Aus dem
Max-Delbrück-Centrum für Molekulare Medizin, Berlin-Buch

DISSERTATION

Isolation and characterization of neural precursor cells in the adult murine dentate gyrus

Zur Erlangung des akademischen Grades

Medical Doctor – Doctor of Philosophy in Medical Neurosciences
(MD/PhD in Medical Neurosciences)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

Harish Babu

aus Kannur, India

Gutachter: 1. Prof. Dr. med. G. Kempermann

2. Prof. Dr. Z. Kokaia

3. Prof. Dr. E. Fuchs

Datum der Promotion: 14. Dezember 2007

Acknowledgments

I would like to…

–sincerely thank my supervisor Dr. Gerd Kempermann for giving the opportunity to work in his lab and work on this topic. I am greatful for his neverending and openended support, advice and patience.

–thank Prof Helmut Kettenmann for the excellent collaboration and valuable support.

–thank Dr. Josef Bischofberger at the University of Freiburg in whose lab some part of the work was carried out and who gave his expert advice on the relevant topics.

–thank the members of the lab and collaborators Anika Bick-Sander, Giselle Cheung, Dan Ehninger, Volker Horvat, Sebastian Jessberger, Friederike Klempin, Rupert Overall, Gerardo Ramirez, Frank Rolfs, Benedikt Römer, Sascha Seibert, Barbara Steiner, Susanne Wolf who at various stages have been helpful scientifically and otherwise and who made the life in the lab easy and fun.

–thank Siegne Knespel, Erike Kotischka, Irene Thun, Ruth Zarmstorff and Ulrike Ziegler for their friendliness and excellent technical help over the years in the lab.

–thank Katja Reuter for her love, support and encouragement.

–thank my parents for their love, inspiration and encouragement. Without their blessings this work would not have been possible.

This list would be endless if I were to mention all the names that have been helpful in one way or other in seeing through this work. Thus, I apologize to everybody reading these lines and not to be mentioned personally. Thanks to all of you.

Special thanks goes to the International Graduate Program Medical Neuroscience of the Charite-Universitätsmedizin Berlin, Germany.

Table of Contents

Index of Figures

For

my parents

Abbreviations

AP	Action potential
APV	2-Amino-5-Phosphopentanoic acid
BDNF	Brain derived neurotrophic factor
BLBP	Brain lipid binding protein
BMP	Bone morphogenic protein
BrdU	Bromodeoxyuridine
CA	Cornu ammonis
CREB	cAMP response element binding protein
CSF	Cerebrospinal fluid
DAB	Diaminobenzidine
DCX	Doublecortin
DG	Dentate Gyrus
DMSO	Dimethysulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
DTT	Dithiothreitol
ECGF	Endothelial cell derived growth factor
EGF	Epidermal growth factor
ELISA	Enzyme linked Immunosorbent assay
EPSC	Excitatory postsynaptic current
FACS	Fluorescent activated cell sorting
FGF	Fibroblast growth factor
GABA	Gamma amino butyric acid
GAD	Glutamic acid decarboxylase
GAP	Growth-associated protein
GBZ	Gabazine (SR95521)
GFAP	Glial fibrillary acidic protein

GFP	Green fluorescent protein
GLAST	Glutamate/aspartate transporter
HRP	Horse radish peroxidase
KA	Kainic acid
LIF	Leukemia inhibitory factor
LTD	Long term depression
LTP	Long term potentiation
Map	Microtubule associated protein
MAPK	mitogen activated protein kinase
NMDA	N-methyl-d-aspartic acid
NT3	Neurotrophin-3
OPD	Ortho-Phenylenediamine
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PSA-NCAM	Polysialic acid-Neural cell adhesion molecule
RMS	Rostral migratory stream
RNA	Ribonucleic acid
RT	Reverse transcriptase
SGZ	Subgranular zone
Shh	Sonic hedgehog
SVZ	Subventricular zone
TBS	Tris buffered saline
TTX	Tetrodotoxin
VEGF	Vascular endothelial growth factor

1 Summary

In much of the brain, no neurons are born in the adulthood but two regions are exceptional to this rule: the dentate gyrus of the hippocampus (where neurons arrive from the subgranular zone) and the olfactory bulb (where neurons arrive from the subventricular zone). Even though several theories regarding the possible functional role of adult neurogenesis have been proposed, the properties of the precursor cells and their regulation in the dentate gyrus remain largely unknown. To get a better insight into this topic the following work has focused on the precursor cells of the adult dentate gyrus by growing them in vitro and studying their properties.

In this thesis work I have focused on the following questions:

1. Can precursor cells from the adult murine dentate gyrus be cultured ex vivo and maintained in a tissue culture environment for prolonged periods of time?
2. Does the adult dentate gyrus harbor stem cells in the true sense of their definition?
3. What are the characteristics of adult hippocampal precursor cells and how do they react to their environment?
4. Do the precursor cells cultured ex vivo generate neurons that are similar in property to their in vivo counterparts?
5. Does neural activity regulate neural precursor cells property in the dentate gyrus?
6. If so, what is the mechanism that realizes this cross talk?

Project 1: In vitro isolation and characterization of precursor cells isolated from adult murine dentate gyrus

Stem cell cultures are key tools of basic and applied research in Regenerative Medicine. Despite widespread interest in adult hippocampal neurogenesis no protocol for long-term stem cell cultures from the hippocampus of adult mice existed. In this thesis work a new strategy is described to obtain serum-free monolayer cultures of neural precursor cells from microdissected dentate gyrus of adult mice. The precursor cells fulfilled the criteria

of stem cells: self-renewal in clonal analyses and multipotency. The precursor cell fate was sensitive to culture conditions with their phenotype highly influenced by factors within the media and externally applied growth factors. Neurons generated from these adherent dentate gyrus precursor cell cultures expressed the characteristic markers like transcription factor Prox1 and calcium binding protein calbindin expressed by mature granule cells in vivo. Similar to granule cells in vivo, treatment with kainic acid or brain derived neurotrophic factor (BDNF) elicited the expression of GABAergic markers, further supporting the correspondence between the in vitro and in vivo phenotype. These data, published in the journal **PLoS ONE** (Babu et al; PLoS ONE 2007 Apr 25; 2: e388), provide a new tool to generate adult murine dentate gyrus stem cell cultures and to analyze functional properties of precursor cells and their differentiated granule cell-like progeny in vitro. Similar to precursor cells isolated from the dentate gyrus, nestin-GFP-expressing cells from corpus callosum/alveus and the SVZ were self-renewing and multipotent in vitro, whereas cells isolated from CA1 were not. These results are presently accepted and ready for publication in the journal **Brain structure and function** (Kronenberg et al; Brain structure and function)

Project 2: Adult hippocampal precursor cells sense synaptic network plasticity to induce neuronal differentiation

Precursor cells in the adult hippocampus reside in a region rich in neuronal network activity. In this thesis work it was hypothesized that neural precursor cells directly respond to changes in local network activity and to synaptic plasticity by initiating neuronal differentiation. Depolarization of hippocampal precursor cell cultures, increased the neuronal differentiation from precursor cells but raised the question whether this effect would reflect a physiological situation. In co-cultures with hippocampal neurons a brief pulse of LTP-inducing stimuli that generated synchronous neuronal network oscillations subsequently increased synaptic strength and led to significantly increased neuronal differentiation from neural precursor cells. This effect was seen only in the presence of synaptically connected mature neurons. When applied directly to precursor

cells, LTP did not induce neuronal differentiation. LTP-induced effects of neurons on precursor cells were mediated by increased release of neurotrophins. Neuronal differentiation was abolished when the neurotrophin signaling was blocked by neutralizing the secreted neurotrophin. These results show that synaptic activity levels influence neural precursor cell differentiation that may have important consequences on the neural network and information processing.

2 Introduction

2.1 Adult neurogenesis

The mammalian brain consists of a vast array of neurons that are born during fetal and early postnatal development. These neurons once born, undergo specific stages of maturation, and are selected, go on to integrate into the neuronal circuitry and form the functional nervous system. In almost all regions of the adult brain the existing neurons are not replaced and thus are left to preserve their numbers. For years, the only forms of structural changes in the brain were thought to be in the form of axonal sprouting, synaptic reorganization and glial proliferation.

Almost four decades ago, pioneering work by Joseph Altman and Gopal Das suggested traces of continued neurogenesis in the adult nervous system (Altman and Das, 1965b, a). This work remained unnoticed for years. But in the late 1970s and early 1980s, studies showed that newborn neurons in the hippocampus survived for long time (several weeks) and appeared to receive functional inputs (Kaplan and Hinds, 1977; Kaplan and Bell, 1983, 1984). Research from then on has collected a wealth of information regarding this process and the regulation of neurogenesis in the adult brain. The addition of new neurons throughout the lifetime of an animal provides a unique model system to study the continued development of the adult brain and also simultaneously delivers a unique tool for replacement and regenerative therapy.

Throughout this thesis work the following nomenclature is used: “stem cells” are cells with demonstrated self-renewal (the ability to generate additional stem cells) and multipotency (the ability to generate neurons, oligodendrocytes and astrocytes); progenitor cells are the progeny of stem cells with limited self-renewal and lineage restriction. “Precursor cells” serves an umbrella term encompassing stem and progenitor cells as well as cells with undetermined but assumed degrees of stemness.

2.1.1 Active neurogenesis is present only in specific regions of the adult brain

Adult neurogenesis, the process of neuronal birth, development and addition in the adult brain, has been shown to occur only in two regions within the adult nervous system. These are 1. The Subventricular zone (SVZ) where cells are born and they migrate to and finally reside in the olfactory bulb and 2. The subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus, where cells are born and new neurons added to the existing adjoining granule cell layer (Fig 1). The presence of the precursor cells has been documented in several regions of the mammalian brain but neurogenesis is restricted to the above-mentioned regions. Throughout the neuraxis precursor cells have been detected that are competent in generating neurons albeit *in vitro*, outside their physiological locale.

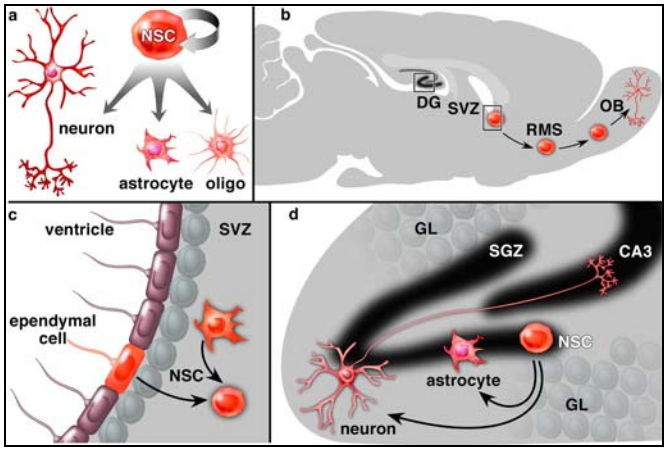

Figure 1: Neural stem cells within the adult murine brain

Neural stem cells reside in two regions of the brain subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus. The neural stem cells are self renewing and generate neuronal astrocytes and oligodendrocytes in these two regions of the brain. Figure adapted from (Taupin and Gage, 2002).

In the SVZ, the cells reside in the lateral wall of the lateral ventricle. The cells divide within this zone generating neuroblasts that migrate several millimeters along the narrow stream called rostral migratory stream (RMS) formed by a glial sheath (Lois and Alvarez-Buylla, 1994; Lois et al., 1996). During their migration the cells continue to

undergo mitosis to increase their numbers. These immature neurons have classical features of migratory neurons such as slender bipolar processes and are positive for several molecular markers previously associated with similar migratory neurons in the developing fetal brain. This process designated as "chain migration" is distinct from the radial and tangential migration, that are key during the early development of the nervous system (Alvarez-Buylla and Garcia-Verdugo, 2002). Once the immature neurons reach the olfactory bulb they become the glomerular and periglomerular interneurons performing functions that are not clear yet. These new neurons secrete GABA (Gamma Amino Butyric Acid) as their primary neurotransmitter. Some of these cells also exhibit dopaminergic phenotype (Hack et al., 2005).

2.1.2 Anatomy of the adult mouse hippocampus

The hippocampus derives its name from the unusual shape that resembles a sea horse (in Greek, "hippo" means horse and "kampos" means sea monster). The hippocampus remains one of the most exhaustively studied areas of the mammalian central nervous system due to its distinctive and readily identifiable morphological structure at both gross and microscopic levels. Hippocampus is a structural and functional component of the limbic system (or called the hippocampal formation). The hippocampal formation includes the following structures, entorhinal cortex, pre and parasubiculum, subiculum, hippocampus along with the DG. To some early imaginative neuroscientist the shape of the hippocampus formation as two interlocked C shapes structures, appeared to resemble the ram's head of the Egyptian god Amon. Thus another name for the hippocampus proper is cornu ammonis, or "Ammon's horn". The cell bodies of the principal neuron layer (three to six cells deep) of the hippocampus, the pyramidal cell layer, is divided into three regions designated CA1, CA2 and CA3 (cornu ammonis) based on the size and appearance of the neurons. The neurons have elaborate dendrites extending to the cell layer in both directions. The apical dendrites originating from the apex of the pyramidal cell body traverse three strata: stratum lucidum, stratum radiatum and stratum lacunosum moleculare.

The external input to the hippocampus arrives largely from the entorhinal cortex, which projects via the perforant path to the subiculum, DG and the CA fields (Fig 2). The layer2 of the entorhinal cortex is the major site of origin of the afferents to DG and CA3. In contrast, the projections to CA1 and subiculum originate from the layer3 of the entorhinal cortex.

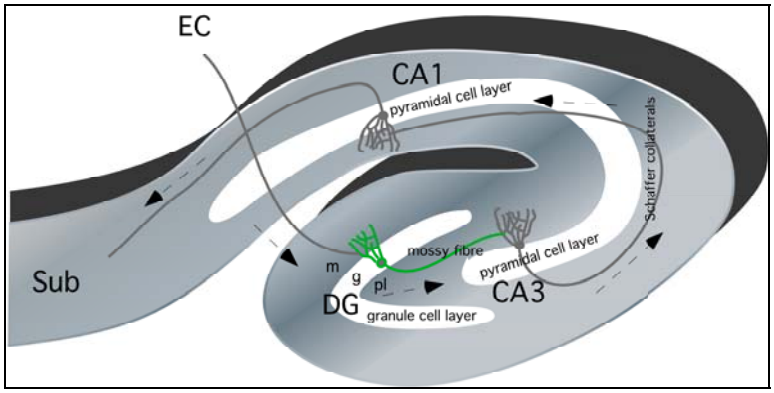

Figure 2: Architectonic divisions of hippocampus

The hippocampus is subdivided into the dentate gyrus CA1, CA3 and subiculum. CA1 and CA3 are composed of pyramidal cells. The input to the hippocampus is from the entorhinal cortex (EC). The dentate gyrus (DG) has three layers: the molecular layer (m), the granule cell layer (g) and the polymorphic layer (pl). The output from the DG is through the mossy fiber to the CA3. The CA3 projects to the CA1 via the Schaeffer collaterals

The principal neurons of the DG on the other hand are the granule cells, which form the granule cell layer. The largely acellular molecular layer is located above the granule cells. Below the granule cell layer is a diffuse cellular layer called polymorphic cell layer (or hilus). The dentate granule cells are bipolar with axons entering the hilar region and the dendrites extending to the molecular layer from the opposite pole of the cell body. The dentate granule cells receive both cortical and hippocampal inputs and these afferent circuitries are finely segregated. The input arriving from the entorhinal cortex (cortical) terminates on the distal dendrites in the outer molecular layer and commissural/associational (hippocampal) fibers arising from the hilar mossy cells form synapses on proximal dendrites in the inner molecular layer. The SGZ receives modulatory serotonergic and catecolaminergic (adrenalin and noradrenalin) inputs. There is also some sparse dopaminergic input the SGZ. The dentate granule cells project unmyelinated axons called mossy fibers establishing synapses with a variety of cell types.

Classically, mossy fibers were shown to give rise to large *en passant* swelling and terminal expansions forming the giant mossy fiber boutons on the proximal dendritic portions of the CA3 pyramidal cells and large hilar neurons (mossy cells). Apart from the giant synapses on the CA3 cells, mossy fibers form numerous distinct synapses on the smooth dendrites of interneurons. In fact majority of the synapses by the mossy fibers are formed by small boutons and filopodial extensions of the giant expansions on the interneurons.

2.1.3 Neurogenesis in the subgranular zone

Within the DG the precursor cells are located in the subgranular layer. These cells divide and generate a large pool of neuroblasts and immature neurons. Most of these immature neurons are eliminated in their first and second week after birth, and presumably a selection process preserves the others (Gage, 2000; Kempermann et al., 2004). Unlike the SVZ, neurons born in the SGZ do not migrate long distances, but are added to the adjacent granule cell layer. These neurons go on to integrate into the existing circuitry, thus performing the jobs that are assigned to them. Just what this job is, is not yet clear.

Once born, the new cells generate axons that traverse the hilus and synapse with the dendrites of the CA3 pyramidal cells (Zhao et al., 2006). At the same time, cells also generate dendrites that radiate to the molecular layer, where they receive input from the incoming fibers of the entorhinal cortex. The newly generated neurons in the DG survive for the rest of life of the organism (Kempermann et al., 2003). Along the course of their development the new neurons start to exhibit membrane characteristic with enhanced capacity to facilitate synaptic plasticity and subsequently upon time showing similar properties to the adjacent granule cells (van Praag et al., 2002; Schmidt-Hieber et al., 2004). GABAergic afferents are one of the first inputs to arrive to the new neurons in the dentate gyrus (Wang et al., 2005; Ge et al., 2006). The structural and functional complexity of the newly born neurons continues to grow with time similar to early

postnatal DG development, but with a protracted time period (Esposito et al., 2005) and in an environment that already bears mature functioning neurons.

2.1.4 Regulation of endogenous neurogenesis in the adult dentate gyrus

Adult neurogenesis is regulated by intrinsic (genetic) and a wide variety of extrinsic (micro environmental) factors. One of the first studies convincingly showing intrinsic regulation was based on the differences in basal neurogenesis levels in various genetic strains of laboratory mice (Kempermann et al., 1997a; Kempermann and Gage, 2002). The genetic background of the animal affected all measures of neurogenesis – proliferation, fate specification and survival. Interestingly, exposure of rodents to an enriched environment consisting of larger housing cages and toys increased the survival of newly born neurons in the SGZ without effecting the SVZ neurogenesis (Kempermann et al., 1997b; Nilsson et al., 1999; Brown et al., 2003). Voluntary physical exercise alone can greatly increase the proliferation and survival of newly born granule neurons (van Praag et al., 1999a). Endothelial cells seem to play a role in this mechanism by increasing vascular endothelial growth factor (VEGF) (Fabel et al., 2003). Astrocytes also regulate the generation of neurons by releasing several pro-neurogenic factors (Song et al., 2002; Lie et al., 2005). Other modes of physical activity such as water maze learning have also been shown to regulate neurogenesis in the adult DG (Gould et al., 1999a; Ehninger and Kempermann, 2006).

The strongest negative regulators of adult neurogenesis known are aging and the serum level of steroid hormones (Cameron and McKay, 1999; Gould et al., 1999b). Aged transgenic mice that lacked the tumor suppressor gene $p16^{INK4a}$ exhibit higher fraction of neural progenitors in their SVZ than littermate controls (Molofsky et al., 2006) suggesting a role for this protein in aging induced suppression of adult neurogenesis. The negative effect of aging is also suggested to be a result of the increased level of steroids found during aging. Adrenelectomized mice have significantly higher levels of neurogenesis compared to sham-operated controls (Cameron and McKay, 1999).

In some animal models of depression and in physical and psychosocial stress experiments, a decrease in neurogenesis has been reported (Magarinos et al., 1996; Gould et al., 1998; Dranovsky and Hen, 2006). This view is supplemented by the observation that adult neurogenesis is positively regulated by clinically relevant antidepressant drugs (Malberg et al., 2000; Czeh et al., 2001). Adult neurogenesis is also affected by a variety of pathological conditions. Seizures induce massive neurogenesis in the DG (Parent et al., 1997) and remains one of the strongest known positive regulator of neurogenesis. The development of newly born neurons is accelerated by seizures but display alteration in dendritic morphology, and are ectopically located with abnormal electrical properties compared to the normal granule cells (Scharfman, 2004; Overstreet-Wadiche et al., 2006). Ischemic brain insults potently stimulate proliferation in both SGZ and SVZ. In a model of focal ischemia in striatum, precursor cells from the SVZ migrated towards the site of dying neurons in the striatum and expressed markers typical of medium spiny neurons – the phenotype of the dying neurons (Arvidsson et al., 2002). But most of these neurons died two to four weeks after the stroke. This study suggested that after ischemia the local microenvironment provides cues for the attraction and the differentiation of precursors cells, but fails to maintain the long-term survival of these cells. Intraventricular infusion of epidermal growth factor (EGF) and fibroblast growth factor–2 (FGF2) after global cerebral ischemia led to increased proliferation, migration, neuronal differentiation and survival of precursors from the caudal periventricular zone to the hippocampus (Nakatomi et al., 2002). The newly populating cells had partially replaced the dying neurons in the CA1 region with the animals demonstrating improvement in hippocampal related behavioral tasks.

Studies in rodent brain exposed to radiation suggested extreme sensitivity of neural precursor cells towards low doses of radiation. Precursor cells failed to differentiate to neurons after they were exposed to radiation even though they retained the capacity for glial differentiation (Monje et al., 2002; Monje and Palmer, 2003). This dysfunction has been linked to microglial inflammatory response, which inhibits neurogenesis. This inhibition, caused partly by the microglial release of IL-6, could be prevented by anti-inflammatory treatment (Ekdahl et al., 2003; Monje et al., 2003). This is interesting considering that resting microglia favors neurogenesis by releasing several

pro-neurogenic mediators such as brain derived neurotrophic factor (BDNF) in the SVZ (Walton et al., 2006). Thus microglia can bidirectionally regulate neurogenesis in a context dependent manner.

Adult neurogenesis is also significantly altered in chronic neurological disorders. In the DG and CA1 of Alzheimer's patients an increased expression of Doublecortin (DCX) and express Polysialated Neural Cell Adhesion Molecule (PSA-NCAM) was noticed (Jin et al., 2004). The proliferation of precursors in the SGZ and SVZ is reduced in Parkinson's disease patients, presumably due to a loss of dopaminergic innervations (Hoglinger et al., 2004).

2.2 Analysis of adult dentate gyrus neural precursor cells *in vitro*

2.2.1 Isolation and characterization of precursor cells *in vitro*

Adult neural stem cells were first isolated from the adult CNS of rodents (Reynolds and Weiss, 1992) and later also from humans (Kukekov et al., 1999; Palmer et al., 2001). But much of the work is done with precursor cells obtained from subventricular zone, leaving questions about precursor cells within DG open. In previous experiments the precursor cells have been allowed to proliferate in the presence of EGF plated onto uncoated surfaces. After couple of days, the cells produce aggregates (of cells) termed "neurospheres". Cells within these aggregates stain for nestin, an intermediate filament protein characteristically found in precursor cells of diverse tissues (Reynolds and Weiss, 1992; Seaberg et al., 2004; Wiese et al., 2004). These cells could be passaged and produced neurons as well as glial cells when the mitogen was withdrawn from the culture. When transplanted, these cells also generate neurons, sidelining the argument of *in vitro* artifact as the basis for these observations. Because of the defined conditions of cell culture and the easy experimental access, manipulations of adult precursor cells in vitro allow the analysis of both intrinsic and extrinsic mechanisms that regulate the various steps involved in neurogenesis.

2.2.2 Neurospheres and monolayer cultures

Neural precursor cells have been traditionally cultured as neurospheres by plating then on an uncoated surface with mitogen containing medium for prolonged propagation. After several days, the cells start to form spheres. Each sphere harbors several hundreds to thousands of cells. As the most energy conserving conformation is in the spherical form, in the absence of any attachment factors the cells remain in the sphere form. The cellular interaction within the sphere is heterogenous (Reynolds and Rietze, 2005). The innermost core of a sphere is presumably "blind" to the culture medium. The growth and fate of these cells is likely to be dependent on the factors secreted by the adjacent cells. Neurospheres nonetheless provide a good model to study the three-dimensional regulation of neural precursor cells.

Adherent monolayer culture, alternatively, allows a more homogenous concentration of the media contents around the cells in the culture dish. Surprisingly despite the fact that the majority of studies on adult neurogenesis and adult neural precursor cells are done in mice, no protocol for long-term monolayer stem cell cultures from the adult murine DG has been reported. Monolayer cultures have been established only for rats and cultures from mouse hippocampus have only been maintained as neurosphere cultures derived from the entire hippocampal formation (Palmer et al., 1997; Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). Consequently, information about stem cells in the adult mouse DG has been either inferred from rat studies or from mouse neurosphere studies that have not assessed whether the model system produced cells that mirrored the *in vivo* situation. An adequate hippocampal stem cell culture model would have to meet two key requirements: The culture would have to build on a reliable precursor cell population with known "stemness" properties and yield cells with truly neuronal properties reflecting the neuronal phenotype of granule cells *in vivo*.

Neurospheres, which are the most widely used form of culturing mouse neural precursor cells, have limitations, because they are heterogeneous (Reynolds and Rietze, 2005). Cells at the core of the sphere differentiate and each neurosphere might actually contain only few precursor cells which become diluted with each ensuing passage (Reynolds and

Rietze, 2005). Functional studies requiring real-time resolution on a single cell level are limited, if not impossible, in the multicellular clusters.

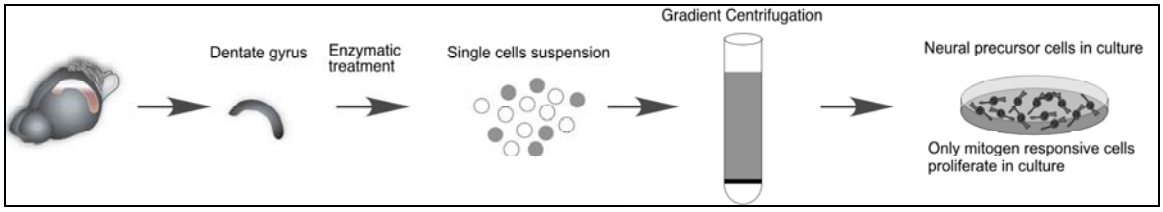

Figure 3: Methodology for the culturing of neural precursor cells in vitro

The desired brain region from which the neural precursor cells are to be cultured is enzymatically digested and plated in mitogen containing serum free medium. The precursor cells proliferate and populate the culture dish.

Adherent monolayer cultures better reveal the morphology of individual cells and fully expose cells to a controlled extracellular environment. Monolayer cultures were originally described for hippocampal stem cells from adult rats or from cells isolated from whole mouse brains but have not yet been adapted for adult mouse hippocampus or subdissected hippocampal DG (Gage et al., 1995; Palmer et al., 1999; Ray and Gage, 2006). Furthermore, it has been disputed whether stem cells in the strict sense of the definition exist in the adult hippocampus (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). With the working hypothesis that a combination of methodological and species-related reasons might have led to the impression that no stem cells existed in the adult hippocampus, a murine monolayer stem cell culture system was developed to directly test this hypothesis (Fig 3). The modified method used to generate monolayer precursor cells in the present thesis work and the results obtained have been published in the journal PLoS ONE (Babu et al., 2007).

2.2.3 Identification of stem cells

What constitutes a stem cell? And how does one distinguish between a stem cell and other cells types in the neighborhood? Stem cells are conceptually defined as cells

that can self renew and have the capacity to generate all three cell types of the brain – neurons, astrocytes and oligodendrocytes. Several proteins have been characterized that are restricted in their expression pattern to precursor cells. Molecular markers are the vital elements in the study of cell differentiation and fate commitment, as defined sets of markers are needed to assign cells to specific types and their hierarchical positions. One of the first precursor cell marker to be identified was nestin (Lendahl et al., 1990), a type IV intermediate filament protein. It is expressed by a wide variety of precursor cells obtained from the neuroepithelium. However, it is expressed by a wide variety of cells outside the bracket of neural precursor cells such as reactive astrocytes, muscle precursor cells and pancreatic precursor cells (Zulewski et al., 2001). Several other markers have also been reported to be associated with precursor cells. The astrocytic protein Glial fibrillary acidic protein (GFAP) is also present in the adult neural stem cells in adult SVZ and SGZ (Doetsch et al., 1999; Laywell et al., 2000; Seri et al., 2001). The stem cells in the neurogenic areas also show characteristics of radial glia cells. Brain lipid binding protein (BLBP) is a marker associated with radial glia in the developing brain and is also expressed by the proliferating cells in the adult DG. It has been shown that BLBP is expressed by both the GFAP expressing "stem cells" and DCX expressing progenitor cells (Steiner et al., 2006). Another gene associated with "stemness" in neural precursor cells, is the transcription factor Sox2 (SRY related high mobility group). Sox2 is expressed in the adult SGZ (Komitova and Eriksson, 2004) and furthermore, transgenic mice in which EGFP (enhanced green fluorescent protein) is driven by Sox2, show EGFP expression in the SGZ of the DG. After isolation by FACS these cells and have been shown to behave as stem cells *in vitro* (D'Amour and Gage, 2003). This result is significant considering that Sox2 is involved in the maintenance of fetal precursor cells in the proliferative state by inhibiting differentiation in these cells (Wegner and Stolt, 2005).

Mature neurons have been generally identified by detecting the neuron specific microtubule associated protein-Map2ab and the pan neuronal marker β-III-tubulin (Tuj1). Doublecortin (DCX), another microtubule-associated protein is also widely used to detect immature migrating neurons. DCX is important in cell division and migration of newly born cells in the developing cortex. DCX is seen in the adult SGZ in a large population of proliferating precursors (Kempermann et al., 2003). This marker is absent from the

putative GFAP expressing stem cells, and appears only in cells committed to the neuronal lineage (Kempermann et al., 2004). Cells expressing DCX also express PSA-NCAM.

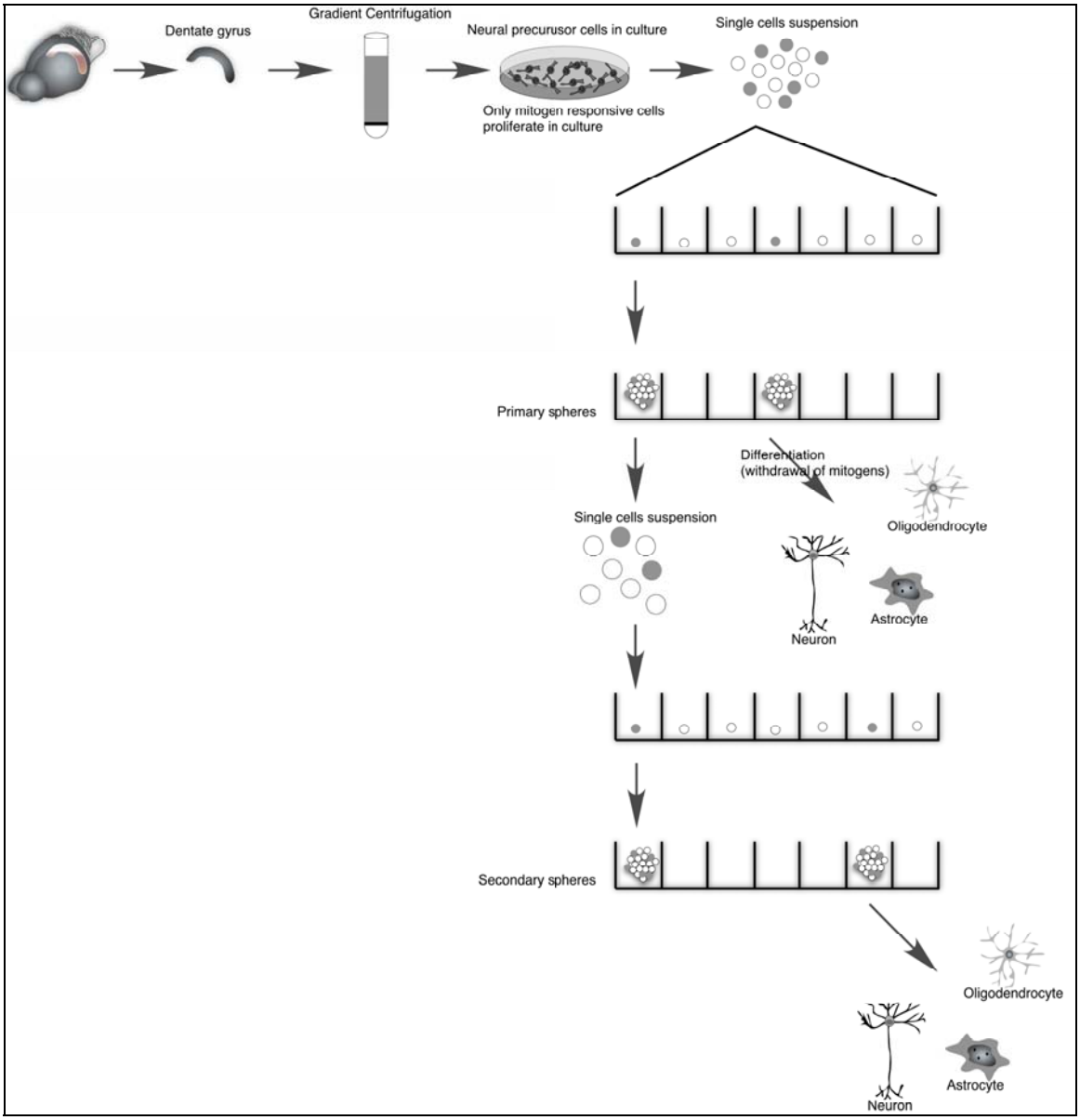

Figure 4: Schematic representation of the assay undertaken to study the self renewal and multipotentiality of adult neural stem cells

The neural precursor cells are isolated from the adult dentate gyrus and then exposed to mitogen for propagation. Neural precursor cells are plated at low density or as single cells to test for self-renewal. Such renewing neural stem cells are then single cell dissociated and replated at low density or as single cells to assess for the self-renewal. At each step a part of the cells are differentiated to check for their ability to generate neurons, astrocytes and oligodendrocytes, proving the multipotentiality of the neural stem cells.

Mature neurons are detected by the presence of neuronal nuclear antigen (NeuN) along with the absence of other non-neuronal and immature markers. In the DG, the prospero related protein (Prox-1) is a marker widely used to distinguish the dentate granule cells from the other neural cell types in the surrounding (Pleasure et al., 2000; Bick-Sander et al., 2006).

Due to their rarity, functional characterization of the precursor cells have been restricted to *ex vivo* approach, by bringing the cells to a tissue culture environment (Fig 4). Defined media is added to the cells that are permissive for cells that proliferate. After several days when the cells proliferate, the precursor cells outnumber other cell types. By controlling the plating density, each neurosphere could be made to arise from a single cell. After trypsinization and single cell dissociation, the cells are again plated with mitogens (Fig 4). Any subsequent formation of secondary neurospheres is indicative of self-renewal of the cells. Demonstration of self-renewal is a strong indicator of the presence of stem cells in the culture. The neurospheres that arise from a single cell are then differentiated. The generation of neurons and glial cells from these suggests the presence of multipotent stem cells (Fig 4).

2.2.4 Regulation of adult neurogenesis: role of neuronal activity

New neurons are born and integrate into the existing machinery of the brain and survive for almost the life of the animal. This has led researchers to believe that neurogenesis could be mechanism of a "hard wiring" form of memory acquisition or consolidation. Neural activity has been shown to regulate neural differentiation from adult precursor cells. Kainic acid, an agonist at the non NMDA receptor subtype of glutamate receptor, induces a potent increase in neurogenesis in the DG (Jessberger et al., 2005). Methods of generalized neural excitation have involved membrane depolarization (Deisseroth et al., 2004) which mimic areas of increased activity involving action potential (AP) firing. Incidentally, AP firing in the dentate gyrus is notably sparse but intrinsic activity, most notably in the sense of oscillations is high. Neural information

processing also involves AP independent process such as EPSCs (Excitatory Postsynaptic Currents), which are synaptic events that occur even in the absence of AP. Due to their strategic location adult precursor cells may very well be influenced by changes in such synaptic activity.

Within the hippocampus, oscillation and synchrony of neuronal networks bring about a wide range of information coding and storage capacity (Buzsaki, 2002; Axmacher et al., 2006). Neurogenesis may have a predetermined instructive role in the network or may alter the general properties of the existing neurons. Adult-born new hippocampal neurons are not only highly excitable but transiently also undergo long-term potentiation (LTP) at relatively lower levels of activating stimuli than older granule cells (Schmidt-Hieber et al., 2004). Such computationally intensive functions demand the intriguing hypothesis that network changes might be directly sensed by the neural precursor cells, relaying this change as a pro neurogenic signal. Or in other words could coherent synchronous oscillatory neural network activity contribute to properties that might influence neuronal differentiation from adult hippocampal precursor cells?

Behavioral activity such as voluntary physical exercise (van Praag et al., 1999b), and environmental enrichment (Duffy et al., 2001) generate synchronized activity in the hippocampus, modulate learning and memory and also LTP. Similarly Environmental enrichment, voluntary physical exercise, aging, and learning and memory paradigms regulate neurogenesis in the DG (Kempermann et al., 1997b; Cameron and McKay, 1999; Gould et al., 1999a; van Praag et al., 1999a; Shors et al., 2002). Thus even though neurogenesis and synaptic plasticity have been suggested to be relevant for hippocampal function, the question whether there is an intersection between these two phenomenon has not been addressed. Precisely whether LTP in itself could regulate precursors cell population kinetics had not been dealt into.

Though the conceptual gap between neurogenesis and behavior is large, targeted modification of neuronal differentiation will potentially influence the coding and storage capacity of the network, to which it is added, and change the behavior of the organism.

In the present study we set out to determine the relationship between synaptic network plasticity and cellular plasticity. To accomplish this we developed a co-culture model consisting of hippocampal neurons and hippocampal precursor cells that allowed control

of neuronal activity levels. Hippocampal neurons are an excellent model to study oscillatory activity and its synaptic relevance. Hippocampal neurons in culture show low synaptic release probability, which increases after brief Ca^{2+} rises through NMDA receptor activation. Although the short-term cellular effects of such stimuli on mature functional neurons are well documented (Malenka, 2003; Malenka and Bear, 2004), the effects of such changes in a network of neurons and neural precursor cells are not known. To study the effect of synaptic plasticity on neural precursor cells we overlaid hippocampal neurons with labeled adult hippocampal precursor cells.

Using this model following questions have been addressed: (1) Can precursor cells from the DG directly detect neuronal network activity? (2) How do precursor cells respond to changes in synaptic activity levels? (3) What is the signaling mechanism that relays between the synaptically active neurons and precursor cells?

3 Materials and Methods

3.1 Tissue dissection

All institutional regulations regarding animal ethics were followed. Adult female mice (C57Bl/6 or CD1) were killed by an overdose of ketamine and then decapitated. After removing the overlying skin and the skull the brains were removed and placed in a cold buffer saline solution (DPBS with 0.6% Glucose). The DPBS consisted of the following ingredients (in mM): $Na_2HPO_4H_2O$, 8; KH2PO_4, 1.4; NaCl, 137; KCl, 3. The brains were then dissected under a stereomicroscope. The brains were split along the interhemispherical cleft. After removing the cerebellum and the spinal cord, the base of the brain and the thalamus were pinched off and the hippocampus was cleared from the rest of the neocortical tissue. The DG was further dissected out of the hippocampus by drawing a wedge along the hippocampal fissure separating the DG from the rest of the hippocampus. The dissected tissue was placed in the cold DPBS. The dissected DG from 4 - 5 animals per preparation was pooled for further procedures.

In order to further refine the microsdissection, brains were removed from the skull in a subset of mice and were placed in cold artificial CSF (aCSF) that was constantly bubbled with 95%O_2 / 5% CO_2. The aCSF consisted of the following ingredients (in mM): NaCl, 124; KCl, 2.5; NaH_2PO_4, 1.25; $CaCl_2$, 1; $MgCl_2$, 1; $NaHCO_3$, 25; D-Glucose, 10. The brains were sliced coronally (300µm) using a vibratome (Leica) and the coronal slices with the hippocampus and the dentate gyrus collected and placed in cold aCSF. The slices were then moved under a dissecting microscope to dissect out the dentate gyrus and free it from the hippocampus and the surrounding ventricular tissue to avoid contamination from the precursor cells that are located also within this region. For extracting SVZ and corpus callosum, the regions were extracted under the microscope and placed in the cold saline buffer analogous to the procedure for DG. These dissections were done with nestin-GFP mice to clearly delineate the regions with precursor cells from one another.

3.2 Tissue digestion

The pooled tissue was dissociated by digestion with a mixture of Papain (2.5U/ml) (Worthington), Dispase (1U/ml) (Roche) and Deoxyribonuclease (250U/ml) (Worthington) for 30 to 40 min at 37°C. The tissue was then triturated and centrifuged at 1000 × g for 3 minutes. The enzymes were discarded and fresh DPBS added and then centrifuged. This washing procedure was carried out three times to prevent any carryover of the enzyme mixture. The course cell suspension was passed through a 40μm cell strainer (Becton Dickinson) to obtain a single cell suspension. The single cell suspension was then subjected to percoll density gradient centrifugation to enrich for precursor cells. The cell suspension was mixed with equal volume of Percoll and was centrifuged at 20,000 × g for 30 min. The stock of isotonic Percoll (Amersham) was prepared by diluting 9 parts of Percoll with 1 part 10X PBS. After the centrifugation the cells were freed of Percoll by washing three times with DPBS. The cells were subsequently collected and transferred to proliferation medium consisting of Neurobasal (Invitrogen), 2% B/27 (Invitrogen), 2 mM Glutamax (Invitrogen), Pen-Strep (Sigma), 20 ng/ml human Fibroblast Growth Factor-2 (FGF2 from R&D or Peprotech) , and 20 ng/ml human Epidermal Growth Factor (EGF from R&D or Peprotech). Based on the sedimentation of the mouse cells in the continuous gradient it was determined that precursor cells could be selectively enriched in the cell pellet formed in a solution of 22% Percoll following low speed centrifugation (1000 × g, 10 minutes, room temperature). The pellet of cells were then mixed with 22% Percoll and centrifuged at 1000 × g for 10 minutes. The stem/progenitor cells and erythrocytes in the pellet are collected and then resuspended in a mixture of 70% Percoll. The erythrocyte pellet was discarded whereas precursor cells were rinsed free of Percoll and plated with proliferation medium and placed in a 5% CO_2 incubator. The medium was changed the following day and then every 2-3 days thereafter.

Test for self-renewal and multipotency: For self renewal experiments, cells were trypsinized, triturated, centrifuged and resuspended in medium. Cells were then either plated at a very low density (often referred to as “clonal density”) or individually aspirated under microscopic control and seeded in separate wells of a microtiter plate.

The presence of only single cells per well was confirmed under the microscope 24h later. In either case, the cells were plated in conditioned medium together with fresh medium in 1:1 ratio with 20ng/ml of both EGF and FGF2. The conditioned medium was prepared from medium incubated for 48 hrs in proliferating precursor cells. The media was centrifuged and the supernatant preserved at 4°C when used on the same or the next day or at -80°C for long term storage.

3.3 Propagation of neural precursor cells

The surface of the culture dishes (polystyrene petri dishes and culture flasks, or glass coverslips in multiwell plates; Nunc/TPP) was first coated with 10 µg/ml Poly-D-Lysine (Sigma) overnight at room temperature. After several rinses in water, the surfaces were left to dry. The surfaces were then coated with 5 µg/ml Laminin (Tebu-bio & Roche) at 37°C overnight. The plates or coverslips were stored at –20°C for future use. After removing excess coating solution, the cells were plated directly onto the surface in proliferation medium. Subsequently, the cultures were fed with new medium every 2-3 days by replacing 75% of the medium. The cells were maintained at a density of 10^4 cells/cm^2. This required the cells to be passaged every 4-5 days. To induce and maintain differentiation the growth medium was replaced with growth medium free of mitogens. To enhance differentiation the medium was supplemented with 0.5µM retinoic acid (Sigma) or 0.5% fetal bovine serum. N2 supplement was from Invitrogen.

Whenever applied, the following growth factors were added in the following concentration:

human Brain Derived Neurotrophic Factor (hBDNF; Peprotech)	100ng/ml
human Insulin (Sigma)	10µg/ml
Bone morphogenic protein (BMP; Peprotech)	50ng/ml
Leukemia inhibitory factor (LIF; Stem cells)	50ng/ml
Vascular endothelial growth factor (VEGF; Peprotech)	100ng/ml
Neurotrophin3 (NT3; Peprotech)	100ng/ml
Endothelial cell growth factor (ECGF; Roche)	10µg/ml

Sonic hedgehog (Shh; R&D) 1μg/ml

The retrovirus for labeling the precursor cells with EGFP was a kind gift of Prof. Wolfgang Uckert (MDC, Berlin). Precursor cells were transduced overnight and the following day were washed and then fresh proliferation medium added. The cells were allowed to proliferate and express EGFP for 2 days after which the precursor cells were fluorescent activated cell sorted (FACS) for EGFP expression. The sorted cells were propagated and used for coculture experiments.

3.4 Immunocytochemistry of cultured cells

All steps were carried out at room temperature except when indicated otherwise. Cells cultured on glass cover slips were fixed with cooled 4% Paraformaldehyde (PFA) in 0.1M phosphate buffer (pH 7.4) for 20-30 min. After washes with Tris-buffered saline (TBS), cells were blocked with 3% Donkey serum (Chemicon) containing 0.2% Triton X-100. Primary antibodies were diluted in blocking buffer and the cells were incubated overnight at 4°C. After 3 washes with TBS, secondary antibodies were diluted in TBS and the cells were incubated for 2 h. The cells were further washed to remove the excess antibody and then mounted on glass slides with PVA-DABCO (Polyvinyl alcohol- diaza-bicyclo-octane).

The following primary antibody and dilutions were used.

Monoclonal: anti-nestin 1:400 (BD pharmingen); anti-RC2 1:50 (DHSB Iowa); anti-βIII tubulin (Tuj-1) 1:1000 (Promega); anti-GAD67 1:1000 (Chemicon); anti-NeuN 1:100 (Chemicon); anti-Map2ab 1:500 (Sigma); anti-CNPase 1:100. Rat anti-BrdU 1:500 (Advance); anti-PSA-NCAM IgM 1:400 (Chemicon)

Polyclonal: rabbit anti-βIII tubulin 1 1:1000 (Covance); guinea pig anti-GFAP 1:1000 (Advanced Immunochemistry); rabbit anti-NG2 1:200 (Chemicon); Rabbit anti Map2 1:2000 (Chemicon); goat anti-Calbindin 1:250 (SWANT); goat anti-calretinin 1:250 (SWANT); rabbit anit-Prox-1 1:5000 (Chemicon); rabbit anti-Synaptoporin 1:200 (Chemicon); rabbit anti-BLBP 1:2000 (kind gift of Nathaniel Heintz Rockefeller

University, New York); mouse anti Synaptophysin 1:100 (Abcam); Rabbit anti-GluR1 1:100 (Chemicon); rabbit anti-GAP43 1:250 (Chemicon).
The fluorophore-coupled secondary antibodies were raised in donkey (Dianova) and used at 1:250. A Leica TCS/SP2 confocal microscope (Leica, Bensheim) was used to evaluate the fluorescent staining using appropriate excitation beams. All analyses were done in sequential scanning mode to avoid channel bleeding and thus the detection of spurious double labeling. Counting was done with a Zeiss Axioplan2 epifluorescence microscope with appropriate filters.

3.5 Glutamic acid decarboxylase induction

Proliferating precursor cells were plated at $1 - 2 \times 10^4$ cells/cm^2. The cells were withdrawn from the proliferation medium and allowed to differentiate for 2-3 weeks. On the day of the experiment, 10 μM KA (Sigma) or 100 ng/ml of BDNF (Peprotech) was added directly to the medium and the cultures were left in the incubator for 30 min. The medium was removed and the cultures were washed twice with fresh medium. They were then incubated in fresh medium for 3h and left in the incubator for additional 3h. Cells were fixed and underwent immunocytochemical procedures as described above, except that 0.25% Triton X-100 was added to the blocking solution and avoided thereafter. Confocal microscopy was used to detect the levels of the Glutamic acid decarboxylase (GAD) induction. The confocal settings were kept identical for both control and the KA and BDNF incubated culture.

3.6 Primary hippocampal cell culture

For culture of primary hippocampal neurons, postnatal mice (postnatal days 0-1) were killed by decapitation and the hippocampus made free from the rest of the cortex. The hippocampus was then subjected to digestion by papain (20U/ml) for 30-40minutes at 37°C. The papain was washed of with Trypsin inhibitor (1mg/ml). The cell suspension was counted and plated on to poly-lysine / laminin coated coverslips at 5×10^4 cells/cm^2.

The hippocampal cells were then plated in the following medium: Neurobasal, B27 (2%) and Glutamax (2mM).

The coculture was established by trypsinizing the EGFP labeled precursor cells and then overlaying the primary hippocampal neurons with precursor cell suspension at a density of 10^5 cells/cm^2 by replacing 75% of the medium. To sequester endogenously secreted BDNF recombinant Fc-TrkB (R&D) and bioneutralizing antibody against BDNF (Santa-Cruz) was added at a concentration of 1-10µg/ml. Rabbit anti-NT3 was used at a concentration of 1-10µg/ml for neutralizing endogenously secreted NT3.

3.7 RT-PCR

Precursor cells were cultured as adherent cultures or as neurospheres and differentiated for 5 – 8 days. RNA was extracted with RNAeasy kit (Quiagen). The RNA was diluted in RNAse free water. After spectrophotometric quantification, RNA from each sample was reverse-transcribed in a final volume of 50 µl. 1µg of the RNA was mixed with 1µg of random primers (Invitrogen) at 70°C for 10 min. Samples were rapidly cooled on ice and RT-Buffer, 0.1 M DTT and dNTPs were added. After addition of reverse transcriptase (Invitrogen) the reaction mixture was incubated for 90 minutes at 42°C. After this, the reaction mixture was incubated at 70°C for 10 min to inactivate the reverse transcriptase. Two µl of the reaction mixture was used for the PCR reaction with gene specific primers. The annealing time was determined individually for all primers to deliver a single product. The PCR protocol consisted of the following reaction cycles: initial denaturing at 95°C for 10 min followed by 40 cycles of 45s at 95°C for, 45s at 57°C, and 1 min at 72°C. The reaction was concluded by a final extension of 10 min at 72°C. The annealing temperature was varied for primers depending on the specific requirements. Products were run on 1-1.5% agarose gels and analyzed under UV light after staining with ethidium bromide. Equal volume of cDNA was added for comparison. The primers used for detecting the mRNA and their annealing temperatures are listed below.

Table 1 PCR primer sequences

Gene		°C	Primers
Nestin	Forward	63	5'-AGGTTTGAAGACGCAGAGGA-3'
	Reverse		5'-TTCGAGAGATTCGAGGGAGA-3'
GFAP	Forward	57	5'-CACGAACGAGTCCCTAGAGC-3'
	Reverse		5'-TCACATCACCACGTCCTTGT-3'
Pax6	Forward	57	5'-ACAGAGTTCTTCGCAACCTGGCTA-3'
	Reverse		5'-ACTGGTACTGAAGCTGCTGCTGAT-3'
BLBP	Forward	57	5'-TTCGGTTGGATGGAGACAAGCTCA-3'
	Reverse		5'-GCTTCATTAGCTGGCTAACTCTGGGA-3'
Vimentin	Forward	57	5'-AGATGGCTCGTCACCTTCGTGAAT-3'
	Reverse		5'-TCCTTCTTGCTGGTACTGCACTGT-3'
NeuroD1	Forward	57	5'-ATTGCGTTGCCTTAGCACTT-3'
	Reverse		5'-TGCATTTCGGTTTTCATCCT-3'
Sox2	Forward	57	5'-CTCTGCACATGAAGGAGCAC-3'
	Reverse		5'-ATGTAGGTCTGCGAGCTGGT-3'
BDNF	Forward	57	5'-GGTATCCAAAGGCCAACTGA-3'
	Reverse		5'-CTTATGAATCGCCAGCCAAT-3'
Camk4	Forward	60	5'-CTTCGAGGTGGAGTCAGAGC-3'
	Reverse		5'-TTTGAGGTCACGATGGACAA-3'
Camk2α	Forward	57	5'- AGGGGCACCACTACCTTATCT -3'
	Reverse		5'- CTTCAGGTCACGATGCACCA -3'
Dcx	Forward	57	5'-GGAAACCGGAGTTGTCAAAA-3'
	Reverse		5'-ATGGAATCGCCAAGTGAATC-3'
TrkB	Forward	57	5'-TGGTGCATTCCATTCACTGT-3'
	Reverse		5'-CTTGGCCATCAGGGTGTAGT-3'
Mash	Forward	57	5'-GTTGGTCAACCTGGGTTTTG-3'
	Reverse		5'-GAACCCGCCATAGAGTTCAA-3'
VGLUT1	Forward	57	5'– TCCCAGGACTGATTCTCACC – 3'

	Reverse		5'– ACACAACAAATGGCCACTGA – 3'
TrkC	Forward	57	5'– CCTGACACAGTGGTCATTGG– 3'
	Reverse		5'– TCCCTCTGGAAATCCTTCCT– 3'

3.8 Immunohistochemistry

Animals were overdosed with ketamine and after the absence of pain reflexes thoracotomy was carried out and the mice were perfused with 0.9% saline to wash out the blood. This was followed by perfusion with 4% (Paraformaldehyde) PFA to fixed the tissue. The brain was dissected free from the skull and placed in 4% PFA overnight at 4°C. The following day the PFA was removed and replaced with 30% Sucrose (in 0.1 M Phosphate buffer) solution. The brains were sliced using a sliding microtome at a thickness of 40 µm with the platform at minus 75°C maintained by dry ice. The slices were transferred to cryoprotectant solution and kept at 4°C for future use.

The slices were transferred from the cryoprotectant solution and washed with multiple washes of TBS. The slices were blocked by placing them in blocking solution (TBS containing 3% donkey serum and 0.1% Tx-100) to minimize non-specific antibody interactions. After blocking for 1 hr at room temperature the slices were incubated in primary antibody for 48 hrs at 4°C. Primary antibodies were diluted in antibody blocking solutions. The slices were subsequently washed three times with TBS at room temperature to wash away the excess unbound antibody. The slices were then incubated in secondary antibodies diluted in antibody blocking solution. The secondary antibodies were coupled to biotin for enhancing the signal whenever appropriate. Streptavidin conjugated horseradish peroxidase (HRP) was subsequently added. The slices were incubated in a solution containing DAB (Diaminobenzidine) when light microscopy was used to detect the antibody staining. Otherwise fluorescent coupled secondary antibodies were used to reveal it using confocal microscopy.

3.9 LTP in cultured hippocampal cells

All stimulations were applied on the day following the initiation of the coculture. The medium was washed off and replaced by Hepes-buffered extracellular saline (ECS) solutions. consisting of the following: NaCl, 140 mM; KCl, 5 mM; $CaCl_2$, 1 mM; Hepes, 25 mM; Glucose, 33 mM; adjusted to pH 7.4 with NaOH. To block GABAergic receptors Bicucullin (20 μM; Sigma) or SR95521 (10 μM; Tocris) was added to the extracellular saline solution. To stimulate opening of synaptic NMDA receptors and oscillations in the neuronal network glycine was added at 100 μM. Strychnine (Sigma) was added at 1μM to prevent the potential stimulation of glycinergic receptors by added glycine. In the control solution, $MgCl_2$ was added instead of glycine at 1mM concentration to maintain the voltage dependent block of NMDA receptors. The stimulations were done at room temperature. APV (50 μM), MK-801 (5 μM) or KN-93 (1 mM) were added directly to the glycine containing solution. After stimulation the cultures were replaced with fresh medium and returned to the CO_2 incubator.

3.10 Electrophysiology

This part of the study was performed at University of Freiburg at Institute of Physiology with the support of Dr. Josef Bischofberger. Whole-cell recordings were performed on cultures after 9-10 days, similar to cell biological experiments. All recordings were made at room temperature. The intracellular solution contained K-Gluconate, 110 mM; KCl, 25 mM; EGTA, 0.1 mM; Hepes, 10 mM; K_2ATP, 4 mM and GTP, 0.3 mM; osmolarity was 290-300 mOsm. The extracellular solution was same as the ECS mentioned in the previous paragraph. The cells were voltage clamped at minus 80mV to detect mEPSCs. Tetrodotoxin (TTX), when used to select for mEPSCs was added at a concentration of 1 mM. The recordings were performed using Axopatch 200A amplifier (Axon Instruments Inc) and records were filtered at 5 KHz, digitized and subsequently analyzed by Stimfit (Christoph Schmidt-Hieber) based on template matching. Series resistance was checked every 5 minutes. Experiments with more than 10% change in series resistance were discarded.

3.11 Calcium imaging

Fura–2 was purchased from MoBiTec and was dissolved in DMSO to generate a stock solution of 5 mM. The final concentration in which the cells to be imaged were incubated was 10 μM 30 min. After this the cells were washed and the dye allowed to de-esterify for 10 min. The cells were imaged under a fluorescent microscope in extracellular solution as mentioned before. The dye was excited using a monochromatic source at wavelengths 330nm and 360nm. The emission was collected at 510nM. The ratio between the emissions from the two excitation was calculated and the intracellular Ca^{2+} concentration deciphered.

3.12 ELISA

BDNF protein was measured with direct ELISA method as described previously but with slight modifications (Balkowiec and Katz, 2002). Sterile ELISA plates (96-well) were treated with Poly-D-Lysine (50 μg/ml) for 1hr at room temperature, rinsed with sterile double distilled water and air-dried. Equal amounts of the supernatant from the various time points after stimulations were adsorbed onto the plates by incubating the medium for 1 hr at 37°C. The plates were then washed with TBS with 0.05% Tween-20 and blocked for 45 min with 5% bovine serum albumin (BSA). Subsequently, anti-BDNF antibody was added for 1hr at room temperature under gentle shaking. After washing the primary antibody the HRP coupled secondary antibody was added for 1hr at room temperature in blocking solution. After several washings the substrate orthophenylenediamine was added in Phosphate Citrate buffer. The plates were read at 490 nm with the reference wavelength of 405 nm in a Wallac multiplate spectrophotometer.

4 Results

4.1 Characterization of precursor cells isolated from the adult murine dentate gyrus

4.1.1 Isolation and characterization of proliferating precursors from micro-dissected adult murine dentate gyrus

Dissection of the entire hippocampus to initiate cultures from neural precursor cells had previously led to controversy because corpus callosum and the SVZ of the lateral ventricles are potential sources of contamination with other stem cells. Here a standardized microdissection procedures was developed to separate the DG from other hippocampal regions along the hippocampal fissure (Fig. 5a-d). Particular care was taken to avoid any contamination by tissue from the SVZ of lateral and 3rd ventricle. In addition, the use of mice expressing EGFP under the neural enhancer element of the nestin promoter assisted in visually separating the nestin-EGFP cells within the DG from the rest of the hippocampus (Yamaguchi et al., 2000); Fig. 5a-c). Within the dissected area, GFP-expressing cells were present only in the region of the SGZ between the hilus and the granule cell layer or SGZ (Fig. 5c). Immediately after isolation any nestin-GFP expressing cells in the cultures could only derive from this region. Care was taken to include the tip of the DG, the area of the hippocampus with the highest density of proliferating cells. The tissue was homogenized and digested with papain. A series of continuous and step Percoll gradients was carried out to isolate the proliferating precursor cells from other cells in the homogenate. Proliferating precursor cells have a high density, allowing these less buoyant cells to be separated from the non-proliferating cells on continuous density gradients spanning between 1.053 - 1.075 g/ml and on centrifugation at >10,000 × g. Centrifugation yielded three visible cell layers (Fig. 5e). The uppermost layer (1.053 g/ml) was composed of cell debris, myelin and differentiated cells. The lowermost layer (> 1.075 g/ml) contained erythrocytes. The middle layer was collected and used to initiate cultures (Fig. 5e).

Immunohistochemistry for nestin, GFAP and PSA-NCAM, markers of hippocampal precursor cells *in vivo* (Filippov et al., 2003; Fukuda et al., 2003), did not reveal putative precursor cells below 1.053 g/ml. The fraction > 1.075 g/ml contained cells expressing nestin and PSA-NCAM (Fig. 5g,h).

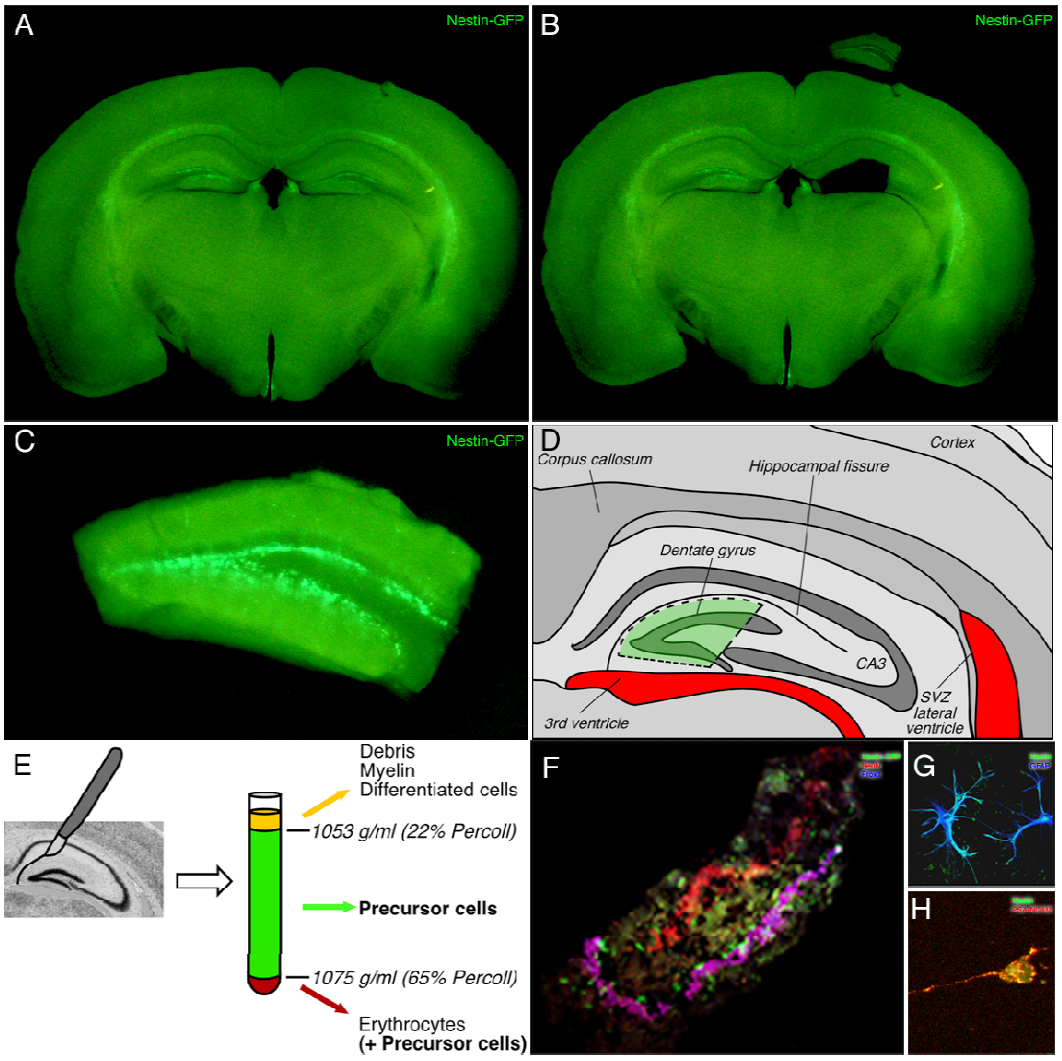

Figure 5: Isolation of endogenous precursor cells from the adult mouse dentate gyrus

A; Coronal section of a mouse hippocampus of a nestin-GFP transgenic mouse. Nestin positive cells can be seen localized in the subgranular zone. **B**; Scheme depicting the dissected region that was used for isolation of the precursor. **C**; the tissue was homogenized and separated on a Percoll density gradient. Proliferating precursors were present in the region with density >1.053gm/ml (>22% Percoll). The Erythrocytes were enriched in the region with density >1.075gm/ml (>65% Percoll). **D**; Part of the tissue was subjected to routine immunochemistry. The dissected region showed the region was indeed Dentate Gyrus, GFP (green), NeuN (Red), Prox1 (blue). **E, F;** One day after isolation and plating on Poly-Lysine/laminin coated

surface, percoll enriched DG precursor cells displayed nestin (green) immunoreactivity that colabeled with either GFAP (blue) or PSA-NCAM (red).

Of all the nucleated cells recovered from the fraction > 1.053g/ml 23.5 ± 1.7% were nestin-positive (mean ± s.e.m.), 42.9 ± 4.6% were positive for GFAP, and 28.0 ± 6.00% of the nestin-positive cells were PSA-NCAM-positive.

Cells were cultured on poly-D-lysine and laminin-coated surfaces in defined medium with mitogens FGF2 and EGF (each 20 ng/ml). After a few days, the cells began to proliferate and populate the culture dish (Fig. 6a). Upon passaging, the cells did not persist in the presence of FGF2 alone, but needed both EGF and FGF2 for continuous proliferation.

Cells were passaged every 5 - 6 days, when the cells reached 80 - 90% confluency. Cell growth and survival in culture are generally highly dependent on cell density. Optimal growth required that cultures maintained a minimum density at plating. Failure to passage the cells in a timely manner (despite medium change) resulted in massive cell death, suggesting that higher cell densities negatively affected stem cell survival even in the presence of extrinsic trophic support.

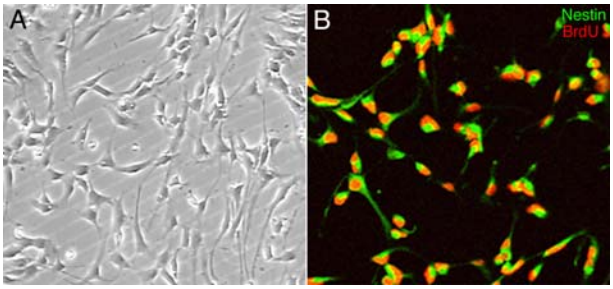

Figure 6: Mouse precursor cells in adherent culture

A; Phase contrast image of mouse precursor cells grown as adherent cultures. Note the phase bright cell characteristic of proliferating precursor cells. **B;** Incubation with BrdU (20μM) revealed they were proliferating and also costained for nestin, a precursor cells marker; Nestin (Green), BrdU (Red).

Incubation with the permanent S-phase marker bromodeoxyuridine (BrdU) revealed up to 95% of the cells to be proliferating (Fig. 6b). BrdU-positive cells displayed spindle shaped morphologies characteristic of precursor cells (Fig. 6b), whereas BrdU-negative

cells showed flat morphologies resembling mature astrocytes. Almost all BrdU-positive cells were also nestin-positive.

The cells could be passaged up to 65 times, beyond which no further attempts were undertaken. Proliferating cells could be frozen down in 10% Dimethyl sulfoxide (DMSO) in proliferation medium and be recovered by rapidly bringing them to 37°C and plating in fresh medium (data not shown).

4.1.2 Clonal analysis of neural precursor cells reveals their stemness properties

As key criterion of stemness, the potential for self-renewal was studied (Reynolds and Rietze, 2005). For clonal analysis, which is technically challenging under adherent conditions, use of the colony-forming capabilities of the detached precursor cells was made (Reynolds and Rietze, 2005). At 10 - 14 days after isolation, the cells were trypsinized and plated at a density of 1000 cells per well (24-well plate) as previously defined to provide optimal clonal sphere growth (Seaberg and van der Kooy, 2002). It was found that 17.5 ± 2.1% of the plated single cells gave rise to clonal neurospheres (Fig. 7a). To assess whether primary neurospheres indeed contained self-renewing precursor cells, single primary neurospheres were dispersed to obtain single cell suspensions and were plated again at clonal density. After 10 - 14 days, approximately 10% of the plated cells generated secondary neurospheres. Thus, precursor cells from the adult murine DG fulfilled the criteria of self-renewal that had been established in previous studies to document differences between the SVZ and DG (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). To unambiguously assess whether the precursor cells indeed had the ability to self-renew, single cells were aspirated under the microscope and placed them into individual wells of a multiwell plate. Their sphere-like colony-forming was assessed 6 days later. Neurospheres were present in 15.6% of the wells. The above studies were performed in C57Bl/6 mice, whereas other reports on murine neural precursor cells had used CD1 mice (Seaberg and van der Kooy, 2002). To rule out strain-related differences that might contribute to the reported differences in stem

cells characteristics, the above experiments with DG precursor cells were undertaken with precursor cells from the outbred strain CD1. There was no difference between precursor cells in their sphere forming capacity from the two strains. Self-renewal was compared with fresh as well as passaged cultures. Self-renewal could be successfully demonstrated at the 20th, 40th, and 60th passage. Irrespective of the passage number, the precursor cells showed immunoreactions for nestin and BrdU. These results suggested that over successive passages precursor cells from the adult DG could self renew.

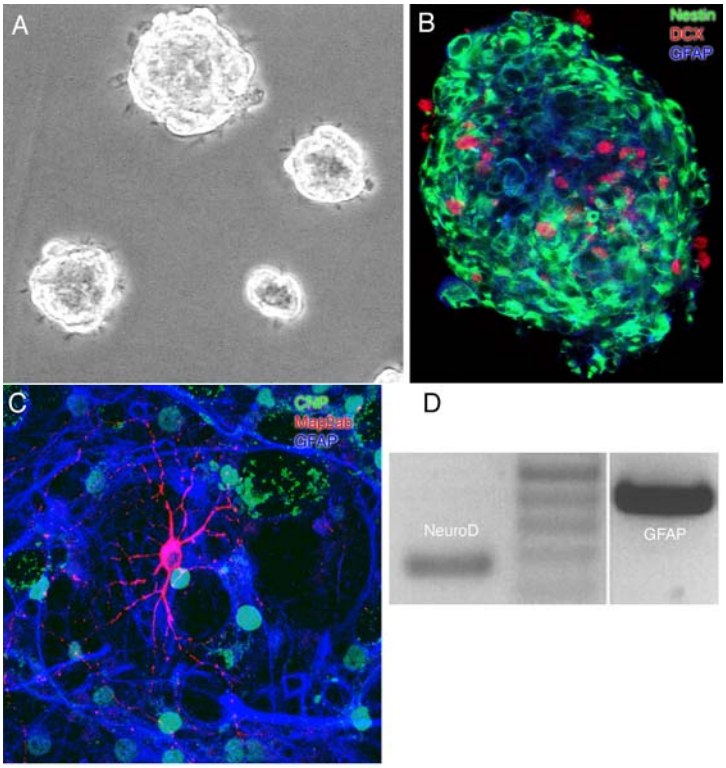

Figure 7: Precursor cells from dentate gyrus show self renewal when plated in clonal density, suggestive of the presence of stem cells

A; Clonally plated single neural precursor cells proliferated gave rise to neurospheres. **B;** Single neurosphere in proliferating conditions showed mixture of cells within them that were in all stages of development. These spheres on fixing and staining showed a differentiated core lacking nestin (Green) but was positive for astrocytic marker GFAP (Blue). Neuronal differentiation aas judged by Doublecortin expression (Red). **C;** The spheres on differentiation differentiated to all three lineages Neurons β-III-tubulin (Red), Astrocytes- (GFAP, Blue), Oligodendrocytes- (CNPase, Green). **D;** Upon differentiation, single clonal spheres upregulated both neuronal and glial genes as shown using RT-PCR for NeuroD and GFAP.

To assess whether clonally derived cell populations were capable of generating differentiated cells of the three neural lineages – neurons, astrocytes, and oligodendrocytes – individual clusters were plated onto laminin-coated surfaces and allowed to differentiate. Differentiation was induced by withdrawing the mitogens and adding fetal bovine serum and retinoic acid. At the time of plating, the core of the clusters was largely composed of differentiated cells, many of which stained for GFAP or DCX, a marker associated with migratory neuroblasts *in vivo* (Fig. 7b; (Kempermann et al., 2003). Immunohistochemistry revealed that 75% of the clonally derived colonies generated neurons (β-III-tubulin), astrocytes (GFAP) and oligodendrocytes (CNPase; Fig. 7c). Differentiating cultures up-regulated mRNA for NeuroD1, a key transcription factor in the neuronal lineage (Fig. 7d). Self-renewing clonal populations demonstrated similar tri-lineage potential at the 20^{th}, 40^{th}, and 60^{th} passage proving that the adherent culture protocol used here could retain the stem cells within them for extended periods of time.

4.1.3 Nestin-GFP expressing cells from SVZ, dentate gyrus, and corpus callosum/alveus are proliferative and multipotent in vitro, whereas those from CA1 are not

To investigate the "stemness" of nestin-GFP-expressing precursor cells in SVZ, corpus callosum and alveus as well as in CA1 adult neural precursor cells were isolated. SVZ, corpus callosum plus alveus, stratum oriens of CA1 and the CA1 pyramidal cell layer were isolated by microdissection (Fig. 8a,b). The cells were single cell homogenized similar to that of dentate gyrus and then plated in serum free medium. Cells from all regions were plated at 1000 cells/ well of a 96 well plate. Well-separated cells were followed for 2-3 weeks and assessed for their potential to proliferate. About 20% of the cells from corpus callosum/alveus and SVZ gave rise to neurospheres (< 50 cells), whereas cells isolated from subregions of CA1 failed to give rise to any colonies (Fig. 8b-f). Thus, cells from corpus callosum/alveus and SVZ proliferated in culture like cells from the dentate gyrus, whereas cells isolated from CA1 (stratum oriens and pyramidal

cell layer) did not (Fig. 8c,e-f). Cultures from CA1 were followed for 12 weeks and failed to proliferate (Fig 8c).

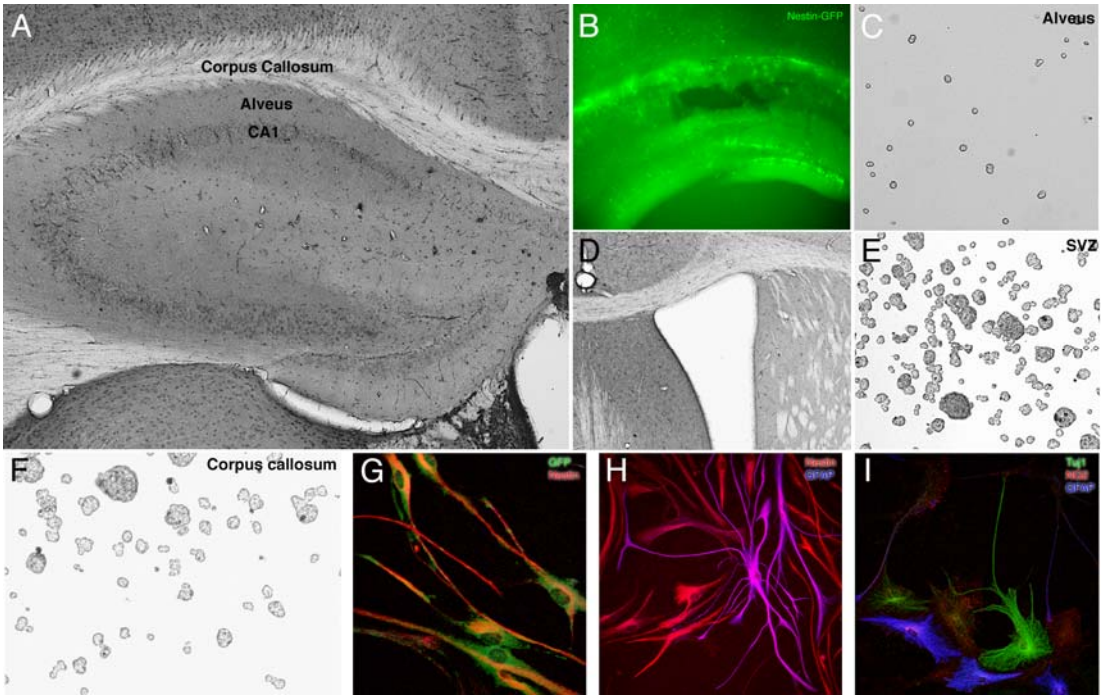

Figure 8: Nestin-GFP-expressing cells from SVZ and corpus callosum/alveus are proliferative and multipotent in vitro while that from CA1 are not

A-F; Corpus callosum plus alveus (CC) and subregions of CA1 were microdissected and precursor cells isolated and cultured. Adult stem cell culture yielded neurospheres from tissue dissected from CC (**F**) and from the SVZ (**D, E**) whereas cells from CA1 subregions failed to proliferate (**B, C**). The hole in the brain slice of a nestin-GFP transgenic mouse after microdissection is shown in (**B**). Note strong GFP fluorescence in corpus callosum/ alveus and in progenitor cells in the dentate gyrus (**B**). **G**; Cells in the adult neural stem cell culture immunoreactive to nestin (red) also show GFP (green) in them. **H**; Cells in adult neural stem cell culture immunoreactive to nestin (red) or GFAP (blue). **I**; Precursor cells isolated from corpus callosum/alveus of (C57Bl/6 mice) can be differentiated into neurons in vitro. The staining shows cells that display immunoreactivity to TuJ1 (ms-monoclonal), an early postmitotic neuronal marker (green). Red: NG2 (Rb-polyclonal); blue: GFAP (Gp-polyclonal).

To assess multipotency the clonal secondary spheres obtained were allowed to undergo differentiation. These differentiation studies were carried out in wild type C57Bl/6 animals so as to prevent any interference with the GFP signal from the nestin-GFP mice (Fig 8g-h). When clonally proliferated cultures (Fig. 8i) were subjected to differentiating

conditions (1% fetal bovine serum with 1μM retinoic acid), cells from the SVZ as well as from corpus callosum/alveus gave rise to both neurons and glia (20% TuJ1-positive neurons, 47% GFAP-positive astrocytes, 22% NG2-positive oligodendrocytes precursor cells; Fig 4i). These data indicated that the precursor cells in the SVZ and the corpus callosum/alveus are capable of self-renewal and give rise to neurons and glial cells *in vitro*.

4.1.4 Proliferating precursor cells exhibit radial glia-like properties and generate neurons after differentiation

The cultured precursor cells displayed features of radial glia as indicated by immunoreaction with antibodies against RC2 antigen (Misson et al., 1988), BLBP and GLAST (Fig. 9a,b). The cells also expressed the transcription factor Sox2 (Fig. 9b), a protein associated with "stemness" in neural precursor cells. In addition, the adherent precursor cells expressed mRNA of nestin, GFAP, Sox2, Pax6, and vimentin (Fig 9c). These set of markers are diagnostic for radial glia, which have the potential to give rise to neurons and astrocytes during development. BLBP and Sox-2 are also expressed by proliferating cells in the SGZ (Steiner et al., 2006)

In the course of differentiation induced by withdrawal of mitogens, the precursor cells started as polygonal to spindle-shaped cells, subsequently acquiring bipolar morphologies. At 10 - 14 days, the immature neurons started to develop multiple elaborate processes positive for β-III-tubulin (Fig. 9d). BrdU incubation and subsequent differentiation yielded neurons positive for the microtubule associated protein 2ab (Map2ab) with BrdU-labeled nucleus (Fig 9e). Cells lacking neuronal marker were strongly positive for GFAP and morphologically resembled protoplasmic or type-II astrocytes (Fig. 9d).

Quantitatively, the generation of neurons was dependent on both cell density and the presence of external soluble factors. We empirically found that the best density for

neuronal lineage commitment was 1-2 × 10^4 cells/cm^2. The proportion of cells in clonal colonies adopting neuronal fates (0.8 - 1%) was far below the rate seen in the neurogenic area of the DG *in vivo*, where about 80% of the new cells are neurons (Kempermann et al., 1997a) but is consistent with the level of *in vitro* neurogenesis from adult neural stem cells in prior reports (Palmer et al., 1995).

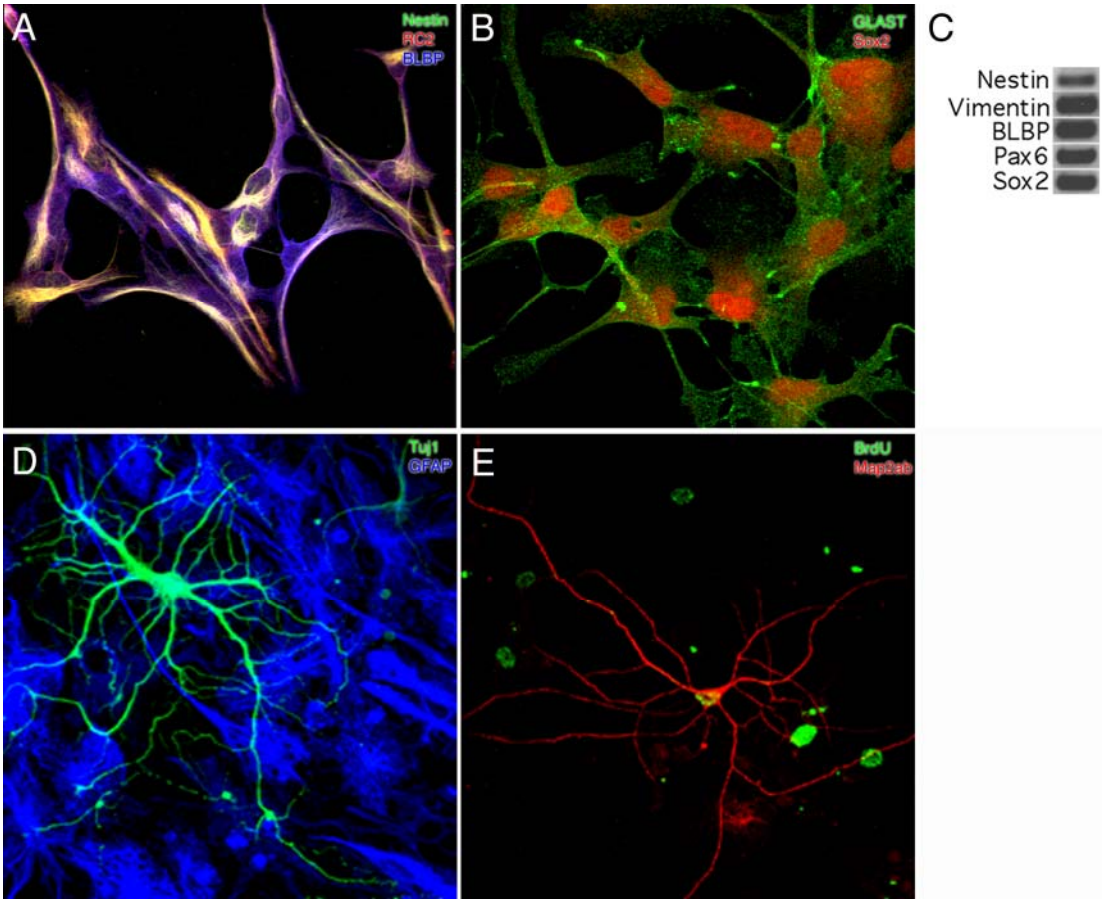

Figure 9: Proliferating precursor cells show features of radial glia and on differentiation generate neurons and astroglia.

A; Proliferating precursor cells express nestin (Green), RC2 (Red), BLBP (Blue). **B;** The precursor cells also immunostained for GLAST (Green) and Sox2 (Red). **C;** RT-PCR analysis of RNA from precursor cells suggests radial glia like phenotype: nestin, GFAP, Vim, BLBP, Pax6, Sox2. **D;** Precursor cells on differentiation generate neurons (β-III-tubulin, Green), Astrocytes (GFAP, Blue). **E;** Addition of BrdU and subsequent differentiation derived BrdU (green) positive neurons (Map2ab, Red).

Further studies are needed to ascertain the combinatorial patterns of stimulation that direct neural precursor cells to a particular lineage. For example, the influences of endothelial cells or astrocytes may provide important neurogenic cues but the exact signaling molecules that promote neurogenesis in this context are still not well defined (Palmer et al., 2000; Louissaint et al., 2002; Song et al., 2002; Shen et al., 2004)

4.1.5 Exogenous factors influence the neuronal differentiation capacity of neural precursor cells

A strong difference in neuronal differentiation was observed between simultaneous withdrawal of EGF and FGF2 compared to sequential growth factor withdrawal. When a low concentration of FGF2 (5 ng/ml) was added to the growth factor-withdrawn precursor cells, a greater number of neurons were generated in comparison to a low concentration of re-added EGF (Fig. 10a). This result is similar to an earlier study that suggested a neurogenic role of FGF2 on rat hippocampal precursor cells (Palmer et al., 1999). Cell density in the culture turned out to be higher with EGF than with FGF2 alone.

Cell density with either EGF or FGF2 alone, however, was significantly greater than without the growth factors, suggesting that both the growth factors exhibited a pro-proliferative effect, with EGF being more potent than FGF2 (Fig 10b). Sustained neuronal differentiation in the presence of low FGF2 (5 ng/ml) resulted in a higher cell density in the culture but led to a decrease in the number of cells that differentiated into neurons (Fig. 10c). When the cells were differentiated after short growth factor stimulation, greater numbers of neurons were generated with FGF2 than with EGF. When precursor cells were differentiated in the presence of EGF alone, the cells showed morphological features of glial cells. Taken together with the previous result that EGF together with FGF2 was needed for maintaining precursor cell renewal, the most parsimonious conclusion is that EGF causes self-renewal of stem cells and FGF2 maintains their neurogenic potential. It is likely that these effects are due to the trophic effects of FGF2 on neural progenitor cells produced upon asymmetric division of stem cells.

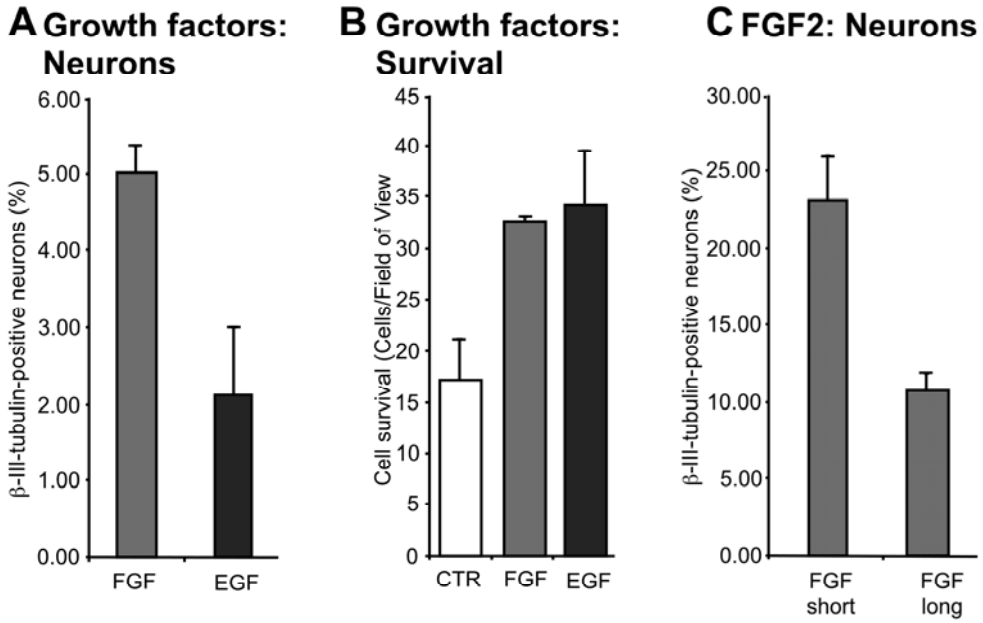

Figure 10: Differential effect of growth factors on neural precursor cells

A; FGF2 increased the neuronal differentiation compared to EGF. Precursor cells differentiated with EGF and FGF were stained with β-III-tubulin at four days after differentiation. **B;** Both FGF2 and EGF increased cell survival in the cultured compared to control. **C;** FGF showed a differential effect on β-III-tubulin positive neuronal differentiation, depending on the duration of treatment. Short treatment led to increased number of β-III-tubulin positive neurons from precursor cells while prolonged FGF2 application led to decrease.

Different serum supplements had differential effects on neural precursor cells when used with the medium. B27 consistently produced an increase in cell survival and the fraction of cells that differentiated into neurons when compared to N2 supplement (Fig. 11a-d). As one of the principle components of N2 is Insulin, N2 was replaced with Insulin and the effect studied. The effects of N2 could only partially be recapitulated by Insulin alone, suggesting that additional factors are responsible for the net effects of N2 (Fig. 11e). But as the added concentration of Insulin (20 µg/ml) has been reported to induce the activation of Insulin like growth factor receptor (IGFR), insulin signaling could be a

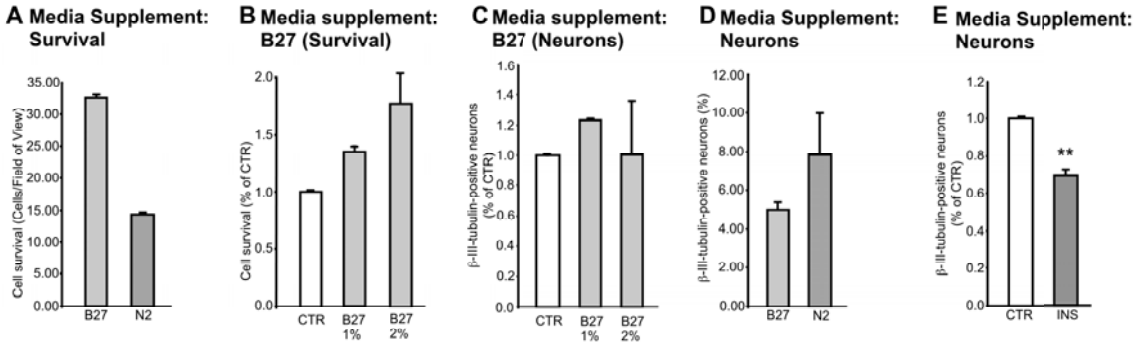

Figure 11: Differential effects of serum supplements on neural precursor cells

There was a general increase in survival with increasing B27 concentration. **A-D;** B27 and N2 had contrasting effects on precursor cells. B27 increased the survival of the precursor cells while N2 increased neuronal differentiation. **E;** Insulin as the key protein component was not sufficient to explain the effects of B27 or N2.

primary mechanism of regulating the neuronal differentiation of adult hippocampal precursor cells (Arsenijevic and Weiss, 1998).

Several growth factors besides EGF and FGF2 have been shown to influence the differentiation of fetal or immortalized precursor cell cultures (Johe et al., 1996); (Mehler et al., 1993). Hippocampal precursor cells were cultured under adherent conditions and exogenous growth factors added to them while withdrawing EGF and FGF2. Neurotrophins serve a broad range of effects during neuronal maturation and survival, acting via the family of tyrosine kinase receptors (Trk). Addition of BDNF showed a robust increase in neuronal differentiation in our culture system. The neurotrophic factor NT3 is expressed at high levels in the DG and NT3 was the only growth factor that showed a high association with adult hippocampal neurogenesis in a genetic screen of recombinant inbred strains of mice (Kempermann et al., 2006). Here, addition of NT3 significantly raised the differentiation of neurons from the precursor cells. There was an approximately 40% rise in neuronal differentiation (Fig. 12). Even stronger, Shh raised the neuronal differentiation by about 80% (Fig. 12). It has been shown previously that Shh had a potent neurogenic effect on precursor cells derived from adult rat hippocampal cells (Lai et al., 2003; Machold et al., 2003; Ahn and Joyner, 2005).

BMP2 and LIF, in contrast led to significant reduction in cells that differentiated into neurons (Fig. 12). These data are consistent with the fact that BMP2 and LIF were

potently anti-neurogenic and induce astrocytic differentiation in several stem cell culture systems (Adachi et al., 2005).

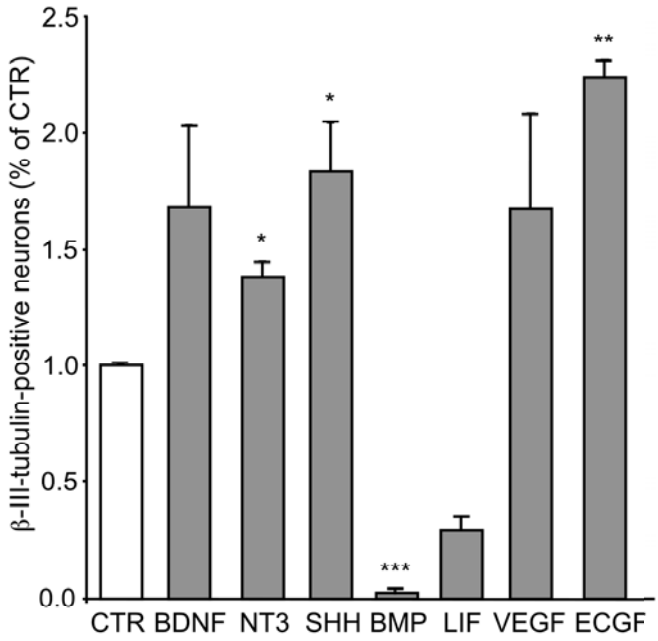

Figure 12: Neurotrophic factors and growth factors had pronounced and differential effects on the neural precursor cells

Precursor cells were incubated with different growth factors and the fraction of neurons generated quantified after 4 days whereas BDNF, NT-3, Shh, VEGF, ECGF caused an increase in β-III-tubulin positive neurons, LIF and BMP showed potent pro-gliogenic effects.

In the intact adult DG *in vivo*, endothelial cells are in close proximity to the precursor cells (Palmer et al., 2000; Louissaint et al., 2002; Shen et al., 2004). Fetal neural stem cells, when cultured together with endothelial cells, generated more neurons than when cultured alone (Shen et al., 2004). In addition, VEGF has been shown to have a strong regulatory effect on adult hippocampal neurogenesis *in vivo* (Jin et al., 2002). Addition of exogenous endothelial derived factors VEGF and ECGF revealed a robust rise in precursor cells that differentiated into neurons (Fig. 12).

4.1.6 Dentate gyrus derived precursor cells differentiate into neurons with granule cell-like properties

To investigate if the new neurons generated *in vitro* would reveal characteristics of their *in vivo* counterpart (granule cells of the DG), differentiated neurons underwent immunocytochemical analysis for a battery of markers associated with DG granule cells (Singec et al., 2002; Brandt et al., 2003). The cultured neurons expressed the transcription factor Prox1, calcium-binding protein calbindin and the synaptic vesicle protein synaptoporin, three markers characteristic of granule neurons *in vivo* (Fig. 13a-c), as well as the pan-neuronal marker NeuN (Fig. 13d). The neurites extending from the differentiated neurons revealed the expression of GAP43, a marker associated with

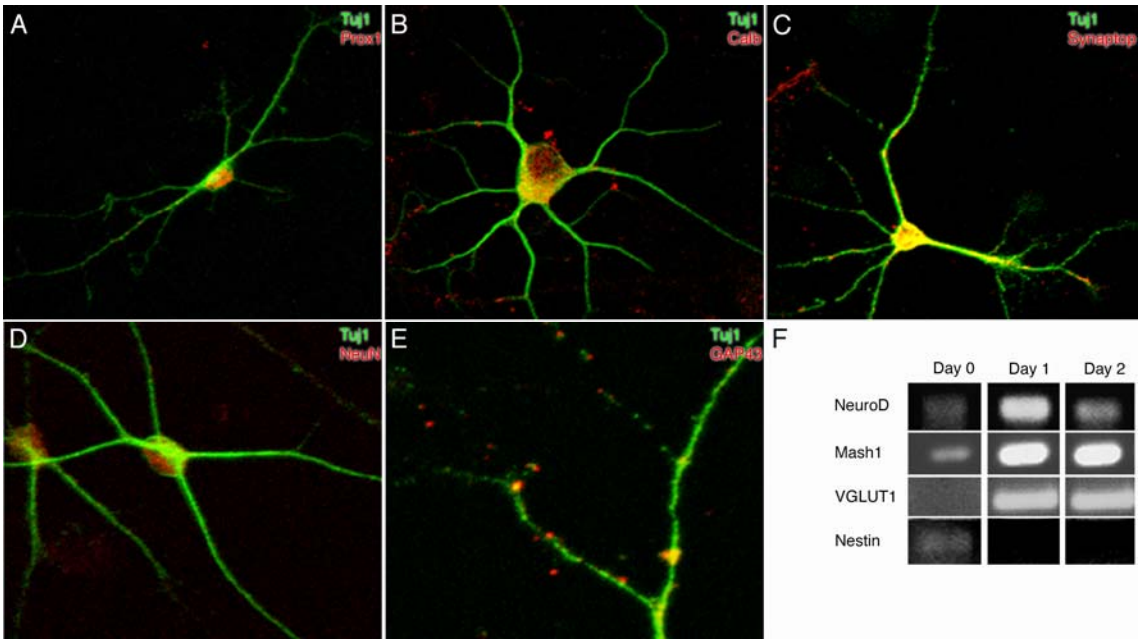

Figure 13: Differentiated neurons show features of granule cells

After differentiation precursor were fixed and stained for markers characteristic of dentate gyrus granule cells. **A;** β-III-tubulin (Green) Prox1(red). **B;** β-III-tubulin (green) Calbindin (Red). **C;** β-III-tubulin (Green) Synaptoporin (Red). **D;** β-III-tubulin (Green) NeuN (Red) **E;** β-III-tubulin (Green) GAP43 (red) **F;** Differentiation led to upregulation of neuronal markers and downregultion of precursor cell markers. Proliferating cultures (day 0) were allowed to differentiate and RNA extracted after times indicated. Differentiation leads to upregulation of neuroD, mash1, VGLUT1 and the downregulation of nestin.

neurites that are outgrowing (Fig. 13e). Analysis by RT-PCR revealed that NeuroD1 and the Vesicular Glutamate Transporter1 (VGLUT1) were up-regulated during differentiation (Fig. 13f), whereas nestin mRNA, indicative of the progenitor cell stage, was downregulated. Proliferating cells showed no or undetectable levels of NeuroD1 and VGLUT1. VGLUT1 is specifically expressed by glutametergic neurons in the adult hippocampus (Takamori et al., 2000)

4.1.8 Induction of GABAergic secondary phenotype in differentiated neurons

To further confirm the in vivo regional identity of the neurons generated from the precursor cells, the ability of dentate granule neurons to express GABA along with glutamate on excitation was exploited. Dentate granule cells are unique neurons in that they can release both glutamate and GABA as neurotransmitters, most evidently in situations of hyper excitability such as seizures (Sandler and Smith, 1991; Gutierrez, 2005). The differentiated neurons from precursor cells were treated with KA (10μM) for 3 h and stained with an antibody specific to Glutamic acid Decarboxylase (GAD67), the enzyme responsible for the GABA synthesis in mature granule neurons. As expected there was an up-regulation of GAD67, the key enzyme of GABA synthesis (Fig. 14) thus, reproducing another granule cell characteristic. Addition of recombinant BDNF (100ng/ml), which also induces the GABAergic phenotype in granule cells, led to a similar upregulation of GAD67 (Fig. 14).

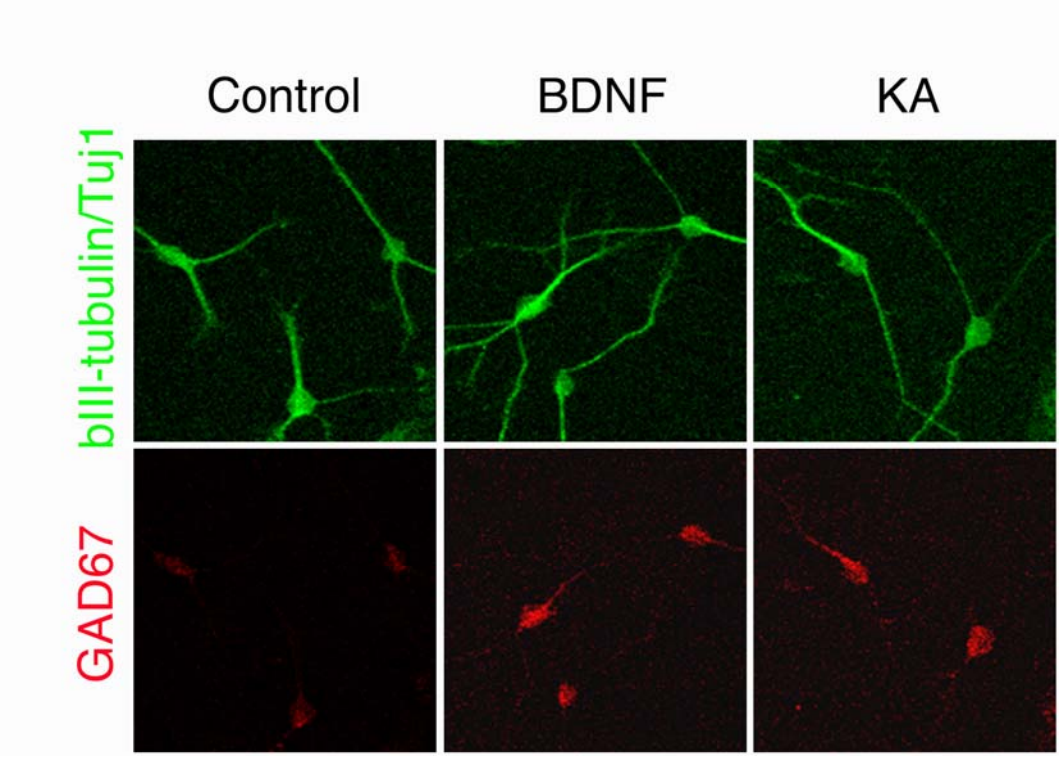

Figure 14: Upregulation of GAD67 by differentiated neurons

Neurons differentiated for 10-12 days were washed once with medium and subjected to 2.5hr stimulation of KA (10μM) or rhBDNF (100ng/ml) in fresh medium and subsequently fixed and evaluated. GAD67 (Red) is upregulated in β-III-tubulin-positive cells (Green), a property associated with granule neurons in vivo.

4.2 Neuronal activity induced regulation of adult neural precursor cells

4.2.1 Adult dentate gyrus precursor cells undergo differentiation in a co-culture model

In the adult DG the precursor cells are located in a region of intense neuronal network activity (Fig 15a-b). The precursor cells have complex morphologies and a subpopulation of them display highly arborizing processes that fan into the molecular layer similar to the mature neurons. The primary inputs to the DG arise from the entorhinal cortex.

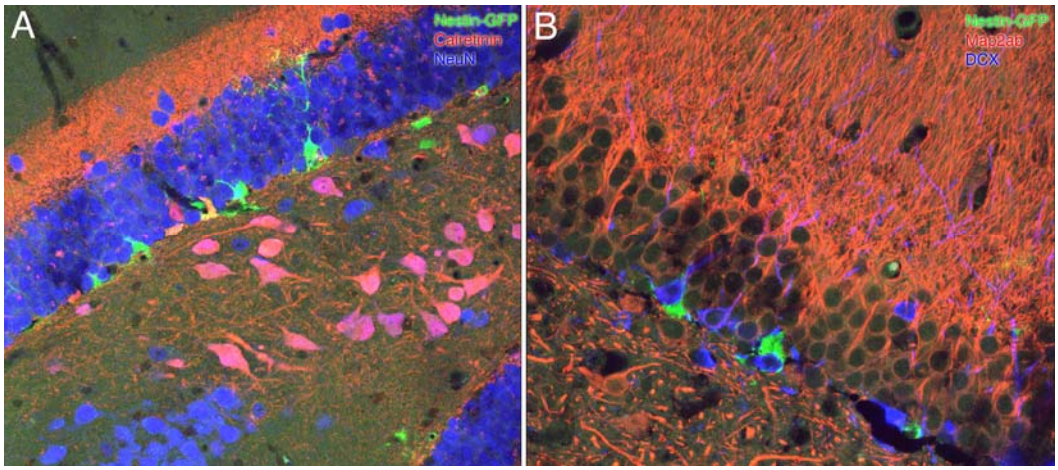

Figure 15: Close association of precursor cells with neurons in the DG

Transgenic adult nestin-GFP mice were perfused and stained for markers associated with neurons in the dentate gyrus. Tissue stained for **A;** GFP (Green), Calretinin (Red), NeuN (Blue) **B;** GFP (Green), Map2ab (Red), DCX (blue).

To reconstruct the intimate *in vivo* relationship between neurons and precursor cells *in vitro*, precursor cells were co-cultured on a layer of neurons and astrocytes. This allows one to subject the precursor cells to defined external conditions making them readily amenable to cellular and molecular analysis. For culturing primary neuronal cell,

postnatal day (0-1) mouse pups were killed and their hippocampus and the adjoining entorhinal cortex cultured for 10-14 days. This time period was chosen by this time neurons have matured, synapse formation is almost complete, and the cells elicit synaptic currents.

To further this study isolated adult DG precursor cells were infected with retrovirus carrying a vector with EGFP, and then subsequently purified by fluorescent activated cell sorting (FACS). These labeled cells were overlaid onto primary neuronal cultures. The co-culture with EGFP-labeled precursor cells enabled us to clearly distinguish the precursor cells and their progeny from the substrate culture. After the required time points, the cultures were fixed and evaluated for neuronal differentiation with antibodies against β-III-tubulin and Map2ab together with EGFP. β-III-tubulin labels the neuronal cell soma and their processes, whereas Map2ab, a structural protein associated specifically with postmitotic neurons, labels primarily the dendritic processes. After 6 days in culture, about 12% of the precursor cells had differentiated into neurons (Fig. 16a). These cells displayed characteristic neuronal morphologies with more than two fine processes (Fig. 16a). The cells that stained for antibody against β-III-tubulin at this stage were also positive for Map2ab. Precursor cells that differentiated into neurons consistently showed a lower intensity of EGFP labeling than their non-neuronal counterparts. Transgene downregulation has been reported previously and it depends on the site of viral integration into the genome host cell (Xu et al., 1989; van Praag et al., 2002).

Synaptophysin is a synaptic vesicle protein that labels presynaptic compartments within the axonal terminals impinging on the dendritic spines. When the differentiated precursor cells were allowed to mature for more than a week, EGFP labeled β-III-tubulin positive neurons had nicely decorated synaptophysin expression along their processes (Fig. 16b). Since dendritic spines represent the principle unitary domain for excitatory input, its presence on an EGFP positive newly differentiated neuron suggested integration into the existing underlying neuronal network.

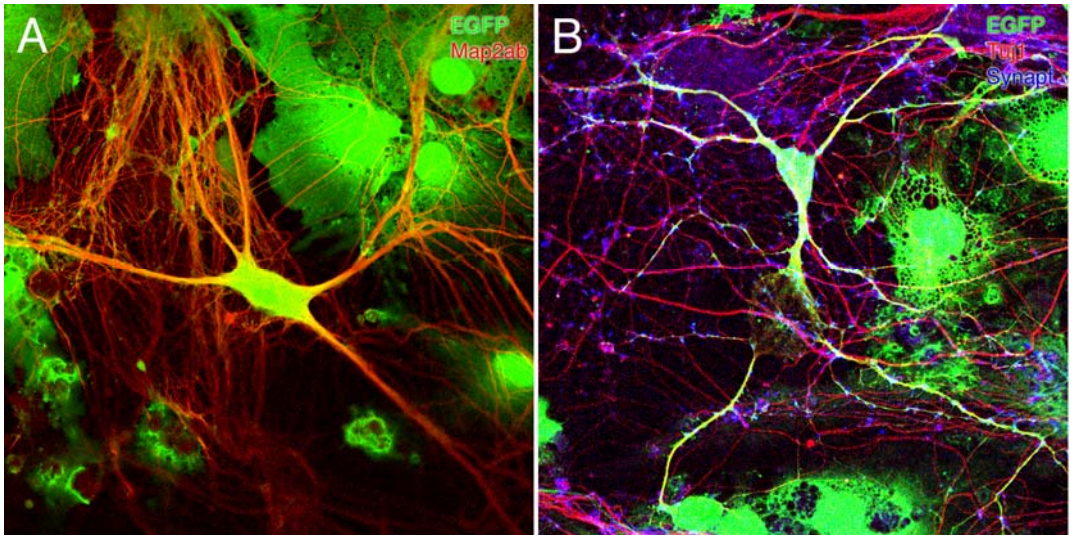

Figure 16: GFP expressing cells produce neurons in the coculture

A; EGFP labeled precursor cells were incubated with primary hippocampal cells. The precursor cells differentiated into neurons that coexpressed EGFP (Green) and Map2ab (Red). Cells that lacked neuronal morphology showed low or no expression of Map2ab while cells with neuronal morphology revealed high levels of Map2ab. **B;** The neurons that were differentiated also were synaptically integrated as revealed by their immunoreactivity against synaptophysin. EGFP (Green), β-III-tubulin (Red), synaptophysin (Blue). Note the synaptophysin puncta on the dendrites and soma of EGFP positive neuron.

4.2.2 Depolarization induces neuronal differentiation from adult precursor cells *in vitro* via activation of L-type calcium channels

To simulate the excitatory conditions in the co-culture the external concentration of potassium ions (K^+) was increased. This increases the neuronal excitation by changing the external ionic milieu casing depolarization of the neuronal membrane. To globally mimic neuronal activity that would putatively surround neuronal precursor cells *in vivo*, the external K^+ concentration was raised to 20 mM. Elevated K^+ concentration depolarizes the membrane as seen in response to neuronal activity (Leslie et al., 2001).

When external K^+ was raised in the cultures, there was a significant increase in cells with a neuronal phenotype as judged by morphological appearance and β-III-tubulin immunocytochemistry (Con 1.0, K^+ 2.78±1.4 $p < 0.05$ Fig. 17a,b). Consistent with previous reports in rat precursor cell cultures (Deisseroth et al., 2004), increased neuronal

activity was a potent regulator of neuronal differentiation from mouse neural precursor. Even though this suggests that precursor cells can sense activity level changes in neural network, the question remained whether this stimulus acts directly or indirectly on the precursor cells.

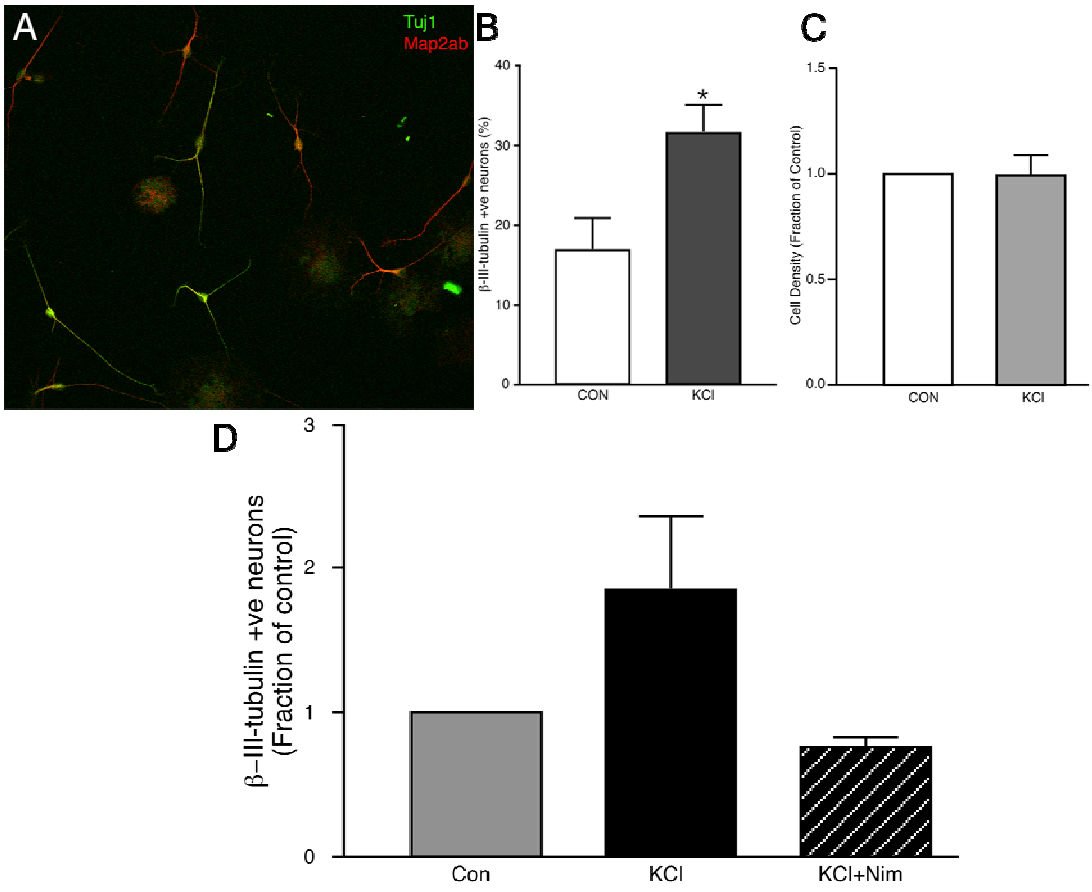

Figure 17: Neuronal activity promotes neuronal differentiation from adult DG precursor cells

A; Depolarization (KCl 20mM) increased the number of neurons generated from adult precursor cells, when cocultured with primary hippocampal neurons. The controls were incubated with equimolar NaCl. **B;** Depolarization induced neuronal differentiation from precursor cells cultured in the absence of neurons. **C;** The net cell density (cells/field of view) in the culture did not change. **D;** The L-type calcium channel blocker Nimodipine inhibited the neuronal differentiation induced by depolarization.

To clarify this question, naive precursors cells free of neuronal cells were subjected to elevated extracellular K^+ (20 mM), and neuronal differentiation was scored after staining for β-III-tubulin and Map2ab. As before, raised KCl led to an increased fraction of β-III-tubulin positive neurons (Con 1.0, KCl; 1.8 ± 0.5; $p< 0.03$; Fig. 17c,d). These cells had

multiple processes similar to neurons. The net cells density did not change suggesting this was not a selection effect of neurons over other cells types (Fig 17c). However, the increase in neuronal differentiation was inhibited by applying Nimodipine to the cells together with elevated K^+, suggesting that L-Type calcium channels were responsible for the effects seen consequent to depolarization (Fig. 17d). These results along with previous reports from rats suggest that neuronal activity regulates differentiation of adult hippocampal precursor cells via L-Type of calcium channels.

4.2.3 Gene expression triggered by depolarization in neural precursor cells

Calcium signaling within cells exhibits considerable polytropism imparted by its high spatial and temporal diversity. For complex multistep processes such as neuronal differentiation, full exploitation of the Ca^{2+} signaling machinery would require differential gene expression. RNA was harvested from precursor cells that underwent depolarization and subsequently RT-PCR was performed and compared with naïve precursor cells. Since Ca^{2+} is vital for differentiation and cell survival, it was hypothesized that some of the genes that are differentially regulated would be elements of the Ca^{2+} signaling toolkit. Camk4 that is regulated by Ca^{2+} and is localized in the nucleus could amplify the signal and thus produce pleotropic changes in the overall gene expression profile. Indeed, depolarized cells exhibited higher levels of RNA for Camk4 compared to controls (Fig. 18). To initiate and maintain any signal in favor of neuronal differentiation, pro-neurogenic transcription factor are a key constituent. Depolarization led to increased expression of NeuroD and Mash, transcription factors involved in dentate granule cells development *in vivo* (Liu et al., 2000; Pleasure et al., 2000; Schwab et al., 2000); Fig. 18). An increase in the transcription of DCX a cytoskeletal-associated protein was also seen in early immature neurons in the adult DG (Fig. 18). The increase was seen as early as 6 hr. One of the principle modifiers of perinatal hippocampal neurogenesis is the neurotrophin BDNF (Maisonpierre et al., 1990). As hypothesized depolarization led to a strong increase in the transcription of BDNF from the precursor cells (Fig. 18).

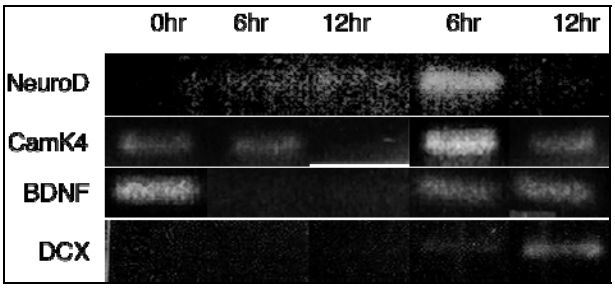

Figure 18: Gene expression changes as a result of depolarization

Depolarization with KCl lead to a robust increase in expression of genes important in the neuronal differentiation such as NeuroD, Camk4, BDNF and DCX.

4.2.4 LTP inducing stimuli leads to robust increase of neuronal differentiation

The above experiments suggested a role of neuronal activity in the regulation of neuronal differentiation from isolated precursor cells as well as in co-culture. But given the very non-specific action of K^+ triggered membrane depolarization, the question left open which kind of physiological neuronal activity pattern would be responsible for regulating neurogenesis. This question was again addressed using the co-culture system, where observed *in vivo* neural activity models can be imposed on neurons and the effects studied on the precursor cells in close proximity. Synchronized bursting and oscillatory activity characterize hippocampal network activity, which are involved in information coding (Lisman, 1997; Izhikevich et al., 2003). Such oscillatory activity leads to changes in synaptic strengths resulting in the induction of LTP, an experimental model for learning and memory. Synchronized oscillatory bursts were imposed in the co-culture model by activating the NMDA receptors present on the substrate neurons. Such NMDA dependent oscillatory activity has been shown to occur in hippocampal neurons (Bacci et al., 1999; Opitz et al., 2002; Arnold et al., 2005). The cultures were stimulated with a buffer lacking Mg^{2+} but added glycine to maximally activate the NMDA receptors allowing extracellular Ca^{2+} to enter via the NMDA receptors. To detect the rise in intracellular Ca^{2+} in the mature substrate neurons, intracellular calcium imaging was

performed with the dye Fura-2. When glycine was applied simultaneous rise in calcium concentration was detected (Fig 19a).

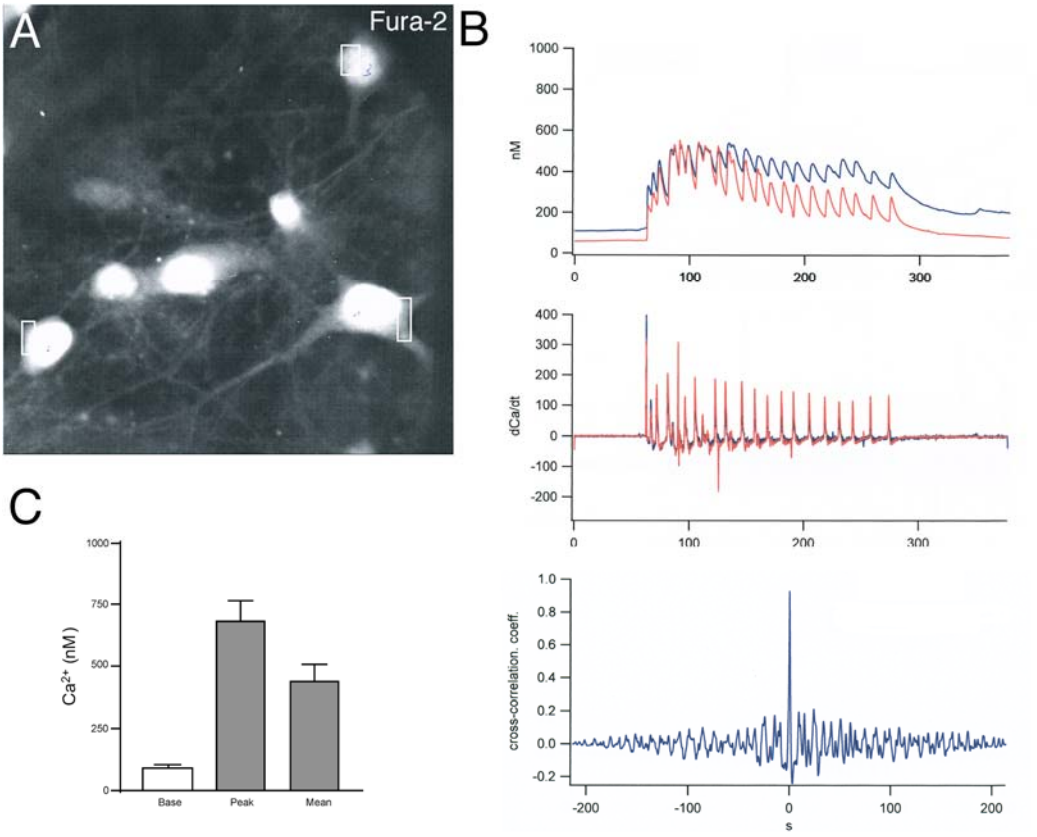

Figure 19: Primary hippocampal neurons show calcium oscillation on stimulating NMDA receptors

A; Example of cultured primary hippocampal neurons loaded with the calcium indicator dye Fura-2. Note that the cell bodies of the neurons can be clearly distinguished. **B;** Example of intracellular Ca^{2+} oscillation in neurons when stimulated with glycine. The Ca^{2+} oscillation in groups of neurons was synchronized. **B;** The change in the calcium concentration as a function of time remains highly synchronized in groups of neurons even though the individual neurons show variations in the definite intracellular Ca^{2+} concentration attained. The cross-correlogram between the two neurons shows that the there is a near perfect synchronization in the oscillation. **C;** The histogram shows the rest peak and the mean rise of intracellular Ca^{2+} attained in neurons when challenged with glycine.

This was not a bulk rise in Ca^{2+} but appeared rather as Ca^{2+} oscillation at 6-7 events per minute that was present as long as glycine was applied. The rise in intracellular Ca^{2+} within hippocampal neurons was quantified with Fura-2 (Fig 19c). The average Ca^{2+}

raised from a basal 89nM to an average of 475nM (Fig 19c). The peak calcium rise reached 680nM in some cases.

How would this oscillatory stimulus affect neuronal differentiation in the co-culture model? EGFP labeled precursor cells were again overlaid on to the hippocampal neuronal cultures. After 16-18 hr when the precursor cells had settled onto the substrate neurons, cultures were subjected to Mg^{2+} free buffer containing glycine to induce oscillatory activity in the neurons. The cultures were fixed 4-6 days later and immunostained for Map2ab and β-III-tubulin expression. There was a robust increase in the fraction of GFP labeled cells that had acquired neuronal phenotype (Con, 7.3±2.7, LTP, 12.4±2.2; $p<0.01$; Fig 20a). To rule out the possibility of LTP-like stimulus directly activating the precursor cells, precursor cells free of the substrate neurons was subjected to glycine and scored for the cells that underwent neuronal differentiation (Fig. 20b).

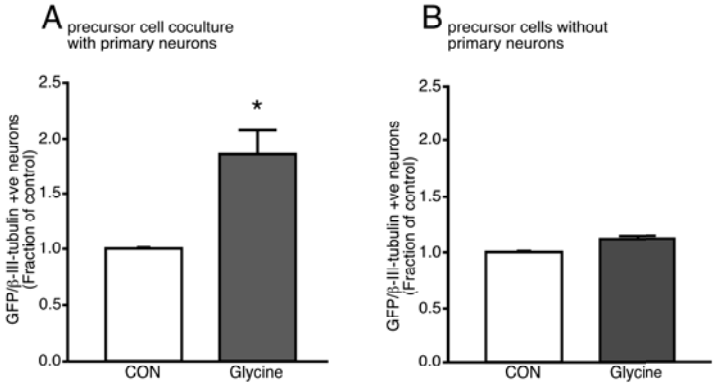

Figure 20: Glycine induced increase in neuronal differentiation

A; Adult neural precursor cells cocultured with neurons showed a significant increase in neuronal differentiation after application of glycine. **B;** Precursor cells cultured in the absence of neurons showed no changes in neuronal differentiation on application of glycine.

As hypothesized, in the isolated precursor cells there was no increase in the fraction of neurons generated (Fig 20b). This clearly demonstrated that neuronal differentiation in the co-culture was a result of the interaction between the neurons and the precursor cells. It is important to note that glycine was applied for only 5 min but produced an increase in neuronal differentiation from EGFP-labeled neural precursor cells measured several days later. This suggests that neural precursor cells could sense the change in the synaptic activity thus bringing about a change in the neuronal differentiation program. To identify

the mechanism for the increase in neuronal differentiation process from precursor cells by brief application of glycine, two possible modes of action were hypothesized.

First, the Ca^{2+} rise within the substrate neurons might cause action potentials to be initiated and propagated along the neighboring neurons. The precursor cells could detect these action potentials, thus initiating a neuronal differentiating program.

Second, the Ca^{2+} rise within neurons would cause synaptic plasticity-related events independent of the action potential generation. These events would be sensed by the neural precursor cell and translated to a possible change in neuronal differentiation.

To investigate whether action potential-related mechanisms are essential for the observed phenomenon, co-cultures were subjected to glycine together with Tetrodotoxin (TTX). TTX is a sodium channel blocker and thus would block the generation of any action potential in the substrate neurons. Surprisingly, the neuronal differentiation remained significantly enhanced compared to controls. This ruled out any direct involvement of action potential dependent mechanism in the enhancement of neuronal differentiation. To test the alternative hypothesis, synaptic communication between neurons as the key step in neuronal differentiation of precursor cells, electrophysiological measurements of synaptic events in the substrate neurons were performed (Fig. 21a). While holding the cells in the voltage clamp mode glycine was applied to cultured neurons and the postsynaptic response mEPSCs recorded from the neurons in the presence of bicuculline (to block inhibitory GABAergic responses) (Fig. 21d). A robust rise was observed in the frequency of the mEPSC compared to pre-glycine (Fig.21b). These increase were observed as early as 5 min after glycine withdrawal and remained potentiated for further 20 min; in some cases as long as for 40 min. Such LTP has been reported by others in hippocampal neurons and remains an experimental model for studying learning and memory processes in a cellular context (Lu et al., 2001; Malenka, 2003). During the application of glycine the neurons revealed an oscillatory period consisting of increased firing activity with periods of relative calm. If the rise in calcium (as seen previously with Fura-2 imaging) was indeed the cause for the LTP-like condition, then sequestering this calcium within the cells should also decrease

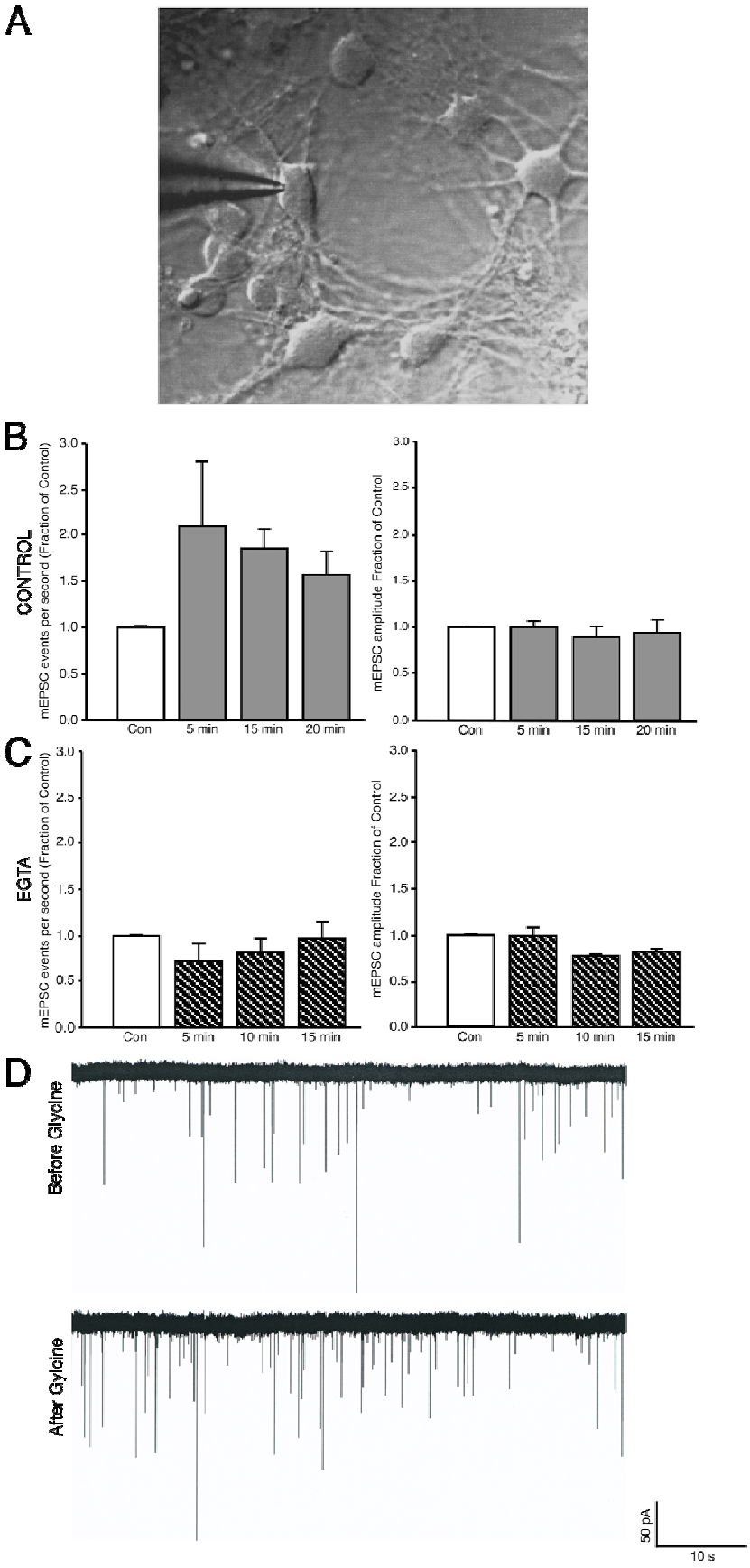
A
B
CONTROL
mEPSC events per second (Fraction of Control)
mEPSC amplitude Fraction of Control
Con
5 min
15 min
20 min
C
EGTA
mEPSC events per second (Fraction of Control)
mEPSC amplitude Fraction of Control
Con
5 min
10 min
15 min
D
Before Glycine
After Glycine
50 pA
10 s

Figure 21: Glycine induced a long term potentiation of mEPSC frequency in cultured neurons

A; Cultured neurons were patch clamp recorded in whole cell configuration. The mEPSCs were recorded from the neurons in the presence of bicuculline to isolate the glutamatergic responses **B;** On application of glycine for 5 minutes the mEPSCs increased in frequency compared to pre glycine (controls) levels. This increase was sustained for as long as 20 minutes after the cessation of glycine. **C;** This increase was completely inhibited when EGTA was included in the patch pipette to chelate the intracellular Ca^{2+}. **D;** Example of neurons that displayed a long term potentiation with glacine application. Note the increase in mEPSCs frequency before and after the application of the glycine.

the LTP. Patch clamp recordings were performed similar to ones described above but this time adding EGTA into the patch pipette. EGTA would chelate the Ca^{2+} within the cell cytoplasm. As expected when EGTA was included in the patch pipettes the neurons failed to elicit any change in the synaptic currents upon glycine stimulation (Fig. 21c). These results suggest that glycine induced synchronous oscillations in the substrate neurons, which resulted in an LTP-like elevation of synaptic strength.

Even though these studies confirm the indirect role of glutamatergic transmission on neuronal differentiation from neural precursor cells, recent results have suggested a key role for excitatory GABA signaling in promoting neuronal differentiation of precursors within the SGZ (Overstreet Wadiche et al., 2005; Tozuka et al., 2005; Wang et al., 2005; Ge et al., 2006). Two observations rule out the likely effect of GABA in the present model. First, the stimulation was done in the presence of bicucullin or SR-95531 – both potent inhibitors of $GABA_A$ receptor. As a result GABAergic transmission to the neural precursor cell could not be an activator of neuronal differentiation. Second, cocultures were incubated in the continued presence of bicucullin or SR-95531 (GBZ) without the glycine induced LTP like stimulus. Surprisingly, there was an increase in the neuronal differentiation from the precursor cells (Con 1.0; GBZ 1,63 ± 0.20 ; $p< 0.03$; Fig 22a,b). It is likely that these affects were a result of seizure like condition brought about by the disinhibition of the neuronal network. Nonetheless this supplements the idea that neuronal differentiation can occur in the absence of GABAergic input to the precursor cells.

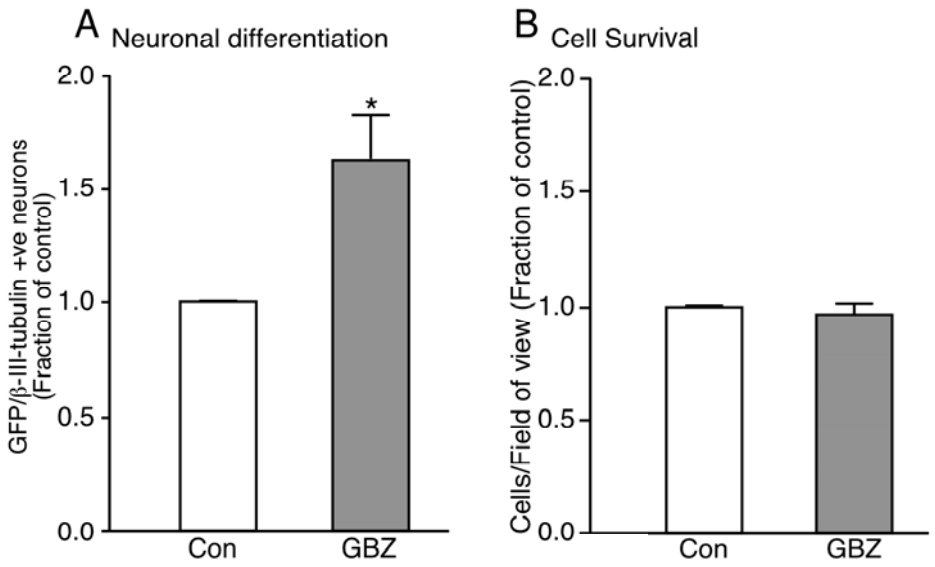

Figure 22: GABAergic neurotransmission is not responsible for the rise in neuronal differentiation following the application of glycine.

A; Cocultures were allowed to differentiate in the presence of the $GABA_A$ receptor antagonist SR-95331. There was an increase in neuronal differentiation suggesting that GABAergic neurotransmission is unlikely to be involved in regulating synaptic plasticity induced neuronal differentiation in cocultures. **B;** The cell survival did not change in the presence of $GABA_A$ receptor inhibitor compared to control.

4.2.5 Blockade of LTP by NMDA antagonists leads to suppression of neurogenesis in vitro

Blocking NMDA receptors *in vitro* abolishes LTP (Liao et al., 2001; Lu et al., 2001). If the culture conditions were indeed inducing neurogenesis because the network of neurons in the cultures underwent LTP, then blocking the induction of LTP should also inhibit the positive regulation of neurogenesis into the neuronal network seen with LTP. To address this question 2-Amino-5-Phosphonopentanoic acid (APV) was added to the stimulation paradigm as above to block any activated NMDA receptors. Patch clamp recordings were performed on the neurons to unambiguously detect whether the decrease in LTP was indeed due to APV administration. As expected APV along with glycine reduced the rise in mEPSC frequency compared to pre-stimulation levels (Fig 23a,b).

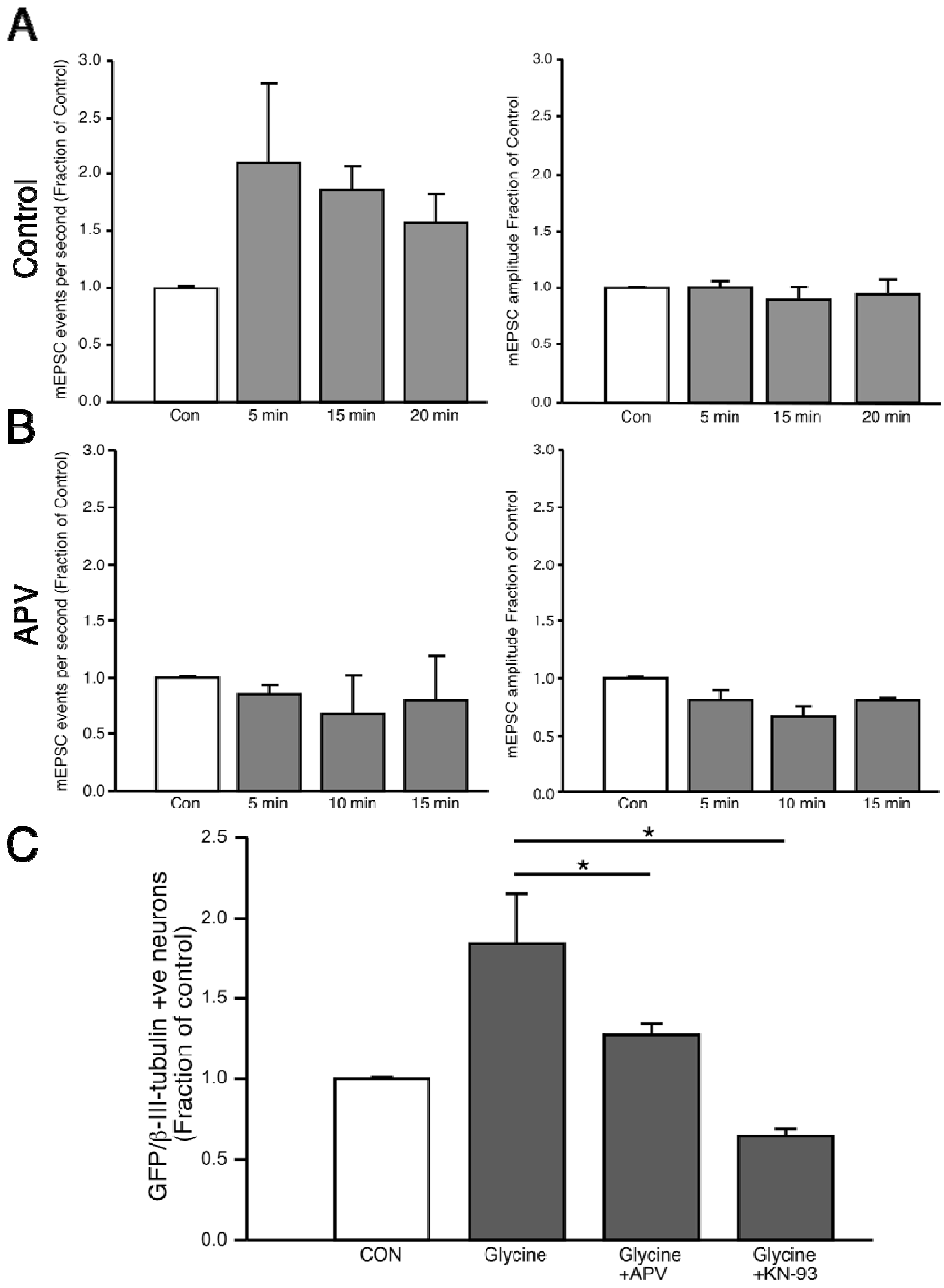

Figure 23: Blocking LTP inhibits the rise in neuronal differentiation in coculture

A; Control LTP similar to the one explained in the previous figure. **B;** When APV was added along with glycine the LTP of mEPSC frequency was abolished. **C;** The cocultures after stimulation with glycine to induce LTP was fixed 4 days later and quantified for EGFP positive neurons. Glycine produced a significant rise in the β-III-tubulin and EGFP double positive neurons. This LTP induced increase in neuronal differentiation was blocked by the APV, inhibitor of NMDA receptors and also by KN-93, an inhibitor of Camk.

As hypothesized, the addition of APV along with glycine in co-cultures abolished the rise in neuronal differentiation (Fig. 23c; Con, 1.0; Glycine, 1.84 ± 0.22; Glycine+APV, 1.27 ± 0.05). NMDA receptor activation induced intracellular Ca^{2+} rise activates the key enzyme Camk2α, which has been shown to be centrally involved in regulating the induction of LTP (Silva et al., 1992; Wu et al., 2006). If indeed LTP-inducing stimuli were responsible for the increase in neuronal differentiation then a block of Camk2α during the induction of LTP should lead to a block in the induction of neuronal differentiation and recruitment into neuronal network. When KN-93, a potent blocker of Camk2α activity, was added to the cultures during the induction of LTP, a significant reduction in the number of new neurons was observed (Con 1.0; Gly 1.84 ± 0.22; Gly+KN93 0.64 ± 0.03; Fig. 23c). As Camk2α is not expressed in precursor cells this observation further supports the view that synaptic activity induced changes were key to the shift in the precursor cells phenotype rather than a direct action of the stimulus. These results suggest that adult neural precursor cells can detect changes in synaptic activity that alters their phenotype.

4.2.6 Neurotrophins regulate the increase in neuronal differentiation from neural precursor cells

The above results suggested that upon stimulation with excitatory stimuli a neuronal network recruits new neurons from a pool of precursor cells. As direct application of these stimuli to isolated precursor cells did not produce similar effects, it seemed plausible that a secreted factor rather then a precursor cell-intrinsic mechanism would underly the induction of neuronal differentiation in response to LTP. Neurotrophins and especially BDNF that are secreted by neurons have been linked to a wide variety of responses related to synaptic plasticity. Several *in vivo* paradigms that increase adult hippocampal neurogenesis also show an increase in the transcription of neurotrophins (Sairanen et al., 2005; Scharfman et al., 2005; Rossi et al., 2006). To examine whether neurotrophins would also regulate neurogenesis in the present paradigm

of excitatory stimulus conditions, primary hippocampal neurons were subjected to LTP-inducing stimuli and the culture medium was harvested at several time points thereafter to detect changes in the levels of secreted neurotrophins. Release of neurotrophins – BDNF and NT3 into the medium was assessed by ELISA. As hypothesized, LTP-induced cultures had significantly higher levels of BDNF at 1 hr after cessation of the stimulus and remained elevated for at least 6 hr (Fig. 24b). This was seen only in the case of BDNF, whereas NT3 acting via TrkC receptor different from TrkB receptors like BDNF, did not show changes in response to LTP (Fig. 24c). This suggested that the co-culture model employed here was a source of neurotrophins, and BDNF level was regulated by the synaptic activity. Detection and transfer of this meaningful signal of elevated neurotrophins would foremost require the presence of a sensor in the form of receptor on the neural precursor cells. The presence of TrkB and TrkC receptors on the precursor cells was investigated by performing RT-PCR studies. Both (TrkB & TrkC) receptors were expressed in precursor cells that increased one day after the start of differentiation (Fig 24c). This suggested that precursor cells, as they differentiate, might be dependent on the levels of the neurotrophins present within the surrounding and precursor cells capable of garnering the neurotrophic signals would differentiate and show prolonged survival. If neurotrophins were indeed the key to the difference in the regulation of neurogenesis then blocking them should reduce the neuronal differentiation after LTP induction. Indeed, scavenging secreted BDNF with bio-neutralizing antibodies led to a decrease in β-III-tubulin /Map2ab-positive neurons (Con 1; α-BDNF 0.23±0.05 $p<0.003$; Fig. 24d). Blocking the effects of BDNF did not produce any change in the net cell survival, indicating a specific effect on the neuronal differentiation (Fig. 24e). Incubating neutralizing antibody against NT3 also resulted in a severe decrease in neuronal differentiation from the precursor cells in coculture without affecting the net cells survival (Fig. 24d,e). These results proves that neurotrophins released by neurons into the coculture medium is responsible for the increase in neuronal differentiation from adult precursor cells seen subsequent to LTP-like stimulus.

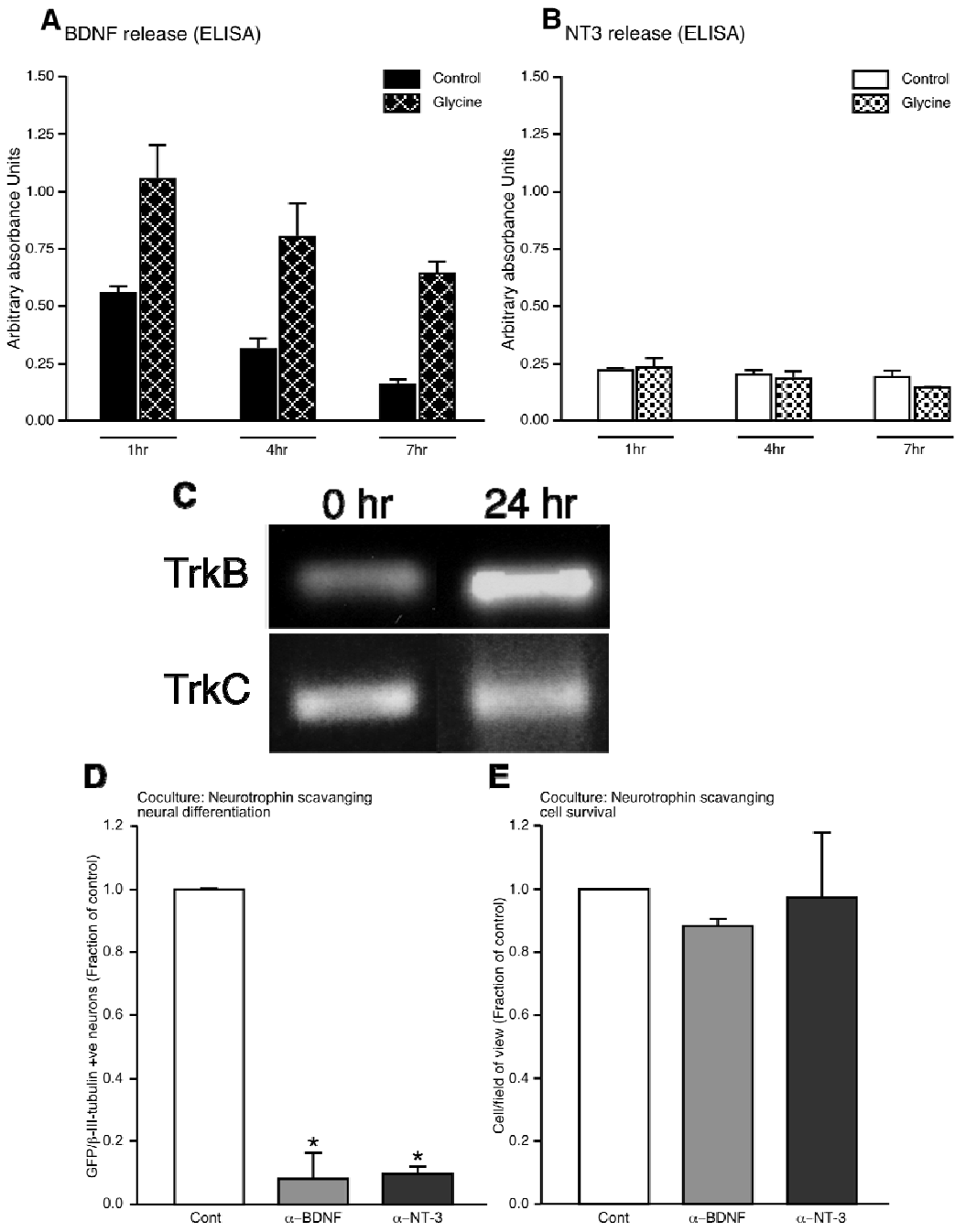

Figure 24: Glycine-induced release of BDNF from primary neurons detected by Trk receptors expressed by neural precursor cells

A; Stimulation of primary hippocampal neurons with glycine for 5 minutes increased the release of BDNF. The media was collected after 1hr, 4hr and 7hr and analyzed by ELISA for the presence of the neurotrophins BDNF and NT3. Glycine induced an increase in BDNF levels in the media at all time points assessed, while NT3 (**B**) levels were not regulated by glycine. **C;** The neural precursor cells expressed the receptors for both the neurotrophins. Both TrkB (BDNF receptor) and TrkC (NT3 receptor) was present at

low levels in proliferating cells but increased upon differentiation. **D;** There was a significant difference in neuronal differentiation when the cocultures were incubated with bio-neutralizing antibodies against BDNF (α-BDNF) and NT3 (α-NT3) after glycine stimulation. **E;** The net cells survival did not change when the neurotrophins when scavenged by bio-neutralizing antibody ($p < 0.005$).

5 Discussion

Precursor cells within the adult dentate gyrus have been linked to be important in hippocampal function but their properties have remained illusive. This thesis work focused on understanding the properties of precursor cells in the adult dentate gyrus by developing a modified precursor cells culture technique based on buoyancy enrichment. The main findings of this thesis work are:

1. Precursor cells from adult murine dentate gyrus can be cultured as monolayer that maintained homogeneity for extended passages and suggested features of radial glia.
2. Neurons generated from the precursor cells revealed immunochemical and functional characteristics similar to their in vivo counterparts, granule cells of the DG.
3. Single cell clonal analysis suggested that precursor cell cultures contained stem cells in the true definition displaying self-renewal and multipotentiality.
4. Neuronal differentiation from the precursor cells was strongly influenced by growth factors in the medium and also by the media composition.
5. Stem cells could be isolated from DG, SVZ and corpus callosum that revealed self-renewal and multipotentiality but not from the CA1 region of the hippocampus.
6. Global neuronal activity imposed by raised K^+ in cocultures of precursor cells with neurons led to increased neuronal differentiation from precursor cells by rapid induction of proneural gene expression.
7. LTP-induced increase in neuronal synaptic strength was associated with significant increase in neuronal differentiation from neural precursor cells in cocultures. Blocking LTP inhibited neuronal differentiation.

8. LTP-induced effects of neurons on precursor cell differentiation in cocultures were mediated by increased release of neurotrophins.

5.1 Characterization of precursor cells isolated from the adult murine dentate gyrus

5.1.1 Are there stem cells in the adult dentate gyrus?

In the present work, it is demonstrated that stem cells can be isolated and propagated from the adult murine hippocampus. A method to reliably obtain monolayer cultures of neural stem cells from the microdissected DG of adult mice is described in detail. There has been controversy regarding the presence or absence of neural stem cells in the DG in comparison to the SVZ (Palmer et al., 1997; Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). Precursor cells isolated from adult rat hippocampus and grown as adherent monolyaers have been shown to have stem cells properties (Palmer et al., 1997) while those grown from the mouse as neurospheres have argued against them (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). In summary, the present data suggest that the differences between the DG and SVZ are not qualitative but rather quantitative. Previous data in rat models and the data obtained in the present set of experiments indicate that the abundance of stem-like cells in the dentate is significantly lower than found in the SVZ (Palmer et al., 1995). Cellular composition of the precursor cell zone is more compact than in the SVZ as SVZ is surrounded by ventricle while the SGZ is surrounded by neurons and glia. Hence any enzymatic digestion leads to severing of the processes leading to poor recovery of the cells from the SGZ than from the SVZ.

The buoyancy enrichment method used in the present protocol allows one to normalize the initial plating density of the immature cell populations and thus to overcome a density dependent growth limitation that likely affected the outcome of previous other studies (Seaberg and van der Kooy, 2002; Bull and Bartlett, 2005). In addition, the dissection method used in the present study included the medial tip of the

DG, which was excluded in earlier studies that failed to detect cells with stem cell-like properties. This region within the DG contains the highest density of proliferating precursor cells *in vivo* (Tashiro et al., 2006*)*.

The present precursor cells isolation protocol is based on established methods (Palmer et al., 1995; Palmer et al., 1997; Seaberg and van der Kooy, 2002; Reynolds and Rietze, 2005) but substantial modifications to the available rat protocols had to be introduced (Table 2). These modifications combined the gradient enrichment procedure which was originally described for rat hippocampal precursor cells with the addition of both EGF and FGF2, the use of B27 as the serum supplement (instead of N2), the control of appropriate plating densities, and the optional use of exogenous factors for the trophic support of cultured cells. It is noteworthy that EGF is mandatory for maintaining stemness properties of precursor cells isolated from the DG. EGF and FGF2 together with B27 serum supplement suppressed differentiation and maintained the precursor cells at a stem cell stage with prolonged self renewal property.

Several studies in the developing telencephalon have suggested that radial glia have the potential to divide and generate neurons (Malatesta et al., 2000; Noctor et al., 2001; Noctor et al., 2002; Malatesta et al., 2003). It had been shown that BrdU incorporating cells in the SGZ in the adult DG have antigenic and morphological profile similar to radial glia (Seki and Arai, 1999; Steiner et al., 2006). The present data suggests that such cells can also be isolated from the adult DG. The observation that EGF is essential for proliferation of stem cells isolated from the adult DG together with other reports that EGF supports radial glial generation from periventricular region (Gregg and Weiss, 2003) suggests that radial glial may be a general feature of stem cells within the neurogenic regions of adult brain. Additional factors such as notch acting synergistically with EGF may be involved in the maintenance of such cells (Gaiano et al., 2000; Yoon et al., 2004).

Neurosphere cultures tend to have a high fraction of differentiated cells within their core. The interaction between differentiating cells and precursor cells may expose the stem cells to several pro-differentiation factors secreted by the cells from the core (Reynolds and Rietze, 2005). The adherent culture system overcomes these problems by allowing

Table 2 Comparison of precursor cell culture protocols from adult hippocampus

	Present study	**(Palmer et al., 1997)**	**(Palmer et al., 1999)**	**(Seaberg and van der Kooy, 2002)**	**(Roy et al., 2000)**	**(Bull and Bartlett, 2005)**
Species	Mouse	Rat	Rat	Mouse	Human	Mouse
Type	Monolayer	Monolayer	Monolayer	Neurospheres	Monolayer	Neurospheres
Region	Subdissected dentate gyrus, including tip	Hippocampus	Hippocampus	Subdissected dentate gyrus, excluding tip	Dentate Gyrus	Dentate Gyrus
Enrichment proc.	Percoll	–	Percoll	–	Promoter based FACS	Promoter based FACS
Plating density	10^4 cells/cm^2	No information	No information	20cells/µl	0.1ml/35mm dish of 10^7cells/ml of cell suspension	7000cells/cm^2
Media	Neurobasal	DMEM-F12	DMEM-F12	DMEM-F12	DMEM-F12	NeuroCult
Supplement	B27	N2	N2	N2/B27	N2, Platelet-depleted Bovine Serum	NeuroCult NSC supplement with 2% BSA
Growth factor	EGF, FGF2	FGF2	FGF2	EGF, FGF2, Heparin	FGF2	EGF, FGF2, Heparin, BDNF
Coating	PDL, Laminin	Polyornithine, Laminin	Polyornithine , Laminin	–	Laminin	–
Test for stemness	17.5 ± 2.1 %	–	21%	0.54 ± 0.1 per 10,000 cells	–	0
Max. passage no.	60	–	–	–	–	3
Functional assay	GAD induction	–	–	–	Electrophysiology	–

the cells to be continuously nurtured by the growth factors in the medium and maintaining homogeneity.

5.1.2 Exogenous growth factors regulate neuronal differentiation from adult neural precursor cells

Growth factors have a potent influence on the neuronal differentiation of precursor cells. The stem cells in the DG have been linked to a privileged niche comprising of factors secreted from various compartments of the brain parenchyma especially astrocytes and endothelial cells (Song et al., 2002; Shen et al., 2004). The identification of the secreted factors that regulate neurogenesis have been of intense research activity. Some of these molecules have been previously implicated in decisive developmental patterning of nervous system. The precursors were highly susceptible to the presence of EGF. Among all the factors we saw that this produced the most potent effect on proliferation. Shh, a morphogen potently increased the neuronal differentiation from precursors. Previous reports have found similar effects both *in vitro* and *in vivo* (Lai et al., 2003; Machold et al., 2003). Factors released by endothelial cells such as VEGF biased the precursor cells to acquire a neuronal phenotype, whereas BMPs and LIF were pro-gliogenic in the culture systems presented here. BMPs have previously been shown to produces a gliogenic niche for the SVZ precursor cells (Lim et al., 2000). The result that multiple factors differentially regulate the precursor cells differentiation suggests that these factors work in concert to streamline the output of DG neurogenesis from the precursors cells stage to the final functional integration. It is proposed that growth factors could regulate the precursor cells in their distinct stages allowing for fine-tuning at every level. Neural precursor cells express TrkB and TrkC receptors for BDNF and NT3 respectively. This was reflected by the positive regulation of neuronal differentiation from precursor cells when incubate with BDNF and NT3. Neurotrophins are released by neurons in the adult brain and is regulated by neuronal activity. Both constitutive and regulated secretion and occurs from neurons eventually producing neuronal differentiation survival (Farhadi et al., 2000). In the present work it is shown that

precursor cells express BDNF at low levels that increases upon differentiation. This expression can be further increased by depolarization though this was seen only for BDNF and NT3 did not show any increase. Sequestering the neurotrophins has a strong negative influence on neuronal differentiation and survival. Thus activity-independent and activity-dependent release of neurotrophins regulates neuronal differentiation.

5.1.3 Neurons generated *in vitro* from the dentate gyrus precursor cells exhibit properties similar to *in vivo* granule neurons

Neurons generated from the precursor cells in our culture system do fulfill the key criteria of functionality in that they generate sodium currents and fire action potential. It was noticed that during maturation the inward currents increased whereas the outward (possibly K^+) currents did not change much. This suggests that such outwards currents may be required in the transition from immature to mature stage of a precursor cell. These outward currents are hyperpolarizing thus might prevent potentially damaging massive Ca^{2+} entry as a result of sustained depolarization. It would be interesting to study the role of the various growth factors reported in the regulation of functional properties of the neurons differentiate. It is highly possible that neurons that "synapse" on precursor cells regulate neuronal differentiation by secretion of neurotransmitter or/and growth factors. It has been shown that GABAergic input is one of the earliest received by the new born neurons (Overstreet Wadiche et al., 2005; Tozuka et al., 2005; Wang et al., 2005; Ge et al., 2006). It has also been shown that some of the factors that have been reported here to have pro-neurogenic effects such as NT-3 and Shh are secreted by GABAergic interneurons (Traiffort et al., 1999). This suggests that GABAergic signaling may have a potent role in sculpting the DG neurogenesis in the adult brain with their dual role of network activity levels controls and release of morphogens in the vicinity of precursor cells. The cells in the cell culture also responded to the neurotrophic factors that have been repeatedly shown to influence various stages of neuronal differentiation during development. It would be interesting to study the various stages these factors influence the life history of a new born fully functional neuron.

Among the neurotransmitters that regulate neurogenesis in the adult brain, GABA has gained importance as GABA inputs are one of the earliest inputs arriving to these neurons (Overstreet Wadiche et al., 2005; Wang et al., 2005; Ge et al., 2006). Synaptically correlated activity is largely mediated by glutamate in the mature hippocampus. Inhibitory transmitters such as GABA also contribute to the excitation of young immature developing neurons by their excitatory nature in these cells as a result of the high intracellular Cl^- concentration (Ganguly et al., 2001). But in *in vivo* studies GABA receptor blockade has failed to detect a decrease in neurogenesis, rather showing an increase in adult hippocampal neurogenesis (Tozuka et al., 2005). These results are not surprising; given the inherent complexity of *in vivo* $GABA_AR$ activation in adult brain, exciting (depolarizing) the newborn neurons, but simultaneously inhibiting the general circuit activity level. When antagonists were applied *in vivo*, there was an increase in the hippocampal neurogenesis (Tozuka et al., 2005). This could be due to an overall increase in the network activity and underlines that even though GABAergic signaling is the first to arrive, glutamatergic signaling in the neuronal networks is a potent regulator of neuronal differentiation, acting likely in an indirect fashion. Transgenic animals which lack the GABA producing enzyme do not completely lack adult neurogenesis (Overstreet Wadiche et al., 2005). This was also seen in the present work, since the incubation of the cocultures with $GABA_AR$ inhibitor, led to increase in neuronal differentiating similar to that seen in the intact hippocampus. This is a significant result with alternative signaling mechanisms collaborating with glutamatergic transmission to regulate neurogenesis. Nonetheless GABA may have a trophic effect in regulating the survival of newly born neurons. Indeed, GABAergic inputs arrive a week after the cells have been born ruling out the possibility of direct GABAergic action in neuronal lineage specification (Overstreet Wadiche et al., 2005; Ge et al., 2006).

5.2 Neuronal activity regulates neuronal differentiation from adult dentate gyrus precursor cells

The results of the present study indicate that structural plasticity brought about by adult neurogenesis is the product of a complex and dynamically changing microenvironment and neurons are a critical causal component. Even though much is known about the role of astrocytes and endothelial cells (Song et al., 2002; Shen et al., 2004) in regulating neuronal differentiation from adult precursor cells, less is known about the role of neurons in this regard. Neural precursor cells within the SGZ of the DG are located in close association to the granule neurons. Upon differentiation the neuronal precursor cells themselves provide these granule neurons. To regulate this process it is likely that this close association is physiologically significant. Early in development, spontaneous activity in the embryonic brain and later in the early postnatal brain experience dependent activity have profound consequences in the formation of neural circuit (Zhang and Poo, 2001). Spatiotemporal patterns of activity play an instructive role on the development of neural patterning and the circuitry of the brain. In this work both global (depolarization by KCl) and synaptic (glycine induced) modifications induced by neuronal activity had a positive effect on differentiation of adult neural precursor cells. How do various forms of activity influence the neural precursor cell pool? Gene expression or protein translation changes induced by activity or by downstream effectors could change the molecular composition of the precursor cells leading to phenotypic fate changes. The present work shows some examples of such changes in the gene expression program. When depolarization was imposed on neural precursor cells by raised extracellular KCl, the precursor cells responded by transcriptional increase for NeuroD, Mash1, BDNF, and Camk4. NeuroD is a potent positive regulator of neurogenesis in various model systems (Ross et al., 2003). NeuroD knockout mice lack any normal dentate granule cells (Liu et al., 2000; Schwab et al., 2000). This malformation is largely due to the defect in transition from precursor cell stage to differentiated granule cells. Overexpression of NeuroD in *xenopus* has led to an increase in the fraction of neurons generated (Lee et al., 1995). NeuroD and Mash1 have been shown to direct the precursor

cells towards a neuronal fate (Cai et al., 2000). These transcription factors have profound changes in the cells that express them, by controlling several downstream genes as a result. Once expressed, NeuroD continues to be expressed well into maturity of granule cells, suggesting it may continue to regulate morphological and functional integration of the dentate granule cells. It is noteworthy that NeuroD was regulated by the influx of Ca^{2+} upon depolarization by raised extracellular KCl. Camk4 can integrate the Ca^{2+} signals arising from multiple sources and thus may represent a nodal point for the transcription mediated mechanisms of activity dependent neuronal differentiation. Camk4 is a nuclear localized kinase, which has previously been shown to regulate hematopoetic progenitor cell fate commitment in *xenopus* (Walters et al., 2002). One of the issues that remains to be solved is the temporal domain of the calcium rise within the precursor cells that is responsible for the change in the phenotypic program. Ca^{2+} spikes can initiate gene transcription programs more effectively than sustained increase in calcium levels (Dolmetsch et al., 1997; Dolmetsch et al., 1998). Cells often respond to stimulus intensity by altering the frequency of intracellular Ca^{2+} rise. Camk and PKC have been linked to decoding this frequency dependent signal of Ca^{2+} rise (De Koninck and Schulman, 1998; Oancea and Meyer, 1998; Mogami et al., 2003). It would be interesting to explore which of these kinases are involved in the neural precursor cells studied here.

The present work suggests that the principle source of Ca^{2+} entry is the L-type of calcium channel. Depolarization induced activation of calcium may also lead to the cleavage and translocation of the C-terminus of L-type calcium channel in the precursor cells (Gomez-Ospina et al., 2006). This nuclear translocation of the C-terminus activates transcription of several genes. It is likely that nuclear translocation of L-type calcium channel is an important mediator in the effects obtained in the present set of experiments. It is also possible that the translocated C terminus of the Ca^{2+} channel may collaborate with the transcription factors to increase the precision of the transcription, thus amplifying the signaling in the process. Phosphorylation of CREB has been detected in the newborn neurons in the DG in the basal condition (Nakagawa et al., 2002a; Nakagawa et al., 2002b). The increase in calcium influx produced by depolarization could intensify the phosphorylation of CREB, thus switching on the transcription machinery (Bito et al., 1996). Another potential candidate is DREAM, a Ca^{2+} binding

protein that is expressed in the nucleus and acts as a transcription factor (Osawa et al., 2001). Several other transcription factors such as NF-AT, MEF2 are also putative candidate in mediating the effects of Ca^{2+} influx via L Type calcium channel (Deisseroth et al., 2003).

5.2.2 Neuronal synaptic modification and calcium oscillation patterns regulate adult neurogenesis *in vitro*

In several developing neuronal circuits Ca^{2+} waves have been shown to be involved in patterning the neuronal circuits. The synchronous oscillations in the neurons could be a result of coupling by gap junctions. The intracellular Ca^{2+} increase through the NMDA receptor could also potentially induce further Ca^{2+} release from the internal stores. The developing cortex undergoes Ca^{2+} oscillations in various patterns depending on the gap junction coupling between various precursor cells (Owens and Kriegstein, 1998). Neural precursor cells isolated from the developing cortex showed Ca^{2+} oscillations that regulated their phenotypic differentiation into GABAergic neurons. Such Ca^{2+} transients have been shown to be involved in neuronal differentiation in *xenopus* embryos (Spitzer et al., 2000). Mature neurons also undergo Ca^{2+} oscillations that regulate synaptic strength and directionality (Berridge, 1998; Wang et al., 2000). Ca^{2+} oscillations lead to the regulation of several gene in the neurons (Borodinsky et al., 2004). Even though the role of Ca^{2+} oscillation in influencing individual cells during different stages of neuronal development is known, how oscillations in one cell type influences the other is not known. This is especially interesting in adult DG where mature functional neurons cosxist with precursor cells. In the present study, the effect of the Ca^{2+} waves in mature neuronal network showed a clear effect on the neighboring neural precursor cells. This study showed that Ca^{2+} oscillations are involved in the differentiation of precursors, which represant a cellular compartments different from the neuronal compartment generating the oscillations. Oscillating neurons were locked in synchronous oscillations and produced a clear increase in the synaptic strength among the

neurons. Induced oscillations could improve the fidelity of the signal conveyed by the neurons and provide a phasic relation to be established between firing times of nearby neurons. In the present study neurotrophins (BDNF) secreted by the neurons as a result of the oscillations led to increased neuronal differentiation from a precursor cell pool. It is not clear from the present study whether the rise in neurotrophin level was a result of the oscillations or stemmed from the subsequent increase in the synaptic strength of neurons. The oscillations could be used by the neuronal network as a means to communicate to the neural pool to make available new neurons for the network. As neuronal activity is present in all areas of the network, higher order functional entity such as synchronicity in activity could give abstraction to the network activity.

5.2.3 Neurotrophins mediate neuronal differentiation in activity-dependent neurogenesis

Evidence for the presence of ionotropic glutamate neurotransmitter receptors on precursor cells is lacking (Song et al., 2005). Precursor cells require an agent that is tightly coupled to the neuronal activity level to decode any neuronal activity. On the precursor level, a detector in the form of a receptor must also be present to relay this signal. Neurotrophins such as BDNF are in a unique position to fulfill this task. BDNF secretion following correlated neuronal activity can increase the sensitivity of gene transcription by collaborating with transcription machinery of the precursor cells.

In adult songbirds, BDNF secreted by endothelial cells was shown to be responsible for the generation of new neurons during the period when they learn new songs (Louissaint et al., 2002). This suggests that BDNF might play an instructive role in deciding the fate of precursor cells in these birds. In the present study this data has been extended to the mammalian brain, albeit here the source of neurotrophins were neurons. Neurons are in an ideal position to regulate neurogenesis by their ability to modulate the BDNF secretion depending on the levels of neuronal activity. In neurons BDNF causes TrkB dependent membrane depolarization within milliseconds (Kafitz et al., 1999). Thus,

BDNF may further induce differentiation from the neural precursor cells by causing depolarization and inducing further Ca^{2+} rise, amplifying the neurogenic signal. As the process of BDNF release and the subsequent biochemical interactions required to relay this information would take longer than Ca^{2+} channels, BDNF (with its depolarizing effect) may also help to sustain the intracellular Ca^{2+} rise in the precursor cells (Blum et al., 2002). This activity could also change the efficacy of non-ionophoretic membrane proteins such as adenylate cyclase (AC). ACs have voltage dependent effects which are independent of Ca^{2+} (Reddy et al., 1995). Studies have shown that activators of AC increase neuronal differentiation from rat hippocampal precursor cells (Takahashi et al., 1999). From the present data it is clear that BDNF also acts as a survival factor for newly born neurons. When BDNF was inactivated (by adding function-blocking antibodies), there was a stark reduction in survival. This survival promoting effects of BDNF is similar in action during early brain development, where BDNF acts to promote survival in developing cortical neurons (Ghosh et al., 1994). BDNF actions on cortical neurons has been linked to its role of activating MAPK and PI3K activity via the TrkB receptor (Dijkhuizen and Ghosh, 2005).

Several studies have shown that the release of BDNF is regulated by the stimulus pattern with high frequency stimulus being most effective (Balkowiec and Katz, 2000). We used stimulus that generated changes similar to the above-referred study, thus supporting the view that BDNF release may be a sensor for general activity. Within 30 min of seizures, BDNF mRNA levels dramatically increase in the DG (Ernfors et al., 1991). Neurotrophins modulate the capability of synapses to undergo LTP/LTD rather than mediate changes in synaptic efficacy (Poo, 2001). BDNF sequestration up to 1hr after the induction of hippocampal LTP caused previously potentiated synaptic transmission to return to baseline (Figurov et al., 1996; Korte et al., 1996). Transgenic animals with lowered BDNF levels display defects in basal transmission and hippocampal LTP. BDNF knockout mice also have decreased basal neurogenesis (Lee et al., 2002; Sairanen et al., 2005). It remains to be seen whether this decrease in DG neurogenesis is due to decreased synaptic plasticity in these mice. If so, BDNF levels may be used to detect levels of activity in a network, and it may drive neuronal

differentiation and survival when demand arises from a neuronal network (Schinder and Poo, 2000).

Neurotrophins also regulate synaptic morphology in an activity-dependent manner, an action that is critical for the development of long lasting LTP and long-term memory function. Neurotrophin secretion is regulated by neuronal activity level (Goodman et al., 1996). The present data suggests that part of the structural plasticity imposed by neurotrophins may be as a result of the new neurons that are added to the circuitry that would allow subsequent stimulus, the capacity to induce LTP in these newborn neurons. This would entitle the circuit to "maintain" its ability to learn new tasks even if the existing neurons are saturated in their resources to sense and respond to new stimuli. A subset of these new neurons may be "bystanders" for the incoming input and only coming into play once the "front liners" are overloaded. This mechanism would allocate a constant supply of young neurons primed to take on positions over an existing network on demand. Neurotrophins released by neurons in a permissive mode is thought to be involved in trophic regulation of synaptic function and the ability to generate LTP (Bonhoeffer, 1996; Poo, 2001). In the event of "increased" neuronal activity there is an elevated level of neurotrophin secretion superseding the existing permissive levels. This perhaps could have a more "instructive" role involving modifications of synaptic functions and contacts as well as generation of new neurons supplementing the existing network with fresh "memory compartments" in the form of new neurons. Within the SVZ system of adult neurogenesis, olfactory sensory stimuli influences the number of newly born neurons in the olfactory bulb (Rochefort et al., 2002). These animals also had improved memory of odors.

To maintain overall levels of neuronal and network activity, synapse specific forms of activity such as LTP and LTD need to be augmented by a wider global process. Addition of new neurons may provide an alternative variable to the existing theories of network homeostasis. Precursor cells capable of generating neurons in vitro exist in multiple brain regions but only two regions in the adult brain generate neurons, the SVZ and SGZ (Lie et al., 2004). This together with the present data implies that the pattern of neuronal activity may be unique in these areas allowing a permissive environment for neurogenesis to progress along the neuronal lineage. Song et al had demonstrated that

astrocytes from the two neurogenic regions differ from the astrocytes in the rest of the nervous system in their neurogenic induction capacities (Song et al., 2002). It remains to be shown whether neurons are also endowed with this inherent difference. One of the important observations from this result is that neutralizing BDNF from the medium in cocultures decreased neuronal differentiation but the net cell survival did not change. Thus even though the BDNF is necessary in generating neurons, the cell survival was differentially regulated. Neurons release some secreted factor other than BDNF or NT3 that was potent in cell survival (Lu et al., 2005) yet failed to generate neurons from the precursor cells. It would be interesting to find out the factor responsible for this effect and its possible role in adult neurogenesis.

5.2.5 Relevance of neuronal activity in neuropsychiatric disorders

Adult neurogenesis is exciting as deregulations of this process might contribute to diseases. Manipulation of neuronal activity could potentially be clinically relevant. Neuronal precursor cells migrate and invade brain regions after pathological insults. Though a link direct link between neurogenesis and pathological condition remains to be confirmed, several studies point to its significance in diseased states. Post mortem studies in patients with chronic depression show structural changes in hippocampus (Rajkowska, 2000; Rajkowska et al., 2005). Depression is associated with hippocampal atrophy and hippocampus is site for adult neurogenesis (Magarinos et al., 1996; Sapolsky, 1996; Sheline et al., 1996; Sheline et al., 2003). Suppression of neurogenesis might contribute to the pathological changes in the hippocampus observed in patients with depression (Gould et al., 1997; Gould et al., 1998). Patients suffering from schizophrenia also have abnormal neuronal synchrony in the hippocampus (Spencer et al., 2003; Spencer et al., 2004). It is possible that antidepressants induced increase neurogenesis may act as a consequence of oscillation induced by glutamatergic signaling. This hypothesis is based on arguments that hippocampal neurogenesis contributes significantly to cognitive functions in the adult brain and that hippocampus is the site for the pathology in these

psychiatric disorders. The number of neurons added into the hippocampus via adult neurogenesis is arguably too low to have any significant impact on the hippocampal function. Several secreted molecules such as BDNF and hormones (Kempermann and Kronenberg, 2003; Saarelainen et al., 2003; Berton et al., 2006) have been linked with psychiatric disorders as well as adult neurogenesis, but they also have roles outside the realms of SGZ. Adult neurogenesis has been shown to be necessary in the beneficial effects of antidepressants (Santarelli et al., 2003). Antidepressants are also potent inducers of BDNF in the adult hippocampus (Castren et al., 2006). Given the present data it is plausible that fluoxetine alters the synaptic plasticity in the mature neurons leading to BDNF release that acts on the precursor cells in the dentate gyrus. Newborn neurons have a higher threshold for action potential generation and could potentially destabilize the existing circuitry if there is an "erroneous" addition to a network. Seizures generate a large pool of new neurons that have been linked to generation of hyper excitability as well as. Electroconvulsive therapy (ECT) is a therapeutic approach in treating psychiatric and neurological disorders. And it is associated with increased neurogenesis in the hippocampus. Further studies are needed to examine the exact activity pattern that produces this effect of raised neurogenesis. Nonetheless, given that the role of neurogenesis remains obscure, there is room to link hippocampal neurogenesis in physiological and pathological processes. Raising the animals in enriched environment or BDNF injection (Komitova et al., 2005) increased the number of differentiated neurons in the stroke region. This suggests again that "enriching" the neuronal input to the precursors cell environment would reduce the failure in the survival of new neurons. BDNF can regulate neuronal activity by positively modulating neuronal activity favoring LTP (Kovalchuk et al., 2002). It would be interesting to examine whether BDNF could be replaced by raising the pattern of neuronal activity around the stroke area. Moreover, patterned electrical activity increases CREB phosphorylation- a factor that determines neuronal survival in a wide variety of neurons (Bito et al., 1996; Bonni et al., 1999).

6 References

Adachi T, Takanaga H, Kunimoto M, Asou H (2005) Influence of LIF and BMP-2 on differentiation and development of glial cells in primary cultures of embryonic rat cerebral hemisphere. J Neurosci Res 79:608-615.

Ahn S, Joyner AL (2005) In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature 437:894-897.

Altman J, Das GD (1965a) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319-335.

Altman J, Das GD (1965b) Post-natal origin of microneurones in the rat brain. Nature 207:953-956.

Alvarez-Buylla A, Garcia-Verdugo JM (2002) Neurogenesis in adult subventricular zone. J Neurosci 22:629-634.

Arnold FJ, Hofmann F, Bengtson CP, Wittmann M, Vanhoutte P, Bading H (2005) Microelectrode array recordings of cultured hippocampal networks reveal a simple model for transcription and protein synthesis-dependent plasticity. J Physiol 564:3-19.

Arsenijevic Y, Weiss S (1998) Insulin-like growth factor-I is a differentiation factor for postmitotic CNS stem cell-derived neuronal precursors: distinct actions from those of brain-derived neurotrophic factor. J Neurosci 18:2118-2128.

Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O (2002) Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med 8:963-970.

Axmacher N, Mormann F, Fernandez G, Elger CE, Fell J (2006) Memory formation by neuronal synchronization. Brain Res Rev 52:170-182.

Babu H, Cheung G, Kettenmann H, Palmer TD, Kempermann G (2007) Enriched monolayer precursor cell cultures from micro-dissected adult mouse dentate gyrus yield functional granule cell-like neurons. PLoS ONE 2:e388.

Bacci A, Verderio C, Pravettoni E, Matteoli M (1999) Synaptic and intrinsic mechanisms shape synchronous oscillations in hippocampal neurons in culture. Eur J Neurosci 11:389-397.

Balkowiec A, Katz DM (2000) Activity-dependent release of endogenous brain-derived neurotrophic factor from primary sensory neurons detected by ELISA in situ. J Neurosci 20:7417-7423.

Balkowiec A, Katz DM (2002) Cellular mechanisms regulating activity-dependent release of native brain-derived neurotrophic factor from hippocampal neurons. J Neurosci 22:10399-10407.

Berridge MJ (1998) Neuronal calcium signaling. Neuron 21:13-26.

Berton O, McClung CA, Dileone RJ, Krishnan V, Renthal W, Russo SJ, Graham D, Tsankova NM, Bolanos CA, Rios M, Monteggia LM, Self DW, Nestler EJ (2006) Essential role of BDNF in the mesolimbic dopamine pathway in social defeat stress. Science 311:864-868.

Bick-Sander A, Steiner B, Wolf SA, Babu H, Kempermann G (2006) Running in pregnancy transiently increases postnatal hippocampal neurogenesis in the offspring. Proc Natl Acad Sci U S A 103:3852-3857.

Bito H, Deisseroth K, Tsien RW (1996) CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. Cell 87:1203-1214.

Blum R, Kafitz KW, Konnerth A (2002) Neurotrophin-evoked depolarization requires the sodium channel Na(V)1.9. Nature 419:687-693.

Bonhoeffer T (1996) Neurotrophins and activity-dependent development of the neocortex. Curr Opin Neurobiol 6:119-126.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286:1358-1362.

Borodinsky LN, Root CM, Cronin JA, Sann SB, Gu X, Spitzer NC (2004) Activity-dependent homeostatic specification of transmitter expression in embryonic neurons. Nature 429:523-530.

Brandt MD, Jessberger S, Steiner B, Kronenberg G, Reuter K, Bick-Sander A, von der Behrens W, Kempermann G (2003) Transient calretinin expression defines early postmitotic step of neuronal differentiation in adult hippocampal neurogenesis of mice. Mol Cell Neurosci 24:603-613.

Brown J, Cooper-Kuhn CM, Kempermann G, Van Praag H, Winkler J, Gage FH, Kuhn HG (2003) Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. Eur J Neurosci 17:2042-2046.

Bull ND, Bartlett PF (2005) The adult mouse hippocampal progenitor is neurogenic but not a stem cell. J Neurosci 25:10815-10821.

Buzsaki G (2002) Theta oscillations in the hippocampus. Neuron 33:325-340.

Cai L, Morrow EM, Cepko CL (2000) Misexpression of basic helix-loop-helix genes in the murine cerebral cortex affects cell fate choices and neuronal survival. Development 127:3021-3030.

Cameron HA, McKay RD (1999) Restoring production of hippocampal neurons in old age. Nat Neurosci 2:894-897.

Castren E, Voikar V, Rantamaki T (2006) Role of neurotrophic factors in depression. Curr Opin Pharmacol.

Czeh B, Michaelis T, Watanabe T, Frahm J, de Biurrun G, van Kampen M, Bartolomucci A, Fuchs E (2001) Stress-induced changes in cerebral metabolites, hippocampal volume, and cell proliferation are prevented by antidepressant treatment with tianeptine. Proc Natl Acad Sci U S A 98:12796-12801.

D'Amour KA, Gage FH (2003) Genetic and functional differences between multipotent neural and pluripotent embryonic stem cells. Proc Natl Acad Sci U S A 100 Suppl 1:11866-11872.

De Koninck P, Schulman H (1998) Sensitivity of CaM kinase II to the frequency of Ca2+ oscillations. Science 279:227-230.

Deisseroth K, Mermelstein PG, Xia H, Tsien RW (2003) Signaling from synapse to nucleus: the logic behind the mechanisms. Curr Opin Neurobiol 13:354-365.

Deisseroth K, Singla S, Toda H, Monje M, Palmer TD, Malenka RC (2004) Excitation-neurogenesis coupling in adult neural stem/progenitor cells. Neuron 42:535-552.

Dijkhuizen PA, Ghosh A (2005) Regulation of dendritic growth by calcium and neurotrophin signaling. Prog Brain Res 147:17-27.

Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. Cell 97:703-716.

Dolmetsch RE, Xu K, Lewis RS (1998) Calcium oscillations increase the efficiency and specificity of gene expression. Nature 392:933-936.

Dolmetsch RE, Lewis RS, Goodnow CC, Healy JI (1997) Differential activation of transcription factors induced by Ca2+ response amplitude and duration. Nature 386:855-858.

Dranovsky A, Hen R (2006) Hippocampal neurogenesis: regulation by stress and antidepressants. Biol Psychiatry 59:1136-1143.

Duffy SN, Craddock KJ, Abel T, Nguyen PV (2001) Environmental enrichment modifies the PKA-dependence of hippocampal LTP and improves hippocampus-dependent memory. Learn Mem 8:26-34.

Ehninger D, Kempermann G (2006) Paradoxical effects of learning the Morris water maze on adult hippocampal neurogenesis in mice may be explained by a combination of stress and physical activity. Genes Brain Behav 5:29-39.

Ekdahl CT, Claasen JH, Bonde S, Kokaia Z, Lindvall O (2003) Inflammation is detrimental for neurogenesis in adult brain. Proc Natl Acad Sci U S A 100:13632-13637.

Ernfors P, Bengzon J, Kokaia Z, Persson H, Lindvall O (1991) Increased levels of messenger RNAs for neurotrophic factors in the brain during kindling epileptogenesis. Neuron 7:165-176.

Esposito MS, Piatti VC, Laplagne DA, Morgenstern NA, Ferrari CC, Pitossi FJ, Schinder AF (2005) Neuronal differentiation in the adult hippocampus recapitulates embryonic development. J Neurosci 25:10074-10086.

Fabel K, Tam B, Kaufer D, Baiker A, Simmons N, Kuo CJ, Palmer TD (2003) VEGF is necessary for exercise-induced adult hippocampal neurogenesis. Eur J Neurosci 18:2803-2812.

Farhadi HF, Mowla SJ, Petrecca K, Morris SJ, Seidah NG, Murphy RA (2000) Neurotrophin-3 sorts to the constitutive secretory pathway of hippocampal neurons and is diverted to the regulated secretory pathway by coexpression with brain-derived neurotrophic factor. J Neurosci 20:4059-4068.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B (1996) Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381:706-709.

Filippov V, Kronenberg G, Pivneva T, Reuter K, Steiner B, Wang LP, Yamaguchi M, Kettenmann H, Kempermann G (2003) Subpopulation of nestin-expressing progenitor cells in the adult murine hippocampus shows electrophysiological and morphological characteristics of astrocytes. Mol Cell Neurosci 23:373-382.

Fukuda S, Kato F, Tozuka Y, Yamaguchi M, Miyamoto Y, Hisatsune T (2003) Two distinct subpopulations of nestin-positive cells in adult mouse dentate gyrus. J Neurosci 23:9357-9366.

Gage FH (2000) Mammalian neural stem cells. Science 287:1433-1438.

Gage FH, Coates PW, Palmer TD, Kuhn HG, Fisher LJ, Suhonen JO, Peterson DA, Suhr ST, Ray J (1995) Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc Natl Acad Sci U S A 92:11879-11883.

Gaiano N, Nye JS, Fishell G (2000) Radial glial identity is promoted by Notch1 signaling in the murine forebrain. Neuron 26:395-404.

Ganguly K, Schinder AF, Wong ST, Poo M (2001) GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell 105:521-532.

Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H (2006) GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 439:589-593.

Ghosh A, Carnahan J, Greenberg ME (1994) Requirement for BDNF in activity-dependent survival of cortical neurons. Science 263:1618-1623.

Gomez-Ospina N, Tsuruta F, Barreto-Chang O, Hu L, Dolmetsch R (2006) The C terminus of the L-type voltage-gated calcium channel ca(v)1.2 encodes a transcription factor. Cell 127:591-606.

Goodman LJ, Valverde J, Lim F, Geschwind MD, Federoff HJ, Geller AI, Hefti F (1996) Regulated release and polarized localization of brain-derived neurotrophic factor in hippocampal neurons. Mol Cell Neurosci 7:222-238.

Gould E, McEwen BS, Tanapat P, Galea LA, Fuchs E (1997) Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. J Neurosci 17:2492-2498.

Gould E, Tanapat P, McEwen BS, Flugge G, Fuchs E (1998) Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proc Natl Acad Sci U S A 95:3168-3171.

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999a) Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 2:260-265.

Gould E, Reeves AJ, Fallah M, Tanapat P, Gross CG, Fuchs E (1999b) Hippocampal neurogenesis in adult Old World primates. Proc Natl Acad Sci U S A 96:5263-5267.

Gregg C, Weiss S (2003) Generation of functional radial glial cells by embryonic and adult forebrain neural stem cells. J Neurosci 23:11587-11601.

Gutierrez R (2005) The dual glutamatergic-GABAergic phenotype of hippocampal granule cells. Trends Neurosci 28:297-303.

Hack MA, Saghatelyan A, de Chevigny A, Pfeifer A, Ashery-Padan R, Lledo PM, Gotz M (2005) Neuronal fate determinants of adult olfactory bulb neurogenesis. Nat Neurosci 8:865-872.

Hoglinger GU, Rizk P, Muriel MP, Duyckaerts C, Oertel WH, Caille I, Hirsch EC (2004) Dopamine depletion impairs precursor cell proliferation in Parkinson disease. Nat Neurosci 7:726-735.

Izhikevich EM, Desai NS, Walcott EC, Hoppensteadt FC (2003) Bursts as a unit of neural information: selective communication via resonance. Trends Neurosci 26:161-167.

Jessberger S, Romer B, Babu H, Kempermann G (2005) Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. Exp Neurol 196:342-351.

Jin K, Zhu Y, Sun Y, Mao XO, Xie L, Greenberg DA (2002) Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo. Proc Natl Acad Sci U S A 99:11946-11950.

Jin K, Peel AL, Mao XO, Xie L, Cottrell BA, Henshall DC, Greenberg DA (2004) Increased hippocampal neurogenesis in Alzheimer's disease. Proc Natl Acad Sci U S A 101:343-347.

Johe KK, Hazel TG, Muller T, Dugich-Djordjevic MM, McKay RD (1996) Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. Genes Dev 10:3129-3140.

Kafitz KW, Rose CR, Thoenen H, Konnerth A (1999) Neurotrophin-evoked rapid excitation through TrkB receptors. Nature 401:918-921.

Kaplan MS, Hinds JW (1977) Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197:1092-1094.

Kaplan MS, Bell DH (1983) Neuronal proliferation in the 9-month-old rodent-radioautographic study of granule cells in the hippocampus. Exp Brain Res 52:1-5.

Kaplan MS, Bell DH (1984) Mitotic neuroblasts in the 9-day-old and 11-month-old rodent hippocampus. J Neurosci 4:1429-1441.

Kempermann G, Gage FH (2002) Genetic influence on phenotypic differentiation in adult hippocampal neurogenesis. Brain Res Dev Brain Res 134:1-12.

Kempermann G, Kronenberg G (2003) Depressed new neurons--adult hippocampal neurogenesis and a cellular plasticity hypothesis of major depression. Biol Psychiatry 54:499-503.

Kempermann G, Kuhn HG, Gage FH (1997a) Genetic influence on neurogenesis in the dentate gyrus of adult mice. Proc Natl Acad Sci U S A 94:10409-10414.

Kempermann G, Kuhn HG, Gage FH (1997b) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493-495.

Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. Trends Neurosci 27:447-452.

Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH (2003) Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. Development 130:391-399.

Kempermann G, Chesler EJ, Lu L, Williams RW, Gage FH (2006) Natural variation and genetic covariance in adult hippocampal neurogenesis. Proc Natl Acad Sci U S A 103:780-785.

Komitova M, Eriksson PS (2004) Sox-2 is expressed by neural progenitors and astroglia in the adult rat brain. Neurosci Lett 369:24-27.

Komitova M, Mattsson B, Johansson BB, Eriksson PS (2005) Enriched environment increases neural stem/progenitor cell proliferation and neurogenesis in the subventricular zone of stroke-lesioned adult rats. Stroke 36:1278-1282.

Korte M, Griesbeck O, Gravel C, Carroll P, Staiger V, Thoenen H, Bonhoeffer T (1996) Virus-mediated gene transfer into hippocampal CA1 region restores long-term potentiation in brain-derived neurotrophic factor mutant mice. Proc Natl Acad Sci U S A 93:12547-12552.

Kovalchuk Y, Hanse E, Kafitz KW, Konnerth A (2002) Postsynaptic Induction of BDNF-Mediated Long-Term Potentiation. Science 295:1729-1734.

Kukekov VG, Laywell ED, Suslov O, Davies K, Scheffler B, Thomas LB, O'Brien TF, Kusakabe M, Steindler DA (1999) Multipotent stem/progenitor cells with similar

properties arise from two neurogenic regions of adult human brain. Exp Neurol 156:333-344.

Lai K, Kaspar BK, Gage FH, Schaffer DV (2003) Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nat Neurosci 6:21-27.

Laywell ED, Rakic P, Kukekov VG, Holland EC, Steindler DA (2000) Identification of a multipotent astrocytic stem cell in the immature and adult mouse brain. Proc Natl Acad Sci U S A 97:13883-13888.

Lee J, Duan W, Mattson MP (2002) Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice. J Neurochem 82:1367-1375.

Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H (1995) Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science 268:836-844.

Lendahl U, Zimmerman LB, McKay RD (1990) CNS stem cells express a new class of intermediate filament protein. Cell 60:585-595.

Leslie KR, Nelson SB, Turrigiano GG (2001) Postsynaptic depolarization scales quantal amplitude in cortical pyramidal neurons. J Neurosci 21:RC170.

Liao D, Scannevin RH, Huganir R (2001) Activation of silent synapses by rapid activity-dependent synaptic recruitment of AMPA receptors. J Neurosci 21:6008-6017.

Lie DC, Song H, Colamarino SA, Ming GL, Gage FH (2004) Neurogenesis in the adult brain: new strategies for central nervous system diseases. Annu Rev Pharmacol Toxicol 44:399-421.

Lie DC, Colamarino SA, Song HJ, Desire L, Mira H, Consiglio A, Lein ES, Jessberger S, Lansford H, Dearie AR, Gage FH (2005) Wnt signalling regulates adult hippocampal neurogenesis. Nature 437:1370-1375.

Lim DA, Tramontin AD, Trevejo JM, Herrera DG, Garcia-Verdugo JM, Alvarez-Buylla An (2000) Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. Neuron 28:713-726.

Lisman JE (1997) Bursts as a unit of neural information: making unreliable synapses reliable. Trends Neurosci 20:38-43.

Liu M, Pleasure SJ, Collins AE, Noebels JL, Naya FJ, Tsai MJ, Lowenstein DH (2000) Loss of BETA2/NeuroD leads to malformation of the dentate gyrus and epilepsy. Proc Natl Acad Sci U S A 97:865-870.

Lois C, Alvarez-Buylla An (1994) Long-distance neuronal migration in the adult mammalian brain. Science 264:1145-1148.

Lois C, Garcia-Verdugo JM, Alvarez-Buylla An (1996) Chain migration of neuronal precursors. Science 271:978-981.

Louissaint A, Jr., Rao S, Leventhal C, Goldman SA (2002) Coordinated interaction of neurogenesis and angiogenesis in the adult songbird brain. Neuron 34:945-960.

Lu J, Wu Y, Sousa N, Almeida OF (2005) SMAD pathway mediation of BDNF and TGF beta 2 regulation of proliferation and differentiation of hippocampal granule neurons. Development 132:3231-3242.

Lu W, Man H, Ju W, Trimble WS, MacDonald JF, Wang YT (2001) Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. Neuron 29:243-254.

Machold R, Hayashi S, Rutlin M, Muzumdar MD, Nery S, Corbin JG, Gritli-Linde A, Dellovade T, Porter JA, Rubin LL, Dudek H, McMahon AP, Fishell G (2003) Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron 39:937-950.

Magarinos AM, McEwen BS, Flugge G, Fuchs E (1996) Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews. J Neurosci 16:3534-3540.

Maisonpierre PC, Belluscio L, Friedman B, Alderson RF, Wiegand SJ, Furth ME, Lindsay RM, Yancopoulos GD (1990) NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. Neuron 5:501-509.

Malatesta P, Hartfuss E, Gotz M (2000) Isolation of radial glial cells by fluorescent-activated cell sorting reveals a neuronal lineage. Development 127:5253-5263.

Malatesta P, Hack MA, Hartfuss E, Kettenmann H, Klinkert W, Kirchhoff F, Gotz M (2003) Neuronal or glial progeny: regional differences in radial glia fate. Neuron 37:751-764.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 20:9104-9110.

Malenka RC (2003) The long-term potential of LTP. Nat Rev Neurosci 4:923-926.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

Mehler MF, Rozental R, Dougherty M, Spray DC, Kessler JA (1993) Cytokine regulation of neuronal differentiation of hippocampal progenitor cells. Nature 362:62-65.

Misson JP, Edwards MA, Yamamoto M, Caviness VS, Jr. (1988) Identification of radial glial cells within the developing murine central nervous system: studies based upon a new immunohistochemical marker. Brain Res Dev Brain Res 44:95-108.

Mogami H, Zhang H, Suzuki Y, Urano T, Saito N, Kojima I, Petersen OH (2003) Decoding of short-lived Ca2+ influx signals into long term substrate phosphorylation through activation of two distinct classes of protein kinase C. J Biol Chem 278:9896-9904.

Molofsky AV, Slutsky SG, Joseph NM, He S, Pardal R, Krishnamurthy J, Sharpless NE, Morrison SJ (2006) Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. Nature 443:448-452.

Monje ML, Palmer T (2003) Radiation injury and neurogenesis. Curr Opin Neurol 16:129-134.

Monje ML, Toda H, Palmer TD (2003) Inflammatory blockade restores adult hippocampal neurogenesis. Science 302:1760-1765.

Monje ML, Mizumatsu S, Fike JR, Palmer TD (2002) Irradiation induces neural precursor-cell dysfunction. Nat Med 8:955-962.

Nakagawa S, Kim JE, Lee R, Chen J, Fujioka T, Malberg J, Tsuji S, Duman RS (2002a) Localization of phosphorylated cAMP response element-binding protein in immature neurons of adult hippocampus. J Neurosci 22:9868-9876.

Nakagawa S, Kim JE, Lee R, Malberg JE, Chen J, Steffen C, Zhang YJ, Nestler EJ, Duman RS (2002b) Regulation of neurogenesis in adult mouse hippocampus by cAMP and the cAMP response element-binding protein. J Neurosci 22:3673-3682.

Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M (2002) Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell 110:429-441.

Nilsson M, Perfilieva E, Johansson U, Orwar O, Eriksson PS (1999) Enriched environment increases neurogenesis in the adult rat dentate gyrus and improves spatial memory. J Neurobiol 39:569-578.

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR (2001) Neurons derived from radial glial cells establish radial units in neocortex. Nature 409:714-720.

Noctor SC, Flint AC, Weissman TA, Wong WS, Clinton BK, Kriegstein AR (2002) Dividing precursor cells of the embryonic cortical ventricular zone have morphological and molecular characteristics of radial glia. J Neurosci 22:3161-3173.

Oancea E, Meyer T (1998) Protein kinase C as a molecular machine for decoding calcium and diacylglycerol signals. Cell 95:307-318.

Opitz T, De Lima AD, Voigt T (2002) Spontaneous development of synchronous oscillatory activity during maturation of cortical networks in vitro. J Neurophysiol 88:2196-2206.

Osawa M, Tong KI, Lilliehook C, Wasco W, Buxbaum JD, Cheng HY, Penninger JM, Ikura M, Ames JB (2001) Calcium-regulated DNA binding and oligomerization of the neuronal calcium-sensing protein, calsenilin/DREAM/KChIP3. J Biol Chem 276:41005-41013.

Overstreet Wadiche L, Bromberg DA, Bensen AL, Westbrook GL (2005) GABAergic signaling to newborn neurons in dentate gyrus. J Neurophysiol 94:4528-4532.

Overstreet-Wadiche LS, Bromberg DA, Bensen AL, Westbrook GL (2006) Seizures accelerate functional integration of adult-generated granule cells. J Neurosci 26:4095-4103.

Owens DF, Kriegstein AR (1998) Patterns of intracellular calcium fluctuation in precursor cells of the neocortical ventricular zone. J Neurosci 18:5374-5388.

Palmer TD, Ray J, Gage FH (1995) FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol Cell Neurosci 6:474-486.

Palmer TD, Takahashi J, Gage FH (1997) The adult rat hippocampus contains primordial neural stem cells. Mol Cell Neurosci 8:389-404.

Palmer TD, Willhoite AR, Gage FH (2000) Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 425:479-494.

Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH (1999) Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J Neurosci 19:8487-8497.

Palmer TD, Schwartz PH, Taupin P, Kaspar B, Stein SA, Gage FH (2001) Cell culture. Progenitor cells from human brain after death. Nature 411:42-43.

Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997) Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. J Neurosci 17:3727-3738.

Pleasure SJ, Collins AE, Lowenstein DH (2000) Unique expression patterns of cell fate molecules delineate sequential stages of dentate gyrus development. J Neurosci 20:6095-6105.

Poo MM (2001) Neurotrophins as synaptic modulators. Nat Rev Neurosci 2:24-32.

Rajkowska G (2000) Postmortem studies in mood disorders indicate altered numbers of neurons and glial cells. Biol Psychiatry 48:766-777.

Rajkowska G, Miguel-Hidalgo JJ, Dubey P, Stockmeier CA, Krishnan KR (2005) Prominent reduction in pyramidal neurons density in the orbitofrontal cortex of elderly depressed patients. Biol Psychiatry 58:297-306.

Ray J, Gage FH (2006) Differential properties of adult rat and mouse brain-derived neural stem/progenitor cells. Mol Cell Neurosci 31:560-573.

Reddy R, Smith D, Wayman G, Wu Z, Villacres EC, Storm DR (1995) Voltage-sensitive adenylyl cyclase activity in cultured neurons. A calcium-independent phenomenon. J Biol Chem 270:14340-14346.

Reynolds BA, Weiss S (1992) Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707-1710.

Reynolds BA, Rietze RL (2005) Neural stem cells and neurospheres--re-evaluating the relationship. Nat Methods 2:333-336.

Rochefort C, Gheusi G, Vincent JD, Lledo PM (2002) Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. J Neurosci 22:2679-2689.

Ross SE, Greenberg ME, Stiles CD (2003) Basic helix-loop-helix factors in cortical development. Neuron 39:13-25.

Rossi C, Angelucci A, Costantin L, Braschi C, Mazzantini M, Babbini F, Fabbri ME, Tessarollo L, Maffei L, Berardi N, Caleo M (2006) Brain-derived neurotrophic factor (BDNF) is required for the enhancement of hippocampal neurogenesis following environmental enrichment. Eur J Neurosci 24:1850-1856.

Roy NS, Wang S, Jiang L, Kang J, Benraiss A, Harrison-Restelli C, Fraser RA, Couldwell WT, Kawaguchi A, Okano H, Nedergaard M, Goldman SA (2000) In vitro neurogenesis by progenitor cells isolated from the adult human hippocampus. Nat Med 6:271-277.

Saarelainen T, Hendolin P, Lucas G, Koponen E, Sairanen M, MacDonald E, Agerman K, Haapasalo A, Nawa H, Aloyz R, Ernfors P, Castren E (2003) Activation of the TrkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. J Neurosci 23:349-357.

Sairanen M, Lucas G, Ernfors P, Castren M, Castren E (2005) Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation, and survival in the adult dentate gyrus. J Neurosci 25:1089-1094.

Sandler R, Smith AD (1991) Coexistence of GABA and glutamate in mossy fiber terminals of the primate hippocampus: an ultrastructural study. J Comp Neurol 303:177-192.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805-809.

Sapolsky RM (1996) Why stress is bad for your brain. Science 273:749-750.

Scharfman H, Goodman J, Macleod A, Phani S, Antonelli C, Croll S (2005) Increased neurogenesis and the ectopic granule cells after intrahippocampal BDNF infusion in adult rats. Exp Neurol 192:348-356.

Scharfman HE (2004) Functional implications of seizure-induced neurogenesis. Adv Exp Med Biol 548:192-212.

Schinder AF, Poo M (2000) The neurotrophin hypothesis for synaptic plasticity. Trends Neurosci 23:639-645.

Schmidt-Hieber C, Jonas P, Bischofberger J (2004) Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature 429:184-187.

Schwab MH, Bartholomae A, Heimrich B, Feldmeyer D, Druffel-Augustin S, Goebbels S, Naya FJ, Zhao S, Frotscher M, Tsai MJ, Nave KA (2000) Neuronal basic helix-loop-helix proteins (NEX and BETA2/Neuro D) regulate terminal granule cell differentiation in the hippocampus. J Neurosci 20:3714-3724.

Seaberg RM, van der Kooy D (2002) Adult rodent neurogenic regions: the ventricular subependyma contains neural stem cells, but the dentate gyrus contains restricted progenitors. J Neurosci 22:1784-1793.

Seaberg RM, Smukler SR, Kieffer TJ, Enikolopov G, Asghar Z, Wheeler MB, Korbutt G, van der Kooy D (2004) Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotechnol 22:1115-1124.

Seki T, Arai Y (1999) Temporal and spacial relationships between PSA-NCAM-expressing, newly generated granule cells, and radial glia-like cells in the adult dentate gyrus. J Comp Neurol 410:503-513.

Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21:7153-7160.

Sheline YI, Gado MH, Kraemer HC (2003) Untreated depression and hippocampal volume loss. Am J Psychiatry 160:1516-1518.

Sheline YI, Wang PW, Gado MH, Csernansky JG, Vannier MW (1996) Hippocampal atrophy in recurrent major depression. Proc Natl Acad Sci U S A 93:3908-3913.

Shen Q, Goderie SK, Jin L, Karanth N, Sun Y, Abramova N, Vincent P, Pumiglia K, Temple S (2004) Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science 304:1338-1340.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E (2002) Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 12:578-584.

Silva AJ, Stevens CF, Tonegawa S, Wang Y (1992) Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science 257:201-206.

Singec I, Knoth R, Ditter M, Hagemeyer CE, Rosenbrock H, Frotscher M, Volk B (2002) Synaptic vesicle protein synaptoporin is differently expressed by subpopulations of mouse hippocampal neurons. J Comp Neurol 452:139-153.

Song H, Stevens CF, Gage FH (2002) Astroglia induce neurogenesis from adult neural stem cells. Nature 417:39-44.

Song H, Kempermann G, Wadiche LO, Zhao C, Schinder AF, Bischofberger J (2005) New neurons in the adult mammalian brain: synaptogenesis and functional integration. J Neurosci 25:10366-10368.

Spencer KM, Nestor PG, Niznikiewicz MA, Salisbury DF, Shenton ME, McCarley RW (2003) Abnormal neural synchrony in schizophrenia. J Neurosci 23:7407-7411.

Spencer KM, Nestor PG, Perlmutter R, Niznikiewicz MA, Klump MC, Frumin M, Shenton ME, McCarley RW (2004) Neural synchrony indexes disordered perception and cognition in schizophrenia. Proc Natl Acad Sci U S A 101:17288-17293.

Spitzer NC, Lautermilch NJ, Smith RD, Gomez TM (2000) Coding of neuronal differentiation by calcium transients. Bioessays 22:811-817.

Steiner B, Klempin F, Wang L, Kott M, Kettenmann H, Kempermann G (2006) Type-2 cells as link between glial and neuronal lineage in adult hippocampal neurogenesis. Glia 54:805-814.

Takahashi J, Palmer TD, Gage FH (1999) Retinoic acid and neurotrophins collaborate to regulate neurogenesis in adult-derived neural stem cell cultures. J Neurobiol 38:65-81.

Takamori S, Rhee JS, Rosenmund C, Jahn R (2000) Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. Nature 407:189-194.

Tashiro A, Sandler VM, Toni N, Zhao C, Gage FH (2006) NMDA-receptor-mediated, cell-specific integration of new neurons in adult dentate gyrus. Nature 442:929-933.

Taupin P, Gage FH (2002) Adult neurogenesis and neural stem cells of the central nervous system in mammals. J Neurosci Res 69:745-749.

Tozuka Y, Fukuda S, Namba T, Seki T, Hisatsune T (2005) GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron 47:803-815.

Traiffort E, Charytoniuk D, Watroba L, Faure H, Sales N, Ruat M (1999) Discrete localizations of hedgehog signalling components in the developing and adult rat nervous system. Eur J Neurosci 11:3199-3214.

van Praag H, Kempermann G, Gage FH (1999a) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2:266-270.

van Praag H, Christie BR, Sejnowski TJ, Gage FH (1999b) Running enhances neurogenesis, learning, and long-term potentiation in mice. Proc Natl Acad Sci U S A 96:13427-13431.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002) Functional neurogenesis in the adult hippocampus. Nature 415:1030-1034.

Walters MJ, Wayman GA, Notis JC, Goodman RH, Soderling TR, Christian JL (2002) Calmodulin-dependent protein kinase IV mediated antagonism of BMP signaling regulates lineage and survival of hematopoietic progenitors. Development 129:1455-1466.

Walton NM, Sutter BM, Laywell ED, Levkoff LH, Kearns SM, Marshall GP, 2nd, Scheffler B, Steindler DA (2006) Microglia instruct subventricular zone neurogenesis. Glia 54:815-825.

Wang LP, Kempermann G, Kettenmann H (2005) A subpopulation of precursor cells in the mouse dentate gyrus receives synaptic GABAergic input. Mol Cell Neurosci 29:181-189.

Wang SS, Denk W, Hausser M (2000) Coincidence detection in single dendritic spines mediated by calcium release. Nat Neurosci 3:1266-1273.

Wegner M, Stolt CC (2005) From stem cells to neurons and glia: a Soxist's view of neural development. Trends Neurosci 28:583-588.

Wiese C, Rolletschek A, Kania G, Blyszczuk P, Tarasov KV, Tarasova Y, Wersto RP, Boheler KR, Wobus AM (2004) Nestin expression--a property of multi-lineage progenitor cells? Cell Mol Life Sci 61:2510-2522.

Wu J, Rowan MJ, Anwyl R (2006) Long-term potentiation is mediated by multiple kinase cascades involving CaMKII or either PKA or p42/44 MAPK in the adult rat dentate gyrus in vitro. J Neurophysiol 95:3519-3527.

Xu L, Yee JK, Wolff JA, Friedmann T (1989) Factors affecting long-term stability of Moloney murine leukemia virus-based vectors. Virology 171:331-341.

Yamaguchi M, Saito H, Suzuki M, Mori K (2000) Visualization of neurogenesis in the central nervous system using nestin promoter-GFP transgenic mice. Neuroreport 11:1991-1996.

Yoon K, Nery S, Rutlin ML, Radtke F, Fishell G, Gaiano N (2004) Fibroblast growth factor receptor signaling promotes radial glial identity and interacts with Notch1 signaling in telencephalic progenitors. J Neurosci 24:9497-9506.

Zhang LI, Poo MM (2001) Electrical activity and development of neural circuits. Nat Neurosci 4 Suppl:1207-1214.

Zhao C, Teng EM, Summers RG, Jr., Ming GL, Gage FH (2006) Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci 26:3-11.

Zulewski H, Abraham EJ, Gerlach MJ, Daniel PB, Moritz W, Muller B, Vallejo M, Thomas MK, Habener JF (2001) Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes. Diabetes 50:521-533.

Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.

Selected Publication

Enriched monolayer precursor cell cultures from micro-dissected adult mouse dentate gyrus yield functional granule cell-like neurons
Harish Babu, Giselle Cheung, Helmut Kettenmann, Theo D Palmer, Gerd Kempermann
PLoS ONE 2007 Apr 25; 2: e388

Running in pregnancy transiently increases postnatal hippocampal neurogenesis in the offspring.
Anika Bick-Sander, Barbara Steiner, Susanne A Wolf, **Harish Babu**, Gerd Kempermann.
Proc Natl Acad Sci USA. 2006 Mar 7; 103(10): 3852-7

Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells.
Sebastian Jessberger, Benedikt Romer, **Harish Babu**, Gerd Kempermann.
Exp Neurol. 2005 Dec; 196(2): 342-51

Local origin and activity-dependent generation of nestin-expressing protoplasmic astrocytes in CA1
Golo Kronenberg, Li-Ping Wang, Martine Geraerts, **Harish Babu**, Michael Synowitz, Paloma Vicens, Gudrun Lutsch, Rainer Glass, Masahiro Yamaguchi, Veerle Baekelandt, Zeger Debyser, Helmut Kettenmann, Gerd Kempermann
(In press: **Brain, Structure and Function**)

Adult hippocampal precursor cells sense synaptic network plasticity to induce neuronal differentiation
Harish Babu, Gerardo Ramirez-Rodriguez Josef Bischofberger, Gerd Kempermann.
(manuscript in preparation)

Additive effects of physical exercise and environmental enrichment on adult hippocampal neurogenesis and gene expression
Susanne A. Wolf, Dan Ehninger, **Harish Babu**, and Gerd Kempermann.
(in revision)

Adult hippocampal neurogenesis predicts the size and plasticity of the infrapyramidal mossy fiber projection.
Benedikt Römer, Sebastian Jessberger, **Harish Babu**, Theo D. Palmer, Eric Wexler, Chunmei Zhao, Fred H. Gage, Linda Overstreet-Wadiche, Moritz D. Brandt, Dan Ehninger, and Gerd Kempermann.
(Manuscript in preparation)

Scientific and other trainings

1) Poster presentation, 08/2004, Route 28 Summits in Neurobiology, Frauenchiemsee, Bavaria, Germany.
2) Poster presentation, 07/2004, 4th Forum of European Neuroscience, Lisbon, Portugal.
3) Poster presentation, 10/2006, Society for Neuroscience 36th Annual meeting, Atlanta, USA.

Eidesstattliche Erklärung

Ich versichere an Eides statt, dass ich die vorliegende Dissertation “Isolation and characterization of neural precursor cells in the adult murine dentate gyrus” selbst und ohne unzulässige Hilfe Dritter verfasst habe, dass sie auch in Teilen keine Kopie anderer Arbeiten darstellt und dass die benutzten Hilfsmittel sowie die Literatur vollständig angegeben sind.

Berlin, den Juni, 2007
Harish Babu

Publications

Enriched monolayer precursor cell cultures from micro-dissected adult mouse dentate gyrus yield functional granule cell-like neurons
Harish Babu, Giselle Cheung, Helmut Kettenmann, Theo D Palmer, Gerd Kempermann
PLoS ONE 2007 Apr 25; 2: e388

Running in pregnancy transiently increases postnatal hippocampal neurogenesis in the offspring.
Anika Bick-Sander, Barbara Steiner, Susanne A Wolf, **Harish Babu**, and Gerd Kempermann.
Proc Natl Acad Sci USA. 2006 Mar 7; 103(10): 3852-7

Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells.
Sebastian Jessberger, Benedikt Römer, **Harish Babu**, Gerd Kempermann.
Exp Neurol. 2005 Dec; 196(2): 342-51

Local origin and activity-dependent generation of nestin-expressing protoplasmic astrocytes in CA1
Golo Kronenberg, Li-Ping Wang, Martine Geraerts, **Harish Babu**, Michael Synowitz, Paloma Vicens, Gudrun Lutsch, Rainer Glass, Masahiro Yamaguchi, Veerle Baekelandt, Zeger Debyser, Helmut Kettenmann, Gerd Kempermann
(In press: **Brain, Structure and Function**)