a row for these cells (Encinas et al., 2011).

Surprisingly, the initial boost only displays a 2.6-fold increase on number of cell. Calculation of the CCL with the determined values (FIGURE 2-8) reveals a length of 8.8 hours, and of 17.6 hours after correction for double-labeling. This is a much slower turn over as estimated for the total BrdU population. Keeping in mind, that this differentiation stages gets highly affect by diverse micro- and macro-environmental factors (Cameron et al., 1995, Brezun and Daszuta, 1999, Kronenberg et al., 2003, Wang et al., 2005, Kempermann et al., 2006, Steiner et al., 2006a, Masuda et al., 2011), I would have suggested a faster rate. With such a long cell cycle adaptations would be rather slow. Thus, type-2a cells have to also arrest from cell cycle, leading to a strong overestimation of CCL. A second inaccuracy of the CCL value is also caused by the assumption that Type-2a cells divide symmetrically into the next differentiation stage. This needs to be further validated and future mathematical modeling will also address the question, whether these cells are capable of asymmetric division.

### ***3.2.2.2. Type-2a cells as origin of ahNG***

Type-2a cells as a very early stage of fate commitment mark a transition step between glial and neuronal differentiation (Steiner et al., 2006). Studies with GFAP-GFP transgenic mice even showed GFAP-expression in some early type-2 cells (type-2a cells), but this was not the case for immunoreactivity, indicating poorer detection of GFAP via histological methods. Typically, neural stem cells within the adult dentate gyrus are identified under usage of GFAP as marker and/or Nestin marker expression plus morphological features, such as radial processes reaching into the molecular layer. Since the difference between type-2a and type-1 cells is not always easy to observe, in particular with different methodological approaches, I could also imagine an intermediate state between type-2 and type-1 cells as origin of ahNG, specifically under the assumption of possible redifferentiation from type-2 into type-1 cells. Maybe the linear model structure of adult neurogenesis is also completely misleading. It is needed to think more in circular structures. This suggests a far more flexible system than assumed before, which can react very diverse to unique demands of the hippocampal network. In a future mathematical model it would be necessary to reassess the existence of two different type-2 cell populations as stated previously (Kronenberg et al., 2003, Kempermann et al., 2004).

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### 3.2.2.3. *Type-2b CCL amounts to 10.44h*

With the same approach used above, CCL of Type-2b and Type-3 cells was estimated. They both increase between 2h and 14h even more than 5-fold. In particular, Type-2b cell increased 5.5-fold in this time period. This results in a CCL of 5.22 hours for Type-2b cells before and 10.44h after correction for double-labeling. Thus, this differentiation stage is the one with very rapid turnover, allowing high proliferation rates in the adult dentate gyrus. Therefore, a quiescent state within this differentiation phase is inconsistent with my data.

Type-2b displays a type of clearly lineage restricted cells (Kempermann et al., 2004). Besides DCX and Nestin, they also express NeuroD1 and Prox1 securely identifying only cells of neural lineage (Steiner et al., 2006a). Besides fate choice, these cells are also highly influenced by several factors, which regulate their appearance. At this differentiation stage regulation via cell death seems to play a major role (Sierra et al., 2010). Nevertheless, it is not clear, if the provoked increase in this cell number caused by for example exercise or other factors (Kronenberg et al., 2003, Encinas et al., 2006) is assumed to be caused by an increase in symmetric Type-2a cell division. In this regard it could make sense to focus on these two differentiation stages together.

In respect to the development over time of the absolute quantity of type-2a and type-2b cells one could assume a population of cells, artificially divided by labels. In this study I found an immediate decrease in the relative proportion of type-2a cells, while the proportion of type-2b cells increased from 2 h to 14h and drops afterwards (FIGURE 2-7) indicating two different populations. Also the estimation of the CCL varies strongly for both differentiation stages, thus, type-2a and type-2b clearly display unique differentiation stages.

### 3.2.3. Type-3 cells

#### 3.2.3.1. *Estimation of population size is rather difficult and reveals a small type-3 cell population*



Type-3 cells are a population rather difficult to identify and separate from other differentiation stages. Morphology is a possible way to distinguish between type-2 and type-3 cells somewhat and also marker expression.

**Type-3** Similar to some type-2 cells and early immature neurons, they express

**Stage 4**

doublecortin and also other markers, such as  $\beta$ III-tubulin or Prox1, not only label type-3 cells. Since confocal analyses are restricted to four different markers up to date, it is impossible to directly address this developmental stage at the same time as the other mitotic and postmitotic stages, without contaminate the data with either type-2b cells, or immature neurons. Plumpe et al. identified DCX cells in great detail, moving from horizontal type-2b cells to type-3 cells with an apical dendrite reaching the molecular layer, but even here 70% of all DCX-positive cells are a merged population of type-3 and early postmitotic cells (Plumpe et al., 2006).

Thus, an indirect mathematical assessment of the number of type-3 cells had to be chosen in my study. Different calculation procedures gave different results (FIGURE 2-14). Both results have in common, that they produce negative values. This does not make any sense in this biological context, and may be caused by huge standard errors of the input values. More interesting, both mathematical methods produce time curves with two peaks, one initially between one and two days, followed by a huge decrease at day three, and another maximum after four (Method A) / five days (Method B). This indicates at least two divisions in a row, but small overall numbers of type-3 cells. DCX-positive cells undergo radial migration and dendritic extensions besides other morphological changes (Plumpe et al., 2006). Keeping this in mind, a fast transition through type-3 cell stage from type-2b (DCX+, Nestin+, SOX2+) through type-3 (DCX+) towards immature neurons (DCX+, CR+, NeuN+) seems plausible as do low turnover rates. Such low turnover rates were also seen from the comparison of my DCX-DAB results and my fluorescence analyses. With one injection of BrdU only 4% of all DCX+ cells were labeled indicating most DCX-positive cells to be immature, or at resting  $G_0$  cell cycle state. We know that apoptosis regulated ahNG at the level of Type-2b (Sierra et al., 2010), thus low Type-3 cell numbers imply the survival of only a very limited number, which is need for functional network integration later on. But it would also be possible that the type-3 cell type displays only a transition stage between mitotic phase and mature phase of ahNG, with changing marker expression.

### ***3.2.3.2. Estimation of CCL for type-3 cells reveals a period of 14.4h for symmetric self-renewal***

Although Type-3 values are very vague I tried to determine CCL. Keeping in mind that we have relabeling distorting the results by ~50% as described above, we end up with a

gross cell cycle length of 14.4 hours. This value only accounts for symmetric self renewal, but could make sense in biological respects. Nevertheless, this value was obtained by so many different calculations (Method A, see Results), that the probability of mayor error is highly increased. Thus, this value is only given as rough idea, without further speculations. Future studies need to especially focus on the Type-3 cell stage, whether it really exists as active mitotic stage, in low numbers, or whether it is only a transition phase.

### ***3.2.3.3. Two different proliferation phases are detected for type-3***

As displayed in FIGURE 2-14 do we receive vary different results for type-3 cells, depending on the calculation method. Both curves, whatsoever, have in common, that we find two peaks. The first peak is most probably caused by labeling of type-2b and type-3 cells during S-phase via BrdU, but the second peak after roughly four days could be caused by progression of cells through differentiation. This peak is post-poned towards type-2 cell maxima by roughly two days. This could mean, that it takes two days to transit from type-2 cell stage to type-3 cell stage. Since the results regarding type-3 cells are based on gross calculations, further evidence must be found under focus on only type-3 cell stages.

### ***3.2.3.4. The cellular picture of type-3 cells remains incomplete raising the need to especially focus on this differentiation stage***

Morphological observations showed type-1 cells directly adjoining type-3+ cells. The two processes are closely relocated, even looking like winding around each other. Since type-3 cells are really rare in ahNG, as described above, these DCX+ cells are assumed to be mainly immature neurons. For scientific correctness, I will further on continue to call the type-3+ cells. This collocation could possibly indicate a signaling process between early immature neurons and type-1 cells, further regulating ahNG. The confocal analyses only provide a first indication, to ascertain direct communication between type-1 and type-3+ cells, maybe via small spines and chemical/electrical synapses stimulation, further experiments using electron microscopy and electrophysiology are necessary.



### 3.2.4. Postmitotic granule cell numbers indicate first termination of the differentiation process after ten days p.i.



Early  
immature  
neuron  
Stage 5

Early immature neurons and premature granule cells are both part of the postmitotic differentiation. It has been stated in previous publications, that it takes four to seven weeks after birth for new neurons to be functionally integrated into the hippocampal network (van Praag et al., 2002). Nevertheless, first NeuN-positive cells were observed as early as 24 hours p.i. indicating rapid transition from type-3 cells to early immature neurons (Brandt et al., 2003). Within the experiments of this thesis NeuN-positive cells were found as early as 14 hours after BrdU, indication, that transition from Type-3 cells in late S-phase to early immature granule cells takes less than 14h. This very low number of cells increased rapidly within the first few days. Six days after the initial administration of BrdU the number of premature granule cells becomes stable indicating equilibrium between newly born neurons and control of neurogenesis through apoptosis at the postmitotic level (FIGURE 2-11). Although we can hardly speak about completely mature granule cells, within days after BrdU we find a strong increase in the proportion of postmitotic cells, such as immature and premature granule cells. As early as ten days p.i. the final fraction of immature and premature cells are established.

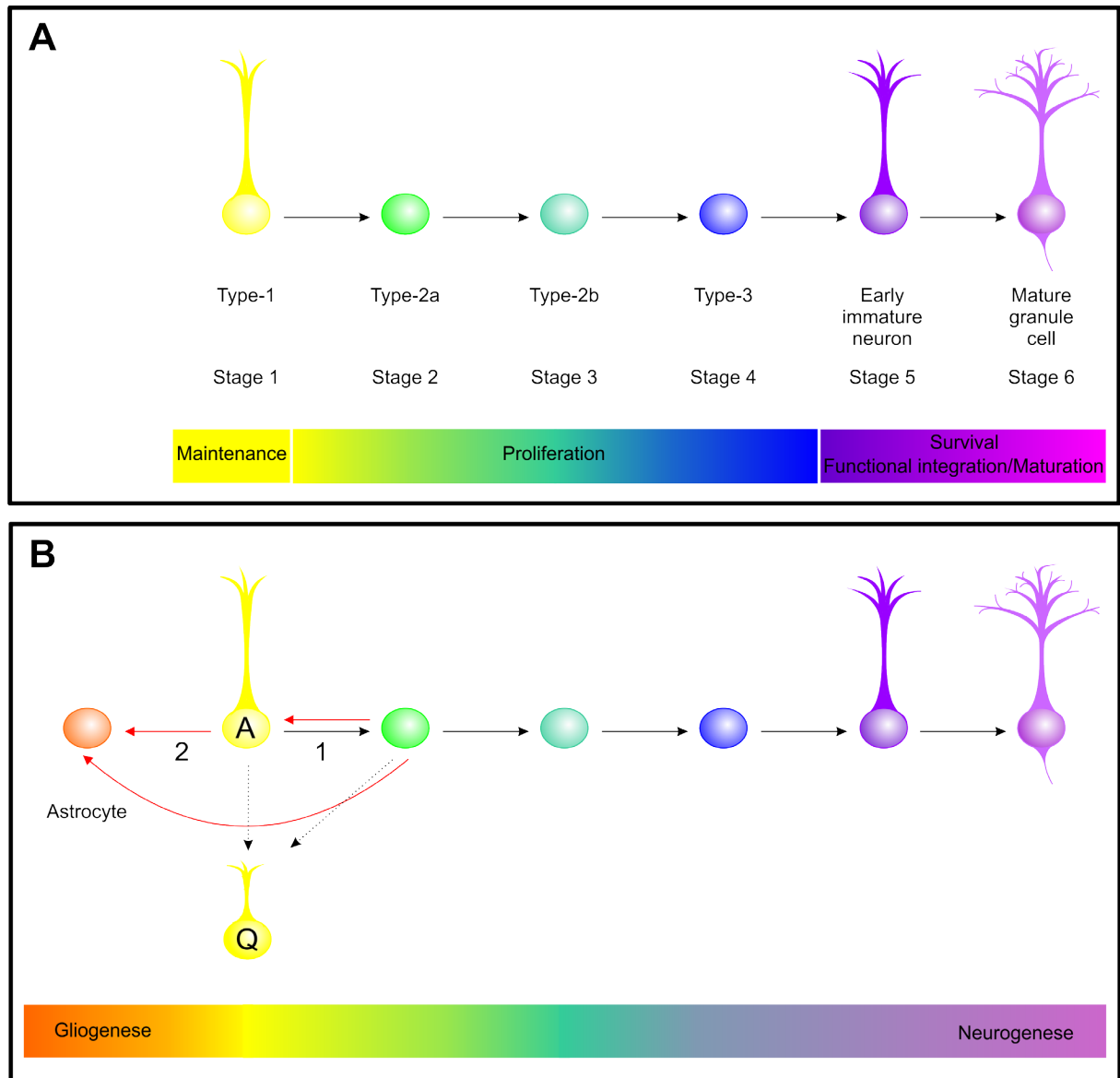
Therefore, I assume a first termination of the differentiation process after ten days, only followed by further functional integration into the hippocampal network. Since Toni et al. found first synapse formation of these new neurons after seven days, one could also hypothesize a much faster maturation (Toni and Sultan, 2011).

### 3.2.5. New Model of ahNG

Overall, the data of this project supports the model of differentiation stages from type-1 to type-2 to type-3 to postmitotic granule cells as stated by Kempermann et al. (Kempermann et al., 2004), but some changes and adaptations should be made. The role of type-1 cells needs to be integrated in more detail, a possible active state in between type-1 and type-2a, which could also even be type-2a, needs to be considered, as well as the separation of type-2a and type-2b (FIGURE 3-3). Very important is also the possibility of redifferentiation from type-2a to type-1, which could solve the discussion

about reappearance of neural stem cells after several divisions and in aging individuals. As mentioned above, Encinas et al. reported the emergence of type-1 cells from the neurogenic pool (Encinas et al., 2011). In contrast, Bonaguidi et al. describes the rare event of symmetric stem cell division in the adult dentate gyrus (Bonaguidi et al., 2011). Additionally, Kempermann (2008) established his “neurogenic reserve hypothesis”, which suggests that the potential for neurogenesis can be maintained in aging individuals under certain conditions, such as activity and enrichment (Kempermann, 2008). These theories are mainly based on type-2 cells, because these cells are often stimulated by activating factors, but redifferentiation could also have a tremendous effect on the preservation of high levels of neurogenesis in old age. Kempermann (2011) tried to solve this conflict between the different publications using the assumption of dependency on external regulators. He claimed, that stem cells are disposed under normal conditions, but have the general potential to be maintained with symmetric division, as stated by Bonaguidi et al., in consequence of specific stimulation (Kempermann, 2011a). This process could also possibly be influenced by social structures, environmental factors and medical treatment. The shift of research focus towards type-1 cells promises the future detection of regulatory mechanisms influencing the appearance and mitotic behavior of neural stem cells within the dentate gyrus. Thus, the question whether redifferentiation really is the key, or a different model of differentiation, needs to be solved in future.

## Discussion - Detailed phenotypic analyses of ahNG



**FIGURE 3-3:** Illustration of different models of the differentiation process within ahNG. **(A)** Old model of ahNG as described in previous publications and as part of the introduction (FIGURE 1-2) **(B)** Illustration of a new model of the differentiation process within ahNG. Type-1 cells (Yellow) divide first asymmetrically into type-2a cell and afterwards symmetrically into astrocytes (Encinas et al., 2011). The pool of these stem cells can be maintained by redifferentiation from type-2a cells (Suh et al., 2007). Type-2a cells can differentiate further into type-2b cells under expression of DCX, or even undergo gliogenesis (Steiner et al., 2006a). Regarding the common features of type-2a and type-2b cells there could be a possibility to combine these two stages under the assumption that no mitosis is needed to perform the step from type-2a towards type-2b cell. Additionally, it is not known yet, whether the quiescent stage (Q) is part of type-1 cell stage or could also be an intermediate, or even type-2a cell stage. Type-2 cells differentiate further into type-3 cells. Type-3 cells finally start to develop dendrite like processes integrating these cells into the hippocampal network. Early immature neurons have established a simple network of processes which is further differentiated and expanded until the final mature granule cell has been originated.

From the present data alone no absolutely certain conclusions about the dynamics of type-3 cells can be drawn, as displayed above. The cells identified in the mitotic analyses could be type-3 cells as well as early immature granule cells. Compared to the decline in the number of type-2 cells the decline of these type-3+ cells is postponed for approximately half a day (FIGURE 2-8). Following the assumption of Hayes and Nowakowski, that a cell cycle in the dentate gyrus takes less than 20 hours, most probably only 12 to 14 hours, the delay would take approximately one cell cycle (Hayes and Nowakowski, 2002). Furthermore, BrdU- and DCX-positive cells increased in number within the first 2 days. Afterwards the absolute number of these cells decreased subsequently. Type-3 cells rise from type-2 / type-2b cells in the previous model (Kempermann et al., 2004). A comparison between the data for type-2b and BrdU- and DCX-positive cells indicates a time shift of approximately one cell cycle. Therefore, we can assume that most BrdU- and DCX-positive cells are type-3 cells and the relative amount of immature granule cells is low. The number of postmitotic cells is expected to increase with time. Brandt et al. analyzed the absolute number of calretinin positive cells over time (Brandt et al., 2003). They found a maximum of these postmitotic cells after 1 week. The data fits nicely to this assumption since the absolute number of all mitotic cell types decreases latest by day 3, while the proportion of early immature neurons rises strongly after 14h with a peak in the absolute number after three days. (FIGURE 2-10 and 2-11).

### **3.3. *Psychosocial influences on ahNG***

The second part of this dissertation combines three small projects estimating different aspects of psychosocial influences on the dynamics of ahNG. Although some interesting results are achieved, this needs to be understood as a first attempt into this relative new field of research. Some studies have analyzed the correlation of social behavior on adult neurogenesis so far, but only few focused on mammals and even less on mice. Thus, this second part tries to get a first impression, if social features, such as group composition, auditory stimuli (communication), and socially strongly influenced disease, such as depression, have any correlation to ahNG.

### ***3.4. Impact of group size on the generation of new granule cell neurons on the adult dentate gyrus***

In this study female mice were housed under standard condition with varying group sizes from 2 till 15 animals per cage. Only cage size was enlarged to one meter times one meter per group for maintenance of minimum space per animal according to the German animal welfare act (Tierschutzgesetz). In this first study on the psychosocial impact on ahNG, we find a group size dependent increase in the number of BrdU-positive cells 24h p.i. Results further show a positive correlation between group size and number of BrdU-positive cells per dentate gyrus with an unclear effect on the distinct differentiation stages (FIGURE 2-16, -17). A trend towards more postmitotic cells 24 hours after BrdU injection with increasing number of new neurons in larger groups is indicated, although not significant (FIGURE 2-17). Relative distribution of cells within each differentiation stage shows also a shift towards later differentiation stages. Thus, increased group size seems to either enhance the speed at which differentiation occurs, or reduces the number of cells within early differentiation stages from resting at their stage and forces them to continue differentiation towards neurons. New in this study is the fact, that we not only focused on net neurogenesis assessed by means of BrdU quantification, but also investigated to changes on single differentiation stages. Although data for the early mitotic phase is still missing, results will be produced soon, highlighting the effect of social environment on ahNG.

#### **3.4.1. Social interaction is needed for enrichment**

Regarding the influence of group size on ahNG it is known from previous studies, that single housed individuals possess higher corticosterone levels, than those housed in groups (Stranahan et al., 2006). Corticosterone (CORT) and other glucocorticoids are known to decrease both proliferation and survival rates in the adult hippocampus (Gould et al., 1992, Cameron and Gould, 1994, Wong and Herbert, 2004). Stress alters glucocorticoid levels within the brain causing a tremendous, but reversible, effect on neurogenesis especially when applied chronic. Also another study with rats revealed negative effects of isolation on spatial learning, ahNG and long-term potentiation. Group housing was able to reverse this effect (Lu et al., 2003). Thus, social interaction seems quite important for stress resistance. However, enrichment seems to be only effective,

## Discussion - Impact of group size on the generation of new granule cell neurons on the adult dentate gyrus

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regarding an increase in the number of BrdU-positive cells, in medium to highly active rats. Individuals with low levels of interaction, exploration and physical activity did not benefit from enrichment (Leal-Galicia et al., 2007). This indicates the necessity of social behavior for enrichment. Only, if an individual participates actively in enrichment and social interaction enhancement of ahNG is possible.

A connection between environmental enrichment and enhanced neurogenesis was described manifold (Kempermann et al., 1997b, van Praag et al., 2000, Kempermann et al., 2002, Brown et al., 2003a, Olson et al., 2006, Tashiro et al., 2007, Fabel et al., 2009, Schloesser et al., 2010). Enrichment is also known to facilitate the recovery from psychosocial stress, e.g. for subordinate animals, but only with intact ahNG (Schloesser et al., 2010). Further studies showed an influence of social hierarchical structures on the survival rate of new neurons (Kozorovitskiy and Gould, 2004). Two weeks after BrdU the dominant animals in this study had significantly more BrdU-positive cells in comparison to subordinate and control animals. Between control animals and subordinate males no difference was observed. In the assessment of the impact of group size on ahNG no behavioral parameters for hierarchy were taken, but on average we can assume that subordinate and dominant behavior level each other out for a group of animals.

Enlarged group size also display one possibility to receive an enrichment effect. Although no effect of enrichment on proliferation was shown in C57BL/6 mice, it has been described for other murine strains and rats (Kempermann et al., 1997b, Kempermann et al., 1998a, Kempermann et al., 1998b, Veena et al., 2009, Beauquis et al., 2010). Environmental enrichment of C57BL/6 mice leads to increased survival rates and, thus, enhanced neurogenesis. This form of enrichment consists of several different stimulations. Since the individual stimuli have never been extracted and observed individually it is highly possible that the stimuli counteract each other. Therefore an increase of proliferation by social stimuli is not unlikely at all even, in this mice strain. Nevertheless, it remains necessary to additionally explore the impact of group size on survival.

Within the realized experiment on group size and neurogenesis in mice a slight non-significant shift towards advanced differentiation stages was observed with an increase in the absolute number of premature neurons for animals housed in groups of 15 individuals.

A recent study by Llorenz-Martin et al. not only showed a relative early enrichment effect after one week, but also analyzed the impact on distinct differentiation stages. Interestingly they found a relevant increase in the number of DCX-positive cells (type-3+ cells) and DCX- / CR-positive cells (early immature neurons), but the proportions of these cell types remained stable (Llorens-Martin et al., 2010). This fits nicely to the results displayed in this thesis. Thus, both environmental and social enrichment seem to act on the transition from type-3 to immature granule cell. Keeping in mind, that 50% of all Calretinin-positive cells (immature granule cells) express Glucocorticoid receptors (Garcia et al., 2004) also an impact of stress reduction caused by social interaction could be possible. Further evidence that the transition from type-3 to immature granule cells gets affected by a combination of social enrichment, stress reduction and CORT levels is given by Mayer et al. They found a strong reduction in DCX-positive cells caused by CORT and reversed by Mifepristone, a glucocorticoid receptor antagonist (Mayer et al., 2006). Therefore, a future study needs to not only investigate the effect of group housing on survival, but also the correlation to plasma corticosterone level, glucocorticoid receptor action and stress reduction. Furthermore the contribution of single differentiation stages will be investigated on their impact on the effect observed in this study.

### ***3.5. Influences of auditory stimuli on ahNG***

Another environmental stimulus with possible impact on adult neurogenesis are excitations of the auditory system. Influence of diverse auditory stimuli on cell proliferation and survival in the adult dentate gyrus as a first step to test behavioral relevant sounds on neurogenesis.

Regular animal house noise was used as a control while silence, white noise and music as behavioral irrelevant stimuli were set in contrast to behavioral relevant pup calls. Pup calls are known to trigger female behavior in a strong way. Pups, which are located outside the nest, start to exhibit ultrasonic vocalizations (USV). As a consequence, the mother searches for the isolated offspring and retrieves it to the nest (Ehret and Haack, 1982, Ehret, 2005). Liu et al. showed for the first time a correlation between communication sounds and plasticity inside the murine brain (Liu and Schreiner, 2007).

Thus, the brain can adapt to sound exposure, but so far, the hippocampus as memory center has not yet been investigated.

### **3.5.1. Proliferation and Survival in ahNG are differently influenced by distinct auditory stimuli**

The present study shows, that all tested auditory stimuli influence ahNG by either effecting proliferation or survival of newborn neurons, or by just enhancing the progenitor pool dependent upon the stimuli exposure. For the proliferation paradigm significant increases of the number of BrdU-positive cells were determined for the groups “silence”, “pup calls” and “Mozart’s music” when compared to regular animal house noise (FIGURE 2-20). Quite impressively we even find a 2-fold increase for both Music and Silence.

Music has been known for several decades to strongly influence cognitive performance, working memory, the cardiovascular system and even improves symptoms of depression and other psychiatric disease (Reinhardt and Lange, 1982, Rauscher et al., 1993, Fukui and Toyoshima, 2008, Lemmer, 2008, Smith et al., 2010, Trappe, 2010). Although a strong correlation between song learning and adult neurogenesis was proven in different bird species by several research groups within the past (Nottebohm et al., 1990, Nottebohm and Liu, 2010), no study was performed, yet, to investigate an impact of music on cell proliferation alone in the adult mammalian dentate gyrus.

AhNG is on the one hand known to be involved in learning and memory consolidation, and on the other hand to be necessary for therapeutic effects in some disease. Thus, music, also positively affecting both, could act via enhanced proliferation. Nevertheless, one needs to keep in mind, that music is very diverse. Therefore, its effects on mammals are strongly dependent upon patterns, rhythm, frequency and speed (Trappe, 2009). Thus, we decided to use a prominent piece of classical music by Mozart (KV 448, see methods), which has been used in several other studies before and has never been reported to cause stress. But, even studies approaching similar questions with identical music excerpts produce different results (Rauscher and Shaw, 1998, Steele et al., 1999). In consequence the results of my study for Mozart’s music should not be generalized without skepticism. Future research will reveal further insight in this regard. Interestingly, the effect on



proliferation lead not to increased neurogenesis but rather newly generated cells of other types.

The “silence” group also caused a similar effect as music on the number of BrdU-positive cells one could argue, both increases in the number of proliferating cells are caused by novelty. Therefore, follow up experiments will need to analyze proliferation after a long exposure period, repeated for several weeks. Thus, novelty could be ruled out. However, no increased proliferation was found for the White noise group that gives further evidence, that novelty may not be the reason for enhanced proliferation.

### ***3.5.1.1. Differentiation stages are affected differently in the proliferation paradigm by distinct auditory stimuli***

When analyzing the data for different cell types and stages, the increased number of BrdU+ cells following “silence” stimuli is split to the pools of type-2a (SOX2+), type-2b, 3 (DCX+) and other cell types, with the majority of cells expressing ‘other’ cell types. The latter observation was also seen for the groups “Mozart’s music” and “pup calls”. In contrast, both groups had a very low type-2b, and type-3 cell pool expressing the neuronal marker DCX (FIGURE 2-22).

The relative distribution clearly shows that both Mozart’s music and pup calls lead to extinction of type-2b and 3+ cells (FIGURE 2-21). This phenomenon can be explained by two different reasons dependent on the stimulus. Under the influence of Mozart’s music cells remain in the early type-2a differentiation stage with possible effect on gliogenesis, since the number of other cells was increased, too.

From previous studies investigating influences of RUN on ahNG, we know, that factors enhancing proliferation not necessarily additionally enhance survival in the same amount, too. One usual approach to answer the question, why this may be the case, state either a transition into resting state of these cells, or claim increased apoptosis rates. New neurons have to be needed in order to be functionally integrated. Keeping in mind, that I found a strong probability for quiescent Type-2a cells (Chapter 3.2.), I suggest a cell cycle exit at the Type-2a differentiation stage. This nicely fits to the dense increase only of SOX2-positive cells. The increase in the number of SOX2-, and DCX-positive Type-2b cells rather indicates regulation via apoptosis, or a more or less complete stop of most type-2a cell differentiation.

In animals exposed to pup calls we also find strongly enhanced proportions of cells in the neuron / glia / others pool. In animals exposed to pup calls, type-2b cells were rarely found with strongly enhanced proportions of cells in the neuron/glia/others pool. This could be explained by an increase in differentiation speed to become mature neurons, or a shift from neurogenesis toward gliogenesis. But here, the number of type-2a cells has not increased as much as seen for ‘Mozart’s music.

In animals exposed to silence we also find an enlarged type-2a cell pool and more new neurons / glia cells / others compared to the control group, while the number of type-2b and type-3 cells remains unchanged. These results can be interpreted in a way that silence prepares the brain for a possible future need of neurogenesis (see impact on survival in the next section). From the pool of type-2a cells new neurons can be formed quite fast. Although silence is categorized as an artificial sound stimulus it rarely occurs in nature, too. Usually, this experience is combined with / followed by a threat (natural disasters, e.g. in an eye of a tornado etc.) or occurs after damage to the auditory system. In both cases rapid learning (highly correlated to ahNG) and quick adaptations to a differently experienced surrounding are very crucial for future survival. The sound of silence has been shown to activate the auditory cortex (Kraemer et al., 2005, Voisin et al., 2006). Thus, my data can be interpreted as a fast and needed adaptation to environmental stimuli.

### ***3.5.1.2. Survival causes a sustainable increase in the number of BrdU-positive cells via increasing net neurogenesis***

To see whether the effects seen for proliferation have long lasting effects, it is important to analyze the number of cells after a longer period of time. From the observation of the dynamics of neurogenesis we know one week is sufficient for passage through the mitotic differentiation stages, thus animals were sacrificed after one week to sound exposure.

The survival of new neurons gets influenced in a very different fashion by the applied auditory stimuli. While the proliferation paradigm reveals specific features unique for each sound, discloses the survival paradigm a more general picture. Here, only the “silence” group significantly increased adult neurogenesis (FIGURE 2-23). Although “Mozart’s music” alters proliferation no change in survival rates display the result we expected. As described above, music is behaviorally irrelevant in mice. Thus, lasting

brain adaptations according to this stimulus would have been very surprising. The sound of “pup calls”, on the other hand was played to naïve, sexually un-experienced females. It has been described recently, that mother and virgin females perceive pup calls in a different way and, thus, also react slightly differently towards them (Liu et al., 2006). To really assess the question whether communication stimulates ahNG one would need to include mothers and also different behavioral relevant sounds, such as male ultrasonic calls (Holy and Guo, 2005). Nevertheless, after one week of exposure to pup calls without the presence of an infant, that needs to be retrieved, a learning effect could proof behavioral irrelevance of this stimulus to the experimental group.

Phenotypic analyses in animals exposed to silence indicate a strong increase in the number of new neurons, while gliogenesis remains unchanged. Additionally, a slight increase in the number of other cell types was observed (FIGURE 2-24). With focus on the distribution of the distinct cell types a shift from S100 $\beta$ -positive cells towards NeuN-positive cells was detected. Thus, most likely the enhanced number of type-2a cells observed for the proliferation paradigm further differentiates towards new neurons. Although silence displays a rather artificial stimulus, too, animals remained in groups and interacted with each other during the exposure. Therefore, regular sound exposure by siblings occurred creating a natural auditory surrounding. In contrast all sound typical for animal facilities, e.g. air conditioning, human noise, etc. were excluded. This could possibly cause several different effects: firstly, as described for proliferation, new neurons could be needed for adaptations to a differently perceived environment, secondly, this could display a sound environment typical for nests in natural mouse-holes, disclosed from noise above ground leading to relaxation and reduction in corticosterone levels as observed in humans, or thirdly, we observe a novelty effect. Thus, further investigations should also include endocrinological examinations.

Altogether we can conclude a strong, stimulus dependent, impact on distinct differentiation stages by auditory stimuli causing either increased proliferation or proliferation and survival.

### ***3.6. Reduced hippocampal neurogenesis in the GR<sup>+/-</sup> genetic mouse model of depression***

#### **3.6.1. Stress connects social impact on ahNG and depression via Glucocorticoid**

In this recent study significant net reduction of ahNG was shown in depression-prone GR<sup>+/-</sup> mice (Kronenberg, 2010). Major depression displays the most prominent disease strongly linked to social factors (George et al., 1989, Bruce and Hoff, 1994). Besides isolation and social networks also stress and HPA axis regulation has a major impact on the prevalence and outcome of major depression. GR<sup>+/-</sup> mice represent a particularly valuable animal model of depression in respect to the corticosteroid receptor hypothesis of major depression (Holsboer, 2000). These mice possess HPA system dysregulation, which displays a hallmark of a subgroup of depressive disorders in humans (Chourbaji et al., 2008). Thus, our observation is in line with the concept that neurogenesis plays a role in the etiopathogenesis of mood disorders and is required for the therapeutic actions of antidepressants (Malberg et al., 2000, Santarelli et al., 2003).

A recent study also identified a small subgroup of neurons regulating HPA axis and linking ahNG to depressive phenotype (Snyder, 2011, Nature). Additionally, the necessity of ahNG for normal stress response was demonstrated in this publication. As described for Project II are Corticosterone (CORT) and other glucocorticoids known to decrease both proliferation and survival rates in the adult hippocampus (Gould et al., 1992, Cameron and Gould, 1994, Wong and Herbert, 2004). Stress alters glucocorticoid levels within the brain causing a tremendous, but reversible, effect on neurogenesis especially when applied chronic. On the other hand showed a study, published a few month ago, that the antidepressant sertraline acts via GR-dependent mechanisms causing increased ahNG (Anacker et al., 2011). Anacker et al. also found a major effect on both DCX-positive cells (type-2b and 3+) and immature neurons. This is in line with the finding of Garcia et al., that 50% of all Calretinin-positive cells (immature granule cells) express Glucocorticoid receptors (Garcia et al., 2004). Although we did not analyze the effect of the GR heterozygote genome on distinct differentiation stages, keeping all the above

information in mind, it seems plausible, that stress reducing group housing, major depression, antidepressants and glucocorticoid action are strongly intertwined.

### **3.6.2. BDNF connects anxiety-like behavior, depression and ahNG, e.g. through trkB receptor stimulation**

Interestingly, the GR<sup>+/-</sup> genetic mouse model of depression also possess a significant reduction of BDNF protein concentrations in the hippocampus (Ridder et al., 2005). Several other studies have linked BDNF, adult neurogenesis, behavior and major depression (Bergami et al., 2008, Li et al., 2008, Rossi et al., 2006). A recent publication has shown that ‘learned safety’ as opposed to learned helplessness is associated with increased survival of new neurons and increased BDNF levels in the dentate gyrus and that conversely, ablation of neurogenesis retards safety learning (Pollak et al., 2008). Furthermore Li et al. showed in the same year, that ablation of the BDNF receptor trkB in neural precursor cells results in impaired neurogenesis. This reduction could neither be reversed by antidepressant treatment nor by wheel running (Li et al., 2008). Lack of trkB in adult progenitors also results in disturbed organization of basic synaptic connections of newly generated neurons and impaired neurogenesis-dependent long-term potentiation, accompanied by compromised survival of newly generated cells and increased anxiety-like behaviors (Bergami et al., 2008). Bergami et al. additionally found an effect mainly on NeuN-positive cells, since these are the differentiation stages which highly develop interconnections within the hippocampal circuit. Thus, they reason a major effect at the transition from immature to mature granule cells.

Since the reduction in neurogenesis in GR<sup>+/-</sup> was only moderate, it seems likely, that in addition to reduced neurogenesis, other mechanisms may also prominently contribute to the depression-related behavioral phenotype of GR<sup>+/-</sup>.

### ***3.7. Interconnection of exemplary social factors on the exact differentiation process in the adult, mammalian dentate gyrus***

Research on the adult dentate gyrus gives new perspectives on cell genesis and deepens our understanding of brain functions, brain plasticity and impressive mammalian flexibility on the cellular level. In addition, intact social behavior seems to be in correlation to hippocampal neurogenesis. My doctoral thesis presents some initial attempts to show a first interaction between adult neurogenesis and psychosocial behavior in the broadest sense.

In this thesis I have shown that the differentiation process is complex and dynamics are very flexible, strongly depending on the involved cell types. Differences in cell cycle length, flexible division pathways (asymmetric vs. symmetric) and non linear progression (e.g. re-differentiation) create a fascinating process. Within my model of the dynamics I only included reliable new information, confirmed by other studies, but an even more complex design will be established in near future.

Such a complex process must be highly regulated. In most studies, regulation usually means a change in the quantity of absolute BrdU-positive cells and only rarely changes in quality, assed by means of distinct differentiation stages, but the kind of regulation within the development of new cells seems to depend on the distinct stages of differentiation as well as on the time course (Llorens-Martin et al., 2010). I here investigated the dynamics of neuronal development within the dentate gyrus in order to be able to predict time phases of different factors influencing different developmental steps within adult neurogenesis. Furthermore I investigated some of these differentiation stages in the context of social influences.

Adult hippocampal neurogenesis is strongly adaptive to/regulated by environmental stimuli (Kempermann et al., 1997b, van Praag et al., 1999b), Van Praag, 1999). We know that voluntary wheel running and living in an ENR both act differently on adult neurogenesis. While enrichment influences proliferation and survival, RUN only increases the proliferating cell numbers at type-2 cell stages (Kronenberg et al., 2003; Olsen et al., 2006). Also for auditory stimuli I find this huge diversity in changes of ahNG. It seems as if only biological relevant cues can cause integration of new neurons,

## Discussion - Interconnection of exemplary social factors on the exact differentiation process in the adult, mammalian dentate gyrus

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while single other factors can trigger earlier mitotic differentiation stages (e.g. increase in type-2a by Music). Also I show that increased group size can slightly, thus insignificantly, increase the number of postmitotic cells after 24h. This could imply a possible shift in dynamics, such as a possible transition already from type-2b cell stages to immature neurons by social stimulation or the leave from  $G_0$  resting state.

A third effect showing regulative influence on ahNG, stress (activating HPA axis, attenuated via activation of GR in hippocampus), as one major social factor leading to psychiatric and neurologic disease, was chosen, and has been shown to diminish the number of dividing cells, especially in models of major depression. Thus, stress may have a negative effect on the dynamics of ahNG, but this needs to be investigated further.

From all this data I clearly reason, that social factors, such as communication, group composition, (social) stress and social-related diseases influence the differentiation process within the adult hippocampus in complex and individual ways. My thesis should therefore be understood as a first attempt into the new field of research investigating single social events and their impact on adult mammalian neurogenesis.

## 4. Methods

### 4.1. Materials

<b>0.1M Borate Buffer (pH 8.5)</b>	3.08 g Boric Acid 500 ml dd H <sub>2</sub> O 5N NaOH to adjust pH to 8.5
<b>Bromodeoxyuridine injection solution (BrdU)</b>	100mg BrdU 10ml 0.9% NaCl
<b>Cryoprotection solution (CPS)</b>	250 ml Glycerol (25%) 250 ml Ethylenglycol (25%) 500 ml 0.1 M PO <sub>4</sub>
<b>Diamino-benzidin (DAB)</b>	10 mg DAB tablet 40 ml TBS
<b>Lactated Ringer</b>	6 g NaCl 0.075 g KCL 0.1 g CaCl <sub>2</sub> 0.1 g NaHCO <sub>3</sub> 2.5 g Lactate 1 L dd H <sub>2</sub> O
<b>0.9% Natriumchloride (0.9% NaCl)</b>	9g NaCl 1l dd H <sub>2</sub> O
<b>Nickelchloride 8%(NiCl)</b>	8 g Nickelchloride (Hexahydrate) 100ml dd H <sub>2</sub> O
<b>Paraformaldehyde (PFA). 4%</b>	500 ml dd H <sub>2</sub> O at 65° 500 ml 0.2 M PO <sub>4</sub> buffer (on Ice) 40 g PFA NaOH to adjust the pH to 7.4



## Methods - Materials

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<b>0.2 M Phosphate Buffer (PBS)</b>	6.35 g NaH <sub>2</sub> PO <sub>4</sub> x 1 H <sub>2</sub> O 27.46 g Na <sub>2</sub> hPO <sub>4</sub> x 2 H <sub>2</sub> O 1 l dd H <sub>2</sub> O adjust ph to 7.4
<b>0.1 M Phosphate Buffer (PBS)</b>	3.175 g NaH <sub>2</sub> PO <sub>4</sub> x 1 H <sub>2</sub> O 13.73 g Na <sub>2</sub> hPO <sub>4</sub> x 2 H <sub>2</sub> O 1 l H <sub>2</sub> O adjust pH to 7.4
<b>0.1 M Potassium Phosphate buffered saline (KPBS)</b>	0.98 g K <sub>2</sub> h <sub>2</sub> PO <sub>4</sub> 5.71 g K <sub>2</sub> hPO <sub>4</sub> 8 g NaCl 1 L dd H <sub>2</sub> O adjust to pH 7.4
<b>Saccharose 30%</b>	150 g Saccharose 500 ml with 0.1M PO <sub>4</sub>
<b>30% Sucrose solution</b>	150 g Sucrose 500 ml 0.1M PO <sub>4</sub>
<b>10x Tris buffered saline (10xTBS)</b>	132.20 g Trizma HCL 19.40 g Trizma Base 90 g NaCl 1 l dd H <sub>2</sub> O
<b>1x Tris buffered saline (1xTBS)</b>	13.22 g Trizma HCL 1.94 g Trizma Base 9 g NaCl 1 l dd H <sub>2</sub> O
<b>TBS-plus</b>	3 ml donkey serum 1 ml Triton X-100 96 ml 1xTBS

## Methods - Materials

### Primary Antibodies: (TABLE 4-A)

Antigen	Species	Dilution	Company
BrdU	Rat, IgG	1:500	Harlan Seralab, Biozol
DCX	Goat, polyclonal	1:200	Santa Cruz Biotechnologies
GFAP	Mouse	1:400	Sigma
GFP	Goat	1:1000	DPC Biermann
GFP	Mouse	1:400	Mobitec
NeuN	Mouse	1:100	Chemicon
S100 $\beta$	Rabbit, polyclonal	1:2500	Swant
S100 $\beta$	Mouse, monoclonal	1:1000	Sigma
CR	Rabbit	1:250	Swant
SOX2	Rabbit, polyclonal	1:400	Chemicon

### Secondary Antibodies: (TABLE 4-B)

Species	Label / Conjugate	Dilution	Company
Donkey anti goat	Biotin-SP	1:250	Dianova
Donkey anti goat	Cy5	1:250	Dianova
Donkey anti mouse	DyLight 405	1:100	Dianova
Donkey anti mouse	Cy2	1:250	Dianova
Donkey anti mouse	Cy5	1:250	Dianova
Donkey anti mouse	FITC	1:250	Dianova
Donkey anti rabbit	Cy2	1:250	Dianova
Donkey anti rabbit	Cy5	1:250	Dianova
Donkey anti rat	Biotin-SP	1:250	Dianova
Donkey anti rat	Rhodamin RedX	1:250	Dianova

### **4.2. Power analysis**

For each experiment power analysis was performed to determine the number of animals required. The needed sample size to compare two means is calculated with the following equation:

$$N \approx (2 * SD^2 * (z_{\alpha} + z_{\beta})^2) / \Delta^2$$

Here N is the number of subjects per group. SD the standard deviation,  $\Delta$  the minimum difference between two population means and  $(z_{\alpha} + z_{\beta})^2$  the power index with  $z_{\alpha}$  as the critical value for the z distribution for the desired value of  $\alpha$  (threshold for significance) and equivalent meaning of  $z_{\beta}$  for the expected value of  $\beta$  (probability of making a type 2 error).

The number of animals per group is based on previous studies of neurogenesis. They indicate a sufficient number of 6 to 10 animals to obtain a significant result, dependent on the expected outcome. For all studies the level of significance is set to  $\alpha = 0.05$ . The desired power is  $\geq 0.80$ .

We postulate a standard deviation between 20% and 30%. We assume a normal distribution and a biological effect between 20% and 40% (much less than 20% are in general not interpreted as biological relevant). Thus, we planned to have between 7 and 10 animals per group.

### **4.3. Animals**

Different strains of mice were used in the different projects of my thesis to adapt them to the needs of the experimental outcome. In Project I transgenic mice expressing Green fluorescent protein (GFP) under the Nestin-promotor (Yamaguchi et al 2000) were used to characterize neural progenitor cells. Since only the results of my Master's thesis are based on the expression of NestinGFP in these animals while the results of my PhD thesis are independent of the genetic label of this class IV intermediate filament, I will not go further into detail. For additional information towards NestinGFP expression see the following references (Yamaguchi, Saito, 2000; Kempermann, 2003, Kronenberg et al. 2003; Filipov V, 2003; Steiner, 2006).

For the experiments within Project II and III mice of the strain C57BL/6 were used. These were either ordered from Charles River WIGA Deutschland GmbH (C57BL/6N, Project II) or from The Jackson Laboratory, USA (C57BL/6J, Project III). In comparison with other strains C57BL/6 animals have distinct advantages in adult neurogenesis research. This special inbred strain typically shows high proliferative activity within the dentate gyrus (Kempermann, 1997a). Thus, these mice are specifically useful for analyses of the effect of social factors on proliferation within the dentate gyrus of adult animals. Additionally, most genetic mouse models were generated from this mouse strain. Hence, it is rather beneficial to use this specific type of mice in all studies for better comparability.

For Project IV, GR<sup>+/-</sup> mice were used, which under express the glucocorticoid receptor (GR), but maintain the regulatory genetic context controlling the GR gene (Ridder, 2005). Briefly, the founders of GR<sup>+/-</sup> mice were developed by using homologous recombination in embryonic stem cells as described by Tronche et al. (Tronche, 1999). GR<sup>+/-</sup> mice were generated by crossing heterozygous C57BL/6N males (backcrossed for >10 generations) with wild-type FVB/N females in order to obtain F1 hybrid mice with exactly the same background. This strain has been used for behavioral and neurochemical characterization studies earlier as described (Ridder et al., 2005; Schulte-Herbrüggen et al., 2007), and makes an important as mouse model of depression (Ridder et al., 2005, Kronenberg et al., 2010).

### **4.4. Housing**

For all experiments mice were held under standard laboratory housing conditions with a 12-hour light / dark cycle and *ad libidum* access to food and water. Delivered mice were given a minimum of five days for habituation after arrival.

All experiments were performed according to national and institutional guidelines and were approved by an official committee (either LaGeSo, Berlin, Germany or the Institutional Animal Care and USE Committee (IACUC) of Emory University).

#### **4.4.1. Project I: Dynamics of adult neurogenesis**

To determine the dynamics of adult neurogenesis 36 male and 37 female NestinGFP transgenic mice on C57BL/6 background were between six and eight weeks of age at the beginning of the experiment (n = 7 per group). The mice were housed in groups of three

to five animals per cage. Cage mates killed two male mice in the course of the experiment. Eight animals, six males and two females, had to be excluded from the study because they were strongly affected by internal hydrocephaly.

Animals received three intraperitoneal injections of 50 mg/kg body weight of DNA synthesis marker 5-Bromo-2'-deoxyuridine (BrdU) on the first day of the experiment at 8 a.m., 2 p.m., 8 p.m. BrdU (Sigma, Deisenhofen, Germany) was dissolved in 0.9% NaCl solution and filtered. At 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 14 days or 21 days after the first BrdU injection groups of mice were killed and their brains processed for immunohistochemistry (FIGURE 4-1 A). All perfusions were done at 8 o'clock in the morning.

### **4.4.2. Project II: Effects of group size on ahNG**

Female C57BL/6N mice were housed in groups of 2, 5, 10 or 15 animals. The Cages measured 1x1 meter independent of the number of animals per group. Except for the size of the cage the subjects were housed under standard condition as described above. All animals were 8 weeks old at the beginning of the experiment.

Animals received three intraperitoneal injections of 50 mg/kg body weight of Thymidine analog BrdU on the seventh day of the experiment at 8 a.m., 2 p.m., 8 p.m. BrdU was dissolved in 0.9% NaCl solution and filtered. 24 hours after the first BrdU Injection all animals were killed with an overdose of Ketamin / Medetomidine mixture and perfused afterwards (FIGURE 4-1 B).

### **4.4.3. Project III: Auditory stimuli on ahNG**

To determine the influence of auditory stimuli on adult neurogenesis 130 female C57BL/6 mice at the age of six to eight weeks were housed in groups of five animals per cage (n = 10 per group). Three mice had to be euthanized in the course of the experiment due to animal care standards. Ten animals were used for measurement of the auditory brainstem response (ABR) in order to determine the average hearing ability of this strain. 120 animals were exposed to various auditory stimulus conditions. They received one intraperitoneal (i.p.) injection of 50 mg/kg body weight of BrdU either 24 hours after the last exposure to an auditory stimulus (proliferation paradigm) or 24 hours before the first (survival paradigm) (FIGURE 4-1 C). The BrdU was dissolved in 0.9% NaCl solution and filtered.

### **4.4.3.1. *Measurement of the auditory brain stem response***

The auditory brain stem response (ABR) was measured in ten C57BL/6J mice at an age of 8 weeks to confirm adequate hearing for this particular strain around the time of the experiments.

The animals were anesthetized with 100 mg/kg Ketamine and 0.3 mg/kg Medetomidine injected intraperitoneal. During the recording of the ABR the eyes were covered with eye ointment to preserve them from damage.

Once the animal was not showing reflexes anymore, silver wires were placed subdermal posterior to the stimulated ear, at the skull midline (ground) and at the contralateral bula.

The recordings were done with a calibrated sound delivery system (Tucker-Davis Technologies, Gainesville, Florida, USA) and BioSig program. Tones were presented to the anesthetized females at 7 kHz, 32 kHz or 65 kHz. All tones were presented at a sampling rate of 223214.0625 samples per second, with 3 ms duration, intensity in the range of 0 dB SPL to 75 dB SPL and an onset / offset ramp of 1.5 ms. Auditory evoked activity was recorded, amplified (x10000), and filtered (10 Hz – 3 kHz). The hearing threshold was defined as the lowest intensity at which reliable responses were recorded.

At the end of the recordings, animals were injected with 0.05 ml lactated Ringers subcutaneously to prevent dehydration. The mice were monitored until they awoke and normal grooming and drinking behavior was observed.

### **4.4.3.2. *Exposure to sound stimuli***

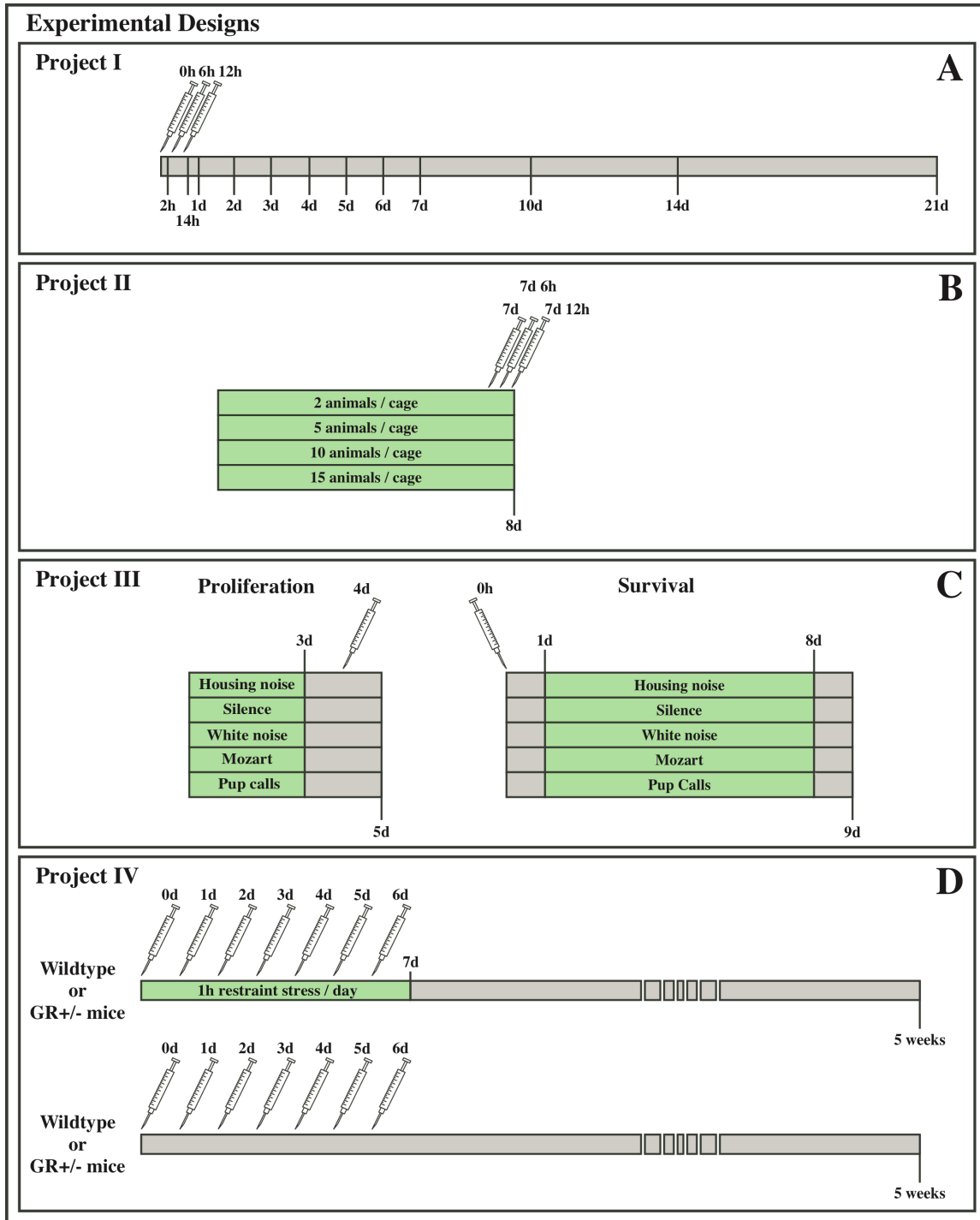
The animals of each group were placed into a sound box (Acoustic Systems, Austin, Texas) for two hours, beginning at 7pm, the start of the dark phase of the light-dark cycle. Dependent on the experimental group, they were either exposed to standard Animal House Noise, silence, White Noise (with a bandwidth of 4 kHz to 80 kHz), pup calls or Mozart's sonata for two pianos in D major (KV 448). The last was processed and transposed into the hearing range of C57BL/6J mice. First a low pass filter was set to 1236 Hz and applied ten times to the music. The wav file was then transposed for 5 octaves into the hearing range of the mice. Afterwards a high pass filter was set to 5953 Hz and applied 2 times. Spectral analysis showed a peak at 10 kHz. More than 90% of the power lay between 5 kHz and 20 kHz. The music was presented with a sample rate of 97656.25. Spectral analysis of the pup calls showed an average frequency of 65 kHz. The

White Noise was applied at an intensity of 70dB. All other stimuli varied in intensity ( $70 \pm 10$ dB SPL). The pup calls were provided by Robert Liu collected and modified as described (Liu et al., 2003).

#### **4.4.4. Project IV: ahNG in the GR<sup>+/-</sup> depression model**

Male GR<sup>+/-</sup> mice and wildtype littermate controls were 4-6 months old at the beginning of experiments (n=10). The experiment was structured in a 2 x 2 design (FIGURE 4-1 D). All animals were injected with BrdU (Sigma-Aldrich, Germany) i.p. at a concentration of 50 mg/kg body weight once daily for seven consecutive days. Directly after BrdU administration 2 experimental groups (one comprise GR<sup>+/-</sup> mice, one comprise wildtype mice) were exposed to restraint stress. Restraint stress consisted of placing a mouse inside a plastic tube (inner diameter: 26 mm) for 1 hour during the dark phase. All animals were killed after an interval of 4 weeks.

## Methods - Housing



**FIGURE 4-1** Experimental Designs. Bars indicate the treatment of the different group, Grey sections indicate standard housing, Green sections the special treatment. Each bar stands for at least one experimental group. Time points displayed above the bars are for better orientation. Time points written below the bars represent the time of perfusion. Syringes imply the injection of 50 mg BrdU/kg body weight. In B and C injection and perfusion time refer to all experimental groups.



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## 4.5. *Immunohistochemistry and Immunofluorescence*

### 4.5.1. Immunohistochemistry

All animals were deeply anesthetized with a mixture of 10% ketamine (0.3 ml / 20 g body weight) and 2% Xylaxin (0.1 ml/20 g bodyweight), except for Project III where 5 mg Nembutal was used. All subjects were perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde (PFA) in 0.1 M KPBS buffer, pH 7.4. Brains were dissected from the skull, postfixed in 4% PFA at 4°C for 24 hours and then transferred into 30% sucrose in 0.1 M phosphate buffer, pH 7.4, for dehydration until they had sunk. For the auditory project all perfusions were done at 7 pm, for all other projects animals were sacrificed at 8 am.

Brains were then cut on a dry ice-cooled copper block with a sliding microtome (Leica, Bensheim) into 40 µm thick coronal sections. Afterwards slides were stored at 4°C in a cryoprotectant solution containing 25% ethylene glycol, 25% glycerol, 25% glycerin and 0.05 M phosphate buffer.

All sections were stained free floating with all antibodies diluted in Tris-buffered saline (TBS), pH 7.4, containing 3% donkey serum and 0.1% Triton X-100. For BrdU-immunohistochemistry one-in-six sections from each brain were transferred into TBS and washed briefly. Sections were pretreated with 0.6% H<sub>2</sub>O<sub>2</sub> to block endogenous tissue peroxidase. Afterwards brain sections were rinsed in TBS, DNA was denatured in 2 N HCl for 30 minutes at 37°C. Sections were then rinsed in 0.1 M borate buffer, pH 8.5, and thoroughly washed in TBS. Brain slices were incubated with the primary antibody overnight at 4°C. Primary and secondary antibodies were diluted in TBS supplemented with 0.1% Triton X-100 and 3% donkey serum (TBS-plus). As primary antibody we used rat anti-BrdU in a concentration of 1:500 (Harlan Seralab, Loughborough, UK). The next day sections were rinsed in TBS and TBS-plus and incubated with the secondary antibody for 2 hours at room temperature. As secondary antibody we used donkey anti-rat-biotin-SP (Dianova, Hamburg, Germany) in a concentration of 1:250. ABC reagent (Vectastain Elite, Vector Laboratories, Burlingame, CA) was applied for 1 hour at a concentration of 9 µl / ml. Diaminobenzidine (DAB) (Sigma, Munich, Germany) was used as a chromogen at a concentration of 0.25 mg / ml in TBS with 0.01% H<sub>2</sub>O<sub>2</sub> and 0.04% nickel chloride. The stained sections were thoroughly washed, incubated in Neoclear for 10 min and mounted with Neomount.

For Doublecortin (DCX) immunohistochemistry another one-in-six sections and for CR immunohistochemistry one-in-twelve sections of each brain were labeled with the same protocol as BrdU. Because DCX and CR immunohistochemistry does not require denaturing of the DNA the HCl incubation step was omitted. As primary antibodies either polyclonal goat anti-doublecortin in a concentration of 1:200 (Santa Cruz Biotechnology, Santa Cruz, USA) for the DCX immunohistochemistry, and rabbit anti-Calretinin in a concentration of 1:250 (Swant, Switzerland) were used. As secondary antibodies donkey anti-goat (or rabbit, respectively) biotin-SP (Dianova) was applied in a concentration of 1:250.

### **4.5.2. Immunofluorescence**

For immunofluorescence, one-in-twelve series of each brain were triple-labeled. The sections were transferred into TBS and washed briefly. The DNA was denatured in 2 N HCl for 30 minutes at 37°C. Sections were then rinsed in 0.1 M borate buffer, pH 8.5, and thoroughly washed in TBS and TBS-plus. The brain slices were incubated with the primary antibodies overnight at 4°C. Primary and secondary antibodies were diluted in TBS supplemented with 0.1% Triton X-100 and 3% donkey serum (TBS-plus). The next day sections were rinsed in TBS and TBS-plus and incubated with secondary antibodies for 4 hours at room temperature in the dark. Sections were then washed with TBS and coverslipped in polyvinyl alcohol with diazabicyclooctane (DABCO) as anti-fading agent. The primary antibodies were applied in the following concentrations: anti-BrdU (rat, 1:500; Harlan Seralab), anti-GFP (goat, 1:1000; DPC Biermann), anti-GFP (mouse, 1:400; Mobitec, Göttingen, Germany), anti-doublecortin (goat, 1:200; Santa Cruz Biotechnologies), anti-GFAP (mouse, 1:400, Sigma), anti-SOX2 (rabbit, 1:400, Chemicon, Temecula, USA), anti-NeuN (mouse, 1:100; Chemicon, Temecula, USA), anti-S100beta (mouse, 1:1000, Sigma), anti-S100beta (rabbit, 1:2500, Swant). For secondary antibodies anti-rat rhodamine-X, anti-rabbit fluorescein isothiocyanate (FITC), anti-rabbit Cy2, anti-rabbit Cy5, anti-mouse FITC, anti-mouse Cy2, anti-mouse Cy5, anti-goat Cy5 (all 1:250; Jackson Immunoresearch, West Grove, USA; distributor: Dianova, Hamburg, Germany) and anti-mouse DyLight 405 (1:100, Dianova) were used (TABLE 4-A and 4-B).

#### ***4.6. Quantification and Imaging***

Quantification of cells labeled with DAB were determined using light microscopy on sections 240  $\mu\text{m}$  apart for BrdU and DCX staining or 480  $\mu\text{m}$  apart for CR immunohistochemistry, covering the entire hippocampus in its rostrocaudal extension as described previously (Kronenberg et al., 2003 & 2007). Briefly, cells located in the granule cell layer and adjacent subgranular zone, defined as a two-cell bodies-wide zone of the hilus along the base of the granule cell layer were counted (Brown et al., 2003). Cells in the uppermost focal plane were excluded to avoid oversampling. For light microscopical analyzes Leica CTR 6000 Microscope was used.

Phenotypic analyses of BrdU-positive cells in the stated differentiation stages was performed using multiple-stained series on sections 480  $\mu\text{m}$  apart, also covering the entire hippocampus, using a spectral confocal microscope (TCS SP2 and TCS SP5; Leica, Nussloch, Germany). Appropriate gain and black level settings were determined on control slices stained with secondary antibodies alone. All images were taken in sequential scanning mode and further processed in either Volocity, for 3D projection or Adobe Photoshop 7.0. Only general contrast adaptations were made and images were not otherwise manipulated.

#### ***4.7. Statistical Analysis***

Statistical analysis were performed either with Statview 5.0.1 for Macintosh (Project IV) or with Origin8 for Windows. Factorial analysis of variance (ANOVA) was performed for all comparisons of morphological data. Two-way ANOVA was followed by Fisher's post-hoc test, where appropriate. Differences were considered statistically significant at  $P < 0.05$ . All graphs are displayed as mean  $\pm$  standard error of the mean (SEM).

### 5. Summary

The term “adult neurogenesis” describes the continuous generation of cohorts of new neurons in some discrete regions of the adult brain, such as the dentate gyrus of the hippocampus. Adult hippocampal neurogenesis (ahNG) has been shown as relevant for typical functions of this brain areal (e.g. memory consolidation), but also influences social behavior and has medical relevance.

The exact dynamics of this neuronal development are still unknown, as are psychosocial impacts on this process. To analyze the temporal pattern of ahNG and quantify the development of the new neurons we analyzed the relative and absolute numbers of cells in all known stages of the neural differentiation, from stem cell phase towards neurons, in mice at different time points after BrdU injection. BrdU permanently labels cells during S-phase of the cell cycle and enabled us to follow a cohort of new cells over time, thus, here a pseudo-longitudinal study was performed. Subsequently, first studies on the relation between social factors and ahNG in mammals are presented. Regulation of the neuronal development is related to, and dependent on inputs from the outer environment. A connection between ahNG and social environment has been demonstrated previously, but detailed information on the altered differentiation process is missing.

In the first part of this thesis I present detailed information on the progression of distinct cell types through the differentiation process of ahNG. Transitions over time are observed allowing the estimation of cell cycle length (CCL) for each cell type involved in ahNG. I found, that the individual cell types are more flexible than assumed before.

In the second part, I investigate the impact of social housing, (behavioral relevant) auditori stimuli and stress on ahNG. While the positive factors were applied to healthy subjects, I used the stress paradigm in the GR<sup>+/-</sup> mice model of depression. Flexible adaptations of different stages in ahNG were also found in these studies on social factors. Each single stimulator affected individual differentiation stages in characteristic ways. While group size, music, silence and pup calls increase BrdU cell counts in proliferation analyses, with distinct effects on each mitotic differentiation stage, does only silence increase survival of new neurons. In the GR<sup>+/-</sup> mouse model I found decreased ahNG, without any effect on the proportion of neurogenesis or gliogenesis.

## Summary

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Based on the information obtained about the dynamics of ahNG a new, innovative model of ahNG is proposed, pushing type-1 and type-2a cells in the center of attention, while diminishing the weight of type-3 cells for the process of differentiation. Type-1 cells, previously assumed as origin of neurogenesis in the hippocampus, are here hypothesized as safeguard of the system, while type-2a cells, highly proliferative, giving rise to gliogenesis and neurogenesis, are hypothesized as the origin of ahNG under normal, healthy conditions. The additional studies give some indications for changes of the model caused by different social factors. The aim is to draw attention to the importance and impact of social environment on adult neurogenesis, brain plasticity and mental health. Thus, future studies will focus more on the promising field of ahNG.

### **6. Zusammenfassung**

Der Begriff “Adulte Neurogenese” beschreibt die kontinuierliche Generierung neuer Nervenzellen in bestimmten Arealen des erwachsenen Gehirns, wie zum Beispiel dem Hippocampus. Adulte hippocampale Neurogenese (ahNG) ist im Säugetier stark in regional spezifische Funktionen, wie z.B. dem semantischen Gedächtnis, involviert, kann aber auch soziales Verhalten beeinflussen und besitzt zusätzlich medizinische Relevanz.

Der exakte Ablauf der neuronalen Entwicklung im Gyrus Dentatus ist bislang ungewiss und soziale Einflüsse auf diesen Prozess wurden noch wenig untersucht. Um den zeitlichen Verlauf der adulten, hippocampalen Neurogenese zu analysieren und die Generierung neuer Körnerzellen zu quantifizieren, wurden als erstes sowohl relative als auch absolute Werte einzelner Entwicklungsstadien zu verschiedenen Zeitpunkten nach BrdU Administration erhoben. BrdU markiert dabei Zellen in der S-Phase des Zellzyklus und ermöglicht es, einer Kohorte neuer Zellen über einen festgelegten Zeitraum zu folgen. Somit handelt es sich bei diesem Teil der Dissertation um eine Pseudo-longitudinale Studie. Im Anschluss werden erstmals Studien präsentiert, welche den Zusammenhang zwischen ahNG und sozialen Faktoren, wie Gruppenzusammensetzung und (z.T. verhaltensrelevanten) auditiven Stimulationen, in gesunden Mäusen untersuchen und dabei vor allem Bezug auf die verschiedenen Differenzierungsstadien nehmen. Neben Untersuchungen im gesunden Organismus wurde zusätzlich der Effekt von Stress in Mäusen mit reduzierter Expression des Glucocorticoid-Rezeptors erforscht. Dieser Mausstamm stellt ein anerkanntes Depressionsmodell dar, mit für Depression typischen, sozialen Verhaltensweisen.

Die Ergebnisse zur Dynamik der ahNG zeigen starke Unterschiede zwischen den Zelltypen der einzelnen Differenzierungsstadien, insbesondere bezüglich der Proliferationsrate und der Zellzykluslänge (CCL). Interessanterweise sind die Unterschiede zwischen den einzelnen Zellstadien noch größer als bisher angenommen, e.g. in Bezug auf den Zellzyklus).

Flexible Anpassungen an den Zeitverlauf der ahNG werden auch im Falle sozialer Stimulation aufgezeigt. Während steigende Gruppengröße, Musik, Stille und Rufe isolierter Säuglinge, die Anzahl BrdU-positiver Zellen in Proliferationsanalysen erhöhte, zeigte nur Stille eine Steigerung der Neurogenese in Bezug auf Überlebensraten

## Zusammenfassung

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neugebildeter Nervenzellen. In unserem GR<sup>+/-</sup> Depressionsmodell fanden wir eine Reduktion der ahNG, ohne Beeinflussung der Neurogenese- oder Gliogenese-Rate.

Basierend auf den Ergebnissen zur Dynamic der ahNG wird ein neues Modell der ahNG vorgestellt, dass erstmalig nicht nur Type-1 Zellen als den bisher vermuteten Ursprung der Neurogenese, sondern auch Type-2a Zellen in das Zentrum stellt. Hingegen wird die Bedeutung der Type-3 Zellen zugunsten von Type-2b und unreifen Körnerzellen gemindert. Type-1 Zellen werden hier als Sicherung des Systems propagiert, während stark proliferierende Type-2a Zellen sowohl Gliogenese als auch Neurogenese begründen. Eine hohe Flexibilität zeichnet das System der Neubildung neuer Nervenzellen aus, wie wir auch mit den Ergebnissen der anderen Studien zeigen konnten. Die weiteren Studien lassen vermuten, dass die Dynamik der ahNG, wie im Model dargestellt, durch soziale Faktoren verändert werden kann.

Diese Untersuchungen sollen Interesse an einem zukünftigen Forschungsfeld wecken, dessen Zweck es sein wird den Zusammenhang zwischen sozialen Umwelteinflüssen, hippocampaler Neurogenese, Gehirnplastizität und mentaler Gesundheit zu lenken.

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## 8. Appendix

### 8.1. Supplementary material

**TABLE 5-B:** Statistical analysis of the number of BrdU-positive cells over time for the different staining methods. Significant differences (Fisher's Post-hoc analysis) have been highlighted.

		Probability (p)		
Group I	Group II	BrdU DAB	BrdU Conf 1	BrdU Conf 2
14h	2 h	<0.0001	0.0099	0.0002
24h	2 h	<0.0001	0.0001	<0.0001
24h	14h	0.0194	0.1279	0.0222
2d	2 h	<0.0001	<0.0001	<0.0001
2d	14h	<0.0001	0.0013	0.0146
2d	24h	0.0019	0.0729	0.8684
3d	2 h	<0.0001	0.0051	0.0000
3d	14h	0.4042	0.8900	0.0053
3d	24h	0.1038	0.1486	0.6489
3d	2d	<0.0001	0.0014	0.7767
4d	2 h	<0.0001	0.1556	0.0000
4d	14h	0.0884	0.2250	0.0156
4d	24h	0.0001	0.0074	0.8880
4d	2d	<0.0001	<0.0001	0.9802
4d	3d	0.0106	0.1633	0.7570
5d	2 h	0.0005	0.9703	0.0011
5d	14h	<0.0001	0.0083	0.4955
5d	24h	<0.0001	0.0001	0.0028
5d	2d	<0.0001	<0.0001	0.0016
5d	3d	<0.0001	0.0041	0.0004
5d	4d	0.0067	0.1509	0.0018
6d	2 h	0.0010	0.4990	0.0980
6d	14h	<0.0001	0.0014	0.0264
6d	24h	<0.0001	<0.0001	<0.0001
6d	2d	<0.0001	<0.0001	<0.0001
6d	3d	<0.0001	0.0006	<0.0001
6d	4d	0.0078	0.0382	<0.0001
6d	5d	0.9592	0.4602	0.0989
7d	2 h	0.0001	0.5625	0.0355
7d	14h	0.0006	0.0019	0.0759
7d	24h	<0.0001	<0.0001	0.0001

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7d	2d	<0.0001	<0.0001	0.0001
7d	3d	<0.0001	0.0008	0.0000
7d	4d	0.0637	0.0477	0.0001
7d	5d	0.4010	0.5234	0.2396
7d	6d	0.3907	0.9225	0.6400
10d	2 h	0.2284	0.1568	0.8619
10d	14h	<0.0001	0.0001	0.0004
10d	24h	<0.0001	<0.0001	<0.0001
10d	2d	<0.0001	<0.0001	<0.0001
10d	3d	<0.0001	<0.0001	<0.0001
10d	4d	<0.0001	0.0056	<0.0001
10d	5d	<0.0001	0.1324	0.0019
10d	6d	<0.0001	0.4541	0.1373
10d	7d	<0.0001	0.3980	0.0527
14d	2 h	0.1311	0.0821	0.4134
14d	14h	<0.0001	<0.0001	<0.0001
14d	24h	<0.0001	<0.0001	<0.0001
14d	2d	<0.0001	<0.0001	<0.0001
14d	3d	<0.0001	<0.0001	<0.0001
14d	4d	<0.0001	0.0021	<0.0001
14d	5d	<0.0001	0.0660	0.0001
14d	6d	<0.0001	0.2811	0.0149
14d	7d	<0.0001	0.2406	0.0042
14d	10d	0.7547	0.7396	0.3221
21d	2 h	0.0130	0.0156	0.4746
21d	14h	<0.0001	<0.0001	<0.0001
21d	24h	<0.0001	<0.0001	<0.0001
21d	2d	<0.0001	<0.0001	<0.0001
21d	3d	<0.0001	<0.0001	<0.0001
21d	4d	<0.0001	0.0002	<0.0001
21d	5d	<0.0001	0.0111	0.0001
21d	6d	<0.0001	0.0755	0.0194
21d	7d	<0.0001	0.0613	0.0056
21d	10d	0.1849	0.2957	0.3747
21d	14d	0.3083	0.4738	0.9175

## Appendix

### 8.2. *Abbreviations*

ABR	Auditory brainstem response	GR	Glucocorticoid receptor
AHN	Animal House Noise	KA	Kainic acid
ahNG	Adult hippocampal neurogenesis	LTP	Long term potentiation
ANOVA	Analyses of variance	n.a.	Not analyzed
ANP	amplifying neuronal progenitors (type-2a cells)	NeuN	Neuronal Nuclei clone A60
BDNF	Brain derived neurotrophic factor	NSC	Neural stem cell
BrdU	5-Bromo-2'-deoxyuridine	PBS	Phosphate Buffer
CA3	Cornu Ammonis region 3	p.i.	Post injection
CCL	Cell cycle length	RMS	rostral migration stream
CNS	Central nervous system	RUN	Running paradigm
CORT	Corticosterone	SD	Standard deviation
CR	Calretinin	SEM	Standard error of the mean
DAB	Diamino-benzidin	SGZ	Subgranular zone
DALY	disability adjusted life years	SOX2	sex determining region-Y-box 2 protein
dB	Dezibel	SPL	Sound Pressure Level
DCX	Doublecortin	SSM	saturate and survival method
DG	Dentate Gyrus	SVZ	Subventricular zone
ENR	Enriched environment	TBS	Tris-buffered saline
GABA	gamma-Aminobutyric acid	TrkB	neurotrophic tyrosine kinase, receptor, type 2
GFAP	Glial fibrillary acidic protein	WHO	World Health Organisation
GFP	Green fluorescent protein	WN	White Noise



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### 8.3. Publications

Kronenberg G\*, Kirste I\*, Inta D, Chourbaji S, Heuser I, Endres M, Gass P; Reduced hippocampal neurogenesis in the GR(+/-) genetic mouse model of depression. *Eur Arch Psychiatry Clin Neurosci.* 2009 Dec;259(8):499-504.

Kronenberg G, Gertz K, Baldinger T, Kirste I, Eckart S, Yildirim F, Ji S, Heuser I, Schröck H, Hörtnagl H, Sohr R, Djoufack PC, Jüttner R, Glass R, Przesdzing I, Kumar J, Freyer D, Hellweg R, Kettenmann H, Fink KB, Endres M; Impact of actin filament stabilization on adult hippocampal and olfactory bulb neurogenesis. *J Neurosci.* 2010 Mar 3;30(9):3419-31

Hellmann J, Jüttner R, Roth C, Bajbouj M, Kirste I, Heuser I, Gertz K, Endres M, Kronenberg G; Repetitive magnetic stimulation of human-derived neuron-like cells activates cAMP-CREB pathway. *Eur Arch Psychiatry Clin Neurosci.* 2011 May 12.

Vollmar, I; Adult Neurogenesis: Why Psychologists and Sociologists should care! *LIFE Newsletter* 2007 Sep 1(2):14-15.

#### Presentations

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|-------------------|---|
| 2-4 Nov. 2011     | World Conference on Regenerative Medicine, Leipzig, Germany   |
| 18–23 July 2009   | Annual Computational Neuroscience Meeting: CNS*2009, Berlin, Germany.   |
| 04 – 08 June 2009 | 11 <sup>th</sup> Annual Meeting of the International Behavioral and Neural Genetics Society, Dresden, Germany |
| 17 April 2009 ,,  | MinD-Hochschul-Netzwerk, MinD Symposium 2009, Munich, Germany   |
| 16 – 20 Oct. 2008 | Autumn Academy of the Max-Planck Research School LIFE, Berlin, Germany  |
| 12 – 17 Oct. 2007 | Autumn Academy of the Max-Planck Research School LIFE, Ann Arbor, MI, USA                                     |
| 03 – 07 Nov. 2007 | Conference of the American Society for Neuroscience, San Diego, CA, USA                                       |
| 8 – 10 Oct. 2006  | Berlin Neuroscience Forum in Liebenwalde, Germany (BNF 2006)  |

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## Appendix

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