Regulation of neural progenitor cell proliferation and fate by proteolytic pathways and inflammatory signals in the brain

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-III):

I. Sippel M, **Rajala* R**, Korhonen L, Bornhauser B, Sokka A-L, Naito M, Lindholm D (2009) Dexamethasone regulates expression of BRUCE/Apollon and the proliferation of neural progenitor cells. *FEBS Letters* **583**(13):2213-2217.

II. Mäkelä J, **Koivuniemi R**, Korhonen L, Lindholm D (2010) Interferon-γ produced by microglia and the neuropeptide PACAP have opposite effects on the viability of neural progenitor cells. *PLoS One* **5**(6):e11091.

III. **Koivuniemi R**, Mäkelä J, Hokkanen M-E, Bruelle C, Ho TH, Ola R, Korhonen L, Schröder J, Kataoka H, Lindholm D (2013) Hepatocyte growth factor activator inhibitor-1 is induced by bone morphogenetic proteins and regulates proliferation and cell fate of neural progenitor cells. *PLoS One* **8**(2):e56117.

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In addition, some unpublished data are presented.

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Author's contribution to the publications:

I. Performed neural progenitor cell isolation and cell culture maintenance, accomplished Usp8 overexpression experiments and subsequent data analysis, and participated in writing of the manuscript

II. Participated in neural progenitor cell isolation and cell culturing, and contributed to siRNA experiments, data analysis and writing of the manuscript

III. Contributed to a design of experiments, performed all the experimental work except *in situ* hybridization and *in utero* electroporation, analyzed the data, and participated in writing of the manuscript

ABSTRACT

Neural progenitor cells (NPCs) are present in the developing and adult neuroepithelium of the brain and are regulated by internal and external signals that influence neurogenesis and tissue homeostasis. NPCs are multipotent tissue stem cells that can arouse all neural cell types, including neurons and glial cells. In culture, NPCs grow preferentially as cell aggregates called neurospheres. This suggests that interactions between cells are essential to regulate NPC behavior and development. Interactions between cells may be facilitated by cell surface-attached proteases and their inhibitors that play an important role in development and during tissue remodeling after injury. Thus, they could regulate also brain development.

Neuroinflammation, an innate immune response of the nervous system, is part of many neurodegenerative diseases. Neuroinflammation involves activation of microglia and production of proinflammatory cytokines. During neuroinflammation, NPCs interact with the immune system and may decrease inflammatory effects in the brain. However, inflammation may have negative effects on NPCs and thus, agents that protect NPCs could serve as a therapeutic potential for neuronal injuries and neurodegenerative diseases by enabling local tissue repair in the brain.

The aim of this thesis was to study the regulation of NPC development by membrane-associated proteins and the effects of inflammation on NPCs. Glucocorticoid hormone (GH) levels increase in inflammation and after stress. GHs have previously been shown to decrease NPC proliferation and neurogenesis. We have studied the effects of a synthetic GH dexamethasone on the cytosolic membrane-associated and anti-apoptotic protein BRUCE, and how BRUCE affects NPC behaviour. In addition, we have studied the secretion of cytokine interferon-gamma (IFNγ) after microglial activation and further the influence of IFNγ on NPCs. To address the role of cell surface-associated protease inhibitors during NPC development, we have studied the expression and function of Kunitz type serine protease inhibitors hepatocyte growth factor activator inhibitors -1 (HAI-1) and -2 (HAI-2) in NPCs.

The results show that dexamethasone enhances degradation of BRUCE by the ubiquitin-proteasome system (UPS), which leads to decreased NPC proliferation. NPC division was negatively affected also by IFNγ produced by microglial cells as well as protease inhibitors HAI-1 and HAI-2. Moreover, IFNγ induced NPC cell death that was rescued by a neuropeptide pituitary adenylate cyclase-activating peptide (PACAP). In the developing NPCs, HAI-1 and HAI-2 expression was increased by bone morphogenetic protein-2 (BMP-2) and BMP-4, which inhibited NPC proliferation and increased glial cell differentiation partly in a HAI-dependent manner.

This thesis provides knowledge about interplay between immune cells and NPCs as well as developmental signaling systems, including proteolytic pathways, that affect NPC behaviour. In NPCs, proteolytic pathways may be regulated by external signals, like cytokines, from the neighboring cells. Proteolysis is involved also in the UPS that regulates the cell cycle machinery and thus, cell division. This thesis also deals with NPC survival, which is of importance for stem cell therapies. Knowledge of reciprocal effects of IFNγ and PACAP on NPCs is relevant when designing treatment for brain inflammation and disease.

TIIVISTELMÄ (Abstract in Finnish)

Aivojen kehityksen aikana lukuisat viestintäreitit solujen välillä sekä solun sisällä säätelevät hermoston kantasoluja. Hermoston kantasolut ovat niin kutsuttuja kudoskantasoluja, joilla on kyky uusiutua. Ne voivat myös erilaistua hermoston eri solutyypeiksi, kuten hermosoluiksi ja hermotukisoluiksi. Hermoston kantasolujen sijainti aivoissa vaikuttaa niiden kehittymiseen ja ne jakautuvat usein vain tietyssä mikroympäristössä, joka säätelee niiden erilaistumista. Viljelmässä hermoston kantasolut kasvavat aggregaatteina, mikä tukee niiden kasvua ja jakautumista viitaten solujen välisten vuorovaikutusten olevan tärkeitä niiden kehitykselle. Solujen välisiä kontakteja sekä vuorovaikutusta ulkoisen ympäristön kanssa voivat välittää solun pinnalla sijaitsevat proteiineja pilkkovat proteaasit sekä niiden estäjät, joilla on tärkeä rooli jo sikiönkehityksen aikana sekä kudosten uusiutumisessa.

Tulehdus on läsnä monissa hermoston tautitiloissa ja sen on näytetty vaikuttavan negatiivisesti hermoston kantasoluihin ja hermosolujen syntymiseen. Aivojen tulehdusreaktioissa mikroglia-solut aktivoituvat ja erittävät tulehdusta edistäviä tulehdusvälittäjäaineita, kuten sytokiinejä, jotka välittävät elimistön immuunijärjestelmän vuorovaikutuksia. Hermoston kantasolut voivat olla vuorovaikutuksessa immuunijärjestelmän kanssa ja saattavat vähentää aivojen tulehdusvastetta. Aivojen tulehdusreaktioissa ja erityisesti aivovaurioissa molekyylejä, jotka suojelevat hermoston kantasoluja, voitaisiin käyttää vaurion hoidossa lisäämään tuhoutuneen kudoksen uusiutumispotentiaalia.

Väitöskirja tutkii hermoston kantasolujen jakautumista ja erilaistumista sekä sitä, miten eri tekijät, kuten sytokiinit ja solukalvoilla sijaitsevat proteiinit vaikuttavat näiden solujen kehittymiseen sekä niiden säätelyyn aivojen tulehdusreaktioissa. Glukokortikoidihormonit ovat osa elimistön immuunijärjestelmän mekanismia, joka vähentää tulehdusvastetta. Niiden määrä lisääntyy stressin tai immuunivasteen seurauksena ja niiden on näytetty vähentävän hermoston kantasolujen jakautumista ja hermosolujen syntymistä. Väitöskirjassa on selvitetty synteettisen glukokortikoidihormoni deksametasonin vaikutusta hermoston kantasolujen ilmentämään BRUCE-proteiiniin sekä sitä, kuinka BRUCE säätelee kantasoluja. Lisäksi väitöskirjassa on tutkittu tulehdusreaktioissa erittyvän sytokiini interferoni-gamman vaikutusta hermoston kantasoluihin sekä solukalvolla sijaitsevien proteaasiestäjien HAI-1 ja HAI-2 ilmentymistä ja toimintaa näissä soluissa.

Deksametasonin osoitettiin lisäävän hermoston kantasoluja säätelevän BRUCE-proteiinin hajotusta proteasomissa, mikä vähensi solujen jakautumista. Myös interferoni-gamma, jota erittyi aivojen mikroglia-soluista, sekä HAI-1 ja HAI-2 vähensivät hermoston kantasolujen jakautumista. Interferoni-gamma myös lisäsi hermoston kantasolujen solukuolemaa, joka kuitenkin estyi suojaavan PACAP-neuropeptidin vaikutuksesta. BMP-sytokiinien BMP2 ja BMP4 osoitettiin lisäävän HAI-proteaasiestäjien ilmentymistä hermoston kantasoluissa. Lisäksi ne vähensivät kantasolujen jakautumista ja lisäsivät niiden erilaistumista hermotukisoluiksi, astrosyyteiksi, osittain HAI-proteiinien välityksellä.

Väitöskirjatyö osoittaa, että hermoston kantasolujen pinnalla esiintyvät proteaasiestäjät säätelevät solujen käyttäytymistä ohjaten niiden kehitystä ja kohtaloa aivojen kehityksen aikana. Lisäksi proteasomihajotuksella on rooli hermoston kantasolujen jakautumisen säätelyssä. Väitöskirja myös osoittaa aivojen mikroglia-solujen vaikuttavan sytokiinien välityksellä hermoston kantasoluihin. Hermoston kantasolut ovat herkkiä tulehdusvälittäjäaineille, ja niiden jakautuminen voi häiriintyä hermoston tautitiloissa ja aivovaurion seurauksena. Siksi niiden kasvun ja jakautumisen säätely on tärkeää etenkin stressi- ja tulehdusreaktioissa ja myös suunniteltaessa kantasoluterapiaa.

ABBREVIATIONS

ALK	activin receptor-like kinase
ALS	amyotrophic lateral sclerosis
Apaf	Apoptosis protease activating factor
Bcl	B cell lymphoma
BDNF	brain-derived neurotrophic factor
BH	Bcl2 homology
bHLH	basic helix-loop-helix
BIR	baculoviral IAP repeat
BIRP	BIR domain-containing protein
BMP	bone morphogenetic protein
BMPR	BMP receptor
BRUCE	BIR repeat-containing ubiquitin-conjugating enzyme
BSA	bovine serum albumin
CBP	CREB binding protein
Cdk	Cyclin-dependent kinase
CKI	Cdk inhibitor
CNS	central nervous system
DG	dentate gyrus
E	embryonic day
EAE	experimental allergic encephalomyelitis
EB	embryoid body
EBSS	Earle´s balanced salt solution
ERK	extracellular signal regulated kinase
ESC	embryonic stem cell
FBS	fetal bovine serum
FCS	fetal calf serum
GC	glucocorticoid
GDNF	glial-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GH	glucocorticoid hormone
GR	glucocorticoid receptor
HAI	hepatocyte growth factor activator inhibitor
HBSS	Hank´s buffered saline solution
HC	hippocampal/hippocampus
Hes	mammalian homologue of Hairy and Enhancer of Split
HGF	hepatocyte growth factor
HGFA	HGF activator
HPA	hypothalamic-pituitary-adrenal
IAP	inhibitor of apoptosis protein
Id	inhibitor of differentiation
IFN	interferon
IL	interleukin
INM	interkinetic nuclear migration
IPC	intermediate progenitor cell
JAK	Janus tyrosine kinase
KD	Kunitz domain
LIF	leukemia inhibitory factor

LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MR	mineralocorticoid receptor
MS	multiple sclerosis
MSP	macrophage-stimulating protein
NE	neuroepithelial
NES	nuclear export sequence
Ngn	neurogenin
NK	natural killer
NO	nitric oxide
NPC	neural progenitor cell
NSC	neural stem cell
P	postnatal day
PACAP	pituitary adenylate cyclase-activating peptide
PAR	protease-activated receptor
PARP	poly-ADP ribose polymerase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PFA	paraformaldehyde
PK	protein kinase
PRR	pattern-recognition receptor
RGC	radial glial cell
ROS	reactive oxygen species
Shh	sonic hedgehog
SPI	serine protease inhibitor
STAT	signal transducers and activators of transcription
SVZ	subventricular zone
TGF	transforming growth factor
Th	T helper cell
TNF	tumour necrosis factor
TTSP	type II transmembrane serine protease
Ub	ubiquitin
UPS	ubiquitin-proteasome system
VZ	ventricular zone

1   INTRODUCTION

The brain development results from series of cellular events, including proliferation, differentiation, migration and cell death of neural stem/progenitor cells (NSC/NPC). These cells are multipotent stem cells with restricted differentiation potential only into neurons and glial cells. NSCs/NPCs are regulated during the brain development by extrinsic and intrinsic signals to guide the proper formation of the complex hierarchy of brain structures. Signals from the cell surroundings are transferred inside the cell to activate intracellular signaling pathways that regulate the cell phenotype and fate. In addition, cell-to-cell signaling via plasma membrane contact may influence cell behavior. NPCs may interact with other brain cells or with each other through proteins expressed on the cell membrane. Moreover, they are affected by secreted factors from other cells.

NSCs are present also in the adult brain, most prominently in the subventricular zone of lateral ventricles and in the dentate gyrus (DG) of hippocampus (Decimo et al., 2012). Adult NSCs serve as a reservoir for cell differentiation and replacement, and endogenous progenitors are valuable in situations of tissue damage that may occur in brain trauma or in neurodegenerative diseases. However, neurogenesis in the brain is affected by various cues, including trophic factors, cytokines and drug treatments, and in several physio- and pathological conditions. Stress is associated with a transient reduction in DG neurogenesis (Decimo et al., 2012). In neurological diseases and disorders, including Alzheimer´s disease, epilepsy, Huntington´s disease and Parkinson´s disease, neurogenesis is often upregulated in the lesioned brain (Taupin 2008). Hence, the stimulation of endogenous NPCs to control neurogenesis could contribute to regeneration of the injured or diseased brain.

Tissue injury in the brain often activates brain immune function that may be noxious for the healthy tissue. Neuroinflammation is involved in the pathogenesis of brain diseases and disorders (Raison et al., 2006; Qian et al., 2010). It affects also NPCs and decreases neurogenesis but, on the other hand, NPCs may promote neuroprotection by modulating the immune reaction (Taupin 2008). Stress can affect immune function by alterations in hormone levels, and chronic stress may also result in brain pathogenesis (Bilbo and Schwarz, 2012).

In order to protect NPCs from harmful effects of inflammation, it is important to study, how NPCs are affected by neuroinflammation and how their survival can be enhanced. Since NPCs in the brain may not tolerate the effects of inflammatory challenge, it is also worth to explore the regenerative capacity of these cells for stem cell therapies. In the case of injury, endogenous NPCs or transplantation of stem cells into the brain may decrease inflammation, restrict cell death through neurotrophic effects and enhance endogenous cell proliferation and recovery processes (Christie and Turnley, 2012; Giusto et al., 2013). Thus, a proper knowledge of the regulation of NPCs is essential for the development of cell therapies and therapeutic agents.

2   REVIEW OF THE LITERATURE

2.1   Brain stem cell development

The mammalian brain is developed from the neuroepithelial lining of the neural tube that is of ectodermal origin. During early development, neuroepithelial (NE) cells that are the primary neural stem cells (NSCs) first undergo symmetrical cell divisions to produce more neuroepithelial cells and to form the thickening germinal layer, called the ventricular zone (VZ) (Smart, 1972). Later, they switch to asymmetric mode of division generating more restricted neural progenitor cells, including radial glial cells (RGCs) and intermediate progenitor cells (IPCs; also called basal progenitors) (**Figure 1**)(Chenn and McConnell, 1995; Huttner and Brand, 1997). With the onset of neurogenesis and following the neural tube closure, neuroepithelial cells transform into RGCs that continue cell divisions asymmetrically. By these self-renewing divisions, RGCs produce two daughter cells: a daughter cell of a same kind and a daughter cell that exhibits an IP- or a neuron-type character (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004; 2007). Thus, through IPCs, RGCs are capable of producing neurons also indirectly. Moreover, RGC-derived IPCs may also generate oligodendrocytes. IPCs divide symmetrically to produce two neurons or two oligodendrocytes depending on the cell fate (Haubensak et al., 2004; Noctor et al., 2004; 2008; Attardo et al., 2008). In addition, a minority of IPCs is able to undergo proliferating symmetric division to expand the progenitor pool (Attardo et al., 2008; Noctor et al., 2008). Thus, IPCs form another proliferating layer near the ventricle, the subventricular zone (SVZ). Finally, at the end of embryonic development, majority of RGCs lose their apical attachment and convert into astrocytes (Schmechel and Rakic, 1979; Noctor et al., 2008). Some of the RGCs remain as a ventricular stem cell population in the adult brain (Kriegstein and Alvarez-Buylla, 2009).
During the development, RGCs also serve as a scaffold for the formation of cortical layers in the brain by guiding the migration of newborn neurons to reach the cortical plate (Rakic, 1971; 1972). Immature neurons migrate along the radial fibers and differentiate into pyramidal cells of the cortex (Gadisseux et al., 1990; Marín-Padilla, 1992).
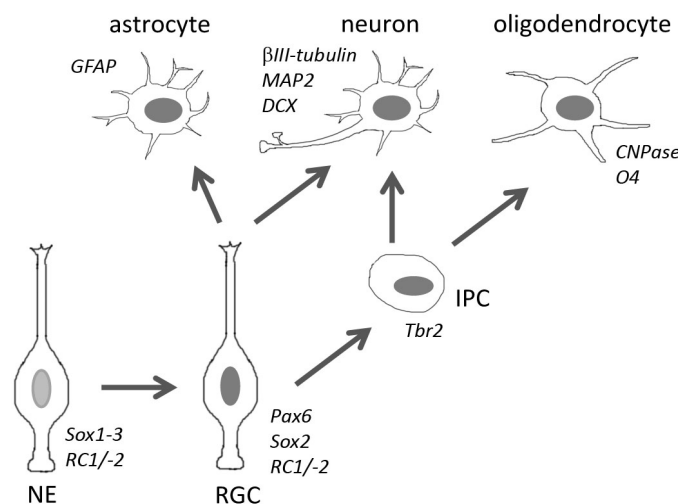
Figure 1. Lineage relationships of neural stem/progenitor cells and their progeny. Neuroepithelial (NE) cells convert into radial glial cells (RGCs) that give rise to intermediate progenitor cells (IPCs) and neurons. During the late embryogenesis RGCs transform into astrocytes. IPCs produce neurons and oligodendrocytes. Cell type-specific markers for stem cells and progenitors are shown in italics. DCX, doublecortin; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein 2; Tbr2, T-brain gene-2.

2.1.1   Neural stem/progenitor cells

2.1.1.1   Neuroepithelial cells

Neuroepithelial cells are anchored to each other by tight junctions (Aaku-Saraste et al., 1996) and form a specialized epithelium that lines the lumen of the cerebral ventricles early in embryonic development. NE cells give rise to all neurons in the mammalian neocortex. They are polarized cells extending from the apical surface of the ventricle into the basal/pial surface to contact the basal lamina (Huttner and Brand, 1997). They go through mitotic divisions at the ventricular surface but their nuclei migrate within the cytoplasm in a manner of interkinetic nuclear migration (INM) (Sauer and Walker, 1959). During DNA synthesis (S) phase, nuclei form a layer above the VZ while nuclei in gap phases 1 (G1) and 2 (G2) travel between these layers in apical-to-basal and basal-to-apical direction, respectively. Neuroepithelial cells are characterized by the expression of transcription factors Sox1, -2 and -3 (Bylund et al., 2003). In addition, they share the expression of RC1 and RC2 epitopes with RGCs (Misson et al., 1988a; Edwards et al., 1990). At the beginning of cortical neurogenesis, NE cells begin to show radial glial cell characteristics.

2.1.1.2   Radial glial cells

RGCs are bipolar cells that share some features with neuroepithelial cells, like apical-basal polarity and contacts with both pial and ventricular surfaces through a radial process. They also undergo INM but their nuclei migrate only within the VZ (Misson et al., 1988b). RGCs, however, acquire morphological changes as well as changes in the expression of intermediate filament proteins and transcription factors. RGCs are connected to each other by adherens junctions and they obtain 24-nm microtubules and 9-nm intermediate filaments accompanied by the growth of the radial fiber (Choi and Lapham, 1978). They also contain glycogen storage granules near the basal end feet (Brückner and Biesold, 1981). RGCs permeate the entire cortical wall and guide the migration of newborn neuroblasts. In contrast to NE cells, RGCs start to elongate their glial processes, which precedes glial transformation (Takahashi et al., 1990).

RGCs are distinguished from neuroepithelial cells by the expression of astroglial markers, including glutamate aspartate transporter (GLAST), brain lipid-binding protein (BLBP), nestin and vimentin (Schnitzer et al., 1981; Tohyama et al., 1992; Feng et al., 1994; Shibata et al., 1997; Perez-Alvarez et al., 2008). Later, they also start to express the astroglial intermediate filament protein glial fibrillary acidic protein (GFAP). In addition, RGCs are characterized by expression of a transcription factor Pax6, which is required for RGC identity and their neuronal differentiation (Götz et al., 1998; Heins et al., 2002; Haubst et al., 2004). RGCs also show high Sox2 expression that declines during the differentiation (Hutton and Pevny, 2011). The maintenance of RGCs is mediated by Notch signaling that inhibits the activation of proneural genes (Gaiano et al., 2000).

2.1.1.3   Intermediate progenitor cells

Intermediate progenitor cells appear in the VZ as a result of RGC division. IPCs retract from the ventricular surface to populate the second germinal layer of the neuroepithelium, the SVZ (Miyata et al., 2004). They lose adherent junctions and lack INM but undergo apical-to-basal translocation within the basal VZ and the SVZ to perform cell division (Farkas and Huttner, 2008). Contrary to NE cells and RGCs that perform cell division in a vertical orientation (perpendicular to the apical surface), IPCs acquire a horizontal (parallel) cleavage plain orientation (Noctor et al., 2008). IPCs have a restricted

potential to differentiate only to a neuronal or glial cell type. Maintenance of IPCs is dependent on interactions with other cells in the developing neuroepithelium. IPCs are identified by T-brain gene-2 (Tbr2) expression, which is required for IPC specification (Sessa et al., 2008; Englund et al., 2005).

2.1.2   Stem cell niche

Stem cells in adult tissues are found in a specific microenvironment called a stem cell niche. The niche supports the survival and renewal of stem cells and regulates their proliferation and differentiation potential by cell signaling of secreted paracrine factors as well as by cell interactions with other cells and with the extracellular matrix. Regulation of stem cell niche is of importance to preserve the stem cell pool and to enable continuous stem cell division for tissue maintenance and its regeneration after injury.

Neural stem cells reside in specific areas in the adult brain, from which the most familiar are the subgranular zone of dentate gyrus in hippocampus and a subependymal zone of the lateral ventricles (reviewed in Kriegstein and Alvarez-Buylla, 2009). In adult hippocampus, neurogenesis occurs locally while the SVZ progenitors migrate along the rostral migratory stream to the olfactory bulb to replace interneurons (Imayoshi et al., 2008). However, neurogenesis have been detected also in other locations in the adult brain, including substantia nigra, amygdala, striatum and neocortex (Gould et al., 1999; Bernier et al., 2002; Zhao et al., 2003). Signaling in the neurogenic stem cell niche is mediated by different cell types residing in the niche, including NSCs, ependymal cells, blood vessel cells and glial cells. Recently, also embryonic neural stem cells have been considered to be regulated by niche-like signals by their neighboring cells, including neuroblasts, endothelial cells and RGCs (Shen et al., 2004; Gama Sosa et al., 2007; Yoon et al., 2008; Nishikawa et al., 2010). Niche-promoting signals may be facilitated via both extrinsic and intrinsic cues. The cerebrospinal fluid provides signals that promote cortical progenitor cell proliferation by contacting the apical domain in the VZ (Lehtinen et al., 2011). In addition, extracellular matrix protein tenascin C has been shown to provide support for neural stem cell development by regulating growth factor signaling (Garcion et al., 2004). Signaling between cells may be facilitated through gap junctions, which form channels with an aqueous pore between the adjacent cells and allow exchange of small molecules, ions and electrical current. RGC division is regulated by gap junction-mediated cell coupling and waves of calcium ions (Lo Turco and Kriegstein, 1991; Bittman et al., 1997; Weissman et al., 2004). Cell-to-cell signaling may also occur through direct cell contact between different cell types (Tung and Lee, 2012).

2.1.3   Proliferation and self-renewal

The purpose of cell proliferation is the self-renewal of a cell to produce similar progeny and to expand the cell population. In the context of neural stem/progenitor cells, proliferation is also required for generation of downstream progenitor cells as well as neurons.

2.1.3.1   Cell division

Cell division is required for growth, development and tissue renewal. Mitotic cell division involves the distribution of identical genetic material into two daughter cells by means of DNA replication, after which the actual cell division takes place. The mitotic cell division is divided into mitosis and cytokinesis. Mitosis divides the nuclear material into daughter nuclei, and cytokinesis divides the cytoplasm and the plasma membrane, generating two daughter cells. During mitosis, the mitotic spindle

guides the replicated chromosomes into the opposite poles of the cell and the daughter nuclei re-form around them.

Cytokinesis begins during the last stage of mitosis with the appearance of a cleavage furrow, a groove at the cell surface that determines the cleavage plane (Eggert et al., 2006). The place and ingression of the cleavage furrow is determined in a consecutive manner by microtubule asters and the mitotic spindle midzone (Bringmann and Hyman, 2005). The cleavage furrow is deepened by the contraction of a contractile ring made of actin filaments associated with myosin proteins that assemble on the cytoplasmic side of the furrow (Tucker, 1971). Assemble of the contractile ring is facilitated by the small GTPase RhoA via activation of its downstream effector proteins (Piekny et al., 2005). Before cleavage, the daughter cells are connected to each other by an intercellular bridge containing a midbody ring made of antiparallel microtubule bundles. In the last step of cytokinesis, abscission, the cytoplasmic connection is closed between the daughter cells via membrane fusion.

A daughter cell fate of a neural stem cell is defined by a cleavage plane orientation and the symmetry of cell division. The symmetric division produces two identical daughter cells, whereas the asymmetric division generates two daughter cells with different fate. Neural stem cell division may be a symmetric proliferative, a symmetric differentiative, an asymmetric self-renewing or an asymmetric differentiative division (**Figure 2**).

RGC → RGC + IPC or IPC + neuron

RGC → IPC + IPC or neuron + neuron
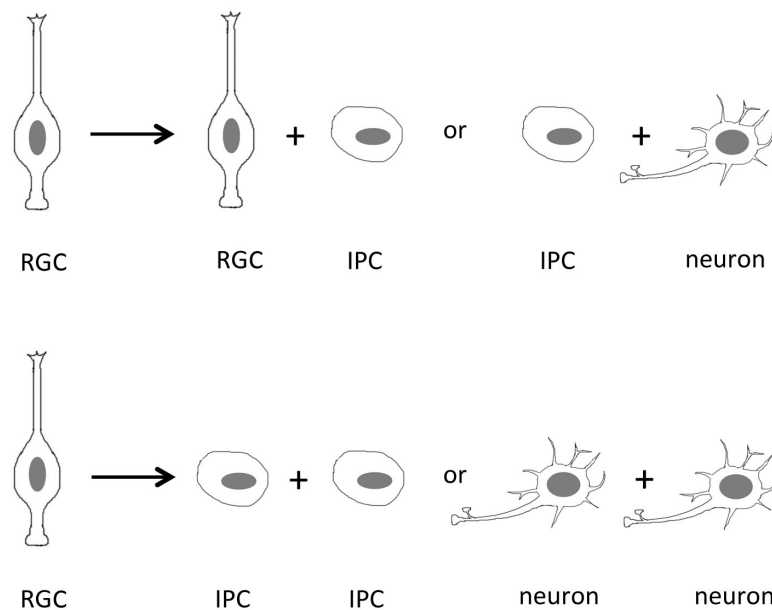
Figure 2. Cell division in neural stem cells. An asymmetric division (above) produces daughter cells with different fates and is either a self-renewing or a differentiative division yielding a similar stem cell and a differentiated cell or two daughter cells that further differentiate, respectively. A symmetric cell division in NSCs (below) generates identical progeny including either proliferative progenitors or differentiating cells. IPC, intermediate progenitor cell; RGC, radial glial cell.

A vertical cleavage occurs perpendicular to the apical surface of the ventricular zone and results in symmetric or asymmetric division of a neural progenitor cell, while a horizontal cleavage plane divides the cell parallel to the apical surface resulting in asymmetric division (Chenn and McConnell, 1995; Kosodo et al., 2004). The fate of a vertically dividing cell depends on whether the apical plasma membrane is bisected or bypassed and thus, whether the apical membrane domain is equally or

unequally distributed to the daughter cell (Kosodo et al., 2004) (**Figure 3**). Symmetric vs. asymmetric division is known to be regulated by Notch inhibitors Numb and Numblike, whose inactivation increases symmetric proliferative divisions and the progenitor cell number in the developing mouse forebrain (Li et al., 2003). Furthermore, Notch activation in the mammalian cerebral cortex progenitors leads to increased proliferative divisions (Mizutani and Saito, 2005). Recent data indicates a role for a transcription factor Pax6 in regulating the orientation of cell division as loss of Pax6 results in increased asymmetric vertical cleavage and an increase in basal progenitor divisions (Asami et al., 2011). The apical-basal polarity of neural progenitors is also essential for the proper symmetry of cell division. Mice lacking the mammalian homologue of the Drosophila lethal giant larvae gene, *Lgl1*, show hyperproliferation of neural progenitors due to disrupted cell polarity and a failure of asymmetric cell divisions (Klezovitch et al., 2004). Moreover, the loss of apical-basal polarity in intermediate progenitor cells allows only symmetric cell division (Attardo et al., 2008).
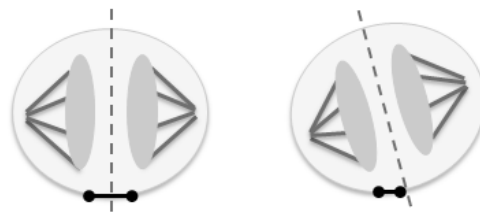

Figure 3. Symmetric and asymmetric cell division in neural stem cells with vertical cleavage plane orientation. On the left, symmetric division with orientation of the mitotic spindle perpendicular to the apical surface results in equal distribution of the apical plasma membrane. On the right, asymmetric division with tilted orientation of the mitotic spindle results in unequal segmentation of the apical plasma membrane. *Modified from Huttner and Kosodo, 2005.*

2.1.3.2　Cell cycle

In the mitotic cell cycle, the mitotic (M) phase alternates with interphase. Interphase comprises gap phases G1 and G2 as well as DNA synthesis (S) phase. In addition, G0 phase comprises a resting phase for a cell that exits the cell cycle from G1 phase. Terminally differentiated cells, like neurons, are permanently in this quiescent state and never enter the cell cycle again. During gap phases G1 and G2, the cell grows and produces more cytoplasm as well as generates new proteins and organelles. In the S phase, a cell duplicates its chromosomes.

The cell cycle is regulated by cell-cycle checkpoints and the cell-cycle clock, the latter of which involves cyclins and cyclin-dependent kinases (Cdks) (**Figure 4**). In G1 phase, Cdk4/6-cyclin D and Cdk2-cyclin E complexes, required for progression through G1, sequentially phosphorylate and inhibit the retinoblastoma protein (Rb) enabling entry into the S phase (Lundberg and Weinberg, 1998; Harbour et al., 1999). Cdk2 then interacts with cyclin A to complete the S phase, after which cyclin A binds Cdk1. This complex runs the cell through G2 phase and then dissociates for Cdk1 to associate with cyclin B, which is required for G2-M transition and initiation of mitosis (Gavet and Pines, 2010).

DNA damage and spindle assembly checkpoints at G1, G2 and M phases determine whether the cell is ready to move forward in the cell cycle (Elledge, 1996). The cell cycle is halted at the checkpoint by stop signals until essential cellular processes are completed for cell to proceed. Concentration and activity of cyclin-cdk complexes are essential to control the entry into the next cell cycle phase.
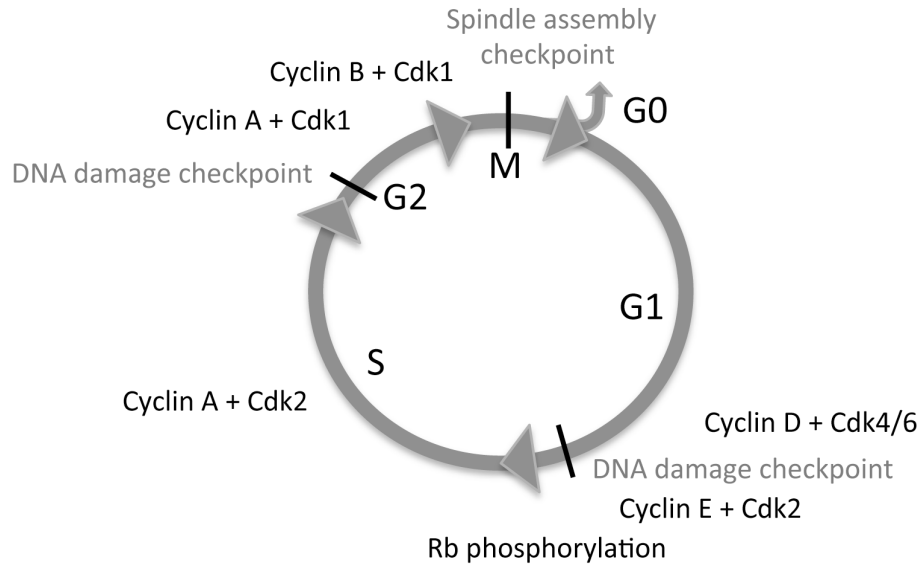
Figure 4. The cell cycle. Cyclin D-cdk4/6 and Cyclin E-cdk2 complexes are involved in G1 phase to sequentially phosphorylate the retinoblastoma protein (Rb) that leads to its inactivation and entry into S phase. In S phase, Cdk2 forms a complex with Cyclin A that drives the cell to the next phase. Progression through G2 phase is facilitated by Cyclin A-cdk1 complex and finally, Cyclin B-cdk1 complex mediates the G2/M transition. After mitosis, the cell may exit the cell cycle into the resting phase, G0. Cell cycle checkpoints at G1, G2 and M phases control cell cycle progression by monitoring proper DNA replication and spindle formation.

2.1.3.3   Cell cycle regulators

Cdks are activated by an association of a cyclin subunit and further by phosphorylation by Cdk-activating kinase. In addition, they are regulated through inhibition by Cdk-inhibitors (CKIs) that are members of the Ink4 or Cip/Kip families. The p53^{Kip2} protein coded by *p53* tumor suppressor gene is a negative regulator of the cell cycle that is induced by DNA damage. p53 ^{Kip2} protein activates p21^{Cip1} expression that is able to block cyclin-cdk complexes and halt the cell cycle. p53 ^{Kip2} protein has been shown to affect neural stem/progenitor cell self-renewal (Meletis et al., 2006; Piltti et al., 2006). However, it is best known for its ability to induce apoptosis (reviewed in Miller et al., 2000). Another tumor suppressor, the retinoblastoma protein Rb inhibits DNA synthesis and induces cell cycle exit. It is involved in the cell cycle machinery of NPCs as it was shown to decrease their proliferative capacity (Piltti et al., 2006). Moreover, a proto-oncogene *c-myc* regulates the cell cycle but has various roles in different cell types and tissues (Grandori et al., 2000; Murphy et al., 2005). It is a transcription factor that affects transcription through several mechanisms (Grandori et al., 2000). It has been shown to increase NPC self-renewal through its ability to bind Myc-interacting zinc finger protein-1 (Miz-1), an inhibitor of CKIs (Kerosuo et al., 2008).

Cyclin D1 belonging to D type cyclins, controls the cell cycle progression in G1 phase (Baldin et al., 1993). Cyclin D1 expression is induced by growth factors and thus, it acts as a sensor for extracellular signals to mediate cell proliferation but also differentiation, migration and tumorigenesis that are regulated independently of the cell cycle machinery (Fu et al., 2004). It is essential also in regulation of NPC proliferation (Sundberg et al., 2006).

2.1.4   Neuronal and glial development

The generation of neurons from neural stem/progenitor cells, or neurogenesis, is regulated by so called proneural genes constituting of basic helix-loop-helix (bHLH) transcription factors (**Figure 5**). These factors have been grouped to distinct families based on similarities in the bHLH domain sequence. In the mouse, only a few genes possess true proneural activity to facilitate progenitor commitment, including *Mash1*, Neurogenins 1-3 (*Ngn1-3*), *Math1* and *Math5* (Ma et al., 1996; Sommer et al., 1995; 1996; Lee 1997; Farah et al., 2000). In addition, the NeuroD and Olig family members share a bHLH domain structure, and are involved in further neuronal differentiation (Lee et al., 1995; Sommer et al., 1996; Farah et al., 2000; Takebayashi et al., 2000).

Proneural genes are expressed already in NE cell stage, where they are induced by neurogenic signals, such as bone morphogenetic protein 2 (BMP2), platelet-derived growth factor (PDGF) and erythropoietin (Johe et al., 1996; Lo et al., 1997; Williams et al., 1997; Shingo et al., 2001). Activated proneural genes regulate neural fate specification and promote progenitor development into neuronal lineages. Proneural genes are negatively regulated by repressor-type HLH factor families Hes (mammalian homologue of Hairy and Enhancer of Split) and Id (inhibitor of differentiation) that have changed or lacking DNA binding ability compared to bHLH proteins (Benezra et al., 1990; Sun et al., 1991; Akazawa et al., 1992; Sasai et al., 1992). Proneural activity is facilitated through Notch signaling by a mechanism of lateral inhibition. Proneural genes prevent their own expression by Notch ligands that activate Notch and upregulate Hes-1 and Hes-5 in neighboring cells, which stay undifferentiated (Kageyama et al., 2005). After Notch-induced upregulation of bHLH genes, their expression is maintained by positive feedback mechanisms of activated downstream genes that facilitate neuronal differentiation. Proneural genes also inhibit glial specifiers and promote cell cycle exit. Moreover, different proneural genes are responsible for the specification of particular neuronal subtypes.

In the murine brain, switch from neurogenesis to gliogenesis occurs at the end of embryonic development and is induced by downregulation of proneural genes (**Figure 5**). Astrocytogenesis is initiated by gliogenic signals, including ciliary neurotrophic factor, fibroblast growth factor, interleukin-6 (IL-6), leukemia inhibitory factor (LIF) and BMPs (Johe et al., 1996; Bonni et al., 1997; Rajan and McKay, 1998; Nakashima et al., 1999; Islam et al., 2009). These gliogenic signals activate the Janus tyrosine kinase/signal transducers and activators of transcription (JAK/STAT) pathway. Phosphorylated STAT transcription factors form a complex with the transcriptional co-activators CREB binding protein (CBP)/p300 onto astrocytic promoters to induce expression of astrocyte-specific genes, like GFAP. The dual role of BMPs in the regulation of both neurogenesis and gliogenesis is explained by competition between Ngn1 and STAT for CBP/p300 association and joining of BMP effector Smad into this complex. During early cortical development, Ngn1 inhibits STAT phosphorylation and combine to CBP/p300 preventing glial gene expression and promoting neuronal gene transcription (Sun et al., 2001). STAT is able to induce expression of negative HLH transcription factors Id2, Id3 and Hes-5 (Gu et al., 2005) leading to reduced proneural gene expression. In addition, BMP2 induces the expression of inhibitory HLH proteins Id1, Id2 and Hes-5 through Smad signaling (Nakashima et al., 2001). Astroglial fate is also regulated by Notch signaling. Inhibitory bHLH factors, Hes1 and Hes5, as well as STAT3 have been shown to be targets of Notch activation (Kamakura et al., 2004) that inhibits neurogenesis and oligodendroglial differentiation and promotes astroglial cell fate (Wang et al., 1998; Tanigaki et al., 2001).

Oligodendrocyte fate determination is regulated by sonic hedgehog (Shh), which upregulates oligodendrocytic markers O4 and chondroitin sulfate proteoglycan NG2 as well as bHLH factors Olig1 and Olig2 that promote oligodendroglial cell identity (Lu et al., 2000; Zhou et al., 2000; Alberta et al.,

2001). However, Olig1 and Olig2 are also involved in motor neuron specification during early development, due to cooperation with Ngn2. Downregulation of Ngn2 and expression of Nkx2.2 in progenitor cells serves as a switch from neuronal differentiation to oligodendrocyte development (Zhou et al., 2001b).
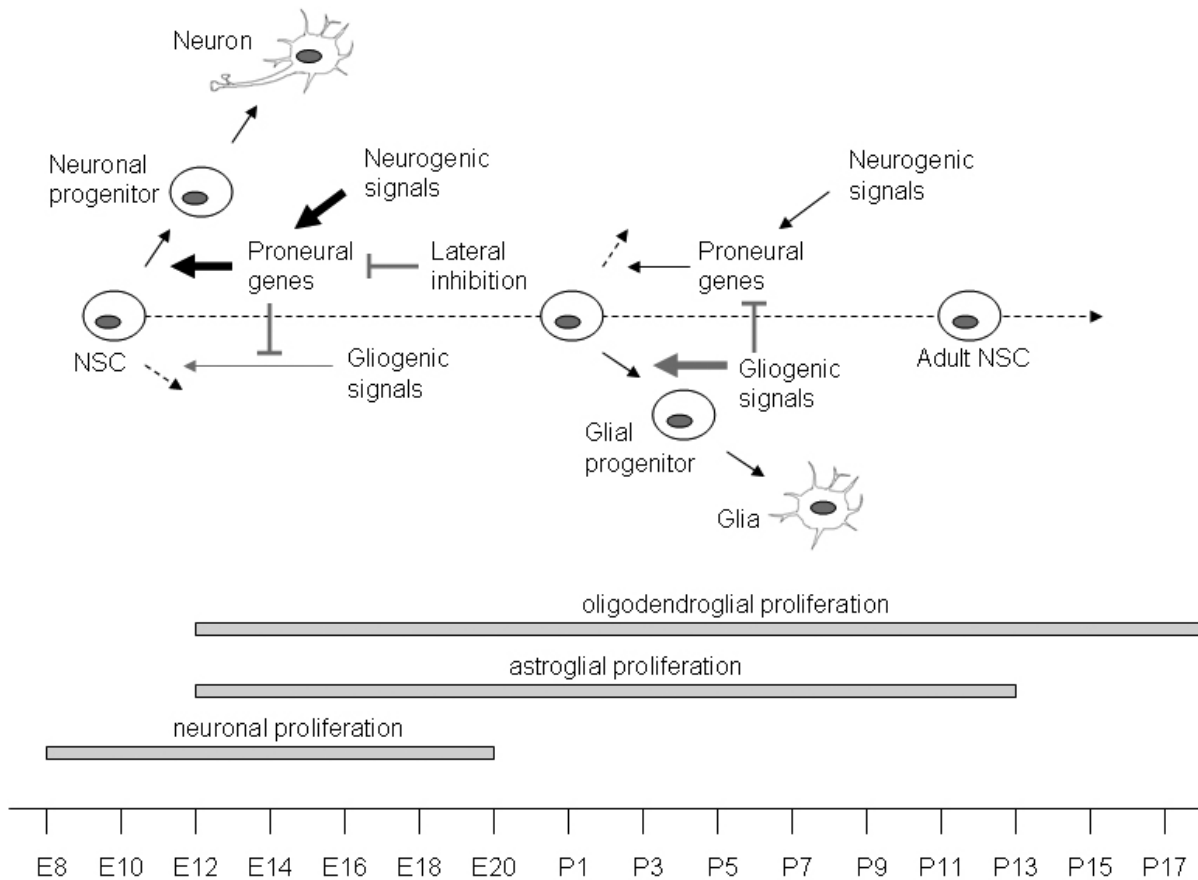
Figure 5. Timeline of neuronal and glial development in rat. During early embryogenesis, neural stem cells produce neurons as a consequence of neurogenic signals that induce proneural gene expression and subsequently inhibit glial differentiation. At the same time, lateral inhibition prevents proneural gene expression in other cells that do not enter the neuronal pathway. The switch from neurogenesis to gliogenesis occurs through gliogenic signals that activate glial differentiation and, in parallel, inhibit neuronal differentiation. In rats, neurogenesis peaks at E14, astrocytogenesis at P2 and oligodendrocytogenesis at P14. E, embryonic day; P, postnatal day. *Modified from Bertrand et al., 2002.*

2.1.5   Cell death

Cell death is traditionally divided into necrosis and apoptosis that were originally distinguished by Kerr et al. (1972). However, recently also other ways of cell death have been described, including oncosis and necroptosis. Apoptosis and oncosis are processes that lead to cell death, while necrosis is an unregulated condition appearing after cell death. Oncosis, or ischemic cell death, is characterized by an apparent cellular swelling that leads to disappearance of the nucleus, while apoptosis exhibits cellular shrinking resulting in nuclear fragmentation (Majno and Joris, 1995). Necrosis is recognized by morphological changes, such as cellular swelling and chromatin condensation that results in cell lysis and inflammation (Wyllie et al., 1980). On the other hand, necroptosis is a regulated form of necrosis

that shares the same morphological features (Degterev et al., 2005; Cho et al., 2009; He et al., 2009; Zhang et al., 2009).

2.1.5.1   Apoptosis

Apoptosis, or programmed cell death, differs from other cell death mechanisms as being a genetically regulated cellular suicide. It is required in maintaining tissue and cellular homeostasis and to control balance between cell proliferation and cell death. Apoptosis can be triggered by several extrinsic and intrinsic mechanisms, which lead to activation of the suicide program. Also lack of signals, like neurotrophin deprivation during neural development, or environmental cues may induce apoptosis. During the development, neural progenitor cells die through apoptosis, which regulates the number of progenitors and eliminates the excess cells. NPCs undergo the so called proliferative apoptosis. NPC death is required for developmental processes, such as neural tube closure (Geelen and Langman, 1977). In addition, proliferative apoptosis regulates the proper balance of proliferation and cell death within the neurogenic zone, as up to 70% of the developing cortical cells die during the embryogenesis (Blaschke et al., 1996; Thomaidou et al., 1997). Thus, apoptosis determines the size and shape of the brain.

The cell morphological features of apoptosis include the nuclear fragmentation and the condensation of nuclear chromatin and cytoplasm into apoptotic bodies, as well as blebbing of the plasma membrane (Kerr et al., 1972). Apoptosis is regulated by the Bcl-2 (B cell lymphoma) family proteins, the adaptor protein Apoptosis protease activating factor (Apaf1) and caspase family of cysteine proteases. Mammalian Bcl-2 family includes both anti-apoptotic members, like Bcl-2 and Bcl-X$_L$, that have multiple Bcl-2 homology domains (BH1-4), and pro-apoptotic members, which are divided into two categories by the presence of several BH domains (e.g. Bax) versus BH3 domain only (e.g. PUMA). Caspases are normally present as an inactive form, a procaspase. They are activated by a proteolytic cleavage that occurs during the caspase cascade, where activated upstream caspases cleave and activate downstream caspases. Mammalian upstream initiator caspases involved in apoptosis include caspases-2, -8, -9 and -10 that activate downstream effector caspases-3, -6, and -7 (Earnshaw et al., 1999).

Apoptosis in NPCs follow either the mitochondrial or the death receptor pathway (De Zio et al., 2005). In the mitochondrial pathway the mitochondrial protein cytochrome c is released into the cytoplasm, where it binds Apaf1 and forms the apoptosome together with dATP, leading to the activation of caspase-9 (Li et al., 1997; Saleh et al., 1999). The death receptor pathway of apoptosis is induced by ligand binding to a cell membrane receptor that bears a cytoplasmic domain called a death domain (Boldin et al., 1995). Well-known death receptor ligands are Fas ligand and tumour necrosis factor α (TNFα) that activate Fas receptor and TNF receptor 1, respectively. Fas receptor binds procaspase-8 via the death domain and forms a death-inducing signaling complex that subsequentially activates caspase-8 (Muzio et al., 1996; Medema et al., 1997). Both the mitochondrial and the death receptor pathway lead finally to the conversion of procaspase-3 into an active caspase-3 and the typical morphological features of apoptosis.

2.2 Bone morphogenetic proteins in development

BMPs consist of more than 20 members in different species within transforming growth factor-β (TGF-β) superfamily that also includes TGF-βs, activins/inhibins, Lefty, Myostatin, anti-Müllerian hormone and growth-differentiation factors. BMPs were first discovered in the process of endochondral bone formation. BMP2, BMP4 and BMP6 relay glucocorticoid-induced differentiation of osteoblasts, BMP6 acting on earlier stage osteoprogenitor cells than BMP2 and BMP4 (Thies et al., 1992; Rickard et al., 1994; Hughes et al., 1995; Boden et al., 1996; 1997). During the development, BMP members exhibit various spatiotemporal expression patterns. They regulate a diverse array of cellular processes, including proliferation, differentiation, cell lineage commitment, survival and apoptosis depending on the activated signaling cascade. BMPs regulate morphogenesis and differentiation in various developing organs, including kidneys, lungs, heart, teeth, skin and hair. BMPs are essential during early embryogenesis and are required for the formation of embryonic mesoderm and extraembryonic tissues as well as for the proper posterior patterning (Mishina et al., 1995; Winnier et al., 1995; Zhang and Bradley, 1996). Homozygous BMP2 mutant mice are embryonic lethal with defects in cardiac development (Zhang and Bradley, 1996). Lack of BMP7 attenuates kidney and eye development and interferes the accurate position of limbs (Dudley et al., 1995; Luo et al., 1995). BMP4 is most prominent BMP family member that regulates tooth formation and differentiation (Neubüser et al., 1997; Zhang et al., 2000b; Gluhak-Heinrich et al., 2010). At the early embryogenesis, BMPs promote epidermal differentiation and inhibit neural induction from the embryonic ectoderm (Hawley et al., 1995; Wilson and Hemmati-Brivanlou, 1995).

2.2.1 BMP signaling

BMPs act as protein dimers and signal through tetramer complexes of specific serine/threonine kinase receptors, BMP receptors, on the cell membrane. BMP receptor (BMPR) complex contains two separate type II and type I receptors that together facilitate the signal transduction. There are different BMP type I receptors, of which most of the BMP members use ALK2 (activin receptor-like kinase 2), ALK3 (BMPR-IA) or ALK6 (BMPR-IB) (**Table I**). All BMPs activate BMP type II receptor BMPRII and may additionally bind to activin type II receptors ActR-II and ActR-IIB. Type II serine/threonine kinases are constantly active and upon ligand binding, they phosphorylate Gly-Ser domain in type I receptors leading to the activation of intracellular signaling cascade (Wrana et al., 1994). There exists also a co-receptor for BMPs, DRAGON, which potentiates BMP signaling by binding to BMP receptors or ligands (Samad et al., 2005). BMPs induce both Smad-dependent and Smad-independent signaling pathways (**Figure 6**) depending on the timing of receptor complex formation. Binding of a ligand to a preformed complex on the cell membrane activates Smad pathway, while ligand-induced receptor complex facilitates mitogen-activated protein kinase (MAPK) signaling pathways, including ERK (extracellular signal regulated kinase), JNK (c-Jun N-terminal kinase), p38 (MAP) kinase, as well as serine threonine kinase mTOR/FRAP (mammalian target of rapamycin/FKBP12-rapamycin-associated protein) and LIM kinase 1 cascades (Yamaguchi et al., 1995; Shibuya et al., 1998; Lou et al., 2000; Hassel et al., 2003; Rajan et al., 2003; Lee-Hoeflich et al., 2004).

TABLE I. Mammalian BMP family members and their type I receptors	
Ligand	**Type I receptor**
Bmp2/4 group	ALK3, ALK6
BMP-2	
BMP-4	
OP-1 group	ALK2, ALK6
BMP-5	
BMP-6/Vgr1	
BMP-7/OP-1	
BMP-8a/OP-2	
BMP-8b/OP-3	
GDF-5 group	ALK6
BMP-14/GDF-5/CDMP-1	
BMP-12/GDF-7	
BMP-13/GDF-6/CDMP-2	
BMP9/10 group	ALK1, ALK2
BMP-9/GDF-2	
BMP-10	
Other members	
BMP-3	NF
BMP-3b/GDF-10	NF
BMP-11/GDF-11	ALK4, ALK5
BMP-15/GDF-9b	ALK6
BMP-16/Nodal	ALK7

Abbreviations: ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; CDMP, cartilage-derived morphogenetic protein; GDF, growth-differentiation factor; NF, not found; OP, osteogenic protein; Vgr, vegetal related.

In Smad-dependent signaling pathway, activated type I receptors phosphorylate certain receptor-activated Smads (R-Smads) that form a heteromeric complex with a common-mediator Smad (co-Smad). This Smad complex translocates into the nucleus and regulates transcription of BMP target genes. Smads are common signal transducers of BMP and TGF-β/activin families. R-Smads include Smad1/2/3/5/8, of which Smad1/5/8 are activated by BMPs (Aoki et al., 2001). There is only one co-Smad in mammals, Smad4. In addition, there is a third class of Smads, inhibitory Smads (I-Smads), including Smad6/7, that negatively regulate BMP signaling through direct interactions with the type I receptors or with an activated R-Smads to prevent R-Smad activation or the R-Smad/co-Smad complex formation, respectively (Imamura et al., 1997; Nakao et al., 1997). Smad6 is specific for BMPs, while Smad7 is a common inhibitor in the whole TGF-β superfamily.
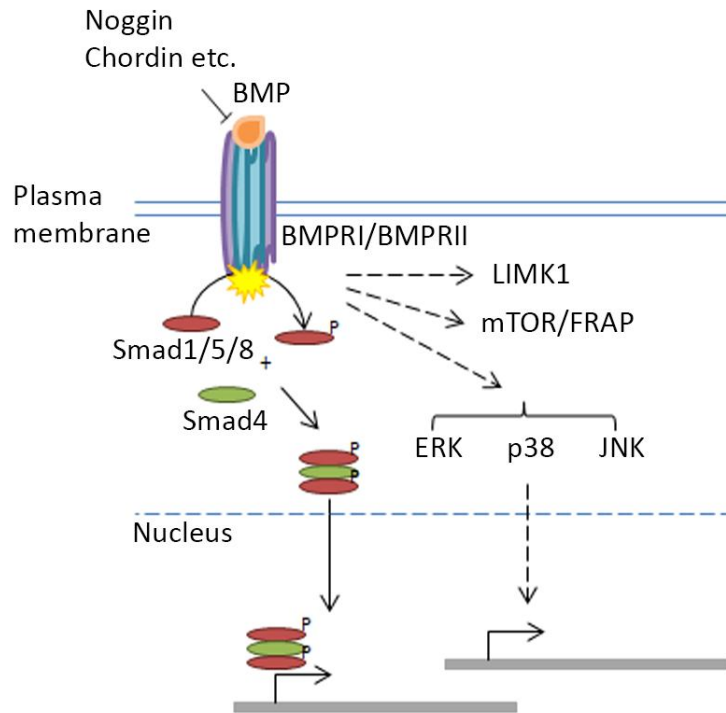
Figure 6. BMP signaling pathways. Ligand binding to a heteromeric complex of BMP type I and type II receptors (BMPRI/BMPRII) phosphorylates Smad1/5/8, which complex with Smad4 and translocate into the nucleus to regulate BMP responsive genes. Alternatively, BMP receptor activation may lead to the activation of MAP kinases ERK, p38 and JNK or LIMK1 and mTOR/FRAP kinases that regulate other target genes. BMP, bone morphogenetic protein; BMPR, BMP receptor; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; LIMK, LIM kinase; mTOR/FRAP, mammalian target of rapamycin/FKBP12-rapamycin-associated protein; P, phosphate.

R-Smads are phosphorylated by type I receptors on their characteristic Ser-Ser-Val/Met-Ser (SSXS motif) in the C-terminal Mad homology 2 domain (Wang et al., 2009). As inactive proteins, R-Smads are localized in the cytoplasm by a nuclear export sequence (NES) (Pierreux et al., 2000; Inman et al., 2002). Once activated, NES in the formed R-Smad/co-Smad complex is interrupted and a nuclear localization sequence in R-Smads guides the complex into the nucleus (Pierreux et al., 2000; Xiao et al., 2000).

Smad complex in the nucleus acts as a transcription factor to regulate transcription of BMP responsive genes by binding directly to the DNA or through interaction with other DNA-binding factors. Smads bind to the specific DNA sequences, a GC-rich GCCGnCGC sequence or Smad-binding element that is either AGAC or GTCT sequence (Kim et al., 1997; Kusanagi et al., 2000). Smads interact with various transcription factors, such as Runx2 and Menin in addition to other nuclear proteins (Zhang et al., 2000a; Sowa et al., 2004). They also bind transcriptional co-activators, including CBP/p300 that has histone acetyl transferase activity, as well as GCN5 and P/CAF N-acetyl transferases that enhance transcriptional activation (Janknecht et al., 1998; Itoh et al., 2000c; Kahata et al., 2004).

BMP signaling is regulated by several extracellular BMP antagonists that bind BMPs and prevent receptor activation and subsequent signal transduction. BMP antagonists include Noggin, Chordin, Follistatin, Follistatin-related gene, twisted gastrulation, Ventroptin and the Dan/cerberus family genes.

2.2.2   BMPs during brain development

Expression of different BMP family members is first detected in the anterior neuroectoderm before neural tube closure at E8.5 during the mouse development (Furuta et al., 1997). Their expression continues throughout the brain development and shows overlapping expression patterns in the dorsomedial telencephalon (Furuta et al., 1997). BMP2, BMP4 and BMP6 expression is localized in the dorsal neural tube, while BMP7 together with BMP4 localizes to the epidermal ectoderm (Tanabe and Jessell, 1996). Studies with other organisms have shown that BMP4 and BMP7 are expressed already in earlier stages of development in the ectoderm and their action must be inhibited in order to convert ectoderm into neuroectoderm (Hawley et al., 1995). In mice, BMP4 induces epidermal fate and concurrently inhibits neural fate (Wilson and Hemmati-Brivanlou, 1995). BMPs inhibit neurulation and later, they are involved in the dorsoventral patterning of the neural tube. Specifically, BMP2 inhibits the bending of neural folds essential for neural tube closure (Ybot-Gonzalez et al., 2007). BMP7 is prominently expressed in the developing mouse hindbrain and affects dorsoventral patterning of this area (Arkell and Beddington, 1997).

Ectodermal BMPs induce further BMP expression in the dorsal neural tube leading to transcriptional activation and establishment of specific dorsal cell types, such as neural crest stem cells and dorsal interneurons (Liem et al., 1995; Selleck et al., 1998; Lee et al., 2000a). BMP expression at the dorsal neural tube forms a gradient towards the ventral side and thus, BMPs also affect the generation of other cell populations with co-operative and antagonistic interactions with Shh that is expressed in the ventral neural tube (Patten et al., 2002). BMPs also regulate rhombencephalic neural crest development by inducing apoptosis in specific rhombomere segments in the developing neural tube (Graham et al., 1994).

2.2.3   Versatile roles of BMPs in neural stem/progenitor cells

BMP2 and BMP4 are expressed in neural stem cell-derived astrocytes, while no expression have been detected in NSCs themselves or in NSC-derived neurons or oligodendrocytes (Lü et al., 2009; Hu et al., 2012). During the brain development, BMPs exert different effects on cell fate in a temporal manner: an early response with apoptosis following neuronal differentiation and finally glial differentiation in late embryogenesis (Mehler et al., 2000). BMP2 and BMP4 induce apoptosis with simultaneous inhibition of proliferation in cortical neural progenitor cells (Mabie et al., 1999). Furthermore, BMPs promote neuronal differentiation in cortical progenitors (Li et al., 1998; Mabie et al., 1999) and BMP7 regulates the formation of dendrites in different embryonic and perinatal neuronal populations (Lein et al., 1995; Le Roux et al., 1999; Withers et al., 2000). BMPs promote astroglial lineage elaboration from embryonic neural progenitor cells with simultaneous inhibition of oligodendrocyte differentiation (Mehler et al., 1995; Gross et al., 1996; Mabie et al., 1999; Bonaguidi et al., 2005).

In adult SVZ stem cell niche, BMPs inhibit neurogenesis, while promoting glial differentiation (Lim et al., 2000). Recently, BMP signaling was shown to induce quiescence in adult hippocampal NSCs to preserve the stem cell pool (Mira et al., 2010). In both adult NSC niches, Noggin antagonizes BMP function by recruiting the quiescent NSCs into the cell cycle and by increasing neuronal differentiation (Lim et al., 2000; Mira et al., 2010).

2.3   Proteolysis

Proteolysis, or protein degradation, is facilitated by enzymes called proteases. Proteolysis is involved in different cellular events, including protein activation, removal of signal sequences or extra residues of proteins, and degradation of unneeded or damaged proteins or the extracellular matrix proteins. Thus, proteases regulate several physiological processes, including development, tissue morphogenesis and repair, digestion, blood coagulation, fibrinolysis, fertilization, immunity and ion and water transport. I will here concentrate on trypsin-like serine proteases hepatocyte growth factor activator (HGFA) and matriptase, and their common inhibitors that are involved in regulation of cellular behaviour. In the last chapter I will also unravel the mechanisms of proteasomal degradation.

2.3.1   Serine proteases

Serine proteases are a family of proteases among five catalytic protease classes: serine, cysteine, aspartic, threonine and metalloproteases (Puente et al., 2003). Serine proteases play versatile roles in both normal physiology and in pathological conditions, such as cancer and degenerative diseases. They consist of nearly or above 200 members in human and mouse, respectively (Puente et al., 2003). Serine residue located in the active site of protease acts as a catalytic domain. Classification of serine proteases is versatile but they include several subgroups and families based on their function and appearance, like membrane-associated or blood serum proteases. The secretion and activation of serine proteases is strictly regulated to limit the excess proteolytic activity. Thus after activation, their action is controlled by cognate endogenous inhibitors, called serine protease inhibitors (SPIs).

2.3.1.1   Hepatocyte growth factor activator

HGFA is a secreted serine protease that was originally purified from bovine serum and identified as an activator for hepatocyte growth factor (HGF) (Shimomura et al., 1992). It is structurally similar to a blood coagulation factor XII (FXII) and belongs to the plasminogen activator (PA)/FXII/HGFA subfamily of the kringle serine protease superfamily (Miyazawa et al., 1993; 1998). HGFA is secreted as a zymogen, pro-HGFA, by the liver hepatocytes and it circulates in the plasma as an inactive form (Shimomura et al., 1993). It has detected, nonetheless, also in gastrointestinal tract, developing kidneys, brain astrocytes, injured and diseased brain, hair follicles and synovial tissues (Yamada et al., 1998; Hayashi et al., 1998; Itoh et al., 2000a; van Adelsberg et al., 2001; Lee et al., 2001; Nagashima et al., 2001). HGFA is activated in response to tissue injury by thrombin and plasma kallikrein (Shimomura et al., 1993; Miyazawa et al., 1996). In tumor tissue, it is activated by kallikrein 1-related peptidases (KLKs) 4 and 5 (Mukai et al., 2008). HGFA activity is regulated also by endogenous inhibitors, including hepatocyte growth factor activator inhibitor (HAI)-1 and HAI-2 as well as protein C inhibitor (Kawaguchi et al., 1997; Shimomura et al., 1997; Suzuki, 2010). By converting pro-HGF into an active αβ heterodimer, HGFA regulates tyrosine kinase receptor c-Met signaling that affects the motility, mitosis and morphogenesis as well as regeneration of various target cells and tissues (Naldini et al., 1991). More recently, HGFA was found to activate macrophage-stimulating protein (MSP) important in regulation of macrophage activity during inflammation (Kawaguchi et al., 2009).

HGFA-deficient mice are viable and develop normally but show impairment in regeneration of the intestinal mucosa after injury (Itoh et al., 2004). Although developmental abnormalities were not observed in HGFA knockout mice, HGFA is, however, required for kidney ontogenesis (van

Adelsberg et al., 2001). In injured liver, HGFA facilitates tissue repair and regeneration through HGF activation and high-affinity binding to heparin (Miyazawa et al., 1996; Kaibori et al., 2002).

HGFA plays a role also in tumorigenesis. Increased expression level of HGFA has been detected in breast cancer, colorectal carcinoma and in renal cell carcinoma compared to normal tissue (Kataoka et al., 2000a; Parr et al., 2004; Yamauchi et al., 2004). Furthermore, HGFA induces tumor cell invasion and tumor growth in glioblastoma cells (Uchinokura et al., 2006).

2.3.1.2 Matriptase

Matriptase (MT-SP1, epithin) is a type II transmembrane serine protease (TTSP) characterized by a short signal anchor near the amino terminus, a large extracellular carboxy terminus with the serine protease domain and in between a stem region containing a single SEA domain, two CUB domains and four low density lipoprotein receptor class A domains (**Figure 7**).
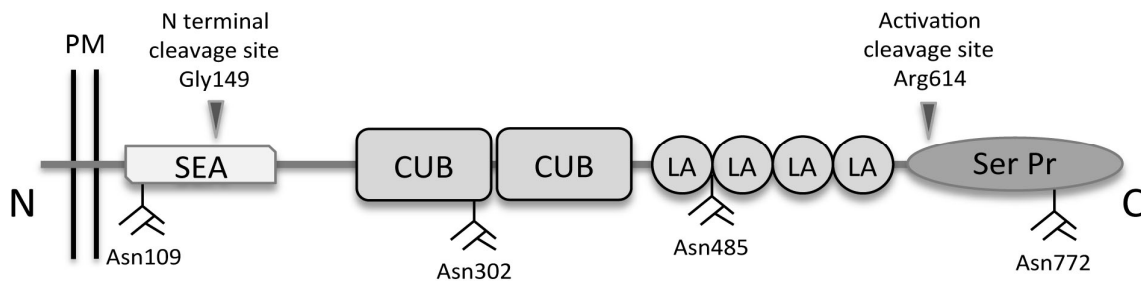

Figure 7. The structure of matriptase. C, carboxyterminus; CUB, complement C1r/C1s/Uegf/Bmp1 domain; LA, low density lipoprotein receptor type A domain; N, aminoterminus; PM, plasma mebrane; SEA, sea urchin sperm protein/enterokinase/agrin domain; Ser Pr, serine protease domain. Also the two cleavage sites and the four N-glycosylation sites are shown. *Modified from List et al., 2006a.*

Among TTSPs, matriptase shares structural similarity and forms a Matriptase subfamily with matriptase-2 and matriptase-3 (Kim et al., 1999; Lin et al., 1999b; Velasco et al., 2002; Szabo et al., 2005). A signal anchor orients matriptase in the plasma membrane as a membrane-associated serine protease. Matriptase is synthesized as catalytically inactive single-chain form, a zymogen. The life cycle of matriptase is unique among the serine proteases, involving an inhibitor-assisted autoactivation, rapid inhibition and finally shedding from the plasma membrane (**Figure 8**). Activation of matriptase is a complicated process that involves two sequential cleavages at the SEA domain and at the activation cleavage site in the serine protease domain (**Figure 7**) (Cho et al., 2001; Oberst et al., 2003b). Only the N terminally cleaved form of matriptase is present at the cell surface (Cho et al., 2001). The cleavage at the serine protease domain converts matriptase into its active two-chain form and occurs through a proteolytic transactivation mechanism between two matriptase zymogen molecules (Oberst et al., 2003b). Activation involves also glycosylation of two N-glycosylation sites, Asn302 and Asn485, and is dependent on the interaction with a cognate inhibitor of matriptase, HAI-1 (Oberst et al., 2003b; 2005). Autoactivation of matriptase can be triggered by a bioactive phospholipids, like sphingosine-1-phosphate (Benaud et al., 2002). The androgen, dihydrotestosterone induces activation of matriptase in LNCaP prostatic adenocarcinoma cells (Kiyomiya et al., 2006). Moreover, a chemical inducer, the polyanionic compound suramin has been shown to stimulate matriptase activation (Lee et al., 2005a).

Extracellular and cytoplasmic acidosis induces matriptase activation in different epithelial and carcinoma cells (Tseng et al., 2010). In addition, matriptase is activated by tissue acidity in different skin disorders (Chen et al., 2011). Shortly after its activation on the cell surface, matriptase is inhibited by high-affinity binding of HAI-1 resulting in the formation of a functionally inactive matriptase-HAI-1 complex (Oberst et al., 2005). In its complexed form, matriptase is shed from the plasma membrane (Lin et al., 1999a). Matriptase is also inhibited by HAI-2 and sunflower trypsin inhibitor (Long et al., 2001; Szabo et al., 2008).
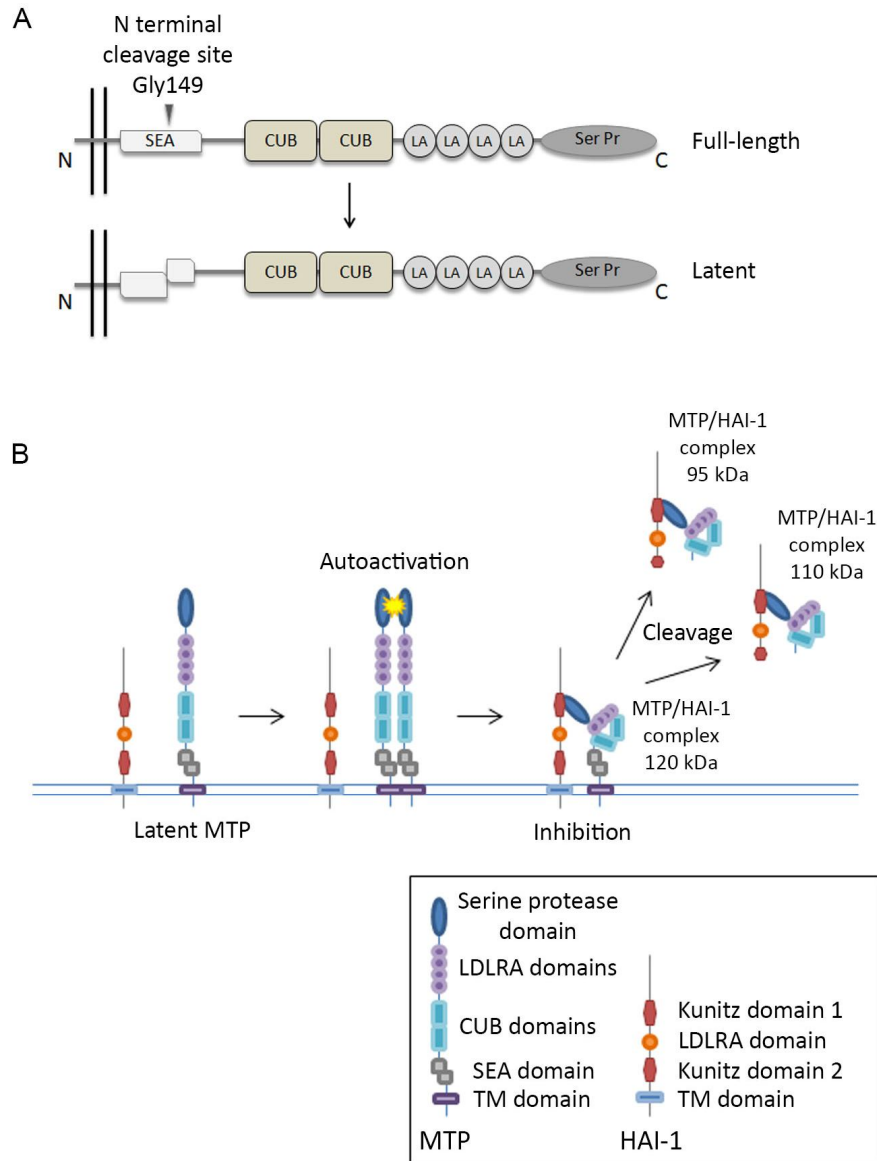
Figure 8. The life cycle of matriptase. (A) Matriptase is synthesized on the rough endoplasmic reticulum (ER) as a 94 kDa full-length zymogen that is processed into a 70 kDa latent form within the ER or Golgi apparatus. (B) On the cell surface, latent matriptase undergoes auto-activation to yield a two-chain active protease that is rapidly inhibited by HAI-1 to form a 120 kDa matriptase-HAI-1 complex. This complex is targeted for proteolytic cleavage and is shed from the plasma membrane as 95 kDa and 110 kDa complexes. LDLRA, LDL receptor A; MTP, matriptase; TM, trans-membrane. *Modified from List et al., 2006a and Wang et al., 2009.*

Matriptase, as other membrane-associated proteases, facilitates its effects through proteolysis, by which it modulates the extracellular matrix and activates target proteins, including latent growth factors, cytokines, hormones and other proteases. The substrate proteins for matriptase found so far include protease activated receptor-2 (PAR-2), HGF, urokinase-type plasminogen activator, prostasin, MSP-1, PDGF-D and Trask (transmembrane and associated with src kinases) (Lee et al., 2000b; Takeuchi et al., 2000; Bhatt et al., 2005; Netzel-Arnett et al., 2006; Bhatt et al., 2007; Ustach et al., 2010).

Matriptase is mainly an epithelial protease and its expression is colocalized with both HAI-1 and HAI-2 in most epithelial tissues (Oberst et al., 2001; 2003a; Szabo et al., 2008). Matriptase is located on the basolateral plasma membrane and it is involved in the tight junction formation and the maintenance of epithelial integrity (Tsuzuki et al., 2005; List et al., 2009; Buzza et al., 2010). The physiologic function of matriptase is shown in matriptase null mice that die within 48 hours of birth due to an interrupted epidermal permeability barrier function and subsequent dehydration and hypothermia (List et al., 2002). The same mice also reveal deficiencies in the development of hair follicles and the thymus (List et al., 2002). Recent studies link matriptase to the regulation of brain development. It mediates the activation of PAR-2 in the nonneural surface ectoderm of the developing neural tube, an event that is essential in neural tube closure (Camerer et al., 2010). Moreover, matriptase induces neural progenitor cell migration and differentiation *in vitro* (Fang et al., 2011). Matriptase is also present in variety of tumors of epithelial origin, including breast, colon, ovarian, cervical, prostate, renal, lung, skin as well as head and neck carcinoma where high expression levels often correlate with the malignant pathology (Oberst et al., 2001; 2002; Tanimoto et al., 2001; Santin et al., 2003; Jin et al., 2005; Lee et al., 2005b; Riddick et al., 2005; Zeng et al., 2005; Cheng et al., 2006; Jin et al., 2006; List et al., 2006b; Baba et al., 2012). In various tumors, matriptase has a role in tumor progression mediating malignant transformation and tumor cell invasion (Suzuki et al., 2004; Förbs et al., 2005; List et al., 2005; Tsui et al., 2008).

2.3.2   Serine protease inhibitors

SPIs can be divided into canonical and non-canonical inhibitors and into serpins based on their mechanism of inhibition (reviewed in Krowarsch et al., 2003). Canonical and serpin inhibitors interact with a cognate protease through a binding loop complementary to the active site of the target, while non-canonical inhibitors bind their target through an N-terminal segment. Serpins, however, also induce a conformational change in the protein structure. SPIs are either single domain proteins or have a tandem repeat of inhibitory domains that varies between 18 serine protease inhibitor families. These inhibitory domains include soybean trypsin inhibitor-type, sunflower trypsin inhibitor-type, bovine pancreatic trypsin inhibitor/Kunitz-type and Kazal-type domains among others.

2.3.2.1   HAI-1 and HAI-2

HAI-1 and HAI-2/placental bikunin are membrane-bound glycoproteins belonging to the Kunitz-type serine protease inhibitor family. HAI-1 was initially identified as a potent inhibitor for secreted protease HGFA (Shimomura et al., 1997). Later, HAI-2 was recognized as another endogenous HGFA inhibitor after its homology to HAI-1 (Kawaguchi et al., 1997). The mature HAI-1 and HAI-2 proteins consist of two extracellular Kunitz inhibitor domains, KD1 and KD2 with varying inhibitory activity, a type-I transmembrane domain and a short cytoplasmic tail (**Figure 9**). In addition, HAI-1 contains a low density lipoprotein receptor (LDLR)-like domain between the KDs (Shimomura et al., 1997). HAI-1 has a splice variant, HAI-1B that contains extra residues in adjacent to KD1, and which shares

substrate specificity and tissue expression with HAI-1 (Kirchhofer et al., 2003). HAI-2 can also be modified by splicing to yield a shorter form of protein that lacks the first KD. This form is the primary isoform in mice, while human tissues mostly express the full-length form (Itoh et al., 1999). Despite lacking the KD1, mouse isoform is able to inhibit HGFA (Kataoka et al., 2002). In addition, there is a third isoform of HAI-2 found in mouse testis that lacks the KD1 and has an insert containing a termination codon between the KDs in mouse cDNA, and thus it lacks also the KD2 in the final protein product (Itoh et al., 1999).
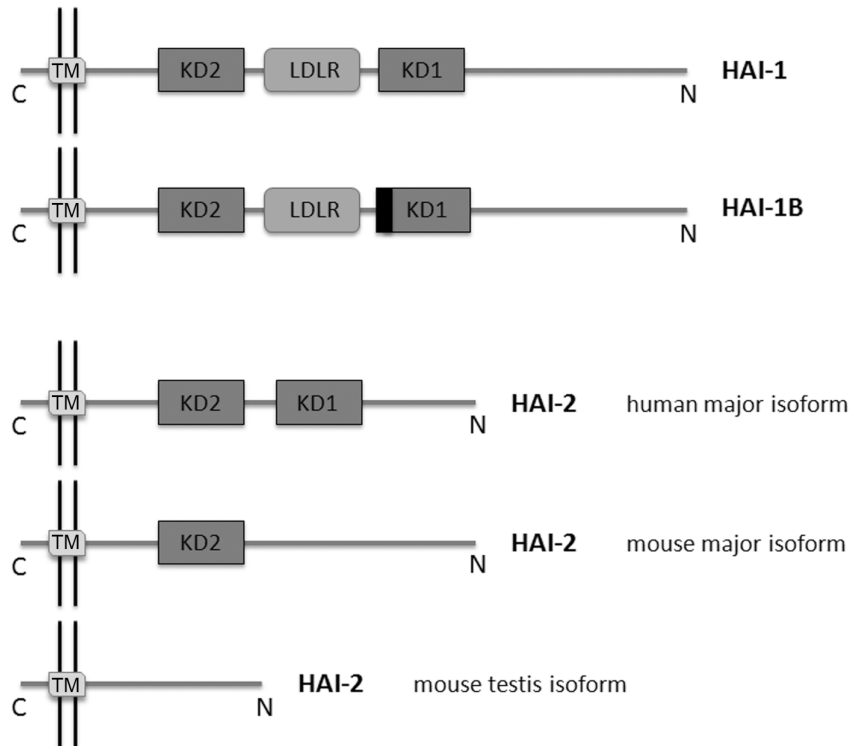

Figure 9. Different HAI-1 and HAI-2 isoforms. C, carboxyterminus; KD, Kunitz domain; LDLR, low density lipoprotein receptor-like domain; N, aminoterminus; TM, transmembrane domain.

HAI-1 and HAI-2 inhibit several trypsin-like proteases that can be either secreted or transmembrane proteins. The inhibitory activity of both HAI-1 and HAI-2 is mostly mediated through KD1 (Qin et al., 1998; Denda et al., 2002). They both show inhibitory activity against HGFA, hepsin, trypsin, matriptase, plasmin and plasma kallikrein, and HAI-2 also inhibits tissue kallikrein and several coagulation factors (Delaria et al., 1997; Kawaguchi et al., 1997; Marlor et al., 1997; Kataoka et al., 2000c; Oberst et al., 2001; Denda et al., 2002; Kirchhofer et al., 2003; 2005; Szabo et al., 2008). In addition, HAI-1 has been recognized as an inhibitor for prostasin (Fan et al., 2005).

Both HAI-1 and HAI-2 co-localize with matriptase in epithelial cells of most adult tissues (Szabo et al., 2008). HAI-1 is expressed in the basolateral plasma membrane of polarized epithelial cells (Kataoka et al., 1999). However, the membrane-associated form of HAI-1 has a short half-life, and it is either secreted to the extracellular medium or recycled to endosomes or apical plasma membrane (Godiksen et al., 2008). In contrary, there is evidence that HAI-2 is expressed beneath the apical surface of some epithelial cells (Kataoka et al., 2000b). Recently, it was shown that HAI-1 is regulated by a proteolytic cleavage by membrane-type matrix metalloprotease MT1-MMP that induces HAI-1 shedding as separate fragments (Domoto et al., 2011).

HAI-1 and HAI-2 are essential for the proper embryonic development, and especially for the formation of the placenta. Homozygous deletion of HAI-1 gene in mice leads to death *in utero* at early developmental stage due to the disrupted basement membrane and lacking branching morphology of the labyrinth layer in the placenta (Tanaka et al., 2005; Fan et al., 2007). HAI-2 deficiency in mice also causes severe developmental defects, including impaired development of the neural tube and abnormal placental labyrinth (Szabo et al. 2009).

Increased expression of HAI-1 is often linked to tissue injury and epithelial regeneration (Kataoka et al., 1999; Itoh et al., 2000b) while downregulation of both HAI-1 and HAI-2 occurs in several cancers. Decreased expression of HAI proteins and subsequent excessive activity of HGFA or matriptase is observed in breast, prostate, colorectal, renal, cerebellar and hepatocellular tumors often leading to malignant progression due to dysregulated protease activity (Kataoka et al., 2000b; Oberst et al., 2002; Kang et al., 2003; Yamauchi et al., 2004; Parr et al., 2004; 2010; Vogel et al., 2006; Tsai et al., 2007; Kongkham et al., 2008; Tung et al., 2009). Reduced expression of HAI proteins enhances tumor invasiveness, which is shown in breast, prostate, lung and pancreatic cancer cells (Cheng et al., 2009; Parr et al., 2010). The role of HAI-1 in tumor cell invasion is linked to regulation of epithelial-mesenchymal transition of certain cancer cells (Cheng et al., 2009). On the other hand, forced expression of either HAI-1 or HAI-2 inhibits tumor cell invasion (Kongkham et al., 2008; List et al., 2005; Tsui et al., 2008; Tung et al., 2009).

2.3.3   Proteasomal degradation

Two major proteolytic pathways mediate protein degradation in the cell: the lysosomal pathway and the ubiquitin-proteasome system (UPS). The lysosomal pathway is mainly involved in the degradation of membrane-associated proteins, while the UPS facilitates cytosolic protein degradation. Autophagy, initially considered as a non-selective degradative process, is part of the lysosomal system, where cytosolic components are enclosed into an autophagosome that subsequently fuses with the lysosome. Recently, also a selective and ubiquitin-dependent form of autophagy was discovered, where proteins are targeted for degradation (Shaid et al., 2012).

2.3.3.1   Ubiquitin-proteasome system

The UPS is involved in protein quality control that regulates the proper folding of proteins and prevents the accumulation of misfolded proteins in the cytoplasm. The UPS can recognize abnormal proteins through molecular chaperones, heat shock proteins (Meacham et al., 2001; Murata et al., 2001; Marques et al., 2006). The UPS has an important role in cell division to target cell cycle regulators, like cyclins and CKIs, for degradation (Benanti, 2012). This enables the correct cell cycle timing and transitions. During neurogenesis, degradation via the UPS regulates Notch signaling and Pax6 expression and thus, controls NSC maintenance and commitment (Tuoc and Stoykova, 2010).

The degradation of proteins by the UPS occurs through a covalent attachment of an ubiquitin (Ub) molecule to the substrate protein and the following proteasomal degradation of the substrate by the 26S proteasome (**Figure 10**). This involves series of enzymatic reactions, where enzymes bind Ub by a thiol ester (Hershko et al., 1983). First, the ubiquitin-activating enzyme, E1, binds and activates Ub in an ATP-dependent manner. Then, in the conjugating reaction, the Ub is transferred by an ubiquitin-conjugating enzyme, E2, from E1 to the ubiquitin ligase (E3). The E3 recognizes the substrate protein and catalyzes the formation of a covalent bond between the Ub and Lys residues of the substrate. Usually, the substrate is polyubiquitinated for the recognition of the 26S proteasome by addition of

several Ub monomers to form a polyubiquitin chain (Hershko et al., 1980; Pickart, 1997). Several Lys residues in the Ub moiety itself serve as substrates for the subsequent ubiquitins and thus, mediate the formation of ubiquitin chains of variable structures (Pickart and Fushman, 2004).
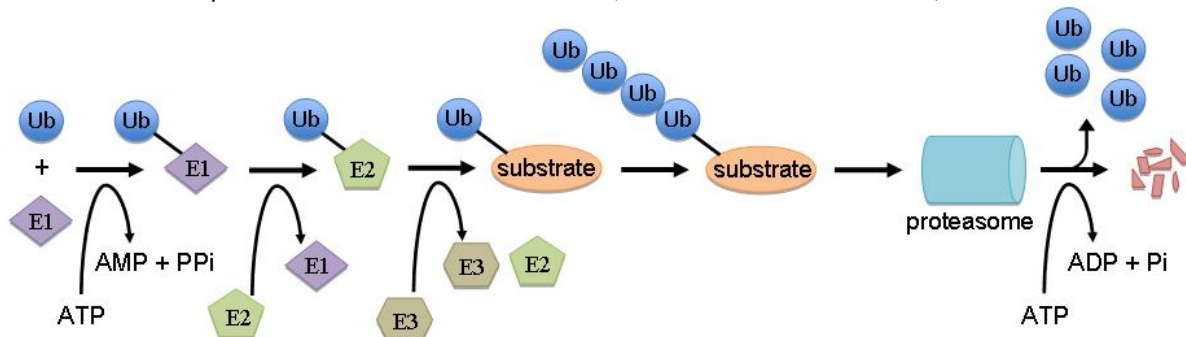

Figure 10. The ubiquitin-proteasome system. In the activation reaction, E1 enzyme binds ubiquitin in an ATP-dependent manner. In the following conjugation reaction, E2 enzyme carries ubiquitin from E1 to E3 enzyme that links ubiquitin to the substrate. Ubiquitin ligation is repeated to yield a polyubiquitinated substrate that is recognized by the proteasome and degraded into peptide fragments with simultaneous ATP hydrolysis. Free ubiquitin is released from the proteasome after protein degradation. Pi, inorganic phosphate; PPi, inorganic diphosphate; Ub, ubiquitin.

The 26S proteasome is composed of the catalytic core, the 20S proteasome, and of the regulatory 19S complex (Eytan et al., 1989; Chu-Ping et al., 1994). The 20S proteasome consists of four piled hexameric rings made of structural α subunits and catalytic β subunits that together form a cylindrical structure. The two outer rings of the cylinder are formed from seven α subunits each, while the inner rings are each made of seven β subunits. The ring-shaped 19S regulatory particle bound to either or both ends of the 20S proteasome regulates substrate entry to the cylinder and serves as a gatekeeper (Smith et al., 2007). The degradation of proteins at the 26S proteasome is dependent on ATP hydrolysis and occurs through series of proteolytic cleavages of the substrate into peptide fragments, which is catalysed by the protease active sites in β subunits. At the proteasome, deubiquitinating enzymes release ubiquitin molecules from the substrate to provide free ubiquitin for the cell.

2.3.3.2 Ubiquitin-conjugating enzyme BRUCE

BRUCE (BIR repeat containing ubiquitin-conjugating enzyme), also called BIRC6, belongs to baculoviral IAP repeat (BIR) domain-containing protein (BIRP) family. BIR domain is a hallmark of inhibitor of apoptosis proteins (IAPs) that form one branch of BIRP protein family. IAPs are best known to regulate apoptosis since they bind and inhibit the activity of caspase cysteine proteases. BRUCE, and other Survivin-like BIRP proteins, differs from these canonical IAPs by having extra amino acid residues at two concerved positions in the BIR domain (Verhagen et al., 2001). BRUCE, however, also exhibits an anti-apoptotic activity (Hao et al., 2004; Ren et al., 2005). In addition, the N-terminal BIR domain of BRUCE mediates substrate binding and together with the C-terminal ubiquitin conjugating domain it possesses chimeric ubiquitin ligase (E3) and ubiquitin-conjugating (E2) enzyme activities (Hauser et al., 1998; Bartke et al., 2004). Thus, BRUCE has a unique merged function of two enzyme activities in ubiquitin conjugation system.

BRUCE is a large 528 kDa membrane-associated protein that localizes to cytosolic membrane structures the *trans*-Golgi network and the vesicular system (Hauser et al., 1998). It is expressed in several adult tissues, being notably abundant in the brain and especially in neurons (Hauser et al., 1998; Sokka et al., 2005). It inhibits apoptosis through ubiquitination of different protein substrates, including

caspases or proapoptotic proteins, like Smac/DIABLO, to facilitate their proteasomal degradation (Bartke et al., 2004; Qiu et al., 2005; Hao et al., 2004). In neurons, it has a protective role against excitotoxic nerve cell injury (Sokka et al., 2005). Moreover, BRUCE is reported to act upstream of tumor suppressor p53 to inhibit mitochondrial apoptosis (Ren et al., 2005). BRUCE itself is regulated through ubiquitination and proteasomal degradation by ubiquitin ligase Nrdp1 or by cleavage by caspases or serine protease HtrA2 (Qiu et al., 2004; 2005). *In vivo* studies suggest a physiological role for BRUCE to function in cell division rather than in apoptosis. BRUCE mutant mice were reported to die perinatally or *in utero* due to defects in placental trophoblast proliferation and deformation of conventional spongiotrophoblast layer during the development (Lotz et al., 2004; Hitz et al., 2005). Some *in vivo* studies, however, showed apoptotic cell death due to *BRUCE* gene-trap insertion mutation (Hao et al., 2004; Ren et al., 2005). The role of BRUCE in cell division is related to regulation of cytokinesis, during which BRUCE associates with the midbody ring to guide proper vesicle targeting and abscission (Pohl et al., 2008).

2.4   Interplay between the central nervous system and immune system

The central nervous system (CNS) and the immune system affect each other through signaling via hormonal cascades and neural pathways. The CNS regulates the immune system through a neuroendocrine system as well as locally at the site of inflammation and in immune organs (**Figure 11**). The immune system and the neuroendocrine system are important for protection against the infection and disease as well as for regulation of normal metabolism. The CNS signals the immune system by way of the hormonal stress response with the production of glucocorticoid (GC) hormones. Furthermore, the autonomic sympathetic nervous system secretes neurotransmitters that activate immune cells through specific receptors. In addition, peripheral nerves release neuropeptides that affect the immune system. On the other hand, the immune system regulates the CNS through production of cytokines by brain immune cells, which can pass the blood-brain barrier in small amounts (Banks et al., 1995).
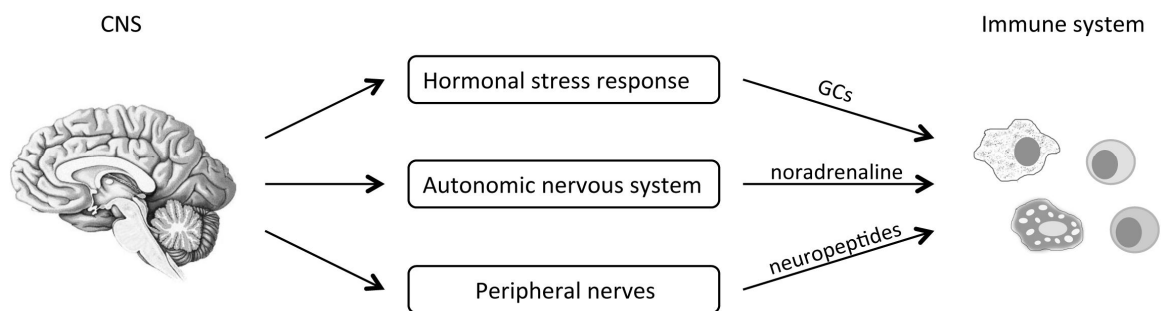

Figure 11. The signaling of the CNS to the immune system. The CNS induces hormonal stress response and glucocorticoid (GC) secretion in response to a stressful stimulus. The autonomic nervous system interacts with the immune system by producing noradrenaline that binds to appropriate receptors in immune cells. Peripheral nerves activate the immune system by secreting neuropeptides. *Modified from Bear et al., 2007, p. 207.*

2.4.1   Glucocorticoid hormones

A classical neuroendocrine response to a stressful stimulus is the production of GC hormones, including cortisol and corticosterone. The secretion of GCs is regulated by a hormonal cascade called hypothalamic-pituitary-adrenal (HPA) axis that begins in the brain and results in the release of these hormones in adrenal gland (**Figure 12**). GCs also regulate their own levels by a feedback mechanism that inhibits the HPA axis. GCs are known to affect several metabolic cascades, and most organs and physiologic systems are sensitive to their action during the stress response. The action of GCs includes the release of stored energy and inhibition of glucose uptake, enhancement of gluconeogenesis and cardiovascular activity, suppression of the immune system and inhibition of reproductive activity. Prolonged excess GC secretion is a hallmark of chronic stress with increased risk of cardiovascular and metabolic diseases, and neuropsychiatric disorders such as depression (Tamashiro et al., 2011; Lee et al., 2012; Datson et al., 2013).
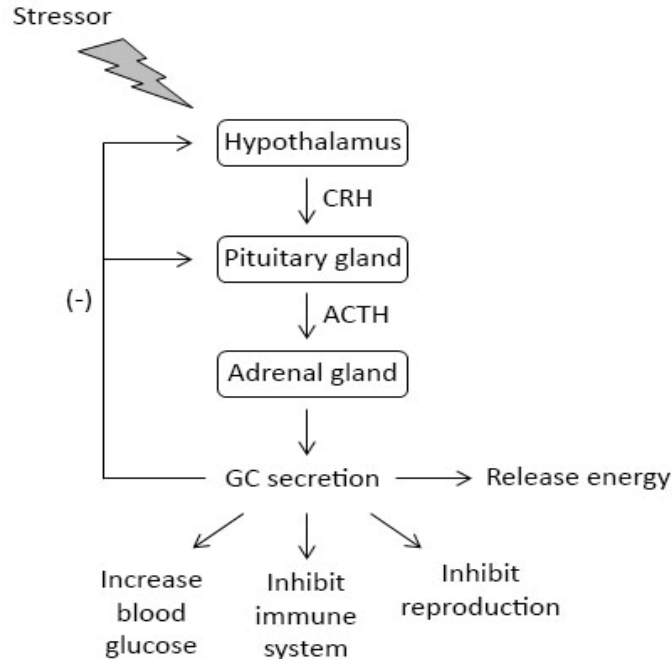
Figure 12. The hypothalamic-pituitary-adrenal (HPA) axis. A stress signal is detected in the brain cortex, which informs the hypothalamus. The hypothalamus secretes corticotropin-releasing hormone (CRH) that stimulates production of adrenocorticotropin hormone (ACTH) in the pituitary gland. ACTH reaches the adrenal gland via bloodstream and induces the expression and secretion of glucocorticoid (GC) hormones. GC secretion is regulated by GCs themselves through a negative feedback loop that inhibits the HPA pathway at the hypothalamus and pituitary level. GCs affect majority of organs and physiologic systems. *Modified from Romero, 2004.*

GCs mediate their effects via cytosolic glucocorticoid and mineralocorticoid receptors (GRs and MRs, respectively). Low levels of GCs bind primarily to MR, while only high levels of GCs bind to GR after MRs are saturated. GRs and MRs act as ligand-dependent transcription factors that regulate target genes either directly or via interaction with other transcription factors.

Endogenous GCs regulate the immune system and inflammation. The physiological levels of GCs inhibit the immune system and have anti-inflammatory actions, while higher pharmacological levels of GCs totally suppress the immune activity. Moreover, most synthetic GCs show higher affinity for GR than natural GCs and thus, have more radical effect on the immune response. Based on these immunosuppressive effects, GCs such as dexamethasone are used to treat autoimmune and inflammatory diseases.

GCs control various aspects of the immune system. They decrease the expression of proinflammatory cytokines like IL-6, TNFα and IL-1β, and upregulate the expression of anti-inflammatory cytokines. GCs also affect mobility of immune cells by inhibiting cell adhesion molecules involved in leukocyte trafficking (Cronstein et al., 1992). Through inflammatory mediators, GCs modulate immune cell maturation and differentiation favoring the development of T helper cell type 2 (Th2)-type adaptive immunity (Franchimont et al., 1998; Agarwal and Marshall, 2001). Furthermore, GCs control lymphocyte selection by triggering cell cycle arrest and apoptosis in lymphoid cells (Forsthoefel and Thompson, 1987; Ma et al., 1992; Reisman and Thompson, 1995; Rhee et al., 1995a; 1995b). Moreover, GCs induce apoptosis in leukemic cells and play an important role in thymocyte selection and

differentiation (Wyllie, 1980; Iwata et al., 1991; Thulasi et al., 1993; Ramdas and Harmon, 1998; Vacchio et al., 1998; Schmidt et al., 2001).

In the adult brain, GCs secreted during the stress response suppress glucose transport in neurons and glial cells and reduce synaptic plasticity in hippocampus (Horner et al., 1990; Virgin et al., 1991; Pavlides et al., 1993). Thus, GC secretion may disrupt memory formation and contribute to neurotoxicity. In addition, GCs have negative impacts on adult hippocampal neurogenesis (Cameron and Gould, 1994). On the other hand, GCs have been reported to activate Trk neurotrophin receptors, and to facilitate neuroprotection in neurons deprived of trophic support (Jeanneteau et al., 2008).

2.4.1.1   Dexamethasone

Dexamethasone is a commonly used synthetic glucocorticoid hormone. It exhibits anti-inflammatory role by downregulating IL-1β and inhibiting binding of transcription factor complexes on TNF-α promoter and thus, distorting TNF-α synthesis (Lee et al., 1988; Steer et al., 2000). Dexamethasone has been suggested to induce the shift from the Th1 type innate immunity to the Th2 phenotype by differentially regulating IL-10 and IL-12 expression after endotoxin stimulation (Visser et al., 1998) and because of its more efficient downregulation of Th1-type cytokines than Th2-type cytokines (Franchimont et al., 1998). Dexamethasone affects immune cell trafficking by suppressing lipopolysaccharide (LPS)-induced adhesiveness of leukocytes to endothelial cells in a GR-dependent manner (Cronstein et al., 1992). In addition, it increases leukocyte mobility through downregulation of an adhesion molecule L-selectin (Nakagawa et al., 1999). Dexamethasone also reduces the expression of chemoattractants required for immune cell accumulation at sites of inflammation, including IL-5, RANTES, eotaxin and CINC/gro, (Ohtsuka et al., 1996; Wingett et al., 1996; Lilly et al., 1997; Sewell et al., 1998).

Dexamethasone has a negative impact on cell division as it controls the expression of many critical cell cycle regulators. Dexamethasone suppresses c-myc transcription and the expression of cyclin D3 and cdks in lymphoid cells, and induces growth arrest at G1 phase (Forsthoefel and Thompson, 1987; Ma et al., 1992; Reisman and Thompson, 1995; Rhee et al., 1995a; 1995b). Dexamethasone also affects neural progenitor cell division by inhibiting their cell cycle through ubiquitin-mediated degradation of cyclin D1 (Sundberg et al., 2006).

2.4.2   Neuropeptide PACAP

Neuropeptides are signaling molecules that are secreted by neuronal cells and, which facilitate cell communication. Neuropeptide PACAP has been discovered as a regulatory peptide in hypothalamus, and it is shown to stimulate adenylyl cyclase activity and cyclic adenosine monophosphate (cAMP) formation in the pituitary (Miyata et al., 1989). PACAP is a neuropeptide that shares three receptors with the vasoactive intestinal polypeptide, named PAC1, VPAC1 and VPAC2 (Harmar et al., 1998). PACAP is expressed in the brain as well as in several peripheral organs. In the brain, it is most abundantly expressed in the hypothalamus but it is also found in various other brain regions (Köves et al., 1991; Ghatei et al., 1993; Hannibal, 2002). Among peripheral organs, PACAP is highly present in most endocrine organs, including pituitary gland and adrenal gland, as well as testis, lymphoid organs and gastrointestinal tract (Arimura et al., 1991; Gaytan et al., 1994; Abad et al., 2002; Mazzocchi et al., 2002). PACAP and its PAC1 receptor are also present in the neurogenic region of the developing brain suggesting a role for PACAP in neurogenesis (Waschek et al., 1998; Jaworski and Proctor, 2000).

PACAP is traditionally known for its ability to act as a neuroprotective and neurotrophic peptide. It protects cortical and retinal neurons against glutamate-induced neurotoxicity (Morio et al., 1996; Shoge et al., 1999) and promotes neuronal survival in different embryonic and postnatal neuronal populations indicating an important role in neuronal development (Lindholm et al., 1998; Lioudyno et al., 1998; Takei et al., 1998; 2000). In cerebellar granule neurons it inhibits apoptosis by regulating caspase-3 activity (Vaudry et al., 2003). Moreover, PACAP protects neurons from ischemic death (Dohi et al., 2002; Reglodi et al., 2002; Tamas et al., 2002; Chen et al., 2006; Stumm et al., 2007). PACAP exhibits neurotrophic effects in dorsal root ganglion neurons and in mesencephalic dopaminergic neurons, where it promotes neurite outgrowth or enhances dopamine uptake and tyrosine hydroxylase expression, respectively (Lioudyno et al., 1998; Takei et al., 1998). Furthermore, it is able to transactivate Trk tyrosine kinase receptors on the intracellular membranes such as Golgi, and increase neuronal cell survival in the absence of neurotrophin binding (Lee et al., 2002; Rajagopal et al., 2004). In addition, it has been shown to act as a non-cholinergic neurotransmitter that is secreted from cholinergic nerve terminals and required for sustained release of catecholamines (Wakade, 1988; Hamelink et at., 2002).

PACAP serves as an anti-inflammatory peptide and displays cytoprotective effects also in cells and tissues outside the brain, including kidneys, intestine, pancreas, heart, lungs and skin. The anti-inflammatory role of PACAP has been shown in several studies with PACAP-deficient mice (Armstrong et al., 2008; Azuma et al., 2008; Nemetz et al., 2008; Kemeny et al., 2010; Elekes et al., 2011). PACAP also protects against autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), which is an animal model of multiple sclerosis (MS) (Kato et al., 2004).

2.4.3   Neuroinflammation

Neuroinflammation is not considered as a classical inflammation with signs of redness and swelling but involves activation of microglia in the nervous system as well as involvement of perivascular macrophages and astrocytes (Graeber et al., 2011). Inflammation involves invasion of circulating immune cells to the tissue, and production of proinflammatory cytokines and chemokines. However, the blood-brain barrier in the brain restricts the entry to the CNS by peripheral immune cells and large molecules, including immunoglobulins, cytokines and complement proteins. In addition to activated microglia, neuroinflammation is characterized by production of neurotransmitters and reactive oxygen species (ROS). It occurs due to tissue trauma or injury or exposure to pathogens. Neuroinflammation is present both in the healthy brain, where it is required for tissue repair as a strictly regulated inflammatory process, and under pathological conditions. It may result in the development or progression of different neuronal disorders, including Alzheimer´s disease, Huntington´s disease, Parkinson´s disease, MS, amyotrophic lateral sclerosis (ALS), epilepsy and major depression (Raison et al., 2006; Qian et al., 2010).

2.4.3.1   Microglia

Microglia belong to glial cells but is distinquished from macroglia, including astrocytes and oligodendrocytes, by their origin, morphology and function. Microglial cells are of myeloid origin and arise from yolk sac macrophages that scatter into the brain during early embryogenesis and differentiate into microglial cells (Saijo and Glass, 2011). In a resting state, as they appear in the healthy brain, microglia are characterized by a ramified appearance with motile processes that continuously monitor the surroundings in the CNS (Nimmerjahn et al., 2005).

Microglia participate in maintenance of brain homeostasis. They support synaptogenesis and neuronal survival as well as the growth of regenerating axons in injured brain by secreting neurotrophic factors, including nerve growth factor, brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF) and neurotrophin-3 (Elkabes et al., 1996; Honda et al., 1999; Batchelor et al., 2002; Bessis et al., 2007). They also play an essential role in synaptic pruning in maturing brain (Paolicelli et al., 2011). Moreover, during the brain development, microglia support neurogenesis by inducing apoptosis and phagocytosing apoptotic cells to balance the neuronal cell number (Ashwell, 1990; Egensperger et al., 1996; Sierra et al., 2010).

Neuroinflammation induces activation of microglia in response to injury, ischemia or inflammatory stimuli, leading to their conformational change into an amoeboid–like phenotype. Reactive microglia migrate along a chemotactic gradient and secrete chemokines to recruit more microglial cells to the site of inflammation (Nolte et al., 1996; Garden and Möller, 2006). Microglia recognize various pathogen-associated molecular patterns found on bacteria and viruses through binding by pattern-recognition receptors (PRRs), including Toll-like receptor 4, which recognizes LPS on the outer membrane of Gram-negative bacteria (Bauman et al., 2009). Activation of PRR-mediated signaling in microglial cells induces the production of inflammatory mediators, like cytokines, chemokines, ROS and nitric oxide (NO) that play a role in innate immunity. On the other hand, microglia also upregulate major histocombatibility complex class II molecules for antigen presentation to T cells (O'Keefe et al., 2002). Thus, microglia facilitate both innate and adaptive immunological responses of the nervous system. Moreover, microglia participate in tissue repair by phagocytosing cellular debris after recognition of endogenous damage-associated molecular patterns released from damaged or dying cells (Chen and Nunez, 2010).

Microglia have been shown to propagate inflammatory response by activating brain astrocytes, which leads to the accumulation of neurotoxic factors (Saijo et al., 2009). Proinflammatory cytokines and neurotoxins NO and ROS secreted by reactive microglia may cause neuronal damage by enhancing oxidative stress and by inducing neuronal cell death. Activation of microglia is described in several neurodegenerative diseases, where they contribute to disease pathogenesis. Death of dopaminergic neurons in Parkinson's disease activates microglia but, on the contrary, reactive microglia also induces dopaminergic neuronal death (Dutta et al., 2008; Cao et al., 2011). Widespread microglial activation is also detected in the brain of patients with ALS (Turner et al., 2004). In Alzheimer's disease, aggregated amyloid-β proteins characteristic in disease pathology, activate microglial receptors (Landreth and Reed-Geaghan, 2009; Yan et al., 2009). Furthermore, microglia is involved in Huntington's disease pathophysiology (Sapp et al., 2001) as well as in the emergence of MS (Priller et al., 2001).

2.4.3.2 Interferon-gamma

Interferon-gamma (IFNγ) belongs to interferon family of cytokines that were originally discovered by their interfering effects on viral replication (Isaacs and Lindenmann, 1987). IFNγ alone constitutes a type II IFN class. It is secreted by an array of immune cells, including lymphocytes, natural killer (NK) cells, B cells and antigen-presenting cells. IFNγ signals through IFNγ receptor (IFNγR) composed of two IFNγR1 chains associated with two IFNγR2 chains (**Figure 13**). IFNγR lacks intrinsic kinase/phosphatase activity but it associates with JAK/STAT signaling machinery for signal transduction. Binding of IFNγ to the receptor induces sequential phosphorylation in JAK2 and JAK1, the latter of which then phosphorylates each IFNγR1 chain (Igarashi et al., 1994; Briscoe et al., 1996). After IFNγR phosphorylation, STAT1 is able to bind to the receptor and is phosphorylated by JAK2

(Briscoe et al., 1996). STAT1 then dissociates from the receptor and enters the nucleus to regulate transcription of IFNγ-regulated genes. IFNγ signaling is regulated by the endosomal pathway that internalizes the IFNγ-IFNγR complex after signal transduction and degrades the ligand (Anderson et al., 1983; Celada and Schreiber, 1987). IFNγ secretion is upregulated by cytokines IL-12 and IL-18 (Munder et al., 1998; Pien et al., 2000; Schindler et al., 2001) and inhibited by IL-4 and IL-10 (Fukao et al., 2000; 2001; Schindler et al., 2001).
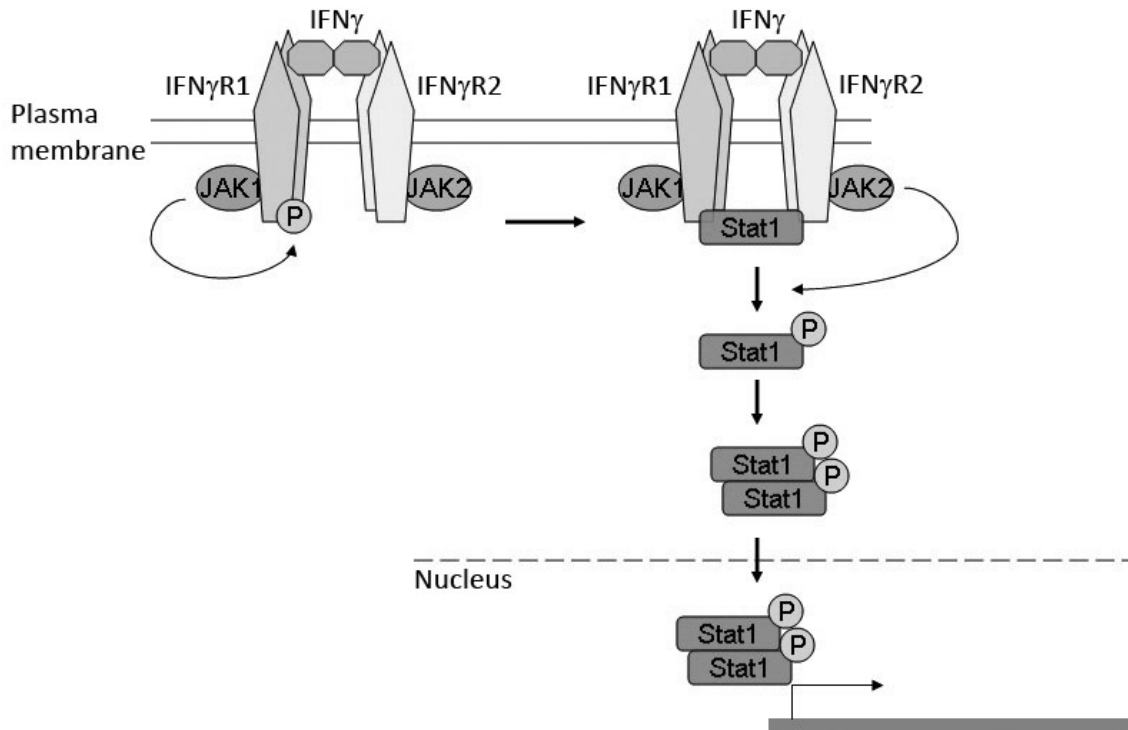

Figure 13. IFNγ-induced JAK/STAT signal transduction. Ligand binding to IFNγR complex induces autophosphorylation of JAK1 and JAK2 with subsequent phosphorylation of IFNγR1 chain by JAK1. STAT1 is then recruited to the receptor and phosphorylated by JAK2. Phosphorylated and dimerized STAT1 translocates to the nucleus and regulates IFNγ-responsive genes. IFN, interferon; IFNR, IFN receptor; JAK, Janus tyrosine kinase; P, phosphate; STAT, signal transducers and activators of transcription.

Alongside the more classical antiviral type I interferons containing IFNα and IFNβ, also IFNγ mediates antiviral defense mechanisms (Müller et al., 1994). IFNγ regulates a plethora of immune functions by enhancing antigen presentation and immune cell trafficking as well as growth, maturation and differentiation of immune cells. Especially, IFNγ primes macrophage responses and activates their microbicidal effector functions by regulating the class I and class II antigen presentation pathways (Fellous et al., 1982; Shirayoshi et al., 1988; Figueiredo et al., 1989; Johnson et al., 1990). It is known to activate various cell types involved in immune function, including macrophages, NK cells and astrocytes (Nathan et al., 1983; Fierz et al., 1985; Carnaud et al., 1999). IFNγ promotes Th1-type immunity but also the transition from innate immunity to adaptive immunity (Gajewski and Fitch, 1988; Collins and Dunnick, 1993; Yoshida et al., 1994). IFNγ also regulates cell growth by inhibiting cell cycle progression (Mandal et al., 1998; Ramana et al., 2000). IFNγ may also induce proliferation or protect from apoptosis (Novelli et al., 1996) by upregulating a number of proapoptotic proteins, such

as interferon regulatory factor 1, Fas and Fas ligand (Sims et al., 1993; Xu et al., 1998). Moreover, IFNγ controls leukocyte attraction via upregulation of several chemokines and adhesion molecules.

IFNγ is involved in the pathology of autoimmune diseases, such as MS (Panitch et al., 1987). However, it is also shown to diminish morbidity in mice with EAE (Billiau et al., 1988).

IFNγ affects the brain at the level of neuroendocrine system as well as neuronal survival and neurogenesis. It reduces secretion of adrenocorticotropin hormone, growth hormone and prolactine from the pituitary in cell culture system (Vankelecom et al., 1990). IFNγ is best known to be toxic for neurons as shown in neuron-glia co-cultures (Jeohn et al., 2000; Sohn et al., 2007) but it can also stimulate neurogenesis, neuronal differentiation and neurite outgrowth (Wong et al., 2004; Song et al., 2005; Baron et al., 2008). Moreover, it affects neural progenitor cells by enhancing neurosphere formation in the embryonic brain, while showing inhibitory effects in the adult brain (Li et al., 2010). Activation of NPC proliferation by IFNγ is mediated through induction of Shh, an essential factor in NPC development (Li et al., 2010; Sun et al., 2010). As being involved in the immune function, in neuroendocrine system and in CNS, IFNγ is a potent mediator of communication between these systems.

3   AIMS OF THE STUDY

Neural stem/progenitor cells provide a platform for the development of the central nervous system and the cells are dependent on diverse signals that reach them from the surrounding. Cell surface-associated proteins may facilitate cell-to-cell interactions or transform extrinsic signals into the cells. Interactions between brain cells and immune cells mediate the interplay of the CNS and the immune system, and are orchestrated by hormones and inflammatory mediators that influence NPC development. The aims of this study were to address the effects of membrane-associated proteins and brain inflammation on NPCs.

The specific aims of this study were:

I   To characterize the role of BRUCE in the glucocorticoid hormone dexamethasone-mediated effects on NPC proliferation

II   To investigate the role of neuroinflammation and the cytokine IFNγ in NPC survival

III   To study the involvement of cell surface protease inhibitors HAI-1 and HAI-2 in the regulation of NPC behavior and cell fate in the developing brain

4   MATERIALS AND METHODS

The detailed descriptions of the materials and methods are found in the original publications.

4.1   Animals

Wistar rats obtained from Harlan (Horst, The Netherlands) and used in studies I-III were mated at 10-12 weeks of age. NMRI mice used in study III were obtained from own breeding. All animal experiments were approved by the local ethical committee and accomplished in accordance with the European Communities Council Directive (86/609/EEC).

4.2   Cell culturing

TABLE II. Cell types used in the study	
Cell type	Publication
E17 hippocampal neurons	III
microglia	II
neural progenitor cells	I-III
p53 deficient mouse embryonic fibroblasts	II
radial glial cells	III

NPCs and hippocampal (HC) neurons were isolated from embryonic day 17-18 (E17-E18) rat brain striatum and hippocampus, respectively. Pregnant rats were anaesthetized with carbon dioxide and killed by decapitation or cervical dislocation. The embryos were collected to sterile phosphate buffered saline (PBS) and placed on ice. The striatal tissue and hippocampi were dissected from the embryonic brains. The striatal tissue was dissociated in a Hank´s buffered saline solution (HBSS) containing 15 mM Hepes, 2 mM glucose, 1.3 mg/ml trypsin, 0.2 mg/ml kynurenic acid, 0.7 mg/ml hyaluronidase and 20 μg/ml DNase. Dissociation enzymes were inactivated with Earle´s balanced salt solution (EBSS) containing 20 mM Hepes and 4% BSA, and the cells were passed through a 70 μm nylon cell strainer, followed by centrifugation at 2000 rpm for 5 min. The cells were resuspended and placed on top of a 30.8% sucrose gradient in HBSS and centrifugated for 7 min at 2000 rpm. After an additional centrifugation at 2000 rpm for 5 min through 20 mM Hepes and 4% BSA in EBSS, cells were resuspended in serum-free cell culture medium containing DMEM/F12 supplemented with L-glutamine, penicillin-streptomycin, B27 and epidermal growth factor (20 ng/ml). Isolated NPCs were plated on nonadherent culture dishes at a density of $5x10^6$ cells per 10 cm dish. Cells formed neurospheres within 2-3 days and were grown for two passages before use in experiments. Neurospheres were dissociated and the medium was changed in every four or five days.

The dissected hippocampi were incubated with 1 mg/ml bovine serum albumin (BSA), 0.5 mg/ml papain and 0.2 mg/ml cystine in PBS for 15 min at +37°C. The cells were gently dissociated in the presence of 20 μg/ml DNase, collected by centrifugation and plated on poly-DL-ornithine-coated 6-well plates at a density of $2x10^6$ cells per well in Neurobasal medium supplemented with B27, L-glutamine and 25 μM glutamate. Medium was replaced after 4-6 hours and the cells were cultivated in Neurobasal medium with L-glutamine and B27 supplement for 7 days.

Microglial cells were prepared from postnatal day (P) 1-2 rat brain cortex. The tissue was minced and passed through a stainless steel mesh, dissociated and washed by centrifugation. Cells were resuspended

in DMEM/F12 medium containing 10% fetal bovine serum (FBS) and penicillin-streptomycin, and cultured for up to three weeks on 75cc flasks with medium addition twice a week.

p53 deficient mouse embryonic fibroblasts were cultured in DMEM medium with 10% FBS, L-glutamine and penicillin-streptomycin, and passaged when 70-80% confluent.

Radial glial cells were differentiated from mouse embryonic stem cells (ESCs). ESCs cultured on mouse embryonic fibroblast feeder cells in knockout-DMEM medium with 15% fetal calf serum (FCS), 1000U/ml LIF, penicillin-streptomycin, non-essential amino acids and β-mercaptoethanol, were deprived of feeders and used for embryoid body (EB) formation. EBs were cultivated in the presence of only 10% FCS and without LIF for four days following addition of 5 µM retinoic acid for additional four days to induce neuronal differentiation. EBs were dissociated and plated onto poly-DL-lysine and laminin-coated dishes in N2B27 medium, a 1:1 mixture of DMEM/F12 and Neurobasal media supplemented with N2 and B27 to induce radial glial phenotype.

4.3   NPC proliferation and differentiation assay

NPC neurospheres were dissociated into single cells and plated onto poly-DL-ornithine-coated 24-well culture dishes and fixed with 4% paraformaldehyde (PFA) after two hours or 5 days in order to study proliferation or differentiation, respectively. Washed cells were subjected for immunocytochemistry using proliferation markers Ki67 or BrdU (after BrdU pulse for 24 hours in cells) and differentiation markers βIII-tubulin, GFAP and CNPase. The number of immunopositive cells was determined using microscopy in four independent fields per coverslip.

4.4   NPC self-renewal and cell cycle analysis

Self-renewal was assayed by plating equal amounts of NPCs and incubating cells for three days followed by estimation of the number of newly formed neurospheres.

For cell cycle analysis, NPCs were washed with ice-cold PBS, fixed with PFA and permeabilized with MeOH. The cells were then pretreated with RNAse A, and stained with propidium iodide. Cells were analyzed with flow cytometry using a FACS calibur flow cytometer.

4.5   NPC viability and cell death assays

NPC survival was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or by counting the number of condensed/apoptotic nuclei stained with Hoechst 33342. Cell death was quantified by staining of fragmented DNA with propidium iodide or terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL).

4.6   Amaxa Nucleofector system

Overexpression and downregulation assays for NPCs were performed using the Amaxa nucleofector transfection system to introduce plasmids, oligonucleotides or siRNA (**Table III**) into cells. Dissociated NPCs were transfected by electroporation according to manufacturer´s protocol and the cell culture medium was replaced 24 hours after transfection. In some overexpression studies, the transfected cells were selected with 600 µg/ml of G418.

TABLE III. Plasmids, oligonucleotides and siRNAs used in the present study.		
DNA/RNA	**Source**	**Publication**
p3xFlag-CMV10-BRUCE	Hao et al., 2004	I
BRUCE AS oligonucleotides	Interactiva	I
BRUCE siRNA	Ambion	I
Cyclin D1 siRNA	Ambion	I
HAI-1 siRNA	Ambion	III
HAI-2 siRNA	Ambion	III
p21 siRNA	Dharmagon	II
p53 siRNA	Dharmagon	II
pCAG-hHAI-1	Koivuniemi et al., 2013	III
pCIneo-hHAI-1	Kataoka et al., 2000c	III
pCIneo-hHAI-1dTM	Miyata et al., 2005	III
pCIneo-hHAI-2	Kataoka et al., 2000c	III
pCIneo-hHAI-2dTM	Kataoka, H.	III
pGEMT-Usp8/Ubpy	Row et al., 2007	I

4.7 ELISA

Microglia conditioned medium was collected after 24 hour-stimulation of microglial cells with LPS and used for enzyme-linked immunosorbent assay (ELISA) with the rat Quantikine kit to measure the amount of IFNγ.

4.8 Immunochemistry

4% PFA-fixed NPCs on 24-well cell culture plates were blocked with 3% BSA in PBS or PBS/0.1%Triton-X-100 (PBS-T) and subjected for primary antibody incubation for overnight at +4°C in blocking solution. On the following day, cells were washed and incubated with fluorescent Alexa or Cy-conjugated secondary antibodies for 1 hour at RT, after which cells were counterstained with 4 µg/ml Hoechst 33342 and mounted.

Paraffin sections from E17 and P1 rat brains were dewaxed in xylene and rehydrated in decreasing ethanol series and water. Sections were subjected for antigen retrieval by boiling the sections in 10 mM citrate, pH 6.0. Sections were blocked with 5% BSA/PBS(-T) and incubated with primary antibodies in 3% BSA/PBS(-T) for overnight at +4°C. Fluorescent Alexa or Cy-conjugated secondary antibodies were diluted in 3% BSA/PBS(-T) and added for sections for 1 hour before counterstaining with 4 µg/ml Hoechst 33342 and mounting.

Frozen sections from E15 and P1 mouse brains were melted and fixed with ice-cold acetone-methanol (1:1). Sections were subjected to antigen retrieval as above and washed with PBS-T before blocking with 5% BSA/PBS-T. Primary antibodies diluted in 3% BSA/PBS-T were added for sections for overnight at +4°C. On the following day, fluorescent secondary Alexa antibodies were added for sections for 1 hour before counterstaining with 4 µg/ml Hoechst 33342. Sections were rinsed with 70% ethanol and mounted.

Primary antibodies used are shown in **Table IV**.

TABLE IV. Primary antibodies used in the study.

Antibody	Host	Source	Publication	Method
β-Actin	rabbit	Sigma	I-III	WB
Bad	rabbit	Cell Signaling	II	WB
Bax	rabbit	Santa Cruz	II	WB
BclX$_L$	rabbit	BD Biosciences	II	WB
BrdU	rat	Sigma	I-III	ICC, IHC
BRUCE	mouse	BD Biosciences	I	ICC, IHC, WB
Caspase 3	rabbit	Cell Signaling	II	WB
Cleaved caspase 3	rabbit	Cell Signaling	II	WB
c-Met	mouse	Cell Signaling	III	WB
CNPase	mouse	Sigma	III	ICC
β-COP	rabbit	Abcam	I	ICC
Cyclin D1	mouse	Santa Cruz	I-III	WB
Cytochrome c	mouse	BD Biosciences	II	ICC
Erk1/2	rabbit	Cell Signaling	III	WB
GFAP	rabbit	Sigma	I, III	ICC, IHC
HAI-1	rabbit	Santa Cruz	III	IHC, WB
HGF	rabbit	Santa Cruz	III	WB
HGFA	goat	Santa Cruz	III	WB
hHAI-2 mAb 2N9	mouse	Kataoka et al., 2000c	III	WB
mHAI-1	goat	R&D Systems	III	ICC
mHAI-1 pAb	rabbit	Tanaka et al., 2005	III	WB
mHAI-2	goat	R&D Systems	III	ICC
mHAI-2 pAb	rabbit	Kataoka et al., 2002	III	WB
IFNγ	rat	R&D Systems	II	cell inhibition
IFNγR2	mouse	Abcam	I	ICC, IHC, WB
Ki67	mouse	BD Biosciences	I-III	ICC, IHC
MG2 mAb	mouse	Hallikas et al., 2006	III	cell inhibition
Nestin	mouse	Millipore	II, III	ICC, IHC
Nestin	goat	R&D Systems	II	ICC, IHC
Nrdp1	rabbit	Bethyl Laboratories	I	IP, WB
p21	mouse	Millipore	II	WB
p53	mouse	Cell Signaling	II	IP, WB
PARP	rabbit	Cell Signaling	II	WB
P-Bad	rabbit	Cell Signaling	II	WB
P-Erk1/2	rabbit	Cell Signaling	III	WB
P-Smad1/5/8	rabbit	Cell Signaling	III	ICC
P-Stat1	rabbit	Cell Signaling	II	ICC, WB
PUMA	rabbit	Cell Signaling	II	WB
P-Vimentin	mouse	Assay Designs	II	WB
α-Spectrin	mouse	Chemicon	I	WB
Stat1	rabbit	Cell Signaling	II	WB
St14	rabbit	Abcam	unpublished data	ICC, WB
Tau	rabbit	Sigma	I	ICC
βIII-tubulin	mouse	Covance	III	ICC
Usp8	rabbit	Abcam	I	WB

Abbreviations: ICC, immunocytochemistry; IHC, immunohistochemistry; IP, immunoprecipitation; WB, western blotting.

4.9 *In situ* hybridization

Whole mount *in situ* hybridization was performed for E17 rat brain sections embedded in agarose that were cut with Leica VT 1000S Vibratome. Sections were preincubated with Proteinase K, hybridized using digoxigenin-labelled cRNA probes and the InSituPro automate and mounted.

4.10 *In utero* electroporation

Exposed E14 or E17 mouse embryos were injected with expression plasmids through the uterine wall into the lateral ventricle of the brain and electroporated using a square-wave pulse generator in a dorsal to lateral direction. After 5 pulses, the uteri were placed back in the abdominal cavity that was sutured. Brains were collected at E15 or P1, respectively, fixed with 4% PFA overnight and immersed with 30% sucrose in PBS.

4.11 Western blotting and immunoprecipitation

NPCs and hippocampal neurons were lysed in ice-cold RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl and 1 mM EDTA) with or without 1% SDS and supplemented with protease inhibitor cocktail. For phosphatase inhibition in some experiments, phosStop solution was added to the lysis buffer. Equal amount of proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and blocked with 5% skimmed milk or BSA, followed by incubation with primary antibodies (**Table IV**) overnight at +4°C. Horseradish peroxidase-conjugated secondary antibodies were added on the membrane for 1 hour, followed by washes and detection using the enhanced chemiluminescence.

Immunoprecipitation of NPCs was performed using the protein A-sepharose or protein G agarose. Lysates in IP lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate) were prepurified with sepharose/agarose and incubated with a primary antibody for overnight. A total of 40 µl of sepharose/agarose was added to lysates for two hours followed by washes with IP washing buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.1% NP-40, and 0.05% sodium deoxycholate). Sepharose/agarose beads were boiled and proteins were subjected for immunoblotting.

4.12 PCR

Total RNA from cultured cells was extracted using the GenElute Mammalian total RNA kit. cDNA was prepared using the reverse transcriptase kit or the SuperScript VILO cDNA synthesis kit for reverse transcriptase (RT)-PCR and quantitative PCR, respectively. Strand-specific cDNA was prepared using the RevertAid Premium reverse transcriptase. In RT-PCR, β-actin was used as a control.

Quantitative PCR was performed with the Power SYBR Green PCR Master mix and using the ABI PRISM 7000 sequence detection system, or with the LightCycler® 480 SYBR Green I Master Mix and using the LightCycler® 480 II system. Samples were run in triplicates and the comparative CT method was used for data analysis. GAPDH was used as an internal control gene.

Primers used for PCR are shown in **Table V**.

TABLE V. Primers used for PCR analysis.

Gene	Forward primer	Reverse primer
Bmp2	5′-TCCATCACGAAGAAGCC-3′	5′-ACTGACTTGTGTTCTGAG-3′
Bmp4	5′-AGCCAACACTGTGAGGAG-3′	5′-TGTCTGGTGGAGGTGAGT-3′
BMPRIA	5′-AGCCTGTCTGTTCATCATT-3′	5′-CAGAGCCTTCATACTTCAT-3′
BMPRIB	5′-TACCTCATCACAGACTATC-3′	5′-CTAATGAACTTGACAGCCA-3′
Bruce	5′-CTCTTACTCCGAGCGAT-3′	5′-CTTAACAGGGGGAGGC-3′
c-Met	5′-ATAGAGTGGAAGCAAGC-3′	5′-GCACTTACAAGCCTATC-3′
HAI-1	5′-GAGCAGAACTTCGTGTG-3′	5′-GTAGTTGTTCTTGTTGCC-3′
HAI-1 qPCR	5′-GCCAGCATCTCTACGGTCT-3′	5′-ACGGCAGCTCGGTCTCA-3′
HAI-2	5′-ATGAAGAATACTGTGTCC-3′	5′-GCTGCTCCTTGTCATC-3′
HAI-2 qPCR	5′-GGCTGTGAGGGAAATGGTAA-3′	5′-ACCATCAATGGTGTTCTCAGTG-3′
Noggin	5′-AGCACTATCTACACATCCG-3′	5′-CACAGACTTGGATGGCTTA-3′
p21	5′-AGGCAGACCAGCCTAACAGA-3′	5′-CAGCACTAAGGAGCCTACCG-3′
SOCS1	5′-AGCAGCTCGAAAAGGCAGTC-3′	5′-ACACTCACTTCCGCACCTTC-3′
SOCS3	5′-ACCAGCGCCACTTCTTCACG-3′	5′-GTGGAGCATCATACTGATCC-3′

4.13 Image analysis and statistics

Fluorescence images were captured with the Leica DM 4500B microscope, the Zeiss Axio Observer Z1 inverted microscope or with the Zeiss LSM 510 Meta confocal microscope. Brightfield images were taken with the Leica DM 4500B microscope.

Western blots and RT-PCR results were quantified with ImageJ software.

Cell counting after immunochemistry was performed manually using microscopy in at least four non-overlapping fields per coverslip.

All experiments were repeated at least three times. Statistical comparisons were performed using Student's t-test or one-way ANOVA and a Bonferroni *post hoc* test. P value $p < 0.05$ was considered as statistically significant.

5   RESULTS AND DISCUSSION

5.1   Stress and neuroinflammation decrease NPC survival and maintenance

5.1.1   Dexamethasone reduces NPC proliferation by downregulating BRUCE

Dexamethasone is a synthetic glucocorticoid hormone used to treat immune disorders due to its immunosuppressive effects. Glucocorticoid production is increased after a stressful stimulus that leads to the use of energy stores and activation of vital functions with simultaneous inhibition of non-necessary functions, like cell growth. Dexamethasone is known to inhibit cell division, which is also observed in NPCs (Sundberg et al., 2006). It reduced the amount of proliferating Ki67-positive cells in culture (I/Fig. 3). In addition, dexamethasone was shown to decrease the expression of BRUCE both at the mRNA and protein level (I/Fig. 3). BRUCE is an anti-apoptotic protein that belongs to a BIRP family of IAP proteins, and which bears an additional ubiquitin-conjugating enzyme activity (Hauser et al., 1998). In the present study, BRUCE was shown to be expressed in the developing neurogenic region of the brain (I/Fig. 1). In cultured NPCs it colocalized with the Golgi marker, β-COP in intracellular membrane structures as well as to differentiated cells exhibiting Tau-positive neuronal character (I/Fig. 1). BIRP proteins, like Survivin, have been shown to affect cell division (reviewed in Johnson and Howerth, 2004) so we addressed the role of BRUCE in both proliferation and cell survival of NPCs. BRUCE downregulation with specific antisense oligonucleotides decreased NPC viability measured by MTT assay but did not induce cell death as a cell death inducer staurosporine did not enhance BRUCE downregulation-mediated decrease in NPC viability (I/Fig. 2). Moreover, BRUCE oligonucleotide transfection did not affect the number of fragmented nuclei in NPC population after staining with propidium iodide (I/Fig. 2). Inhibition of BRUCE with siRNA, on the other hand, affected NPC proliferation and decreased the amount of Ki67-positive cells in culture (I/Fig. 2).

In NPCs, BRUCE may be involved in the regulation of cytokinesis, as shown previously by Pohl et al. (2008) in other cell cultures. Dexamethasone-induced decrease in NPC proliferation was attenuated by overexpression of BRUCE (I/Fig .3), further confirming the role of BRUCE in NPC division. Infact, *in vivo* studies have indicated an important role for BRUCE in cell proliferation during early embryogenesis (Lotz et al., 2004; Hitz et al., 2005). BRUCE acts in the final steps of protein ubiquitination, which proposes that it may regulate inhibitory cell cycle proteins by ubiquitination to promote cell division. In line with this, BRUCE is able to inhibit tumor suppressor p53^{Kip2}, a protein that also controls cell proliferation, in a process of apoptosis (Ren et al., 2005).

Dexamethasone affected BRUCE both at the mRNA and protein level through activation of glucocorticoid receptors, shown by simultaneous treatment of cells with GR antagonist, mifepristone, that inhibited dexamethasone-mediated effects on BRUCE expression (I/Fig. 3). BRUCE mRNA expression was, however, recovered after 12 hours of dexamethasone treatment (I/Fig. 3), suggesting that dexamethasone induces a rapid downregulation by regulating mRNA production or stability directly. On the other hand, BRUCE protein level was reduced more slowly and showed no recovery in 24 hours (I/Fig. 3). BRUCE is known to be regulated by the proteasomal degradation pathway (Qiu et al., 2004) suggesting that dexamethasone may affect BRUCE protein level by directing it to the proteasome.

5.1.2   Activated microglia produce IFNγ that decreases NPC viability

Activation of microglia is a hallmark of neuroinflammation and can be experimentally induced by LPS, a component of bacterial cell wall that is recognized by microglial cells (Bauman et al., 2009). Microglia may induce cell death in brain cells by producing inflammatory factors and neurotoxins. To study, whether neuroinflammation affects NPCs, LPS was used for microglial activation in a cell culture system. LPS stimulation for 24 hours (500 ng/ml) increased the production of an inflammatory mediator IFNγ in microglial culture almost 10-fold (II/Fig. 7). This further resulted in decreased NPC viability after NPC treatment with microglia-conditioned medium (II/Fig. 7). However, LPS itself in different concentrations (10-500 ng/ml) did not affect NPC survival (II/Fig. 7). The effect of microglia-conditioned medium on NPC viability was rescued by neutralization of IFNγ with 0.25 μg/ml anti-IFNγ blocking antibodies (II/Fig. 7), indicating that microglia mediates its effects on NPCs via IFNγ secretion. These results demonstrate that neuroinflammation exerts a negative impact on NPCs.

5.2   Cytokine signaling decreases NPC proliferation and affects NPC cell fate

5.2.1   IFNγ reduces NPC proliferation

Cytokines are intercellular signaling molecules secreted by various cell types that activate intracellular signaling cascades through binding of cell membrane receptors. IFNγ is an inflammatory cytokine that propagates inflammation by activating IFNγRs on the cell membrane. By immunochemistry, we observed that IFNγRs are present in the embryonic neuroepithelium and in NPCs (II/Fig. 1), and further studied the influence of exogenous IFNγ on NPCs in the cell culture. IFNs signal through JAK/STAT pathway. In NPCs, 100 ng/ml IFNγ was shown to increase phosphorylation of Stat1 after 30 minutes and to induce its translocation into the nucleus, shown by western blotting and immunostaining (II/Fig. 2). Being active in NPCs, IFNγ signaling decreased the total number of Nestin-positive NPCs and reduced the amount of BrdU-positive cells (II/Fig. 2 and 3). Furthermore, IFNγ decreased the number of Ki67-positive NPCs as well as the formation of secondary neurospheres (II/Fig. 2 and 3). Moreover, cell cycle analysis revealed that IFNγ decreased the amount of NPCs in S phase and increased those in G0 phase.

The effect of IFNγ on NPC proliferation was independent of cell cycle regulators Cyclin D1 or p53^{Kip2} since their expression levels were not changed by IFNγ (II/Fig. 3). Especially, we detected no changes in NPC proliferation or survival during p53^{Kip2} inhibition by siRNA (II/Fig. 3). In addition, cell viability was decreased by IFNγ also in p53^{Kip2}-deficient mouse embryonic fibroblasts (II/Fig. 3), indicating that IFNγ affects cell viability independently of p53^{Kip2}. However, the IFNγ-mediated decrease in NPC proliferation was induced by activation of a CKI p21^{Cip1} (II/Fig. 3). Downregulation of p21^{Cip1} with specific siRNA rescued the IFNγ-induced effect on NPC proliferation (II/Fig. 3). p21^{Cip1} has been reported to mediate IFNγ-induced growth inhibition also in cancer cells (Gooch et al., 2000). Moreover, studies of p21^{Cip1} knockout mice reveal a role for p21^{Cip1} to inhibit neural progenitor proliferation in adults (Kippin et al., 2005; Pechnick et al., 2008).

Taken together, IFNγ negatively regulates NPC proliferation and maintenance by activating p21^{Cip1}, independent of its upstream regulator p53^{Kip2} but possibly also by inducing cell cycle exit. NPCs may exist as quiescent cells in the adult brain and maintain their ability to self-renew and differentiate. After

brain injury, they are recruited back into the cell cycle to facilitate neurogenesis. Inflammatory mediators, like chemokines, have been shown to inhibit proliferation and to induce quiescence of human NPCs (Krathwohl and Kaiser, 2004). Thus, IFNγ may also induce quiescence and promote NPC maintenance in some circumstances. In contrast to negative effects of IFNγ on NPC proliferation presented here, it has been shown to promote embryonic NPC proliferation (Li et al., 2010). On the other hand, Li et al. (2010) also showed that endogenous IFNγ inhibits neurogenesis in the non-inflammatory brain. IFNγ is usually secreted by immune cells in response to immune activation. However, low levels of IFNγ can be produced also by non-immune cells, like cerebrovascular endothelial cells (Wei et al., 2000). Thus, IFNγ may be important in facilitating niche-specific survival signals in non-inflammatory brain but may reduce NPC survival in inflammatory conditions. IFNγ may hence regulate NPC behavior by different ways in different conditions and depending on the exact cell population or the cytokine concentration.

5.2.2   IFNγ decreases NPC survival and induces cell death

Reduced cell proliferation may be a consequence of cell death or cell cycle exit due to quiescence or differentiation. IFNγ has been demonstrated to induce neuronal cell death (Jeohn et al., 2000; Sohn et al., 2007). Thus, we studied in more detail the mechanisms of IFNγ on NPC behavior. Already a concentration of 1 ng/ml for 48 hours decreased NPC viability in MTT assay (II/Fig. 2). In addition, staining of fragmented nuclei with TUNEL or propidium iodide revealed that IFNγ induces NPC cell death (II/Fig. 4). To study cell death mechanism in IFNγ-treated NPCs, we addressed the expression of different proapoptotic proteins. The results indicated that IFNγ-induced cell death in NPCs involves cleavage of caspase-3 as well as poly-ADP ribose polymerase (PARP) (II/Fig. 4), a protein required for DNA repair. PARP can be cleaved and inhibited by caspases, which may contribute to inability to repair the damaged DNA and finally lead to apoptosis.

In our further studies, we observed that IFNγ upregulates the proapoptotic protein PUMA, while it has no effect on the expression of other Bcl-2 family proteins, including Bcl-X$_L$ and Bax (II/Fig. 6). Moreover, IFNγ-mediated cell death assayed by TUNEL staining was rescued by treating NPCs with a non-specific caspase inhibitor, boc-aspartyl(OMe)-fluoro-methylketone (BAF) (II/Fig. 4). These results propose that IFNγ mediates NPC cell death through apoptosis. In line with this, cytochrome c released in the cytoplasm was detected in IFNγ-treated cells further suggesting that IFNγ induces mitochondrial cell death pathway.

5.2.3   BMPs decrease NPC proliferation and induce their differentiation into astrocytes

During the embryonic development BMPs are essential cytokines that regulate various cell fates in the nervous system. We detected expression of BMP2 and BMP4 as well as their BMP type I receptors BMPRIA and BMPRIB, and antagonist Noggin in NPCs *in vitro* (III/Fig. 6). Furthermore, BMP2 concomitantly with BMP type I receptors was expressed in the embryonic neuroepithelium, while BMP4 expression was not detected *in vivo* (III/Fig. 6). Stimulation of NPCs with 25 ng/ml of exogenous BMP induced Smad1/5/8 phosphorylation (III/Fig. 4) and resulted in decreased number of BrdU- and Ki67-positive cells (III/Fig. 4 and data not shown). Moreover, BMP signaling led to decreased Cyclin D1 expression that is known to be essential in NPC proliferation (**Figure 14**; Sundberg et al., 2006). BMPs are known to regulate astrocytic cell fate in neuronal progenitor

populations (Mehler et al., 1995; Gross et al., 1996; Mabie et al., 1999), so we addressed the effect of BMPs on NPC differentiation. In line with previous observations, we detected increase in the amount of GFAP-positive astrocytes by BMP2 and BMP4 with no signs of increased β-tubulin or CNPase-positive neuroblast or oligodendroglia populations, respectively (III/Fig. 5).
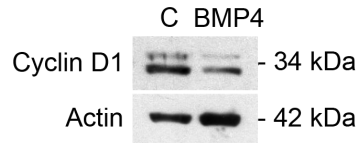

Figure 14. Immunoblot of NPCs. 25 ng/ml of BMP4 decreases Cyclin D1 protein expression. Two forms of Cyclin D1 are shown as 36 kDa and 30-31 kDa bands. β-actin was used as a loading control.

5.3   PACAP is able to rescue the decreased NPC survival in neuroinflammatory conditions

Inflammatory conditions negatively affected NPC viability, which emphasizes the significance of protection of NPCs after neuronal injury to promote their maintenance and recruitment in these circumstances. To find potent protective factors, we studied a plethora of neurotrophic factors, cytokines, chemokines and immunosuppressive drugs, including BDNF, GDNF, IL-6, IL-10, type I IFNs, granulocyte macrophage colony-stimulating factor (GM-CSF), TGFβ, indometacin, minocycline and forskolin. None of these factors were able to protect NPC survival against IFNγ (II/Fig. 5).

Neuropeptides are protective factors against neurotoxicity in neurons. Hence we studied a neuropeptide PACAP in neuroinflammatory conditions. The results revealed that PACAP protects NPCs since it rescued IFNγ-induced effect on cell viability (II/Fig. 5). However, it did not block IFNγ-induced Stat1 phosphorylation (II/Fig. 6). PACAP has been shown to inhibit apoptosis and caspase-3 activation (Vaudry et al., 2003). Expression studies of apoptotic proteins revealed that PACAP inhibits pro-apoptotic protein PUMA and cleavage of caspase-3, which were both increased by IFNγ (II/Fig. 6). Moreover, PACAP induced phosphorylation and inactivation of a pro-apoptotic protein Bad but it did not affect other anti- or pro-apoptotic proteins studied, including Bcl-X$_L$ and Bax (II/Fig. 6).

PACAP acts through PAC1 receptor that is expressed in the developing neuroepithelium (Waschek et al., 1998). In neuroepithelial cells, PACAP can activate intracellular protein kinase (PK) pathways, including PKA and PKC via the PAC1 (Zhou et al., 2001a). PACAP has been shown to protect NSC survival independent of PKA-signaling but to mediate NSC self-renewal and differentiation via PKA (Ohta et al., 2006). In addition, PACAP facilitates NSC differentiation through PKC-signaling (Watanabe et al., 2006). We hence studied if PACAP acts through protein kinase pathways to mediate NPC survival. Inhibition of PKC pathway with a specific antagonist, Gö6976, decreased the survival effect of PACAP during IFNγ treatment, while blocking of PKA-signaling with an inhibitor H89 did not (II/Fig. 6). These results indicate that PKC signaling is involved in PACAP-mediated cell survival in NPCs.

PACAP is known to improve NSC proliferation in the adult brain (Mercer et al., 2004; Ohta et al., 2006). Hence, we studied the effect of PACAP also on NPC proliferation. We observed increase in cell proliferation by PACAP but it was not able to rescue the decreased cell proliferation induced by IFNγ (II/Fig. 6). Thus, PACAP serves as a protective factor only against IFNγ-mediated NPC cell death. As shown above, microglial cells produce IFNγ that reduces NPC viability. Microglia mediates the brain

immune function, which is upregulated in many neurodegenerative diseases. Recently, microglia have been shown to promote neurogenesis after immune challenge and this effect was dependent on another cytokine than IFNγ (Battista et al., 2006). This suggests that microglia may exert different functions in the brain depending on the secreted factors. NPCs themselves can protect against brain inflammation (Einstein et al., 2003; Pluchino et al., 2005) and may promote neurogenesis in the pathological brain. Thus, it is important to protect NPCs from negative influences of the immune system. We addressed the protective effect of PACAP on NPCs during contact with microglia-secreted factors in conditioned medium. In line with results presented above, PACAP was able to protect NPCs also for decreased cell viability by microglia-produced IFNγ (II/Fig. 7).

We have shown that PACAP is a potential rescue factor against NPC cell death in inflammatory situations. PACAP may thus serve as a therapeutic agent for brain inflammation in neurological disorders as it has been shown to passage the blood-brain barrier (Dogrukol-Ak et al., 2004). It may also be useful in brain stem cell therapies to promote NPC survival. The safety and tolerability of PACAP has been studied in several human trials that have revealed adverse side effects after systemic administration, including pain and altered hormone secretion (Chiodera et al., 1996; Murck et al., 2007; Schytz et al., 2010; Amin et al., 2012). In addition, PACAP shows poor metabolic stability, insufficient distribution, low bioavailability and fast blood clearance because of its peptidic nature (Bourgault et al., 2011). Thus, the pharmacokinetic and pharmacological properties of this natural peptide must be first improved to develop a clinically appropriate immunoprotective drug.

5.4 Proteolytic pathways in NPCs

5.4.1 BRUCE is degraded by the ubiquitin-proteasome system in NPCs

BRUCE is a target of proteasomal degradation and it is shown to be regulated by an ubiquitin ligase Nrdp1 in the apoptotic pathway (Qiu et al., 2004). To study more closely the regulation of BRUCE by dexamethasone in NPCs, we used a proteasome inhibitor MG132 simultaneously with hormone treatment. Inhibition of the proteasome was able to rescue BRUCE expression (I/Fig. 4), indicating that dexamethasone induces proteasomal degradation of BRUCE in these cells. Nrdp1 stability is regulated by a deubiquitinating enzyme Usp8 (Wu et al., 2004) that was expressed in NPCs and upregulated by dexamethasone treatment after 3 hours (I/Fig. 4). Mifepristone inhibited the upregulation of Usp8 by dexamethasone (I/Fig. 4) referring to glucocorticoid receptor-mediated regulation. However, dexamethasone did not affect Nrdp1 expression (I/Fig. 4). Thus, we studied possible interactions between these proteins in NPCs by immunoprecipitation of Nrdp1. The results revealed that Usp8 and BRUCE were coimmunoprecipitated with Nrdp1 especially in dexamethasone-treated NPCs (I/Fig. 4) suggesting that dexamethasone-induced upregulation of Usp8 may be required for interaction and possible proteasomal degradation of BRUCE by this system. Moreover, subsequent Usp8 overexpression experiments indicated that Usp8 downregulates BRUCE protein and inhibits NPC proliferation (I/Fig. 4). In conclusion, BRUCE is a potent target for ubiquitination by Nrdp1 in NPCs. Furthermore, dexamethasone may induce BRUCE degradation by regulating the Usp8-Nrdp1 pathway. Altogether, the data indicates that dexamethasone may regulate BRUCE in different ways at the mRNA and protein levels. In addition to proteasomal pathway, BRUCE is regulated by a protease cleavage of an IAP antagonist Omi/HtrA2 (Qiu et al., 2005). Hence, it would be interesting to study whether other proteolytic pathways also affect BRUCE in NPCs.

5.4.2   BMPs regulate HAI-1 and HAI-2 expression

HAI-1 and HAI-2 are cell surface-associated proteins that regulate proteolytic reactions on the plasma membrane by inhibiting various target proteases. The upstream signaling of HAI proteins is, however, not known. To search for putative regulators of HAI-1 and HAI-2 we used different growth factors known to be essential in stem cell regulation, including Wnt3a, TGFβ and BMP4. BMP4 was found to upregulate the expression of HAI-1 and HAI-2 at the mRNA level (III/Fig. 3). As BMP4 forms a subgroup with BMP2 in the BMP family and they share the same type I receptors, we asked, whether BMP2 also regulates HAI proteins. Indeed, BMP2 upregulated HAI-1 both at the mRNA and protein level and increased also HAI-2 mRNA levels (III/Fig. 3). BMPs affect transcription of various target genes via Smad or MAPK signaling pathways. One of the target genes of BMPs include a transcription factor GATA2 (Dalgin et al., 2007), which is a potent regulator of HAI-1 and HAI-2 gene expression, as its binding site is present in the promoter regions of both HAI proteins (Itoh et al., 2001). This proposes that GATA2 may mediate BMP-induced increases in HAI-1 and HAI-2 expression and that HAI-1 and HAI-2 are regulated at the gene expression level. In conclusion, cell surface protease inhibitors HAI-1 and HAI-2 are targets of cytokine signaling in NPCs.

5.4.3   Serine protease inhibitors HAI-1 and HAI-2 reduce NPC proliferation

HAI-1 and HAI-2 are transmembrane serine protease inhibitors that are abundantly expressed in epithelial tissues (Szabo et al., 2008), and hence we studied their existence at the embryonic and postnatal neuroepithelium. Staining with specific antibodies showed expression of these proteins at the neurogenic area of the brain and their colocalization in neural progenitor population was indicated with Nestin double-expression (III/Fig. 1). Moreover, HAI-1 and HAI-2 expression was detected in cultured NPCs with proliferation and progenitor markers Ki67 and Nestin, respectively (III/Fig. 1). Closer look of proliferating and differentiated NPCs revealed their differential expression pattern, HAI-1 expression being constant during differentiation, while HAI-2 expression was downregulated in differentiated cells (III/Fig. 1). Since both HAI proteins were present in NPCs, we addressed their function in these cells using overexpression constructs of the full-length as well as truncated forms of proteins, the latter lacking the transmembrane (TM) domain. Forced expression of HAI-1 and HAI-2 in NPCs led to decreased amount of Ki67-positive cells (III/Fig. 2). No differences were observed between the full-length and TM-lacking isoforms of the proteins, indicating that the extracellular part of the proteins, containing the Kunitz domains, is responsible for the effect on proliferation. This suggests that inhibition of target proteases via KDs is required for proper NPC division. Subsequently to decreased proliferative capacity of NPCs, HAI-1 and HAI-2 overexpression resulted in decreased expression of Cyclin D1 (III/Fig. 2) suggesting that they may induce cell cycle arrest or exit in NPCs. In addition, overexpression of HAI-1 decreased phosphorylation of ERK1 and -2 (ERK1/2) (III/Fig. 2), whose activity is known to be essential in maintenance of brain stem cells (Enarsson et al., 2002).
To study, whether endogenous HAI proteins affect NPC proliferation, we used specific siRNAs to downregulate their expression. Reduction of both HAI-1 and HAI-2 levels by siRNA increased the number of BrdU-positive NPCs (III/Fig. 2). Furthermore, inhibition of HAI-1 with a specific monoclonal antibody, MG2, led to a similar result (III/Fig. 2). Collectively, these findings suggest that HAI proteins could inhibit NPC proliferation by regulating important cell cycle proteins and by affecting intracellular signaling pathways subsequently to protease inhibition.
As indicated above, HAI proteins and BMP2 and BMP4 have similar impact on NPC proliferation and BMPs were shown to increase HAI expression. This raised a possibility that HAI proteins mediate the

effects of BMPs on NPC proliferation. To prove this hypothesis, we simultaneously treated NPCs with BMP4 and HAI-1 or HAI-2 siRNA. Inhibition of HAI-1 and HAI-2 separately was able to partially rescue the amount of BrdU-positive dividing cells that was decreased by BMP (III/Fig. 4). These results show that BMP2 and BMP4 at least partially mediate their effects on cell proliferation via HAI proteins, which describes a novel signaling mechanism where cytokine signaling may regulate cellular behavior via transmembrane proteins other than cell adhesion molecules.

5.4.4   HAI-1 induces astrogliogenesis and regulates NPCs *in vivo*

BMPs were shown to upregulate HAI expression and to induce astroglial differentiation of NPCs. Thus, we studied if HAI proteins are also involved in regulation of NPC differentiation. Overexpression of HAI-1 resulted in increased number of GFAP-positive astroglia in culture (III/Fig. 5). However, the amount of βIII-tubulin-positive neuroblasts or CNPase-positive oligodendrocytes was not affected (III/Fig. 5). In contrast to HAI-1, HAI-2 overexpression did not have a significant effect on NPC differentiation (III/Fig. 5). To further study if HAI-1 is responsible for BMP-induced effect on increased astrogliogenesis, we treated BMP2-stimulated cells simultaneously with HAI-1 siRNA. The results indicated that blocking of HAI-1 expression inhibits BMP-induced astroglial formation in culture (III/Fig. 5), demonstrating that at least BMP2 regulates astrogliogenesis concomitantly with HAI-1.

HAI-1 was shown to be a more potent regulator of NPCs, possibly due to partial redundancy of HAI-2 to HAI-1 that is also suggested before (Szabo et al., 2008; 2009). Thus, we focused on HAI-1 in order to address the *in vivo* effects of these protease inhibitors in neural progenitor cells. Since HAI-1 knockout mice are embryonic lethal already during the early developmental stage and HAI-1 heterozygotes display normal physiology (Tanaka et al., 2005), we decided to use *in utero* electroporation method to achieve HAI-1 overexpression in the brains of older embryos. HAI-1 expression vector under the pCAG promoter together with a control GFP vector was introduced into the embryonic E14 or E17 mouse brain to study the proliferation and differentiation of targeted cells, respectively. The achieved results confirmed the *in vitro* effects of HAI-1 in NPCs, including decreased amount of Ki67-positive cells in the embryonic E15-old neuroepithelium (III/Fig. 7) and increased number of GFAP-positive astroglia in P1 brains (III/Fig. 8). Taken together, these results show that HAI-1 is an important regulator of proliferation and astroglial cell fate in NPCs.

5.4.5   Potent targets of HAI proteins in NPCs

HAI-1 and HAI-2 share several common target proteases. They were originally identified as potent inhibitors of HGFA (Kawaguchi et al., 1997; Shimomura et al., 1997) that exerts its effects on cell behavior and tissue regeneration through activation of HGF zymogen (Shimomura et al., 1992). We thus studied the expression and/or function of HGFA and HGF in NPCs as potent targets of HAI protein inhibition. Both HGF and HGFA were present in proliferating NPCs (III/Suppl.Fig. 1). However, HAI overexpression did not affect HGFA expression levels (III/Suppl.Fig. 1) proposing that it is not a target for HAI inhibition. Moreover, HGF receptor c-Met was nearly absent in NPCs (III/Suppl.Fig. 1). To further elucidate the possible role of HGF in NPCs, we studied the effect of c-Met inhibitor SU11274 and exogenous HGF on NPC proliferation. Neither of these factors affected the amount of BrdU-positive cells in culture (III/Suppl.Fig. 1). To exclude the possibility that HAI proteins may inhibit HGF function, we used HAI siRNAs concurrently with exogenous HGF in the study of NPC proliferation. We observed no changes in NPC proliferation by HGF (III/Suppl.Fig. 1).

These results indicate that HGF signaling is not involved in the regulation of NPC proliferation in the present conditions and that HAI proteins do not facilitate their effects on NPC cell division through HGFA/HGF. As HGF is known to be activated by HGFA in injured tissue (Miyazawa et al., 1996), the role of HGF signaling would be interesting to assess in the situation of brain injury or inflammation. On the other hand, HGF has been shown to affect neuronal maturation (Korhonen et al., 2000) suggesting that it might be involved in NPC differentiation and/or migration. This, however, insists further studies.

Lack of HGF signaling in NPCs in the present study proposes that HAI proteins may have other target protease(s) in these cells. Matriptase is a membrane-associated serine protease that is abundantly coexpressed with HAI-1 and HAI-2 in epithelial tissues (Szabo et al., 2008). Matriptase is involved in neural tube closure during the brain development but it is regulated by a complex manner requiring both HAI-1 and HAI-2 inhibitors as well as hepsin and prostasin as activators (Szabo et al., 2009; Camerer et a., 2010). This suggests that matriptase may serve as a potent target for HAI proteins in NPCs and could be involved in NPC proliferation. We detected expression of matriptase especially in proliferating NPCs and the expression was localized to the same cells with HAI proteins (**Figure 15A-B**). Furthermore, matriptase expression was downregulated after forced expression of HAI-1 and HAI-2 (**Figure 15C**). These expression studies suggest that matriptase may be a target of HAI protein inhibition in NPCs. Moreover, matriptase could regulate NPC proliferation and/or differentiation but this requires further analysis.
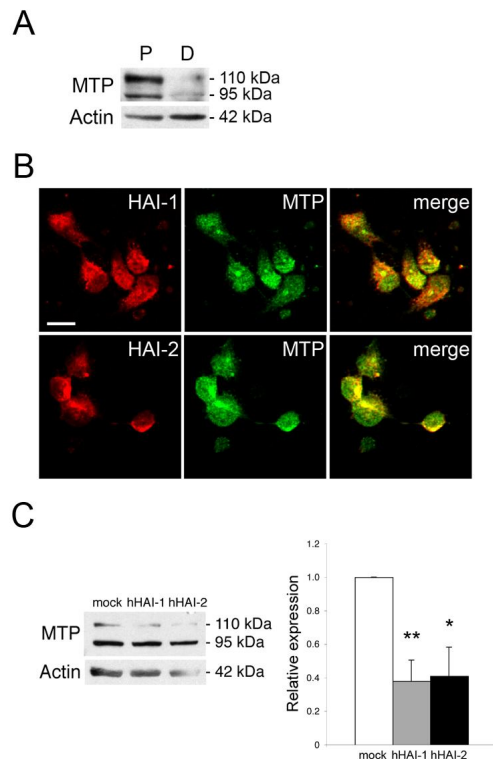

Figure 15. Expression of matriptase (MTP) in NPCs. A. MTP is expressed in proliferating (P) NPCs as a complexed form with HAI-1, shown as 95 kDa and 110 kDa bands. MTP expression declines during NPC differentiation (D). β-actin was used as a loading control. **B.** Confocal microscopy shows colocalization of MTP with HAI-1 and HAI-2 in dissociated NPCs. Scale bar, 20 μm. **C.** MTP expression decreases after HAI-1 and HAI-2 overexpression. β-actin was used as a loading control. Quantification of 95 kDa and 110 kDa bands is shown on the right. Values are means ±SEM, n=3. ** p < 0.01, * p< 0.05 by Student's t-test.

6   CONCLUSIONS AND FUTURE PROSPECTS

In this thesis work I have aimed to study NPCs and how they are affected by brain inflammation and proteolytic pathways. NPC development is regulated by diverse signals through interactions with other cells in their local environment. The knowledge of the mechanisms governing the maintenance and differentiation of NPCs is relevant in the situations of brain injury and disease, where neurons degenerate and NPCs could serve as a reservoir for cell replacement. Neurodegenerative diseases and brain trauma are associated with inflammation that can have an impact on cell survival and behavior. The precise outcome of brain inflammation whether friend or foe for brain regeneration is hard to control or predict.

The thesis work unveils an important interplay between neural progenitor cells and brain microglia that could play a role in neuroinflammation and in brain diseases. This interaction involves the cytokine IFNγ produced from microglia with unfavorable influence on NPC proliferation and survival. IFNγ was found to inhibit NPC proliferation via effects on the cell cycle machinery and to decrease cell viability by inducing cell death signals in the NPCs. The effects on NPC cell viability were counteracted by the neuropeptide PACAP that also decreased microglial secretion of inflammatory mediators. PACAP could be used as a potential factor in treatment of brain inflammation and in neurodegenerative diseases, in which activated microglia contribute to disease progression. However, it will be important to study the potential role of PACAP in the *in vivo* setting of neuroinflammation and brain diseases. In addition, the use of PACAP as a clinically competent drug would require modifications to improve pharmacological properties of this natural peptide.

Cell proliferation is linked to the proper activity of the UPS through regulation of key proteins involved in the cell cycle. We observed that the expression levels of BRUCE are negatively regulated by the synthetic glucocorticoid hormone dexamethasone, possibly via the UPS. BRUCE is a ubiquitin-conjugating enzyme and was crucial for the regulation of NPC division. BRUCE could mediate its effect on cell proliferation by targeting cell cycle regulators or the components of the midbody ring involved in cytokinesis. The precise targets for BRUCE in NPCs and how it influences NPC development needs to be studied more in the future.

The present work also unravels a novel signaling system in the NPCs to guide cell development and differentiation. The BMP family of cytokines are essential factors in many developmental pathways. We observed that BMPs regulate the cell membrane-anchored protease inhibitors HAI-1 and HAI-2 expressed by the NPCs and also control cell proliferation and fate. The effects of BMPs were observed both in cell cultures and in developing neuroepithelium *in vivo* and were found to be age-dependent. At an earlier developmental stage the BMPs reduced proliferation and at later stages the BMPs induced differentiation of NPCs into astrocytes. The precise mechanisms underlying these cell responses remain to be studied further, but as *bona fide* protease inhibitors, HAI-1 and HAI-2 may influence several signaling cascades important for cell fate decision and cell proliferation. However, the target protease(s) in the NPCs regulated by the HAI proteins warrant more studies. The HAI proteins and their downstream proteases could serve as therapeutic targets to modulate proteolytic cascades in the NPCs, and provide a valuable tool for stem cell therapies in various brain diseases.

Overall, the regulation of NPCs is important in conditions like stress and inflammation that affect neurogenesis. Chronic stress and inflammation may result in various neurological diseases and disorders or, vice versa, they may occur secondary to pathogenesis and brain trauma. NPCs are also a potent target for stem cell therapy to facilitate neurogenesis and brain recovery in disease.
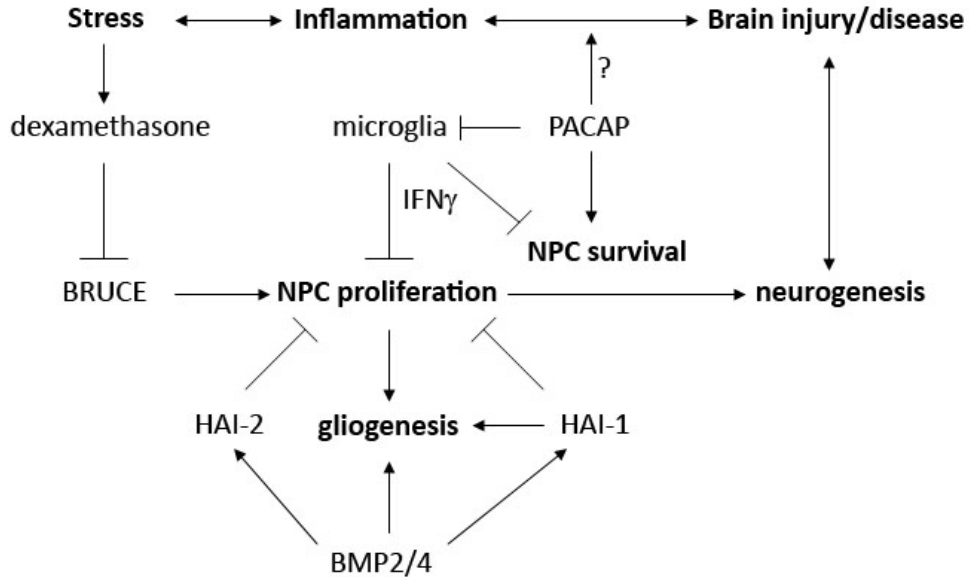
Figure 16. Conclusions. Stress is associated with decreased neurogenesis, and it may affect the immune system by changing glucocorticoid hormone (GH) levels. A synthetic GH, dexamethasone, was shown to inhibit the expression of protein BRUCE that is required for proper neural progenitor cell (NPC) proliferation.

Brain damage can activate the immune system leading to neuroinflammation that is involved in the pathogenesis of brain diseases and disorders. During neuroinflammation, microglia produce proinflammatory cytokines such as interferon(IFN)-γ that reduces NPC proliferation and survival. Neuropeptide PACAP has a dual role in promoting NPC survival by inhibiting microglial secretion of IFNγ and by counteracting the IFNγ-induced cell death. Because of its protective role, PACAP could be useful in treatment of brain inflammation and disease, as well as in brain stem cell therapy.

In addition to proinflammatory cytokines, other cytokines, such as bone morphogenetic proteins (BMPs), regulate NPC behaviour. BMP2 and BMP4 increase HAI-1 and HAI-2 protein expression in developing NPCs resulting in reduced proliferative capacity of these cells. Moreover, HAI-1 mediates the effects of BMP2/4 on NPC differentiation by inducing gliogenesis.

Increased NPC proliferation and survival contributes to neurogenesis that is affected in neurological diseases. Stem cell therapies by stimulating the endogenous NPCs or by transplantation of stem cells into the brain could enhance neurogenesis and recovery in brain injury or disease.

7 ACKNOWLEDGEMENTS

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