N-syndecan and HB-GAM in neural migration and differentiation: modulation of the growth factor activity in brain

Anni Hienola

Neuroscience Center
Institute of Biotechnology
Department of Biological and Environmental Sciences
Faculty of Biosciences
University of Helsinki

Finnish Graduate School of Neuroscience

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Supervised by:

Professor Heikki Rauvala
Neuroscience Center
University of Helsinki, Finland

Reviewed by:

Docent Juha Partanen
Institute of Biotechnology
University of Helsinki, Finland

and

Docent Kirsi Sainio
Faculty of Medicine
University of Helsinki, Finland

Opponent:

Professor Takashi Muramatsu
Faculty of Psychological and Physical Science
Aichi Gakuin University, Aichi, Japan

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V. N-syndecan deficiency impairs neural migration in brain. [5]**Hienola A**, Tumova S, Kulesskiy E, Rauvala H. J Cell Biol 2006, 174(4), 569–80.

[1]Author analyzed the morphology of the HB-GAM overexpressing and knockout mouse hippocampal structures.

[2]Author analyzed the morphology of the syndecan-3 knockout mouse hippocampal structures.

[3]Author designed all experimental setups, was responsible for the breeding and genotyping of the knockout animals, tissue sample collection, sample preparation and analysis in all *in vitro* and *in vivo* experiments described in the paper. Author organized and wrote the manuscript with input from co-authors.

[4]Author designed and performed the *in vitro* experiments concerning the functional roles of the individual TSR domains and the di-TSR fragment of HB-GAM in neural cell migration, in relation to the function of the full length HB-GAM.

[5]Author designed and performed all the *in vivo* and *in vitro* analyses of neural cell migration in the N-syndecan knockouts, the immunolocalization experiments in forebrain cells and in the cerebrum, and the HB-EGF/N-syndecan binding assays. Author assembled and wrote the manuscript with input from co-authors.

Abbreviations

BrdU	5-bromo-2-deoxyuridine
CASK	calcium/calmodulin-dependent serine protein kinase
CNS	central nervous system
CP	cortical plate
CSPG	chondroitin sulfate proteoglycan
E	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	EGF receptor
FAK	focal adhesion kinase
FGF	fibroblast growth factor
FGFR	FGF receptor
GABA	gamma-aminobutyric acid
GAG	glycosaminoglycan
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GPI	glycosyl-phosphatidylinositol
HB-EGF	heparin-binding EGF-like growth factor
HB-GAM	heparin-binding growth-associated molecule
HSPG	heparan sulfate proteoglycan
IZ	intermediate zone
LTP	long term potentiation
MCR-4	melanocortin receptor-4
MZ	marginal zone
NE	neuroepithelial
NMR	nuclear magnetic resonance
NSC	neural stem cell
PDZ	postsynaptic density-95/discs large/ZO-1
PG	proteoglycan
RG	radial glia
RMS	rostral migratory stream
RPTP-β/ζ	receptor-type protein tyrosine phosphatase β/ζ
SVZ	subventricular zone
TGF	tumor growth factor
TSR	thrombospondin type 1 repeat
VZ	ventricular zone

1 Summary

The juvenile sea squirt wanders through the sea searching for a suitable rock or hunk of coral to cling to and make its home for life. For this task it has a rudimentary nervous system. When it finds its spot and takes root, it doesn't need its brain any more so it eats it. It's rather like getting tenure. Daniel C. Dennett (from Consciousness Explained, 1991)

The little sea squirt needs its brain for a task that is very simple and short. When the task is completed, the sea squirt starts a new life in a vegetative state, after having a nourishing meal. The little brain is more tightly structured than our massive primate brains. The number of neurons is exact, no leeway in neural proliferation is tolerated. Each neuroblast migrates exactly to the correct position, and only a certain number of connections with the right companions is allowed. In comparison, growth of a mammalian brain is a merry mess. The reason is obvious: Squirt brain needs to perform only a few, predictable functions, before becoming waste. The more mobile and complex mammals engage their brains in tasks requiring quick adaptation and plasticity in a constantly changing environment.

Although the regulation of nervous system development varies between species, many regulatory elements remain the same. For example, all multicellular animals possess a collection of proteoglycans (PG); proteins with attached, complex sugar chains called glycosaminoglycans (GAG). In development, PGs participate in the organization of the animal body, like in the construction of parts of the nervous system.

The PGs capture water with their GAG chains, forming a biochemically active gel at the surface of the cell, and in the extracellular matrix (ECM). In the nervous system, this gel traps inside it different molecules: growth factors and ECM-associated proteins. They regulate the proliferation of neural stem cells (NSC), guide the migration of neurons, and coordinate the formation of neuronal connections.

In this work I have followed the role of two molecules contributing to the complexity of mammalian brain development. N-syndecan is a transmembrane heparan sulfate proteoglycan (HSPG) with cell signaling functions. Heparin–binding growth–associated molecule (HB-GAM) is an ECM–associated protein with high expression in the peri-natal nervous system, and high affinity to HS and heparin. N-syndecan is a receptor for several growth factors and for HB-GAM. HB-GAM induces specific signaling via N-syndecan, activating c-Src, calcium/calmodulin-dependent serine protein kinase (CASK) and cortactin.

By studying the gene knockouts of HB-GAM and N-syndecan in mice, I have found that HB-GAM and N-syndecan are involved as a receptor-ligand–pair in neural migration and differentiation. HB-GAM competes with the growth factors fibroblast growth factor (FGF)-2 and heparin–binding epidermal growth factor (HB-EGF) in HS-binding, causing NSCs to stop proliferation and to differentiate, and affects HB-EGF–induced EGF receptor (EGFR) signaling in neural cells during migration. N-syndecan signaling affects the motility of young neurons, by boosting EGFR–mediated cell migration. In addition, these two receptors form a complex at the surface of the neurons, probably creating a motility-regulating structure.

2 Review of literature

2.1 Development of forebrain structures

Neurulation begins very early in the mammalian development. The process starts with the dorsal induction of the neural plate, a layer of cells that becomes the source of all neurons in the developing animal. The formation of the neural plate is already visible three days after implantation, on embryonic day 7.5 (E7.5) in the mouse. A groove appears along the mid-axis of the plate and the edges start to bend inwards, forming a tube of neural cells.

A complicated sequence of molecular signals induces the differentiation of new cell types in the neural tube. These signals define the rostro-caudal and dorso-ventral axes of the early embryo. As a result, the neural tube starts to differentiate in to distinct central nervous system (CNS) structures. Many of these inducer signals have been identified, and are reviewed for example by Copp et al. (2003).

Formation of anterior brain structures begins with bulging of the head process away from the foregut and growing heart. In the mouse the first, closed brain cavity exists already 8,5 days after fertilization. During the next 12 hours many recognizable primitive brain structures form. Mesencephalon, myelencephalon, telencephalon, optic stalks and vesicles are easily spotted (figure 1). The walls of the brain cavities consist at this stage of one cell layer (figure 2), where cells undergo rapid, symmetrical mitosis. As a result the brain cavities expand like balloons.

Later, new layers are formed by the earliest cells escaping from the mitotic cycle in the ventricular layer. Chronologically the second layer to form is the marginal layer, where the cells are called Cajal-Retzius cells. They are characteristically very large and multipolar. The third layer formed by new neurons is the subplate, which acts as a barrier between the germinal areas and the future cortical plate (CP). The mitotic cells in the ventricular layer below the subplate start piling up, eventually creating two separate layers, where the mitotic cell somas lay; the ventricular zone (VZ) and the subventricular zone (SVZ).

From very early on the brain surface, or pia, and the ventricle wall are connected with bipolar, longitudinal stem cells with thick processes. These cells have their soma in the ependymal layer, where they undergo cell divisions. Later, when the other layers become visible, these cells are called the radial glia, although they are not — strictly speaking — differentiated glial cells but NSCs (Levison et al., 2005).

2.2 Neural stem cells

Our understanding of NSCs has dramatically changed during the last two or three decades, and the general concept of 'stem cell' has been reviewed in other tissue types as well. Most mammalian tissues retain a small population of constantly dividing cells capable of producing terminally differentiating daughter cells. These stem cell stocks renew themselves and replenish the mature cell population specific for that tissue type, when the mature cells die due to natural causes or tissue damage. It was long assumed that the mammalian CNS is an exception to this behavior, and that the mature brain does not have proliferating cells or, indeed, stem cells.

2.2.1 Embryonic NSCs

Early brain cavity walls consist solely of NSCs. Later, the embryonic NSCs are confined to the VZ and SVZ (Vetter and Dorsky, 2005). When neurogenesis is over, the VZ is thought to shrink and form the thin ependymal cell layer in the adult ventricle wall, while the SVZ persists as a shallow structure with distinct histological morphology adjacent to the ependymal wall.

The NSCs are capable of producing all neural cell types in the brain: astrocytes, oligodendrocytes, and neurons. They divide either

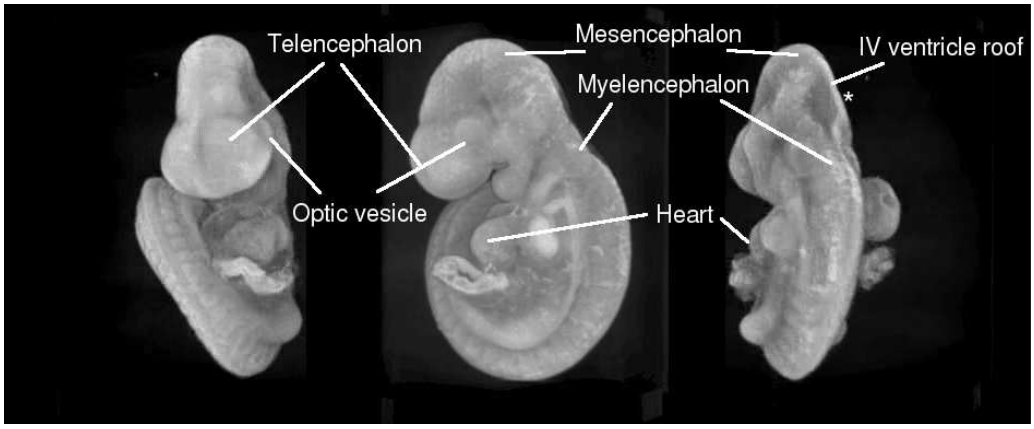

Figure 1: Mouse 9.5 day embryo.
Telencephalic and mesencephalic vesicles are the most prominent brain structures visible. Myencephalon continues caudally from the the IV ventricle, which is still unclosed (asterisk). Optic vesicles and the heart are other clear anatomical structures at this developmental stage. Photos reprinted with permission from Edinburgh Mouse Atlas Project.

symmetrically — the term 'stem cell division' is sometimes used — or asymmetrically (Vetter and Dorsky, 2005). In symmetric division the two daughter cells are virtually identical and both continue as true NSCs. When asymmetric cell division occurs, one daughter cell continues as a NSC, while the other becomes a committed neural precursor. The committed precursor can further divide and produce more committed precursors, but it has lost its capability to produce other neural cell types (Vetter and Dorsky, 2005).

Radial glia (RG) is a good example of an NSC population undergoing mostly asymmetric cell divisions. RG produces the majority of the pyramidal neurons all over the brain (Malatesta et al., 2003; Anthony et al., 2004; Noctor et al., 2004). In the cerebral cortex, the RG is attached to both the VZ ependymal layer, and to the pial surface, and the nuclei of RG cells undergo interkinetic movement within the VZ. When the nucleus reaches the ependymal layer, the cell divides, producing a neuronal daughter cell, while the RG cell still reaches both bordering layers of the cortex (Noctor et al., 2004).

The occurrence of asymmetric cell divisions is fundamental for the production of different glial and neuronal cell lineages in the brain. The basic principles involved in defining the stem cell asymmetry during cell division have been studied in *Drosophila melanogaster* neuroepithelial (NE) cells. Intracellular localization of key molecules controlling asymmetry in a NSC, just before the cell division, determines the future fate of the daughter cells (for review, see Wodarz and Huttner, 2003).

To summarize, several proteins localize either to the apical or to the basal side of the stem cell. Some of these proteins have been identified, amongst them is a protein named Numb, which is found in the basal side of the cell. A division plane separates the apical and basal compartments, leaving one daughter cell with a very high amount of Numb protein (Spana et al., 1995). Numb asymmetry is crucial for the formation of cell lineages in the fly brain, as Numb deletion abolishes the asymmetric cell divisions in the neural epithelium (Buescher et al., 1998).

The molecules determining asymmetry in stem cells are phylogenetically very conserved, and in vertebrate stem cells similar functional-

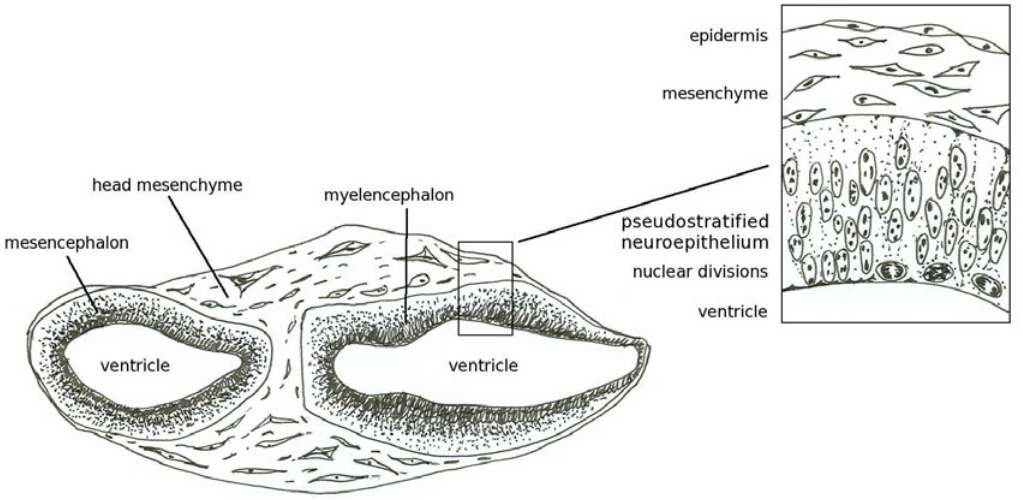

Figure 2: Organization of the early brain cavity wall.
At day 9 after fertilization the brain cavities are clearly visible. Neural tissue forms beneath the head epidermis and mesenchyme and consists of one cell layer, pseudostratified neuroepithelium. Nuclei are slightly scarcer in the MZ, than in the rest of the layer, which is occupied by characteristic spindle-shaped and radially oriented cells. The nuclei of these cells undergo interkinetic nuclear migration, which is closely associated with their mitotic cycle. Mitotic divisions of nuclei are located closest to the ventricle. After mitosis, the nuclei of the daughter cells ascend, while the cells go through the rest of the phases of the cell cycle. Some of them will descend their nuclei again to start a new mitosis.

ity has recently been demonstrated (Shen et al., 2002). Conditional deletion of Numb and Numblike (another vertebrate homolog for fly Numb) in the dorsal forebrain in mice enhances NSC proliferation and inhibits differentiation. This leads to dramatic morphological changes in the forebrain, including enlargement of the ventricles, and partial loss of corpus callosum (Li et al., 2003).

Interestingly, the asymmetry of cell divisions appears not to depend only on intracellular localization of key proteins, but also on the distribution of cell surface receptors. EGFR is one of the asymmetrically distributed receptors during NSC divisions. In early NSCs (E13-15), high levels of EGFR drive the daughter cells towards differentiation and induce migration. In the older NSCs (E16-17) EGFR induces more specifically astrocytic fate, in expense of differentiating oligodendrocytes (Burrows et al., 1997; Caric et al., 2001; Sun et al., 2005).

The fact that cell surface molecules could contribute to stem cell asymmetry raises the idea that ECM molecules could influence the polarity of stem cells. In the case of EGFR and some other receptor tyrosine kinases we already know that a link exists between them and integrins (reviewed in Giancotti and Tarone, 2003, and Cabodi et al., 2004), which are receptors for ECM proteins.

2.2.2 Adult NSCs

There is a proposed continuum from embryonic stem cells to adult stem cells in the persistent germinal areas of the mammalian brain. While the ventricular neurogenetic areas in the embryonic brain consist of VZ and SVZ, only SVZ is identifiable in the adult brain.

The continuum-theory is supported by the finding that both embryonic and adult NSCs express an overlapping array of NSC-specific re-

ceptors and respond to the same growth factor stimuli (Lie et al., 2005; Sailer et al., 2005). However, the stem cells in the adult SVZ are distinct from embryonic NSCs, in terms of gene expression and differentiation (Abramova et al., 2005; Aiba et al., 2005).

Identification of adult NSCs has raised hope for finding new therapeutic methods to treat aging-associated neurodegenerative diseases and other CNS injuries. Neurogenesis is triggered by trauma in the mammalian CNS, and consequently all three neural cell types are produced (Magavi et al., 2000; Kernie et al., 2001; Yang et al., 2006). However, the mammalian CNS does not, in practical terms, show much direct replacement or recovery of damaged neuronal tissue *per se*. Recovery from ischemic insults and mechanical trauma often involves isolation and scarring of the damaged tissue, and partial functional recovery by slow, plastic changes in the neuronal networks (Kernie et al., 2001). The significance, or benefit, of neurogenesis after traumatic injury remains obscure.

In senility-associated diseases, however, NSCs can offer functional recovery by directly replacing the destroyed neurons. Fetal mesencephalic cells have been used for replacing dopaminergic cells in the substantia nigra of Parkinson's disease patients. The results have been variable, and the source of the cell grafts raises practical and ethical issues (Lindvall and Bjorklund, 2004; Winkler et al., 2005). Currently, hopes lie in the possibility to either create an expandable source of NSCs, or isolate patients own NSCs, induce them to form desired neurons, and transplant them back to the area of injury.

A more ambitious idea would be to induce the proliferation and differentiation of patients own, dormant NSCs in situ. This technique, as intuitive as it sounds, carries the real risk of pushing the endogenous, primitive cells into malignant growth, or awakening already existing, dormant tumor cells. It is currently understood, that stem and cancer cells share similar regulational mechanisms, and that cancer etiology might lie in the lineage of stem and progenitor cells (Feinberg et al., 2006). On the other hand, metastatic, solitary tumor cells might pass unnoticed in otherwise healthy organs for years, and spring back to action upon receiving the right stimulus, e.g. from a growth factor (Kirsch et al., 2000).

2.2.3 Stem cell niche

Currently it is believed that the behavior of NSCs depends on the composition of their surrounding matrix, which they secrete themselves and by their neighbouring cells. Thus, NCSs receive different types of signals from their immediate microenvironment. These signals define the niche of the dividing stem and precursor cells. Several key players participating in the definition of the stem cell niche have been identified. These include many growth factors, ECM proteins, cell surface receptors, and cytokines.

NSC-trophic factors FGF-2, FGF-8 and Wnt associate with HSPGs found in the ECM and at the cell surfaces. Presence of cell surface HSPGs in the stem cells is required for the full activity of these growth factors (Ford-Perriss et al., 2003). With a combination of FGF-2 and EGF it is possible *in vitro* to simulate conditions, that keep NSCs as symmetrically dividing stem cells (Conti et al., 2005), which may prove to be valuable finding for the possible NSC therapies.

In both embryonic and adult NSC microenvironment, the ECM proteins laminin, fibronectin, and collagens affect the NSC maintenance and differentiation (Whittemore et al., 1999; Garcion et al., 2001; Kearns et al., 2003; Tate et al., 2004). Whereas the ECM receptors (integrins) heavily modulate the responses of a cell to the surrounding matrix molecules (Tate et al., 2004; Andressen et al., 2005; Flanagan et al., 2006). In addition, integrins are known to cross-talk with cell surface growth factor receptors (Cabodi et al., 2004; Campos et al., 2006).

In NSCs integrins play a crucial role in enhancing growth factor induced proliferation as well as differentiation (Cabodi et al., 2004; Tate et al., 2004; Leone et al., 2005).

2.2.4 NSCs *in vitro*

In vitro a NSC can be induced to proliferate, so that the daughter cells forme a morula-like structure called a neurosphere. These clonal neurospheres can form in the presence of either FGF-2 or EGF, or both (Conti et al., 2005). The spheres can grow quite big, to the extent where the innermost cells die because of lack of nutrients. If a sphere can attach to a suitable matrix, like laminin or other ECM proteins, the undermost cells in the sphere flatten and start spreading, providing an anchor to the rest of the sphere (Kearns et al., 2003). The cells in the sphere start migrating away from the sphere core, along anchor structures, which resemble closely the RG in the growing cerebral cortex. The manner of the migration also looks similar, when observed with time-laps video (Wichterle et al., 1997; Jacques et al., 1998; Kearns et al., 2003). This causes the sphere to form an NSC disc that can become very large in two dimensions.

Some of the conflicting findings concerning the fate determination and plasticity of NSCs may be due to cell culture conditions changing the characteristics of the stem cells (Machon et al., 2005). A recent study demonstrates, how a neural precursor population *in vivo* differentiates to neurons and astrocytes, while neurospheres derived from the same precursor pool show trilineage differentiation, producing oligodendrocytes in addition to the other two cell types (Machon et al., 2005). The authors of this work warn researches about misleading lineage studies relying only on *in vitro* evidence.

2.3 Neural cell migration

In the light of recent studies neural migration in the developing mammalian brain is enormously complex. In many brain structures most of the neurons are born very near, or in their future home, and by superficial examination appear to have straightforward and simple modes of locomotion over relatively short distances. However, the improved *in vivo* observation techniques (Tsai et al., 2005; Watson et al., 2006) have revealed many aspects of neural movement as well as new sources and routes for neurons (Nadarajah and Parnavelas, 2002; Tabata and Nakajima, 2003).

The role of RG as a movement-supporting structure for new pyramidal cells has been nearly rewritten during the last five years. RG was previously thought to function solely as guidance structure in the cerebral cortex, spanning from the ventricle wall to the pial surface. Newly born pyramidal cells in the VZ attach to the glial threads and start locomotive ascent to the CP (Polleux and Anton, 2005). After fulfilling this role, the RG was previously thought to differentiate to astrocytes (Pixley and de Vellis, 1984). As was discussed in the previous chapter, RG is actually a source for pyramidal cells (Levison et al., 2005), and according to new data, pyramidal cells undergo at least three different types of movement during their ascension, only one of these involving the usage of RG as a supporting structure (Tabata and Nakajima, 2003). What still seems to be true is that the terminal differentiation of RG leads to formation of astrocytes (Levison et al., 2005).

While cortical pyramidal neurons arise from the cortical VZ and SVZ, the GABAergic (GABA, gamma-butyric acid) interneurons originate from non-cortical brain areas (Rakic, 1995; Anderson et al., 1997; Tamamaki et al., 1997; Tan et al., 1998; Lavdas et al., 1999). Lateral, medial and caudal ganglionic eminences are ventral proliferative areas of the ventricle, and the cells originating there migrate along several different routes to their targets in the

cortex (Tamamaki et al., 1997; Lavdas et al., 1999; Ang et al., 2003).

2.3.1 Radial migration in the cerebral cortex

The most studied migration pathway is the so called radial pathway, taken by the post-mitotic pyramidal, or projection, neurons in the cerebral cortex (Kornack and Rakic, 1995; Tan et al., 1998; Hatten, 1999). The first post-mitotic structure to form in the cerebrum is the transient preplate. The first born neurons in the VZ migrate into the preplate, splitting it into the subplate and the marginal layer. The CP is formed in between these two layers. The marginal layer contains the characteristic Cajal-Retzius cells and later becomes lamina I, while the subplate gradually vanishes. In humans, subplate has disappeared on the week 38 post-menstrual age (de Graaf-Peters and Hadders-Algra, 2006), but in mice preplate persists until three weeks after birth (Wood et al., 1992).

In radial migration the neurons are born in the nearby VZ, and migrate through the subplate to the CP, forming radial columns. The ascending neurons migrate through the CP and stop before the Cajal-Retzius cells, staying on top of the previously arrived neurons. In this manner the most recently born neurons always form the uppermost layer of the CP (figure 3). The cortical laminae have been birth-dated in mice, and according to these data the cells in the lamina VI are born on E11, while cells in lamina II undergo their last mitosis around E17 (Takahashi et al., 1999).

The movements of the nucleus play a surprisingly important role in neuronal migration. The nucleus of a young neuron is huge in relation to its cytosol; it can occupy 50% of the whole cell volume (Lambert de Rouvroit and Goffinet, 2001; Schaar and McConnell, 2005). Moving a load of this size over long distances obviously influences the form of migration. In the cortex two mechanically distinct ways of migration can be identified; translocation and

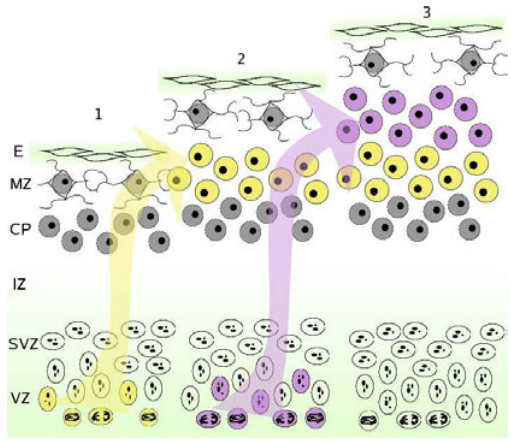

Figure 3: Layering in the embryonic cerebral cortex.
Radially migrating neurons accumulate in the CP in a so called inside-out-manner. The gray cells in the CP represent the first-born neurons. (1) The yellow cells in the VZ represent the source of post-mitotic cells, which migrate radially through the previously existing layer of cells in the CP (2) and form a new layer (yellow) between the old one and the MZ. The next cell cycle (purple cells) produces again post-mitotic neurons (3) that ascend to the top of the CP (purple layer). E, epidermis.

locomotion (Lambert de Rouvroit and Goffinet, 2001; Schaar and McConnell, 2005).

Translocation typically occurs in the early phases of corticogenesis, when the cortex is still quite thin and the distance from the VZ to the CP is short. A neuron reaches with a process to the pial surface, while the nucleus stays in the VZ. After the process is established, the nucleus is lifted inside the process by a microtubule cage to the level of the CP, and the cell detaches from the VZ and the pia (Schaar and McConnell, 2005).

Radial locomotion is a typical mode of migration when the cortex is quite thick and RG is used as guidance aid. A neuron grows a short leading process, wraps it around a glial process, and inches its way up to the target level. In this form of migration the nucleus is moved by the microtubule net in saltatory fashion into the space formed by the proceeding leading neurite

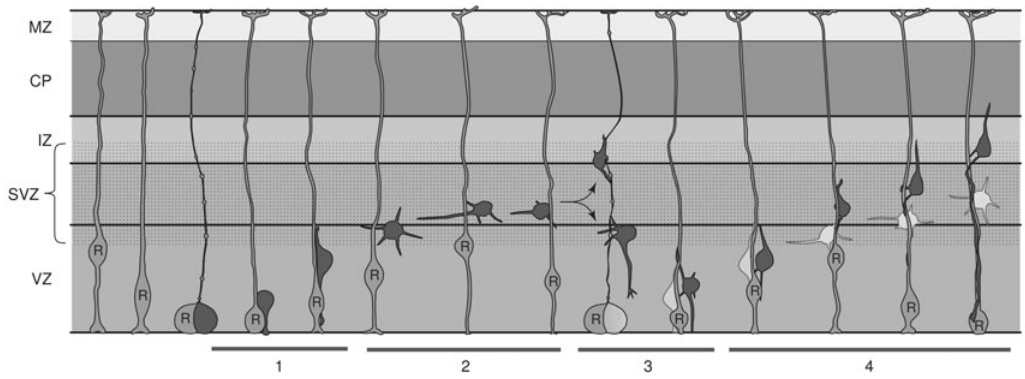

Figure 4: The four phases of radial neuronal migration in the cerebrum.
In the first phase pyramidal neurons (dark gray) ascend along RG (R). In the second phase the neurons obtain a multipolar morphology and pause in the IZ. Some pyramidal cells undergo a third phase, and descend back to the VZ. Finally, in the fourth phase the cells undergo the typical, radial migration to the CP, guided by RG cells. RG generates neural cells (light gray), while undergoing interkinetic nuclear movement. Reprinted from Kriegstein and Noctor (2004), with permission from Elsevier.

(Schaar and McConnell, 2005).

Radial locomotion can be further divided into three, or sometimes four, distinct phases (figure 4). After an initial glia-aided climb to the SVZ, most of the pyramidal cells ascend in the SVZ in a manner called multipolar migration, which is a relatively slow method of migration and does not require the presence of supporting cell structures (Tabata and Nakajima, 2003).

Cells undergoing multipolar migration display dynamic protrusion and retraction of multiple processes without any noticeable preference for direction (Tabata and Nakajima, 2003). After reaching the intermediate zone (IZ), the cells assume bipolar morphology, attach to a radial process and start the classical radial locomotion. The bipolar locomotion occurs almost twice as fast as the multipolar migration. Sometimes, the neuron does not enter the IZ, but dips back to the VZ. From there it finally ascends all the way to the CP by radial locomotion (Tabata and Nakajima, 2003).

2.3.2 Tangential migration of interneurons

The major cortical interneuron route is the tangential route, which exists only during the development of the cerebrum. In mouse, these cells are born in the VZ and SVZ of the lateral, medial and caudal ganglionic eminence and migrate through the striatum to the cortex (figure 5) (Kriegstein and Noctor, 2004). There they find their way to the marginal layer and migrate tangentially until they find their correct cortical column. Then the interneurons take a dip to the SVZ of that column and bounce back to the cortical layer, where they finally settle and differentiate (Ang et al., 2003). The first neurons to migrate like this are born already at E11 in mouse (Anderson et al., 1997).

This complex behavior is still very much a mystery. It is not known, why the cells should search for the ventricular layers before homing to their targets that lay superficial to the SVZ. It is remarkable that interneurons obey the same inside-out layering in the cortex as the pyramidal cells do. How do they receive the layer information, and how do they initially migrate against the ascending stream of radially migrating pyramidal cells? Two possibilities spring

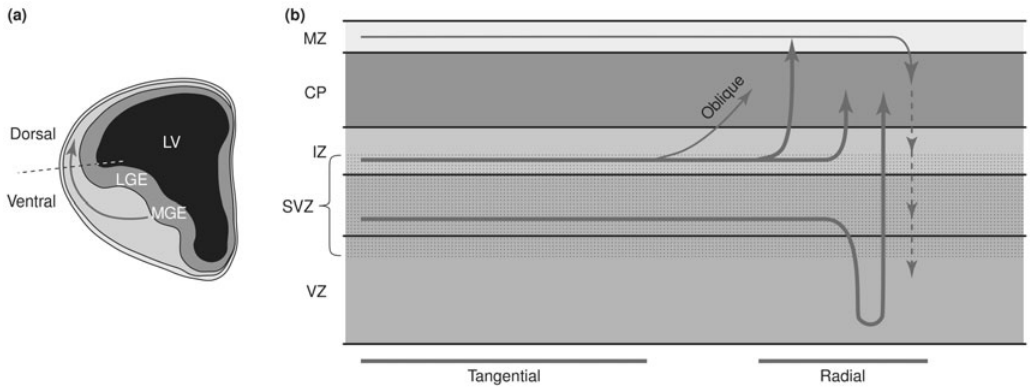

Figure 5: Migration of interneurons in the cerebrum.
(a) Cortical interneurons originate mainly in the medial ganglionic eminence (MGE) of the telencephalon, and start tangential migration across the cortico-striatal junction (broken line) towards dorsal telencephalon. (b) Interneurons migrate in the cortex mostly in the IZ and SVZ, but some are found in the MZ as well. Cells in the MZ descend to the CP, and sometimes even further to the deeper layers (broken line). Cells in the IZ ascend to the CP with or without RG. Cells migrating in the SVZ typically turn to descend towards the ventricle, make a U-turn, and start ascending again guided by the RG. Reprinted from Kriegstein and Noctor (2004), with permission from Elsevier.

to mind immediately: either both pyramidal and interneurons receive their layer information in the VZ or SVZ of the cortex, or these two neuronal populations have distinct chemoguidance factors. Some evidence exists for the latter. Glial cell line–derived neurotrophic factor (GDNF) has been identified as a chemoattractant for tangentially migrating GABAergic cells (Pozas and Ibanez, 2005), and recently also for interneurons in the rostral migratory stream (RMS), but not for cells arising from the cortical SVZ (Paratcha et al., 2006).

2.3.3 Migration in the RMS

One major migration route for interneurons survives until, and through, adulthood in rodents and in humans — the RMS. The cells on this route are born in the rostral parts of the ventrolateral SVZ, and form a network of migrating cells already in the SVZ (Lois et al., 1996; Peretto et al., 1997). The cells come together in the rostral tip of the SVZ and move along glial structures towards the olfactory bulb (figure 6) (Doetsch et al., 1997; Peretto et al., 1997). The neurogenetic structure in the SVZ persists throughout adult life, supplying the olfactory bulb with new interneurons via RMS (Doetsch et al., 1997; Jankovski et al., 1998; Craig et al., 1999).

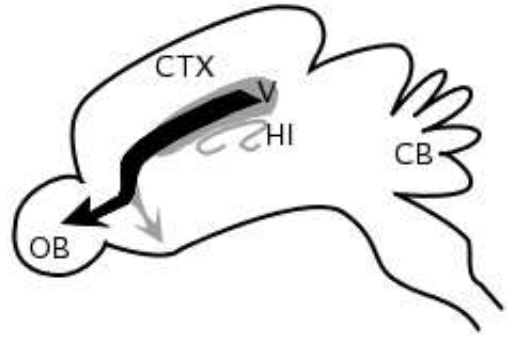

Figure 6: RMS in the mouse brain.
The rostral and ventral parts of the brain ventricles (V) produce interneurons, which gather via neurophilic chain migration to the rostral tip of the ventricles. From there they take the migration route either rostrally toward the olfactory bulb (OB) or ventrally towards the ventral cortical areas. Hippocampus (HI), cortex (CTX), cerebellum (CB).

2.3.4 Intracellular events during neuronal migration

Mobility is one of the most important qualities of a neuron. The obvious necessity of movement during CNS development converts to less visible mobile plasticity during brain maturation, regeneration, and even in learning and memory formation. Neurons share the same cytoskeletal structures required for cell migration and process growth as other cell types, but posses many specific molecular solutions as well. The need for fast axonal transport over long distances (several meters in large mammals) is one of the neuronal features, which requires highly specialized protein complexes in association with the traditional cell cytoskeleton.

The neuron cytoskeleton basically consists of two physically separate structures: the microtubule and the actin cytoskeleton. The actin cytoskeleton is a very dynamic, constantly changing structure closely associated with the cell plasma membrane. It mainly provides the force and direction in cell spreading and withdrawal. The microtubule cytoskeleton is a more stable structure surrounding the nucleus like a cage, and participating in vesicle transport within the cell soma and inside the neurites. The microtubule mesh is needed for neurite outgrowth and probably participates in stabilizing the growing neurites (for review, see Dehmelt and Halpain, 2004). Actin and microtubule cytoskeletons interact with each other in neurite initiation, growth cone spreading, and in neurite elongation, but the involved linkage mechanisms are still largely unresolved (Lambert de Rouvroit and Goffinet, 2001; Dehmelt and Halpain, 2004; Schaar and McConnell, 2005).

Mechanistically neuronal locomotion can be roughly divided into two distinctly regulated events. Firstly, the leading neurite extends rapidly and stabilizes after reaching several microns ahead of the soma. Secondly, the nucleus and soma are displaced forward to the widened space, while the trailing process retracts (Komuro and Rakic, 1995; Wichterle et al., 1997; Schaar and McConnell, 2005). These events are sequentially repeated and both are required for successful migration.

Nuclear movements are controlled by the microtubule net. Doublecortin and LIS1 are amongst the most studied microtubule associated proteins, because their mutations are either linked to or directly responsible for several human disorders, known as lissencephalies (see section 2.3.6). In mouse, Doublecortin polymerizes microtubules, and stabilizes their mesh (Francis et al., 1999; Gleeson et al., 1999a; Horesh et al., 1999), while LIS1 acts from within a protein complex, linking microtubule bundles to the nuclear membrane and to the microtubule organizing center (Feng et al., 2000; Sasaki et al., 2000). Together with dynein, LIS1 provides the traction force to the microtubules, which draws the nucleus forward towards the microtubule organizing center.

2.3.5 Signal transduction to the cytoskeleton

The actin cytoskeleton comes very close to the plasma membrane at the sites where the cell adheres to outside objects. When the adhesion is to the ECM, the contact points are called focal adhesions or focal contacts (for review, see Petit and Thiery, 2000). Several groups of transmembrane adhesion molecules mediate the plasma membrane contact with the actin cytoskeleton, among them the integrins, cadherins, and immunoglobulin superfamily cell adhesion molecules (IgCAMs). Of these receptors the integrins create focal contacts with ECM proteins, the others are cell-cell adhesion molecules.

The important outcome of an integrin-ECM contact is that it leads to elevated tyrosine phosphorylation in the cell (Kornberg et al., 1991; Klinghoffer et al., 1999). One relevant signaling molecule that is activated, is the focal adhesion kinase (FAK) (Wennerberg et al.,

2000). FAK phosphorylation is crucial for cell spreading and migration, and appears to control the turnover of focal contact components (Burridge et al., 1992; Ilic et al., 1995; Carragher et al., 2003). Another important activated signaling molecule is c-Src (Klinghoffer et al., 1999; Arias-Salgado et al., 2003; Hsia et al., 2005). Both kinases regulate the remodelling of the actin cytoskeleton, usually as a complex, but c-Src can function independently as well (Hsia et al., 2005). The remodeling takes place by phosphorylation of cortactin (Thomas et al., 1995; Head et al., 2003; Agerer et al., 2005).

Cortactin binds and cross-links filamentous actin. It is activated by many extracellular signals via phosphorylation of multiple tyrosine residues (Huang et al., 1997, 1998). Phosphorylated cortactin induces motility in several cell types. Its tight connection to the actin cytoskeleton and to the plasma membrane–associated signaling complexes makes it a powerful, general organizer in cell motility and regulation of cell morphology.

2.3.6 Pathology in neuronal migration

Neuronal migration can fail in several ways, and the outcome reflects the type of the failure. The failures are often linked to the regulation of the cytoskeleton, microtubule remodeling, or chemotactic behavior of the cell. The composition of the ECM is crucial for successful movement, and the outside signals secreted by other cells have to be correct. Errors in neuronal migration often lead to visible cortical malformations, sometimes to epileptical seizures, and even to mental retardation. The common cortical malformations and rough scetches of their mechanisms are illustrated in figure 7.

If a neuron fails to grow a leading process, it cannot start the migration at all and will stay in the germinal areas, the VZ and the SVZ. This leads to peri-ventricular heterotopias, in which patches of misplaced neurons are located very close to the ventricle (figure 7B) (Lambert de Rouvroit and Goffinet, 2001). This type of pathogenesis is usually connected with defects in the actin cytoskeleton. In humans, mutations in filamin (an actin-binding protein) have been identified as possible causes for these malformations (Fox et al., 1998). Normally filamin crosslinks actin filaments into a bundle, and can also bind the cytoplasmic tails of integrins, linking actin network to cell membrane. Absence of filamin appears to disrupt the elongation of the neurites (Fox et al., 1998).

In another type of migration failure, a neuron can initiate migration normally, but its microtubule network cannot move the nucleus, i.e. the interkinetic nuclear movement is defective. This happens in the so called classical, type 1, lissencephaly, and is very often caused by mutations in the LIS1 or doublecortin gene (Reiner et al., 1993; Gleeson et al., 1999b). Doublecortin mutations cause the X-linked variation of type 1 lissencephaly in human males, resulting in the so called doublecortex phenotype in the carrier females. The neurons affected by these mutations settle below the cortical subplate and never form a proper CP (figure 7C).

Preplate cells guide the migrating neurons to accumulate inside the plate, to split it into the subplate and the marginal zone (MZ), and to form the CP. If the preplate does not express the guidance cues, or if the migrating neurons lack a means of responding to the stimulus, the cells cannot enter the preplate, but accumulate below it (Sheppard and Pearlman, 1997). By some unknown mechanism the cells then obtain partially the identity of preplate cells. The next wave of neurons cannot, in turn, cross this layer of neurons, and thus the normal inside-out gradient of neurons turns outside-in (figure 7D). This results in inverted cortical layering below the subplate and is similar to the phenotype of the classical mouse mutant *reeler* (Caviness and Sidman, 1973; Caviness and Rakic, 1978; Caviness, 1982).

The Cajal-Retzius cells in the preplate and in the MZ secrete an ECM protein named reelin, a huge glycoprotein that binds the apolipoprotein E2 receptor on the migrating neurons (Shep-

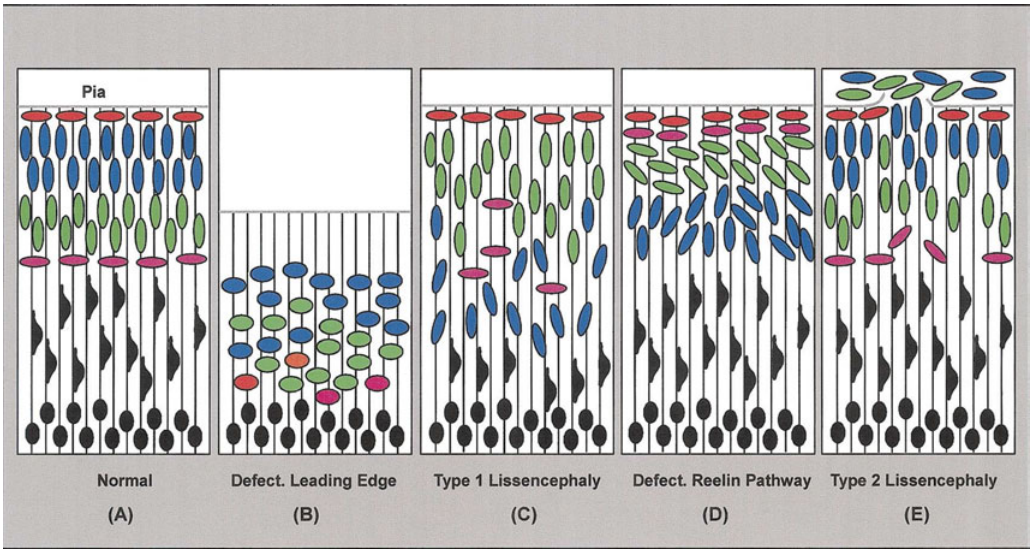

Figure 7: Defects in radial migration.
Cortical malformations are often due to failures in neuronal migration. Panel A shows the the normal patterning of the cerebral cortex, with migrating neurons (black), subplate (purple), neurons that have previously migrated (blue and green), and Cajal-Retzius–cells (red). In B, the neurons fail to grow a leading edge, cannot start migration, and accumulate in shallow, disorganized patches called peri-ventricular heterotopias. In C (type 1 Lissencephaly), the neurons migrate, but because of defective microtubule network, the interkinetic movement of the nucleus does not function normally. They cannot pass the earlier layers, and stay below them. In D the migrating neurons cannot pass the subplate, and accumulate in an "inverted" manner below it. Finally, in E (type 2 lissencephaly) the neurons lack a stopping signal, and "leak" through the Cajal-Retzius–layer (red) and the pial membrane outside of the cortex. Reprinted from (Lambert de Rouvroit and Goffinet, 2001), with permission from Elsevier.

pard and Pearlman, 1997; Hiesberger et al., 1999; Trommsdorff et al., 1999). The resulting signaling cascade normally causes the neurons to stop below the marginal layer and form the superficial layer of the CP. In addition to reelin, mutations in the other proteins in the signaling cascade produce a similar phenotype as seen in *reeler* (Sheldon et al., 1997; Trommsdorff et al., 1999).

If the MZ stop-signals do not work, as in type 2 lissencephaly (or cobblestone lissencephaly), the neurons do not know when to stop, and the pial membrane leaks. The neurons accumulate outside the superficial layer, and form characteristic cobblestone structures to the meninges (figure 7 E).

Because lissencephalies are caused by disruptions in the intracellular microtubule function or in the migratory machinery, they are sometimes associated with other severe symptoms, like muscular dystrophia in the case of Walker-Warburg syndrome (Vajsar and Schachter, 2006), or facial deformities in the case of Miller-Dieker syndrome (Yingling et al., 2003). These brain abnormalities cause stalled cognitive development already at the age of a few months, seizures, microcephaly, and sometimes cerebral palsy.

2.4 Neural cell differentiation

All neural cells in the brain — neurons, astrocytes and oligodendrocytes — arise from the ancestral pool of NE cells lining the embryonic

brain ventricles. RG appears in the cortex very early, sharing many molecular and morphological characteristics with the NE cells (Hockfield and McKay, 1985; Frederiksen and McKay, 1988; Misson et al., 1988; Lendahl et al., 1990), but in addition express many molecules found in astrocytes, e.g. vimentin (Dahl et al., 1981; Pixley and de Vellis, 1984). The RG behaves as stem cells, producing neurons as well as astrocytes. Later on in life, in the adult germinal areas of the brain, the NSCs appear as fully differentiated astrocytes (Doetsch et al., 1999; Laywell et al., 2000). The hypothesis of neural lineages has changed substantially during the last two decades, and this change in current view is illustrated in the figure 8.

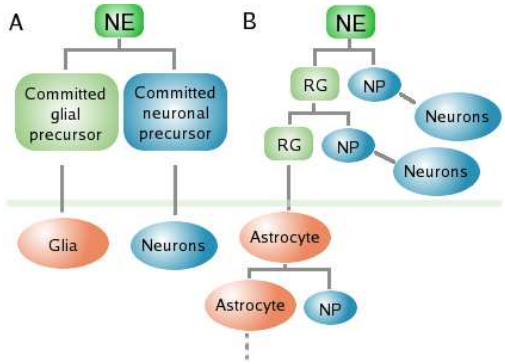

Figure 8: Neural cell lineage hypothesis.
All neural cells in the brain have their ancestry in the NE cells. (A) Historically, the glia and neurons were thought to have distinct lineages of committed precursor cells, which had differentiated from the NE. (B) The current hypothesis sees a continuum in the neural cell lineages. Initially, NE gives raise to RG and neural precursors (NP), which further differentiate into neurons. Later, the RG differentiates into astrocytes, which continue as adult NSCs, producing NP via asymmetric cell division. Green horizontal line marks the end of embryonic neurogenesis.

Segmentation of the brain largely determines the future fate of precursor cells in the different segments. To give a crude example, if the cerebral cortex is divided in the ventral and dorsal segments, then the ventral germinal areas give rise to interneurons and oligodendrocytes, while the dorsal germinal areas produced pyramidal neurons. Both areas produce astrocytes as well. Several intrinsic and extracellular factors have been identified, which induce regional identity in the telencephalon (Schuurmans and Guillemot, 2002).

2.4.1 Neurons

Interneurons and pyramidal neurons seem to arise from different progenitor pools that are regionally separated. Neuronal subtypes inside both groups differentiate in a less clear manner. In the cerebrum, pyramidal neurons differentiate according to their layer identity, and they achieve this by being born at the right moment, in a simplified scenario (Takahashi et al., 1996; Noctor et al., 2004). Interneurons appear to follow the same rule of birthday–dependent layering (Tamamaki et al., 1997; Ang et al., 2003), but their movements are more obscure and thus their final differentiation has been quite hard to follow.

The ability of cortical neuronal precursors to obtain different layer identities decreases during development. Precursors isolated from an early cortex and transplanted to an older one can form more superficial layers, like II-III, than they were originally destined to. However, precursors isolated from an older cortex and transplanted to a younger one, cannot differentiate to the deep layer neurons, but instead may wait several cell cycles before they assemble to the layer structure (Desai and McConnell, 2000).

After a neuron has reached the region it was destined to by intrinsic or extrinsic factors, it has to establish synaptic connections in order to survive. Growing an axon and pin-pointing a potential target are young neurons' first tasks. Axon guidance and synapse formation are well studied areas, where the role of ECM and cell surface PGs has proven to be most crucial (Kinnunen et al., 1996; Tisay and Key, 1999; Pizzorusso et al., 2002; Inatani et al., 2003; Pavlov et al., 2004; Hayashi et al., 2005).

Heparan sulfate (HS) and chondroitin sulfate (CS) appear to have almost opposing effects on neurite growth and guidance, HS being mostly an inducing and CS being an inhibiting factor (Liu et al., 1998; Inatani et al., 2003; Hayashi et al., 2005; Tropea et al., 2003). Both GAGs are required for the structural development of the CNS. In CNS or peripheral nervous system (PNS) synapse formation, the exact role of HSPGs has been elusive. Very recently breakthrough findings in *Drosophila* Syndecan mutants have been published. Fly syndecan controls the number of neuromuscular junctions forming; syndecan overexpression increases and attenuation directly decreases the number of synaptic junctions (Johnson et al., 2006).

2.4.2 Astrocytes

Astrocytes are probably born in all germinal areas of the brain. In the cerebrum their differentiation starts a little later than the differentiation of neurons, and the germinal areas of astrocytes and neurons differ somewhat. The early mainly VZ gives rise to neurons, while astrocytes appear when the SVZ has formed (Levison et al., 2005). *In vitro* the growth factors EGF and FGF-2 have gained fame in differentiating between neuronal and glial fate of neural precursors (Levison et al., 2005; Vetter and Dorsky, 2005). FGF-2 appears to promote the growth of mostly neuronal precursors, while EGF stimulated cultures produce a mixed population of neurons and glia (Craig et al., 1996; Burrows et al., 1997; Kuhn et al., 1997).

The temporal switch from neuron-producing to astrocyte-producing precursors has proven to be puzzling. In numerous experiments isolated and cultured NSCs have followed this temporal behavior in the absence of any exogenous factors (Irvin et al., 2003). This suggests the existence of a timed cell intrinsic signal which the precursors obey. However, the isolated stem cell cultures always contain a population of differentiated neurons, which could provide an extrinsic cue also *in vitro*. A recent study (Barnabe-Heider et al., 2005) provides support for the hypothesis of extrinsic factors in NSC regulation: Cardiotrophin-1 is secreted by postmitotic young neurons, and initiates the differentiation of surrounding astrocytes. In the absence of cardiotrophin-1 the production of astrocytes is significantly delayed and reduced.

Neuron-astrocyte -crosstalk is essential not only in cell differentiation, but later in regulating the synaptic connections of neurons. Astrocytes as neuronal signal modifiers is a rather new idea, and direct evidence for this has only recently started to accumulate. Astrocytes express many receptors for neuronal signaling molecules, and previously it was thought that astrocytes function like vacuum-cleaners, removing the excess of neurotransmitters from near the synaptic connections (Sakatani et al., 1992; Martin et al., 1993; Merzak et al., 1996). Recently it has become clear that astrocytes respond to transmitter stimuli, and in addition, stimulate neurons by releasing their own transmitters (Poitry-Yamate et al., 2002; Huang et al., 2004; Perea and Araque, 2005; Panatier et al., 2006). What the physiological meaning of this new level of complexity in neural signaling is, remains to be solved, but the current consensus is reviewed in Fellin and Carmignoto (2004) and Kozlov et al. (2006).

2.4.3 Oligodendrocytes

Oligodendrocytes are mostly born in the ventral telencephalon, from where they migrate to the white matter (Rakic and Zecevic, 2003). Little is known about the lineage of oligodendrocytes but they are certainly different from Schwann cells — their PNS relatives. In mice and rats, oligodendrocytes arise at a peri-natal stage; mature forms are already seen a few days before birth, but most of the maturation and invasion of the white matter happens during the first weeks after birth (Trapp et al., 1997). Mature oligodendrocytes populate the areas of heavy axonal transport, basically nerves and axonal

bundles, i.e., the white matter (Rogister et al., 1999).

Oligodendrocytes have apparently one major role in the CNS — they act as insulators surrounding conducting axons. The structure of the insulating material is such that an axon potential cannot propagate evenly through the length of the axonal membrane, but "jumps" short distances over insulated parts of the axon. These nodes of conductivity are called the nodes of Ranvier, and they form the basis for the fast axon potentials needed for long distance conductivity to and from the PNS.

2.5 Extracellular matrix of the brain

The ECM can be viewed as a multidimensional mesh occupying the inter-cellular space in the brain tissue. With a little bit of imagination one can understand some of the most fundamental functions of the ECM by observing its physical structure. It is a construction of highly adhesive, filamentous, and often multimeric proteins, offering structural support to the brain tissue as well as carrying out a multitude of biological tasks.

The mesh proteins themselves provide physical contact points for growing neurites, mature dendritic and axonal structures, astro- and microglial protrusions, and oligodendrocytes (Burridge et al., 1992; Buttery and ffrench Constant, 1999; Andressen et al., 2005). The mesh traps water-soluble small molecules, like growth factors, slowing down their perfusion in the tissue or stopping it altogether. The adhesiveness of the ECM proteins to the trapped molecules determines the dynamics of their perfusion, generating short distance gradients of these molecules between the brain cells (Gustafsson and Fassler, 2000). The ECM mesh can store soluble proteins, PGs, GAGs and so on, but it can also release them and present them to the cell surfaces, activating receptors (Gustafsson and Fassler, 2000).

Matrix molecules have their own receptors at the cell surfaces, mainly integrins, but also PGs like N-syndecan and glypican-1, and cell adhesion molecules, like connectin 1. Many ECM molecules bind to other receptors as well, and can induce either specific intracellular responses or influence indirectly the activity of certain signaling cascades (Akita et al., 2004; Stern et al., 2006; Zacharias and Rauch, 2006). Many ECM molecules are PGs, glycoproteins or pure GAG chains, which makes their biochemistry very complex. Enzymes which cleave glycan chains produce divergent variations in the length and composition of the polymers, and the cleaved particles can have signaling functions of their own.

The ECM-cell cross-talk is important to the matrix assembly as well. Cell surfaces provide contact points with relevant receptors for the ECM molecules, thus creating organization sites for the matrix. In fact, without proper cell surface receptors, the ECM proteins may fail to form cell-surrounding matrix structures. This can lead to ECM disruption and further to tissue organization problems, as has been documented for the epidermal basement membrane (DiPersio et al., 1997; Williamson et al., 1997; Sasaki et al., 1998).

2.5.1 Molecules constructing the brain ECM

Already by the morula stage of an embryo, the cells of a multicellular animal secrete a mixture of proteins that surround the individual cells. This material is degradable by certain proteases, disrupting the morula back to single cells (Iwakura and Nozaki, 1985). This adhesive matrix is the predecessor of the ECM and basement membrane throughout the developing animal. In the brain the composition and the volume of the ECM changes during development, indicating changing biological functions. According to some estimations, the volume of the ECM is about 40% of the immature, and 20% of the adult total brain volume (Wright et al., 2002).

The structural proteins of the ECM are mostly glycoproteins or long GAG polymers. Hyaluronan is a polymer consisting of GAG disaccharides. It is present in cartilage and brain ECM, and most of our knowledge comes from cartilage studies (McDonald and Hascall, 2002). Hyaluronan is a long, linear molecule that has binding sites for certain proteins, such as CSPSs. Hyaluronan binds these proteins at their polypeptide termini, creating lamp-brush-like structures (Morgelin et al., 1994). CS is heavily hydrated, and a long hyaluronan molecule binding several CSPGs will accumulate large amounts of water. This leads to swelling and expansion of the lamp-brush-structure, and eventually forms the gelatinous goo known as ECM (Rauch, 2004).

In cartilage, this swelling is restricted by filamentous collagen, which gives cartilage its elasticity (Knudson and Knudson, 2001). In the brain, the CSPGs are not as heavily glycosylated as in cartilage, and thus swell less. Eventually, the skull acts as a final barrier for ECM hydration (Rauch, 2004).

Hyaluronan is considered to be the most fundamental ECM constructing molecule in the brain and cartilage, as it serves as a large binding platform for various hydrophilic molecules. In the developing brain neurocan, a lectican-family CSPG, binds to hyaluronan and tenascin-C, a polymeric glycoprotein (Jones and Jones, 2000; Rauch et al., 2001). This trio, together with other related molecules, forms the anlage of brain ECM, as neurocan and tenascin-C bring more functionality and biological variety to the basic structure (figure 9).

During development, these molecules are replaced by others belonging to the same protein families; neurocan is replaced by another lectican, brevican, and tenascin-C are replaced by tenascin-R (Chiquet-Ehrismann et al., 1995; Yamaguchi, 1996; Milev et al., 1998b; Bruckner et al., 2000). Although both neurocan and tenascin-C are found in ECM structures outside the brain, it seems that brevican and tenascin-R are brain ECM specific molecules (Jaworski et al., 1994; Yamaguchi, 1996).

Previously, hyaluronan was held as a mere space-filling molecule, without any significance other than structural. Currently, it is appreciated as a physiologically important molecule, at least involved in the development of immune responses (Termeer et al., 2003). This finding further supports the view that ECM as an anlage has other functions than simply holding the brain together.

2.5.2 ECM associated molecules

In addition to the basic ECM structure, many molecules associate with the ECM in different levels of organization. Some are an inherent part of the structure, such as the glycoproteins, laminin and fibronectin (Rauvala et al., 1989), whilst others contribute less to the general structure and more to the dynamical biological functions of the brain cells. A complex, modular structure is typical for an ECM-associated protein. ECM proteins often oligomerize, bind other ECM proteins, cell surface proteins — especially integrins, and oligosaccharide structures. Thus, the amino acid sequences of ECM structural proteins are filled with domains for different types of interactions (figure 10) (Hohenester and Engel, 2002).

The importance of basic ECM molecules, like laminin and fibronectin, in general tissue organization is demonstrated by the multiple null-mutations produced in mice, of which many are pre- or peri-natally lethal (George et al., 1993; Xu et al., 1994; Gustafsson and Fassler, 2000). Lethality in fibronectin and laminin mutations could be due to implantation defects, or it could be associated with dramatic failures in mesodermal assembly in mice (Gustafsson and Fassler, 2000).

Many secreted PGs are considered to be ECM-associated molecules. Of HSPGs, perlecan is abundantly expressed at E10 in mouse brain, in the ECM surrounding the neural tube. It is not, however, present in the ECM immedi-

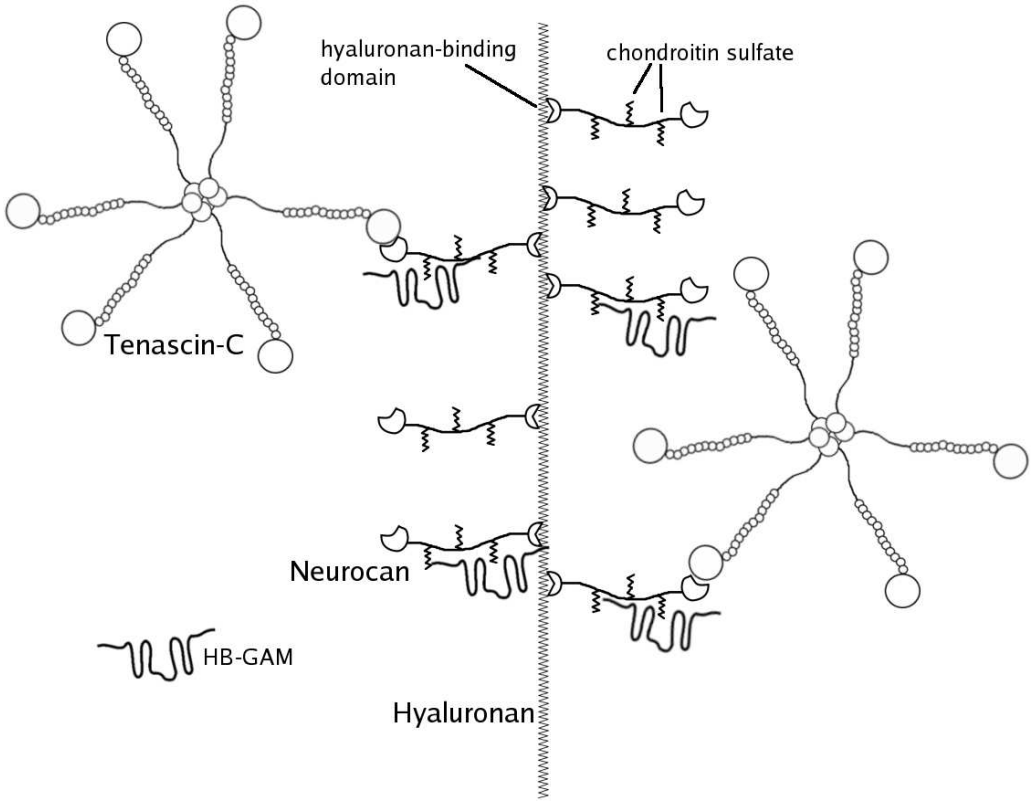

Figure 9: Fundamental ECM structures: Hyaluronan and PGs.
Hyaluronan is a linear polysaccharide with attachment sites for the N-termini of CSPGs like neurocan. In the developing brain ECM, neurocan in turn binds tenascin-C, as illustrated in the figure in its characteristic oligomeric form. It is important to note that neurocan, and later brevican, bind tenascins with their protein core, which leaves the CS chains free for other types of interactions (Milev et al., 1998a). HB-GAM binds many lecticans, including neurocan, and thus associates directly with the ECM (Milev et al., 1998a).

ately surrounding the proliferating cells in the neuroepithelium (Ford-Perriss et al., 2003). In *Drosophila,* perlecan contributes to the formation of growth factor or morphogen gradients, especially of FGF and Hedgehog (Park et al., 2003). In the mammalian brain its importance in gradient regulation is not so well established.

Agrin is an elusive HSPG found in the brain ECM. It plays an important role in the development and establishment of neuromuscular junctions by aggregating acetylcholine receptors in neuromuscular synapses; hence the name, agrin. In fact, agrin mouse knockouts die due to neuromuscular failures immediately after birth (Gautam et al., 1996; Serpinskaya et al., 1999). Agrin is expressed in the ECM of the adult brain, too, but the early lethality of the knockout mice has made it very difficult to find functions for agrin in adult synaptic plasticity.

In the adult neuronal network CSPGs have been found to play a curious role in establishing the so called perineuronal nets around neurons. Amongst these CSPGs are versican and brevican (Koppe et al., 1997), neurocan (Matsui et al., 1998), aggrecan (Matthews et al., 2002), tenascin-R (Bruckner et al., 2000;

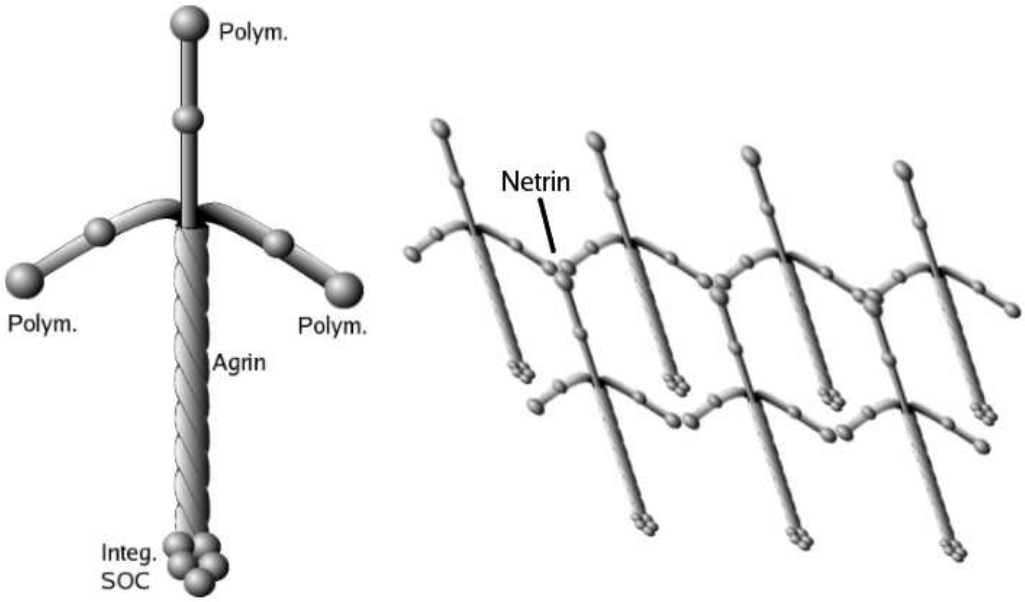

Figure 10: Model of a typical ECM molecule: structure of laminin.
Laminin is a trimer glycoprotein with multiple polymerization (Polym.) and binding sites for many molecules, including other ECM proteins. Only some of these sites are shown in the figure. The complex end of the trimer bundle binds cell surface molecules like integrins (Integ.), sulfated carbohydrates (SOC) i.e. PGs, and agrin. When laminin polymerizes, the junctions of the net provide additional binding sites for many ECM-associated proteins, like netrins. The mesh itself can act as a steric regulator e.g. for diffusion (Hohenester and Engel, 2002).

Haunso et al., 2000), and phosphacan (Wintergerst et al., 1996; Haunso et al., 1999). The nets can be viewed as hard, physical boundaries tightly surrounding neurons and effectively hindering any morphological changes, like growth of filopodia, in the neurons (reviewed in Murakami and Ohtsuka, 2003). In neuronal injuries it is quite clear that this type of inhibition is a major obstacle for plastic recovery and regeneration. In many studies applying chondroitinases to CNS injury sites has been a successful method for removing this inhibition (Zuo et al., 2002; Tropea et al., 2003; Yick et al., 2003).

A study by Pizzorusso et al. (2002) demonstrated a remarkable recovery of plasticity in the visual cortex of an adult rat after chondroitinase treatment. In the ocular dominance model the one eye of a young animal is covered for the so called critical period, when visual input through the eye molds the neuronal connections in the respective visual cortex. The experiment leads to blindness of the covered eye. In this model the eye still receiving input occupies — in addition to its normal cortical areas — partially areas of the cortex belonging to the blinded eye. After the critical period is over, plasticity of the cortex vanishes and uncovering the blinded eye does not induce new neuronal connections. Thus the eye remains blind. After chondroitinase treatment of the affected visual cortex the plasticity is, amazingly, gained, and visual input through the 'blind' eye remodels the corresponding visual cortex again, gaining functionality.

Although the presence of CSPGs especially in the adult CNS appears to be mostly a nuisance to neurologists, the perineuronal nets

probably have a very important protective role in the CNS (Celio and Blumcke, 1994). Thus, therapy using an enzyme treatment to destroy the whole sugar barrier surrounding a neuron is probably not without unwanted consequences.

2.5.3 Cell adhesion to matrix

As has been noted above, many ECM proteins possess binding sites for cell surface molecules, and stabilize the matrix surrounding the cells by binding these receptors. On the other hand, the cells grip to the matrix molecules and use them as contact points to stabilize their processes during cell movements. The major cell surface receptors for the ECM are the integrins. In addition to ECM proteins, integrins cross-talk with other cell surface molecules, which further influences the nature of integrin-ECM contacts.

The currently identified group of integrins consists of 18 α- and 8 β-subunits. The subunits form heterodimers, with over 20 known combinations (for a review of the family of integrins, see Juliano, 2002). In the CNS the β1 and β2-subunits predominate, especially at the early stages of brain development (Cousin et al., 1997; Pinkstaff et al., 1999; Rodriguez et al., 2000). Integrins bind mainly ECM proteins fibronectin, laminin, vitronectin and fibrinogen (Bronner-Fraser, 1985, 1987; Buck et al., 1986; Hofer et al., 1990), while at the cell surface integrins interact with the immunoglobulin superfamily adhesion molecules, including intercellular adhesion molecules and vascular cell adhesion molecule (Elices et al., 1990; Birdsall et al., 1992). Many αxβ1-integrin knockout mice die prenatally, and show severe malformations in their cerebrum, such as laminar disorganization, neuronal displacement and proliferation defects (for a review of the phenotypes, see Schmid and Anton, 2003).

Integrin αvβ8 dimers have a crucial function in establishing the intracerebral vasculature. Full depletion of either of the subunit genes results in peri-natal lethality due to massive hemorrhages in the brain (McCarty et al., 2002; Zhu et al., 2002). Surprisingly, removing any of the subunits from the vascular endothelial cells did not cause disruptions of the capillaries or otherwise change the vascular structure in the brain. Only when the genes coding the subunits were disrupted in the neural cells, the mutant mice started to display hemorrhages similar to those found in the full knockouts (McCarty et al., 2005; Proctor et al., 2005). These studies demonstrate, how important the matrix-stabilizing cell-ECM contacts are in the living brain, not only for the immediately affected cells but for all surrounding structures dependent on the ECM mesh. In the case of this particular integrin dimer, it appears that the NE and early glial cells are mostly responsible for stabilizing the integrin-ECM contacts, that support angiogenesis.

HSPGs participate in cell-cell and cell-matrix adhesion at different levels. Direct evidence for the establishment of cell-cell contacts solely by HSPGs does not exist, although syndecan-1 and 4 over-expression is known to increase formation of cell aggregates due to HS chains (Stanley et al., 1995). The majority of ECM proteins possess heparin and HS binding sites, and HSPGs bind them with relatively weak affinities (Ingham et al., 1990; Sweeney et al., 1998; Herndon et al., 1999). The weak binding in itself appears to be enough to induce cytoskeletal changes (Dehio et al., 1998; Herndon et al., 1999; Okamoto et al., 2003), but usually HSPGs, especially syndecans, are considered to function as co-receptors for integrins and other ECM protein receptors (figure 11).

In the co-receptor-model HSPGs bind ECM proteins with weak affinity and pull them closer to the cell surface, where the proteins are bound by integrins, launching cell signaling cascades. This co-operation is studied especially in carcinogenesis and metastasis, where syndecan-1 appears to be important (Leppa et al., 1992; Beauvais and Rapraeger, 2004). In cell spreading and movement the HSPGs and integrins also interact. Signaling through both

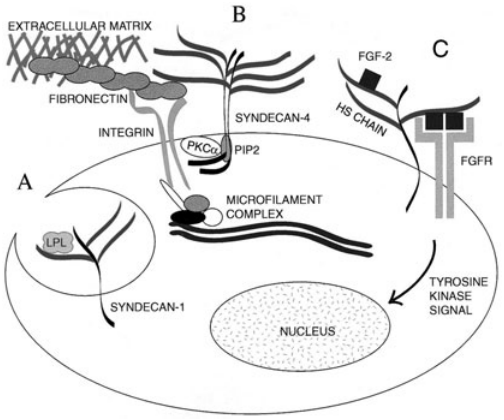

Figure 11: Integrins and syndecan-4 co-operate in cell adhesion.
Syndecans participate in many functions in the cell surface, like endocytosis (A), cell adhesion (B), and growth factor activity (C). Integrin alone can attach the cell to fibronectin matrix, but it is not enough for the formation of stress fibres and a link to the cytoskeleton. Syndecan-4 is required for the formation of a functional focal contact. It binds fibronectin and is recruited to the sites of ECM–cell contacts, where it establishes a signaling cascade to the cell cytoskeleton (Yoneda and Couchman, 2003). Reprinted from Tumova et al. (2000), with permission from Elsevier.

β1-integrin and syndecan-4 is required for cell spreading on fibronectin (Saoncella et al., 1999; Thodeti et al., 2003), although some controversy exists as to what extent the HS chains are necessary in this model (Echtermeyer et al., 1999). Syndecan-1 is reported to regulate the expression of integrins in the epithelial cells (Stepp et al., 2002), but the mechanism for this is still unclear.

2.6 Proteoglycans

> "Proteoglycans: cell biologists have had a love-hate relationship with these molecules almost since their discovery", Lander and Selleck (2000).

A PG in a simplistic form is a protein with one or more chains of sugar residues attached. The simplicity ends there. The sugar residues are polysaccharides consisting of GAGs possessing many types of chemical modifications, like sulfations (Kreuger et al., 2006). Among the proteins known to carry these GAGs there are transmembrane proteins, GPI anchored proteins, and secreted proteins. Many of the membrane attached proteins show a tendency to be shed from the membrane by proteases, which increases the biochemical complexity of the protein cores alone (Sanderson et al., 2005; Wang et al., 2005).

The story of the sugars is even more complex and very difficult to approach. One major difficulty is the lack of reasonably easy and quick methods for sequencing the existing GAG chains and mapping their chemical modifications.

Despite the difficulties in analyzing the GAGs, we already know that GAG synthesis and modification are tissue and cell type specific (Herndon et al., 1999; Shuo et al., 2004). Many of the enzymes involved in GAG synthesis and modification are known, and their expression has been mapped in different developmental stages and in different tissue types (Lin et al., 2000; McLaughlin et al., 2003; Bulow and Hobert, 2004). The GAG-carrying proteins are also very much developmentally regulated and are usually tissue or cell specific (Yamaguchi, 1996; Saunders et al., 1997; Toba et al., 2002).

GAG chains share basic structural similarities. They are negatively charged linear polysaccharides, composed of disaccharide repeats. These disaccharide units contain a uronic acid and a hexosamino sugar, which are modified during their synthesis, resulting in tissue and cell specific diversity (Raman et al., 2005; Kreuger et al., 2006). The modifications are most notably O- and N-sulfations and N-acetylations (McLaughlin et al., 2003; Kreuger et al., 2006).

The amino sugar component varies in GAGs and is the basis of their division in to different groups. GAGs with glucosamine are heparin, HS, and hyaluronan. GAGs with galactosamine

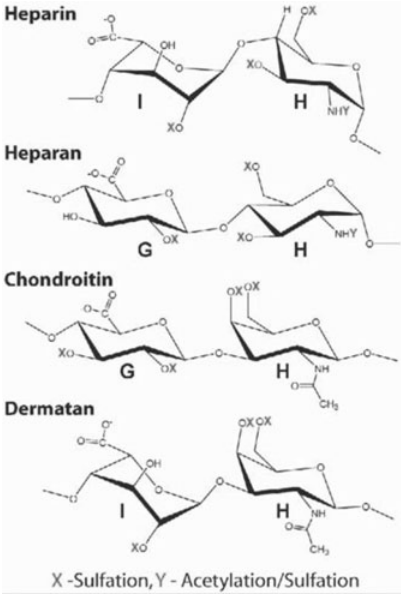

Figure 12: Heparin and GAG disaccharides. Heparin and and other GAGs have differing disaccharide units. 'H' stands for the amino sugar component, which is glucosamine in heparin and HS, and galactosamine in CS and dermatan sulfate. 'I' and 'G' are iduronic acid and glucuronic acid, respectively. These disaccharides are not restricted to one GAG type, but variation exists. I.e. HS can have iduronic acid residues, which are predominantly found in heparin. Reprinted from Raman et al. (2005) with permission from Elsevier.

are CS and dermatan sulfates (figure 12). Other GAG compositions and groupings exist, but escape the focus of this review.

Virtually all mammalian cells express some PGs. In many occasions, the necessity of these molecules to the normal development and function of organs has been demonstrated. Although GAGs and their carrier proteins are physically linked, the interactions between these two molecular worlds — sugars and peptides — are not straightforward.

Moreover, although CS is predominantly found in just CSPGs, some syndecans have mixed glycosylation, presenting CS chains in addition to HS (Deepa et al., 2004; Shuo et al., 2004). This kind of mixed glycosylation of PG core proteins is not a new finding, but appears to be specific for syndecans. The functional meaning of this mix-up might relate to differential growth factor binding in CS/HS-syndecans (Deepa et al., 2004).

2.6.1 Chondroitin sulfate

CS are a substantial part of the brain ECM. There are many different carrier proteins for CS, most of them are soluble. In the developing brain CSPGs participate in growth factor modulation, inducing NSC proliferation, neural migration and guiding axonal and dendritic growth. Occasionally, especially in neurite outgrowth, CSPGs appear to act as counterweights for HSPGs, in that axonal growth is inhibited in high-CS areas, whereas in high-HS areas axon growth is promoted (Tisay and Key, 1999).

At the time of writing this thesis, CS has captured researchers interest by being the nuisance substance in axonal regeneration and in neuronal plasticity (Zuo et al., 2002; Rhodes and Fawcett, 2004). In rats, CSPGs are expressed heavily during nerve damage by the surrounding glial cells, and they are crucial in formation of the so called glial scar, which prevents the regrowth of axons over the injured area (Yick et al., 2003; Hayashi et al., 2005). In areas of synaptic connectivity in murine and human brain, CSPGs form the so called perineuronal nets (Celio and Blumcke, 1994; Koppe et al., 1997); rigid structures which tightly hug the soma of pyramidal neurons. These envelopes appear to effectively block any local plastic changes in the neurons, preventing rewiring and new connections (Murakami and Ohtsuka, 2003).

2.6.2 CSPGs

Receptor type protein tyrosine phosphatase-β/ζ and phosphacan Phosphacan is one of the major CSPGs in the murine CNS. It is a splice variant of the receptor type protein phos-

phatase (RPTP)-β/ζ, lacking the transmembrane and intracellular phosphatase domains. Another splice variant of the receptor exists in mouse, sRPTP-β/ζ, a form lacking most of the extracellular CS glycosylation sites (Levy et al., 1993; Maurel et al., 1994). Although RPTP-β/ζ and its splice variants are mostly expressed in the glial cells, their temporal regulation differs during murine brain development (Canoll et al., 1996). The full-length receptor is most abundant during early brain development, and it is found in the proliferating areas of the cerebral cortex. Phosphacan expression, on the other hand, increases during development, and is most prominent in post-natal and adult murine brains (Canoll et al., 1996; Hayashi et al., 2005). The short form of the receptor is present in relatively low amounts throughout pre- and post-natal brain development (Canoll et al., 1996).

RPTP-β/ζ is also a known receptor for HB-GAM in neural migration (Maeda and Noda, 1998). Phosphacan binds HB-GAM, too, and it is possible that these two soluble molecules modulate the activity of the other in the ECM, or that they have a cooperational, thus far unknown, function in brain development.

2.6.3 Heparan sulfate and heparin

HS and heparin share the basic GAG structure, heparin having a higher sulfation percentage than HS (Raman et al., 2005; Kreuger et al., 2006). Heparin is used routinely as an anticoagulant, and many other therapeutic applications exist or are under investigation. Thus, heparin is commercially available in large amounts, and it can, to some extent, mimic the functions of HS. The main repeat in heparin molecule resembles the protein binding repeat in HS. According to structural studies carried with nuclear magnetic resonance (NMR), the high sulfation level of heparin increases its affinity in protein binding (Mulloy and Forster, 2000; Raman et al., 2005). Heparin is thus utilized as an HS mimetic in many HS–protein-complex models.

HS is, however, distinct from heparin, although this distinction is often neglected in research setups. In addition to structural and functional differences, it has to be kept in mind that the only natural source for heparin in a living animal are the mast cells, while HS is expressed by virtually all cells in multicellular animals. Moreover, heparin exists as a secreted oligosaccharide, while HS is usually bound to a PG core through an O-glycosidic linkage.

HSPGs are particularly important in modulating the gradients of different growth factors and cytokines in tissue. In the brain these factors are often guidance factors for neuronal migration and axonal growth, but can also regulate the general organization of the CNS structures (McLaughlin et al., 2003; Rhiner et al., 2005; Fisher et al., 2006). The importance of HSPGs in brain organization was quite dramatically demonstrated with a conditional knockout mouse lacking the enzyme exostosis type 1 (EXT1) in brain (Inatani et al., 2003). EXT1 is responsible for building the disaccharide repeats in GAGs, and without it no HS is synthesized. The full mouse knockout of EXT1 is not viable beyond gastrulation (Lin et al., 2000), and in a mouse, where the mutation is targeted to the CNS, whole brain structures are missing or badly malformed (Inatani et al., 2003).

In the ECM of the brain, HSPGs are represented mostly by perlecan and agrin. The majority of the cell surface HSPGs belong to one of two families of PGs; the syndecans or the glypicans.

2.6.4 HSPGs

Glypicans Glypicans are named after their GPI anchor to the cell surface, they are represented by six genes in mammals, two in *Drosophila melanogaster* and one in *Caenorhabditis elegans*. The protein cores are not specially homologous, except for many conserved cysteine residues, but the glycosylation sites, which are located close to the cell

membrane, seem to be preserved in all glypicans (Veugelers et al., 1999). The 14 conserved cysteines suggest that glypicans are very globular and do not extend far from the cell membrane.

The family of glypicans has a crucial role in developmental morphogenesis in flies and in humans. In *Drosophila* the glypicans Dally and Dally-like affect the Wnt signaling pathway and bind the fly tumor growth factor (TGF)-β homolog, and their mutations cause severe patterning and growth defects in antenna, eye and genitals (Jackson et al., 1997; Baeg et al., 2001). In humans a mutation in glypican-3 gene causes Simpson-Golabi-Behmel-syndrome (Pilia et al., 1996), which has made glypican-3 the most studied HSPG in the glypican family. The X-linked syndrome is characterized by pre- and post-natal overgrowth, but covers a variety of malformations too. There is no clear correlation between different mutations in the glypican-3 gene and the different phenotypes seen in the patients. Thus, the syndrome is thought to be caused by the lack of functional glypican-3 protein together with familial genetic factors affecting the phenotype (Hughes-Benzie et al., 1996).

Many of the murine glypicans are expressed in the developing CNS. In mouse, glypican-1 and 4 are found in the proliferative areas of the ventricles, but glypican-1 is present in the postmitotic neurons as well (Litwack et al., 1998; Hagihara et al., 2000). Glypican-2 is expressed in the immature mouse neurons, especially in their growing axons, and it is down-regulated when the axons reach their targets (Ivins et al., 1997). Interestingly, glypican-5 is up-regulated in the mature nervous system (Saunders et al., 1997). These differences in the neural expression raise the question, whether the members of the glypican family could have opposite functions during neural proliferation and differentiation.

Syndecans The first syndecan to be identified, syndecan-1, was isolated from epithelial cells and named after the Greek word 'syndein' — to bind together — because it was believed to bind ECM molecules to the actin cytoskeleton of the cells (Rapraeger and Bernfield, 1983; Koda and Bernfield, 1984; Saunders et al., 1989). This assumption has later turned out to be more or less the correct one. Syndecans are transmembrane PGs with intracellular sequences which bind both cortactin and the PDZ-domain (postsynaptic density-95 / discs large / ZO-1) of CASK. Cortactin and CASK are proteins well known for their interactions with the actin cytoskeleton (see also 15) (Kinnunen et al., 1998b; Cohen et al., 1998; Hsueh et al., 1998). The family of mammalian syndecans has four members, while invertebrates have only one. In the zebrafish, genes encoding syndecan-2 and 4 homologs have been identified (Chen et al., 2004; Whiteford and Couchman, 2006), while a predicted syndecan-3 (N-syndecan) gene is found but no known protein products exist (GenBank accession nr XM_695352). Syndecans are highly conserved across different species, and inside the gene families, sharing similar extracellular structures (Rapraeger, 2001).

Curiously, syndecans 1, 3 and 4 have glycosylation sites for CS very close to the plasma membrane. At least syndecan-1 and 4 are known in some circumstances to carry a mixed population of HS and CS chains (Deepa et al., 2004). Apparently, the heterogeneity adds to the ligand binding strength of these syndecans, as has been demonstrated with HB-GAM, midkine and FGF-2 (Deepa et al., 2004). To date, heterogeneous glycosylation in N-syndecan is just speculation, but intuitively it appears more probable than not.

Syndecans and glypicans in protein binding The structural distinction between syndecans and glypicans leads to functional distinctions in many HSPG-mediated events (figure 13). Distal or proximal presentation of the HS chains to the cell membranes is important for modulation of growth factor activity and cell-cell contacts.

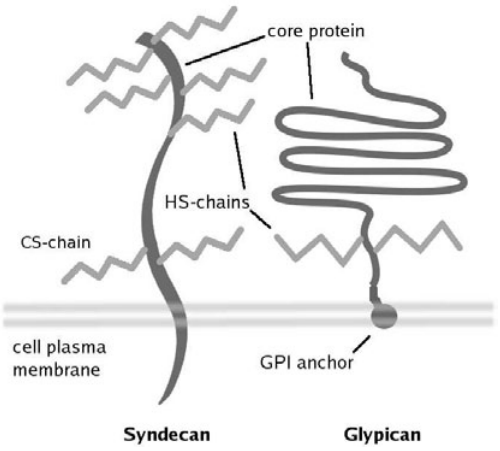

Figure 13: Structural differences between syndecans and glypicans.
Typically syndecans have a streight protein core, with glycosylation sites distal to cell membrane. Glypicans are more globular and have most of their glycosylation sites close to the cell membrane.

Thus, both syndecans and glypicans seem to be able to present FGF-2 to its receptor, while only syndecans seem to affect the strength of cell-cell adhesion during leukemia cell invasion (Steinfeld et al., 1996; Liu et al., 1998).

The predicted, relatively straight structure of syndecans, with the distal GAG attachment sites, makes the syndecans likely binding partners with insoluble ECM molecules. Laminin, fibronectin and collagens are known to bind the syndecan ectodomains, immobilizing the transmembrane PGs to the membrane, where they can further bind to the cytoskeletal proteins. Both syndecans and glypicans bind the soluble ECM associated molecules, like growth factors and cytokines.

The most notable group of growth factors binding HSPGs are the FGFs. FGF receptor (FGFR) activation provides the classic scheme of HSPG-dependent growth factor binding, where either syndecan or glypican acts as a co-receptor for one of the FGFRs (see chapter 2.7.1). Some of the FGF family proteins are surprisingly specific to certain types of HSPGs, like in the case of FGF-1 and FGF-2. In mouse, FGF-1 is expressed at an earlier phase of embryonic development than FGF-2 and it appears to favor syndecan-1 as a co-receptor, while FGF-2 has a clearly higher affinity for N-syndecan (Chernousov and Carey, 1993; Nurcombe et al., 1993). Recent knockout mouse studies have revealed, how essential the interplay between HSPGs and FGFs actually is. Removing the enzyme responsible for a specific step in syndecan glycosylation, EXT1, produces a remarkably similar phenotype compared with the FGF-8 knockout in the mouse brain (Meyers et al., 1998; Inatani et al., 2003).

Modulations in the sulfation pattern of the HS GAGs is an important aspect in growth factor binding. This was nicely demonstrated in two recent papers describing the syndecan glycosylation and biological functions in *C. elegans*. It appears that certain sulfation patterns are required for axonal midline crossing and for motoneuron migration, and that syndecan in *C. elegans* is the major target of these GAG modifications (Bulow and Hobert, 2004; Rhiner et al., 2005).

Cytokines HB-GAM and midkine form a distinct group of ECM-associated proteins on their own (Merenmies and Rauvala, 1990; Tsutsui et al., 1991). HB-GAM and midkine induced neurite outgrowth and migration of different cell types are mediated by at least HSPGs N-syndecan and syndecan-1, and by a CSPG, RPTP-β/ζ (Kinnunen et al., 1996; Asai et al., 1997; Nakanishi et al., 1997; Imai et al., 1998; Maeda and Noda, 1998; Milev et al., 1998a). In addition, in the case of midkine, some integrins and Low-density protein receptor-related protein are probably involved in its signaling functions (Muramatsu et al., 2000, 2004).

2.6.5 Proteoglycans and NSCs

Cell surface-bound HSPGs syndecans and glypicans are both represented in the early NSC environment of mice, but in a very specific manner. Syndecan-1 and N-syndecan are the

major syndecans expressed at E10 mouse neuroepithelium, while glypican-4 of glypicans is the most abundant there (Ford-Perriss et al., 2003). Syndecan-1 and glypican-4 expression is largely overlapping; both are expressed by NSCs and contribute to the stem cell niche (Ford-Perriss et al., 2003). N-syndecan, on the other hand, is also richly present in the neuroepithelium at E10, but it is not found at the NSC surfaces, but in the differentiated neurons (Inatani et al., 2001; Ford-Perriss et al., 2003). Syndecan-1 is downregulated in the differentiated cells.

Phosphacan promotes specifically FGF-2–driven cell proliferation and colocalizes with FGF-2 in the developing forebrain (Milev et al., 1998c). Very recently, a general NSC promoting function for different types of mixed CSPG preparations was published. The CSPGs appear to induce proliferation only in the FGF–2 induced NSC population, but not in the EGF–induced cells (Ida et al., 2006).

2.7 Growth factors and their receptors

After initial patterning of the mouse embryonic brain structures, neurogenesis is very much dependent on a few key growth factors and their receptors. Many of these growth factors depend on the cell surface proteglycans, or on the composition of the nearby ECM, in receptor activation (Brickman et al., 1995; Ornitz, 2000; Chu et al., 2005). Receptors can cluster and form signaling pools at the cell surface. These receptor clusters are often found in so called lipid rafts, and they concentrate many important signaling molecules, amplifying their effects (Liang et al., 2001; Ma et al., 2003; Hur et al., 2004). This amplification may even be directed to different parts of the cell. In neurons, localization of the lipid rafts has a clear effect on the direction of neurite growth (Guirland et al., 2004).

FGFs and EGF -family growth factors can, *in vitro*, maintain NSCs in the absence of other growth factors. FGF-8 functions as an organizer protein in the mouse mesencephalon, and lack of it leads to the absense of dopaminergic neurons and to cerebellar malformation (Chi et al., 2003). FGF-2 acts as a general proliferation and differentiation factor for mouse embryonic and adult NSCs, driving them towards neuronal fate, while EGF promotes proliferation and differentiation mostly of glia (Kilpatrick and Bartlett, 1995; Kuhn et al., 1997; Tropepe et al., 1999).

EGFR expression starts after neurogenesis has been going on for awhile, and no amount of exogenous EGF can induce glial fate in the earlier NSCs (Burrows et al., 1997; Tropepe et al., 1999). Moreover, many growth factors share the same receptor, and can induce different cellular responces, while the same ligand can be shared by several receptors, causing a wider array of different responses. It is very common that a family of growth factors has a corresponding, large family of receptors with distinct affinities and expression patterns (Simon, 2000).

2.7.1 FGFRs and their ligands

FGFRs and their ligands are among the oldest known growth factor systems in vertebrates. The receptor family has four members, while the number of different ligands is, at the moment, 23. Not all FGFs are found in all species (Reuss and von Bohlen und Halbach, 2003; Thisse and Thisse, 2005). Humans have 22 FGFs, as do mice. Humans miss FGF-15 while mice lack FGF-19. FGF-1, -2 and -8 are probably the most studied growth factors in the context of the CNS, while FGFR1 is the receptor most commonly referred to (Reuss and von Bohlen und Halbach, 2003; Thisse and Thisse, 2005). FGFs and FGFRs have several functions in the developing and mature CNS. They participate in neural tissue induction, NSC proliferation, neural differentiation, axonal elongation and branching, and even in learning and memory (Reuss and von Bohlen und Halbach, 2003;

Thisse and Thisse, 2005).

FGFRs are protein tyrosine kinases. Homology between the four receptors is high in the cytoplasmic part. Combined with their similarity in signaling, this has meant that FGFR1 is often used to model the general behavior of all the family members (Johnson et al., 1991; Johnson and Williams, 1993). In essence, the differences in signaling are more about the strength of kinase activity than about selecting signaling proteins (Raffioni et al., 1999).

FGFR tyrosine kinases have several target proteins, including Src, PLCγ, Crk and SNT-1. Conclusive data about the specific role of these targets have been hard to obtain. It appears that FGFRs behave very differently depending on the cell type. PLCγ and src are not involved in mediating the mitogenicity of FGFs, but it is not clear, if they are involved in cell motility (Goldschmidt-Clermont et al., 1991; Landgren et al., 1998). PLCγ signaling could link with the modulation of actin cytoskeleton via profilin, but it is not directly involved in cell motility (Goldschmidt-Clermont et al., 1991; Landgren et al., 1998). Src kinase activation could be another route to cell motility via cortactin, but the evidence for FGFR interaction with src and cortactin is contradictory (Zhan et al., 1993, 1994; Landgren et al., 1995).

Again, in mitogenicity, singling out a particular signaling route is difficult. Crk is activated by FGFRs and does not affect cell motility but mitogenesis, although apparently only in certain cell lines (Mohammadi et al., 1996a,b; Larsson et al., 1999). The best candidate for mediating mitogenic signals is the SNT-1/FRS-2 route, which directly connects FGFR to the Ras/MAP kinase pathway, an important route in growth factor induced proliferation (Wang et al., 1996; Kouhara et al., 1997).

Most FGFs bind more than one receptor, and many bind all of them, but there are distinctions in affinities, which become essential in the dynamics of FGF/FGFR-complex formation. The extracellular part of every FGFR has a domain which can vary due to alternative splicing of the mRNA (Johnson et al., 1991). This variance in the genetic structure adds to the diversity of FGFRs and is the basis for the significantly distinct FGF/FGFR affinities (Ornitz et al., 1996; Ornitz, 2005).

Many FGFs and FGFRs are already involved in the neuronal patterning of an early mouse embryo. Especially FGF-2 has become famous for its function in cortical development and expansion of the NSC pool (Cattaneo and McKay, 1990; Murphy et al., 1990; Vaccarino et al., 1995; Tropepe et al., 1999). FGF-2's potency in neural development is well demonstrated by its ability to drive mouse NSCs to proliferation and to induce neuronal differentiation in the precursors without the aid of other growth factors (Ray et al., 1993; Kilpatrick and Bartlett, 1995; Vaccarino et al., 1995). Moreover, FGF-2 is also a remarkably important stimulator for adult neurogenic cells (Zheng et al., 2004; Xu et al., 2005).

FGF-2 binds all four FGFRs, and three of them — FGFR-1, -2, and -3 — are expressed in somewhat overlapping patterns in the developing mouse and chicken telencephalon (Orr-Urtreger et al., 1993; Patstone et al., 1993; Peters et al., 1993). FGFR-1 is abundant in the VZ of the developing cortex and hippocampal primordium and its expression strongly overlaps with the expression of FGF-2 (Vaccarino et al., 1999; Ohkubo et al., 2004). It is thus likely to be the key receptor in mediating the effects of FGF-2 on NSCs. However, the general organizing role of FGFR-1 and -2 in early embryos makes it very difficult to discern their specific roles in brain development, as the full mouse knockouts are prenatally lethal (Yamaguchi et al., 1994; Arman et al., 1998). A conditional knockout has been created for FGFR-1, which has substantially helped research concerning the functions of the receptors in the CNS (Partanen et al., 1998; Trokovic et al., 2003; Ohkubo et al., 2004).

2.7.2 EGF receptors and their ligands

In mammals, the EGF receptor family has four members named ErbB1-4, ErbB1 being a synonym for EGFR. Of these four receptors ErbB1 binds the traditionally known EGF family growth factors, EGF, TGF-α, amphiregulin and HB-EGF (Kaser et al., 1992; Iwamoto and Mekada, 2000; Falk and Frisen, 2002; Piao et al., 2005). ErbB2 does not have any known ligands, and it is suspected to act as a coreceptor for the other family members. ErbB3 and 4 are neuregulin receptors, although ErbB3 binds EGF as well (Zhu et al., 1995; Ma et al., 2003). Because ErbB1, or EGFR, is the main receptor for the neural EGF-family growth factors, the rest of this section discusses only EGFR.

EGFR is expressed in the cortex when the SVZ forms, and persists in the adult germinal areas (Burrows et al., 1997). EGFR is the predominant growth factor receptor in the population of interneuron progenitors that supply the mouse olfactory bulb with replacement neurons throughout adult life (Gritti et al., 1999). It is noteworthy that aging reduces the expression level of EGFR in this cell population, causing reduced fine olfactory discrimination (discrimination between very similar odors) in older mice (Enwere et al., 2004). This reduction is due to a decreased supply of new interneurons to the olfactory bulb.

EGFR acts in neural cells as a scatter-factor — a receptor that induces migratory behavior in the cell. EGFR itself probably does not give directional information to the cell but causes it to switch from stationary to mobile behavior (Caric et al., 2001; Boockvar et al., 2003; Aguirre et al., 2005). This ability is probably connected to the crosstalk between EGFR and some cell surface integrins which form complexes with the receptor and contribute to its signaling (Cabodi et al., 2004).

In the developing brain three ligands for EGFR are found: EGF, HB-EGF and TGF-α (Lazar and Blum, 1992). TGF-α and HB-EGF are abundant ligands in the adult mouse brain, although their expression is regionally different (Seroogy et al., 1991; Piao et al., 2005). Amphiregulin is a mitogen for adult mouse NSCs, and it is present in the choroid plexus along the ventricles and in the hippocampus of the adult murine brain (Falk and Frisen, 2002). Both EGF and HB-EGF are expressed during neural proliferation and migration (Seroogy et al., 1991; Kaser et al., 1992; Lazar and Blum, 1992; Nakagawa et al., 1998; Piao et al., 2005). HB-EGF is known to function as a chemotactic molecule for neurons, and it cannot activate EGFR without the presence of HS (Besner et al., 1992; Aviezer and Yayon, 1994; Caric et al., 2001).

Although the amount of EGFR is not very high at any point during neural development or adulthood, it is found to have an ubiquitous role in gathering and amplifying different cell signals (Zwick et al., 1999). c-Src kinase has been identified as an important shared signaling molecule between EGFR and many other receptors, like N-syndecan (Hur et al., 2004). In addition, cortactin is reported to link the cytoplasmic signaling complexes of EGFR and other receptors, amplifying the signal and preventing endocytosis of ligand-bound EGFR (Lynch et al., 2003; Timpson et al., 2005).

2.7.3 HS in activation of FGFR and EGFR

FGFs bind strongly to heparin and HS, and the presence of HS is required for FGFR activation by FGFs (Rapraeger et al., 1991; Yayon et al., 1991). FGF/FGFR coupling serves as the classical model for HS-dependent growth factor receptor activation and its structural mechanism has been reviewed in Mohammadi et al. (2005). After the FGF/FGFR complex is formed, the complex further dimerizes with the aid of a heparin molecule or an HS chain, which binds a groove in the FGF/FGFR unit. This binding stabilizes the forming dimer and activates the receptor tyrosine kinases. Figure 14 summarizes the current view of the ternary structure of the complex.

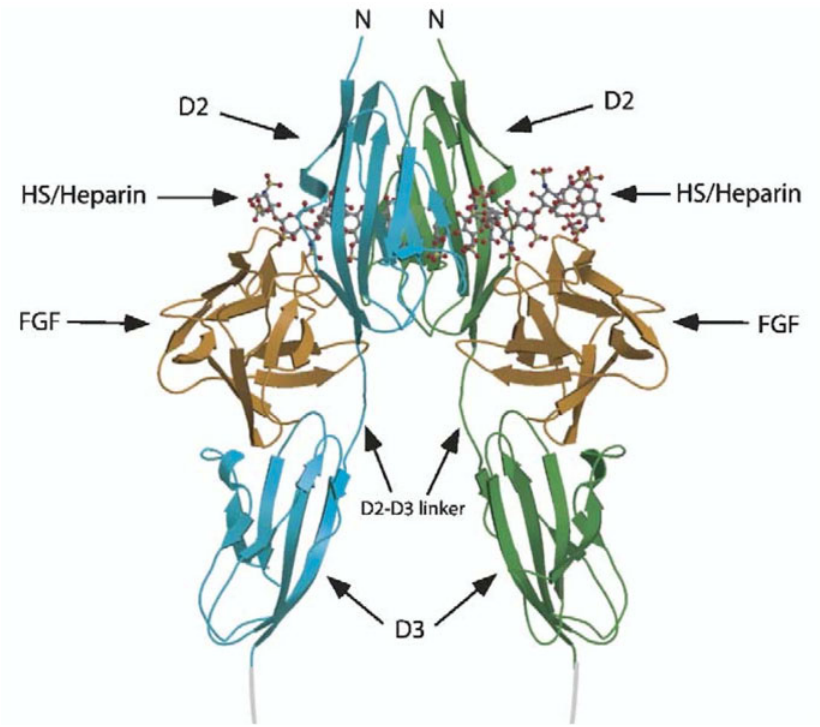

Figure 14: FGFR-heparin-FGF–binding, ternary structure.
Heparin/HS dimerizes FGFR by binding the D2–domains of the receptor, and FGF, which in turn binds the receptor's D3–domain. Transmembrane domains of the receptor dimer are left out from the picture. Reprinted from Mohammadi et al. (2005), with permission from Elsevier.

Although all FGFs have a high affinity to HS, the specific affinities can vary. Current data suggest that HS modulates FGFR activation and signaling in a rather complicated way. Different FGF/FGFR combinations seem to pick and choose distinct HS sequences amongst the available ones, thus the availability of certain subtypes of HS would have an acute effect on the function of the ligand-receptor complex. Another possibility is that a certain subtype of HS chains can stabilize the FGF/FGFR complex more efficiently than other HS chains, thus leading to stronger signaling. How this structurally takes place is still disputed (Pellegrini et al., 2000; Mohammadi et al., 2005).

FGFR isoforms have differing affinities to HS as well. In Brickman et al. (1995) a HSPG was found in mouse NE cells that specifically bound to FGFR-1 but not to the other available receptor, FGFR-3.

In HS dependent EGFR activation the mechanism is slightly different. Only one EGFR ligand — HB-EGF — seems to require the presence of heparin or HS to activate the receptor (Higashiyama et al., 1993; Aviezer and Yayon, 1994; Shishido et al., 1995). Apparently, HS and heparin act on the ligand and not on the ligand-receptor complex, in contrast with the FGFR activation.

HB-EGF has two domains; the heparin binding domain and the EGF-like domain (Higashiyama et al., 1992). Deletion of the hep-

arin binding domain creates a continuously active form of HB-EGF, suggesting that the domain masks the EGFR-binding domain of the growth factor (Takazaki et al., 2004). When HS or heparin binds HB-EGF it reveals the EGFR-binding part of the protein, promoting EGFR activation.

2.8 N-syndecan

N-syndecan, or syndecan-3, has come to the field of syndecan research quite late (Gould et al., 1992). In mice and rats, it is mostly expressed in the developing nervous system but also in the adult hippocampal structures (Nolo et al., 1995; Carey et al., 1997; Nakanishi et al., 1997; Kinnunen et al., 1998a; Toba et al., 2002). The other syndecan family members, especially syndecan-1, have received most of the attention of researches studying HSPGs in oncogenesis, inflammation and other pathological conditions (Endo et al., 1997; Bellin et al., 2003; Elenius et al., 2004; Reiland et al., 2004). N-syndecan expression appears to be very strictly regulated in the adult murine brain by neuronal activity (Lauri et al., 1999). This offers some interesting opportunities to further study its role in feeding behavior and obesity, in Alzheimer's disease, and in recovery from neuronal injuries (Reizes et al., 2001; Elimova et al., 2004; Strader et al., 2004; Beckman et al., 2006).

During brain development, the functions of N-syndecan have not been distinguished from the functions of HSPGs, although it is well known that N-syndecan differs in some very significant ways from the other abundant syndecan in brain, syndecan-1 (Bandtlow and Zimmermann, 2000; Bellin et al., 2003). They have differing abilities to bind ECM molecules and to modulate the cytoskeleton via cell signaling. In short, syndecan-1 has higher affinity to the ECM, than N-syndecan, and most of the functions of N-syndecan are related to cell signaling, instead of interactions with the ECM (Bernfield et al., 1999). This area has so far received very little attention.

2.8.1 Developmental expression of N-syndecan

The expression of N-syndecan starts very early in the mouse embryo, around E9.5, when it is expressed in the caudal axons growing towards the spinal cord. A few days later it is found in the dorsal root ganglia and in the growing axons in midbrain and in the thalamo-cortical pathways (Nolo et al., 1995; Imai et al., 1998). N-syndecan is, in addition, found in the early proliferating areas of the cerebral cortex, but it is expressed in the post-mitotic neural precursors (Ford-Perriss et al., 2003). More explicitly, N-syndecan localizes to the growing processes in these neurons. Outside the cerebrum, N-syndecan is very strongly expressed in the rat olfactory placode at E15, where it is found in the growing axons of the olfactory epithelium and in the migrating neurons (Toba et al., 2002).

Expression of N-syndecan in the rodent brain and spinal cord gradually increases during neuronal differentiation and peaks at around one week after birth (Carey et al., 1997). The expression peak coincides with the period of heavy neuronal and glial migration in the cerebrum. Neuronal pruning and establishment of synaptic connections also take place during the two weeks after birth.

Outside the CNS, N-syndecan is expressed in different cell types in the developing skeletal bones. In the chick embryo, N-syndecan is found in the proliferating chondrocytes of cartilage (Shimazu et al., 1996). The amount of N-syndecan mRNA is noticeably decreased during chondrocyte maturation. In mice, N-syndecan is expressed prenatally by mineralized chondrocytes and by immature osteoblasts migrating towards ossification sites (Imai et al., 1998).

2.8.2 N-syndecan in CNS plasticity and behavior

N-syndecan gene expression is upregulated by high-frequency stimulation that induces long term potentiation (LTP) in rat hippocampal slices (Lauri et al., 1999). The upregulation is strongly associated with the formation of LTP, as inhibition of N-syndecan results in enhanced LTP in rats (Lauri et al., 1999).

A detailed experiment with syndecan-1 overexpressing, or syndtrophin, mice suddenly revealed a physiological function of N-syndecan in feeding behavior. Mice overexpressing syndecan-1 in the hypothalamic nuclei become obese after reaching maturity. The reason for obesity is hyperphagia (i.e., over-eating), rather than hormonal or metabolical changes (Reizes et al., 2001). Both syndecan-1 and N-syndecan bind the melanocortin receptor 4 (MCR-4)–inhibiting peptide and, at the cell surface, present the inhibitor to MCR-4. The cells expressing melanocortin receptor 4 appear to control the feelings of hunger and satiety in animals, and probably in humans too (for review see Porte et al., 1998).

Transgenic syndecan-1 is not shed from the cell surface of the hypothalamic neurons, and thus constantly inhibits the MCR-4, inducing hyperphagia (Reizes et al., 2001). The normal syndecan-1 ectodomain was hypothesized to shed, and induce satiety during feeding. Indeed, in wildtype animals, the amount of cell surface N-syndecan increases during fasting, and it is shed during feeding (Reizes et al., 2001). The shed extracellular domain of N-syndecan is suggested to remove the inhibiting blocker peptide from the melanocortin receptor, and the blocker is replaced with satiety inducing peptide. In N-syndecan knockout mice the hypothalamic nuclei naturally lack N-syndecans contribution to the hunger-satiety control, shifting the balance towards satiety, as the inhibitor peptide is no longer presented to MCR-4. After fasting, N-syndecan knockout mice display remarkedly decreased hunger-induced hyperphagia in comparison with wildtype mice (Reizes et al., 2001).

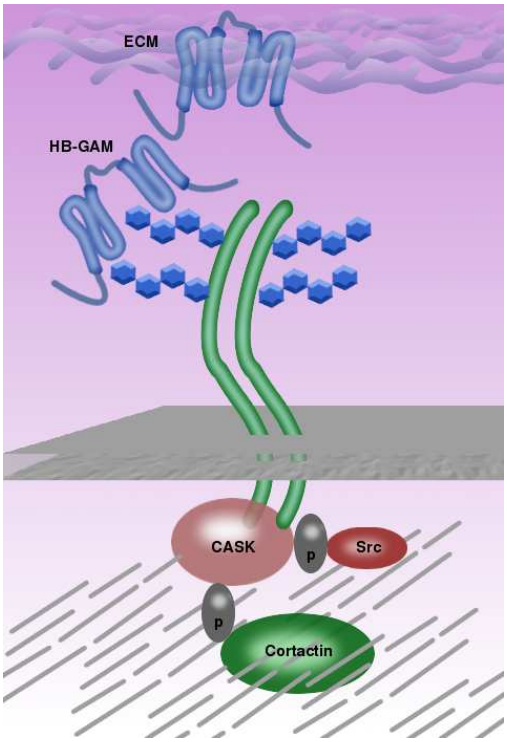

Figure 15: N-syndecan signaling.
ECM–associated HB-GAM binds to HS chains in N-syndecan. This dimerizes the receptor, and the intracellular PDZ-domain–binding part of N-syndecan activates CASK/cortactin, and induces c-Src or c-Fyn phosphorylation. CASK and cortactin as a complex further induce reassembling of the actin filaments of the cytoskeleton (Kinnunen et al., 1998b; Lauri et al., 1999). (p, phosphorylation.)

2.8.3 N-syndecan in cell migration

As was mentioned above, expression data available for N-syndecan suggest functions in neuronal migration. Although lacking for neurons, in other cell types concrete evidence exists for N-syndecan mediated migration. Osteoblast recruitment by HB-GAM during endochondrial ossification depends on and is mediated by N-syndecan (Imai et al., 1998). N-syndecan me-

diated neurite outgrowth is dependent on c-Src phosphorylation (figure 15), and the same signaling cascade could be linked to cell migration. In addition, N-syndecan is expressed by the migrating neurons in the olfactory placode (Toba et al., 2002), which provides indirect evidence for N-syndecan's function in neural migration.

2.9 HB-GAM

HB-GAM (also called pleiotrophin) and midkine form a two-member family of heparin-binding ECM associated cytokines, distinct from the heparin-binding growth factors like FGFs (Merenmies and Rauvala, 1990; Muramatsu and Muramatsu, 1991; Muramatsu, 1993). They are not classical growth factors in the sense that most of their biological functions require them to be matrix bound. In the case of HB-GAM only immobilized protein induces neurite outgrowth in primary mouse and rat forebrain cells, and the soluble protein actually inhibits neuritogenesis.

HB-GAM and midkine share 50% homology in amino acid sequence, and a similar three dimensional structure. Basically, both proteins have two distinctly folded domains, consisting of three parallel β-sheets (Iwasaki et al., 1997; Kilpelainen et al., 2000). These domains are connected with a flexible linker and both C- and N- termini have a non-folding lysine-rich sequence. The β-sheets are homologous to the so called thrombospondin type 1 (TSR)-domain structure (Kilpelainen et al., 2000), a sequence that is found in several proteins associated with heparin-binding and CNS development (figure 16).

Of these two TSR-domains, the C-terminal domain is the most conserved between HB-GAM and midkine. Interestingly, this is the domain that retains many of the biological functions of midkine (Asai et al., 1997; Akhter et al., 1998), while both domains appear to be needed in heparin binding.

HB-GAM and midkine are very conserved amongst species (Kadomatsu and Muramatsu, 2004). Recently, the *Drosophila* homologs of midkine and HB-GAM, called miple1 and miple2 (*mi*dkine and *ple*iotrophin), were identified based on structural homology (Englund et al., 2006). The amino acid sequences are 20% and 24% homologous to human midkine and HB-GAM, respectively, while their structural similarity based on domain structure is higher.

2.9.1 Developmental expression of HB-GAM

HB-GAM immunoreactivity is detectable shortly after the start of neurulation in mouse and rat embryos (Rauvala et al., 1994; Mitsiadis et al., 1995). In the neurogenetic areas of the cerebrum HB-GAM is secreted by the NSCs and by differentiated glia and neurons (Wewetzer et al., 1995; Jung et al., 2004). HB-GAM expression peaks, as happens with N-syndecan, approximately one week after birth in mice and after 10 days in rats (Nolo et al., 1995).

HB-GAM expression overlaps with N-syndecan expression in the developing murine CNS (Kinnunen et al., 1998a), but in the limb buds and osteogenetic bones the pattern of HB-GAM is different (Mitsiadis et al., 1995; Imai et al., 1998). In limb buds HB-GAM is present in the proximal mesenchyme and in differentiating muscle, but not in the quickly proliferating distal mesenchyme. In rat limb buds, HB-GAM inhibits the FGF-2–induced proliferation in the mesenchyme, promoting differentiation (Szabat and Rauvala, 1996).

Later, in the developing long bones, HB-GAM is expressed again in the border of undifferentiated and differentiated tissue, this time in the chondrocytes of the cartilage templates (Imai et al., 1998). HB-GAM is also found in partially mineralized bone, where N-syndecan is also present. HB-GAM functions in the cartilage template as an osteoblast recruiter. N-syndecan–expressing osteoblast precursors mi-

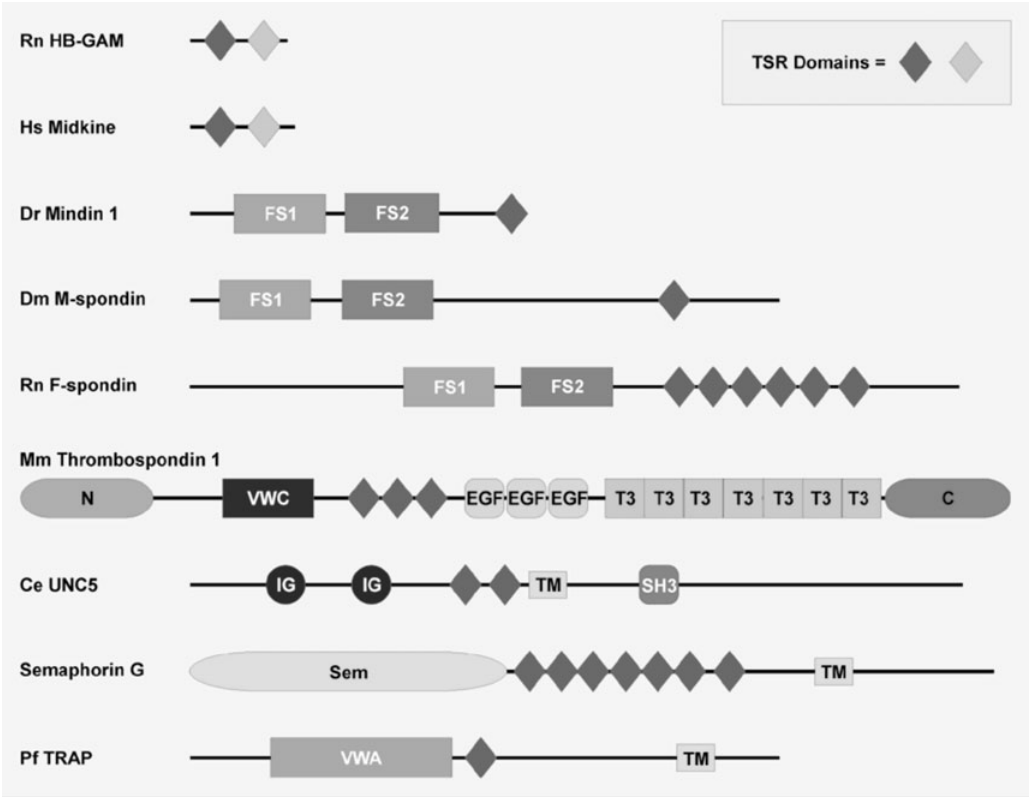

Figure 16: TSR-family proteins.
HB-GAM and its homolog midkine carry two β-sheet domains similar to the TSR-domain, a structure shared by many different proteins. Many of these proteins are expressed in the CNS and participate in axon guidance and growth, like in the case of Semaphorin G and F-spondin.

grate towards HB-GAM in the cartilage, where they differentiate and form mineralized bone mass (Imai et al., 1998).

2.9.2 HB-GAM in CNS plasticity, learning and memory

HB-GAM gene expression is regulated by synaptic activity in the brain, as was shown with LTP induction in rat hippocampal slices (Lauri et al., 1996). High frequency stimulation, which induces LTP, also induced increased expression of HB-GAM, which was localized in the CA1 (*Corny Ammonis* field 1) area in the hippocampus. The N-methyl-D-asparate (NMDA) receptor blockers, which inhibit LTP, prevented this increase. Furthermore, HB-GAM itself attenuates LTP in hippocampal slices, as exogenous HB-GAM can prevent LTP or increase the threshold for LTP formation (Lauri et al., 1998). HB-GAM appears not to interfere with synaptic base line activity and its effect is limited to the early stages of LTP induction, making it a specific modulator of synaptic plasticity in the hippocampus.

2.9.3 HB-GAM in bone

HB-GAM is strongly expressed during development in the mesenchymal growth-arrested

cells in growing limbs (Szabat and Rauvala, 1996). Later, its expression is restricted to the chondrocytes (Imai et al., 1998), cells that are responsible for the formation of cartilage and the target matrix for ossification during bone formation. HB-GAM expression persists in this matrix also during mineralization and osteoid formation. In fact, HB-GAM acts as a guidance cue for migrating osteoblasts, and the corresponding receptor is probably N-syndecan, which is abundantly expressed in these cells (Imai et al., 1998). Osteoblasts produce osteoids in the cartilage matrix and form new bone by endochondral ossification.

Although HB-GAM is not expressed in normal adult bone, it has an important role in the homeostasis of bone tissue during strain and in bone injuries (Petersen et al., 2004; Li et al., 2005a). Mice over-expressing HB-GAM under a bone-specific promoter show higher mineralization of the bone, while growing longer weight-bearing bones like tibia and femur, in comparison to their wildtype littermates (Tare et al., 2002; Li et al., 2005a). On the other hand, HB-GAM over-expression appears to reduce the stiffness of the long bones in aged animals, and mineralization during fracture healing is clearly defective (Li et al., 2005a). It is possible that HB-GAM interferes with bone morphogenic protein-2 driven bone homeostasis and causes problems in older transgenic animals.

According to one report, HB-GAM knockouts do not show any bone phenotype, indicating that HB-GAM is not necessary for bone growth or homeostasis (Lehmann et al., 2004). However, in this particular study the authors did not pay specific attention to the weight bearing bones or bone turnover. Mechanical strain induces HB-GAM expression in osteocytes and further promotes osteoblast recruitment and differentiation (Liedert et al., 2004). In addition, in studies made in our laboratory we have found a clear bone turnover defect in HB-GAM knockouts, revealed during physical strain in the weight bearing long bones (Imai, Kuja-Panula, Hienola, and Rauvala, manuscript in preparation).

2.9.4 HB-GAM in pathological conditions

HB-GAM appears to be consistently upregulated in many types of CNS injuries or pathogenesis (Nakagawara et al., 1995; Wisniewski et al., 1996; Yeh et al., 1998). The up-regulation coincides with the upregulation of many neuroprotective growth factors, like FGFs, of which many bind heparin. Is HB-GAM a neuroprotective molecule as such in CNS injuries, or is it modulating the functions of other heparin-binding proteins that coexist at the sites of injury, contributing to the pathogenesis?

HB-GAM expression is up-regulated in senile plaques associated with Alzheimer's disease and Down's syndrome (Wisniewski et al., 1996). While no genetic linkage between HB-GAM and the forementioned diseases has been detected, the heparin binding ability of HB-GAM might play a role in the development of plaques or in their clearing. At the moment, the formation and role of senile plaques in the disease is a matter of dispute. Amyloid deposits are clearly associated with senility, but are the deposits causing the disease or are they a result of dumping potentially harmful substances out of neurons, i.e. a defense mechanism? In short, whether HB-GAM is beneficial or harmful in senile plaques, is an open question.

Hippocampal and cortical ischaemic injury induces HB-GAM gene expression in the reactive astrocytes and in the affected pyramidal neurons (Takeda et al., 1995; Yeh et al., 1998). Although the HB-GAM mRNA is induced also in the neurons, the protein itself is either not translated or it is actively transported via axons and dendrites, as it is not seen in the pyramidal cells with immunostaining (Takeda et al., 1995; Yeh et al., 1998). HB-GAM participates in the regulation of neuronal plasticity, thus it is plausible that it should be transported to the sites of neuronal connections, where changes in

synaptic responses might determine the fate of the injured neurons.

After ischaemic insult, HB-GAM is not only induced in astrocytes and neurons, but in the microvasculature at or surrounding the site of injury (Yeh et al., 1998). This has lead to speculation about the possible role for HB-GAM in angiogenesis. In fact, HB-GAM expression is associated with neovasculature after ischaemic insults in other tissue types too, like in myocardium (Christman et al., 2005).

The embryonic and early post-natal expression pattern of HB-GAM has lead to speculation that HB-GAM could participate in carcinogenesis, or be a marker protein for malignant cells. This possibility has been central among the earliest studies involving HB-GAM and midkine, and numerous experiments have proven some of the speculations correct. In general, HB-GAM is not very widely expressed in different types of human carcinomas, but high levels are found in early stages of neuroblastomas, benign ganglioneuromas and in breast carcinomas (Wellstein et al., 1992; Nakagawara et al., 1995). This is somewhat in contrast to midkine expression, which is relatively high in malignant cells of many tissue types, and is associated with poor prognosis, especially in neuroblastomas (Nakagawara et al., 1995).

3 Aims of the study

- Morphometrical and phenotypic analysis of the HB-GAM overexpressing and knockout mice
- Morphometrical analysis of the N-syndecan knockout mice
- Assessment of NSC proliferation in HB-GAM knockout embryos
- Functional analysis of the protein sub-domains of HB-GAM
- Analysis of neural migration in N-syndecan knockout mice
- Characterization of modulation of FGF-2 activity by N-syndecan and HB-GAM in NSCs

Morphometrical and phenotypic analysis of the HB-GAM overexpressing and knockout mice The goal was to produce a thorough morphological analysis of HB-GAM overexpressing and knockout mice. In the overexpressing mice, analysis was focused to the CNS. In knockout mice the focus was on the CNS and skeleton in several developmental stages.

Morphometrical analysis of the N-syndecan knockout mice The goal was to analyze the morphological changes in the N-syndecan knockout CNS at several different developmental stages.

Assessment of NSC proliferation in HB-GAM knockout embryos After histochemical examination of the CNS in the adult HB-GAM knockout mice, we continued to study the neural proliferation and migration in the embryonic HB-GAM knockout CNS both *in vivo* and *in vitro*.

Functional analysis of the protein sub-domains of HB-GAM The goal was to analyze the biological roles of the individual protein domains of HB-GAM. The two TSR-domains were PCR-cloned and the resulting recombinant proteins were used to analyze their functions in neurite outgrowth and neural migration, together with the full-length protein. The purpose was to clarify, if the two repeat structures could confer any individual biological activity.

Analysis of neural migration in N-syndecan knockout mice Our goal was to reveal the function of N-syndecan in neuronal migration during mouse forebrain development, by analyzing the migration phenotype in the N-syndecan knockout mice. We concentrated on the cortical radial migration and migration from the SVZ to the olfactory bulb, along the RMS.

Characterization of modulation of FGF-2 activity by N-syndecan and HB-GAM in NSCs We examined the biological significance of N-syndecan's co-receptor function in the FGFR1–dependent proliferation of NSCs, and how HB-GAM could modulate this function.

4 Experimental procedures

Method					
Culture of living brain slices	I	II			V
Immunohistochemistry		II	III	IV	V
Cortical primary cell culture			III	IV	V
BrdU labeling *in vivo*			III		V
BrdU labeling *in vitro*			III		
Analysis of neurite outgrowth			III	IV	V
Boyden chamber cell migration			III	IV	V
Stereological methods: Selector	I	II	III	IV	V
Stereological methods: optical fractionator					V

Table 1: The referred methods.
The methods used in this work are described in the original publications listed here, in order of appearance.

5 Results

5.1 Histological analysis of the HB-GAM overexpressing, and HB-GAM and N-syndecan knockout mice

Both N-syndecan and HB-GAM are expressed in the routes of axonal growth and neuronal migration in the brain, therefore knocking out these genes could have affected the neuronal assembly in the animals. Changes in the bone structure were also a possibility, based on previous findings made in our laboratory (Imai et al., 1998). In previous studies FGF-2 binding to N-syndecan had been reported, and experiments made in our laboratory had verified that at least in the developing limb buds, HB-GAM plays an important role in modifying FGF signaling, probably via HS binding (Szabat and Rauvala, 1996). We decided to collect representative samples of different organs at different developmental stages from the HB-GAM and N-syndecan knockout mice and analyze them by histological methods. In addition, the hippocampi of the HB-GAM overexpressing mice was analyzed with stereological methods.

5.1.1 Gross morphology in HB-GAM overexpressing and knockout mice (I)

The HB-GAM knockouts were originally produced in the C57BL/6J$\times$129/Ola hybrid mouse strain, and they did not show any clear abnormalities in gross morphology, or in behavior (Amet et al., 2001). To reduce the genetic variation in the mouse line, we bred the HB-GAM knockout mice into the inbred mouse strain 129S2/SvHsd (I). Once more, the knockouts were fertile and had viable offspring and normal litter size. The weight of the animals did not differ from the wildtype littermates and we could not detect any obvious defects in their gait, posture or in other immediately visible behavior.

The HB-GAM overexpressing mice were produced using a transgene construct with the PDGFβ -chain promoter. The adult transgenic mice appeared normal in their morphology and behavior, and produced litters with normal numbers of pups (I).

The morphology of different brain structures in both mutants was examined on a more general level. We paid especially close attention to the large axonal pathways connecting the cortical lobes to the other parts of the brain. These included corpus callosum, thalamo-cortical pathways, striatal pathways, and pathways connecting the cerebellum to the thalamic and cortical structures. These observations were carried out in adult brain samples as well as in peri-natal samples, and in neither case detectable differences were found (Hienola and Rauvala, unpublished results). In the same study, the dimensions of the adult knockout brains were measured, and were found to be identical to the wildtype brains (Hienola and Rauvala, unpublished results).

We then turned our focus to early development of the HB-GAM knockout limbs, in the hope of revealing a phenotype linked to previous observations made by Szabat and Rauvala (1996). To our disappointment we could not detect any defects in the formation of mesenchymal structures in the limb buds or growing digits, and the growth pace of the limbs appeared normal during embryogenesis (Hienola, Imai, and Rauvala, unpublished results).

However, in the adult knockouts longitudinal sections from the long bones revealed severe structural changes in the cartilage surface in the joints and in the callous bone material. The long, weight-bearing bones were slightly shorter in the knockouts, while the size of the vertebrae did not differ from normal. This lead to further analysis of the bone turnover in the HB-GAM knockouts, and it turned out that the strain dependent bone renewal was indeed defective in the knockouts, leading to osteoporotic symptoms (Imai, Kuja-Panula, Hienola, and Rauvala, manuscript in preparation).

5.1.2 Hippocampal morphology in HB-GAM overexpressing and knockout mice (I)

The LTP phenotype in the HB-GAM overexpressing and knockout mice directed the analysis to the hippocampus of these animals (I, figures 2 and 4). Photographed serial sections were used to analyze the cell densities in the areas CA1, CA3 and dentate gyrus. There were no detectable differences in the neuronal densities or neuronal morphology in the hippocampi of the HB-GAM overexpressing or the knockout mice. The general morphology of the neurons was examined using anti-neurofilament antibodies, but no axonal abnormalities were found (Hienola and Rauvala, unpublished results).

5.1.3 Gross morphology in N-syndecan knockouts (II)

Observation of freely behaving N-syndecan knockouts showed no obvious abnormalities. The animals bred normally and the size of the litters did not differ from normal. The mice had normal body-weight at the age of three months (Hienola and Rauvala, unpublished results).

As in the case of HB-GAM knockout mice, N-syndecan knockouts had a hippocampal plasticity phenotype (II, figure 4), which required thorough analysis of the hippocampal structure. The analysis was performed from serial sections from the hippocampal areas CA1 and CA3 and dentate gyrus. No differences in the neuronal number was observed and the morphology of the pyramidal cells and their processes appeared normal (II, figure 2).

A similar morphological analysis was performed with N-syndecan knockouts as was done with the HB-GAM knockouts, and with similar results. The axonal pathways appeared normal in the knockouts and the major thalamic and hypothalamic nuclei did not differ in size or in cell number from the wildtype samples (Hienola and Rauvala, unpublished results).

5.2 HB-GAM and NSCs

After studying the axonal pathways in the HB-GAM knockout mice, attention was turned to the cortical structures. It was quickly learned that the neuronal number in the knockout mice differed from that in the wildtype mice. The aspect of NSC regulation was brought up, as HB-GAM was known to compete for HS binding with FGF-2, a well established growth factor for NSCs. The most important effects of HB-GAM in NSCs have been summarized in table 2.

5.2.1 HB-GAM inhibits FGF-2 driven stem cell proliferation (III)

NSCs were derived from mouse embryonic cortical preparations to demonstrate HB-GAMs ability to interfere with growth factor stimulation in these cells. FGF-2 stimulated NSC proliferation was measured using 5-bromo-2-deoxyuridine (BrdU) labeling *in vitro*, and a strong elevation in the number of proliferating cells was repeatedly observed (III, figure 3B). Adding HB-GAM to the cell culture medium inhibited the proliferation response in FGF-2 stimulated cultures but not in EGF stimulated cultures (III, figure 3B, E). In fact, it was clear that HB-GAM caused slight inhibition in cell proliferation even in growth factor starved cortical cells (III, figure 3C).

Formation of neurospheres is a characteristic phenomenon of NSC cultures. HB-GAM was able to inhibit the clonal formation of neurospheres in the presence of FGF-2 but not if the clone formation was stimulated with EGF (III, figure 3D, E). The number and size of neurospheres was also significantly diminished, if HB-GAM was present in the culture medium (III, figure 4). This was most clearly seen, when the neurospheres were allowed to attach to laminin and grow as discs (III, figure 5). The average diameter of the discs was visibly smaller in HB-GAM containing cultures, but not — surprisingly — in cultures where midkine was added (III, figures 3C, 3D, and

Stimulus	HB-GAM	FGF-2	HB-GAM	EGF	HB-GAM	HB-GAM/H	Midkine
Control	BSA	BSA	FGF-2	BSA	EGF	FGF-2	FGF-2
Disc growth	$\Leftrightarrow$	$\Uparrow$	$\Downarrow$	N/A	N/A	N/A	$\Leftrightarrow$
Clone formation	$\Leftrightarrow$	$\Uparrow$	$\Downarrow$	$\Uparrow$	$\Leftrightarrow$	$\Uparrow$	$\Leftrightarrow$
β-III-tubulin$^+$	$\uparrow$	$\Downarrow$	$\Uparrow$	$\Downarrow$	$\Leftrightarrow$	$\Downarrow$	N/A
GFAP$^+$	$\uparrow$	$\downarrow$	$\Leftrightarrow$	N/A	N/A	N/A	N/A
Nestin$^+$	$\Leftrightarrow$	$\Uparrow$	$\Downarrow$	$\Uparrow$	$\Leftrightarrow$	$\Uparrow$	N/A

Table 2: HB-GAM in NSC proliferation arrest and differentiation.
This table shows the relative changes in some commonly followed traits of NSCs caused by HB-GAM. Exogenous FGF-2 is a well known stimulator for NSCs in cell culture. HB-GAM, on the other hand, does not affect the growth of NSCs very strongly, *per se*. However, HB-GAM is a strong inhibitor of FGF-2 in cell culture, and thus indirectly reduces the proliferation of NSCs. Low-molecular weight heparin (H) removes the inhibition. In addition, it either indirectly or directly enhances differentiation of neural cells. On EGF stimulated NSCs, HB-GAM doesn't have any observable effect in clonal growth. A noteworthy observation is, that midkine, a molecule related to HB-GAM, does not have the same effect on FGF-2 stimulation, as HB-GAM does. $\Uparrow$ and $\Downarrow$ represent relatively strong change in trait, in comparison with the control stimulus, while $\uparrow$ and $\downarrow$ show modest change. $\Leftrightarrow$ means no change in trait, N/A stands for data that is not available (III).

5). Very interestingly, adding low molecular weight heparin reversed the inhibitory effect of HB-GAM in these assays (III, figure 3D, and data not shown). This strongly suggested that HB-GAM somehow interfered with the binding of FGF-2 to its HS co-receptor, and thus inhibited the FGFR activation.

5.2.2 HB-GAM promotes neural differentiation (III)

Inhibition in neurosphere formation and in BrdU incorporation *in vitro* is achieved either by increasing cell death or by halting the stem cell cycle. We systematically searched for apoptotic and necrotic cells on culture plates, but could not see increased cell death in HB-GAM treated cell cultures. Because HB-GAM inhibited proliferation specifically in FGF-2 stimulated NSCs, we looked for increased differentiation in these cells. As model systems, we used clonal neurospheres and NSC discs.

When neurospheres are starved of growth factors, they start differentiating by attaching to the culture plate and flattening. Within a few days the morphology of the cells had clearly changed to resemble spreading neurons or astrocytes, and they no longer grew in a clump of cells but separated from each other. A certain percentage of neurospheres always starts to differentiate even in the presence of growth factors. In our conditions approximately 80% of the neurospheres remained undifferentiated after three days in the presence of FGF-2. HB-GAM negated the stem cell stimulation, reducing the amount of undifferentiated clones to < 50% in three days (III, figure 4). Once again it was found that low molecular weight heparin reverted the HB-GAM induced differentiation completely (III, figure 4).

Nestin is a well known cytoskeletal protein in NSCs, and its expression goes down when neural differentiation starts. NSC discs grown with FGF-2 were immunostained with anti-nestin, in the presence or absence of HB-GAM. The differentiation clearly started earlier in the discs with additional HB-GAM in the medium, the number of nestin positive cells reduced to 40% in five days, while it remained at 75% in cultures containing only FGF-2 (III, figure 6). Once again, midkine failed to induce any differentiation in this assay (III, figure 6). And, once again, additional heparin in the disc cultures prevented HB-GAM-induced differentia-

tion (III, figure 6).

We examined further the differentiation pattern that HB-GAM was clearly inducing in NSCs. Differentiated disc cultures were stained with antibodies against glial fibrillary acidic protein (GFAP) and anti-β-tubulin to distinguish between the cells of astrocytic and neuronal lineages. The discs were allowed To differentiate for five days and the relative amount of cells expressing the immunomarkers was counted. There was no clear difference in the amount of GFAP positive, or astrocytic, cells between cultures with FGF-2 or combination of FGF-2 and HB-GAM. However, the amount of β -tubulin positive, or neuronal, cells was elevated in the cultures, where HB-GAM was present (III). 47% of the cells counted displayed a neuronal phenotype, when only 28% were β-tubulin positive in discs differentiated without HB-GAM (III). We did not at this point analyze further the possible neuronal subtypes induced by HB-GAM.

In addition to the *in vitro* findings, we saw a decreased expression of medium and heavy chain neurofilaments in HB-GAM knockout P10 pups, in comparison with their wildtype littermates (III, Supplementary figure 1). The amount of light chain neurofilament was approximately the same at all observed ages (P3, P10, and 2 months). The medium and heavy chain neurofilament expression is associated with neuronal maturity, and a temporal drop in their levels indicates delayed maturation (Steinschneider et al., 1996).

5.2.3 HB-GAM modulates cortical cell density (III)

HB-GAM is expressed from early on in the germinal areas of the expanding cortex, around the NSCs in the VZ (see 2.9.1). We reasoned that if HB-GAM could reduce the stem cell -stimulating effect of FGF-2 in culture conditions, it might achieve this *in vivo*, too.

The first finding to support this idea was the increased proliferation rate of NSCs isolated from HB-GAM knockout embryos. Within 24 hours the HB-GAM deficient cells accumulated 50% more BrdU in their nuclei than the corresponding wildtype cells (III, figure 3B). The next step was to observe the proliferation rate of NSCs *in vivo*, in the areas where HB-GAM is normally expressed.

We injected timed pregnant females carrying litters (E12,5) with mixed genotypes — heterozygotes, knockouts and wildtypes. The injection protocol was designed such that all cells undergoing stem cell cycle at the chosen age had a chance of incorporating BrdU. In practice this meant keeping the systemic level of BrdU in the females constant for six hours with three sequential injections. After this the females were sacrificed and the embryos were collected for immunohistochemistry.

The six–hour time window captured a population of proliferating cells in the VZ of the embryos. This population was 50% bigger in the knockout embryos than in the wildtypes (III, figure 2C-E). The difference in the immunostained sections was visible to the eye, and remained visible also in the later embryonic and post-natal ages with ordinary histochemical staining methods.

The continuum was to estimate the neuronal number in the cerebral cortex of the adult knockout mice and compare that to the wildtype cortex. The task was done using a stereological method called selector-method, where cell numbers can be estimated from thin sections, thus allowing the usage of photographs. We photographed random samples from serially sectioned brains and calculated the cell density in the parietal cortex of the mice. This location was chosen, because it was easy to define repeatedly in different individual brains. The cell densities in the hind limb motor cortex were analyzed later to make sure that the phenotype was found in other cortical areas as well.

The phenotypes in both areas were virtually identical. In short, HB-GAM knockouts had significantly elevated neuronal density in

all cortical layers, when compared to the wild-type littermate controls (III, figure 1A). The elevation was most striking in the layers II-IV (III, figure 1B). The size distribution of the extra neurons did not differ from the cells in the normal cortex and didn't thus provide any hints about the possible neuronal sublineage differences in the knockout brains (III, figure 1C). We confirmed with immunostaining that the extra cells were indeed pyramidal neurons, which are born in the VZ near their future cortical location, and not GABAergic interneurons, which are born elsewhere in the brain and travel to the cortex later in development (III, figure 1E, F).

5.3 Functional dissection of HB-GAM

HB-GAM and midkine are structurally similar heparin binding proteins. They both have independently folding domains homologous to TSR-repeat (Iwasaki et al., 1997; Kilpelainen et al., 2000). The N-terminal TSR repeat (TSR-N), the C-terminal TSR repeat (TSR-C), and the fragment containing both TSR domains (di-TSR) were PCR-cloned and analyzed in several functional assays (IV, table 1). The most important effects of the TSR-fragments of HB-GAM are summarized in table 3.

5.3.1 TSR domains in neurite outgrowth (IV)

HB-GAM was originally isolated as a strong inducer of neurite outgrowth. The cloned TSR fragments were tested in an essentially similar manner, plating hippocampal neurons on cell culture plastic coated with the recombinant proteins. The number of cells with neurites was then counted. Di-TSR fragment induced neurite outgrowth with approximately same intensity as HB-GAM (IV, figure 6A). The single TSR-C and TSR-N domains did not induce any neurites, not even at very high concentrations (up to 10 μM; IV, figure 6A).

It was possible, however, that the cloned peptides had differences in the coating efficiency, thus resulting in biased data. Therefore it was necessary to try the peptides as soluble inhibitors to neurite outgrowth, induced by matrix bound HB-GAM. Consistently, the di-TSR fragment displayed inhibition similar to HB-GAM, while the single TSR-C and TSR-N peptides failed to act as inhibitors (IV, figure 6B).

Interestingly, it appeared that the linker sequence between the TSR domains was replaceable with an artificial linker sequence — consisting solely of glycine residues — without any loss to the biological activity (IV, figure 6B). This agreed with the HS/heparin binding and NMR data, which suggested that the linker sequence was not involved in the HS/heparin binding.

5.3.2 TSR domains in neural migration (IV)

The haptotactic migration assays are usually made using proteins immobilized on pierced polycarbonate membranes. The coating efficiency of HB-GAM's di-TSR and single TSR fragments was so poor on this polymer that the haptotactic properties of the fragments could not be studied directly. Instead, HB-GAM bound to this material fairly well, and we could assay the interference of soluble TSR fragments with HB-GAM-induced haptotaxis.

Soluble HB-GAM inhibited neural cell migration to immobilized HB-GAM, and, as expected, produced a half-maximal inhibition at equimolar concentration (IV, figure 8). The soluble di-TSR fragment behaved in a manner identical to the full length HB-GAM (IV, figure 8). To our surprise — and contradicting the results obtained from the neurite outgrowth assay — TSR-C domain was almost as active as the di-TSR fragment in forebrain cell migration (IV, figure 8). TSR-N did not inhibit migration in any detectable way.

Fragment	C-TSR	N-TSR	di-TSR
Inhibition of neurite outgrowth	–	–	+
Induction of neurite outgrowth	–	–	+
Inhibition of neural migration	+	–	+

Table 3: TSR-fragments of HB-GAM in neurite outgrowth and inhibition of neural migration. The effects of the three available TSR-fragments of HB-GAM were tested in freshly isolated embryonic forebrain neurons. In neural migration studies the coating efficiency of the TSR-fragments was so poor, that their effects were studied only in the inhibition of HB-GAM–induced migration. All experiments were done using BSA as a negative, and HB-GAM as a positive control. – indicates no effect, + indicates an effect similar to HB-GAM (III, IV).

5.3.3 TSR domains in growth factor modulation (III)

The inhibitory effect of HB-GAM in FGF-2 and HB-EGF signaling is retained in the truncated di-TSR fragment of HB-GAM. The di-TSR fragment functions essentially as the full length HB-GAM in attenuating NSC proliferation (III, figure 3C), in suppressing FGFR phosphorylation (III, figure 7), and in inhibiting HB-EGF chemotaxis (Hienola and Rauvala, unpublished results). In addition, the di-TSR induced inhibition is reversible by the addition of low molecular weight heparin (Hienola and Rauvala, unpublished results), similarly to the modulation of the activity of the full length HB-GAM.

The single TSR domains of HB-GAM do not possess such inhibitory properties (Hienola and Rauvala, unpublished results), which argues the importance of HS binding in the HB-GAM/growth factor interactions. These findings strongly suggest that HB-GAM competes with the ECM and cell surface associated HS binding growth factors, dynamically regulating their activity during murine CNS development.

5.4 Growth factors binding N-syndecan

5.4.1 FGF-2 in neural proliferation (III)

FGF-2 is one of the major proliferation inducers in NSCs, its functionality being highly dependent on HS mediated receptor binding. HB-GAM probably displaces FGF-2 from HS residues at the cell surface, and thus inhibits FGFR signaling in NSCs. The half-maximal inhibitory concentration of HB-GAM is approximately 10-fold (molar) to FGF-2, which means that the experimental inhibitory conditions can exist *in vivo*.

Although in previous studies FGF-2 binding to N-syndecan has been reported and confirmed, this binding appears not to be important for NSC proliferation, as the neural proliferation is normal in N-syndecan knockouts, both *in vitro* and *in vivo* (V). Of syndecans, the neural stem and progenitor cells do express at least syndecan-1, that could either compensate the loss of N-syndecan, or act alone as the required co-receptor for FGF-2. HB-GAM binds HSPGs other than N-syndecan in the brain and could thus inhibit FGF-2 binding to HS, regardless of the core proteins involved.

5.4.2 HB-EGF (V)

HB-EGF binds N-syndecan ectodomain IgG fusion protein (ENS-IgG) with similar affinity as HB-GAM. An ELISA assay was used to determine the relative difference between HB-GAM's and HB-EGF's affinity to ENS-IgG. Using the IgG tag to determine the absolute amount of ENS, we bound ENS-IgG to immobilized HB-GAM and HB-EGF. From the specific binding curve the K_d values for both proteins were calculated using Scatchard analysis.

Analyzed phenotype	N-syn ko
Migration to HB-GAM	⇓
Migration to Midkine	⇓
Migration to HB-EGF	⇓
EGF induced motility	⇓
HB-GAM induced p-c-Src	⇔
Radial migration	⇓
Migration in RMS	⇓
Neural proliferation	⇔

Table 4: N-syndecan mediated migration.
The table summarizes how N-syndecan deficiency affects the migration of neural cells *in vitro* and *in vivo*, when compared to the wildtype phenotype. The changes are displayed as arrows showing the relative chance in trait. ⇑ and ⇓ represent relatively strong change in trait, in comparison with the control stimulus. ⇔ means no change in trait (V).

We estimated the K_d of HB-GAM to be 14 nM to ENS and HB-EGF bound ENS with similar affinity, with estimated K_d 16 nM (V, figure 8).

HB-GAM competes strongly with HB-EGF in ENS binding, as was shown using biotinylated proteins binding immobilized ENS-IgG. The competition goes the other way, too, HB-EGF being capable of replacing HB-GAM during ENS binding (V, figure 8).

5.5 N-syndecan and HB-GAM in neural cell migration

N-syndecan participates in neural migration on several levels. It has signaling functions of its own, which directly affect the actin-cytoskeleton. It acts as a guidance receptor in haptotaxis, and in chemotaxis. It also clusters with EGFR, which is known because of its motility–promoting properties in neurons. Some findings are summarized in table 4.

5.5.1 HB-GAM haptotaxis (IV, V)

HB-GAM is expressed along the major migratory routes in the brain, including RG in the cerebrum and rostral migratory stream to the olfactory bulb. Co-localization with N-syndecan is strong in these structures. HB-GAMs other known receptor, RPTPβ/ζ, has been previously reported to mediate HB-GAM guided haptotactic migration of forebrain neurons (Maeda and Noda, 1998), and N-syndecan was shown to mediate HB-GAM guided migration of osteoblasts (Imai et al., 1998), but the involvement of HB-GAM and N-syndecan as a ligand-receptor pair in neuronal migration has not been previously studied.

In modified Boyden chamber assays (figure 17) immobilized HB-GAM induced strong migration response in primary forebrain neurons, displaying a 200% increase at ≈1 μM coating concentration, and saturation point at ≈4 μM coating concentration, which produced an average of 400% increase in the number of migrating cells (V, figure 3A). However, the coating efficiency of HB-GAM to the polycarbonate membrane was left unresolved. Adding an equimolar concentration of HB-GAM to the migration assay medium decreased the number of migrating cells to less than 50%, which indicates that the relative amount of immobilized HB-GAM was not as high as it usually is on ordinary cell culture plastic (IV). When primary CNS neurons from N-syndecan knockout embryos were used, the number of migrating neurons was dramatically reduced, producing only approximately 200% increase in the saturating concentrations of HB-GAM and no response at 1 μM concentration (V, figure 3A).

5.5.2 Migration in N-syndecan knockout brain (V)

The major migratory routes that we examined were the RMS and radial migration in the cerebral cortex of the N-syndecan knockout mice. Timed-pregnant female mice were injected with BrdU at litter age E15, when the last neurons originating from th VZ are born. These cells migrate through all embryonic cortical layers, arriving at the superficial part of the CP within days and staying there. At the

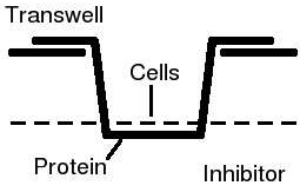

Figure 17: Transwell, or modified Boyden–chamber assay.
Migration chambers rest slightly elevated on multiwell plates. The chamber bottom is made of porous polycarbonate membrane with defined pore-size. The lower surface of the membrane is coated with the haptotactic protein, like HB-GAM, and the well is filled with cell culture medium, until the membrane is submerged. Possible inhibitors are in the medium. Cells of interest are pipetted to the upper side of the membrane, inside the chamber. The number of cells migrating through the pores is counted after 6-24 hours.

litter age E18 the mothers were sacrificed and the embryonic brains collected for examination. The scattering of BrdU labeled cells in the cortex was determined with BrdU antibodies. Many of the labeled cells were still on route to the CP but a significant part — over 50% — of all cells had reached the CP.

The density of BrdU labeled cells in the VZ/SVZ, IZ and CP layers was estimated and the data was plotted to see relative differences in the location of labeled cells between normal embryos and N-syndecan knockouts. The knockouts clearly had fewer cells present in their CP than the wildtype mice (V, figure 1B, C). Respectively, the BrdU cell density in the layers VZ/SVZ was significantly higher in knockouts when compared with wildtypes, indicating a delay in the accumulation of labeled cells in the superficial layers (V, figure 1B, C).

This delay in cell accumulation was visible also in the adult knockouts, where the density differences in laminae VI and II/III in the total amount of neural cells had stayed almost the same as was observed at E18 (V, figure 1A). The cortical sections from P3 knockout and wildtype pups were stained with anti-p75 antibody, which specifically stains neurons. The amount of radially migrating neurons was significantly less in the knockouts at P3, when the radial migration is still strongly ongoing (figure 18).

The other migration route of interest, RMS, was examined a little differently. We cultured living parasagittal brain slices collected from P3 mouse brains, containing the RMS and olfactory bulb. DiI, a autofluorescent lipid-soluble marker, was injected to the rostral part of the RMS, effectively labeling a population of cells traveling along the route to the olfactory bulb. After 24 hours the slices were fixed, washed, and cut to cryosections for observation (V, figure 2A, B). The amount of DiI-labeled cells that had accumulated in the olfactory bulb during the 24 hours was estimated, and there was a clear defect in the N-syndecan knockout slices. The amount of DiI-labeled cells was half of that in the wildtype olfactory bulbs, indicating a severe delay to the cells migration along RMS (V, figure 2C, D).

However, we could not see any clear volume or cell density differences between adult knockout and wildtype olfactory bulbs. The nature of migration in RMS is such that it continues throughout the adult life in rodents, serving as a cell renewal route at least for the olfactory interneurons, if not for other types of neurons as well. This continuous renewal and replacement of cells could compensate and mask the migration phenotype that is seen in the RMS of the developing knockout brain.

5.5.3 EGFR induced migration (V)

EGFR induces migratory behavior in all neural cells, and also in post-natal, dormant neuronal progenitors. EGFR activation was used to test, if N-syndecan knockout cells had normal migratory behavior, when induced by receptor systems other than N-syndecan. Surprisingly,

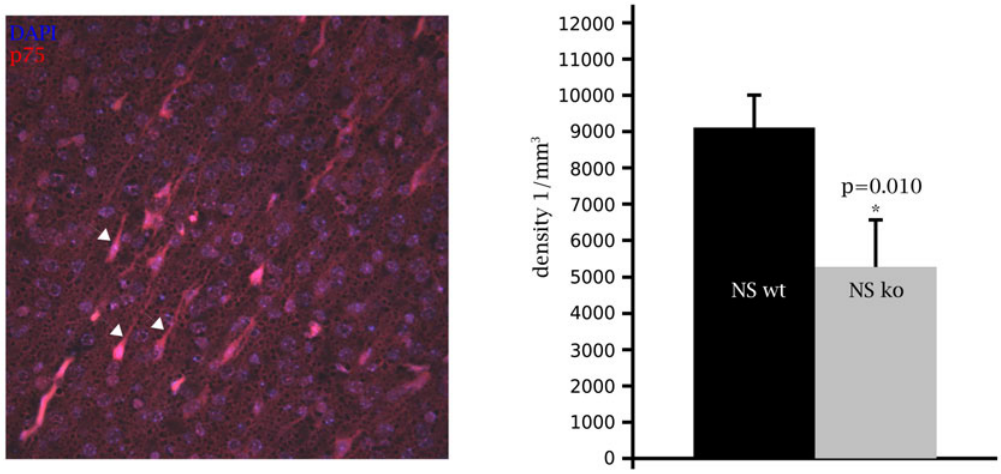

Figure 18: p75 immunostained cells in the mouse P3 cortex.
N-syndecan knockout pups have fewer p75 positive neurons with migratory morpholoy (arrowheads) at P3 in their cerebral cortex, than their wildtype littermates. The estimation was done using optical disector from the IZ of the crebral cortex, using DAPI as a nuclear stain. 4 N-syndecan knockout pups and 4 wildtype pups were analysed. P-value was calculated with t-test. p75 antibody was a kind gift from Dr. Eero Castrén Neuroscience Center.

N-syndecan seems to play a crucial role in EGFR induced neural migration. N-syndecan deficient forebrain cells do not respond to the migration-enhancing stimulus caused by EGFR activation. In a migration chamber assay 50 ng/ml of EGF induces a 200% increase in the migration of normal neural cells. The same activation induces only a 20% increase in the N-syndecan knockout neural cells (V, figure 3B).

In the normal cells this induction was EGFR receptor mediated, as AG1478 — a specific EGFR inhibitor — reduced migration close to the control level. With a src-family kinase inhibitor, PP2, the EGFR mediated migration was clearly reduced, too, but inhibition of the mitotic signaling pathway involving MEK with U0126 did not have any effect on the migration (V, figure 3C).

We continued to examine, if the N-syndecan knockout embryos were deficient in EGFR induced migration *in vivo*. First we attempted to inject EGF to the lateral ventricles of E15 knockout and wildtype embryos via surgical opening in the abdominal wall of the anesthetized mothers. This approach turned out not to be very successful, due to high embryonic lethality. The next approach was to pulse-label late dividing neurons with BrdU by injecting BrdU to timed-pregnant mice. The embryonic brains were collected 30 minutes after the injection and the brains were sliced. The slices were then cultured for 24 hours with or without EGF in the culture medium. There was a significantly increased accumulation of BrdU-labeled cells in the CP of the EGF treated cortices in comparison to the non-treated brain slices. In this semi *in vivo* assay, N-syndecan deficient brain slices were not responding to EGF stimulus as the wildtype slices did, showing clearly less accumulation of BrdU-labeled cells in CP (V, figure 1G). The number of BrdU positive cells did not differ between the genotypes, indicating otherwise normal mitotic response to the treatment (V).

5.5.4 HB-EGF chemotaxis (V)

Soluble HB-EGF is a chemotactic protein for migratory neural cells. In our experiments HB-EGF induced dose-dependent migration in embryonic forebrain cells, reaching saturation point — 300% increase — approximately at 10 nM concentration (100 ng/ml). In N-syndecan knockout cells, however, HB-EGF did not induce very strong migration, even at 10 nM concentration the relative induction stayed under 100% (V, figure 6A).

Because HB-GAM previously competed with HB-EGF in N-syndecan binding, we decided to test, if HB-GAM could inhibit HB-EGF chemotaxis in a modified Boyden chamber assay. Soluble HB-GAM added to the cell culture medium produced half maximal inhibition at molar concentration approximately 3-fold to HB-EGF, and reached saturation at 30-fold concentration, when migration settled around 20 to 30% of non-inhibited migration (V, figure 6B). Thus, it appears that *in vitro* HB-GAM and HB-EGF binding to N-syndecan and competition therein has functional meaning in neural cell migration.

The inhibition also caused a significant decrease in HB-EGF–induced EGFR signaling. A ten-fold molar excess of HB-GAM brought HB-EGF–induced tyrosine phosphorylation to 30% of the levels without inhibition (V, figure 6C). This result is physiologically significant, as when compared with HB-EGF, HB-GAM concentration is very high around migrating neurons.

5.5.5 Clustering of EGFR and N-syndecan (V)

Because there appeared to be a close connection with N-syndecan and EGFR in neural motility, we examined, if these two receptors coincide at the cell surface. First, we isolated from neural cell membranes the low-density lipid fraction called lipid rafts — possible signaling centers in cell plasma membranes that differ from the rest of the membrane in their lipid structure. EGFR was previously shown to be found in the lipid rafts (Hur et al., 2004). We managed to locate all the significant molecules associated with N-syndecan and EGFR signaling, and relevant to our experiments, including HB-GAM and HB-EGF, and several signaling molecules (V, figure 7B).

In neurons growing on HB-GAM, using confocal microscopy, N-syndecan and EGFR could be localized with immunostaining to the bases of the growing neurites (V, figure 7A). This, however, was not enough to show definite physical interaction between these two receptors. We therefore applied a microscopy method named FRET, which reveal physical coupling of two fluorochrome labeled molecules. N-syndecan and EGFR clustered somewhat in neurites of cells growing on HB-GAM, but not so strongly on laminin. When HB-EGF was added to the medium, the clustering clearly increased in the cells (V, figure 7C-G).

6 Discussion

N-syndecan as a PG participates in forming a very significant regulatory structure on the cell surface — the mesh of GAGs. In very simplified sense this landscape resembles a patchy forest with tall trees and their high branches covering the dense bushes below. N-syndecan as a straight and rather long molecule plays the part of a tree in this landscape, while globular glypicans resemble the bushes.

In cell biology the analogies to the macroscopic world always fail at some point and the limit of this one is reached here. The hydrophilic oligosaccharides form a much denser structure than any living tree branches, as they entrap water and water soluble molecules and probably form patches of weak gel rather than a mesh.

HB-GAM associates very strongly to the ECM surrounding the cells and its diffusion in the ECM is quite low (Lauri et al., 1998). So, HB-GAM is a rather immobile protein, which

largely stays where it was secreted after synthesis. Its low perfusion in the ECM allows it to form very steep gradients, which is crucial for HB-GAM to act as an axonal growth factor and a migration cue. The low mobility results in one more important property of HB-GAM — its local concentration can be very high.

When N-syndecan comes in contact with brain ECM, it encounters and rapidly binds HB-GAM. N-syndecan also binds many other small proteins that are in the vicinity. The high local concentration of HB-GAM and its high affinity to N-syndecan gives HB-GAM a chance to 'elbow' other binding candidates off efficiently. For FGFs or HB-EGF to bind N-syndecan, either their local concentrations have to be very high, or HB-GAM has to be down-regulated, or replaced somehow, as all three proteins have similar affinities to heparin and N-syndecan (V; Raulo et al., 1992).

6.1 HB-GAM in NSC proliferation and differentiation

HB-GAM is secreted by NSCs, already at E10 (Furuta et al., 2004; Jung et al., 2004). It also very strongly binds one of the major ECM PGs in embryonic brain, neurocan (Milev et al., 1998a; Rauch et al., 2001). The low perfusion of HB-GAM in the ECM might actually be due to this binding. Nevertheless, low perfusion makes HB-GAM accumulate in the proximity of the stem cells, and the probability of HB-GAM binding the cell surface HSPGs increases. Thus, HB-GAM participates in the creation of the stem cell niche.

This niche becomes more and more restricting to the stem cell cycle, and some NSCs escape the cycle, becoming either more committed, asymmetrically dividing precursor cells, or post-mitotic precursors. HB-GAM functions in the cell cycle escape. It occupies the binding sites of FGF-2 at the surface of NSCs, namely in the HSPG co-receptors, that FGF-2 requires for activating its receptor. HB-GAM inhibits the phosphorylation of FGFR1, proliferation ceases, and the cell starts differentiating (III, figure 19).

In this work, the HB-GAM–induced proliferation arrest was followed by observing two commonly followed NSC traits, the clone formation, and the growth of NSC spheres and discs. In all experiments the outcome was the same: HB-GAM alone has no effect on the growth of NSCs. Without growth factors, NSCs start to differentiate, regardless of HB-GAMs presence (III). So, HB-GAM *per se* does not have a strong effect on non-stimulated NSCs, it does not induce immediate growth arrest, or increase cell death (III).

The interesting things start to happen, when the NSCs are stimulated to continue their stem cell cycle. Upon growth factor induced stimulation, isolated NSCs proliferate, they form neurospheres — structures filled with proliferating stem cells — and grow as large stem cell discs on substrates like laminin or fibronectin. HB-GAM inhibits all of these traits of NSCs, if the stimulating growth factor is FGF-2 (III). When heparin is added to the experiments, the inhibitory effect of HB-GAM disappears which is a strong support for the HSPG-competition hypothesis (III, figure 3). HB-GAM's TSR-fragment behaves in an identical manner to the full-length protein, giving further confidence, that the heparin-binding ability plays a crucial role here (III).

EGF stimulated NSCs are immune to HB-GAM–induced inhibitory effects. EGF does not need HSPGs or heparin to bind its receptor, so HB-GAM does not have a place to interfere. On the other hand, midkine — a structurally related molecule to HB-GAM — does not inhibit FGF-2–induced NSC proliferation (III, figure 3). We have no clear explanation for this, except that midkine probably does not bind the same HSPGs as HB-GAM, or not with similar affinity.

The differentiation of NSCs manifests as decreasing levels of nestin expression and increasing levels in the expression of various neural markers, like GFAP for astrocytes, neuro-

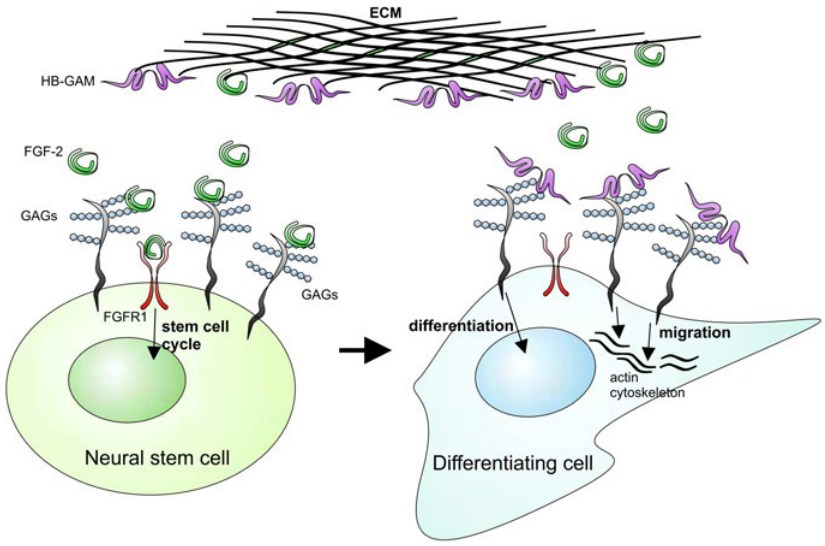

Figure 19: HB-GAM in NSC differentiation.
NSCs are responsive to FGFR1 signaling, which keeps them in an undifferentiated, proliferative state, i.e. in stem cell cycle. FGF-2 needs HSPGs at the cell surface to activate FGFR1, and syndecan-1 or glypicans in NSCs are the probable co-receptor candidates. HB-GAM binds these HSPGs as well, and because of its high local concentration (see text), it can compete with FGF-2 and replace it. This competition leads to attenuation of FGFR1 signaling, and further to neural cell differentiation. It is possible, that HB-GAM could induce a differentiation-promoting signaling cascade via the HSPG-receptors, but according to our findings, N-syndecan is not among these PGs. Cell differentiation is in addition a self-promoting phenomenon, as the differentiated cells start to secrete even more HB-GAM, inducing a differentiating microenvironment for their neighbors (III).

filaments and β-III-tubulin for neurons (III). In non-stimulated NSCs, exogenous HB-GAM slightly increases the number of GFAP and β-III-tubulin–positive cells, but this increase is marginal. In FGF-2 stimulated cultures, HB-GAM causes strong differentiation in the cells, decreasing the number of nestin expressing cells and increasing the amount of neuronal cells, but not of GFAP–positive cells (III). Measuring GFAP–expressing cells is probably not a very good method to study neural differentiation, as GFAP is expressed by several NSCs as well.

Nevertheless, HB-GAM–induced differentiation tends to drive the cells towards neuronal fate more effectively than simple removal of the growth factor, and letting the NSCs differentiate without stimulus. This finding indicates a more active role for HB-GAM in neuronal differentiation. N-syndecan expression is already quite prominent in the more mature neural cells, and HB-GAM could indeed induce specific differentiation response in the N-syndecan–expressing cells. On the other hand, another HB-GAM receptor RPTP-β/ζ could function in neuronal differentiation. The effects of HB-GAM on cultured NSCs are summarized in the table 2 of Results section.

6.2 N-syndecan and HB-GAM in neural migration

Earlier studies have defined the structural superfamily of TSR-proteins, to which HB-

GAM together with its cousin midkine belongs (Kilpelainen et al., 2000). HB-GAM has two TSR-like peptide domains that undergo very clear conformational changes upon heparin binding, as has been shown in nuclear magnetic resonance (NMR) studies. The rest of the molecule — the lysine-rich N- and C-terminal tails and the linker between the TSR-domains — has practically no detectable structure (Kilpelainen et al., 2000). These parts of the protein are probably functional, but according to observations in this study, they are not relevant to HB-GAM's biological functions, while it is matrix-bound (IV). When soluble, HB-GAM and its partial peptides may stimulate angiogenesis and cell transformation (Polykratis et al., 2004; Zhang et al., 2006).

As was described previously (see 5.3), the TSR-fragment of HB-GAM behaves in an identical manner to HB-GAM in NSC growth modulation. However, the single C- and N-domains do not bind heparin or N-syndecan (IV) and do not have any inhibitive effects on FGF-2 stimulated proliferation of NSCs (Hienola and Rauvala, unpublished results). It was therefore interesting to see, if this would hold true in HB-GAM induced haptotaxis as well.

HB-GAM induces strong haptotactic migration in embryonic neurons (IV, V). The di-TSR-fragment does the same, and both haptotactic stimulations can be inhibited by adding equimolar concentrations of either HB-GAM or the di-TSR-fragment in the modified Boyden chamber assay. Surprisingly — and contradicting the results obtained from neurite outgrowth assay — the single C-terminal TSR-domain of HB-GAM was about as inhibitive as the double-TSR fragment in forebrain cell migration (IV, figure 8). The N-terminal TSR-domain did not inhibit migration in any detectable way. Difference in the receptor system could be the explanation, as HB-GAM binding to RPTP-β/ζ might not be similar to binding to N-syndecan (Maeda and Noda, 1998).

To date, no published data exists concerning the role of HB-GAMs individual domain structures in RPTP-β/ζ binding. On the other hand, the C-terminus of both HB-GAM and midkine seem to be very conserved in comparison to the N-terminus, and in both proteins the C-terminus retains the observed oncological activities of the full length molecule, while the N-terminus appears to have lost them (see Kadomatsu and Muramatsu, 2004, for review).

6.2.1 N-syndecan signaling in migration — far-reaching associations

Interaction of HB-GAM and N-syndecan in neurite outgrowth has been previously established, and in this work we continued to explore this interaction in neural migration. The basic findings are summarized in table 4 of the Results section. One of the important findings was that HB-GAM–induced c-Src phosphorylation in neural cells is apparently N-syndecan–dependent, and thus very specific. The importance of this specificity will become clearer later during this discussion.

N-syndecan deficient neurons fail to migrate to HB-GAM in an haptotactic migration assay (V, figure 3). The decreased c-Src activation could at least partially explain this failure, as c-Src is an important mediator in cell-motility–related signaling. However, a similar, although not as strong, decline in the haptotaxis of N-syndecan–deficient neurons to midkine was detected (V). The c-Src phosphorylation was not examined in these cells on midkine matrix, so it is not possible to say anything definite about this finding. Considering midkines binding to N-syndecan, and midkine function in neurite outgrowth induction, it is tempting to claim, that both HB-GAM and midkine would, in normal cells, induce c-Src phosphorylation, and the lack of it would thus explain the defective migration on both proteins.

For the purposes of this work, it was decided to use EGF to test the general motility of N-syndecan deficient cells. Initially it was thought that the overall motility of these cells should be fairly normal, and that the mi-

gration defects *in vitro* and *in vivo* would be due to specific lack of HB-GAM–N-syndecan–dependent migration. This confidence proved to be misplaced. Surprisingly, EGFR–induced migration was dramatically defective in N-syndecan knockout neurons (V). Investigation of the available literature gave hints about the involvement of c-Src activation — or the lack of it — in EGFR induced cell motility (Hur et al., 2004). The scale of migratory failure in N-syndecan knockout cells was, however, so large that the defective c-Src activation was not a sufficient explanation.

6.2.2 Physical interactions of N-syndecan and EGFR

N-syndecan and EGFR cluster on the plasma membrane of neurons, specifically at the bases of growing neurites, and patchily along the neurites (V). This clustering occurs spontaneously, to some extent, but is greatly enhanced, if the cells are grown on HB-GAM coated plates (V). Contact with matrix–bound HB-GAM appears to boost the machinery that causes the clustering of the two receptors. Enhancement of c-Src phosphorylation modulates the signaling strength of EGFR (Hur et al., 2004), but does this recruit EGFR closer to the source of the signal? An important finding in this work was, that both receptors, and almost all molecules relevant for their signaling, were located in the lipid rafts (V). Microtubules and the actin–network appeared to directly regulate the formation of lipid rafts and the recruitment of signaling molecules in them (Head et al., 2006). N-syndecan signaling activates c-Src, CASK, and cortactin, which would provide a link to the events that restructure the signaling centers in the growing neurites (Kinnunen et al., 1998b).

HB-EGF is a heparin-dependent ligand for EGFR, and according to our findings, it binds glycosylated N-syndecan ectodomain with affinity similar to HB-GAM (V). HB-EGF is also known for its chemotactic properties in neural migration. While matrix–bound HB-GAM clearly enhances the motility of neurons, soluble HB-GAM has quite the opposite effect on migration (IV, V). *In vitro* this effect is relatively easy to explain. Soluble HB-GAM occupies the free N-syndecan at the cell surfaces, preventing the receptor from binding the matrix–bound HB-GAM, and thus removing the haptotactic stimulus. HB-GAM can also inhibit the chemotactic migration of neural cells to HB-EGF (V). This, again, is relatively easily explained by competition in N-syndecan binding. But how do these two separate phenomena play out *in vivo*? It seems that HB-GAM has opposite functions in cell migration.

Here it is important to remember, that the contradictory effects of HB-GAM on cell migration can all be explained with the experimental setups *in vitro*. There is no evidence that HB-GAM would have an inhibitory effect on HB-EGF migration *in vivo*. In fact, for this to be the case, the cell migration should be increased in the HB-GAM knockout mice, a phenotype that we could not observe. Admittedly, this kind of phenotype would probably be masked by the changed cell proliferation in the HB-GAM knockout brains, and would be very difficult to confirm. What we do know, is that both HB-EGF–induced chemotaxis, and HS-independent EGFR-activation are hampered in N-syndecan knockout brain tissue (V). To us these results give a picture of the migration-mechanism, where the motility–inducing machinery is very important, and where the chemotactic cues cannot work, if this machinery is broken.

Furthermore, when the physical clustering of N-syndecan and EGFR was examined, a increased clustering could be seen firstly on HB-GAM coated plates, and secondly upon HB-EGF stimulation (V). These effects seemed to be cumulative, indicating that matrix–bound HB-GAM did not inhibit the stimulation by HB-EGF. Thus, both ligands in their own way promote the motility of neural cells, while their receptors cluster either by sharing a ligand (HB-EGF) or sharing a signaling cascade (c-

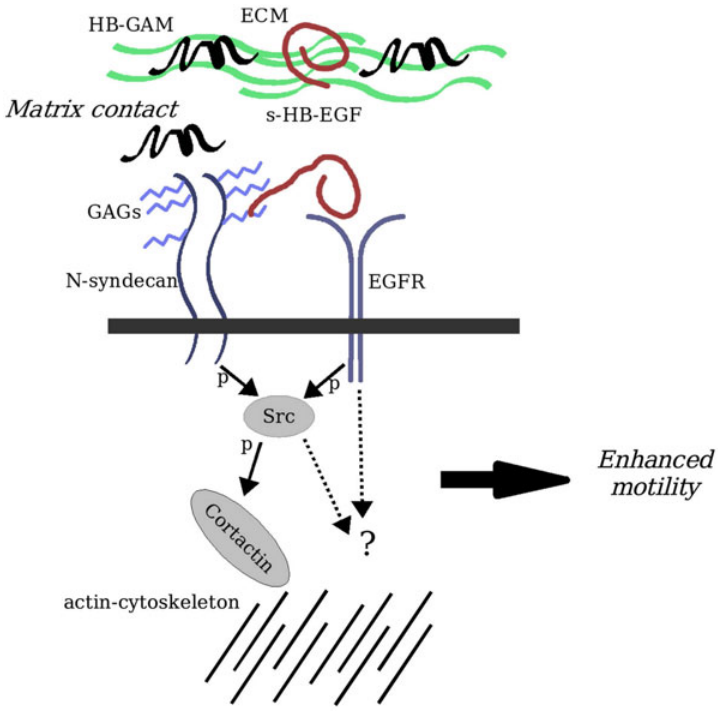

Figure 20: N-syndecan interaction with HB-GAM and EGFR in cell migration.
The ECM harbors many proteins that bind N-syndecan. ECM-associated HB-GAM induces c-Src phosphorylation in neural cells via N-syndecan, and this signaling pathway is further linked to the modulation of the actin cytoskeleton, and neurite outgrowth. In addition to HB-GAM, several growth factors bind N-syndecan as well. HB-EGF is a heparin-dependent ligand of EGFR, and N-syndecan can function as a co-receptor for HB-EGF. At the moment we think, that matrix-bound HB-GAM induces EGFR and N-syndecan clustering at the cell surface, and this clustering could further enhance the c-Src signaling pathway, and promote cell motility. In addition to this, HB-EGF uses N-syndecan as a co-receptor to bind EGFR, and gives the chemotactic stimulus to the migrating cell (V).

Src and CASK), or both (figure 20).

The maximal expression of HB-GAM and N-syndecan in the brain is almost at the same time, approximately 10 days after birth (Nolo et al., 1995). This period in brain development is associated with post-mitotic migration of neurons and astrocytes, myelin formation, pruning of extra connections and neurons, and general molding of the plastic functions of the CNS. Coincidently, the peak of HB-EGF expression overlaps with HB-GAM and N-syndecan, around one week after birth (Caric et al., 2001). As N-syndecan deficiency appears to attenuate the migration of both pyramidal neurons (radial migration; V) and interneurons (RMS migration; V), we believe that the signaling and binding properties of N-syndecan are generally required for normal cell motility in brain, and do not significantly affect the adhesion or 'gripping' of neural cells to their guidance cells.

6.3 N-syndecan and HB-GAM in pathophysiology in the brain

Although HB-GAM and N-syndecan are involved in the basic developmental mechanisms of the brain, namely stem cell differentiation and neural migration, there are no known related pathological conditions, where HB-GAM or N-syndecan would play a part. The polymorphism databases do not recognize recorded mutations in human N-syndecan or HB-GAM genes, but in mouse strains six non-synonymous single-nucleotide variants in HB-GAM are known. The polymorphisms concentrate to the exons 2 and 3 of HB-GAM (http://www.ncbi.nlm.nih.gov/SNP/). These records offer an opportunity to research association of brain pathogenesis with HB-GAM polymorphisms in mice, but it would be even more interesting to find such polymorphisms in the human genome as well.

6.3.1 HB-GAM and N-syndecan in neuronal plasticity

HB-GAM and N-syndecan are both associated with neuronal plasticity in the hippocampus, and elsewhere (I, II, see 2.8.2). The altered LTP response is connected with poor performance in hippocampus-dependent learning and memory tasks (I, II). N-syndecan especially regulates mouse feeding behavior. In a recent study, lack of N-syndecan turned out to be beneficial for mice on high-fat diet, as their food-intake was clearly reduced. This study suggests that inhibiting N-syndecan might have therapeutic importance in treatment of obesity caused by high-fat diet (Strader et al., 2004).

The specific attenuation of LTP by HB-GAM produces measurable phenotypes in the HB-GAM over-expressing and knockout mice. In the over-expressing animals the LTP induction is very much weakened and LTP lasts for a shorter period of time, when compared to the wildtype littermates (I). In the knockouts the phenotype is very much the opposite, the LTP threshold is much lower and the LTP lasts at least as long as in the wildtype littermates (I; Amet et al., 2001). On the other hand, HB-GAM associates with plastic changes in early GABAergic interneurons, and changes in HB-GAM activity could cause observable changes in learning and memory formation between different mouse strains (I; Pavlov et al., 2006).

Enhanced LTP has been previously associated with better performance in learning and memory formation in mice with neurotransmitter receptor mutations. Many controversial studies exist, and direct linking of LTP and memory performance has very little support today. In the case of HB-GAM mutant mice this controversy is especially visible. The HB-GAM over-expressing mice with attenuated LTP perform a lot better in hippocampus-dependent memory formation and learning tasks than their normal littermates. And as in LTP, the learning phenotype is exactly the opposite in the HB-GAM knockouts, where the enhanced LTP induction is associated with lower performance in memory and learning tasks (I).

6.3.2 HB-GAM as an oncogene

Oncogenic properties are often associated with HB-GAM, although the actual mechanisms for HB-GAM's oncogenicity have not been revealed (Kadomatsu and Muramatsu, 2004). Brain gliomas are especially difficult malignant tumors to treat, as they penetrate the brain tissue with spider-like networks, occupying relatively large areas of otherwise healthy brain-tissue. Recently, suppression of the RPTP-β/ζ and HB-GAM with either antibodies or siRNA–techniques has proven to reduce the growth of malignant gliomas (Foehr et al., 2006; Grzelinski et al., 2006; Ulbricht et al., 2006). It is not yet completely clear, if the HB-GAM polymorphism contributes to the formation of tumors.

6.3.3 Senility–associated diseases

Diseases associated with progressive senility, like Alzheimer's, are also on the map, when we discuss about HB-GAM and N-syndecan in brain pathology. HB-GAM accumulates in the brain plaques, or lesions, in the brain tissue of Alzheimer's disease and Down's syndrome patients. Accumulation occurs only in association with neuronal injury, and thus could be just an injury response by the damaged neurons (Wisniewski et al., 1996).

N-syndecan, on the other hand, could play an important role to either direction in the development of amyloid-diseases. HS is known to affect the formation of amyloid fibrils in patients with Alzheimer's disease, but there are currently contradicting findings about the specific role of brain HS in fibril-formation (Castillo et al., 1997, 1999; Li et al., 2005b; Beckman et al., 2006; Gralle et al., 2006). Usually HS is associated with enhanced fibril-formation, but some recent findings suggest that short HS-chains could be inhibitive to the process. So, this area of research definitely needs more attention, before anything conclusive can be said.

N-syndecan and HB-GAM are expressed in the deep nuclei of the brain. The dopaminergic substantia nigra is a small nucleus near the base of the brain, but it has an enormous number of dopaminergic connections to the cerebrum and cerebellum, and lesions in the nigra cause Parkinsonian symptoms. In Parkinson's disease the dopaminergic activity in the nigra declines, for an unknown reason.

HB-GAM is expressed by dopaminergic precursor cells, and is found to promote the differentiation of dopaminergic cells in mesencephalic cultures enriched in dopaminergic precursor cells (Hida et al., 2003; Jung et al., 2004; Mourlevat et al., 2005). These findings lead to the tempting thought of testing HB-GAM as an anti-parkinsonian agent. N-syndecan is expressed in substantia nigra as well, and could very well be the effective receptor for HB-GAM.

It is becoming clearer, that many senility–diseases are multi-gene diseases, and the association studies are very complex. Most of the participating players, either proteins, RNA, oligosaccharides, or environmental factors are not yet known. Handling the amount of information required has only recently become practical, and the first large-scale association studies in many diseases have just begun. To get the full benefit of modern genome and proteome techniques, a more detailed mapping of both HB-GAM and N-syndecan genes in mice and humans is required. That will give us a step-ladder to reach the next level in the complexity, that is necessarily involved in the studies of brain development, plasticity, and pathology.

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