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Making neurons from stem cells: Molecular mechanisms and spider silk substrates

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Illustration of Neural Stem Cells on Spider Silk Proteins.
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Making Neurons From Stem Cells: Molecular Mechanisms and Spider Silk Substrates

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ABSTRACT

The understanding of the function of the nervous system and the brain is one of the major intellectual challenges in life sciences. Neurological and psychiatric disorders are in addition major issues for the society, and new approaches are needed to learn more about the brain and to develop new treatments. The development of the mammalian brain is a highly regulated process that involves extra- and intracellular signaling to efficiently regulate gene expression in a precise spatial and temporal manner. The understanding of the differentiation mechanisms into neurons, glia and other cell types in the developing forebrain however is still incomplete. Studies of embryonic telencephalic neural stem cells (NSCs) *in vitro* may increase the understanding of the molecular mechanisms of brain development, and aid in developing new protocols for defined differentiation of stem cells for clinical use.

This thesis is aimed at investigating the mechanisms underlying bone morphogenetic protein (BMP4)-mediated differentiation of NSCs, and to explore the use of recombinant spider silk protein-based matrices in combination with signaling factors, especially BMP4, to generate functional neural cell circuits *in vitro*.

In the first study, we discovered that BMP4 treatment of NSCs resulted in a dramatic increase in the expression of the BMP4-inhibitor Noggin. BMP4 mediated non-neural differentiation into mesenchymal cells at low seeding densities, neuronal differentiation at high seeding densities, and astrocyte differentiation in any condition. As the Noggin levels increased linearly at higher densities, we hypothesized that the endogenous Noggin production predominantly mediated an inhibition of mesenchymal differentiation. We further observed that BMP4 stimulation induced an AMPA responsive neuron population at high seeding densities, and that this population was increased by co-stimulation of the signaling factor Wnt3a. By applying whole transcriptome sequencing, we aimed at elucidating the molecular mechanisms responsible for the increased neuronal differentiation by BMP4+Wnt3a. This approach, however, revealed an unexpected increase in the expression of genes associated with inhibitory GABAergic neurons, and also functional GABA-responsive neurons in the culture. RNA knockdown experiments demonstrated that this GABAergic component was dependent on the expression of the neurogenic bHLH factor Hes6.

To apply these novel protocols for differentiation of NSCs into functional neurons, we introduced a novel way of culturing NSCs on substrates generated from recombinant spider silk protein (4RepCT). Spider silk protein is a promising biomaterial due to its biocompatibility, biodegradability, and possibility to use in various forms both in 2D and 3D. NSCs cultured in 2D cultures on 4RepCT “film” structures showed no significant differences in cell proliferation, viability, or differentiation potential compared to control cultures in optimized conditions. 4RepCT substrates generated as “foam” structures could be used for 3D culturing of NSCs, and these NSC cultures differentiated nicely into astrocytes and neurons. Calcium imaging assays revealed that BMP4+Wnt3a-treatment of NSCs grown in 3D 4RepCT-matrices resulted in efficient generation of functional excitatory neurons.

These studies have thus revealed new molecular mechanisms underlying neural differentiation of cortical stem cells, and point to the versatility of using spider silk protein-based substrates for stem cell cultures. Future studies aim at testing these new concepts *in vivo* for improved treatment of neurological disease.

LIST OF PUBLICATIONS

I. Andersson T, Duckworth JK, Fritz N, **Lewicka M**, Södersten E, Uhlén P, Hermanson O. (2011) Noggin and Wnt3a enable BMP4-dependent differentiation of telencephalic stem cells into GluR-agonist responsive neurons. Mol. Cell. Neurosci. 47:10-8.

II. **Lewicka M**, Rebellato P, Uhlén P, Hermanson O. (2013) BMP4-induced neuronal differentiation of cortical stem cells involves a Hes6-dependent GABAergic component. Manuscript.

III. **Lewicka M,** Hermanson O, Rising AU. (2012) Recombinant spider silk matrices for neural stem cell cultures. Biomaterials 33:7712-7717.

IV. **Lewicka M**, Rebellato P, Uhlén P, Rising A, Hermanson O. (2013) Building brains in spider webs: Stem cell-derived GluR- agonist responsive neurons grown in 3D matrices of recombinant spider silk protein. Manuscript to be submitted.

PUBLICATION NOT INCLUDED IN THE THESIS

I. Leão RN, Reis A, Emirandetti A, **Lewicka M**, Hermanson O, Fisahn A. (2010) A voltage-sensitive dye-based assay for the identification of differentiated neurons derived from embryonic neural stem cell cultures. PLoS One 5:e13833.

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LIST OF ABBREVIATIONS

AMPA	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
BMP	Bone morphogenetic protein
CxxC5	C-X-C motif chemokine 5
CNS	Central nervous system
CNTF	Ciliary neurotropic factor
E	Embryonic day
ECM	Extra cellular matrix
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GABA	Gama-Aminobutyric Acid Receptor Associated Protein
GFAP	Glial fibrillary acidic protein
GluR	Ionotropic glutamate receptor
Hes6	bHLH transcription cofactor / hairy and enhancer of split 6
IKVAV	Ile-Lys-Val-Ala-Val peptides
Fibro	Fibronectin
NSC	Neural stem cells
MAP2	Microtubule associated protein 2
MBP	Myelin basic protein
P or PORN	Poly-L-ornithine
PDMS	Polydimethylosiloxane
PNS	Peripheral nervous system
PS	Polystyrene
REST	Repressor element 1 silencing transcription factor
4RepCT	Spider silk protein, Consisting of 4 tandem repeats and the C-terminal domain of the major ampullate spidroin 1
RGD	Arg-Gly-Asp peptides
Shh	Sonic hedgehog
SMA	α-Smooth muscle actin
Syntyg	Synaptotagmin
TGFβ	Transforming growth factor β
TuJ1	Tubulin beta 3
T3	Thyroide hormone 3
Wnt	Wingless-related mouse mammary tumor virus (MMTV) integration sites

1 INTRODUCTION

1.1 Neural induction

Brain development is a complex process consisting of a series of spatiotemporal events that still riddle researchers.

The fertilized egg undergoes several rounds of cell division, cell fate specification and cell migration to build a complex organism. Lineage specification and cell migration result in the development of the morula, followed by the blastocyst. During gastrulation, the cell mass of blastula becomes a multilayered gastrula with all three germ layers: ectoderm, mesoderm and endoderm. Further on, cell division results in thickening of the ectoderm and creation of the neural plate. The neural plate forms the neural tube, which during development will become the brain and spinal cord.

Cell fate depends on morphogens – signaling molecules that are secreted by organizers during development (Fig. 1). The organizer concept was introduced in the 1920s by Spemann and Mangold (Mangold 1924) and was later followed by identification of the neural fate inducing genes noggin, chordin and follistatin (Smith and Harland 1992; Piccolo, Sasai et al. 1996; Fainsoda, Deißlerb et al. 1997). BMP is expressed in the ventral side of the embryo where the ectoderm is formed, whereas noggin and chordin expressed in the dorsal part are involved in neural induction (Hemmati-Brivaniou 1995; Sasai, Lu et al. 1995; Weinstein and Hemmati-Brivanlou 1999). In the 90's Echelard described a morphogen that is responsible for ventral patterning of the brain, namely, Sonic hedgehog (Shh) (Echelard, Epstein et al. 1993). Expression of Shh in the notochord plays an important role in early dorso-ventral patterning of the brain, which relies on opposing effects by Shh and dorsally expressed genes - bone morphogenetic protein (BMP) and wingless-related integration site (Wnt) (Robertis, Larrain et al. 2000). Shh induces development of ventral structures, for example, motor neurons, BMP stimulates growth of dorsal structures, for example, choroid plexus and the hippocampus, whereas Wnt have been shown to be involved in the establishment of dorsal cortical structures (Fig.1). The rostro-caudal development of midbrain and hindbrain is driven by genes such as Wnt1, engrailed 1(en1) and fibroblast growth factor 8 (FGF8) (Lee, Danielian et al. 1997).

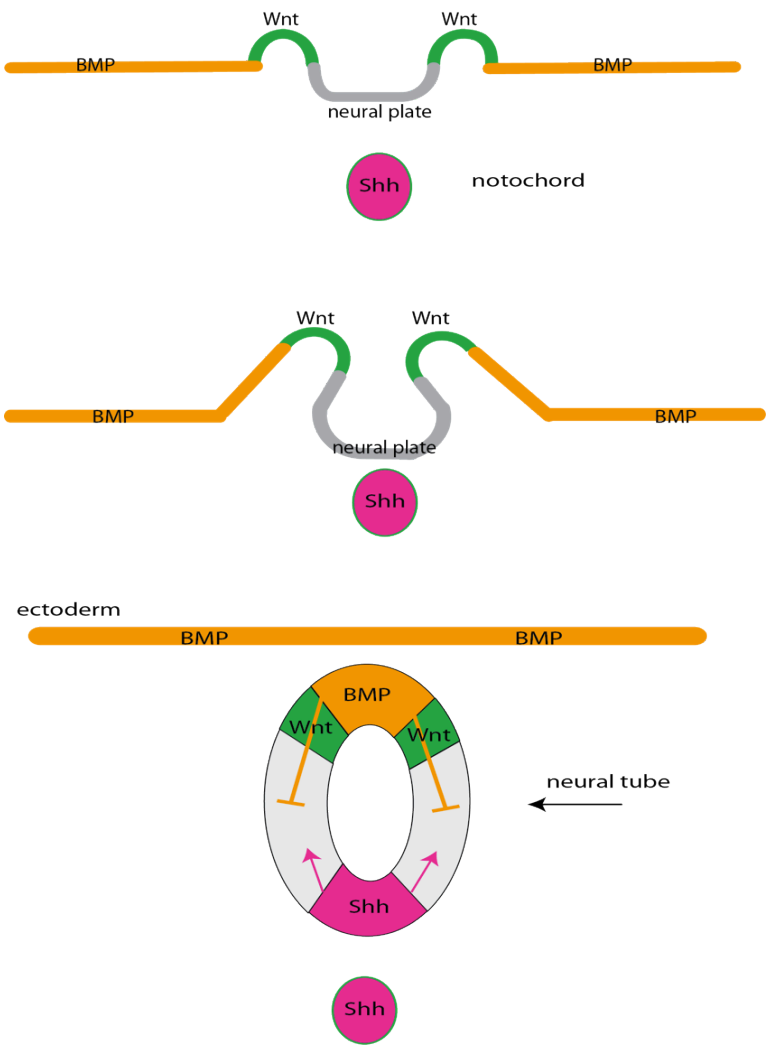

Fig. 1 BMP and Wnt involvement in neural tube closure.
(Modified from Sanes et al. 2004)

Initial neural fate determination is dependent on inhibition of BMP signaling and at later stages it regulates CNS development.

The opposing gradient of BMP and Shh creates dorsoventral patterning of the neural tube. BMP proteins are secreted in the developing spinal cord from the roof plate (dorsal) opposing ventral Shh secretion from the floor plate. (Ericson, Muhr J. et al. 1995). Loss of BMP signaling in the spinal cord is associated with absence of Math1 positive cells, which are the most dorsal cells in the spinal cord (Chesnutt, Burrus et al. 2004).

1.2 BMP and Wnt signaling in telencephalon development

BMP4 and Wnt3a, two out of many morphogens involved in regional specification of the telencephalon are a focus of this thesis.

1.2.1 Bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor β (TGFβ) superfamily, which is evolutionarily conserved in vertebrates (Danesh, Villasenor et al. 2009). In 1956, Urist showed BMP activity in bone and cartilage formation (Urist 1965); however, the proteins were identified in the late 80's (Luyten, Cunningham et al. 1989; Wozney 1989). Nowadays, the BMP family contains 20 members and is well known for its involvement in developmental and pathophysiological processes (Liu and Niswander 2005).

Signaling by BMPs is mediated by serine-threonine kinase (RSTKs) receptors type I and II which are a group of catalytic receptors that includes members of the transforming growth factor β (TGFβ) and activin receptors (ALK1-7) (Fig. 2). Binding of the ligand to the receptors results in phosphorylation of receptor I mediated by receptor II. RSTKs initiate intracellular signaling by activation of Smad proteins (Massague 1998; Miyazono 2000; Wu and Hill 2009) that will bind to a common mediator, Smad4 (Wrana 2000). This event is followed by Smad4 translocation to the nucleus which activates downstream gene transcription regulation (Wrana 2000).

The functions of BMP signaling for development of telencephalon are highlighted through the actions of the extracellular BMP antagonists noggin, chordin and gremlin (Liu and Niswander 2005). Noggin prevents BMP2, -4 and -7 from binding to its receptors (Zimmerman, Jesu s-Escobar et al. 1996). Equilibrium of BMP and noggin factors is necessary to maintain normal development.

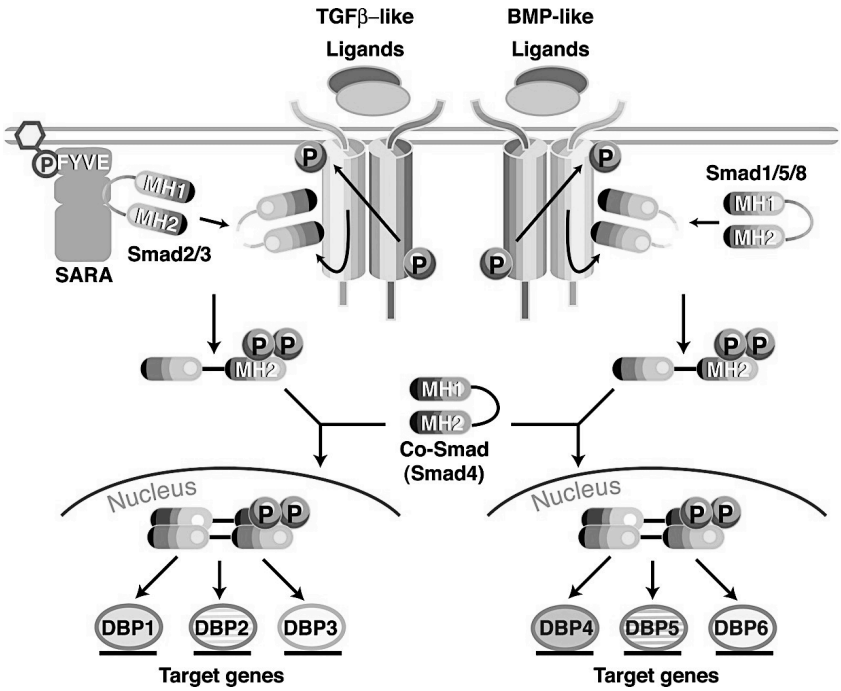

Fig. 2 Signal transduction by TGF-beta family

(Attisano and Wrana 2002)

BMP signaling is required for a wide range of neurodevelopmental processes and general development (Table. 1).

Neurodevelopmental processes:	**References**
Embryonic cell fate specification: neural or non-neural ectoderm	(Chen, Zhao et al. 2004; Liu and Niswander 2005; Danesh, Villasenor et al. 2009; Inestrosa and Arenas 2010)
Neurulation	
Dorso-ventral patterning of the brain and spinal cord	
Forebrain and cerebellum development	
Neurite outgrowth	
Synapse formation	
Cell migration	
Cell proliferation	
Apoptosis	
Organogenesis	(Hogan 1996; Danesh, Villasenor et al. 2009)

Table. 1 Role of BMP signaling in development

BMP4 is expressed in the early developing brain in the most dorsal neuroectoderm and dorsal forebrain (Furuta, Piston et al. 1997). The significance of BMP4 protein function has been shown in knockdown animals where BMP2 and BMP4 removal resulted in early embryonic lethality at E6.5 and E9.5. Severe brain malformations and little or no mesoderm were also spotted due to BMP4's involvement in early embryonic organ and tissue development (Winnier, Blessing et al. 1995; Danesh, Villasenor et al. 2009). Moreover, BMP4 overexpression in transgenic animals enhance astrocytic differentiation and inhibits the generation of oligodendrocytes (Gomes, Mehler et al. 2003).

In addition to the involvement of BMPs in development, BMP4 signaling has also been linked to pathophysiology. Compromised BMP activity results in impaired choroid plexus formation (Smith and Harland 1992) and uncontrolled BMP stimulation turns cortical primordium into choroid plexus (Panchision, Pickel et al. 2001). BMP4 may be involved in Fibrodysplasia Ossificans Progressiva (FOP) pathogenesis of bone formation (Duprez, Bell et al. 1996; Kan, Hu et al. 2004). Moreover, BMP signaling has been associated with hepatocellular and ovarian cancer (Kan, Hu et al. 2004; Kallioniemi 2012). BMP signaling in cancer has dual involvement in the disease progression, both promoting due to its regulation of cell proliferation and mobility and opposing via regulating differentiation and apoptosis (Kallioniemi 2012).

1.2.2 Wnt signaling / β-catenin signaling pathway in nervous system

Wnt signaling stands for wingless-related mouse mammary tumor virus (MMTV) integration site. The Wnt gene was first identified as a wingless mutation in *Drosophila melanogaster* (wg-1) (Chopra 1976; Lawrence 1977) and later in 1982 as a proto-oncogene Int-1 (later called Wnt1) involved in the development of mammary tumors (Varmus 1982). Finally in 1987, Rijsweijk et al. showed that wg-1 and Int-1 proteins are gene homologs, which gave rise to the new family Wnt–wingless (Rijsewijk, Schuermann et al. 1987; Nusse, Brown et al. 1991).

Wnt signaling is initiated by Wnt ligand binding to a Frizzled receptor (MacDonald, Tamai et al. 2009). Today 19 different Wnt ligands are known, which can be subdivided into two groups:

1. Canonical/Wnt1 class - eg. Wnt1, Wnt3a, Wnt8a/b.
2. Non-canonical/Wnt5a class- eg. Wnt4, wnt5a, Wnt11

Wnts are know to activate at least three main pathways (Fig. 3):

1. Wnt/β-catenin pathway
2. Wnt/PCP pathway
3. Wnt/Ca^{2+} pathway

The second and third pathways do not signal via β-catenin, and are thus known as Wnt/β-catenin independent pathways.

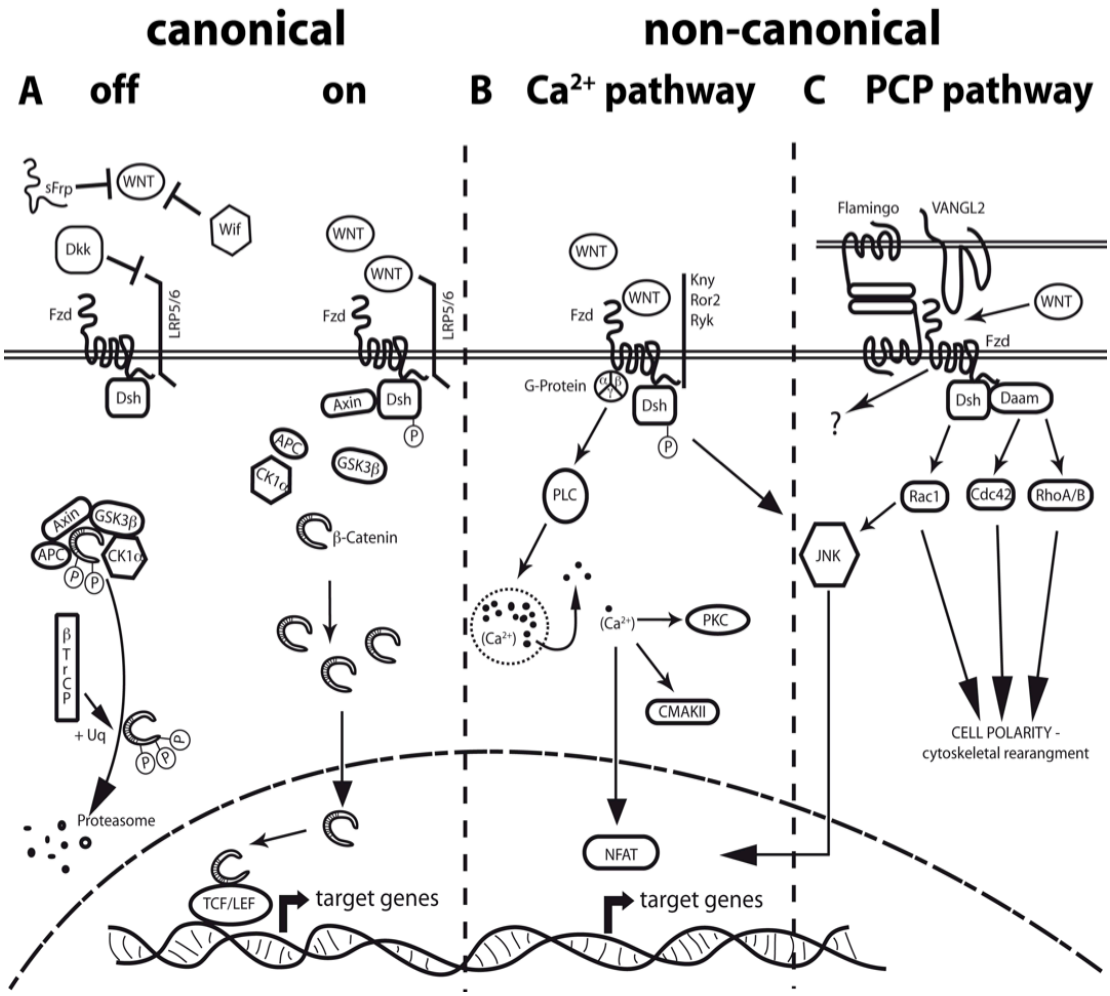

Fig. 3 Wnt pathways: A. Canonical Wnt/β-catenin pathway, B. Non-canonical Wnt/Ca^{2+} pathway, C. Non-canonical Wnt/PCP pathways (Liebner and Plate 2010)

Wnt/β-catenin pathway can be activated in presence or absence of Wnt ligand (Fig. 3). In the absence of Wnt-ligand, β-catenin is phosphorylated by kinases from the destruction complex, Ck1α and GSK3, and degraded in the proteasome (Fig. 3A. off state). Ligand binding to Frizzled and LRP5/6, initiate Dishevelled interaction with

Frizzled, followed by phosphorylation of LRP5/6 and inhibition of GSK3, which disassemble destruction complex. This allows β-catenin to enter the nucleus and regulate gene expression with TCF/LEF (Fig. 3A. on state) (Grove 2011). Cell cycle progression/proliferation, cell fate specification, and differentiation are regulated by some genes identified in the Wnt/β-catenin pathway (Willert and Nusse 2012).
Wnt/PCP pathway is involved in neural tube closure and rearrangement of the cytoskeleton. Wnt/Ca2+ signaling pathway uses calcium as a second messenger in Wnt/ β-catenin independent signaling.

The canonical Wnts activate the Wnt/β-catenin pathway, which was believed to be independent of the non-canonical one (Ciani and Salinas 2005). However, more detailed studies have shown that different Wnts interact with variable ligands, which leads to activation of the canonical Wnt/β-catenin signaling pathways by the non-canonical class of Wnts, which is context-dependent (Ciani and Salinas 2005; Grumolato, Liu et al. 2010).

The Wnt/β-catenin pathways have been identified to be involved in early nervous system development, maintenance and regeneration (Table. 2).

Developmental processes:	**References**
Anterior-posterior neural tube patterning	(Robertis, Larrain et al. 2000; Hendrickx, Van et al. 2009; Hikasa, Ezan et al. 2010),
Regionalization of the forebrain	(Jang, Bonaguidi et al. 2013),
Dorso-ventral forebrain patterning	
Hippocampal development	
Midbrain-hindbrain boundary formation	(Himmelstein, Bi et al. 2010
Hindbrain and neural crest growth	(Hari, Brault et al. 2002)
Dorsalization in the spinal cord	(Ciani and Salinas 2005)
Neural tube closure	
Tissue organization/polarity	(Kleber and Sommer 2004; Kalani, Cheshier et al. 2008; Hirabayashi, Suzki et al. 2009)
Cell renewal	
Cell cycle progression/proliferation	(Willert and Nusse 2012)
Cell fate specification and differentiation	(Kleber and Sommer 2004(Munji, Choe et al. 2011))
Oligodendrocytes maturation	(Di Pasquale and Brivanlou 2009; Feigenson 2011; Steventon and Mayor 2012)
Angiogenesis	(Daneman, Agalliu et al. 2009)
Apoptosis	Ille and Sommer 2005
Axon guidance	(Ciani and Salinas 2005)
Path finding	
Axon remodeling	
Dendrite morphogenesis	
Synapse formation	
Adult neurogenesis	(Vanderhaeghen 2009; Inestrosa and Arenas 2010)
Tissue repair	(Galliot and Chera 2010; Abo and Clevers 2012; Faigle and Song 2013)
Brain vasculature	(Liebner and Plate 2010)

Table. 2 The role of Wnt/β-catenin pathways in development, maintenance and regeneration

Canonical Wnt/β-catenin pathway and Wnts expression are conveyed in developing nervous system starting with neural tube closure followed by blocking of the early neural induction of FGFs under the anterior-posterior patterning. Thus, inhibition of Wnts is necessary for early forebrain development and diencephalon (Satoh, Kasai et al. 2004).

The Wnt/β-catenin pathways are essential for gene transduction due to the fact that Wnt3a knockdown animals are missing the hippocampus and are often lethal at embryonic stage (Amerongen and Berns 2006). Moreover, overexpression or abnormalities in Wnt signaling are related to brain pathophysiology, such as mood disorders (Duman and Aghajanian 2012) and cancer (Polakis 2000; Polakis 2012).

1.2.3 BMP and Wnt crosstalk

The human body is built from numerous amounts of cell types that require communication with each other. BMPs and Wnts are two out of several signaling pathways involved during early development towards sustainability (Rajan, Panchision et al. 2003; Inestrosa and Arenas 2010) of the nervous system and its regeneration.
Both pathways depend on cell-surface receptors and reach the nucleus by interchanging their cytoplasmic components. Often, these proteins work as opposing forces to each other, as in brain and spinal cord development. Their involvement in cell fate determination, proliferation and synaptic physiology (Inestrosa and Arenas 2010) makes them a subject of interest. Hence, studying their full functions demands further research.

In our studies, we used BMP4 and Wnt3a to treat cortical cell cultures due to their direct involvement in cerebral cortex development.

BMP4 and Wnt3a are the main signaling centres of the telencephalon development at mid-gestation stage (Rubenstein 2001) together with fibroblast growth factor 8 (FGF8) and Shh (Fig 4 A). The opposing gradient of morphogens in dorsoventral patterning of the telencephalon creates different zones of neuronal progenitors that have definite localization and properties (Rubenstein 2001). TGF-beta and Wnt activities coordinate regulation of neural tube patterning and closure (Chesnutt, Burrus et al. 2004).
Moreover, previous studies in the lab demonstrated C-X-C motif chemokine 5 (CxxC5) involvement in interaction between BMP4 and Wnt3a. BMP4 stimulation of NSCs

increase Cxxc5 expression that is putative inhibitor of Wnt and modulates Wnt-signaling in a cell density (Andersson, Södersten et al. 2009) dependent manner.

The cross talk between BMPs and Wnts and their involvement in patterning of cerebral cortex have been describe before (Shimogori, Banuchi et al. 2004; Andersson, Södersten et al. 2009). However, the key events in this cross talk have not been fully identified.

However, it is known that BMP, FGF, WNT signaling are involved in early cortical development by interacting with empty spiracles homolog 2 (Emx2). In early cortical primordium Emx2 regulates BMP activity by inhibiting noggin (Shimogori, Banuchi et al. 2004). This resulted in Wnt signaling inhibition by repression of FGF8/17, which is involved in anterior-posterior axis formation of neocortex development (Fig. 4 B).

Moreover, studies showed that BMP4 signaling is required for the canonical Wnt signaling to be involved in oligodendrocyte development in the spinal cord and suggested that Wnt signals upstream of BMP (Feigenson 2011).

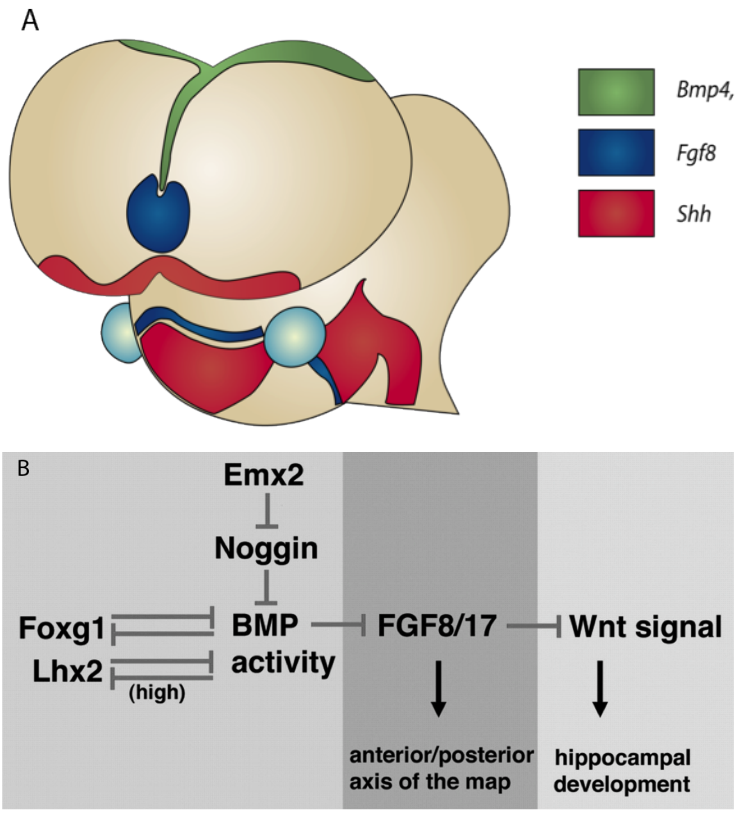

Fig. 4 A. Bmp4 and Wnt3a expression in the developing mouse telencephalon, modified from (Rubenstein 2001); B. Preliminary model of signalling interaction that pattern the mouse cerebral cortex, from (Shimogori, Banuchi et al. 2004)

1. 3 Stem cells

The self-renewing ability and the capacity to differentiate into specialized cell types are the major characteristics of stem cells. The 21st century discoveries in the stem cell field and the Nobel in Physiology or Medicine awarded to Yamanaka and Gordon in 2012 for induced pluripotent stem cells (iPSC), still leave an open arena for further research. Stem cells are promising tools for basic research, however, better understanding of its molecular properties and application to regenerative medicine need to be investigated.

Stem cells can be categorized into:

1. Embryonic stem cells – totipotent cells derived from early stage embryos (from morula to blastocyst) and can generate all cells in the body including extraembryonic tissue.
2. Fetal stem cells – pluripotent cells derived from the fetus and are tissue specific.
3. Adult stem cells – undifferentiated cells derived from adult organisms and are lineage-restricted depending on their origin that can be multipotent and unipotent

Potency of stem cells can be classified into subgroups:

1. Totipotent – differentiate into all cell types, only embryonic stem cells
2. Pluripotent – differentiate into cells of all three germ layers except extra-embryonic tissue.
3. Multipotent – also called progenitor cells, can differentiate into multipotent cells from the same germ layer origin.
4. Unipotent – have self-renewing properties of their own cell population.

1.3.1 Neural stem cells

Neural stem cells (NSCs) are multipotent cells derived from nervous system that have been identified in rodent and humans (McKay 1997; Gage 2000). NSCs can be found in embryonic, mammalian adult CNS (Temple 2001; Miller and Gauthier-Fisher 2009) and the peripheral nervous system (PNS) (Anderson 1992) (Table. 3). Dorsal telencephalon derived NSCs differentiate into neurons, astrocytes and oligodendrocytes (Teixeira, Duckworth et al. 2007). NSCs originating from the neural crest generate Schwann cells, sympathetic and sensory neurons of the PNS (Anderson 1992).

Location	NSC location
CNS embryo	Basal forebrain, cerebral cortex, hippocampus, cerebellum, neural crest, spinal cord
CNS adult	Nasal epithelium, olfactory bulbs, subventricular zone, hippocampus, retina, spinal cord
PNS	Neural crest

Table. 3 Neural stem cell location in embryonic and adult nervous system.
From (Anderson 1992; McKay 1997; Gage 2000; Miller and Gauthier-Fisher 2009)

In our studies we used primary, telencephalon stem cells (NSCs) cultured *in vitro* as adherent monolayers (Hermanson, Kristen et al. 2002). During embryonic development neural stem cells are located just above the ventricles and anterior to the neural tube. The cells can be cultured *in vitro* in well-defined media without fetal bovine serum (FBS) and maintain in multipotency under fibroblast growth factor 2 (FGF2) addition. When FGF2 is withdrawn and cells are exposed to the signaling factors BMP4, ciliary neurotropic factor (CNTF), triiodothyronine (T3), NSCs differentiate into neurons, astrocytes and oligodendrocytes respectively (Fig. 5).

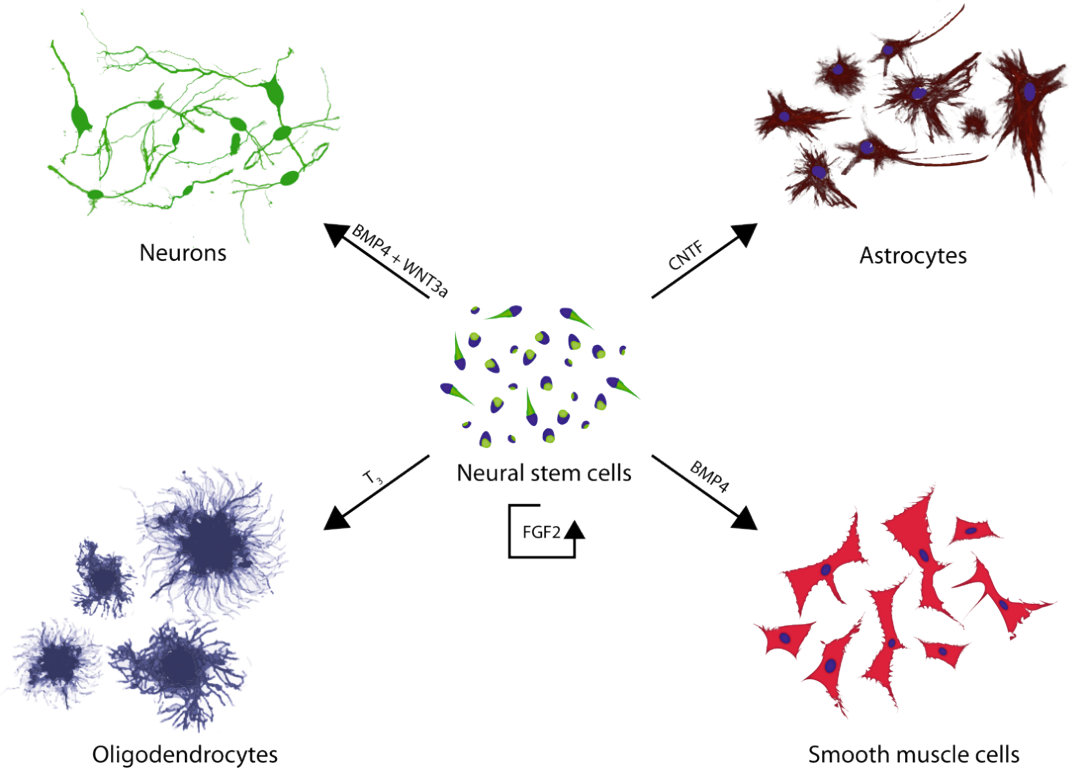

Fig. 5 Neural stem cell differentiation *in vitro*
Modified from (Teixeira, Duckworth et al. 2007)

Additionally, smooth muscle cells can be obtained by FBS treatment (Tsai and McKaym 2000; Teixeira, Duckworth et al. 2007). Also, BMP4 and Wnt3a co-treatment leads to neuronal differentiation and proliferation (Kleber and Sommer 2004; Lee, Kleber et al. 2004; Kleber, Lee et al. 2005; Leao, Reis et al. 2010; Andersson, Duckworth et al. 2011; Lewicka, Hermanson et al. 2012).

NSCs have shown promising results in preclinical regenerative medicine studies on a rat traumatic brain injury model (Lundberg, Södersten et al. 2012). The NSCs can contribute to adult neurogenesis when they are appropriately stimulated by its neurogenic environment (Ninkovic and Gotz 2007; Miller 2008; Miller and Gauthier-Fisher 2009), thus making them excellent tool in regenerative medicine.

1.3.2 Neural stem cells in cerebral cortex development

In cortical development and the stem cell surrounding it, several key factors such as FoxP2 (Tsui, Vessey et al. 2013) and CxxC5 (Andersson, Södersten et al. 2009), influence the destiny of neurons during neurogenesis. Moreover, a balance between intrinsic developmental events and environmental signaling defines the neuronal or glial fates in the developing cortex (Miller and Gauthier 2007).

During mid-embryogenesis, the cerebral cortex is formed from neuroepithelial stem cells located at the telencephalic vesicles. NSCs are located on periventricular generative zones and give rise to neurons and radial glia followed by astrocytes and oligodendrocytes. BMPs play an important environmental role in developmental events together with FGFß and Shh, which are involved in NSC expansion, self-renewal, restriction and incipient lineage commitment (Mehler 2002). The telencephalon formation starts at around E10.5 in the mouse embryo from the uniformed ventricle in the caudal part of the telencephalic vesicles and can be subdivided into cortex (the dorsla pallium) and basal ganglia (ventral subpallium). The neurogenesis occurs during mice E12 to E18, followed by astrocytes around E16 and oligodendrocytes formation starts postnatal. Cortex develops from ventricular zone in the dorsocaudal part of the telencephalic vesicles (Sur and Rubenstein 2005).

Asymmetric division of progenitor cells in the epithelium results in thickening of the neural tube (McKay 1997) that give rise to neural progenitors (NPs) in the ventricular zone (VZ). NPs undergo symmetric divisions and generate two daughter cells. The

symmetric expansion creates the subventricular zone (SVZ) just above VZ. Many of the neocortical projection neurons are generated from terminal division of the dorsal telencephalon at the VZ and SVZ (Kwan, Sestan et al. 2012). The earliest neurons appear around E10.5 in the mouse and some NPs become radial glia cells (RGC). RGCs can function as progenitors, undergo neurogenic asymmetric divisions in the VZ and give rise to intermediate progenitors (IPs) that will migrate away from VZ to the SVZ to undergo further neurogenic divisions. Moreover RGCs provide support for the radial migration of the cells to their destination in the mantle layers in the neocortex. Studies have shown that progenitor RGC will give rise to glutamatergic pyramidal-projection neurons in the cortex (Kriegstein, Castañeda-Castellanos et al. 2004; Noctor, Martinez-Cerdeno et al. 2008).

The first rounds of mitosis generate the first post mitotic neurons, called preplate neurons (PP) a superficial part of the cerebral wall. The preplate will be divided into superficial layer (marginal zone) and subplate (Kwan, Sestan et al. 2012) by inward radial migration of the cortical neurons and the new cortical plate layer is formed. The inside-out mechanism of cortical neuronal growth results in the earliest born neurons being located in the deep layer 6 and the latest born neurons in the superficial layer 2 (Kriegstein, Castañeda-Castellanos et al. 2004; Noctor, Martinez-Cerdeno et al. 2008) (Fig. 6).

Accumulation of IPs and newborn neurons form the cortical plate, and cell division, migration and maturation result in an increase in the cerebral cortex surface area and finally the folding of the cortex (Molyneaux, Arlotta et al. 2007).

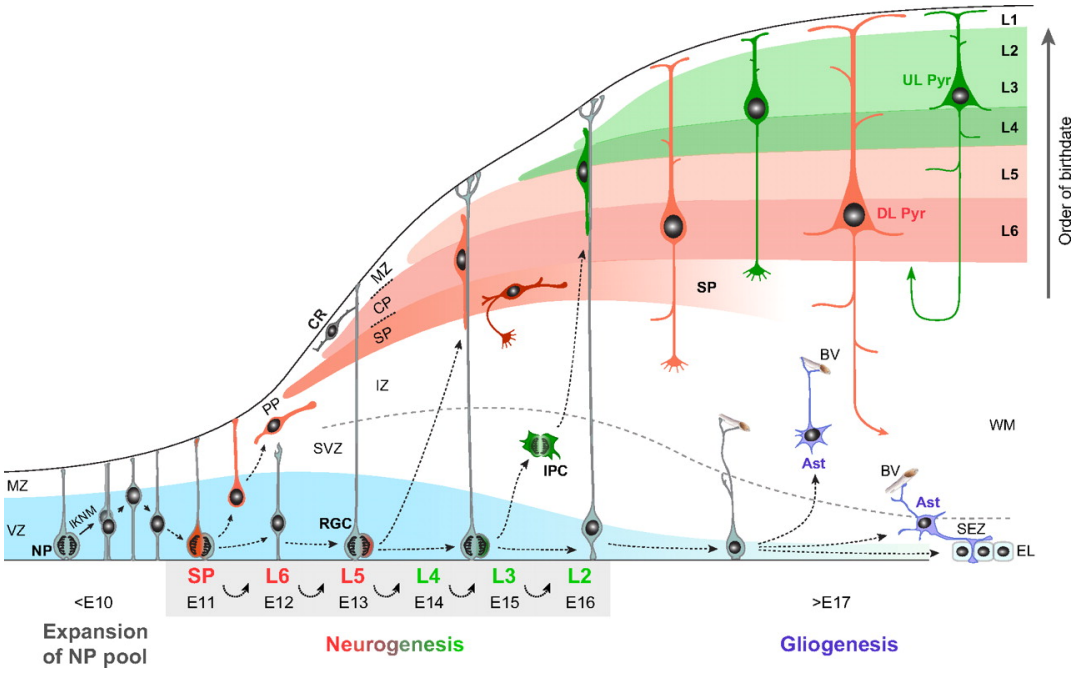

Fig. 6 Neuron generation and migration in the mouse cortex (Kwan, Sestan et al. 2012)

1. 4 Stem cells microenvironment

1.4.1 Stem cell niche

The human body is built from four types of primary tissue:

1. Epithelial – eg. skin
2. Connective – eg. blood, ligaments and tendons
3. Muscle – cardiac, skeletal, smooth
4. Nervous – neurons and glia.

All four types of tissue stated above are controlled directly by physical, chemical and biological processes. Stem cell population originates in a "niche" – defined by elements of local environment regulating the stem cell state by architectural components, physical engagement between cells, cell-cell and ECM-cell signaling, paracrine and endocrine stimuli, neural input and metabolic products of tissue activity (Scadden 2006). Moreover the niche can influence tissue generation, maintenance and repair.

In 1907, Ross Harrison established cell culture and years after, altered animal research models to study cells in petri dishes in two dimensions (2D)(Landecker 2007). However, cells in traditional culture can only proliferate in x-y direction in contrast to *in vivo* events, which are happening in 3D x-y-z axis (Discher, Mooney et al. 2009; Schwartz 2010). Stem cells grown on polystyrene (PS) culture dishes in 2D are deprived of their normal environment. To make the experimental design resemble *in vivo* conditions, it is important to mimic some of the cells' natural habitat, for example by using extracellular matrix (ECM) components (Badylak, Freytes et al. 2009; Chen 2010).

When culturing cells *in vitro*, the following factors are important to consider:

1. Chemical and biochemical environmental factors: cell adhesion molecules (ECM), cytokines, integrins, hormones and growth factors.
2. Physical environmental factors: force, stiffness and rigidity.
3. Spatial and temporal environmental factors.

All these factors influence cellular adhesion, migration, proliferation, differentiation, as well as signaling pathways (Schmeichel and Bissell 2003; Engler, Sen et al. 2006; Scadden 2006; Widhe, Bysell et al. 2010; Brizzi, Tarone et al. 2012). The stem cell

niche controls NSC behaviour and defines their function (Miller and Gauthier-Fisher 2009).

Endo and colleagues have shown that the matrix protein anosmin is necessary for cranial neural crest formation together with FGF, BMP, and Wnt modulation (Endo, Ishiwata-Endo et al. 2012). Their data support the importance of the *in vivo* niche environment.

Extrinsic environmental factors can influence neural precursor cell proliferation during embryonic central nervous system development (Bartkowska, Paquin et al. 2007).
Further, the importance of cell and ECM molecule interactions can be observed in brain impairments such as schizophrenia, memory impairment, sarcoma tumor (Brennaman, Kochlamazashvili et al. 2011; Bartus, James et al. 2012; Berretta 2012; Cui, Freeman et al. 2013; Fong, Lamhamedi-Cherradi et al. 2013).

Incorporating 3D cell substrate in *in vitro* experiments became possible after Elsale and Bard's pioneer experiment in 1972. They used collagen I matrices polymerized *in vitro* to study 3D fibrous network. Since then, 3D scaffolds have become popular for studying cell behavior and physiology in a more physical environment. Yet, there are only few simple and reproducible protocols to generate stable 3D scaffolds compatible for stem cell cultures.

1.4.2 Biomaterials for tissue engineering

The aim of tissue engineering is to develop biocompatible materials to restore, maintain and improve human health (Vacanti 1993). A biomaterial is any material, substrate or compound that interacts with a biological system. They can be natural polymers, including collagen I, fibrin, matrigel and silk; synthetic polymers, such as polyglycolic acid, polylactic acid and polyethylene glycol; or ceramics, metals and semiconductors (Widhe, Bysell et al. 2010). The advantage of natural materials is their biomimetic properties. However, the unknown composition and having animal or cancer origin, does not allow for full experimental control. Synthetic substrates allow tailor made design and structure characterization depending on the requirements.

Early steps in the biomaterials field, were initiated by Romans, Chinese and Aztecs more than 2000 years ago where they established the usage of gold in dentistry

implants. Nowadays, we take dental, orthopedic and cardiovascular implants for granted, just like contact lenses and surgical sutures, even though it took a long time to obtain such knowledge.

Depending on the need, biomaterials require specific physical or chemical properties eg. flexibility, strength, non-toxicity, and biodegradability.

The alternatives of 3D scaffolds for tissue engineering on the market are vast due to its physiological relevance (Pampaloni, Reynaud et al. 2007; Dutta and Dutta 2009; Justice, Badr et al. 2009). Its microenvironment can alter the cell phenotype by being a stimulus, effecting proliferation, viability and differentiation (Teixeira, Hermanson et al. 2010; Wojcik-Stanaszek, Gregor et al. 2011) due to changes in stiffness (Teixeira, Ilkhanizadeh et al. 2009), mechanotransduction (Vogel 2006; Hoffman, Grashoff et al. 2011) or material functionalization (Pashuck and Stevens 2012; Widhe, Johansson et al. 2013). In a context dependent manner – cell type, tissue, and organism - we need to custom make cell-material interactions. The spatial and temporal control of a cell's physical and chemical niche is an important (Lutolf, Gilbert et al. 2009) driving force of biomaterials research (Huebsch and Mooney 2009), presenting a complex range of applications in humans (Place, Evans et al. 2009).

1.4.3 Spider silk protein

Humans have used silk polymers from silkworms and spiders for a long time. About 5000 years ago, Chinese started farming the silkworm *Bombyx mori* for cloth production and it was boosted in the 18th century by stocking and glove manufacturing. Moreover, the extraordinary properties of the nests of orb weaving spiders (Araneidae) led to their use in fishing as well as in medical applications, to stop hemorrhage or for wound healing. The mechanical and chemical assets of spider silk became of interest for regenerative medicine and triggered the production of the recombinant spider silk on an industrial scale.

All spiders produce silk in the major ampullate gland that is strong, extensible, lightweight, viscoelastic and biodegradable (Omenetto and Kaplan 2010; Eisoldt, Smith et al. 2011; Widhe, Johansson et al. 2012). These properties makes spider silk superior to most man-made fibers (Table. 4).

Material	**Strength, G_{max} (GPa)**	**Extension ε_{max}**	**Toughness (MJ m^{-3})**
Dragline silk	0.8-1.5	0.15-0.39	96-230
Bombyx mori silk	0.6	0.18	70
Tendon collagen	0.15	0.12	7.5
Kevlar 49 fiber	3.6	0.027	50
Elastin	0.002	1.5	2
High tensile steel	1.5	0.008	6

Table. 4 Tensile properties of the materials, modified from (Omenetto and Kaplan 2010)

Genetic research revealed variability in silk protein among different species and prioritized recombinant silk spidroins production from certain spiders. The solubility of silk aggregates allows us to construct different scaffold formats, eg. fibers, meshes, films, foams, gels, sponges, textiles and hydrogels that are thermally and chemically stable (Omenetto and Kaplan 2010; Widhe, Johansson et al. 2012) (Table. 5)

Spider silk formats	Features	Publication
Films	Smooth surface with small granules (~10μm), 2μm thick, coherent and flexible	Widhe et a., 2010
Foams	Heterogeneous mounds and craters (30-200μm in diameter), 1-2mm thick	Widhe et a., 2010;
Fibers	Fiber bundles of varying thickness, 0,1-20μm irregular grooves and ridges	Widhe et a., 2010;
Meshes	Fiber bundles of varying thickness, 10-70μm irregular grooves and ridges	Widhe et a., 2010;
Gels: hydrogels, porous sponges	High water content, nonlinear viscoelastic material response, low stiffness and strength	Kluge et al., 2008
Spheres/capsules	Stable thin polymer shell	Kluge et al., 2008
Textiles	Fibers 10-60μm in diameter	Kluge et al., 2008

Table. 5 Spider silk biomaterial formats

Moreover, the silk proteins can self-assemble into functional materials (Kluge, Rabotyagova et al. 2008; Widhe, Bysell et al. 2010), which make them suitable for cell culture experiments. Spider silk proteins thus allow surface modification, eg. addition of Arg-Gly-Asp (RGD) peptides, a cell-binding domain that reportedly increases oligodendrocyte differentiation; Ile-Lys-Val-Ala-Val peptides (IKVAV) – that reportedly stimulates differentiation towards astrocytes (Kluge, Rabotyagova et al. 2008; Widhe, Johansson et al. 2013). Moreover, spider silk protein could be used as a drug delivery carrier, for instance as microcapsules.

Previous evidence demonstrated the applicability of spider silk protein in regenerative medicine. There is no tissue response to the material: it is well accepted by the host and does not cause chronic inflammation (Brown and Phillips 2007; Fredriksson, Hedhammar et al. 2009; Rising 2013). Moreover, migration of new cells into the wound has been observed.

In the present studies (Paper III and IV), spider silk protein was therefore chosen due to its ideal biomaterial properties and satisfactory qualities for regenerative medicine (Vepari and Kaplan 2007; Widhe, Bysell et al. 2010). The recombinant spider silk (4RepCT) used in the study, was produced by Spiber^{TM}. The protein was obtained from the spider *Euprosthenops australis*, which makes one of the strongest and most extensible draglines (Rising, Johansson et al. 2007; Hedhammar, Rising et al. 2008).

2 AIMS OF THE THESIS

The present thesis focuses on understanding of the molecular mechanisms of stem cell differentiation into mature and functional cortical cell populations. The nature and structure of the cortex motivated us to verify new materials for stem cell culture and perform part of the study in a more physiologically relevant 3D environment.

Specific aims:

- Understanding the mechanisms underlying cell density-dependent BMP4-mediated differentiation of NSCs (Paper I).

- Understanding the mechanisms underlying BMP4 and Wnt3a-mediated neuronal differentiation of NSCs (Paper II).

- Identifying novel biocompatible substrates for efficient culturing of neural stem cells (Paper III).

- Generating functional neuronal circuits from neural stem cells on 3D substrate generated from recombinant spider silk protein (Paper IV).

3 RESULTS AND DISCUSSION

3.1 Paper I: Noggin and Wnt3a enable BMP4-dependent differentiation of telencephalic stem cells into GluR-agonist responsive neurons.

Signaling factors such as BMP, Wnt, FGF and Shh regulate spatial and temporal development of the telencephalon. *In vitro* experiments have shown that cortical stem cell differentiation relies on signaling as well as on contact dependent mechanisms (Tsai and McKaym 2000; Andersson, Duckworth et al. 2011). In both cases the BMP4 involvement is not well understood.

We investigated BMP4 signaling mechanism on neural stem cells in high-density (HD) and low-density (LD) plating. Bone morphogenetic protein 4 (BMP4) stimulation of NSCs plated at HD resulted in increased neuronal differentiation. However, NSCs plated at LD responded differently, and did not show any increased neuronal differentiation but instead significantly increased numbers of mesenchymal smooth muscle cells. At both conditions, BMP4 induced astrocytic differentiation. Gene expression profiling studies using microarrays 3h after BMP4 stimulation revealed a dramatic increase in the expression of the BMP4 antagonist noggin, in addition to increased expression of other factors such as the Wnt-signaling inhibitor CXXC5 with links to DNA methylation, and Spark like 1.

Studies using noggin inhibitor together with conditioned media experiments indicated that noggin was produced by NSCs stimulated with BMP4 and the levels of noggin in the media correlated with the seeding density. Theses results suggested that high noggin levels in the cell culture media in HD cultures inhibited predominantly the mesenchymal differentiation, but did not explain the increase in neuronal differentiation.

We further observed differences when NSCs were co-treated with Wnt3a in addition to BMP4. Co-treatment resulted in increased yield of mature neurons and their physiological activity. Physiological activity was assessed by calcium imaging studies, stimulation with ATP and the glutamate receptor (GluR) agonist AMPA. Only around

5% of BMP4-induced neurons responded to glutamate receptor (GluR) agonist compared to over 25% after BMP4+Wnt3a treatment.
Ca^{2+} studies and immunocytochemistry results showed increase neuronal maturation of the BMP4+Wnt3a co-treated cells that were expressing markers for postmitotic neurons MAP2 and synaptotagmin - a membrane protein of synaptic vesicles.

This study improved our understanding of the roles for BMP4, Wnt3a and noggin in telencephalic stem cell differentiation. It should be noted that noggin and cell contact in HD culture are not solely the key factors for the switch between mesenchymal and neuronal fate. Certain transcriptional co-factors could be involved as well, such as CBP (Wang, Gallagher et al. 2012) that regulates chromatin structure.

The involvement of Wnt3a in the BMP4-induced differentiation to mature and functional pyramidal-like neurons emphasized the importance of spatial organization of signalling factors, as the complementary expression pattern of BMP4 and Wnt3a *in vivo*, required for proper development of hippocampal pyramidal neurons, thus could be at least to an extent mimicked by *in vitro* administration of the two factors.

3.2 Paper II: BMP4-induced neuronal differentiation of cortical stem cells involves a Hes6-dependent GABAergic component.

The co-dependence of BMP4 and Wnt3a signaling plays important roles throughout the development of the telencephalon. However mechanisms underlying the differentiation events still need further investigation. To study the molecular differentiation program of cortical development *in vitro*, we continued to use embryonic cortical stem cells and growth factors Bmp4 and Wnt3a that regulate the development of the telencephalon *in vivo*. While our previous study suggested that levels of noggin and other factors may inhibit mesenchymal differentiation by BMP4 signaling, the factors or mechanisms underlying the increased neuronal differentiation by BMP4 alone and co-treatment with BMP4 and Wnt3a remained unidentified.

Whole transcriptome sequencing by RNA-seq of NSCs stimulated with BMP4 or BMP4+Wnt3a for 3 weeks showed that markers representing all cortical layers were expressed to a significant extent in these cultures (Tbr1, Cux2, Lhx2, Lmo4, Otx1, Tle4, Lxn etc).

Moreover, excitatory neuronal markers Glud1, Gria2, Grina, Grm3 were also observed, in line with the finding that BMP4 and BMP4+Wnt3a could induced differentiation into glutamate receptor agonist-responsive neurons. Surprisingly, markers for inhibitory (GABAergic) neurons were also observed in the RNA-Seq (Gabarap, CCK, NPY, Dlx5).

To investigate if this gene expression correlated with an increase in GABAergic cells we used calcium imaging experiments. While we found few cells to be GABA-responsive after 2-3 weeks of differentiation, we found a transient population of GABA-responsive cells at days 5-7 of treatment which diminished with further differentiation.

This observation was validated with gene expression studies that confirmed the increased and many times transient expression of genes specific for GABAergic neurons: Dlx2, DLX5, CCK, NPY, GAD1.

To investigate the factors underlying the facilitated neuronal differentiation after BMP4 and BMP4+Wnt3a treatment in more general terms, we first investigated the expression of REST, a DNA binding protein and a well known transcriptional repressor of neuronal genes. The expression of REST, however, did not correlate with neuronal differentiation (Hermanson 2008). Although REST is a well-studied factor, it is well-known from the literature that many different factors can influence balance between intrinsic development and niche formation, resulting in changing of the cell fate (Bartkowska, Paquin et al. 2007; Miller and Gauthier 2007; Wang, Weaver et al. 2010; Wang, Gallagher et al. 2012; Tsui, Vessey et al. 2013).

To further focus on the mechanisms underlying BMP4 or BMP4+Wnt3a involvement in the GABAergic differentiation, we implicated siRNA-mediated knockdown of Dlx5, identified in the RNA-Seq experiments, and Spark like 1, identified in the early microarray experiments, but no differences in GABAergic gene expression were observed after such knockdowns compared to control cultures (Itaba-Matsumoto, Maegawa et al. 2007) (Gongidi, Ring et al. 2004)

We noted an enrichment in the neurogenic bHLH-factor Hes6 (Jhas, Ciura et al. 2006; Methot, Hermann et al. 2013) in our RNA-Seq experiments, and a temporal study of the expression of Hes6 at several points after BMP4 and BMP4+Wnt3a treatment revealed a reproducible increase with around 100% of Hes6 mRNA compared to control cultures. Indeed, Hes6 siRNA knockdown resulted in a decrease in the expression of GABAergic associated genes (Dlx2, Gad1) after 24h treatment with BMP4 or BMP4+Wnt3a.

Hes6 pro-neurogenic involvement in cortical GABAergic neuron differentiation in *in vitro* cell cultures highlighted the importance of niche signaling. For further understanding of GABAergic cells growth during development available animal models could be used. This will elicit our understanding of human diseases related to the cortical malformations such as microcephaly and lissencephaly.

3.3 Paper III: Recombinant spider silk matrices for neural stem cell cultures.

The mechanical and physical properties of recombinant spider silk protein suggest it to be an ideal biomaterial. We decided to conduct biocompatibility studies and culture NSCs on spider silk protein (4RepCT) to determine the possible application of substrate usage in preclinical therapeutic application.

In standard/control experiment, NSCs are cultured on culture plates pre-coated with Poly-L-ornithine (P) and fibronectin (F). Plates with 4RepCT were initially pre-coated similar to controls, but also not pre-coated, only rinsed twice with PBS and pre-incubated with DMEM/F12 media at 37^0C, were used.
Surprisingly, we found that 4RepCT substrate is as efficient to culture NSCs in 2D without any additional coating. Since the recombinant spider silk protein, generated as a thin "film" substrate, did not cover the whole petri dish, we noted a sharp line of NSCs on the edge of the spider silk substrate but no NSCs grew on the uncoated polystyrene plastic. The result suggested that the recombinant spider silk protein 4RepCT generated as a film substrate supported NSC survival and expansion.

NSCs seeded on 4RepCT substrates expanded well in presence of the FGF2 for 48h however they appeared to be in lower numbers compared to control. The cells expressed nestin that is a well-known marker for undifferentiated NSCs and low (astrocytes) or no staining for other markers (neurons and oligodendrocytes) were detected. To quantify the observations, we performed proliferation studies with EdU assay. Observations showed no changes between the control and experimental matrices after 72h-96h, pointing to differences in time for cell adhesion rather than proliferation. Cells cultured on spider silk substrates showed no significant differences in cell viability compared to control in Live/Dead assay studies as well as through microscopic observation under of DAPI stained cells, presenting condensed or pyknotic nuclei.

To investigate whether the NSCs retained the multipotent phenotype when grown on 4RepCT film structures, we stimulated the cultures with various differentiation-inducing factors. NSCs stimulated with CNTF differentiated into astrocytes on 4RepCT similar to controls. Similarly, NSCs stimulated with BMP4 or BMP4+Wnt3a on

4RepCT showed neuronal differentiation potential as efficient as the controls as assessed by markers and morphology.

NSCs stimulation with T3 for 7 days to obtain oligodendrocytes on spider silk protein showed lower proportion ($p<0.05$) of MBP+ cells compared to control matrices.

As efficient as in standard protocols, biocompatibility of spider silk protein with NSCs opens new frontiers towards novel biocompatibility applications for other types of stem cells, such as embryonic stem (ES) and induced pluripotent stem (iPS) cells. It should, however, be noted that the underlying mechanism for the survival and self-renewal of the NSCs demonstrated when grown on uncoated 4RepCT structures is still unknown. It is tempting to hypothesize that the 4RepCT substrate stimulated the NSCs to produce extracellular matrix components allowing attachment to the scaffold. Future experiments may investigate whether 4RepCT exposure triggers changes in gene expression in the undifferentiated NSC population.

Substrates generated from recombinant spider silk protein, such as 4RepCT, are incredibly strong and modifiable, and offer a variety of possibilities to generate new structures such as 3D sponges for stem cell culture and pre-clinical optimization studies. Spider silk protein features and NSCs applicability in medical and therapeutic application such as drug screening make those an ideal tool for regenerative medicine studies (Rising 2013). Usage of spider silk protein in cell culture studies has been a growing trend. New formats of material design – foams, hydrogels – as well as other promising properties, strengthens its application in regenerative medicine studies.

3.4 Paper IV: Building brains in spider webs: Stem cell-derived GluR- agonist responsive neurons grown in 3D matrices of recombinant spider silk protein.

The two dimensional structure (2D) of cell culture dishes create obstacles for neuronal outgrowth, axonal branching, cell-cell interactions and cell-ECM interactions. Further, its mechanical and physical properties do not mimic the *in vivo* environment. The future of regenerative medicine relies on 3D-designed biomaterial substrates that reflect the *in vivo* environment. Moreover, it is important to use 3D scaffolds in preclinical application for better understanding of the ontogeny of neurons, signaling mechanisms and cell activity (Schmeichel and Bissell 2003; Nam, Onodera et al. 2010; Ribeiro, Vargo et al. 2012).

We therefore investigated the physiological activity of NSCs differentiated with BMP4 and BMP4+Wnt3a for 3 weeks on 3D scaffolds generated from recombinant spider silk protein (4RepCT) and shaped in a "foam" (sponge-like) structure. These experiments showed an increased Ca^{2+} activity of AMPA-responsive neurons differentiated on the 3D 4RepCT matrix. NSCs treated with Bmp4+Wnt3a on foam were in average actually two times more active than on plastic, although the variation of the results excluded statistical significance.

Immunocytochemistry studies revealed a 37% increase in mature MAP2+ neurons differentiated with BMP4+Wnt3a on 3D spider silk protein compared to cells cultured on 2D polystyrene plates. During BMP4 differentiation, we observed a 43% increase of neurons cultured on foam compared to plastic.

Our data highlight the possibilities of 3D substrates in enhancing differentiation and possibly the formation of neuronal networks *in vitro*. Moreover we further believe that a 3D substrate microenvironment promotes the neuronal activity, cell-cell interactions and cell-ECM interactions, which result in cell morphology and function closer to the one observed *in vivo*. It is possible that this 3D approach could help us understand several diseases that involve dysfunctional neuronal networks such as mood related and other psychiatric disease and apply this technique in drug screening to improve treatment in personalized medicine.

4 MATERIALS AND METHODS

Stem cell culture

Neural stem cells (NSCs) were isolated from cerebral cortices of timed pregnant Sparque Daley E15 rat embryos and cultured as described before (Brunkhorst et al., 2005; Hermanson et al., 2002; Johe et al., 1996). Briefly, NSCs were mechanically dissociated in well-defined, serum free DMEM/F12 medium (Gibco) enriched with N2 supplements. Cells were seeded in adherent monolayers on dishes pre-coated with poly-L-ornithine and fibronectin (Sigma-Aldrich). Cells used in the experiments were from the second passage at a density of 10 000cells/cm^2 (high density) and 500 cells/cm^2 (low density). The cells were maintained in proliferative state and/or expanded using 10ng/ml FGF2 (R&D). During differentiation, FGF2 was withdrawn from the culture and replaced with differentiation factors: BMP4 -/+ Wnt3a (R&D, 10ng/ml) to obtain neurons; CNTF (R&D, 10ng/ml), T3 for astrocytic differentiation (R&D, 10ng/ml) and FBS to obtain smooth muscle (Sigma-Aldrich). Cultures were treated with BMP4 and CHAPS (Wnt3a control, Sigma-Aldrich) then growth factor(s) were added every 24h while fresh media was replaced every 48h.

Ethical protocols

Northern Stockholm's animal research ethics committee granted ethical permission for all experiments including tissues and animals.

siRNA knowdown by nucleofection

Neural stem cells were nucleofected using ON-TARGETplus rat siRNA smartPOOL (Thermo Scientific). Experiment was performed with a Nucleofector (LONZA) using Rat NSC nukleofector kit (cat: # VPG 1005) according to manufacturer's instructions. Cells were expanded to 90% confluency and 2 x 3 x 100 mm plates were used per nucleofection. Cells were nucleofected with 1-3 ug of siRNA (depending on siRNA) using program A-33. Thus, 10'000 cells / cm^2 were seeded with FGF2 for 24h before the differentiation starts, followed by collection at time points of interest. Control siECEFP experiments with same siRNA concentration of interest were performed to each experiment then used for results normalization. Titration of siRNA was performed at the beginning to assess the optimal concentration and efficiency of nucleofection was measured by gene expression for each independent experiment.

Immunocytochemistry

Cell cultures were rinsed once in Phosphate-Buffered Saline (PBS) (Sigma-Aldrich) and then fixed with 10% Formalin (Sigma-Aldrich) for 20 min at room temperature (RT) followed by permeabilization for 3 X 5 min with PBS/0.1% Triton-X 100 (Sigma Aldrich). Primary antibodies were diluted in PBS/0.1% Triton-X 100/1% bovine serum albumin (BSA; Sigma Aldrich) then incubated overnight at 4°C. Primary antibodies sources and dilutions were as follows: rabbit polyclonal anti-glia fibrillary acidic protein (GFAP; 1:500; DAKO), mouse monoclonal anti-Neuronal Class III B-Tubulin (TuJ1; 1:500; Covance), mouse anti-intermediate filament protein nestin (1:500; BD Pharmingen), rat anti-MBP monoclonal (MAB386; 1:250; Chemicon). The cells were washed 6 X 5 min each with PBS/0.1% Triton-X 100. Secondary antibodies were incubated in PBS/0.1% Triton-X 100/1% BSA at room temperature for 1h. Secondary antibodies were species-specific labeled with Alexa-488 or Alexa-594 (1:500; Molecular Probes). Next, the samples were washed 3 X 5 min each in PBS and mounted with Vectashield including DAPI (Vector Laboratories, Inc) or excluding DAPI. Fluorescent images were acquired with Axioskop software using Zeiss Axioskop2 microscope coupled to an MRm (Zeiss) camera at a 10X and 20X magnification.

Viability assay

After second passage, NSCs were seeded at a density of 10'000cells/cm^2 in 35 mm plates pre-coated dishes and expanded in N2 media + FGF2 for 24h. The NSCs were then differentiated with BMP4 or BMP4+Wnt3a for 5, 7 and 14 days. Cell viability was assessed using a Live/Dead kit on 35mm dishes (Cat: #04511, Sigma). Briefly, NSCs were washed twice with RT PBS and a control setup incubated with 70% ethanol was used in parallel to each experiment. NSCs were incubated with 500 µl of 10x (Ethidium bromide + Calcein in PBS) solution for 5 min at 37°C then rinsed shortly with RT PBS. Quantification of the dead cells relative to the number of live cells from five independent locations on each plate was calculated manually and presented as mean with +/-S.E.M.

Proliferation studies

EdU assay. NSCs were incubated for 30 min in EdU (Click-iT EdU Kit, Invitrogen) according to manufacturers recommendations. Briefly, NSCs were fixed for 20 min

with 10% formalin, permeabilized 3 X 5 min with 0.5% Triton-X 100 followed by two washes in PBS with 3% BSA. 0.5 ml Click-iT reaction cocktail (1X Click-iT reaction buffer, CuSO4, Alexa Fluor azide, reaction buffer additive) was added to each well followed by 30 min incubation at RT. Finally, cells were washed twice with PBS, stained 10 min with Hoechst then mounted for visualization. Quantifications of the fractions of EdU-positive cells relative to the number of Hoechst-stained cells was presented on a chart as a mean with +/- S.E.M.

BrdU assay. 50μM Bromodesoxyuridine (BdrU, BD Pharmigen^{TM}) was added to cells media and incubated for 1h at 37°C followed by media aspiration, two washes with PBS (Gibco) and fixation with 10% Formalin (Sigma-Aldrich) for 20 min at room temperature (RT). Next, cells were washed 3 X 5 min with cold PBS and incubated on a shaker 60 min in 1 M HCl at RT. Further, plates were washed twice with PBS, permeabilized for 3 X 5 min with PBS/0.1% Triton-X 100 (Sigma-Aldrich) then incubated with primary anti-BrdU antibody (Abcam, 1/500 cat: # 6326) in PBS/0.1% Triton-X 100/1% bovine serum albumin (BSA; Sigma-Aldrich) overnight at 4°C. Subsequently, six washes 5 min each with PBS/0.1% Triton-X 100 and secondary antibodies incubation in PBS/0.1% Triton-X 100/1% bovine serum albumin (BSA; Sigma-Aldrich) for 1h at RT. Secondary antibodies were washed out 3 X 5 min with PBS and cells were covered with mounting media Vectashield including DAPI (Vector Laboratories, Inc). Fluorescent images were acquired with Axioskop software using Zeiss Axioskop2 microscope coupled to an MRm (Zeiss) camera at a 10X and 20X magnifications. . Quantifications of the fractions of BrdU-positive cells relative to the number of DAPI-stained cells was presented on a chart as a mean with +/- S.E.M.

RNA isolation and quantitative/semi quantitative RT-PCR

RNA was isolated using RNeasy kit (Qiagen) standard protocol. DNA contamination was removed using RNase free DNase I (Qiagen). First strand cDNA was obtained using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). For quantitative RT-PCR, Platinum SYBR green mix (Invitrogen) was used and run on Applied Biosystems 7300 Real Time PCR system . Primers used for the quantitative RT-PCR were designed to span introns whenever possible. To further exclude DNA contamination, -RT (no reverse transcriptase) samples were done as controls. Data were analyzed using standard curve method and house keeping TATA-binding protein (TBP) was used to normalize the results for each experiment.

Gene expression profiling using whole genome transcriptome (RNA-Seq) approach.

Total RNA was extracted from NSCs stimulated with BMP4 or BMP4+Wnt3a for 3 weeks. We used the TrueSeq RNA sample Preparation kit (Catalog # RS-122 2001) to generate mRNA libraries from total RNA. Triplicates of each sample were sequenced and analyzed by Solexa/Illumina Hi-seq. After pre-filtering the raw data by removing sequenced adapters and low quality reads, the sequence tags were aligned to the rat genome (rn4) with Bowtie alignment tool (Langmead, Trapnell et al. 2009). We used rpkmforgenes program to estimate the expression of each gene.

Statistics

For every experiment in all studied conditions, 5 random pictures obtained at
20X magnification with the Zeiss Axioskop2 microscope were picked for manual quantification. The nucleus was stained with DAPI/Hoechst, each micrograph depicted around 150 to 1000 cells in fields of a diameter of around 500 mm. GraphPad Prism 4.0 (GraphPad Software, San Diego, California) was used for statistical analysis of the data. One-way ANOVA followed by unpaired t-test were used for cell counts in wells. P-values < 0.05 were considered significant.

Production of protein matrices

The recombinant miniature spider silk protein 4RepCT was produced in *Escherichia coli* and purified as described previously (Hedhammar, Rising et al. 2008) including depletion of lipopolysaccharides (LPS). The protein was purified and sterilized by passage through 0.22 mm filter and concentrated to 3 mg/mL by ultrafiltration (Amicon Ultra, Millipore) before preparation of matrices. The protein was assembled in two different formats: film and foam (Widhe, Bysell et al. 2010). The matrices were air dried over night at RT under sterile conditions and stored at RT. Before the matrices were used in experiments they were washed twice with sterile PBS and pre-incubated with serum-free DMEM:F12 media (Gibco) for 1 h at 37 °C with 5% CO_2. For more detail information about surface morphology, scanning electron micrographs and in depth analysis of the substrates, please see (Widhe, Bysell et al. 2010).

Calcium recordings

All Calcium measurement experiments on neurons and astrocytes derived from NSCs were carried out in Kreps-Ringer buffer with or without Ca^{2+} at 37°C in a heat-

controlled chamber (Warner Instruments). Cells were incubated with Ca^{2+}sensitive fluorescence indicator Fluo-3/AM (5 μM, Life Technologies) mixed with 0.1% Pluronic f-127 (Life Technologies) in Krebs solutions. The solution was added for 20 min to the cells medium at 37°C. All Ca^{2+}measurements were carried out in Krebs-Ringer with a cooled EMCCD QuantEM 512:SC camera (Photometrics) combined with an upright microscope (Zeiss Axio Examiner.D1) set with a W-Plan apochromat 20X/1.0 objective (Carl Zeiss). Filter wheel (Sutter Instrument) was used to obtain excitation at 495 nm at sampling frequency 0.5 Hz. MetaFluor (Molecular Devices) was used to control all devices and to analyze acquired images. Cells were constantly perfused (1 ml/min) with Krebs–Ringer's solution using a peristaltic pump (Ismatec). The drugs used for Ca2+ recordings were AMPA (10 μM; Tocris) and ATP (10 μM; Sigma) and applied in the bath as describe in previous protocol (Andersson, 2011). The MATLAB (The MathWorks Inc.) software was used to determinate response to AMPA and ATP as described previously (Uhlen, 2004). Response more then 30% compared with the baseline was considered as a response.

5 CONCLUSIONS

- BMP4-mediated differentiation of NSCs involved a regulation of the BMP-inhibitor noggin that may underlie the different outcomes of BMP4-stimulation in low versus high density-seeded cultures. (Paper I)

- Wnt3a acted with BMP4 in a synergistic way in neuronal differentiation process, and increased the fraction of glutamate receptor agonist-responsive neurons in NSC cultures. (Paper I)

- BMP4 treatment of NSCs further induced a GABAergic differentiation event with or without Wnt3a that required intact expression of the neurogenic transcription factor Hes6. (Paper II)

- Matrices generated from recombinant spider silk protein were found to be almost completely biocompatible with NSC cultures, with or without coating of extracellular matrix proteins, and except for a slight negative effect on oligodendrocyte differentiation, NSCs seemed to retain full differentiation potential when expanded on these substrates in the presence of FGF2. (Paper III)

- 3D matrices generated from recombinant spider silk protein provided an excellent substrate for BMP4+Wnt3a-mediated differentiation of NSCs into functional neurons. (Paper IV)

We propose that the improved technical protocols and increased understanding for BMP4-mediated differentiation of NSCs *in vitro* will contribute to improved models for drug development. For example, NSCs derived from patients (e.g., by iPS cells) can in relatively short time form neuronal 3D circuits that possibly more reliably and accurately mimic neuronal activity *in vivo*. We aim at in the very near future investigate the appropriateness of the spider silk-based 3D NSC cultures and BMP4+Wnt3a-induced neuronal cultures *in vivo* by transplantations in animal models of stroke and irradiation damage.

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