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D i s s e r t a t i o n

To the acquisition of the academic degree
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from Master of Science in Pharmacy Olga Chechneva
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Dedicated

To my mother

for her love, care and patience

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Abstract

Adult neurogenesis plays a role in many physiological (memory formation) and pathological (stroke, depression) processes. Increased neurogenesis in response to brain injury was recently reported and is considered a mechanism of regeneration after neuronal loss. There are evidences that numerous factors like glutamate, inflammation, and growth factors are involved in the post-insult increase of precursors proliferation and differentiation. However the mechanism of injury-induced neurogenesis is still poorly understood.

In the present study early neurogenesis *in vitro* in rat organotypic hippocampal slice cultures (OHC) was characterized and the complex interplay between neuronal damage, microglia activation, cell proliferation, neurogenesis and the role of inflammation after oxygen-glucose deprivation (OGD) was investigated. In addition the effect of exogenous growth factors, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) on proliferation and neurogenesis in OHC in the absence and presence of injury was studied.

Proliferation was assessed by bromodeoxyuridine (BrdU) incorporation, neurogenesis by BrdU-double labeling with doublecortin (DCX) or ß-III Tubulin, neuronal damage by propidium iodide uptake, microglia by OX-42 staining, pro-inflammatory cytokines by real-time RT-PCR.

Massive cell proliferation was observed in the glial envelope covering the slice. Glial envelope was formed by GFAP or GFAP/Nestin positive astrocytes and activated microglia during slice cultivation. The proliferation was lower inside of the slice culture. bFGF and EGF showed mitogenic properties in our system by increasing the number of BrdU+ cells.

In addition to the dentate gyrus (DG) OHC included a second neurogenic zone: the posterior periventricle (pPV), which is a part of the lateral ventricle wall. This structure lining the stratum oriens contained Nestin+ precursors. Morphological and functional differences

could be identified between DG and pPV precursor populations. Nestin+ cells with stellar morphology were found in the DG, while elongated Nestin+ cells were present in the pPV. bFGF treatment induced a fast but short-lasting neurogenic response in the DG while the pPV showed a more pronounced and long lasting neurogenic effect of bFGF.

After exposure of OHC to 40 min OGD microglia activation and upregulation of IL-1ß, TNF-α and IL-6 mRNA (2h after OGD) preceded the development of neuronal damage (6h after OGD) that was followed by an increase in cell proliferation (16h after OGD). BDNF, EGF or NGF treatment attenuated OGD-induced proliferation. Neurogenesis was inhibited at 3d after OGD in both neurogenic zones, however the restoration of neurogenesis was already observed at 6d. At this time point a significant increase of newly generated neurons was found in the pPV compared to control. Number of BrdU/ß-III Tubulin+ neurons was significantly increased in the pPV of OGD-exposed OHC by bFGF application.

MK-801, indomethacin or minocycline prevented the OGD-induced neuronal damage, cell proliferation and caused a decrease of OX-42+ microglia in the damaged area. Under control conditions MK-801, indomethacin or minocycline induced neurogenesis in the pPV. After OGD number of BrdU/DCX+ cells was decreased in the pPV by MK-801 treatment. However indomethacin or minocycline did not affect OGD-induced neurogenesis in the pPV.

In conclusion, this study is the first to show that i) two neurogenic zones, the DG and the pPV, are present in interface OHC; ii) the DG and pPV contain neural precursors with different neurogenic properties. High neurogenesis occur in the pPV while low neurogenesis is present in the DG of OHC; iii) inflammation is mounted in OHC at early time point after OGD. It is associated with activation, migration and proliferation of microglia; iv) due to microenvironmental changes neurogenesis in both neurogenic zones is inhibited early after OGD (3d) and restored later on (6d). OGD stimulates neurogenesis in

the pPV; v) neuroprotection against OGD-induced damage in OHC by anti-inflammatory treatment is associated with intact neurogenesis. Taking together, these *in vitro* data represent the evidences of injury-regulated neurogenesis in relation with inflammation in OHC that could be useful for further understanding of mechanisms of neurogenesis and anti-inflammatory treatment strategies after cerebral ischemia.

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1. Introduction

Neuronal loss occurs after different acute (stroke, trauma) or chronic (Parkinson disease, Alzheimer disease) neurodegenerative diseases. The recent discovery of adult neurogenesis opens the fascinating possibility to consider endogenous stem cells proliferation and neurogenesis as a potential mechanism of neuronal repair.

The presence of a large number of dividing cells in the central nervous system (CNS) has been known for almost a century (Allen, 1912) and considered as process of cell renewal. While glial cells can be reproduced by simple cell division, stem cells proliferation and neuronal differentiation of progenitor cells are required for neuron generation.

1.1. Neurogenesis in the CNS

Several neurogenic zones have been reported to retain an ability to generate neurons early after birth, however neurogenesis in these regions is diminished following postnatal development .

Fig. 1.1. Neurogenesis in the adult mammalian brain. Schematic sagittal section through the adult mouse brain. DG, dentate gyrus; LV, lateral ventricle; OB, olfactory bulb; RMS, rostral migratory stream; SGZ, subgranular zone; SVZ, subventricular zone. This figure was adapted from Doetsch and Scharff, 2001.

There are regions in the adult rodent brain (germinal layers) where neurogenesis has been observed to occur throughout life (Fig. 1.1) the subventricular zone (SVZ) lining the lateral ventricle (LV) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) with additional reports of neurogenesis in the Ammon´s horn and substantia nigra .

1.1.1. Neurogenesis in the subventricular zone

In the SVZ, cells are born over a large area adjacent to the LV. From their site of birth, chains of young neurons migrate along a complex network of tangential pathways and join the rostral migratory stream (RMS), which leads to the olfactory bulb, where new cells differentiate into two types of local interneurons, granule and periglomerular neurons .

1.1.2. Neurogenesis in the hippocampus

Cells born in the SGZ migrate a short distance and differentiate into new granule neurons within the DG . The newborn neurons in the SGZ are long-lived and generate appropriate projections within the existing hippocampal architecture . Since the hippocampus plays an important role in learning and memory and exhibits a high degree of structural plasticity , neurogenesis in the adult hippocampus may be important for learning and memory in mammalians.

1.1.3. Stem cells and their origin

Stem cells have been characterized as cells with long-term self-renewal (self-renewal throughout the lifetime of the animal) and multipotentiality. Being proliferating, stem cells give rise to progenitor cells, which exhibit less self-renewal ability and unipotentiality or multipotentiality. The general term to refer to either a mixed or unknown proliferating population is precursors . Progenitor cells then differentiate into neuronal or glial lineage, giving rise to neurons or astrocytes and oligodendrocytes, respectively .

The identity of stem cells is still under discussion. One of the hypothesis confirmed astrocyte origin of stem cells has been proposed by Alvarez-Buylla with colleges . They described the structure of germinal zones and characterized stem cells as glial fibrillary acidic protein (GFAP)+ B-type cells with astrocyte morphology. Alvarez-Buylla et al. also reported the presence of neuroblasts (A-type), rapidly dividing transiently-amplifying cells (C-type) and

ependymal cells (E-type) in the SVZ. An intermediate precursors (D-type), which generate new granule neurons (G-type) were identified in the SGZ of the DG . Other hypothesis suggested that new neuron could derive from radial glia or subependymal cells of LV wall (LVw) . Neural precursor cells situate in a permissive microenvironment (niche) in the neurogenic regions. The phenotype that is adopted by neural precursors depends on the location of the stem cells in the brain and on the type of cells that are needed in that area .

1.1.4. Experimental detection of cell phenotype

Immunohistochemistry is mainly used to define the cell phenotype. Labeling of specific cell structures (receptors, filaments) and metabolites (nuclear, cytoplasmic or synaptic proteins) could define not only cell phenotype but also e.g. cell maturation stage, physiological functions etc. The characteristics of several cell markers are shown in Table 1.

Table. 1. Cell markers used for immunohistochemical cell characterization

Cell marker	Cell type	Characteristic	References
Stem and progenitor cells			
Nestin	Neural stem cells Astrocytes Undifferentiated neuroepithelial cells	Intermediate filament protein	

Cell marker	Cell type	Characteristic	References
Mash-1	Neuronal Progenitors	Transcription factor	
Prox-1	Neuronal Progenitors	Transcription factor	
Proliferating cells			
Bromodeoxyuridine (BrdU)	Mitotic cells in S-phase Cells undergoing to DNA repair Apoptotic cells reentering the cell cycle	Tymidine analog which incorporates into the DNA of cells during S-phase of cell cycle	
Proliferating cell nuclear antigen (PCNA)	Mitotic cells in G1, S and partially G2/M phase Cells undergoing DNA repair	Co-factor for DNA polymerase delta in S-phase as well as during DNA synthesis associated with mechanisms involved in DNA damage repair	
Neuronal cells			
Doublecortin (DCX)*	Proliferating progenitors Neuroblasts	Microtubule-associated protein expressed	

	Migrating neurons Differentiating neurons	transiently early after division
ß-III Tubulin	Young immature neurons Mature neurons	Neuron-specific marker. Starts to be expressed after birht and continues throughout the neuronal life
Neuronal nuclei (NeuN)	Mature neurons	Neuron-specific nuclear protein expressed on post-mitotic neurons
Microtubule associated protein-2 (MAP-2)	Mature neurons	Cytoskeletal protein, regulats the assembly and stability of neuronal microtubules
Glial cells		
Glial fibrillary acidic protein (GFAP)	Mature astrocytes Progenitor cells	Intermediate filament composed in large part of glial fibrillary acidic protein
S-100ß	Immature and mature astrocytes	Cytosolic Ca^{2+}-binding protein, not "glia specific" marker
Griffonia simplicifolia Isolectin B4 (IB4)	Ramified and amoeboid microglia	Isolectin binds with galactose containing glycoconjugates (lectin receptors)
CD11b/c (OX-42)	Microglia Macrophages	Surface complement receptors type 3

*Hippocampal granule neurons still express DCX in mature stage, for 2-4 weeks after birth.

To find out the phenotype of a newly generated cell, proliferating cells are labeled with DNA replication marker bromodeoxyuridine (BrdU), a thymidine analog, which incorporates into the DNA of all cells during the S-phase of cell cycle . Further cell fate can be investigated by double labeling of BrdU with a specific cell marker.

1.1.5. Factors regulating neurogenesis

Neurogenesis is a complex phenomenon and is affected by many factors (see Table 2). Neurogenesis in mammals can be upregulated/induced by injuries , growth factors (GFs), neurotransmitters and physical exercise while neurogenesis can be downregulated by

depression, stress and age . Much research effort has been invested into understanding the mechanism and regulation of adult neurogenesis, and considerable progress has been made over the past 10 years . GFs have been shown to play a significant role in the mechanism of neurogenesis .

1.1.6. Growth factors

Numerous GFs are involved in the regulation of cell proliferation, neurogenesis and cell survival in the postnatal and adult brain . The GFs can be classified in 1) GFs families e.g. fibroblast growth factor (FGF) family, transforming growth factor family etc.; 2) neurotrophins e.g bran-derived neurotrophic factor (BDNF), nerve growth factor (NGF) etc.; 3) GFs like epidermal growth factor (EGF), stem cells factor (SCF) etc.

Glial cells as supportive cells in the nervous system and endothelial cells are the main source of GFs in the brain parenchyma. The biological functions of GFs are mediated by binding of these secreted proteins to their transmembrane tyrosine kinase receptors that causes the formation of tyrosine kinase dimers .

Table 2. Factors affecting neurogenesis in the hippocampus and SVZ

Factor	Neurogenesis		References
	DG	SVZ	
bFGF	Increase	increase	
BDNF	increase	increase	
EGF	decrease	decrease	
Glutamate	decrease	n.d.	
MK-801	increase	increase	
Glucocorticoids	decrease	n.d	
Age	decrease	decrease	
Depression	decrease	n.d.	
Enriched environment	increase	increase	
Wheel running	increase	increase	

Nitric oxide donor	increase	increase
Global ischemia	increase	n.d.
Focal ischemia	increase	increase

n.d., not determined; bFGF, basic fibroblast growth factor; BDNF, brain-derived neurotrophic factor; EGF, epidermal growth factor.

The ligand-mediated aggregation of the receptors allows them to phosphorylate one another on intracellular domains and then catalyzes the formation of large signaling complexes through the recruitment of cytosolic and membrane associated proteins. The newly formed complex of proteins activates several kinase pathways: phosphatidylinositol-3-kinase; phospholipase C-γ and protein kinase C (PKC), which control cell proliferation and differentiation, cell growth, synaptogenesis, activity dependent plasticity and cell survival . All neurotrophins bind with low affinity $p75^{NTR}$ receptors that regulate nuclear factor-κB downstream pathway and control cell division and apoptosis . Some characteristics of GFs, whose mitogenic and neurogenic effects were examined in this work, are present in the Table 3.

Table 3.Characteristics of bFGF, EGF, BDNF and NGF

Growth factor	Type of receptors	Characteristic	References
bFGF	Family of four receptors tyrosine kinases: FGFR1-4	1. mRNA-encoding bFGF is detected at early stages of rodent CNS development; 2. bFGF and it's family of receptors is widely distributed in the adult CNS, especially in the hippocampus and SVZ; 3. Stimulate proliferation of neural precursors and neuronal differentiation; 4. Mitogen for precursor cells isolated from brain parenchyma.	
EGF	Tyrosine kinase EGFR	1. EGF and EGFR not so widely expressed as FGF family; 2. EGF receptors are expressed in the germinal zones by E15, postnataly in the SVZ and SGZ; 3. Stimulate proliferation of precursors in the developing and adult brain; 4. Stimulate glial rather than neuronal differentiation.	
BDNF	High affinity tyrosine kinase receptors,TrKB, and low affinity p75 receptors	1. High TrkB expression in the subependimal layer of the LV; 2. Regulate development of large amount of neuronal cell types; 3. Stimulate neurogenesis in the SVZ;	

		4. Promote the survival of newly generated and post-mitotic neurons.
NGF	High affinity tyrosine kinase receptors,TrKA, and low affinity p75 receptors	1. Stimulate neuronal differentiation of neural precursors; 2. Stimulate neurite outgrowth; 3. Regulate neuroprotection and sell survival; 4. Trophic function.

bFGF, basic fibroblast growth factor; BDNF, brain-derived neurotrophic factor; EGF, epidermal growth factor; NGF, nerve growth factor.

Since GFs possess a neurogenic effect and can sustain neuronal survival, GF therapy might provide an opportunity for pharmacological manipulations of endogenous neurogenesis and treatment of diseases associated with neuronal loss such as cerebral ischemia.

1.2. Cerebral ischemia

1.2.1. Pathophysiology of ischemic cell death

With an incidence of approximately 250-400 cases in 100 000 and a mortality rate of around 30%, cerebral ischemia remains the third leading cause of death in industrialized countries . Clinically, two major forms of ischemic damage can be distinguished: stroke and hemorrhage. Stroke, is the complete or partial interruption of cerebral blood flow whereas hemorrhage results from bleeding into the parenchyma, e.g. from a ruptured aneurysm or subarachnoidal artery.

The experimental ischemia models can be divided into two: global and focal ischemia. Global ischemia results from entire carotid artery occlusion or abruptly stopped blood flow after cardiac arrest that induces delayed neuronal death of selectively vulnerable CA1 neurons of the hippocampus . In contrast, occlusion of a single middle cerebral artery (MCAO; e.g. by thrombus) is an example of focal cerebral ischemia. Focal ischemia gives rise to irreversible damage in the core region and a partially reversible injury in the surrounding penumbral zone . Different forms of ischemic insult are dependent on the amount and distribution of spared tissue as well as the type and severity of cell damage occurrence.

Ischemic injury causes severe neurodegeneration and consequently a loss of normal brain

functions resulting from a complex sequence of pathophysiological events including excitotoxicity, inflammation and programmed cell death (Fig. 1.2).

Oxidative phosphorylation is the main mechanism of energy production in the brain tissue. Energy is required for cell metabolism and maintenance of the ion gradient. Depletion of energy due to restriction of glucose and oxygen delivery leads to the loss of membrane potential and depolarization of neurons and glia , activation of voltage-dependent Ca^{2+} channels and release of excitatory amino acids (glutamate) into the extracellular space.

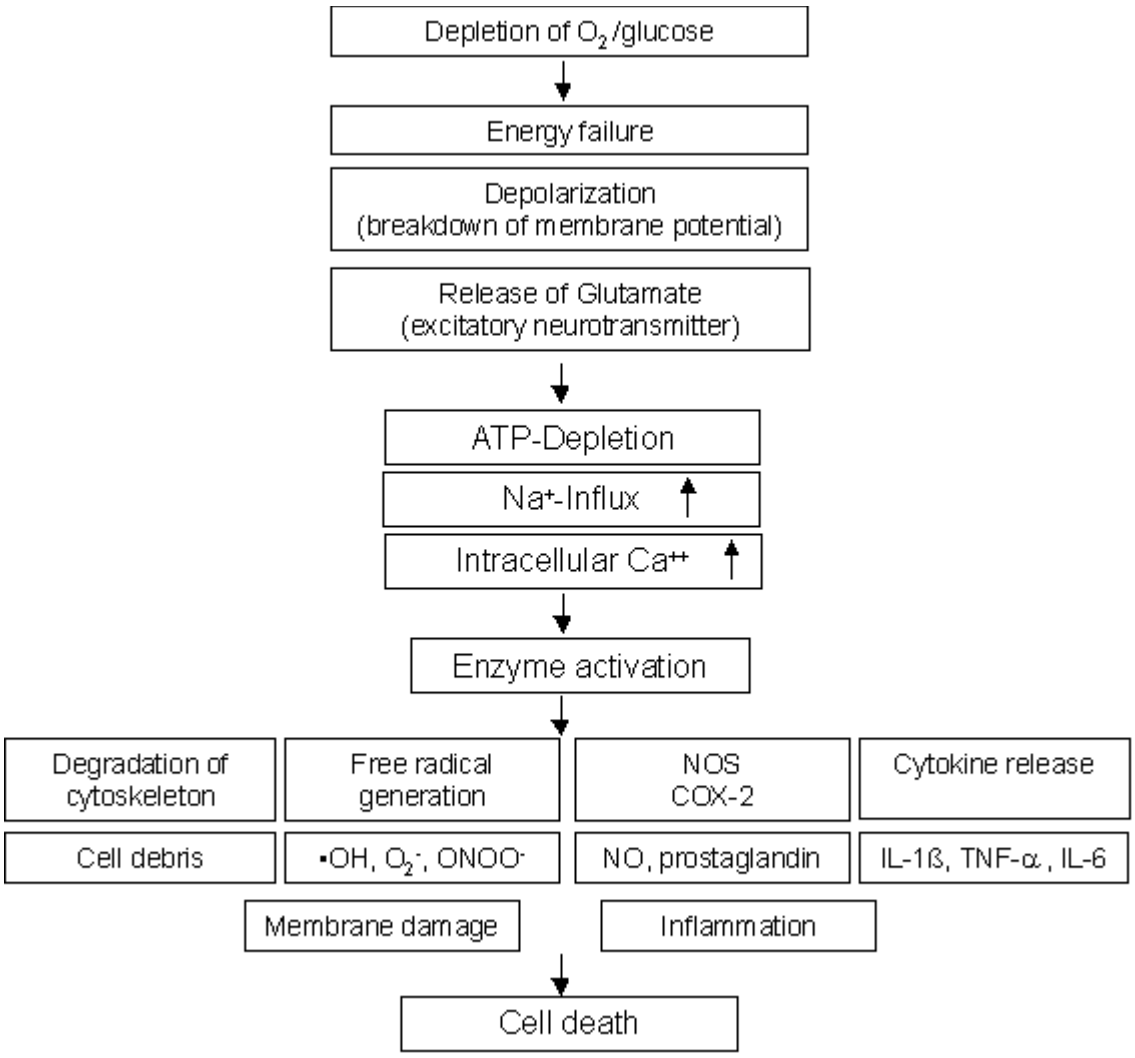

Fig. 1.2. Simplified overview of pathophysiological mechanism in ischemic cell death. Deprivation of glucose and oxygen leads to energy failure and depolarization of the cell membrane. Activation of glutamate receptors results in ATP-depletion and significantly increases intracellular Na^+ and Ca^{2+} levels. Intracellular Ca^{2+} overactivates numerous enzyme systems (proteases, lipases, endonucleases, etc.). Free radicals are generated, which damage membranes (lipolysis), mitochondria and DNA, triggering programming cells death (apoptosis) and formation of cell debris. Free radicals induce the release of pro-inflammatory mediators that leads to microglia activation and inflammatory reaction. Complex sequences result in irreversible cell damage and cell death. Diffusion of glutamate into the extracellular space can propagate a series of waves of depolarization and spread of damage. IL, interleukin;

NO, nitric oxide; COX, cyclooxygenase; TNF, tumor necrosis factor. Adapted from K. Dinkel and modified.

As a result of glutamate-mediated overactivation, Na^+ and Cl^- influx into the cell that is followed by passive transport of water and cell swelling. Release of pro-inflammatory mediators interleukin (IL)-1ß and tumor necrosis factor (TNF)-α from activated glia as well as damaged neurons stimulate the inflammatory reaction in the ischemic tissue. Activation of NMDA and AMPA/kainite receptors contribute to Ca^{2+} overload and activation of photolytic enzymes that degrade cytoskeletal proteins. Phospholipase A_2 and cyclooxygenase (COX)-2 generate free-radical species, which induce lipid peroxidation and membrane damage . Activation of neuronal nitric oxide synthase (NOS) produces nitric oxide (NO), which reacts with a super-oxide anion to form the highly reactive peroxynitrite, that promotes tissue damage . It was demonstrated that application of NMDA receptor antagonists, Ca^{2+} or Na^+ blockers as well as free radical scavengers provide protection to neurons against ischemia-induced neuronal death . Once neuronal damage has developed, an increase in cell proliferation and neurogenesis occurs.

1.2.2. Neurogenesis after cerebral ischemia

Increased cell proliferation and neurogenesis in response to cerebral ischemia was reported and considered as a compensatory mechanism of neuronal loss . Global ischemia gives rise to enhanced cell proliferation in the rodent SGZ . Focal ischemia (MCAO) induces neurogenesis in the SGZ and the SVZ where from cells migrate to the damaged striatum . By focal cortical injury neurogenesis can be stimulated in the cerebral cortex with additional increase of neurogenesis in the DG .

The mechanisms regulating ischemia-induced neurogenesis are only partly understood. Expression of developmental transcription factors by precursors proliferating following ischemic insult provides evidence that neurogenesis could proceed as it does during embryonic

development . Ischemia induces a number of factors that might underlie the neurogenic response to insult, including erythropoetin , vascular endothelial growth factor , SCF, heparin-binding EGF and basic FGF (bFGF) . bFGF and SCF receptors are induced in the SVZ and SGZ after focal ischemia . In addition to neurogenic effect, GFs can sustain the survival of damaged neurons. It was shown that preconditioning is a phenomenon in which a brief ischemic insult induces tolerance against a subsequent severe ischemic insult stimulates GFs expression and neurogenesis in adult rat hippocampus .

Glutamate receptor activation seems to play a prominent role in mediating the neurogenic response to ischemia . Blockade of both NMDA and AMPA receptors at the time of global ischemia prevented the increase of neurogenesis in the SGZ as well as the CA1 neuronal death . Neurogenesis following ischemic insult could be mediated by the glutamate-evoked increased levels of certain GFs, e.g. bFGF and BDNF . However, Liu et al. proposed that decrease in NMDA receptor signaling observed for 2 weeks after transient forebrain ischemia can contribute to increase of neurogenesis in the DG (Liu et al., 1998; Westerberg et al., 1989; Ogawa et al., 1991). Neuronal death within the hippocampus also could provide neurogenic stimulus after injury .

Although increased neurogenesis in the brain in response to ischemic injury occurs, the majority of injury-generated neurons die early after birth . Post-injury microenvironment may not support the complete regeneration of the damaged area either due to significant neuronal injury and/or the associated inflammatory reaction .

Since only a fraction of lost neurons are actually replaced, the capacity of endogenous regeneration seems to be rather limited, making neuroprotective and neurogenic therapy essential to support functional recovery after cerebral ischemia .

So far only one study have demonstrated that application of bFGF and EGF after global ischemia in rats gives rise to substantial regeneration of hippocampal CA1 pyramidal neurons . Nakatomi et al. proposed that newly generated neurons were derived from the posterior

periventricle (pPV), which is adjacent to the hippocampus as a part of the LVw and contains precursor cells displaying *in vitro* stem cell properties . However, the same therapeutical protocol of GF application failed to provide an increase of early neurogenesis but increased the infarct volume after focal ischemia in rats . Thus, GF's therapeutical effects are highly dependent on type of damage, GFs combinations, concentrations and schedule of treatment.

1.3. Inflammation after cerebral ischemia

1.3.1. Mechanism of inflammation

Numerous mechanisms regulating cell death, cell survival and tissue regeneration are activated after ischemic brain injury . One of the processes that play a role in the delayed progression of the damage is post-ischemic inflammation . It is characterized by activation of microglia, infiltration of peripheral macrophages and neutrophils into the brain initiated by local expression of cytokines, chemokines, and adhesion molecules .

In the normal CNS, microglia are present in a resting state and possess a characteristic ramified morphology. With the onset of CNS injury, microglia become activated in association with a change in stellate morphology . Microglial cells are activated gaining phagocytotic and antigen presenting capacity expressing major histocompatibility complex (MHC) class II molecules . Microglia proliferate and migrate toward the site of injury where they remove debris after irreversible damage . Once activated, microglia are capable of secreting cytotoxic substances such as glutamate, NO, reactive oxygen species (ROS; O_2^-, H_2O_2, OH^-), matrix metalloproteinases and their inhibitors and pro-inflammatory cytokines (TNF-α, IL-1ß and IL-6). In the injured brain, microglia rather than astrocytes appear to be the main source of pro-inflammatory and anti-inflammatory cytokines and mediators . Astrocytes are far less competent in presenting antigen than are microglial cells . Astrocytes express pro-inflammatory cytokines and become ″activated″, demonstrating cell hypertrophy and increased immunoreactivity for GFAP .

Pro-inflammatory cytokines are synthesized in low levels in the normal CNS and are strongly upregulated after brain insult . Pro-inflammatory cytokines activate inducible NOS (iNOS) and COX-2 that regulate the production of NO, prostaglandins (PGs) and pro-inflammatory cytokines .

NO activates a number of intracellular signaling pathways, like mitogen-activated protein kinases (MAPK, e.g. p38 MAPK, PKC) and may ultimately lead to neuronal death via inhibition of mitochondrial respiration .

Expression of COX has recently emerged as an important determinant of the cytotoxicity associated with inflammation by producing superoxide and prostanoids (PGs and tromboxans) . COX-1 is constitutively expressed in many cell types in which it produces prostanoids that subserve normal physiological functions. COX-2 normally is not present in most cells. Upregulation of COX-2 mRNA is detected in the ischemic hemisphere beginning 6h after ischemia . COX-2 reaction products are responsible for many of the cytotoxic effects of inflammation . PGs can induce the production of IL-6 by activating the p38 MAPK and PKC pathways and stimulating glutamate release .

1.3.2. Anti-inflammatory mediators and treatment

Microglia activation has been proposed to be both detrimental and beneficial in certain disorders of neurodegeneration, inflammation, and ischemia . Phagocytosis of cellular debris from degenerated neurons support wound healing and neuronal regrowth, secretion of certain cytokines and GFs can aid neuronal regeneration . Neurotrophins like NGF, BDNF and neurotrophin-3 have been shown to be capable of inhibiting the MHC class II inducibility in microglia . Mattson et al. demonstrated suppression of glutamate-induced H_2O_2 by bFGF, NGF and BDNF . These GFs could increase activity of superoxide dismutases and glutation reductases, inducing thus anti-oxidative mechanisms. Upregulation of macrophage colony-stimulating factor receptors and triggering receptor expressed on myeloid cells-2 in activated

microglia has been implicated in neuroprotective microglia activity.

While the expression of inflammatory cytokines that may contribute to ischemic injury has been repeatedly demonstrated, cytokines may also provide ″neuroprotection″ in certain conditions by promoting growth, repair, and ultimately, enhanced functional recovery . Indeed, microglia-derived IL-1ß is required for the production of ciliary neurotrophic factor and insulin-like growth factor 1 by astrocytes, which promote the repair of injured neurons . Moreover, on the model of excitotoxic injury Bernandino et al. demonstrated a TNF-2 receptor mediated neuroprotective effect of TNF-α .

Inhibition of inflammation by the tetracycline derivative minocycline or non-steroidal anti-inflammatory drug indomethacin was neuroprotective in a model of cerebral ischemia *in vivo* . Minocycline, blocks microglia activation and proliferation through the inhibition of p38 MAPK, while indomethacin reduces COX activity . Since an immune response normally accompanies brain injury, it is quite possible that inflammatory molecules have a definite impact on the proliferative and differentiation potential of neural progenitors.

1.3.3. Inflammation and neurogenesis

The innate immune response in the brain is able to positively or negatively modulate neurogenesis in the CNS depending on the cytokines and GFs that are predominantly expressed in the cellular environment.

Recent reports show that inflammation impairs hippocampal neurogenesis . Ekdahl and co-workers showed that seizure-induced neurogenesis was suppressed by lipopolysaccharide administration but restored by minocycline application . They presented a significant negative correlation between the number of new neurons and the number of activated microglia. Monje et al. showed that administration of indomethacin could restore the hippocampal neurogenesis inhibited by irradiation . IL-6, which is produced by activated microglia, was shown to play a key role in this mechanism . In addition, in a model of photothrombotic infarct pre- and post-insult, anti-inflammatory treatment with indomethacin could increase hippocampal

neurogenesis . In contrast, Sasaki et al. showed that delayed treatment with COX inhibitors blunted the enhanced proliferation of neural progenitors in the DG after global ischemia . Moreover, they observed a decrease in proliferating cells in the DG of heterozygous and homozygous COX-2 knockout mice compared to wild-type mice after ischemia. Hence the role of microglia and inflammation in damage-regulated neurogenesis seems to depend on the etiology and the severity of the injury and thus requires further investigation.

1.4. Organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures (OHC) are used for the investigation of neuronal damage, neuroprotection and synaptic transmission . OHC represent an *in vitro* model that contains the different neural cell types and retains the complex three-dimensional organization of the nervous tissue. Cultured slices maintain their cellular architectures and interneuronal connections, and neurons survive during the long-term culture and physiologically mature over this period, allowing an extended survival study . They contain only resident microglia and influx of peripheral leukocytes does not occur in this *in vitro* preparation. OHC provide good experimental access to model pathophysiological pathways in living tissues and facilitate the design of therapeutic agents. In particular, OHC combined with OGD could provide a surrogate system for investigation of neuronal cell loss following ischemic injury to the brain. Furthermore this system is suitable for detecting metabolism-related effects.

Little is known about neurogenesis in OHC. The topic was recently investigated in roller-tube and interface OHC by Raineteau and Kamada . In roller-tube OHC mature granular BrdU/NeuN+ neurons were found in the SGZ and granular cell layer (GCL) of the DG two weeks after BrdU application. The number of BrdU/NeuN+ neurons corresponded to the number found in the hippocampus of adult rodents *in vivo*. Neurogenesis was detected in the DG and could be stimulated by epidermal growth factor application . In the interface OHC neural progenitors were identified exclusively in the suprapyramidal region of the GCL of the

DG. Mature BrdU/NeuN+ cells were found in the DG four weeks after BrdU application . Thus, it was shown that neurogenesis in OHC is limited to the DG where the newly generated neurons appear and maturate.

Taking together, OHC were considered as an excellent model for the *in vitro* investigation of interplay between neuronal damage, inflammation and neurogenesis in the CNS after OGD. This study was performed on interface OHC maintained on permeable membranes ("Stoppini method") and all manipulation as well as GFs treatment were tested under serum free conditions.

1.5. Aim of the project

Based on our knowledge the aim of this thesis was to investigate the pathophysiological events and neurogenesis in organotypic hippocampal slice cultures after oxygen-glucose deprivation.

Specific aims include:

1. To identify and characterize neurogenic zones and neural precursors in OHC. To characterize cell proliferation, distribution of proliferating cells in OHC under control conditions. To elucidate the effect of exogenous growth factors on cell proliferation and neurogenesis.

2. To investigate the interplay between cell damage, microglia activation, cell proliferation and neurogenesis after OGD. To define the temporal relation between cell damage and proliferation. To elucidate the effect of exogenous growth factors on cell proliferation and neurogenesis after OGD.

3. To investigate the effect of inflammation in OGD-induced cell damage, microglia activation, cell proliferation and neurogenesis by using anti-inflammatory treatment with indomethacin and minocycline. To compare the effects of anti-inflammatory

compounds with effects of NMDA receptor antagonists.

2. Materials and Methods

2.1. Materials

2.1.1. Lab instruments and materials

Amersham Pharmacia Biotech: Buckinghamshire, UK	GeneQuant*pro* photometer
Applied Biosystem: (Foster City, CA, USA)	ABI PRISM™ Optical Adhesive Covers 6700 ABI PRISMTM 96-Well Optical Reaction Plate with Barcode (code 128) LightCycler ABI Prism® 7000 SDS
Billups & Rothenberg, Inc.: (Del Mar, CA, USA)	Modular Incubator Airtight Chamber
Bio-Rad: (München, Germany)	Thermal cycler, My cycler
Campden Instruments Ltd.: (Leicester, UK)	Vibratome NVSLM1
Carl Zeiss: (Jena, Germany)	Zeiss LSM 5 Pascal confocal laser scan microscopy Axiovert 100TV transmission light microscope
Leica Instruments: (Nussloch, Germany)	Cryostat Leica CM 3050
Millipore: (Molsheim, France)	0.4 μm porous Millicell membrane
Nikon : (Tokyo, Japan)	Nikon Eclipse TE 300 fluorescences microscope
The Mickle Laboratory Engineering Co.: (Guildford, UK)	McIlwain tissue chopper

2.1.2. Chemicals

Biochrom AG:	1mM L-glutamine, 200 mM
(Berlin, Germany)	0.5% gentamycine,10 mg/ml
Carl Roth GmbH + Co. KG:	D-(+)-Saccharose
(Karlsruhe, Germany)	Glycerin
	Hydrochloric acid (HCl)
	Paraformaldehyde
	Triton X
Chemicon International, Inc.:	bFGF
(Temecula, CA, USA)	Recombinant human NGF-beta
Fermentas GmbH:	Ribonuclease inhibitor, 40 U/µl
(St. Leon-Rot, Germany)	
Invitek GmbH:	50xdNTP Master Mix
(Berlin, Germany)	
Promega:	M-MLV Reverse Transcriptase Buffer
(Madison, WI, USA)	M-MLV Reverse Transcriptase, 200 U/µl
	Random Primers, 0.5ng/µl
Qiagen:	Quanti Test SYBR Green PCR Master Mix
(Hilden, Germany)	RNeasy Mini Kit
R&D systems GmbH:	Recombinant human BDNF
(Wiesbaden-Nordenstadt, Germany)	
Roche Molecular Biochemicals:	EGF, ß-urogastron
(Mannheim, Germany)	
Serva:	α-D-Glucose
(Heidelberg, Germany)	
Sigma:	3,3´-diaminobenzidine
(Deißenhofen, Germany)	BrdU
	Mannitol
	Indomethacin
	Minocycline hydrochloride
	Propidium iodide (PI)
Thermo Shandon:	Histomount

Tocris Bioscience: (Avonmouth, UK)	MK-801 (dizocilpine)
Vector Laboratories: (Burlingame, CA, USA)	Phosphatase – avidin-biotin complex Vectastain Elite ABC-kit

2.1.3. Antibodies

BD Transduction Laboratories: (San Jose, CA, USA)	Monoclonal mouse anti-Nestin
Chemicon International, Inc.: (Temecula, CA, USA)	Monoclonal mouse anti-GFAP Monoclonal mouse anti-neuronal nuclei (NeuN)
Dianova: (Hamburg, Germany)	Biotinylated donkey anti-mouse
Immunologicals Direct : (Raleigh, NC, USA)	Monoclonal rat anti-BrdU
Jackson Immunoresearch Laboratories, Inc.: (West Grove, PA, USA)	Cy3 donkey anti-mouse IgG Cy3 donkey anti-goat IgG Cy2 donkey anti-rat IgG
Promega: (Madison, WI, USA)	Monoclonal mouse anti-ß-III Tubulin
Santa Cruz Biotechnology: (Santa Cruz, CA, USA)	Polyclonal goat anti-doublecortin (DCX)
Serotec: (Oxford, UK)	Monoclonal mouse anti-OX-42
Sigma: (Deißenhofen, Germany)	Extra Avidin Cy3 Monoclonal rabbit anti-GFAP
Vector Laboratories: (Burlingame, CA, USA)	IB4

2.1.4. Cell culture medium and components

Cell Concept GmbH: (Umkirch, Germany)	**Preparation medium:** MEM-Hanks 25 mM Hepes Phenol red pH 7.35 **Culture medium:** 66% MEM-Hanks 33% Hanks´ balanced salt solution (HBSS) 17mM Hepes 350 mg/ml $NaHCO_3$ 5 mM glucose pH 7.8
Gibco: (Eggenstein, Germany)	25% Heat-inactivated horse serum Neurobasal-A B27 supplement
Serum-based medium:	75% Culture meduim 1mM L-glutamine 0.5% Gentamycine10 mg/ml 25% Heat-inactivated horse serum
Serum-free medium:	75% Culture medium 1mM L-glutamine 0.5% Gentamycine 25%Neurobasal-A with 0.5% B27 supplement

2.1.5. Buffers and solutions

Ringer's solution	124 mM NaCl, 4.8 mM KCl, 1.3 mM $MgSO_4x7H_2O$, 1.2 mM KH_2PO_4, 25.6 mM $NaHCO_3$, 2 mM $CaCl_2x2H_2O$, 10 mM glucose, pH 7.4
OGD-medium	124 mM NaCl, 4.8 mM KCl, 1.3 mM $MgSO_4x7H_2O$, 1.2 mM KH_2PO_4, 25.6 mM $NaHCO_3$, 2 mM $CaCl_2x2H_2O$, 10 mM glucose, pH 7.4
PBS	137 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2PHO_4, 1.4 mM KH_2PO_4, pH 7.4
TBS	99 mM Tris, 15 mM NaCl, pH 7.4
30% Sucrose	30% solution of D-(+)-saccharose in PBS
4% PFA	4% solution of paraformaldehyde in PBS

2.1.6. Primer sequences

Gene name	5`- 3´primer sequence	Primer concentration (μM)	$MgCl_2$ (mM)	Amplicon length (bp)	Accession, nucleotide number
Cyclophilin A	FW :AGCACTGGGGAGAAAGGATT RW :AGCCACTCAGTCTTGGCAGT	0.05	2.5	248	NM017101
IL-1ß	FW :CACCTCTCAAGCAGAGCACAG RW :GGGTTCCATGGTGAAGTCAAC	0.2	2.5	79	M98820
TNF-α	FW :AAATGGGCTCCCTCTCATCAGTTC RW :TCTGCTTGGTGGTTTGCTACGAC	0.2	3.5	269	X666539
IL-6	FW: TCCTACCCCAACTTCCAATGCTC RW : TTGGATGGTCTTGGTCCTTAGCC	0.2	2.5	79	M26744

Note. FW, forward primer; RW, reverse primer; IL, interleukin; TNF-α, Tumor necrosis factor α.

2.2. Methods

2.2.1. Tissue preparation

2.2.1.1. Preparation of organotypic hippocampal slice cultures

Organotypic hippocampal cultures were prepared according to the standard interface method from postnatal day 7-9 (P7-9) Wistar rats (Harlan Winkelmann GmbH Borchen, Germany). After decapitation, the brain was removed, hippocampi were dissected and transversely sliced into a 350 μm thickness on a McIlwain tissue chopper. The slices were then carefully separated with two pairs of fine forceps and transferred to sterile, 0.4 μm porous Millicell membrane, using a glass Pasteur pipette. Slices were maintained in culture at 37^0C in 1 ml of serum-based medium for 2-3d. Thereafter cultures were transferred to serum-free medium and kept in a tissue culture incubator for 12d at 33^0C in 5% CO_2 with medium change twice a week.

OHC were selected with PI before the experiment to exclude damaged cultures. PI was added to the culture medium in final concentration of 2 μg/ml for 12h. PI negative OHC were selected using fluorescence microscopy.

2.2.1.2. Preparation of organotypic cortex-hippocampal cultures

OCC were prepared from P7-9 Wistar rats using a modification of the method by Plenz and Kitai . After decapitation, the brain was removed and placed into a sterile Petri dish. Coronal sections of 400 μm were cut using a vibratome and dissected to dorso-lateral cortical and hippocampal tissues (Fig. 2.1). Slices were plated onto Millicell culture inserts and maintained for 10d under the same conditions as OHC.

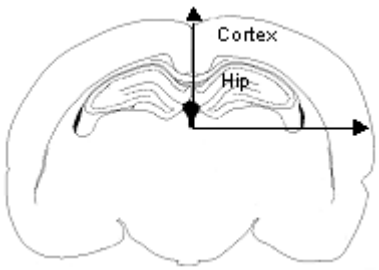

Fig. 2.1. Schematic drawing of a rat brain coronal section demonstrating the anatomy of OCC.

2.2.2. Oxygen and glucose deprivation

OGD was used as an *in vitro* model of cerebral ischemia. For OGD, the inserts with OHC were placed into 1ml of Ringer solution containing mannitol (10mM) instead of glucose (OGD-medium) in sterile 6-well culture plates. Before use, OGD-medium was saturated by flushing a gas mixture of 95% N_2/5% CO_2 for 10 min. The cultures were then placed into an airtight chamber and exposed to 10 min of 95% N_2/5% CO_2 gas flow. After that the chamber was sealed and placed for 40 min in an incubator where the temperature was maintained at 37^0C. Control cultures were maintained for the same time under normoxic atmosphere in glucose-containing Ringer's solution. After OGD, OHC were returned to their original culture conditions.

2.2.3. Experimental setups

2.2.3.1. Cell proliferation and neurogenesis in OHC

OHC were prepared from P7-9 Wistar rats and maintained in Millicell semiporous membrane for 12-14d. Slices were selected using PI before proliferating cells were labeled by treatment with 20µM BrdU for 2d (Fig. 2.2). BrdU+ cells were detected at 1d, 3d, 6d and 10d after BrdU was applied first. BrdU/DCX+ cells were detected at 3d and 6d, BrdU/ß-III Tubulin+ cells at 6d and 10d and BrdU/NeuN+ cells at 10d.

2.2.3.2. Growth factor treatment

OHC were treated with GF to a final concentration of BDNF 25 ng/ml, bFGF 5 ng/ml; EGF 10 ng/ml or NGF 10 ng/ml for 2d or 5d (Fig. 2.2). GF was added 24h after the start of BrdU application. BrdU+ cells were detected at 3d and 6d after BrdU was applied first. BrdU/ß-III Tubulin+ cells were detected at 6d. For bFGF neurogenesis was assessed also at 3d with BrdU/DCX immunohistochemistry.

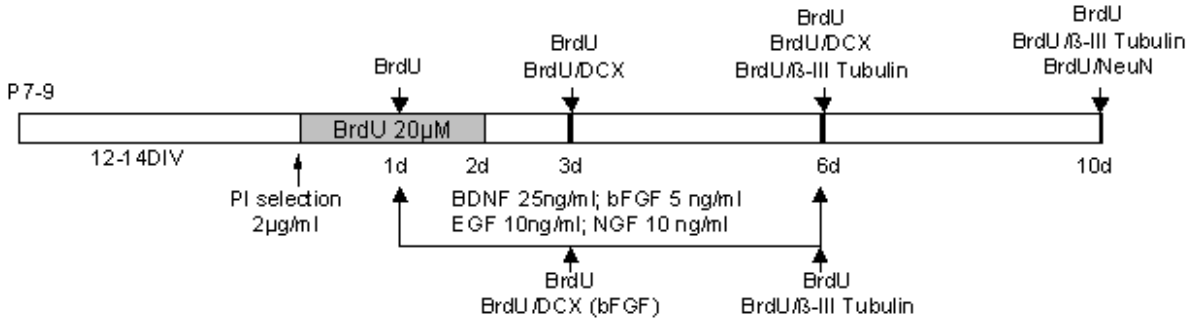

Fig. 2.2. Experimental setup used to investigate basal and GF modulated proliferation and neurogenesis in OHC.

2.2.3.3. Cell damage, microglia, cell proliferation and pro-inflammatory cytokines after OGD

To study the temporal profile of cell damage, microglia activation, cell proliferation and pro-inflammatory cytokine levels early after insult, OHC were subjected to analysis at 0, 2, 6, 16 and 24h after OGD (Fig. 2.3).

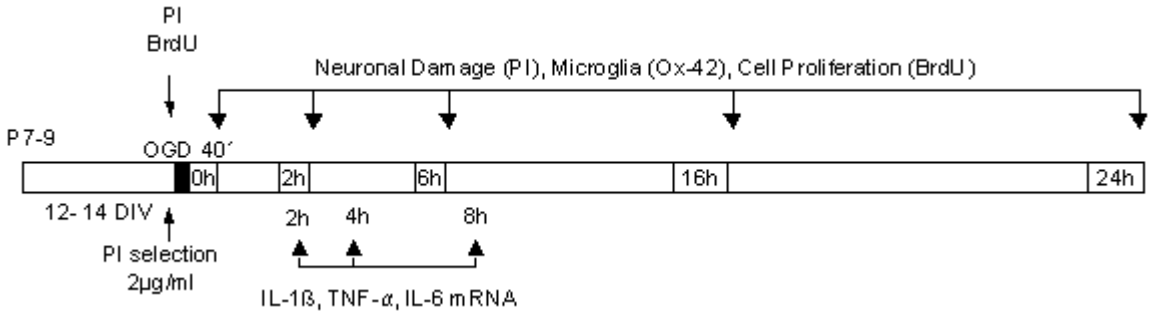

Fig. 2.3. Experimental setup used to study temporal profile of neuronal damage, microglia activation, cell proliferation and pro-inflammatory cytokines mRNA level after OGD.

PI and BrdU were added to the OGD-medium to a final concentration 2 µg/ml and 20 µM respectively and were present during OGD. Furthermore, cultures were incubated under normal conditions with PI (2 µg/ml) and BrdU (20 µM) containing medium. PI uptake was assessed at 0, 2, 6, 16 and 24h after OGD. Thereafter OHC were fixed, cryosected and immunohistochemically stained for microglia and proliferating cells detection. Damage was assumed as an integral density of PI uptake in the CA1 or DG. OX-42+ and BrdU+ cells were quantified in the CA1 and DG areas. IL-1ß, TNF-α and IL-6 mRNA levels were assessed by real-time RT-PCR using SYBR green. Total RNA was isolated from the OHC at 2, 4 or 8h after OGD. As a house-keeping gene peptidylprolyl isomerase A (cyclophilin A) was detected.

2.2.3.4. Cell proliferation and neurogenesis after OGD

To investigate neurogenesis in OHC after insult, slices were incubated with BrdU for 2d after OGD (Fig. 2.4). Early neurogenesis was detected at 3d with BrdU/DCX immunostaining, late neurogenesis at 6d with BrdU/DCX and BrdU/ß-III Tubulin labeling.

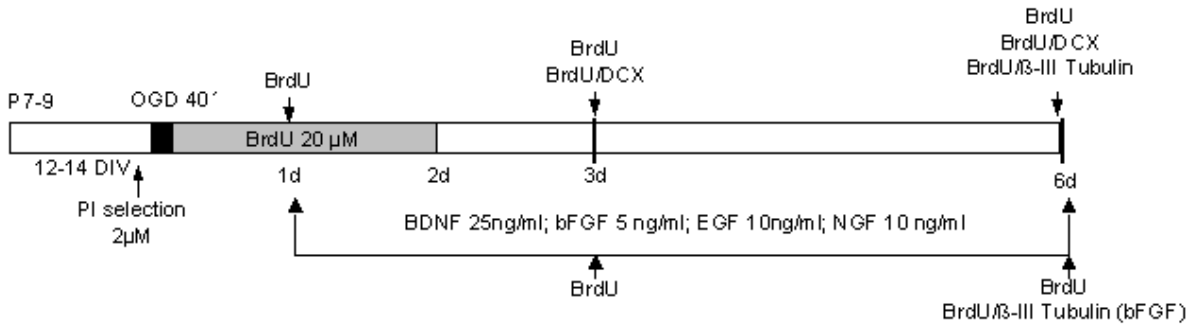

Fig. 2.4. Experimental setup used to study cell proliferation and neurogenesis after OGD and with or without growth factors treatment.

2.2.3.5. Growth factor treatment after OGD

To study how the exogenous GFs can influence OHC regulated cell proliferation and neurogenesis, GF was added into the culture medium in a final concentration of BDNF 25 ng/ml, bFGF 5 ng/ml, EGF 10 ng/ml or NGF 10 ng/ml for 2d or 5d in 24h after OGD (Fig. 2.4). BrdU+ cells were detected at 3d and 6d after OGD. BrdU/ß-III Tubulin+ cells were detected at 6d after 5d of bFGF treatment.

2.2.3.6. Anti-inflammatory compounds treatment

To investigate the contribution of inflammation in neuronal damage, cell proliferation, microglia activation and neurogenesis, OHC were treated with indomethacin or minocycline hydrochloride. Compounds were added to the culture medium at a final concentration of 10µM or 50µM for 2h before, during, and 2d after OGD. Since indomethacin was dissolved in ethanol, the appropriate vehicle-control (0.1% ethanol) was included. As a positive control, the well known NMDA-receptor antagonist MK-801 (10µM) was used. OHC were treated with BrdU immediately after OGD for 2d. Neuronal damage and microglia were detected 24h after OGD, proliferating cells and newly generated BrdU/DCX neurons were defined at 6d.

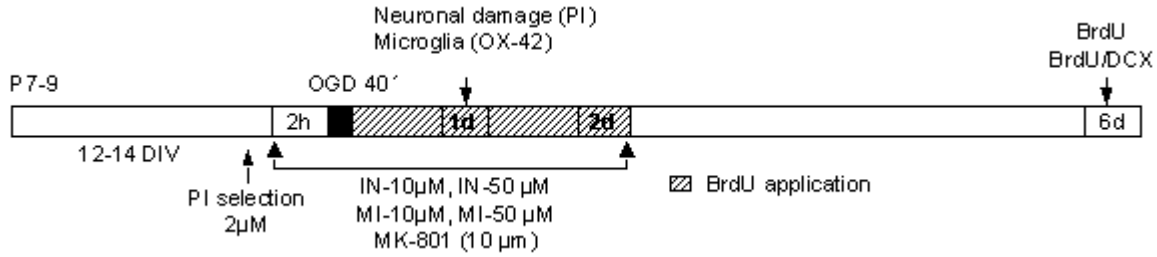

Fig. 2.5. Experimental setup used to investigate the role of inflammation in neuronal damage, cell proliferation and neurogenesis. IN, indomethacine; MI, minocycline.

2.2.4. Cell damage

Cell damage was evaluated by cellular uptake of PI. The fluorescent dye intercalated into the DNA of cells that lost membrane integrity and therefore represents a marker of cell degeneration. OHC were incubated with PI (10µM) for 2h before measurement. To follow after the temporal changes of cell damage after OGD PI uptake was measured at 1d, 5d, 10d and 15d. For PI intensity detection slices were excited with a 510–560 nm light and the emitted fluorescence acquired at 610 nm using a rhodamine filter on an inverted fluorescence microscope (Eclipse TE 300). Images were taken using a CCD camera and analyzed using image analysis software (LUCIA, Nikon). Damage was given as an integral density of PI intensity in the area.

2.2.5. Immunohistochemistry

2.2.5.1. Microglia

Microglia were detected by immunolabeling of CD11b/c surface receptors - OX-42, which are complement receptors type 3. Although CD11b/c is one of the earliest markers to be upregulated on activated microglia, it is also expressed in resting microglia . Cultures were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4) for 40 min. After 24h incubation with 30% sucrose, OHC were cryosectioned in 20µm coronal slices. After endogenous peroxidase quenching with 0.6% peroxide hydrochloride slices were washed with tris-buffered saline (TBS). Thereafter the nonspecific binding was blocked with 10% donkey

normal serum (DNS) and 0.5% Triton-X in TBS for 4h at room temperature (RT). Subsequently, slices were incubated with primary antibody monoclonal mouse anti-OX-42 (1:100) in blocking solution overnight at 4^0C. The slices were rinsed with TBS, incubated with the secondary antibody biotinylated donkey anti-mouse (1:500) for 1h, and reacted with alkaline phosphatase – avidin-biotin complex (1:400) and 3,3´-diaminobenzidine. After washing, slices were left to dry and coverslipped with histomount. Microglia were quantified using the transmission light microscope (Zeiss) and Axiovision Software (Zeiss).

2.2.5.2. Proliferating cells

Proliferating cells were detected by using the DNA replication marker bromodeoxyuridine (BrdU), a thymidine analog which incorporates into the DNA of all cells during the S-phase of cell cycle . OHC were incubated with BrdU (20µM). Fixed and cryosectioned slices were used for immunohistochemistry. DNA denaturation was achieved by treatment with 2N HCl at 36°C for 60 min. Slices were rinsed with PBS and incubated with 10% DNS and Triton-X 0.5% in PBS for 3h, at RT, thereafter with rat anti-BrdU (1:300) primary antibody overnight at 4°C. BrdU+ cell were visualized by Cy2 donkey-anti rat IgG (1:500). Slices were washed and coverslipped with PBS-Glycerin (2:3).

2.2.5.3. Newly generated neurons

To determine the fate of newly generated neurons the following neuronal markers were chosen: the early neuronal markers DCX, ß-III Tubulin and the mature neuronal marker NeuN (see Tab. 2.1). OHC at age 12-14DIV were incubated for 2d in medium containing 20µM BrdU. Cultures were washed then with PBS and fixed at 1d, 5d or 10d post-treatment. After 24h incubation with 30% sucrose and freezing, cultures were cryosectioned and 20µm coronal-sectioned slices were used for immunodetection. DNA denaturation was achieved by treatment with 2N HCl at 36°C for 60 min. Thereafter OHC were incubated for 3h with blocking solution

(10% normal serum, 0.5% Triton X-100 in PBS) and overnight with rat monoclonal anti-BrdU IgG2a and an antibody against DCX or ß-III Tubulin or NeuN in PBS containing 5% normal serum and 0.5% Triton X-100 at 4°C. Slices were incubated for 2h at room temperature with Cy2 donkey anti-rat IgG (1:500) and Cy3 donkey anti-goat IgG (1:500) for BrdU/DCX+, Cy2 donkey anti rat IgG (1:500) and Cy3 donkey anti mouse IgG (1:500) for BrdU/ß-III Tubulin and BrdU/NeuN, then washed with PBS and coverslipped. Controls included omission of both or one of the primary antibodies.

Table 2.1. Cell specific markers used for immunohistochemistry.

Primary antibody	Dilution	Cell type
Monoclonal rat anti-BrdU	1:300	proliferating cells
Monoclonal mouse anti-Nestin	1:500	astrocytes, precursor cells
Monoclonal mouse anti-GFAP	1:500	astrocytes
Monoclonal rabbit anti-GFAP	1:500	astrocytes
Polyclonal goat anti-DCX	1:300	neuroblasts
Monoclonal mouse anti-ß-III Tubulin	1:1000	immature neurons
Monoclonal mouse anti-NeuN	1:1000	mature neurons
IB4	1:40	microglia

2.2.5.4. Other cell types

To characterize the different cell types present in OHC, double and single immunolabeling of unsectioned, coronal- or cross-sectioned slices was performed using the following cell markers: GFAP, Nestin, DCX, ß-III Tubulin, NeuN, BrdU (see Tab. 2.1). As secondary antibodies Cy2 or Cy3 donkey anti-mouse IgG and Cy3 donkey anti-goat IgG (1:500) were used. Microglia were detected with IB_4 and visualized with Extra Avidin Cy3 (1:500).

2.2.6. Isolation of RNA

Total RNA was isolated from OHC with the total RNA isolation kit (RNeasy) from Qiagen. Briefly, the slices were transferred one by one into the 1.5 ml tube with a brush (from 3 to 6 slices per group) and disrupted in 350 µl Buffer RLT with a pestle. One volume of 70% Ethanol was then added, 700 µl of sample was applied to an RNeasy mini spin column in a 2-ml collection tube, and centrifuged for 15 sec at 10,000 x g. Thereafter 700 µl of Buffer RW1 was pipeted onto the RNeasy column, and centrifuged for 15 sec at 10,000 x g for washing. RNeasy column was transferred into a new 2-ml collection tube, 500 µl Buffer RPE was pipeted onto the RNeasy column, and centrifuged for 15 sec at 10,000 x g for washing. After the second washing with 500 µl Buffer RPE, the RNeasy column was centrifuged for 2 min at maximum speed to dry the RNeasy membrane. The RNeasy column was then transferred into a new 1.5-ml collection tube, 50 µl RNase-free water was pipeted onto the RNeasy membrane and centrifuged for 1 min at 10,000 x g to elute RNA contained solution. DNase digestion was made using the RNase-Free DNase set (Qiagen). For that 5 µl of RNase-free Buffer and 1 µl RNase-free DNaseI were added to the tube with RNA and incubated for 1h at 37^0C. The rest of DNaseI was removed by incubation with DNase inactivation reagent for 2 min at RT. After centrifugation at 10, 000 x g for 1 min the supernatant containing total RNA was transferred to the RNA-free tube and stored at -80^0C. Before use in experiment RNA concentration was measured at a wavelength 260 nm using a photometer.

2.2.7. Real-time reverse transcription polymerase chain reaction (real time RT-PCR)

For real time RT-PCR not PI-treated OHC were used.

RNA was reverse-transcribed as follows:

0.5 ng/µl random primers	1 µl
Template RNA (1 µg)	15 µl

↓ 70^0C, 5 min

add

M-MLV Reverse Transcriptase Buffer x5	5 µl
M-MLV Reverse Transcriptase	1 µl
Ribonuclease Inhibitor	0.6 µl
dNTP Master Mix	1 µl
RNAse free water	1,4 µl

↓ 37^0C, 1h

Store cDNA at $+4^0$C

cDNA was amplified using the primers based on rat peptidylprolyl isomerase A (cyclophilin A), rat IL-1ß, rat IL-6 and rat TNF-α sequence. The probe without template served as a negative control.

PCR was carried out with Quanti Test SYBR Green PCR Master Mix (Qiagen) using 200 ng of cDNA in each reaction. Briefly, quantitative PCR was performed using LightCycler ABI Prism® 7000 SDS. PCR conditions for amplifications were: denaturation for 15 min at 95^0C; 40 cycles at 95^0C for 30 sec, 55^0C for 30 sec and 72^0C for 1 min. Amplification specificity was analyzed using a melting curve and by TBE agarose (1,5 %) gel electrophoresis using ethidium bromide. Documentation was done by a still video system. Results were analyzed using LightCycler Software v. 2.0 (Applied Biosystems) after establishing a reaction efficiency for each pair of primers. Quantification was carried out using ΔC_T method. The results are presented as relative increase (folds) compared to control.

2.2.7. Cell quantification and statistical analysis

Image analysis of BrdU and double immunofluorescent labeling was performed by Zeiss LSM 5 Pascal confocal laser scan microscopy (Zeiss, Jena, Germany) with excitation/emission wavelengths of 535/565 nm (Cy3, red) and 470/505 nm (Cy2, green). BrdU+ cells were quantified in the areas CA1 (0.24 mm^2; 6 squares of 0.04 mm^2), CA3 (0.16 mm^2; 4 squares of 0.04mm^2) or DG (0.24 mm^2; 6 squares of 0.04 mm^2) in cryosectioned slices (Fig. 2.6).

Doublelabeled neurons were quantified in one confocal plane throughout the area CA3, CA1, DG or the pPV. Based on the BrdU staining pattern (evenly stained BrdU+ nucleus vs

punctuated) neurons detected in the stratum oriens and glial envelope (GE) were considered to be derived from the pPV and thus were included in the pPV group. Doublelabeling was proven using the z-stack analysis and Prizma Colocalization software (Zeiss).

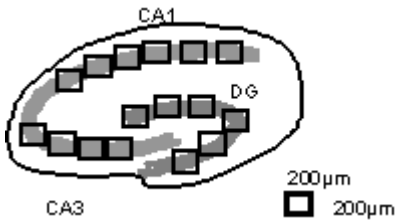

Fig. 2.6. Schematic drawing of BrdU+ cells quantification in OHC.

All data are given as the mean±SD. Experiments were repeated at least three times. The statistical significance of differences between means was evaluated by ANOVA followed by post-hoc Newman-Keuls test for multiple comparison, with $P<0.05$ considered significant.

3. Results

3.1. Characterization of OHC

3.1.1. Identification and in situ distribution of CNS cell types in OHC

OHC were prepared from P7-9 Wistar rats and cultivated for two weeks before experiments were performed. To characterize the three-dimensional structure of OHC, different cell types were detected by immunohistochemistry in unsectioned ("whole mounts") and cross-sectioned 12-14 DIV slices. Astrocytes (GFAP+), precursor cells (Nestin+), migrating neuroblasts (DCX+), young and mature neurons (ß-III Tubulin+), mature neurons (NeuN+) and microglia (IB4+) were labeled by the respective markers. Images were taken in different regions of OHC (Fig. 3.1A). During cultivation OHC flattened down from an original 350 µm to about 150-180 µm. A glial envelope (GE) had formed, which ensheathed the slice and probably functioned as a protective layer (Fig. 3.1B, 3.1B´). The 4-7 µm GE cell layer contained GFAP+ and GFAP/Nestin+ astrocytes, and Nestin+ cells with stellar morphology. These cells were evenly scattered on the top of slice surface (Fig. 3.1C) and formed a dense network at the "OHC–culture membrane border" (Fig. 3.1.D). There were also numerous microglia present in the GE. The majority of microglia had the rounded morphology of activated cells (Fig. 3.1E) while inside the slice mostly ramified microglia could be observed (Fig. 3.1F). Morphologically different Nestin+ cell populations could be detected in OHC: (1) relatively small cells (6-9 µm) with stellar morphology located in the GE and the molecular layer (ML; Fig. 3.1C, D, G), (2) larger Nestin+ cells (13-16µm) in the DG (Fig. 3.1G) and (3), Nestin+ elongated cells (8-9 µm; Fig. 3.1H, green). This third type of Nestin+ cells formed the border between the stratum oriens and the GE. Interestingly, immature DCX+ (Fig. 3.1H, red) or DCX/ß-III Tubulin+ (Fig. 3.1I) neurons were also found here. Mature neurons (NeuN+) formed the pyramidal cell layer or were occasionally distributed randomly in the stratum oriens (Fig. 3.1J). However NeuN+ cells were not observed at the "stratum oriens-GE border".

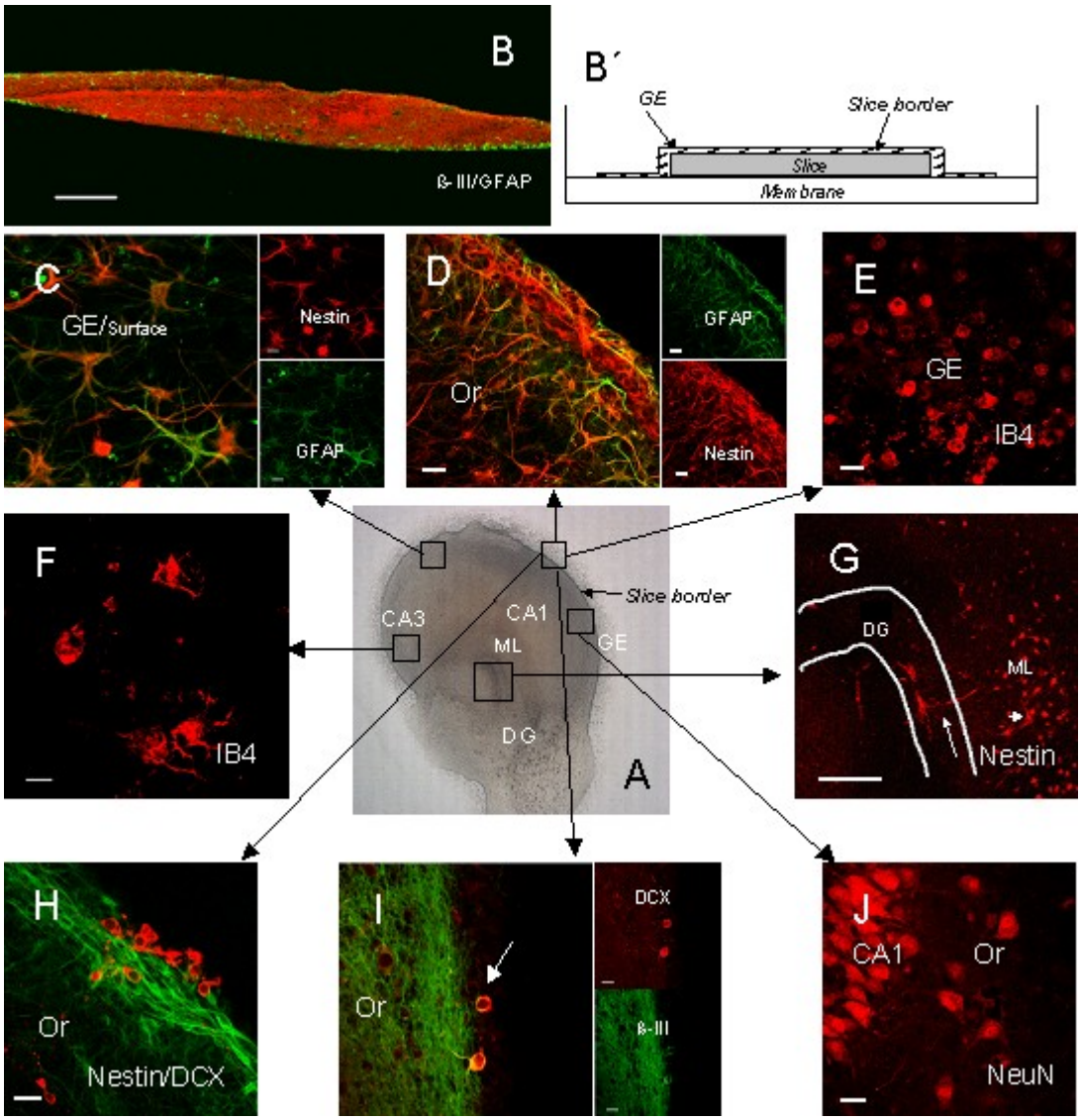

Fig. 3.1. Identification and in situ distribution of CNS cell types in OHC. Transmission light image of 14 DIV OHC (A). Different cell types were detected by immunohistochemistry in unsectioned ("whole mounts") and cross-sectioned 14 DIV slices. The ß-III Tubulin/GFAP (ß-III Tubulin – red; GFAP - green) staining image (B) and schematic drawing (B´) show the GE ensheathing the ß-III Tubulin+ hippocampal slice. The GE contains GFAP, GFAP/Nestin+ astrocytes and Nestin+ cells on the surface of the slice (C; Nestin – red; GFAP - green) and at the "OHC–culture membrane border" (D). Activated microglia in the GE (E) and resting microglia inside of the slice culture (F). Smaller Nestin+ cells with stellar morphology in the ML (G, arrow head) and larger Nestin+ cells in the DG (G, arrow). Elongated Nestin+ cells (green) and DCX+ neuroblasts (red) at the "stratum oriens-GE border" (H). DCX+ neuroblasts (red) expressed ß-III Tubulin (green) at the "stratum oriens-GE border" (I, arrow). Mature NeuN+ cells in the pyramidal cell layer and stratum oriens (J). Scale bar 200µm for B; 100µm for G; 20µm for D, E, H, I, J; 10µm for C and F.

3.1.2. Distribution and appearance of proliferating cells in OHC

To investigate proliferation in OHC, dividing cells were labeled with 20µM BrdU for 2d (see Fig. 2.2). Single BrdU immunohistochemistry revealed massive cell proliferation in the GE

(surface of the slice; Fig. 3.2A) compared to low proliferation inside the slice (Fig. 3.2B). BrdU+ cells were quantified in 20µm cryosectioned slices at 1d, 3d, 6d and 10d after BrdU application. Massive increase in cell proliferation was found at 3d compared to 1d after BrdU was first applied (Fig. 3.2C). The number of proliferating cells did not change significantly from 3d to 6d or from 6d to 10d of incubation. The relatively high level of basal proliferation detected in OHC was probably due to the young age of the tissue at the time of preparation (P7-9).

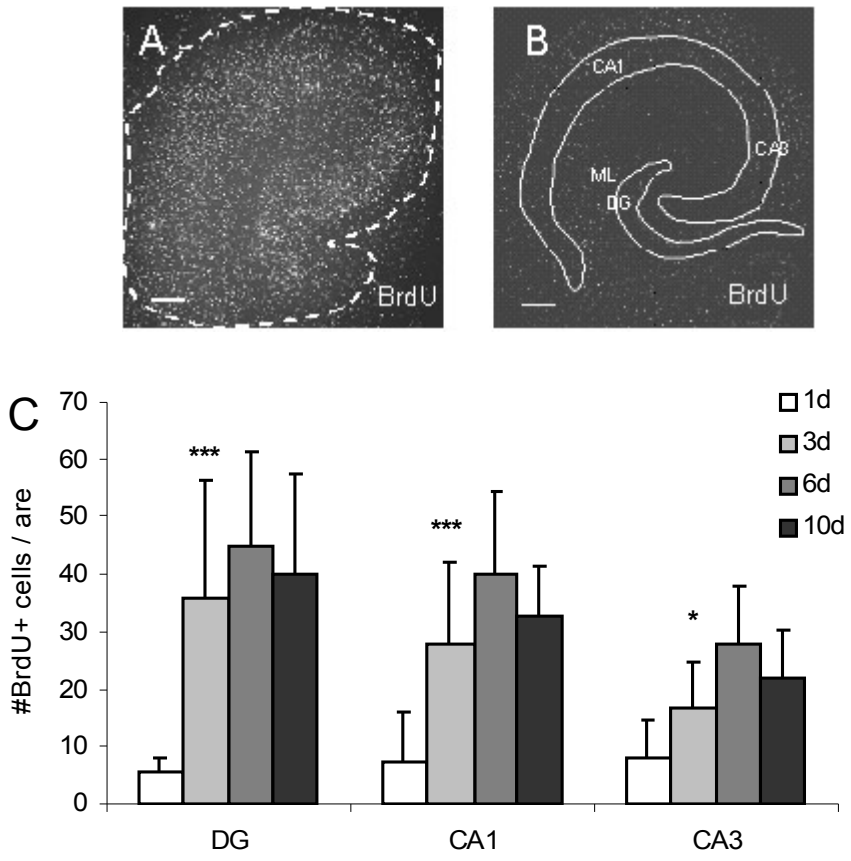

Fig. 3.2. Distribution and quantification of proliferation in OHC. OHC were treated with BrdU for 2d thereafter proliferating cells were detected by immunohistochemistry. Images show massive cell proliferation in the GE (A) compared to the inner layers of OHC (B). (C) Proliferating cells were quantified in 20µm sectioned slices at 3d, 6d and 10d after BrdU application. BrdU, bromodeoxyuridine; CA, cornu ammonis; DG, dentate gyrus; ML, molecular layer. Scale bar 200µm for A and B.

3.1.3. Cell proliferation and growth factor treatment

Next we choosed to examine the effect of several GFs on cell proliferation. OHC were

treated with BDNF (25 ng/ml), bFGF (5 ng/ml), EGF (10 ng/ml) or NGF (10 ng/ml) for 2d or 5d (see Fig. 2.2).

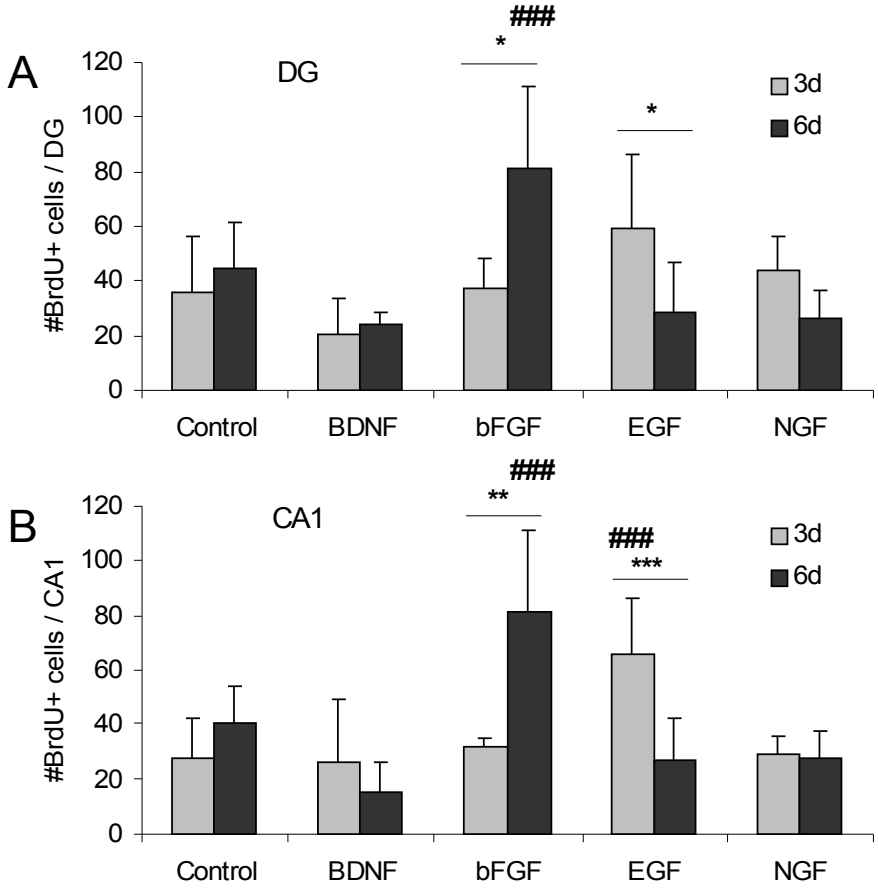

Fig. 3.3. Effect of GF treatment on cell proliferation in OHC. OHC were treated with one of the following GF in a final concentration of BDNF 25 ng/ml, bFGF 5ng/ml, EGF 10 ng/ml or NGF 10ng/ml for 2d or 5d. GF was added into the culture medium 24h after the start of BrdU treatment. BrdU+ cells were quantified at 3d and 6d in the DG (A) and in the CA1 area (B). "*" represents the significant differences in number of BrdU+ cells between 2d and 5d GF treatment, "#" represents the significant differences in number of BrdU+ cells between control and GF-treated grous.The data are expressed as the mean±SD, *P<0.05; **P<0.01; ***; ###P<0.001 (ANOVA followed by post-hoc Newman-Keuls test).

Proliferating cells were detected at 3d (2d treatment) or 6d (5d treatment) after BrdU was first applied. BDNF, bFGF or NGF applied for 2d failed to increase cell proliferation in the DG (Fig. 3.3A) or CA1 whereas short EGF treatment stimulated proliferation in the CA1 (Fig. 3.3B). The number of BrdU+ cells was significantly increased in both areas by longer

treatment (5d) with bFGF (Fig. 3.3). Other factors did not change the proliferation pattern neither in the DG nor in the CA1. The differences in cell proliferation between short and long GF application were found. bFGF applied for 5d could enhance proliferation compared to shorter bFGF treatment (2d). In contrast, long EGF application resulted in reduction in number of BrdU+ cells seen at 3d. Thus, from the several GFs used here only bFGF and EGF could show the time-dependent mitogenic properties under control conditions. Next we investigated neurogenesis in OHC.

3.2. Neurogenesis in OHC

3.2.1. Identification of a second neurogenic zone in OHC

In order to identify 1) whether the immature neurons (DCX+ and/or ß-III Tubulin+) located at the border between the stratum oriens and the GE originated from the LVw precursors and 2) whether these precursors appeared only after trauma (separation of hippocampi during preparation) or also in the absence of injury combined cortex-hippocampal organotypic slice cultures (OCC) were prepared (see Fig. 2.1). These cultures contained the hippocampus, the cortex and the intact posterior part of the LVw, where the precursor cells could be located (Fig. 3.4A). Nestin+ or BrdU/DCX+ cells were detected at 3d and BrdU/ß-III Tubulin+ cells at 6d after BrdU application. Although Nestin+ cells were scattered throughout the slice culture, above the stratum oriens they formed a defined structure appearing as a borderline between the dorsal hippocampus and the cortex (Fig. 3.4B), thus resembling the "stratum oriens-GE border" in OHC. This structure is the pPV, part of the LVw, which lines the hippocampus and contains elongated Nestin+ cells (Fig. 3.4C). DCX+ cells in the pPV and in the DG could be detected in OCC (Fig. 3.4D).

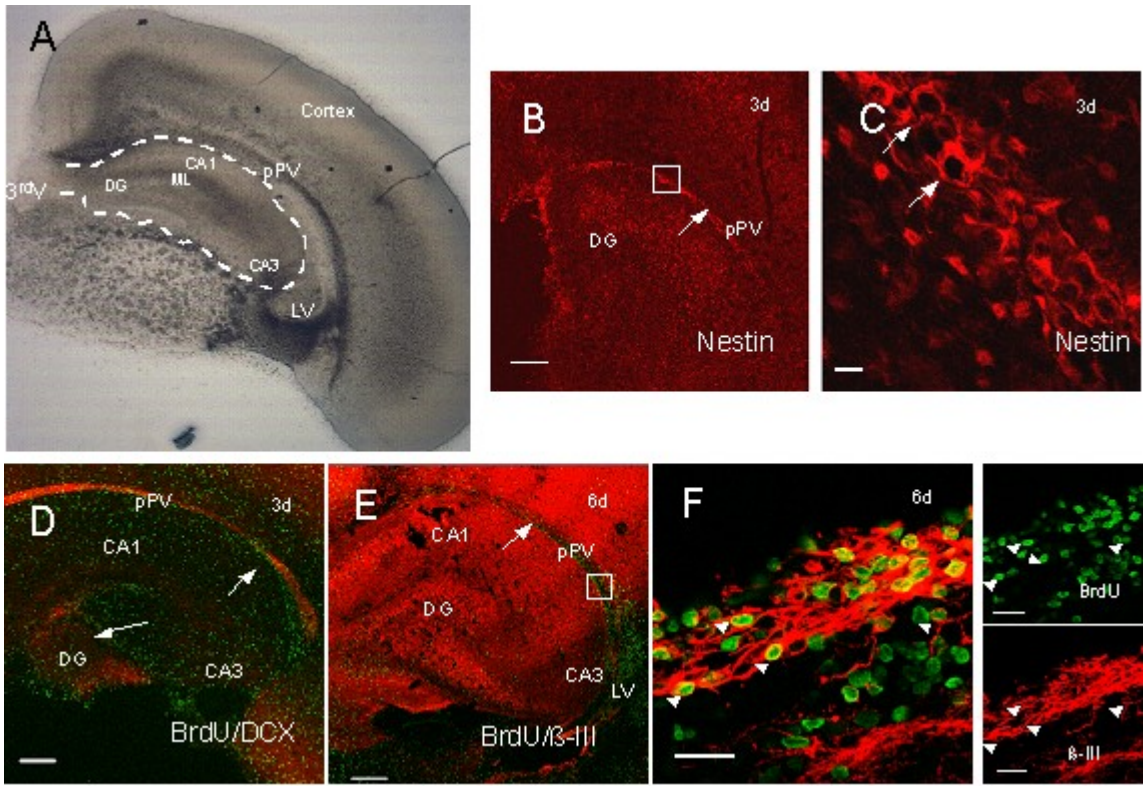

Fig. 3.4. Neurogenesis in OCC. OCC were prepared form P7-9 rats. 10 DIV OCC were treated with BrdU thereafter Nestin and BrdU/DCX immunohistochemistry was performed at 3d and BrdU/ß-III Tubulin at 6d after BrdU application. (A) Transmission light image of OCC. Nestin immunohistochemistry showing the pPV at the "cortex–hippocampus border" (B, arrow). The box in B is magnified in C demonstrating the elongated Nestin+ cells (arrow) in the pPV. DCX (red) immunoreactivity in the DG and the pPV (D, arrows). ß-III Tubulin staining (E, red) showing the pPV (arrow) between hippocampus and cortex. The box in E is magnified in F demonstrating the BrdU/ß-III Tubulin+ neurons (arrows; BrdU – green; ß-III Tubulin – red) in the dorso-lateral part of the pPV at 6d after BrdU application. Scale bar 200µm for B, D and E; 20µm for F; 10µm for C.

Moreover the intensity of DCX immunoreactivity was stronger in the pPV compared to the DG. Since LV was mostly ß-III Tubulin negative, it appeared as a gap between hippocampus and cortex (Fig. 3.4E). Newly generated neurons (BrdU/DCX+ or BrdU/ß-III Tubulin+) could be detected at the dorso-lateral part of the pPV (Fig. 3.4E, square). BrdU/DCX+ cells found 3d after BrdU application seemed to be isolated from each other (data not shown), whereas at 6d many BrdU+ cells expressed ß-III Tubulin and formed groups of ß-III Tubulin+ cells (Fig. 3.4F).

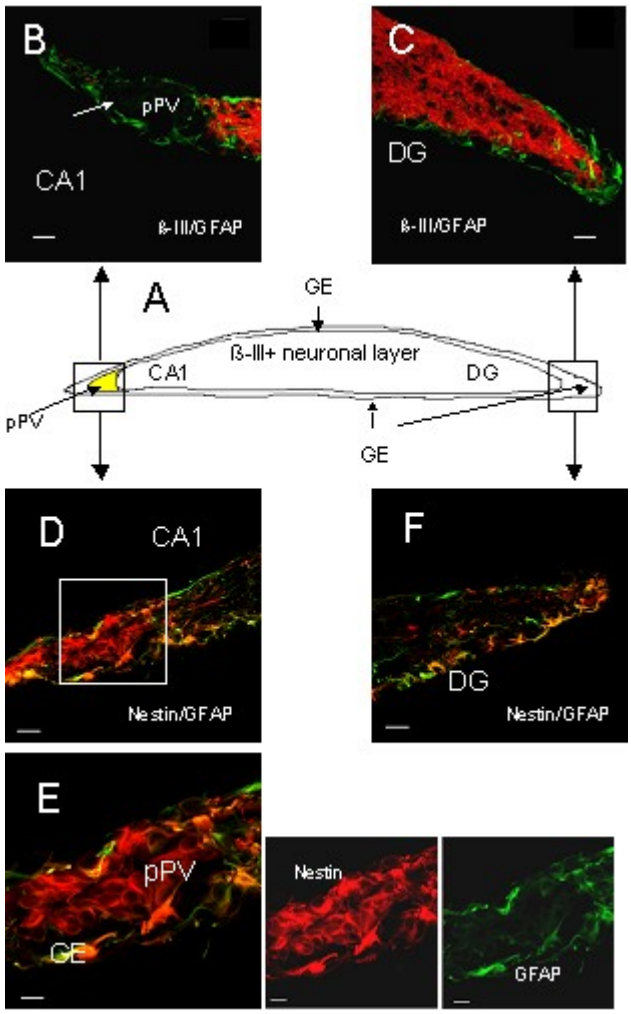

Fig. 3.5. The pPV as a neurogenic zone in OHC. ß-III Tubulin/GFAP and Nestin/GFAP immunohistochemistry was performed in 14 DIV cross sectioned OHC. (A) Schematic drawing of cross sectioned OHC and the location of the pPV. The pPV was the ß-III Tubulin negative structure (B, arrow; ß-III Tubulin – red; GFAP - green) at the "CA1 side" which was not present at the "DG side" (C) of OHC. Nestin+ cells (red) in the pPV (D). The region in the box in D is shown at a higher magnification in E demonstrating the elongated morphology of Nestin+ cells in the pPV. Nestin+ cells with stellar morphology in the GE at the "DG-side" (F; Nestin – red; GFAP - green). Scale bar: 20µm for B, C, D, F; 10µm for E.

Thus, in OCC of P7-9 rats, the pPV lining the hippocampus contained Nestin+ precursors, DCX+ neuroblasts and newly generated ß-III Tubulin+ neurons. Similar to OCC the pPV could be also identified in OHC as a mostly ß-III Tubulin negative area at the dorsal part of the culture ("CA1-side"; Fig. 3.5A) as demonstrated by ß-III Tubulin/GFAP staining (Fig. 3.5B). This structure was not present at the ventral part of the culture ("DG-side"; Fig. 3.5C). Elongated Nestin+ cells probably originating from the pPV were present in this structure (Fig. 3.5D, E). At the "DG-side" only Nestin+ cells with stellar morphology and Nestin/GFAP+ cells from the GE

could be observed (Fig. 3.5F). These data showed that in addition to the DG the pPV represents a second neurogenic zone in OHC. Since Nestin+ precursors in the pPV and the DG had a different morphology, we investigated whether there were also functional differences.

3.2.2. Qualitative comparison of neurogenesis in the DG and the pPV of OHC

Next, the basal neurogenesis in the two neurogenic zones identified in OHC was compared. Newly generated neurons were detected at different time points after BrdU application by the early neuronal marker DCX (3d and 6d) or ß-III Tubulin (6d and 10d). DCX labeling of OHC was predominant in the GCL of the DG (Fig. 3.6A). However some DCX+ cells were also found in the pyramidal cell layer, stratum oriens and at the "stratum oriens-GE border". These cells probably originated from pPV precursors.

3.2.2.1. Neurons generated in the DG assumed a granular cell morphology and showed mostly a punctuated nuclear BrdU pattern

BrdU/DCX+ neurons could be found in the DG at 3d after BrdU application (Fig. 3.6B). BrdU/DCX+ cells were located mostly in the SGZ however some cells were observed in the hilus where they assumed the morphology of migrating neuroblasts (Fig. 3.6C).

The BrdU staining in the majority of doublelabeled neurons appearing in the DG showed a punctuated pattern in the BrdU+ nuclei. At 6d after BrdU application BrdU/ß-III Tubulin+ neurons with a granular cell morphology in the GCL were detected (Fig. 3.6D). These cells were also found later on at 10d (Fig. 3.6E). Very few newly generated neurons which expressed the mature neuronal marker NeuN at this time point were observed in the DG (Fig. 3.6F).

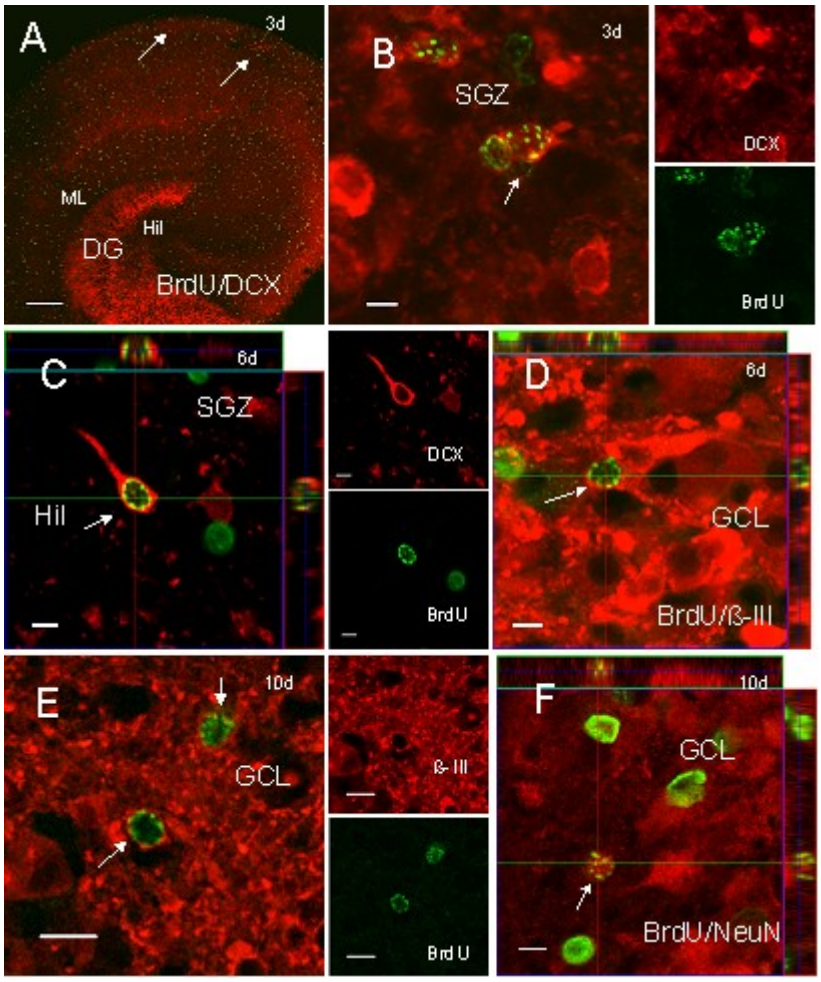

Fig. 3.6. Neurogenesis in the DG of OHC. After 2d BrdU treatment of 14 DIV OHC, newly generated neurons were detected by DCX at 3d and 6d or ß-III Tubulin at 6d and 10d after BrdU application. (A) DCX immunoreactivity in OHC. Arrows indicate DCX+ cells at the "stratum oriens-GE border". BrdU/DCX+ cells in the SGZ of the DG at 3d (B, arrow; BrdU – green; DCX - red). Note the punctuated pattern of BrdU labeled nuclei of newly generated neurons in the DG. Migrating BrdU/DCX+ neurons in the hilus at 6d (C, arrow). BrdU/ß-III Tubulin+ neurons in the GCL of the DG at 6d (D, arrow) and 10d (E, arrow; BrdU – green; ß-III Tubulin – red). Mature BrdU/NeuN+ neuron in the DG at 10d (F, arrow; BrdU – green; NeuN – red). Scale bar: 200µm for A; 10µm for E; 5µm for B, C, D and F.

3.2.2.2. Neurons from pPV were present in the pPV and the stratum oriens and had evenly stained BrdU+ nuclei

In the pPV BrdU/DCX+ cells were observed at an early (3d) and later time point (6d). They were found in the stratum oriens (Fig. 3.7A, B) or in the pPV (Fig. 3.7C). At 6d BrdU/ß-III Tubulin+ cells with neuroblast morphology were also detected in the pPV (Fig. 3.7D) or stratum oriens (Fig. 3.7E). The BrdU staining pattern of the nucleus DCX+ neurons generated in the pPV differed from the one in the DG. In the majority of newly generated neurons in the

pPV the BrdU labeling was not punctuated, but the whole nucleus was evenly stained. Only few newly generated neurons with a punctuated BrdU staining pattern were found here (data not shown). This criterion was essential to differentiate between DG and pPV derived neurons. At 10d BrdU/ß-III Tubulin+ cells showed a different morphology ranging from round neuroblasts with or without cell processes to developing neurons (Fig. 3.7F).

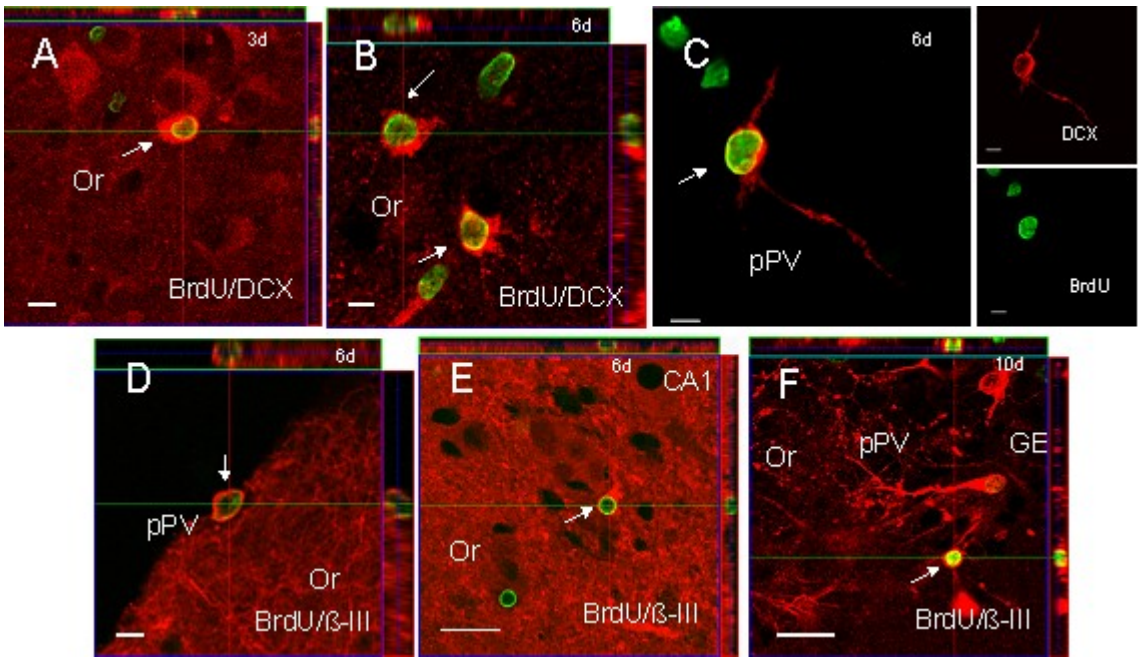

Fig. 3.7. Neurogenesis in the pPV of OHC. 14 DIV OHC were treated with BrdU for 2d thereafter newly generated neurons were detected by DCX at 3d and 6d or ß-III Tubulin at 6d and 10d after BrdU application. BrdU/DCX+ neurons in the stratum oriens at 3d (A, arrow) and at 6d (B, arrow; BrdU – green; DCX – red). BrdU/DCX+ neurons in the pPV at 6d (C, arrow). Note the even pattern of BrdU labeling of the nuclei of newly generated neurons in the pPV. BrdU/ß-III Tubulin+ neurons with neuroblast morphology in the pPV (D, arrow; BrdU – green; ß-III Tubulin – red) and the stratum oriens at 6d (E, arrow). BrdU/ß-III Tubulin+ neurons with neuroblast or migrating phenotype in the pPV (F, arrows). Scale bar: 20µm for E, F; 10 µm for A; 5µm for B, C and D.

Since BrdU/ß-III Tubulin staining included the whole neuronal cell body and processes, differences in the size of newly generated neurons (data not shown) could be also observed. The majority of neurons seen were small and could be defined as interneurons. However numerous large, "pyramidal-cell like" neurons (15µm) were also found close to the stratum oriens.

3.2.3. Quantitative comparison of neurogenesis in the DG and the pPV

To compare neurogenesis in the two neurogenic regions of OHC, BrdU/DCX+ and BrdU/ß-III Tubulin+ neurons were quantified in DG, CA1, CA3 and pPV area at 3d, 6d and 10d after BrdU application. Based on the BrdU staining pattern (evenly stained BrdU+ nucleus), neurons appearing in the stratum oriens and the GE were counted as pPV-derived cells. The number of newly generated neurons in the DG did not increase from 3d to 6d (BrdU/DCX) or from 6d to 10d (BrdU/ß-III Tubulin) (Fig. 3.8). A slight decrease of BrdU/DCX+ cells from 3d to 6d in newly generated neurons was observed. In comparison to the DG the pPV contained more BrdU/DCX+ neurons at 3d however their number also decreased from 3d to 6d (Fig. 3.8).

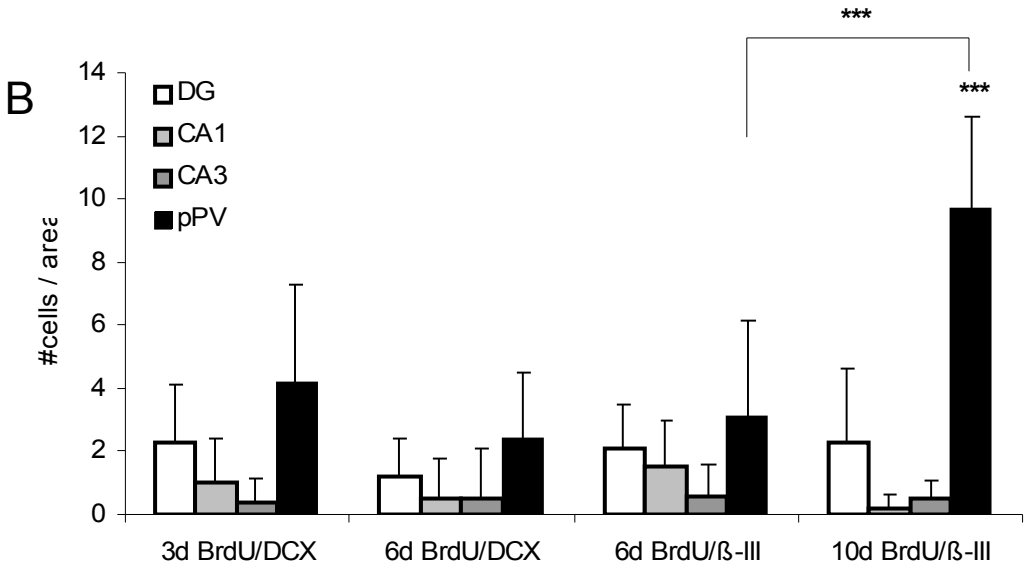

Fig. 3.8. Quantitative analysis of basal and bFGF-induced neurogenesis in OHC. 12-14 DIV OHC were treated with BrdU for 2d thereafter newly generated neurons were detected with DCX at 3d and 6d or with ß-III Tubulin at 6d and 10d after BrdU application. The data are expressed as the mean±SD, ** P <0.01 and ***P<0.001 (ANOVA followed by post-hoc Newman-Keuls test).

A significant increase of neurogenesis (BrdU/ß-III Tubulin) in the pPV was observed at 10d in comparison to 6d. A rather low basal neurogenesis was observed in the DG vs the pPV. We hypothesized that this difference could be indicative of a functional difference between the two

populations of precursors. Therefore we wanted to compare the response to the exogenous neurogenic stimulus - GFs.

3.2.4. The DG and the pPV precursors showed a different response to growth factor treatment

The neurogenic properties of DG and pPV precursors were investigated by the application of GFs . The neurogenic response to GFs, survival and development of newly generated neurons was detected after longer (5d) GF treatment by BrdU/ß-III Tubulin labeling (see Fig. 2.2). GFs failed to alter neurogenesis in the DG (Fig. 3.9A). bFGF could increase the number of BrdU/ß-III Tubulin+ neurons in the pPV whereas EGF treatment resulted in significant decrease of neurogenesis in this zone. BDNF or NGF application did not change the basal neurogenesis in the pPV.

Since only bFGF showed the significant neurogenic effect in our system, we further studied the influence of short (2d) and long (5d) bFGF treatment on neurogenesis and migration of newly generated neurons in the CA1 and CA3 pyramidal layers. A fast neurogenic response to bFGF after 2d treatment in the DG ($P=0.109$; control vs bFGF) and in the pPV with a significant increase of neurogenesis in the pPV was found (Fig. 3.9B). Later on (5d treatment) lower numbers of newly generated neurons (BrdU/ß-III Tubulin) were found in both neurogenic zones. At this time point a neurogenic effect was still observed in the pPV while in the DG the number of newly generated neurons (BrdU/ß-III Tubulin) was decreased and not different from the untreated control.

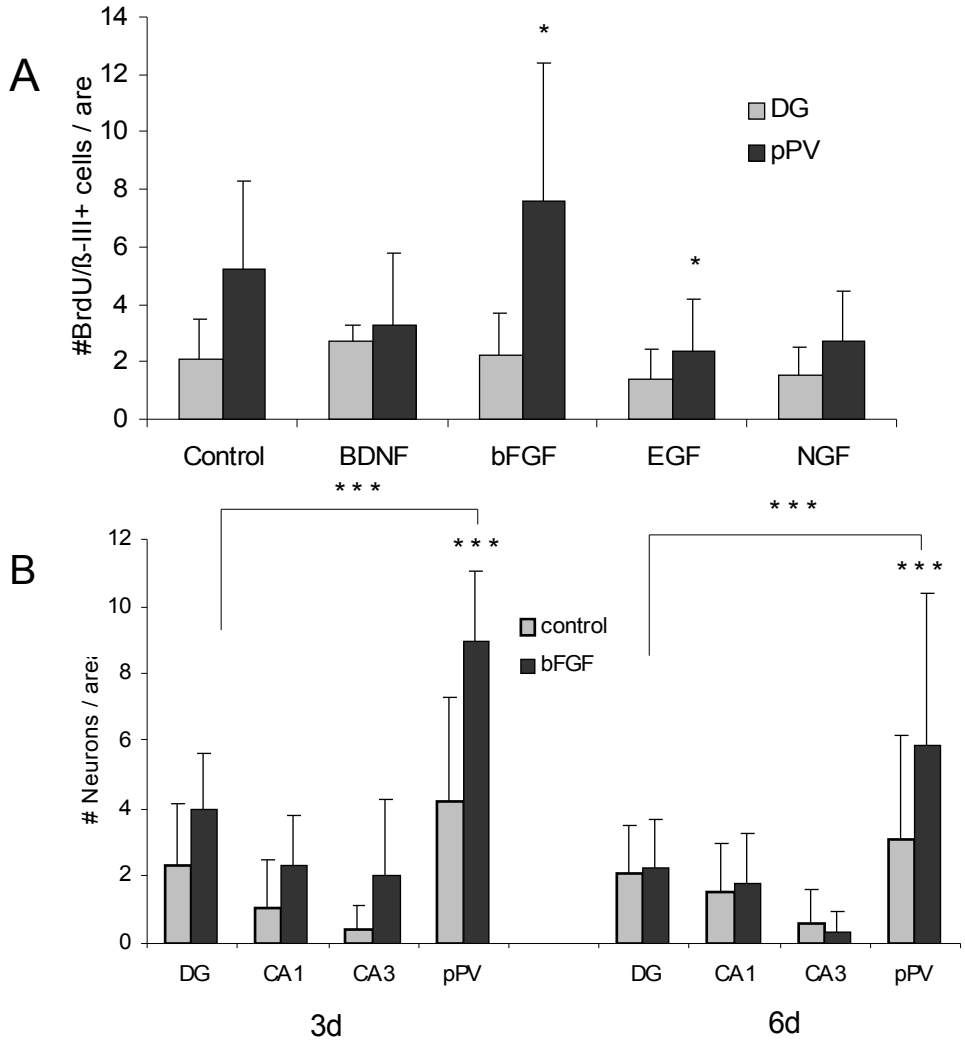

Fig. 3.9. Quantitative analysis of GF-regulated neurogenesis in OHC. (A) Effect of long (5d) GF treatment on neurogenesis in the DG and pPV. GF was added into the culture medium in 24h after BrdU was applied first in a final concentration of BDNF 25 ng/ml, bFGF 5ng/ml, EGF 10 ng/ml; NGF 10 ng/ml for 5d. (B) Effect of short (2d) and long (5d) bFGF treatment on neurogenesis and cell migration in OHC. OHC were treated with bFGF for 2d (short treatment) and 5d (long treatment). Newly generated neurons were detected at 3d by DCX and at 6d by ß-III Tubulin. The data are expressed as the mean±SD, *P <0.05 and ***P<0.001 (ANOVA followed by post-hoc Newman-Keuls test).

In the 2d bFGF treated OHC more BrdU/DCX+ cells compared to control were observed in the CA1 or CA3 areas suggesting that bFGF could stimulate the migration of newly generated DCX+ cells from the pPV in the pyramidal layer. This effect was abolished by long bFGF application. These results suggest that the duration of GF therapy could be one of the parameters, which influence the neurogenic effect of exogenous GFs.

Taking together, two neurogenic zones in OHC: the DG and the pPV were described here.

Low basal neurogenesis was observed in the DG vs higher basal neurogenesis in the pPV. Neurons appearing in the SGZ of the DG assumed a granular cell morphology and could be found in the GCL of the DG. Neurons from the pPV stayed isolated from each other and were present in the pPV or distributed along the "stratum oriens-GE" and "DG-GE" borders. The precursor cells in the two neurogenic zones showed morphological and functional differences. bFGF treatment induced a fast and short-timed neurogenesis in the DG while the pPV precursors showed a stronger and long lasting neurogenic response to bFGF stimulation.

3.3. Cell damage, microglia activation and cell proliferation after OGD

12-14 DIV OHC were exposed to 40 min OGD (see Fig. 2.3). Cell damage, microglia activation, and cell proliferation was detected by PI uptake, OX-42, and BrdU immunoreactivity respectively at different time points after OGD.

3.3.1. Cell damage after OGD

A gradual increase in necrotic cell death was observed from 0 to 24h after insult (Fig. 3.10).

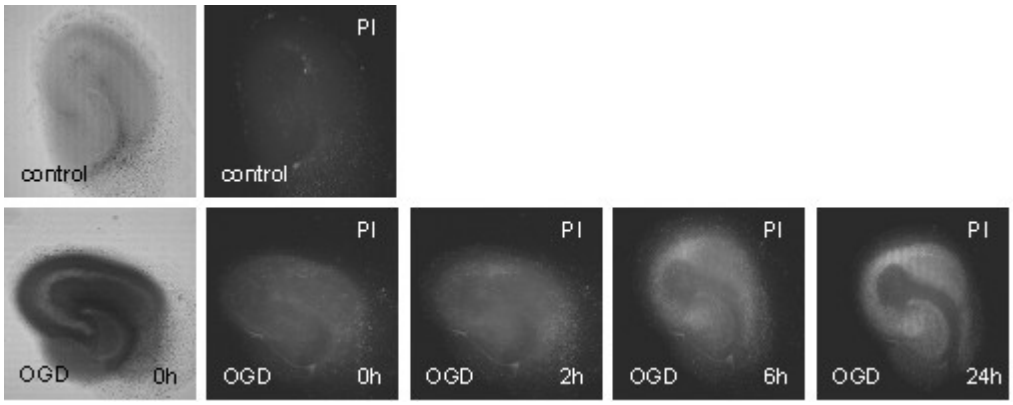

Fig. 3.10. Cell damage occurring in OHC after OGD. OHC were exposed to 40 min OGD. Cell damage was evaluated by PI uptake at 0, 2, 6, 16 and 24h after insult. Transmission light images show OHC under control and OGD conditions. Fluorescent light images show PI uptake at different time points after OGD.

At 24h after insult pyramidal layer and GCL were strongly PI+. In the CA1 pyramidal

layer significant neuronal damage was observed at 6h (Fig. 3.11A) whereas in the DG it occurred at 16h after OGD (Fig. 3.11B).

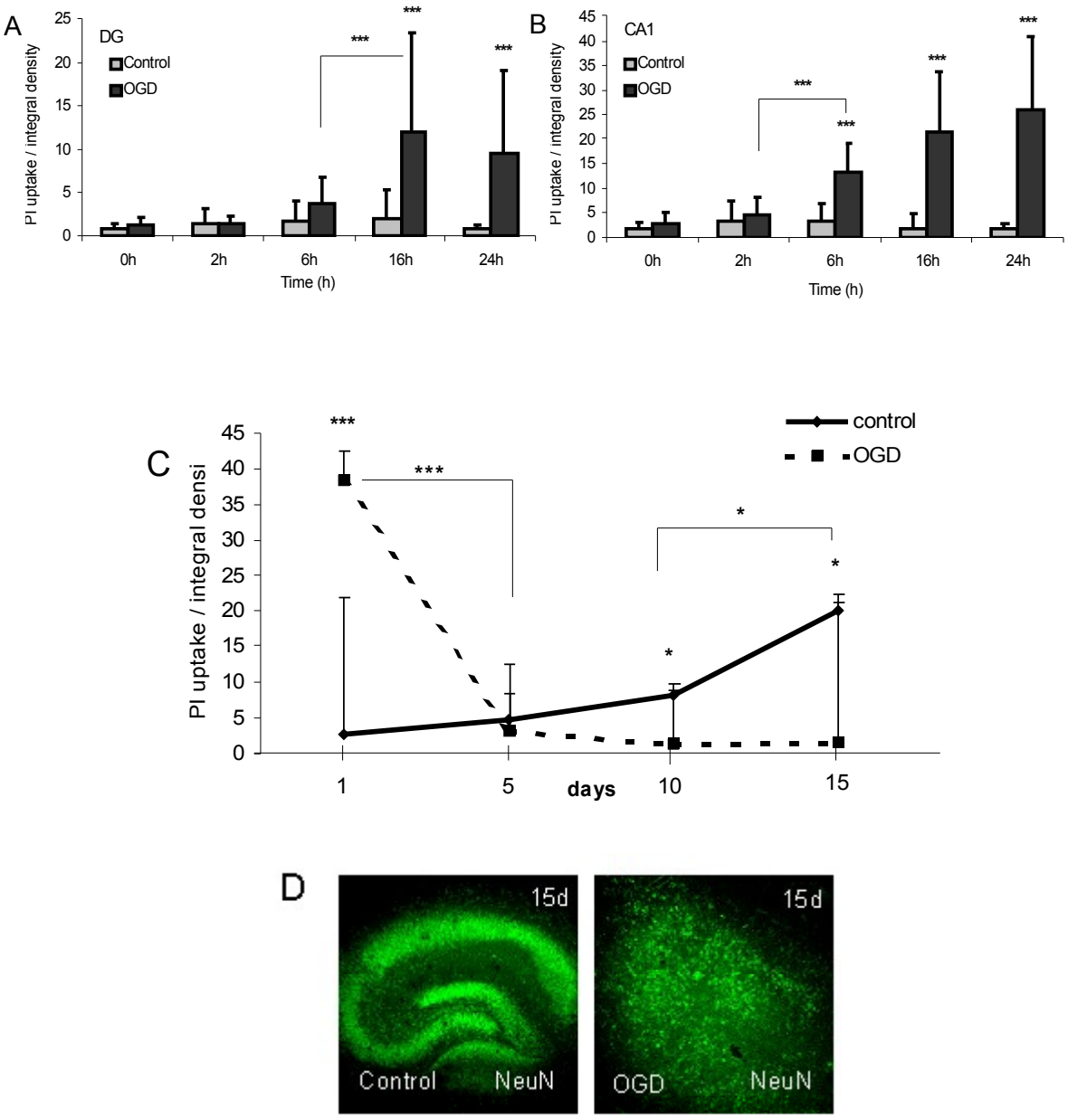

Fig. 3.11. Quantification of cell damage in OHC after OGD. Integral density of PI uptake in the DG (A) and CA1 was quantified using Lucia Software. (C) Temporal profile of damage in pyramidal cell layer in control and OGD-treated OHC maintained for 15d after beginning of experiment. The data are expressed as the mean±SD, ***P<0.001 (ANOVA followed by post-hoc Newman-Keuls test). (D) NeuN staining of OHC maintained in culture for 15d (29DIV) after beginning the experiment. Scale bar 200 μM.

OHC maintained under control conditions for more than 5d (17-19DIV) after beginning of experiment (Fig. 3.11C) did not show the increase of neuronal degeneration in the

pyramidal cell layer. Later on, at 10d (22-24DIV) significant increase of PI uptake in the CA1-CA3 layers with further increase at 15d was defined. Due to the death and elimination of injured cell, cell damage in the pyramidal layer was decreased at 5d after OGD compared to 1d (Fig. 3.11C). Low PI incorporation was observed also at 10d and 15d after OGD. NeuN staining of OHC maintained for 15d (29DIV) after OGD showed almost complete neuronal degeneration of the slice (Fig. 3.11D, right panel) whereas under control conditions (left panel) pyramidal and granular neuronal layers could be seen.

3.3.2. Activation of microglia after OGD

The majority of resident microglia in OHC in the absence of injury was found on the surface of the slice where, in addition to astrocytes, it formed the GE ensheathing the slice culture (see Fig. 3.1).

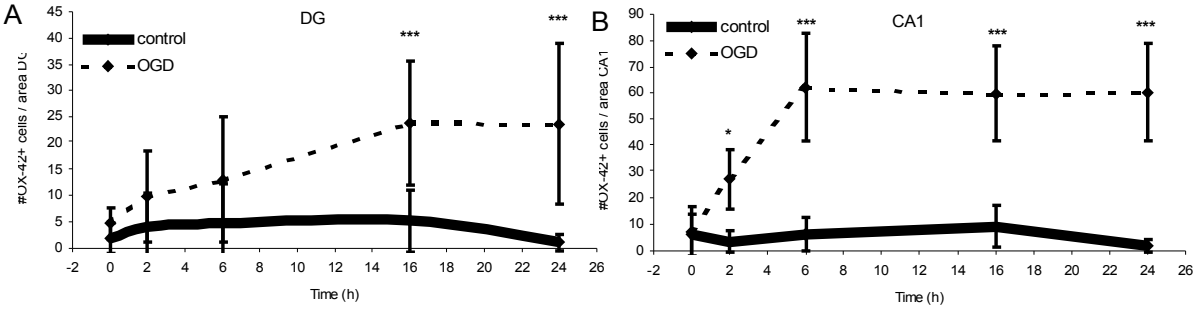

Fig. 3.12. Temporal profile of microglia accumulation in theDG and CA1 after OGD. The data are expressed as the mean±SD, ***P<0.001 (ANOVA followed by post-hoc Newman-Keuls test).

At the same time, inside of the slice, low numbers of OX-42+ microglia were observed (Fig. 3.12). A significant increase in microglia was found in the CA1 area at 2h after OGD with a further increase at 6h (Fig. 3.12B). The increase of microglia in the DG was gradual and reached significance at 16h (Fig. 3.12A). OX-42 immunostaining showed the morphological changes of microglia in OHC from ramified cells seen under control conditions (Fig. 3.13A) to amoeboid activated microglia after OGD (Fig. 3.13B).

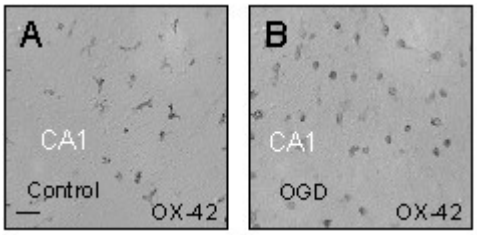

Fig. 3.13. Change of morphology of microglia after OGD. OX-42 immunolabelling shows ramified microglia present in the CA1 under control conditions and activated amoeboid microglia after OGD. Scale bar 50 µm.

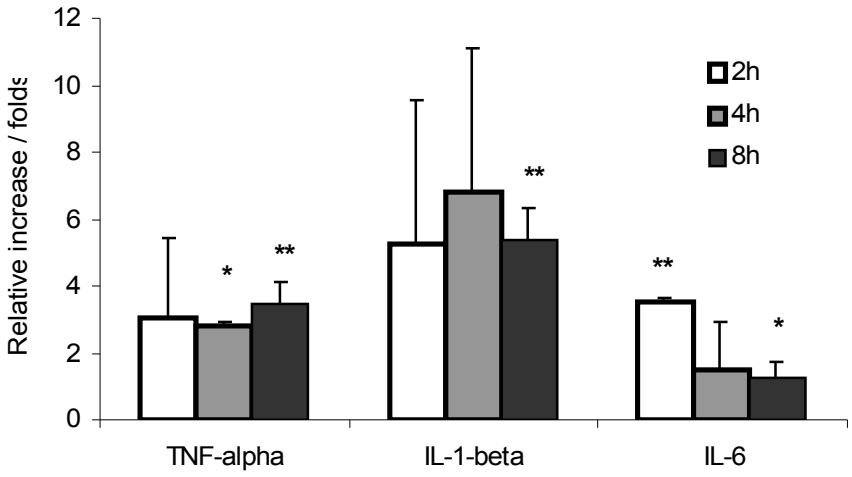

Fig. 3.14. Upregulation of pro-inflammatory cytokines after OGD. mRNA level of pro-inflammatory cytokines TNF-α, IL-1ß and IL-6 was assessed at 2, 4 and 8h after OGD. The data are expressed as the mean±SD, * P<0.05, ** P <0.01 and ***P<0.001 (ANOVA followed by post-hoc Newman-Keuls test).

At 2h after OGD, early microglia activation was accompanied by an upregulation of pro-inflammatory cytokines mRNA level (Fig. 3.14). An increase of TNF-α (4x), IL-1ß (7x) and IL-6 (4x) mRNA was detected. Increased mRNA level of these pro-inflammatory cytokines was observed also at 4 and 8h after OGD.

3.3.3. Cell proliferation after OGD

A high cell proliferation was always found in the GE (see Fig. 3.2A). A low number of BrdU+ proliferating cells was seen after 40 min of BrdU treatment (OGD period) in cryosectioned OHC under control conditions (Fig. 3.15A, B).

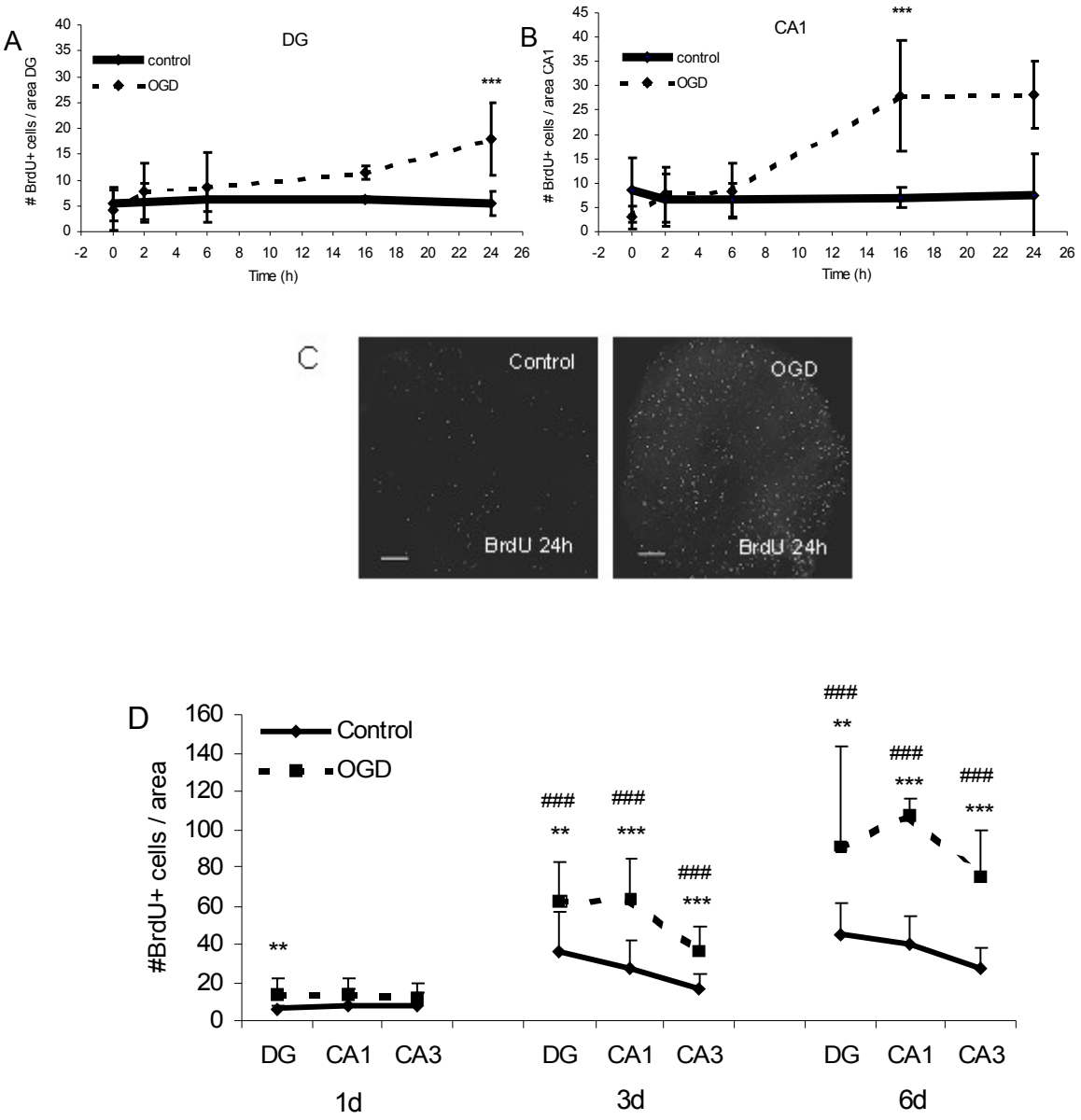

Fig. 3.15. Increase of cell proliferation in OHC after OGD. Quantification of BrdU+ cells in the CA1 (A) and DG (B) at early face (0-24h) after OGD. (C) BrdU immunolabelling shows the distribution of BrdU+ cells in OHC under control conditions (left panel) and the increase of cell proliferation 24h after OGD (right panel) compared to control. Scale bar 200 µm. (D) Quantitative comparison of cell proliferation in OHC at 1d, 3d and 6d after OGD. "*" represents the significant differences in number of BrdU+ cells between control and OGD in one area, "#" represents the significant differences in number of BrdU+ cells between 1d and 3d or 3d and 6d in one area. The data are expressed as the mean±SD, *P<0.05, $^{**,\#\#}$P<0.01 and ***$^{,\#\#\#}$P<0.001 (ANOVA followed by post-hoc Newman-Keuls test).

Longer incubation with BrdU (for 24h) did not result in an increase of proliferating cells, neither in the CA1 area nor in the DG. After OGD, a significant increase of BrdU+ cells compared to control was revealed at 16h in the CA1 area (Fig. 3.15B) and at 24h in the DG (Fig. 3.15A). Massive increase of cell proliferation at 24h after OGD (Fig. 3.15C, right panel) compared to control (Fig. 3.15C, left panel) could be seen in the whole hippocampal

formation. At later time points after OGD (3d, 6d; 2d of BrdU application) further increase of BrdU+ cells was found (Fig. 3.15D). Moreover the number of proliferating cells increased significantly from 1d to 3d and from 3d to 6d of incubation after OGD.

3.3.4. Effect of growth factors on cell proliferation after OGD

To investigate the effect of exogenous GFs on cell proliferation under OGD conditions we treated OHC with BDNF 25 ng/ml, bFGF 5 ng/ml, EGF 10 ng/ml or NGF 10 ng/ml using the standard protocol (see Fig. 2.4). A decrease in BrdU+ cells compared to OGD was found in the DG and CA1 after short or long BDNF treatment (Fig. 3.16).

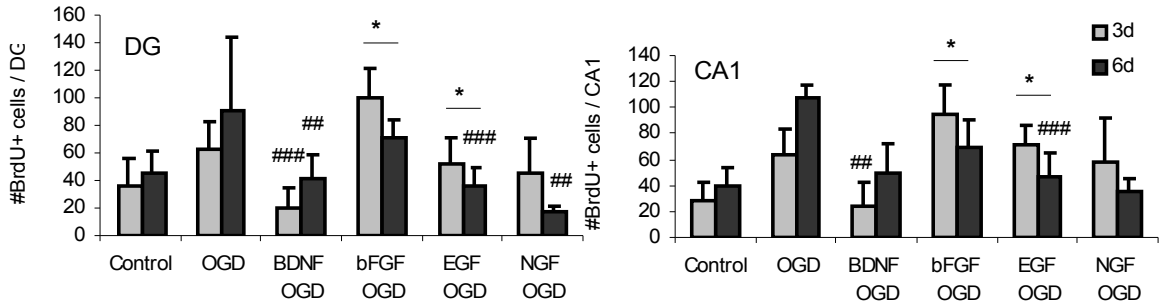

Fig. 3.16. Quantitative analysis of cell proliferation in OHC treated with exogenous GF after OGD. OHC were incubated with BrdU for 2d after OGD and were treated for 2d or 5d with one of the following GF in a final concentration of BDNF 25 ng/ml, bFGF 5 ng/ml, EGF 10 ng/ml or NGF 10 ng/ml. GF was added in culture medium at 24h after OGD and BrdU+ cells were detected at 3d and 6d after OGD. "*" represents the significant differences in number of BrdU+ cells between 3d and 6d GF treatment, "$^{\#}$" represents the significant differences in number of BrdU+ cells between OGD and OGD+GF treatment at the same time point. The data are expressed as the mean±SD, *P<0.05, $^{**,\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 (ANOVA followed by post-hoc Newman-Keuls test).

Long EGF or NGF application reduced cell proliferation in the both areas. In the cases of bFGF and EGF significant decrease of BrdU+ cells was observed in long treated cultures compared to short GF application. After having established the course of events after OGD and effect of GFs on OGD-regulated cell proliferation we were interested in the effect of injury on neurogenesis.

3.4. Neurogenesis in OHC after OGD

To label proliferating cells, OHC were treated with BrdU for 2d after OGD. Early (3d) and later (6d) neurogenesis was investigated by BrdU/DCX and BrdU/ß-III Tubulin immunohistochemistry (see Fig. 2.4). ß-III Tubulin immunostaining showed the neuronal network of OHC under control conditions (Fig. 3.17A) and the cyto-architecture of CA1 pyramidal layer (Fig. 3.17B).

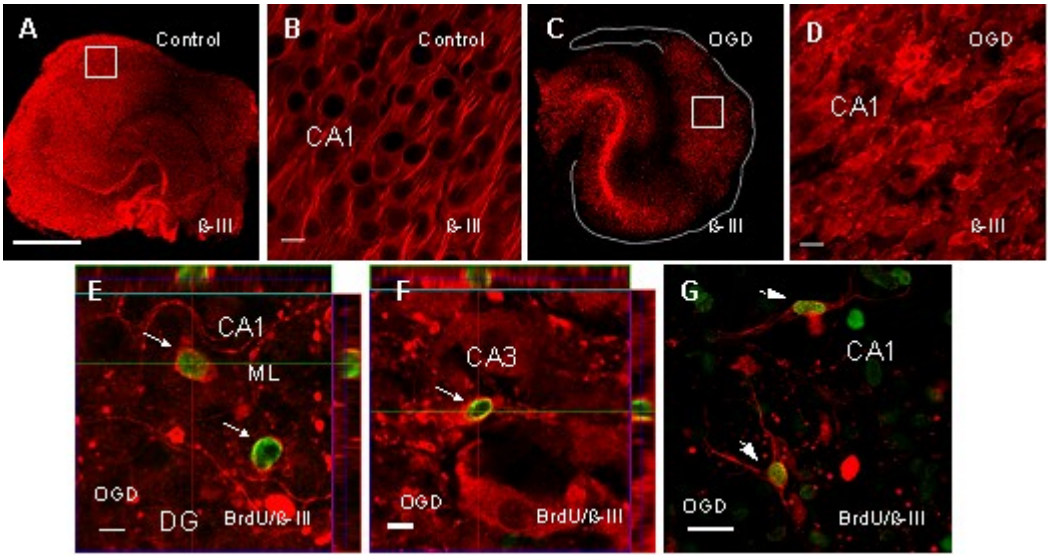

Fig. 3.17. Neuronal structure of OHC and newly generated neurons after OGD. BrdU/DCX+ neuron in the pPV-stratum oriens area. ß-III Tubulin staining shows neuronal architecture of OHC under control conditions (A) and after OGD (C). The regions in the box in A and C are shown at a higher magnification in B and D respectively, demonstrating morphology of pyramidal CA1 neurons under control conditions and after OGD. (E) BrdU/ß-III Tubulin+ neurons with neuroblast morphology in the molecular layer of OHC after OGD. (F) BrdU/ß-III Tubulin+ neuron in the pyramidal CA3 layer of OHC after OGD. (G) BrdU/ß-III Tubulin+ neurons in the CA1 layer of OHC after OGD seem to assume the morphology of pyramidal cells. Scale bar: 500µm for A and C; 20µm for G; 10 µm for B, D, E and F.

After OGD the neuronal network was destroyed (Fig. 3.17C) since pyramidal CA1 neurons had degenerated and showed atrophy of dendritic processes as well as shrinkage of cell bodies (Fig. 3.17D). However, newly generated neurons were observed in OGD-exposed cultures (Fig. 3.17E, F). They were found in the molecular layer, the pyramidal cell layer, the pPV, stratum oriens and DG. The majority of newly generated neurons at 6d after OGD displayed neuroblast morphology. BrdU/ß-III Tubulin neurons with pyramidal cell morphology were

rarely found in the CA1 area of OGD exposed OHC (Fig. 3.17G).

Quantification of BrdU/DCX and BrdU/ß-III Tubulin+ cells showed low basal neurogenesis in the DG compared to the pPV (Fig. 3.18). Early after OGD (3d) a decrease of BrdU/DCX+ neurons was defined in both neurogenic zones.

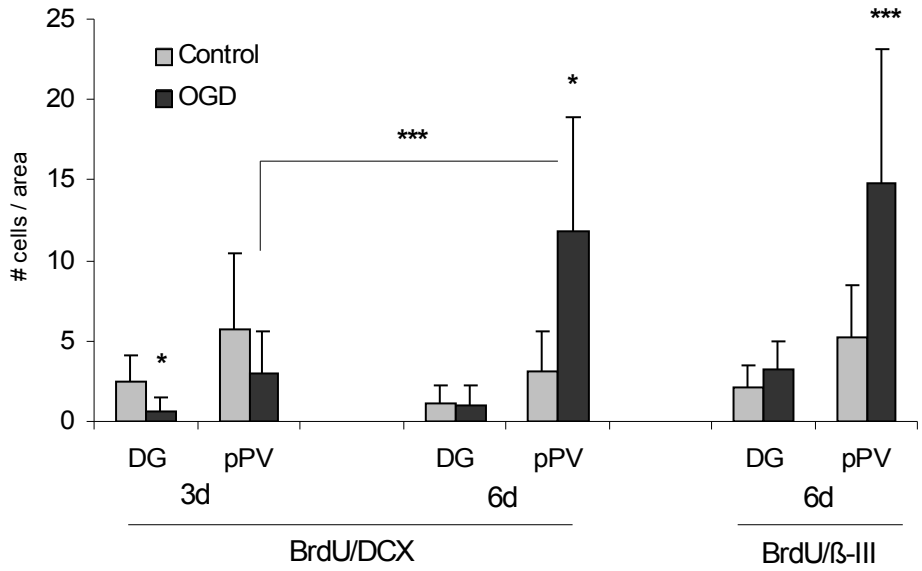

Fig. 3.18. Neurogenesis in OHC after OGD. Quantitative analysis of neurogenesis in OHC under control and OGD conditions at 3d and 6d after OGD. The data are expressed as the mean±SD, *P<0.05; ***P<0.001 (ANOVA followed by post-hoc Newman-Keuls test).

Moreover the downregulation of DCX expression was seen in the DG of OGD exposed cultures (data not shown). However at a later time point (6d) restoration of neurogenesis was detected and a significant increase in the number of BrdU/DCX+ or BrdU/ß-III Tubulin+ cells was found in the pPV (Fig. 3.18).

3.4.1. bFGF increases neurogenesis in the pPV after OGD

To investigate whether the application of exogenous GFs can boost neurogenesis after insult in our system, we treated OHC with bFGF 5ng/ml for 5d (protocol see Fig. 2.4). At 6d after OGD we observed significant increase in number of BrdU/ß-III Tubulin+ neurons in the

pPV of OGD-treated OHC compared to control (Fig. 3.19). bFGF applied after OGD showed considerable neurogenic effect in the pPV, stimulating thus the tissue regeneration after neuronal loss.

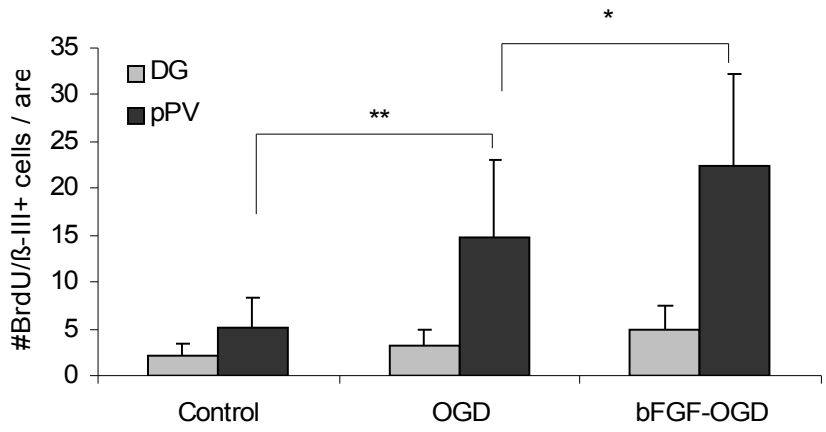

Fig. 3.19. Increase of neurogenesis by bFGF treatment after OGD. OHC were incubated with BrdU for 2d after OGD and treated for 5d with bFGF in a final concentration of 5 ng/ml. bFGF was added into the culture medium at 24h after OGD and BrdU/ß-III Tubulin+ neurons were detected at 6d after OGD in the DG and pPV. The data are expressed as the mean±SD, *P<0.05, **P<0.01 (ANOVA followed by post-hoc Newman-Keuls test).

Taken together, these data showed the temporal relationship between neuronal damage, microglia activation, inflammation, cell proliferation and neurogenesis (Fig. 3.20). The inflammatory reaction was already evident 2h after insult. At the same time point accumulation of microglia occurred in the CA1 area and reached the maximal level at 6h accompanied by significant neuronal damage. At 16h we found a considerable increase of cell proliferation. In the DG these effects were detected later compared to the pPV. Neurogenesis in OHC was detected in the DG and the pPV.

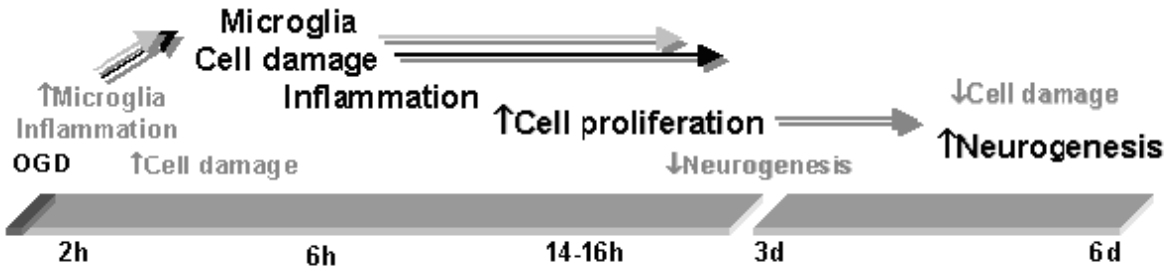

Fig. 3.20. Temporal interplay between microglia activation, inflammation, cell damage, cell proliferation and neurogenesis in OHC after OGD

Neurogenesis in the pPV was significantly higher than the one in the DG. At 3d after insult, neurogenesis was inhibited but the increase of neurogenesis was already observed at 6d. At this time point neurogenesis was significantly increased in the pPV, while attenuation of cell damage has been found. Continual increase in cell proliferation took place and could be downregulated by exogenous GFs treatment. We further assessed the contribution of inflammation in OGD-induced changes and neurogenesis by blocking the inflammation with anti-inflammatory compounds.

3.5. Anti-inflammatory treatment

To investigate the contribution of inflammation to the complex sequence of pathological events and neurogenesis, OHC were treated with anti-inflammatory compounds: the nonselective COX inhibitor indomethacin or the inhibitor of p38 MAPK minocycline at a final concentration of 10 or 50 μM. A MK-801 (10μM) treated group was included as a positive control for neuroprotection. Compounds were added for 2h before, during and until 2d after OGD (see Fig. 2.5). Cell damage and the number of OX-42+ microglia were detected 24h after insult. Cell proliferation and neurogenesis was assessed at 6d after OGD with the early neuronal marker DCX.

3.5.1. Anti-inflammatory treatment reduces neuronal damage after OGD

Neither indomethacin nor minocycline induced neuronal damage in OHC under control conditions (Fig. 3.21). MK-801, indomethacin or minocycline treatment significantly prevented OGD-induced neuronal injury. The effect of indomethacin or minocycline was less pronounced compared to MK-801.

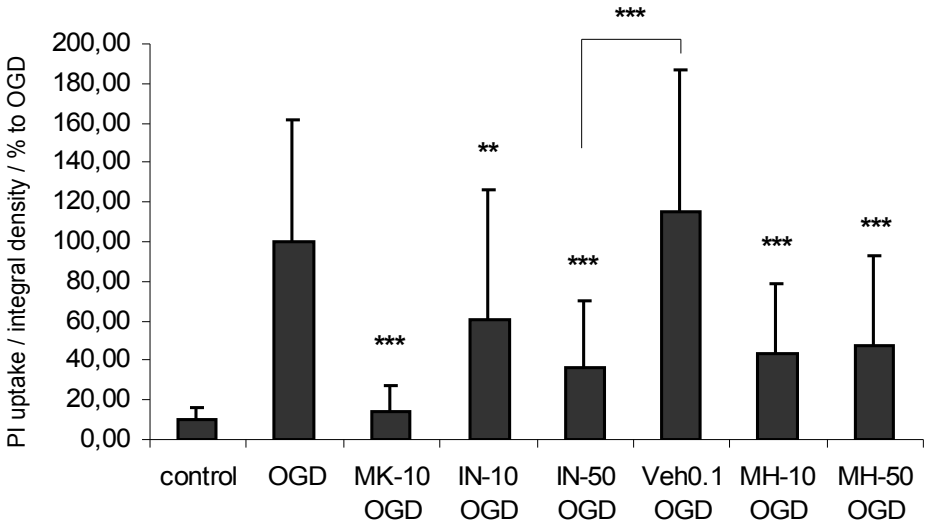

Fig. 3.21. Effect of anti-inflammatory treatment on cell damage after OGD. 12-14DIV OHC were treated with indomethacin (10 or 50μM) or minocycline (10 or 50μM) for 2h before, during and 2d after OGD. MK-801 (10μM) was used as a positive control of neuroprotection. At 24h after OGD, cell damage was assessed by PI uptake. The data are expressed as the mean±SD, **P<0.01; ***P<0.001 (ANOVA followed by post-hoc Newman-Keuls test). IN, indomethacin; MI, minocycline; MK, MK-801; Veh, 0.1% ethanol.

3.5.2. Anti-inflammatory treatment reduces the numbers of microglia after OGD

A decreased number of microglia in the DG of OGD-exposed cultures treated with MK-801, indomethacin or minocycline was observed (Fig. 3.22). A significant reduction of microglia was also defined in the CA1 area after treatment with MK-801, indomethacin (10 μM) and minocyclin (50 μM). Treatment with indomethacin (50 μM) resulted in a slight decrease of microglia in the CA1 area (P=0.106, vs OGD) whereas minocycline (10 μM) did not display this effect.

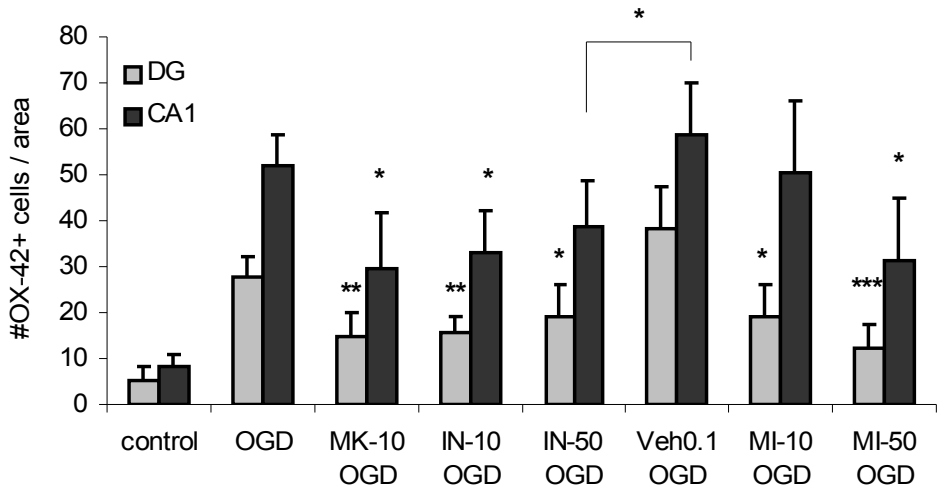

Fig. 3.22. Effect of anti-inflammatory treatment on microglia after OGD. 12-14DIV OHC were treated with indomethacin (10 or 50µM) or minocycline (10 or 50µM) for 2h before, during and 2d after OGD. MK-801 (10µM) was used as a positive control of neuroprotection. At 24h after OGD, microglia was assessed by OX-42 immunostaining. The data are expressed as the mean±SD, *P<0.05; **P<0.01; ***P<0.001 (ANOVA followed by post-hoc Newman-Keuls test). IN, indomethacin; MI, minocycline; MK, MK-801; Veh, 0.1% ethanol.

3.5.3. Anti-inflammatory treatment reduces cell proliferation after OGD

We also observed that MK-801, indomethacin or minocycline abolished OGD-induced cell proliferation in the DG and CA1 (Fig. 3.23). This effect has been seen at 6d after OGD when the high level of proliferation was found.

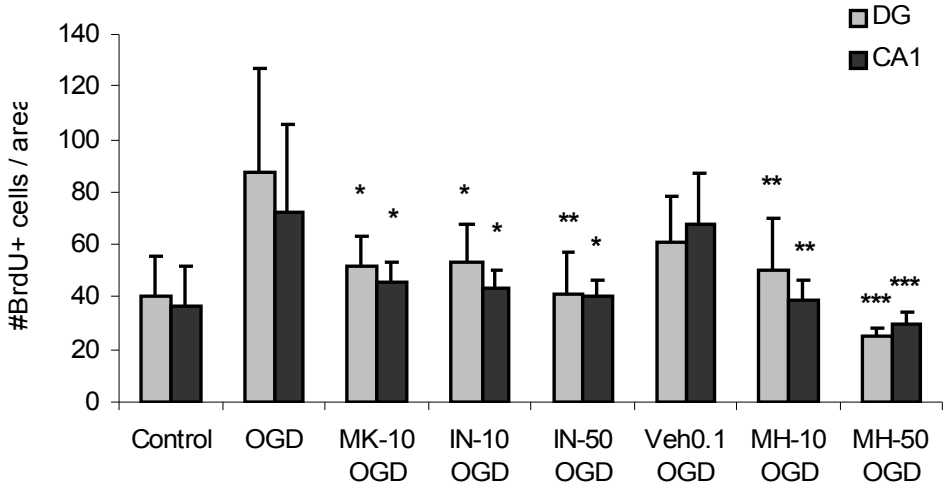

Fig. 3.23. Effect of anti-inflammatory treatment on cell proliferation after OGD. 12-14DIV OHC were treated with indomethacin (10 or 50µM) or minocycline (10 or 50µM) or MK-801 (10µM) for 2h before, during and 2d after OGD. Proliferating cells were labeled by BrdU for 2d after OGD. BrdU+ and BrdU/DCX+ cells were detected at 6d. The data are expressed as the mean±SD, *P<0.05; **P<0.01; ***P<0.001 (ANOVA followed by post-hoc Newman-Keuls test). IN: indomethacin; MI: minocycline; MK, MK-801; Veh, 0.1%

ethanol.

3.5.4. Anti-inflammatory treatment and neurogenesis after OGD

3.5.4.1. MK-801 treatment and neurogenesis

MK-801 at a final concentration of 10 μM increased (P=0.045, MK-801 vs control) neurogenesis in the pPV under control conditions (Fig. 3.24). A reduction in BrdU/ß-III Tubulin+ cells was observed in OGD-exposed cultures treated with MK-801. DG neurogenesis was not affected by MK-801 treatment.

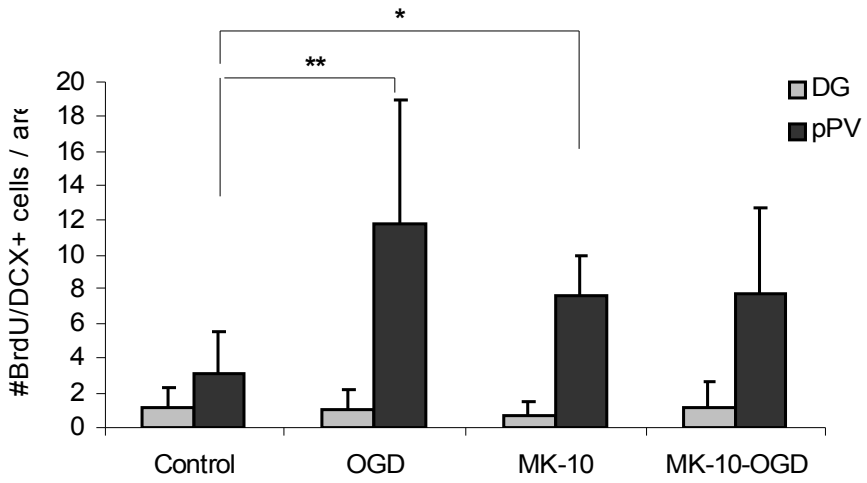

Fig. 3.24. Effect of MK-801 treatment on neurogenesis after OGD. OHC were treated for 2h before, during and 2d after OGD with MK-801 in final concentration of 10μM. Cultures were incubated with BrdU for 2d after OGD and newly generated neurons were detected at 6d with BrdU/DCX immnunolabeling. Influence of MK-801 treatment on basal and OGD-induced neurogenesis. The data are expressed as the mean±SD, *P<0.05; **P<0.01; ***P<0.001 (ANOVA followed by post-hoc Newman-Keuls test). MK, MK-801.

3.5.4.2. Anti-inflammatory treatment and neurogenesis

Indomethacin (Fig. 3.25A) or minocycline (Fig. 3.25B) showed neurogenic properties, increasing the number of BrdU/DCX+ cells in the pPV under control conditions (P<0.001, indomethacin 10μM vs control; P=0.048, indomethacin 50μM vs control; P=0.009, minocycline 10μM vs control). A higher concentration of minocycline did not show this effect (P=0.653, minocycline 50 μM vs control). Neurogenesis in the pPV was significantly increased at 6d after OGD compared to control (P<0.001, OGD vs control).

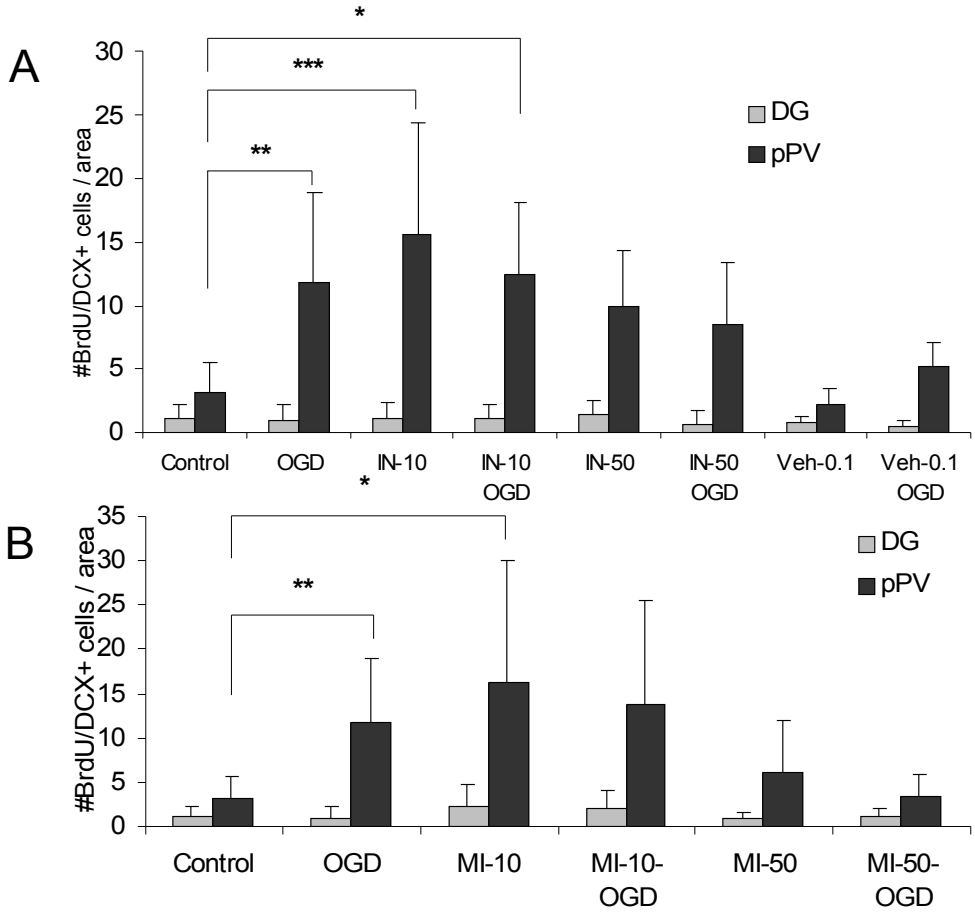

Fig. 3.25. Effect of anti-inflammatory treatment on neurogenesis after OGD. OHC were treated for 2h before, during and 2d after OGD with indomethacin (A) or minocycline (B) in a final concentration of 10μM or 50 μM. Cultures were incubated with BrdU for 2d after OGD and newly generated neurons were detected at 6d with BrdU/DCX immnunolabeling. The data are expressed as the mean±SD, *P<0.05; **P<0.01; ***P<0.001 (ANOVA followed by post-hoc Newman-Keuls test). IN: indomethacin; MI: minocycline; Veh, 0.1% ethanol.

OGD-induced neurogenesis in the pPV whereas indomethacin (10 or 50 μM) or minocycline (10 μM) did not affect it. However neurogenesis was reduced after OGD in the vehicle-control group (0.1% ethanol) (P=0.106, vehicle vs OGD) or the group treated with a higher concentration of minocycline (P=0.058, minocycline 50μM vs OGD). In the DG no difference in the number of BrdU/DCX+ cells was detected between control and treated groups.

Thus, our results demonstrated that blockade of NMDA receptors by MK-801 as well as inhibition of COX by indomethacin or p38 MAPK by minocycline prevented OGD-induced neuronal injury, microglia activation and migration. That in turn reduced injury-

induced cell proliferation. Indomethacin (10 or 50 μM) or minocycline (10 μM) displayed a neuroprotective effect, and did not interfere with OGD-induced neurogenesis. Whereas MK-801 slightly decreased neurogenesis under OGD conditions.

4. Discussion

In the first part of this study basal cell proliferation and neurogenesis in interface OHC was characterized. OHC were prepared from P7-9 rats and maintained *in vitro* for 14d before the proliferating cells were labeled with BrdU.

4.1. Glial envelope and proliferating cells in OHC

The initial trauma during preparation and subsequent cultivation stimulated astrocytes to proliferate and form a glial barrier ensheathing the slice (glial envelope). The presence of activated astrocytes expressing Nestin and activated microglia indicated a protective function of the GE . We found massive cell proliferation in the GE (surface of the slice) compared to low proliferation inside the slice. However, due to the young age of animals used, the cell proliferation inside the slice was higher compared to the proliferation found in the adult hippocampus in vivo . Roughly the same proliferation rate could be detected in DG, CA1 or CA3 areas. This has also been reported in roller-tube OHC . The cell proliferation significantly increased between 1d and 3d of incubation indicating the difference in the duration of BrdU presence. However the number of proliferating cells did not change over time during the further incubation period from 3d to 10d. Cell proliferation in OHC was affected by exogenous bFGF or EGF (Table 4.1). While EGF could increase the number of BrdU+ cells after short application (2d), longer incubation (5d) was needed to stimulate proliferation by bFGF. At the same time long EGF treatment resulted in significant reduction of BrdU+ cells after initial increase whether due to the apoptosis and death or migration of newly generated cells. Exogenous BDNF or NGF did not interfere with basal cell proliferation in our system either due to the low binding affinity of GFs receptors or sufficient level of endogenous GFs in the tissue .

4.2. Identification of a second neurogenic zone in OHC and morphological differences of DG and pPV precursors

In addition to the Nestin+ astrocytes and the small Nestin+ cells with stellar morphology in the GE, other morphologically different Nestin+ cell populations could be observed in OHC. The DG contained larger Nestin+ cells with stellar morphology while elongated Nestin+ cells were present in the "stratum oriens-GE border". This structure could be the remainder of the secondary dentate matrix and dentate migration path. However in P7-9 rat hippocampus these structures are already diminished and neurogenesis in the secondary dentate matrix is not longer present during the cultivation of OHC . Therefore we hypothesized that elongated Nestin+ cells might be derived from the pPV. The pPV is adjacent to the hippocampus as a part of the embryonic LV which contains precursor cells displaying *in vitro* stem cell properties . In the absence of injury stem cells in the adult brain pPV are dormant and can proliferate and migrate into the CA1 area in response to global ischemia following GF treatment . Elongated Nestin+ cells in the pPV were observed in sham operated and ischemic adult rats (C. Pforte, unpublished observation). Here we found that these cells generated DCX and ß-III Tubulin+ neurons which formed the structure of the pPV between the cortex and the hippocampus in OCC. After separating the hippocampi from the cortex during slice preparation a part of the pPV was also present in the OHC. Cross sections of OHC showed that the GFAP- and ß-III Tubulin-negative tissue above the dorsal hippocampus ("CA1-side") contained elongated Nestin+ cells. The morphology (elongated Nestin+) and in situ appearance (cell clusters forming a dense network) of cells was similar to Nestin+ precursors of the pPV. Thus, in the OHC besides the DG we could identify the pPV as a second neurogenic zone. These zones are characterized by two morphologically different populations of precursors.

4.3. Functional differences of DG and pPV precursors

4.3.1. Low neurogenesis in the DG compared to high neurogenesis in the pPV

We revealed also functional differences between two precursor populations. Low neurogenesis was observed in the DG in contrast to higher neurogenesis in the pPV. The number of newly generated neurons in the pPV increased in number up to 10d after BrdU application and significantly exceeded the number of newly generated neurons in the DG. In the DG the amount of newborn neurons (basal neurogenesis) did not change during the whole experimental period (10d). It has been described that neurogenesis in the DG in roller-tube cultures is reduced in the presence of serum . In our system the presence of serum significantly decreased neurogenesis in the pPV while DG basal neurogenesis was not affected (data not shown).

4.3.2. Punctuated pattern of BrdU+ nuclei of newly generated neurons in the DG compared to even stained neuronal nuclei in the pPV

Neuronal precursors in the pPV seemed to display the characteristics of quickly dividing cells. This could also explain the different pattern of BrdU immunolabeling in the nucleus of cells generated in the DG and pPV. The S phase of the cell cycle of pPV precursors is probably faster than that of precursors in the DG. Thus, 2d of BrdU incubation may be long enough to label a sufficient number of newly generated neurons. Since very rarely neurons in the pPV showed punctuated pattern of BrdU labeling, we suggest that these cells were generated after removing of BrdU from the culture medium. The long cell cycle of DG precursors maybe prevented abundant BrdU incorporation and thus caused the punctuated BrdU staining pattern in the nucleus in a majority of newly generated neurons in the DG.

In contrast Liu et al. observing the punctuate labeling of BrdU in many cells in the DG after global ischemia suggested the dilution of label by several cycles of cell division . In this case DG precursors in our system should have high proliferating rate since punctuated pattern

of BrdU labeling could be seen already in BrdU/DCX+ cells detected 3d after BrdU application. However basal DG neurogenesis in our juvenile interface OHC was lower compared to roller-tube OHC and in vivo studies . The difference in the number of newly generated neurons in roller-tube cultures could be explained by differences in age (P7 vs P5), species (rat vs mice) of the animals, culture conditions and experimental setup (14d vs 17d), BrdU treatment protocol (2d vs 3d), thickness of analyzing slices (20µm vs 50-100µm) and neurogenic markers used (DCX, ß-III Tubulin vs NeuN). The permanent interface air-tissue system might influence the activity of DG precursors in differently compared to continuous alternation of feeding and aeration in the roller-tube cultures . Neurogenesis in the DG was lower compared to neurogenesis in vivo. Evidently, the neuronal precursor cells in the DG are restricted in the potential while the glial precursors dominate under *in vitro* conditions. This may have occurred because different environmental cues present in the DG and dentate hilus influence the developmental fate of CNS progenitors.

4.3.3. Maturation of newly generated neurons in the DG and pPV

In the DG newly generated neurons could be observed at 3d after BrdU application. At 10d they displayed a granular cell morphology, however only few of them expressed NeuN. In spite of the long incubation period (10d) the majority of BrdU/ß-III Tubulin+ neurons in pPV retained a neuroblast morphology. We assumed that newly generated neurons could not complete the maturation and died shortly after generation however the increase observed on 10d suggests that majority of cells survive. Hence, the maturation of newly generated neurons in the pPV seems to be slower compared to the DG and longer time may be required for the complete maturation. A comparable delay of glial development in organotypic slice cultures was reported by del Rio . Furthermore, for the neuronal differentiation of neuroblasts the migration to the olfactory bulb through the RMS from the SVZ or into the GCL from the SGZ is thought to be necessary . However in our system neuroblasts generated in the pPV did not formed migratory

stream, remaining in the pPV, migrating to the stratum oriens or along the slice border or into the CA3 and CA1 pyramidal layer. The morphological characteristics (shape of the cell body, size) of the majority of newly generated neurons in pPV indicated that they seemed to be interneurons. The use of markers specific for neuronal subpopulations as well as following the fate beyond 10d are both suitable approaches to elucidate the fate of newly generated neurons as well as their ability to integrate into the neuronal circuitry.

4.3.4. Effect of growth factors on neurogenesis in OHC

Neurogenesis in the DG and the pPV was stimulated by short (2d) bFGF treatment, however a significant increase was revealed only in the pPV (see Table 4.1). The bFGF-induced increase of neurogenesis in the DG at 2d proved to be only temporary since the number of newly generated neurons in the DG decreased later on (5d treatment). Either the newly generated neurons might not survive or they migrated from the DG to the pyramidal cell layer . In contrast to the DG, the bFGF effect on neurogenesis in the pPV pointed at more potent neurogenic and survival properties of pPV precursors. Similar to the effect of EGF on cell proliferation extended EGF treatment (5d) reduced neurogenesis in the pPV with no effect on DG neurogenesis. That could point either on induction of apoptosis and elimination of neuronal progenitors or stimulation of progenitors to differentiate in glial cell lineage by EGF treatment (Kuhn et al., 1997). Neither BDNF nor NGF could affect neurogenesis in our system.

Table 4.1. Effect of exogenouse GFs on basal cell proliferation and neurogenesis in OHC

Parameter	bFGF		EGF		BDNF		NGF	
	2d	5d	2d	5d	2d	5d	2d	5d
Proliferation	No effect	Increase	Increase (CA1)	Decrease	No effect	No effect	No effect	No effect
Neurogenesis	Increase	Increase (pPV)	n.d.	Decrease (pPV)	n.d.	No effect	n.d.	No effect

n.d., not determined

Here we described the neurogenesis *in vitro* in OHC. We showed for the first time that in

addition to the DG with the pPV there is a second neurogenic zone present in OHC (Fig. 4.1). Stellar larger Nestin+ cells were found in the DG while elongated Nestin+ cells were observed in the pPV. Precursors from the DG showed a fast but insignificant response to bFGF stimulation. pPV precursors had a fast and efficient response to bFGF and seemed to possess more potent neurogenic properties.

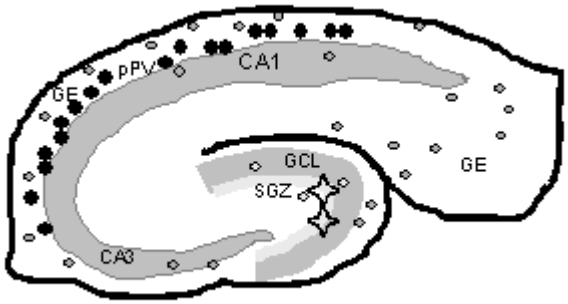

Fig. 4.1. Schematic drawing of the neurogenic zones present in OHC. Stars represent precursors in the DG; big black dots represent precursors in the pPV; small white dots represent newly generated neurons in the DG; small grey dots represent newly generated neurons in the pPV.

EGF was able to reduce pPV neurogenesis without affecting the DG neuronal precursors. Hence, the difference between the DG and pPV response to GFs could be explained by either a differential regulation of these two neuronal precursor populations by GFs or different proliferation rate/turnover. Since pPV precursors did not express GFAP they could not be characterized as SVZ stem cells but may be as subependymal cells reported by Seaberg and van der Kooy . These authors showed significant stem cell properties of subependymal cells isolated from the areas surrounding the hippocampus (posterior LV, 3rd ventricle and "hippocampal arch"). However, the *in vitro* assays of multipotenciality and self-renewal are much dependent form the experimental protocol used for isolation of precursor cells (Kempermann G., personal communication). Moreover the finding that ependimal cells do not incorporate mitotic markers at any of the survival times studied indicates that these cells are not the primary precursors of

the newborn neurons (Gage et al., 2004). Thus, we assumed that the elongated Nestin+ cells observed in the pPV could be the rapidly dividing transit-amplifying cells (type C) which are derived from the SVZ astrocytes (stem cells, type B) and lack the expression of GFAP but still able to divide.

4.4. Cell damage, microglia activation and cell proliferation after OGD

Based on the reported observation that neurogenesis in the DG and SVZ is increased in response to neuronal damage we used OHC to investigate the complex interplay between neuronal damage, microglia activation, inflammation, cell proliferation and neurogenesis after OGD.

4.4.1. Microglia activation and neuronal damage preceded cell proliferation after OGD

Resident microglia are activated rapidly after the onset of ischemia . Microglia transform into phagocytes and remove necrotic neurons but sparing eventually surviving neurons even in close vicinity of necrotic neurons . Here we described an increase in activated microglia in the CA1 area as early as 2h after the insult. Inflammation in OHC was mounted at an early time point after OGD (2h) and associated with upregulation of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 mRNA level. At 6h after OGD a significant loss of CA1 pyramidal neurons simultaneously with a massive increase of microglia in the CA1 area was identified. At a later time point neuronal injury and microglia activation were revealed in the DG confirming a higher vulnerability of CA1 pyramidal neurons to OGD compared to DG granular neurons. Since a significant increase in BrdU+ cells in the CA1 was found at 16h after OGD, the fast increase of microglia seen (2-6h after OGD) was clearly due to cell migration and not proliferation. The microglia are probably derived from the GE where activated microglia were present even under control conditions . Accumulation of microglia in the damaged area could suggest a detrimental (inflammation and secondary damage) as well

as a beneficial (neuronal support) role of this cell population in the CNS tissue after injury .

4.4.2. MK-801, indomethacin or minocycline treatment showed a neuroprotective effect

We demonstrated that treatment with indomethacin or minocycline resulted in a marked neuroprotective effect against OGD-induced injury in OHC. Administration of indomethacin after OGD in OHC was first reported by Arai et al. . They, however, could not detect a neuroprotective effect of indomethacin, maybe due to a shorter duration of treatment (from 40 min before until 40 min after the OGD). In our study indomethacin or minocycline also reduced the number of microglia in the CA1 and DG after OGD. The neuroprotective effect of indomethacin is likely to result from COX-2 inhibition in turn inhibiting PGs production. Involvement of the other effects of indomethacin, such as direct scavenging of NO radicals or activation of peroxisome proliferator-activated receptors, could also contribute to neuroprotection . Minocycline inhibits the p38 MAPK pathway, which is implicated in signal transduction leading to iNOS expression in glial cells and apoptosis of neurons . Reduction of COX-2 expression, prostaglandin synthesis and inhibition of the induction of IL-1ß-converting enzyme by minocycline was also reported . There is evidence that COX-2 is upregulated in late stages of injury and COX-2 reaction products contribute to NMDA-mediated cytotoxicity whereas NMDA-receptors are directly involved in OGD-induced neuronal injury . Therefore blockage of NMDA receptors by MK-801 showed a more prominent neuroprotective effect compared to indomethacin or minocycline. Evidence that MK-801 strongly reduces cytokine expression , especially TNF-α either by preventing neuronal damage or direct effect on TNF-α production, also contribute to neuroprotective activity of this NMDA-receptor antagonist.

4.4.3. Cell proliferation and cell damaged at later time points after OGD

Phagocytosis of injured cells by activated microglia results in the removal of apoptotic

cells and cell debris from damaged tissue and subsequent stimulation of tissue reconstruction . GFs like bFGF, BDNF, erythropoietin and hypoxia induced factor have been described to be released from activated microglia and astrocytes after injury . They upregulate cell cycle proteins, which in turn control cell proliferation, differentiation and survival. Microglia proliferate earlier than astrocytes in response to cerebral ischemia . At 16h after OGD we found a significant increase of BrdU+ cells in the CA1 area (24h in the DG). Neuroprotection with MK-801, indomethacin or minocycline reduced the increased microglia proliferation seen after OGD. Continued increase of cell proliferation from 1d to 6d after the insult has been seen. In contrast cell damage decreased gradually from 1d to 5-6d after OGD probably due to the removal of injured neurons by activated microglia. Low cell damage has been also seen at 10d and 15d after OGD. At that time point neuronal structure of OGD-exposed OHC was completely destroyed compared to control cultures and massive astrogliosis as well as present of activated microglia took place.

4.4.4. Effect of exogenous growth factors on cell proliferation after OGD

Increase of cell proliferation after OGD was abolished by exogenous GFs treatment (see Table 4.2). Short BDNF application as well as long BDNF, NGF or EGF treatment reduced the number of BrdU+ cells seen after OGD. Since the neuronal degeneration in OHC is associated with high proliferative activity, reduction of cell proliferation could indicate earlier reported neuroprotective properties of GFs . Neuroprotective effect of BDNF could arise via its ability to block the mechanism by which pathophysiologycal Ca^{2+} influx through the NMDA receptor causes membrane PKC inactivation, seen after cerebral ischemia .

Table 4.2. Effect of exogenous GFs on cell proliferation in OHC after OGD

bFGF		EGF		BDNF		NGF	
2d	5d	2d	5d	2d	5d	2d	5d
No effect	No effect Decrease*	No effect	Decrease Decrease*	Decrease	Decrease	No effect	Decrease (DG)

*Compared to 2d treatment.

4.5. Neurogenesis in OHC after OGD

At 3d after OGD, neurogenesis in both neurogenic zones (DG and pPV) was inhibited either due to the disturbance of the neurogenic microenvironment by inflammation/neuronal degeneration, to a direct effect of activated microglia on progenitor cells , or due to massive glutamate release . We observed that in the DG of OGD-exposed slices microglial cells formed clusters localized directly in the SGZ. Numerous activated microglia were also present in the pPV. In accordance to previous studies the IL-6 upregulation detected after OGD could be responsible for the inhibition of neurogenesis ovserved early after injury . The inflammatory microenvironment could support the dominant proliferation of glial rather than neuronal precursors thus reducing neurogenesis .

After neuronal degeneration had peaked, mechanisms of tissue reparation could take place. The attenuation of the inflammatory reaction as well as the loss of glutamatergic neurons could promote neurogenesis . At 6d after OGD, restoration of neurogenesis in both neurogenic zones was detected. Moreover in the pPV, but not in the DG, neurogenesis after OGD was significantly increased supporting the hypothesis of functional differences between the DG and pPV precursors . Since we studied neurogenesis early after injury using the cellular markers of immature neurons we excluded that BrdU might label cells postmitotic neurons, which can reenter the cell cycle as a prelude to apoptosis after brain injury .

Increase of newly generated BrdU/β-III Tubulin+ neurons could be also observed at 10d after OGD (data not shown). However, at that time point the majority of injured neurons underwent to degeneration (β-III Tubulin+ staining) and post-ischemic increase of neurogenesis fails to provide the regeneration of lost tissue demonstrating limited capacity of endogenous repair mechanisms. In our system bFGF applied for 5d after OGD was able to stimulate neurogenesis in the pPV as detected 6d after the insult. A beneficial effect of GFs

was recently showed by Nakatomi et al. where combination of bFGF and EGF infused bilaterally into the ventricle of ischemic rats stimulated morphological and functional recovery of CA1 pyramidal neurons . Thus, GF therapy can represent a promising approach for regeneration of neuronal loss.

4.6. Neuroprotection and neurogenesis

4.6.1. MK-801 increased basal and reduced OGD-induced neurogenesis in the pPV

Neurogenesis in the developing and adult brain is negatively regulated, in part, by NMDA receptors having a negative impact . We observed an increase of basal neurogenesis in the pPV after MK-801 treatment. Stimulation of neurogenesis by NMDA receptor antagonists has also been shown *in vivo* . The neuroprotective effect of MK-801 was associated with a reduction in OGD-induced neurogenesis in the pPV indicating probably the survival of glutamatergic neurons, which have the projection to progenitor cells.

4.6.2. Indomethacin or minocycline increased basal neurogenesis and did not interfere with OGD-induced neurogenesis in the pPV

Indomethacin (10 or 50 μM) or minocycline (10 μM) stimulated basal neurogenesis in the pPV but not in the DG. The induction of basal neurogenesis by anti-inflammatory compounds could be explained by an ongoing latent pro-inflammatory state of OHC even under control conditions thus causing some depression of neurogenesis. However a neurogenic effect of anti-inflammatory compounds was also not found in the DG *in vivo* .

Although indomethacin (10, 50μM) or minocycline (10μM) reduced neuronal damage after OGD, they did not interfere with OGD-induced neurogenesis. Similar to our data Kluska et al. demonstrated the increase of hippocampal neurogenesis in the adult rats treated 30 min prior and 16d after phototrombotic insult . In contrast, Sasaki with co-authors showed an inhibition of ischemia-induced proliferation of progenitors by indomethacin application after

global ischemia. However, the further fate of these progenitors was not clarified . Moreover in this *in vivo* study indomethacin was injected at the proliferation peak of DG progenitors (on 8-9d after ischemia), when the neuronal damage had already developed and mechanisms of neurogenesis were activated. This was clearly different from our experimental design (pre- and post- insult treatment). We observed an inhibition of neurogenesis after OGD only with a high concentration of minocycline (50µM). One possible hypothesis is that a certain degree of inflammation is needed for injury-induced neurogenesis. However, at this stage definitive conclusions regarding the role of microglia in this process cannot be drawn since the anti-inflammatory compounds (indomethacin or minocycline) do not provide selective microglia inhibition, but can also affect neurons and astrocytes.

Since previous studies on the role of inflammation and neurogenesis using different experimental setups have not shown a consistent uniform picture, one has to consider that the role of inflammation might depend on the type and degree of injury as well as the time point of interference.

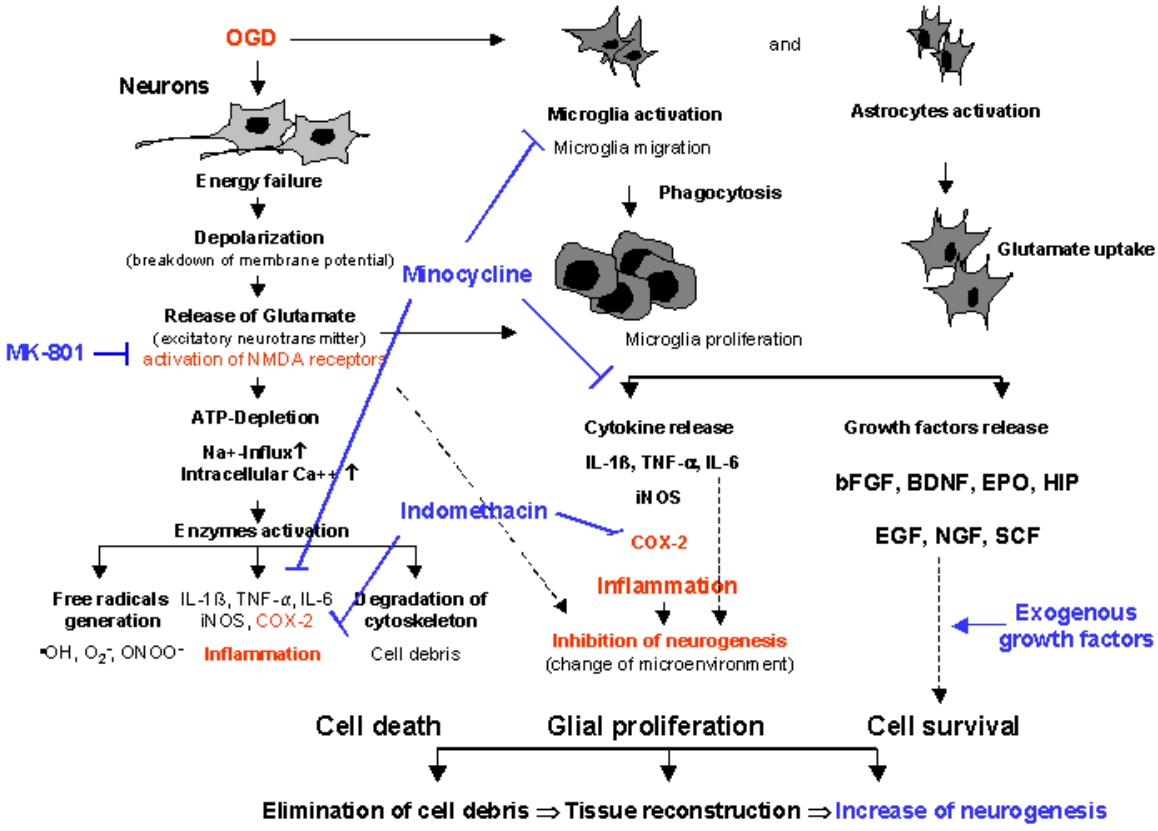

Fig. 4.2. Mechanism of involvement of inflammation in neuronal damage and neurogenesis in OHC after OGD.

4.7. Conclusion

Neuronal precursors with different neurogenic properties which were described *in vivo* are present in OHC. Morphological and functional differences of these cells could reflect their different role in the regeneration after neuronal loss. The sequence of events in the (patho)physiological response after OGD provide evidence that inflammatory reaction is involved in OGD-induced neuronal damage (Fig. 4.2). The inflammation occurred early after insult is associated with production of pro-inflammatory cytokines by activated microglia, astrocytes and injured neurons as well as production of iNOS and activation of COX-2. The level of neuronal degeneration seems to determinate the cytotoxic potential of microglia . We could confirm the previously described neuroprotective effects of anti-inflammatory compounds against OGD-induced neuronal damage in OHC. However we cannot conclude that microglia are the main culprits of neuronal degeneration since anti-inflammatory compounds (indomethacin or minocycline) could not provide selective microglia inhibition but can affect also neurons and astrocytes. Activated microglia eliminate debris of death cells and together with astrocytes uptake the glutamate released in response to injury. Neurotrophic factors released from activated microglia and astrocytes rescuer the damaged cell and sustain their survival. At early face of insult the inflammatory reaction contributes to the change of neurogenic microenvironment that does not support the proliferation of neuronal precursors. However neurogenesis as a compensatory mechanism of neuronal loss is activated later, after the pick of degeneration went down. We suggest that depending on the time point inflammation probably has both negative and positive effects on neurogenesis. Moreover, this *in vitro* study shows that an anti-inflammatory approach being neuroprotective seems not to interfere with insult-induced neurogenesis as a potential repair mechanism.

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6. Abbreviations

AMPA	2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BrdU	bromodeoxyuridine
CA	cornu ammonis
CNS	central nervous system
COX	cyclooxygenase
d	day
DCX	doublecortin
DG	dentate gyrus
DIV	days *in vitro*
DNS	donkey normal serum
EGF	epidermal growth factor
FGF	fibroblast growth factor
GCL	granular cell layer
GE	glial envelope
GF/GFs	growth factor/growth factors
GFAP	glial fibrillary acidic protein
IB4	*Griffonia simplicifolia* Isolectin B_4
IL	interleukin
IN	indomethacin
LV	lateral ventricle
LVw	lateral ventricle wall
MAP-2	microtubule assotiated protein
MAPK	mitogen activated protein kinase
MCAO	middle cerebral artery occlusion
MI	minocycline
MK	MK-801
ML	molecular layer
NeuN	neuronal niclei
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NOS	nitric oxide synthase
OB	olfactory bulb
OCC	cortex-hippocampal organotypic slice cultures
OGD	oxygen-glucose deprivation
OHC	organotypic hippocampal slice cultures
P	postnatal day
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PFA	paraformaldehyde
PGs	prostaglandins
PI	propidium iodide
PKC	protein kinase C
pPV	posterior periventricle
RMS	rostral migratory streem
RT	room temperature

SCF	stem cells factor
SGZ	subgranular zone
SVZ	subventricular zone
TBS	tris buffered saline
TNF	tumor necrosis factor

Appendices

I. ZUSAMMENFASSUNG

Adulte neurogenese spielt eine Rolle bei vielen physiologischen (z.B. Gedächtnisbildung) und pathologischen (z.B. Schlaganfall, Depression) Prozessen. In neueren Studien wurde eine erhöhte Neurogenese als Antwort auf eine Verletzung des Gehirns gezeigt, die als ein Mechanismus der Regeneration nach dem Verlust von Neuronen angesehen wird. Es gibt Hinweise darauf, dass eine Vielzahl von Faktoren, wie z.B. Glutamat, Entzündungs- und Wachstumsfaktoren, zum Anstieg der Proliferation und Differenzierung von Vorläuferzellen nach einem Insult beitragen. Der Mechanismus, der durch eine Verletzung Neurogenese induziert, ist bisher jedoch weitgehend unklar.

In der vorliegenden Studie wurde die frühe Neurogenese *in vitro* an organotypischen Hippokampusschnittkulturen der Ratte (OHC) charakterisiert und das komplizierte Wechselspiel zwischen neuronaler Schädigung, Mikroglia-Aktivierung, Zellproliferation, Neurogenese und der Rolle der Entzündung nach Sauerstoff-Glukose Entzug (OGD) untersucht. Außerdem wurde die Wirkung exogener Wachstumsfaktoren, nämlich des basalen Fibroblastenwachstumfaktors (bFGF), des epidermalen Wachstumfaktors (EGF), des vom Gehirn gebildeten neurotrophen Faktors und des Nervenwachstumfaktors, auf die Proliferation und die Neurogenese in OHC bei Anwesenheit und Abwesenheit einer Schädigung überprüft.

Die Proliferation wurde über die Aufnahme von Bromodeoxyuridin (BrdU), die Neurogenese durch eine Doppelmarkierung mit Bromodeoxyuridin und Doublecortin (DCX) oder ß-III Tubulin, die neuronale Schädigung über die Propidiumiodidaufnahme, Mikroglia durch eine OX-42 Färbung und proentzündliche Zytokine mittels Echtzeit-RT-PCR bestimmt.

Es wurde eine massive Zellproliferation in der glialen Umhüllung des Schnittes beobachtet. Die gliale Umhüllung wurde während der Kultivierung der Schnitte von GFAP

bzw. GFAP/Nestin-positiven Astrozyten sowie aktivierter Mikroglia gebildet. Die Proliferation innerhalb der Schnittkultur war geringer. bFGF und EGF zeigten mitogene Eigenschaften und erhöhten die Zahl BrdU-positiver Zellen. Es konnte gezeigt werden, daß es in OHC zusätzlich zum Gyrus dentatus (DG) eine weitere neurogene Zone gibt: den posterioren Periventrikel (pPV), der einen Teil der lateralen Ventrikelwand darstellt. Diese an das Stratum oriens grenzende Struktur enthielt Nestin-positive Vorläuferzellen. Es konnten morphologische und funktionelle Unterschiede zwischen DG und pPV Vorläuferzellpopulationen identifiziert werden. Im DG wurden Nestin-positive Zellen mit einer sternförmigen Morphologie gefunden, während im pPV elliptisch geformte Nestin-positive Zellen auftraten. Eine bFGF-Behandlung verursachte eine schnelle aber kurzlebige neurogene Antwort im DG, wobei bFGF im pPV einen ausgeprägteren und langlebigeren neurogenen Effekt bewirkte.

Wenn die OHC einer 40 min OGD ausgesetzt wurden, war eine Aktivierung der Mikroglia und die Hochregulation von IL-1ß-, TNF-α- und IL-6-mRNA (2h nach OGD) zu beobachteten, die der Entwicklung der neuronalen Schädigung (6h nach OGD) und einem Anstieg der Zellproliferation (16h nach OGD) voranging. Eine Behandlung mit BDNF, EGF oder NGF verminderte die OGD-induzierte Proliferation. Die Neurogenese war an Tag 3 nach OGD in beiden neurogenen Zonen gehemmt. Die Wiederherstellung der Neurogenese konnte aber bereits am Tag 6 beobachtet werden. Zu diesem Zeitpunkt wurde eine im Vergleich zur Kontrolle signifikante Erhöhung der Anzahl neugebildeter Neurone im pPV gefunden. Die Zahl BrdU/ß-III Tubulin-positiven Neurone im pPV OGD-exponierter OHC konnte durch die Gabe von bFGF erheblich gesteigert werden.

MK-801, Indomethacin oder Minozyklin verhinderten die OGD-induzierte neuronale Schädigung, Zellproliferation und verursachten eine Abnahme von OX-42 positiver Mikroglia im geschädigten Bereich. Unter Kontrollbedingungen induzierten MK-801, Indomethacin und Minozyklin Neurogenese im pPV. Nach OGD verminderte eine MK-801 Behandlung die

Zahl der BrdU/DCX-positiven Zellen des pPV. Indomethacin oder Minozyklin beeinflussten die OGD-induzierte Neurogenese im pPV jedoch nicht.

Zusammenfassend zeigt diese Studie erstmals i) dass es in als Oberflächenschnitte kultivierten OHC zwei neurogene Zonen gibt, den DG und den pPV ii) das DG und pPV neuronale Vorläuferzellen mit unterschiedlichen neurogenen Eigenschaften enthalten. Die Neurogenese im pPV war hoch, während die Neurogenese im DG von OHC niedrig war; iii) eine Entzündung tritt in OHC schon zu einem frühen Zeitpunkt nach OGD auf. Sie ist mit der Aktivierung, Migration und Proliferation von Mikroglia verbunden; iv) wegen Änderungen des Mikromilieus wird die Neurogenese in beiden neurogenen Zonen früh nach OGD (3 Tage) gehemmt und später (6 Tage) wieder hergestellt. OGD regt die Neurogenese im pPV an; v) Neuroprotektion gegen OGD-induzierte Schädigung durch anti-inflammatorische Behandlung ist in OHC von intakter Neurogenese begleitet. Somit lieferten die *in vitro* Daten wichtige Hinweise, die für ein weiteres Verständnis der Mechanismen der Neurogenese und der anti-inflammatorischen Behandlung nach Schlaganfall nützlich sein könnten.

II. SELBSTÄNDIGKEITSERKLÄRUNG

Erklärung

Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation mit dem Thema

„Neurodegeneration und Neurogenese in organotypischen hippokampalen Schnittkulturen nach Hypoxie/Hypoglykämie“

selbständig verfaßt, nicht schon als Dissertation verwendet habe und die benutzten Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zur Erlangung des akademischen Grades doctor rerum naturalium (Dr.rer.nat.) an anderen Einrichtungen eingereicht habe.

Lion-Feuchtwangerstr. 25, Magdeburg, 24.10.2005

Diplom- Pharmazeutin - Olga Chechneva

III. PUBLICATIONS

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