Heparin-binding growth-associated molecule (HB-GAM) in activity-dependent neuronal plasticity in hippocampus

Ivan Pavlov

Neuroscience Center and Department of Biological and Environmental Sciences,
University of Helsinki

Finnish Graduate School of Neuroscience (FGSN)

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Supervised by:

Professor Heikki Rauvala, MD, PhD
Neuroscience Center,
University of Helsinki, Finland

and

Docent Tomi Taira, PhD
Neuroscience Center,
Department of Biological and Environmental Sciences
University of Helsinki, Finland

Reviewed by:

Docent Claudio Rivera, PhD
Institute of Biotechnology,
University of Helsinki, Finland

and

Docent Heikki Tanila, MD, PhD
Department of Neuroscience and Neurology,
University of Kuopio, Finland

Opponent:

Dr. Ceri H. Davies, PhD
Psychiatry Centre of Excellence for Drug Discovery,
GlaxoSmithKline, Harlow, Essex, United Kingdom

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List of original publications

This thesis is based on the following articles, referred in the text by their Roman numerals

I. ***Pavlov* I****, Voikar* V, Kaksonen M, Lauri SE, Hienola A, Taira T, Rauvala H.* Role of heparin-binding growth-associated molecule (HB-GAM) in hippocampal LTP and spatial learning revealed by studies on overexpressing and knockout mice. *Mol Cell Neurosci.* 2002 Jun;20(2):330-42. (* equal contributors).

II. *Kaksonen* M,* ***Pavlov* I****, Voikar* V, Lauri SE, Hienola A, Riekki R, Lakso M, Taira T, Rauvala H.* Syndecan-3-deficient mice exhibit enhanced LTP and impaired hippocampus-dependent memory. *Mol Cell Neurosci.* 2002 Sep;21(1):158-72. (* equal contributors).

III. ***Pavlov I****, Rauvala H, Taira T.* Enhanced hippocampal GABAergic inhibition in mice overexpressing heparin-binding growth-associated molecule (HB-GAM). (*Neuroscience*, In Press).

IV. *Raulo E., Tumova S.,* ***Pavlov I.****, Pekkanen M., Hienola A., Klankki E., Taira T., Kilpeläinen I. and Rauvala H.* The two thrombospondin type I repeat domains of heparin-binding growth-associated molecule are necessary and sufficient for the interaction with hippocampal neurons. (*J Biol Chem.*, In Press; 2005 Sep 9; [Epub ahead of print]).

Abbreviations

ALK, anaplastic lymphoma kinase

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate

CA, *Conru Ammonis*

CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II

CASK, Ca^{2+}, calmodulin-associated serine/threonine kinase

CNS, central nervous system

CSPG, chondroitin sulfate proteoglycan

ECM, extracellular matrix

FGF, fibroblast growth factor

fEPSP, field excitatory postsynaptic potential

GABA, γ-aminobutyric acid

GAG, glycosaminoglycan

HB-GAM, heparin-binding growth-associated molecule

HFS, high-frequency stimulation

HS, heparan sulfate

HSPG, heparan sulfate proteoglycan

LIN-2, abnormal cell LINeage-2

LTD, long-term depression

LTP, long-term potentiation

mIPSC, miniature inhibitory postsynaptic current

MK, midkine

MMP, matrix metalloproteinases

NCAM, neural cell adhesion molecule

NMDA, *N*-methyl-D-aspartate

PDZ, PSD-95/Dlg/ZO-1 (postsynaptic density-95/disc large/zona occludens)

PPF, paired-pulse facilitation

RPTPβ/ζ, receptor-like protein tyrosine phosphatase ζ/β

sIPSC, spontaneous inhibitory postsynaptic current

tPA, tissue plasminogen activator

TSP, thrombospondin

TSR, thrombospondin type I repeat

Abstract

Cell adhesion and extracellular matrix (ECM) molecules play a significant role in neuronal plasticity both during development and in the adult. Plastic changes in which ECM components are implicated may underlie important nervous system functions, such as memory formation and learning. Heparin-binding growth-associated molecule (HB-GAM, also known as pleiotrophin), is an ECM protein involved in neurite outgrowth, axonal guidance and synaptogenesis during perinatal period. In the adult brain HB-GAM expression is restricted to the regions which display pronounced synaptic plasticity (e.g., hippocampal CA3-CA1 areas, cerebral cortex laminae II-IV, olfactory bulb). Expression of HB-GAM is regulated in an activity-dependent manner and is also induced in response to neuronal injury.

In this work mutant mice were used to study the *in vivo* function of HB-GAM and its receptor syndecan-3 in hippocampal synaptic plasticity and in hippocampus-dependent behavioral tasks. Phenotypic analysis of HB-GAM null mutants and mice overexpressing HB-GAM revealed that opposite genetic manipulations result in reverse changes in synaptic plasticity as well as behavior in the mutants. Electrophysiological recordings showed that mice lacking HB-GAM have an increased level of long-term potentiation (LTP) in the area CA1 of hippocampus and impaired spatial learning, whereas animals with enhanced level of HB-GAM expression have attenuated LTP, but outperformed their wild-type controls in spatial learning. It was also found that $GABA_A$ receptor-mediated synaptic transmission is altered in the transgenic mice overexpressing HB-GAM. The results suggest that these animals have accentuated hippocampal GABAergic inhibition, which may contribute to the altered glutamatergic synaptic plasticity.

Structural studies of HB-GAM demonstrated that this protein belongs to the thrombospondin type I repeat (TSR) superfamily and contains two β-sheet domains connected by a flexible linker. It was found that di-domain structure is necessary for biological activity of HB-GAM and electrophysiological phenotype displayed by the HB-GAM mutants. The individual domains displayed weaker binding to heparan sulfate and failed to promote neurite outgrowth as well as affect hippocampal LTP.

Effects of HB-GAM on hippocampal synaptic plasticity are believed to be mediated by one of its (co-)receptor molecules, namely syndecan-3. In support of that, HB-GAM did not attenuate LTP in mice deficient in syndecan-3 as it did in wild-type controls. In addition, syndecan-3 knockout mice displayed electrophysiological and behavioral phenotype similar to that of HB-GAM knockouts (i.e. enhanced LTP and impaired learning in Morris water-maze). Thus HB-GAM and syndecan-3 are important modulators of synaptic plasticity in hippocampus and play a role in regulation of learning-related behavior.

Introduction

The ability of neurons to modify the efficacy of synaptic transmission is important for various aspects of neural function. Dramatic changes in the synaptic connectivity occur during the perinatal period when new contacts are being elaborated. Refinement of synaptic connectivity in the course of development critically depend on electrical activity of the neurons and involves cooperative and competitive interactions between converging inputs, leading to stabilization or elimination of the immature connections (Zhang and Poo, 2001). However, neural plasticity is not only confined to the developing brain but is also an essential property of the mature nervous system where it is a prerequisite for adaptation to the ever changing world. It is also considered to be the biological substrate for memory formation. In the adult brain processes similar to those used during development are thought to be employed for lasting activity-dependent changes in synaptic efficacy, namely long-term potentiation (LTP) and long-term depression (LTD).

Evidently, the conversion of transient electrical signals into persistent modifications in synaptic structure requires intimate coupling between electrical and molecular signalling within the neuron and its microenvironment. Here an important question is: What are the molecular mechanisms that detect the neuronal activity patterns, and link them to functional and structural changes at the synapses? Recent studies have pointed out the importance of cell surface adhesion molecules, soluble growth factors, and in particular, extracellular matrix (ECM)-associated factors, in the formation of functional neuronal connections during development, as well as in neuronal plasticity in the adult (e.g. Luthi et al., 1994; Lauri et al., 1998; see also Dityatev and Schachner, 2003). These molecules mediate transsynaptic signals in response to neuronal activity in order to coordinate simultaneous pre- and postsynaptic modifications (e.g. Contractor et al., 2002). One of such ECM-associated components implicated both in the nervous system development and adult plasticity is heparin-binding growth associated molecule (HB-GAM). This study is concentrated on the role of HB-GAM and its receptor syndecan-3 in the hippocampal activity-dependent synaptic plasticity and learning and memory.

Review of the literature

Special role of hippocampus in learning and memory

Based on studies on amnesic patients such as HM, long-term memory has been divided into declarative and procedural type (for review see Squire, 2004). Declarative memory contains memory for facts and events and can be consciously brought in mind, whereas procedural memory expresses itself as perceptual biases and improved performance upon repetition. Most types of declarative memory depend on intact functions of the hippocampus and patients with hippocampal damage suffer anterograde amnesia and display inability to remember e.g., particular facts, names and places (Scoville and Milner, 1957). In contrast, procedural memory includes several memory systems that are all independent of the hippocampus.

Initial experimental studies in rodents emphasized the special contribution of the hippocampus for spatial learning and claimed that non-spatial tasks do not require the hippocampus. The discovery of hippocampal “place cells” made a significant advancement in understanding the role of hippocampus in memory (O'Keefe and Dostrovsky, 1971). It was demonstrated that hippocampal pyramidal cells are involved in encoding the information about the particular spatial location of the animal (Keefe, 1979).

More recent experimental studies have shown the importance of the hippocampus in nonspatial tasks that require flexible use of learned association and thus compare to human declarative memory. These include odor-based transitive inference and social transmission of food preferences. The lesion experiments suggested that animals with hippocampal damages had impaired ability to explore other options and adopt new behavior (reviewed by Eichenbaum and Cohen, 2001).

Behavioral analysis of learning and memory

The intricate nature of the relationship between different forms of memory in complex behavior complicates the interpretation of behavioral results in animal studies. Nevertheless, there are several tests which measure the analogue of human

declarative memory (memories of places, objects, odors) in rodents (Sweatt, 2003).

Spatial learning is hippocampus-dependent in both humans and rodents. A variety of paradigms exist to investigate spatial learning, for instance, Barnes maze (Barnes, 1979), but the hallmark in hippocampus-dependent behavioral studies is Morris water maze. In this test animals use spatial cues in the testing room to find a hidden underwater platform in a circular swimming pool (Morris, 1984). The test is based on the motivation of the animal to escape water and climb the platform as quickly as possible. Many other types of mazes (radial maze, T-maze, Y-maze) are used to study learning and memory (e.g. Olton and Papas, 1979). In the working memory tests in the radial maze animals are trained to remember unique episodes in the maze for goal-directed behavior as they visit radial arms of the maze learning the places of the food rewards. Other brain areas are also involved in the radial maze memory tasks besides hippocampus (e.g. prefrontal cortex, which has strong connections to hippocampus). Though, it is usually difficult to discriminate between the effects on learning, memory and recall in animal experimental models the variations in experimental design allow to address diverse aspects of learning behavior (Eichenbaum and Cohen, 2001).

The characteristic feature of declarative memory is its associativity, meaning that learning occurs in some context, and the memory episode associates with this context. Thus, it was hypothesized that associative molecular mechanisms (e.g. similar to those used in LTP induction) are important for learning and memory. Fear conditioning and taste aversion are widely used associative learning paradigms. Fear conditioning test evaluates the ability of the animal to associate environmental cues and stimuli to aversive stimulus (foot shock) and is based on the tendency of mice to freeze in response to fearful stimuli. There are two forms of fear conditioning: context-dependent (foot shock is associated with particular environment) and cued fear conditioning (foot shock is associated with a certain stimulus, e.g. auditory tone). Fear conditioning tasks are generally dependent on the amygdala. The contextual and cued fear is assessed by measuring the duration of freezing in the test conditions and in the altered context. Cued conditioning task is usually used to assess general hippocampus-independent associative learning that is amygdala-dependent. Contextual fear conditioning task in addition involves hippocampus-dependent mechanisms (Phillips and LeDoux, 1992; Holland and Bouton, 1999). Other tests of hippocampus-dependent forms of fear conditioning exist, for example, contextual discrimination and trace fear conditioning (Frankland et al., 1998; Huerta et al., 2000).

It is not unusual that revealing the aberrant behavior especially in the case of 'mild' phenotype can be problematic. In addition, changes in some forms of behavior may alter performance in other tests and thus result in erroneous interpretations of the results. For instance, increased anxiety could be the reason for low performance in Barnes maze though having no affect on learning behavior in Morris water maze (Gerlai and Clayton, 1999). Thus, it is often required that several tests from the same behavioral domain are done to evaluate the involvement of the gene under the study in particular behavior. The increasing body of data generated by the mutant mice studies requires that the results should be comparable between different laboratories. This resulted in the creation of standard procedures and test batteries for behavioral studies (Brown et al., 2000; Nolan et al., 2000; Crawley and Paylor, 1997). However, each new mutant can display novel behavioral responses which are not detected by the standard test arrays. Further, a number of tasks in behavioral screening lack ethological relevance and may be insensitive to the differences between mutant animals and their wild-type controls (Gerlai and Clayton, 1999).

Synaptic plasticity in hippocampus

Long-term potentiation

LTP is defined as lasting use-dependent increase in the efficacy of synaptic transmission. Originally discovered by Bliss and Lomo (Bliss and Lomo, 1973) in dentate gyrus in response to high-frequency stimulation (HFS) of the perforant path of anesthetized rabbits, LTP was subsequently found in all excitatory pathways of hippocampus as well as some other brain regions (Racine et al., 1995; Rogan et al., 1997). Thus, the ability of synapses to display long-term changes in the efficacy of neurotransmission is generally viewed as a fundamental property of the majority of synapses. The mechanisms underlying LTP induction may vary. Some forms of lasting potentiation require *N*-methyl-D-aspartate (NMDA)-receptor activation while others do not. If not indicated otherwise, here we will discuss the NMDA-dependent form of LTP induced by HFS in the pyramidal cells of the CA1 area of hippocampus (fig. 1). Time course of LTP is generally divided into several phases: the post-tetanic potentiation (first several minutes following tetanic stimulation), early LTP (up to ~60 minutes after induction) and late LTP (potentiation lasting longer that 1 hour).

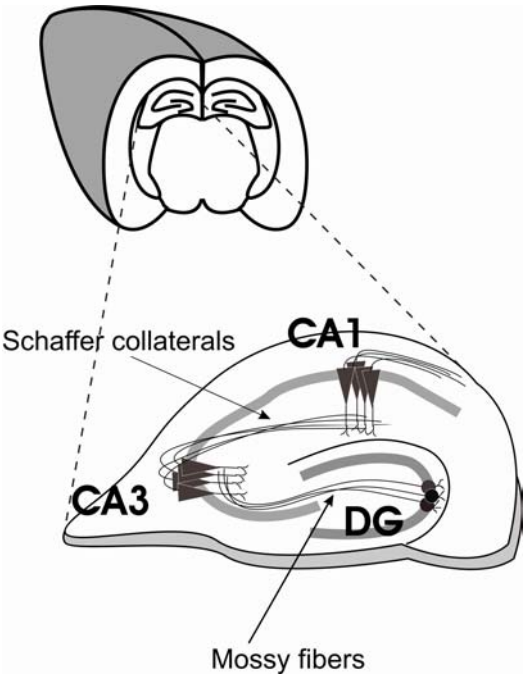

Figure 1. Schematic representation of the synaptic connectivity in the transverse hippocampal slice. Granule cells of the dentate gyrus (DG) send their axons (mossy fibers) to the proximal dendrites of the pyramidal cells in the CA3 region. The CA3 primary neurons form excitatory synaptic input to the CA1 pyramidal cells by en passant synapses of Schaffer collaterals on the apical dendrites.

Triggering mechanisms of LTP induction in the area CA1 are well described (for recent review see Lynch, 2004). During low-frequency basal synaptic transmission glutamate released from the presynaptic terminal activates postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor channels which generate synaptic response when the neurons are near their resting potentials. The NMDA receptors, another type of glutamate receptors, are voltage sensitive. Their ion channels are blocked by the extracellular Mg^{2+} when the membrane potential is close to the resting values (fig. 2 a). Thus, unlike AMPA receptors, NMDA channels contribute little to the basal synaptic transmission. In order to remove Mg^{2+} block from the channels the membrane should be depolarized. This is achieved during high frequency stimulation of the presynaptic fibers. NMDA channels are permeable to Ca^{2+} and their activation let Ca^{2+} to enter the cell (fig. 2 b). The rise of intracellular Ca^{2+} is crucial for LTP induction as it triggers the activation of several signalling pathways required for the increase in the synaptic strength.

The maintenance of LTP is less well understood. However, several cellular and molecular mechanisms have been implicated in this process both at the pre- and post-synaptic sites of the contact (fig. 2 c). Activation of CaMKII as well as several other postsynaptic protein kinases (e.g. PKC, PKA, MAPK, fyn, src) seem to be critical for stabilizing LTP at least at the early stages. Initial activation of kinases by Ca^{2+} leads to their autophosphorylation and thus the process becomes independent of transient Ca^{2+} influx (Soderling and Derkach, 2000). Lasting changes of synaptic strength apparently involve regulation of AMPA receptor function and trafficking. Evidence that LTP is accompanied by an increase in single channel conductance of AMPA receptors was provided by Benke and co-authors (Benke et al., 1998). Single-channel conductance of functional AMPA receptors increases as the result of their CaMKII-mediated phosphorylation (Derkach et al., 1999). Additional AMPA receptor subunits are driven into the synapses after LTP-inducing stimulation *in vitro* as well as during experience-dependent plasticity *in vivo*. Conversely, LTD is associated with AMPA receptor withdrawal from the postsynaptic site (Hayashi et al., 2000). Insertion of new AMPA receptors into the plasma membrane of hippocampal neurons requires transient synaptic activation of the NMDA receptors similar to that occurring during LTP induction (Pickard et al., 2001).

Regulation of glutamate uptake has been recently suggested to be important to maintain LTP (Levenson et al., 2002). Increased uptake may be necessary to protect receptors in the potentiated synapses from desensitization. Moreover, glutamate uptake limits transmitter spillover from the synaptic cleft and thus is crucial for maintaining the specificity of LTP (Tsvetkov et al., 2004).

Another possible mechanism underlying changes in synaptic strength during LTP may be alteration in release kinetics. Due to the particular kinetics of glutamate binding to the AMPA receptors rapid elevations of glutamate concentration during transmitter release more effectively activate AMPA receptors that slower changes in the transmitter concentrations (Renger et al., 2001). Alternatively, fusion pore size may be changed affecting the amount of glutamate released by the single vesicle (reviewed by Krupa and Liu, 2004). A number of studies indicate that postsynaptic cell can communicate with the presynaptic compartment and affect release parameters (e.g. release probability and quantal size) via secreted diffusible factors, retrograde messengers. Most popular candidates for retrograde messengers include membrane-permeable nitric oxide (NO), superoxide anion (O^{2-}), carbon monoxide (CO), arachidonic acid (AA), and neurotrophic factors (e.g. brain-derived neurotrophic factor [BDNF])

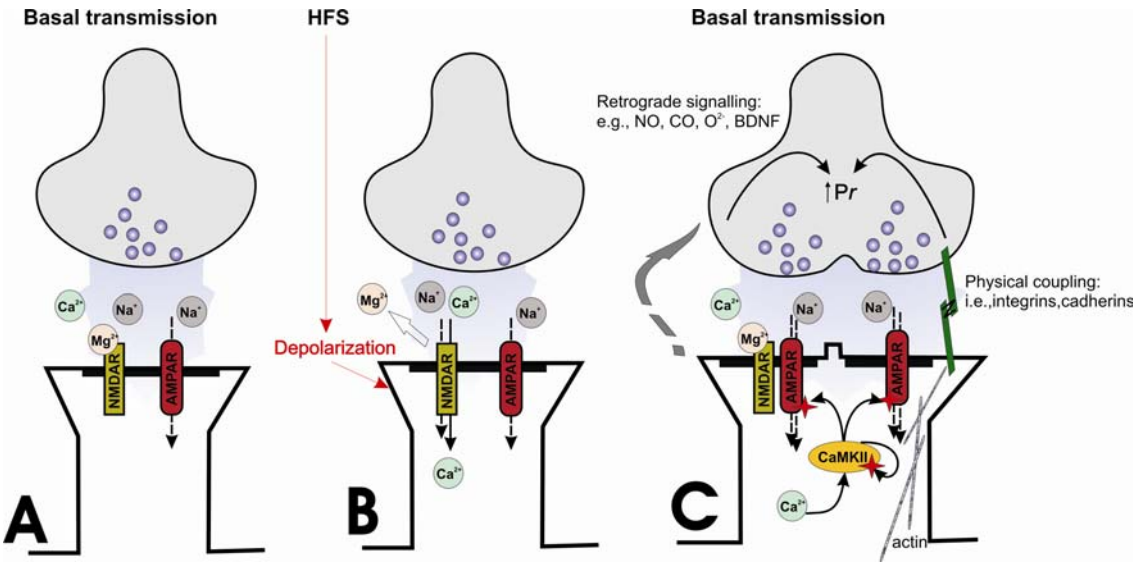

Figure 2. Summary diagram illustrating mechanisms underlying LTP in the area CA1 of hippocampus. A-B) LTP induction requires depolarization of the postsynaptic membrane and relieve of Mg^{2+} block of the NMDA receptor channels. Activation of NMDA receptors allows Ca^{2+} influx into the cell, which is critical to trigger LTP. C) LTP expression may rely on both pre- and postsynaptic changes. Several mechanisms have been reported to account for the changes in the efficacy of synaptic transmission following LTP induction including increase in single-channel conductance of AMPA receptors by their phosphorylation, recruitment of additional AMPA receptors, upregulation of transmitter release. Alterations in the glutamate release may be mediated by retrograde signalling via diffusible factors and molecules involved in physical coupling of the presynaptic terminal and postsynaptic site. Further, structural changes have been demonstrated to accompany LTP suggesting the role of cytoskeleton and molecules involved in cell-cell and cell-extracellular matrix interactions.

(Medina and Izquierdo, 1995). Though no direct evidence exists so far, protein complexes that physically link the pre- and postsynaptic areas can participate in retrograde signalling by means of conformational changes. Such complexes may be formed by adhesion molecules (e.g. integrins or cadherins) known to be important modulators of LTP (Chan et al., 2003; Tang et al., 1998). In addition, cell-adhesion molecules through their links to the cytoskeleton affect structural remodelling of synapses during LTP (Wheal et al., 1998).

It is generally agreed that LTP produces lasting changes in synaptic morphology (see reviews by Yuste and Bonhoeffer, 2001). However, despite recent advances in imaging techniques it is still the matter of controversy whether structural changes of the synaptic connections during LTP involve only alterations in the shape of synaptic contacts, or the increase in synapse number. The last can also occur as the result of splitting the existing synapses and/or formation of new contacts (Fiala et al., 2002; Hering and Sheng, 2001; Ostroff et al., 2002). In addition, maintenance of the late-LTP is dependent on gene expression and protein synthesis (Kandel, 2001).

Intriguingly, certain forms of synaptic plasticity in adults and activity-dependent mechanisms of synaptogenesis display striking similarities. Though activity blockade does not prevent formation of functional synaptic contacts, selective stabilization of some inputs and elimination of others depend on correlated activity both in central and peripheral synapses (Bouwman et al., 2004; Lauri et al., 2003; Zhang and Poo, 2001; Verhage et al., 2000). One of the most important common features for the activity-dependent input refinement and LTP is their NMDA-dependency. NMDA receptors serve as molecular detector of temporal correlation of pre- and postsynaptic activation, and both processes require activation of NMDA receptors to be initiated (Cline, 2001; Hahm et al., 1991; Shi et al., 2001). Both processes also crucially depend on CaMKII activation (Wu et al., 1996). Further, postsynaptic receptor trafficking involved in LTP expression is mechanistically similar to the functional synapse maturation when physiologically “silent synapses” acquire AMPA receptors. Thus it was hypothesized that LTP-like phenomena could be instrumental for the maturation of excitatory synapses (Durand et al., 1996; Liao et al., 1995). Remarkably, spontaneous neural activity is sufficient to selectively deliver GluR4-containing AMPA receptors into developing synapses (Zhu et al., 2000). Apparently, activity-

dependent processes utilize common molecular mechanisms early in development and in the adult (Constantine and Cline, 1998), thus many signalling molecules involved in development of synaptic contacts are also important modulators of synaptic plasticity in the adults.

Role of inhibition in synaptic plasticity

GABA (γ-aminobutyric acid)-mediated synaptic inhibition plays a critical role in the control of excitation in the hippocampus. The GABAergic network controls excitability and coordinates spatiotemporal integration properties of hippocampal principal neurons. Though GABAergic interneurons comprise a relatively small subpopulation of hippocampal neurons their extensive arborisation allows a single interneuron to synapse many pyramidal cells forming up to 12 contacts with each postsynaptic neuron (Buhl et al., 1994a; Buhl et al., 1994b). Some interneurons terminate mainly on the perisomatic region of principal hippocampal cells while others terminate on the dendritic area of pyramidal neurons (Miles et al., 1996). In addition, interneurons may target other interneurons creating highly interconnected inhibitory network (Acsady et al., 1996; Gulyas et al., 1996). GABAergic neurons in hippocampus provide two basic types of inhibition of CA1 pyramidal cells in response to Schaffer collateral stimulation: feed-forward and feed-back (recurrent) inhibition (fig. 3). In the case of feed-forward inhibition, GABAergic neurons are directly activated by the axons projected from the CA3 principal neurons and thus inhibit CA1 pyramidal cells. Otherwise, excitatory input of CA3 projections activates CA1 pyramidal cells. The latter send their axon collaterals to the interneurons which in turn recurrently inhibits CA1 pyramidal cells. Feed-back inhibition is mediated primarily by the perisomatic inhibition of pyramidal neurons (Parra et al., 1998). GABAergic transmission is mediated by ligand-gated ionotropic $GABA_A$ receptor channels permeable for Cl^- (HCO_3^-) and K^+-permeable metabotropic $GABA_B$ receptors. $GABA_A$ receptors mediate fast GABAergic neurotransmission. Activation of $GABA_B$ receptors mediates slow K^+ currents and causes prolonged neuronal hyperpolarization. In addition to postsynaptic localization, $GABA_B$ receptors are expressed in the presynaptic terminals, where they function as autoreceptors suppressing transmitter release (Davies and Collingridge, 1996).

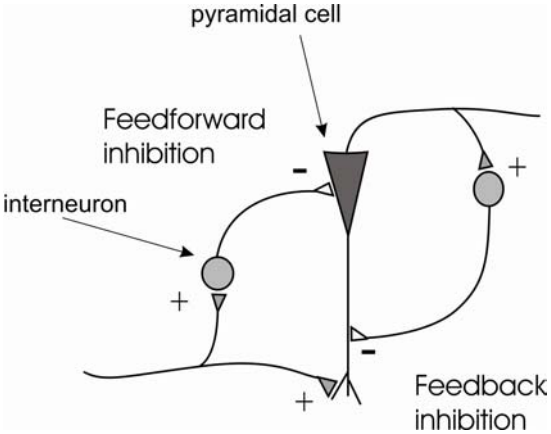

Figure 3. Feedforward and feedback inhibitory circuits in the CA1 area of hippocampus. "+" – excitatory synapse, "-" – inhibitory synapse.

GABAergic transmission is involved in induction and expression mechanisms of long-term plasticity in the hippocampus as well as in the other brain areas (e.g. visual cortex) (Feldman, 2000; Steele and Mauk, 1999). Modulation of synaptic plasticity by GABA receptor-mediated transmission is dependent on temporal pattern and intensity of stimulation (Chapman et al., 1998; Staubli et al., 1999). Blockade of $GABA_A$ receptor-mediated responses in hippocampus generally produces enhanced LTP (Chapman et al., 1998; Wigstrom and Gustafsson, 1985). Conversely, upregulation of GABAergic neurotransmission suppresses LTP (Levkovitz et al., 1999). Repetitive stimulation with high-frequencies result in the fatigue of synaptic inhibition (McCarren and Alger, 1985), thus leading to increased depolarization during tetanic stimulation. The mechanism of facilitated depolarization involves $GABA_B$ receptor-mediated autoinhibition of GABA release (Davies et al., 1991; Mott and Lewis, 1991). However, presynaptic $GABA_B$ receptor activation has been demonstrated to be important only for theta-burst stimulation-induced LTP but not for HFS-induced potentiation (Staubli et al., 1999). Alternatively, as demonstrated in several studies, $GABA_A$ responses may produce depolarization in CA1 hippocampal neurons during high-frequency stimulation (Kaila et al., 1997; Taira et al., 1997). These data suggest that GABA-mediated transmission can provide excitatory drive in the adult hippocampus and play a facilitatory role in LTP induction (c.f. Autere et al., 1999). This depolarizing action of GABA also seems to be regulated by $GABA_B$ receptor activation (Brown et al., 2003; Cobb et al., 1999). However, the role of excitatory GABA in plasticity of glutamatergic synapses still remains unclear.

$GABA_A$ receptor-mediated responses switch gradually from depolarizing to hyperpolarizing

towards the end of the second postnatal week (Lamsa et al., 2000; Rivera et al., 1999). In the developing brain the depolarization provided by activation of GABA receptors is sufficient to remove the voltage-dependent Mg^{2+} block from NMDA channels, which makes the GABAergic system also an attractive candidate for the regulation of synaptic plasticity early in postnatal life (Ben-Ari, 2002; Leinekugel et al., 1995). Indeed, GABAergic transmission contributes differently to the induction of LTD in the area CA1 of hippocampus during the course of maturation. At the early stages of development depolarization provided by $GABA_A$ receptor-mediated currents promote activation of NMDARs, thus shifting the threshold for the LTD induction and making the synapses more prone for activity-dependent plasticity (Pavlov et al., 2004). Different effects of $GABA_A$ receptor blockade on LTD has been also demonstrated for the juvenile and adult rats (Wagner and Alger, 1995). Recent studies also revealed the role of $GABA_A$ receptor-mediated inhibition in the developmental shift of LTP induction efficiency (Meredith et al., 2003).

Mutant mice approach to study plasticity, learning and memory

Transgenic and gene-targeted mutant mice provide an important tool to study the role of a particular gene in the brain function *in vivo*. Combining results of behavioral studies with data obtained by the use of *in vitro* methods allows to get insights into the molecular and cellular mechanisms underlying complex forms of behavior.

An extensive progress made by molecular genetics, particularly in developing methods to produce genetically modified organisms, boosted the field of neuroscience during the past decade. A brief overview of the available approaches in the mutant studies is presented in the Table 1. The use of genetically modified mice allows to analyse the functions of a particular gene in behavior and relate the results to the *in vitro* studies. Cellular and molecular mechanisms underlying hippocampal synaptic plasticity are widely suggested to be implicated in memory formation. Thus, many studies have focused on the link between hippocampal synaptic plasticity and performance in learning and memory tasks (reviewed by Chen and Tonegawa, 1997; Lynch, 2004). The first mutant mice used in the studies of molecular mechanisms underlying learning and memory in hippocampus were Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Silva et al., 1992b; Silva et al., 1992a) and fyn (Grant et al., 1992) knockouts. Both displayed impaired LTP and deficit in spatial learning in the Morris water maze. Later the role of many other molecules (including all major glutamate receptor subunits) in LTP and learning and memory has been evaluated using knockout and transgenic studies. Recent results of microarray analysis of memory-related genes recognized more genes, which previously have not been related to synaptic plasticity or learning behavior (Cavallaro et al., 2002; Robles et al., 2003). Among those genes are the ones that encode molecules responsible for cell-cell and cell-matrix interactions, extracellular signalling molecules, growth factors and their receptors.

The major drawback of conventional gene targeting and transgenic approach is that irreversible changes in the genotype often complicate the interpretation of phenotypic analysis and may even preclude the study of the mutant animal. Developmental compensation for the loss-of-function or gain-of-function of the particular gene could mask its function and result in no phenotype in the mutant animal or lead to the phenotype caused by altered expression of other gene(s). Also, ablation of certain genes may cause severe dysfunction in the course of development leading to the perinatal lethality. In some instances the potential problems may be circumvented by the use of inducible and tissue-specific gene expression systems which allow to control expression temporally and spatially (Picciotto and Wickman, 1998; Williams and Wagner, 2000). Whatever the case, it is clear that studies of mutants should be complemented by other approaches and the interpretation of the phenotype of the mutant mice should be correlated to the results of other experimental paradigms.

A great concern of all mutant studies is that the phenotypic changes crucially depend on genetic background. Thus, controlling genetic background is essential in the studies of mutants (Crawley et al., 1997). It is a common practice to analyse the mutations in a hybrid background thus eliminating homozygosity of alleles which potentially may be responsible for abnormalities. However F1 mice are not always ideal and in some cases it may be advantageous to perform the study of certain phenotype in an inbred strain. Strain-dependent differences in hippocampal synaptic plasticity as well as behavioral variability have been well documented (Bampton et al., 1999; Nguyen et al., 2000 a, b; Voikar et al., 2001; Wolfer et al., 2002). Nevertheless, different ability to perform in behavioral tests and different electrophysiological characteristics of inbred lines provide an additional tool to study certain aspects of gene function. For example, it is preferable to backcross the mutation, which is supposed to reduce a particular function, into an inbred line displaying ‘high-scores’ in the relevant behavioral task.

Table 1.

Technique	Description	Possible pitfalls
Random mutagenesis	Mutations are produced at random and their rate enhanced e.g. by X-rays. Widely used for instance in *Drosophila* studies.	Large populations of animals need to be analysed
Transgenesis	One or more exogenous copies of the gene of interest are introduced into genome in order to produce constitutively active ("gain-of-function mutants") or dominant negative ("loss-of-function mutants") forms of a specific protein.	Insertional effects (function of another gene is affected by the transgene), ectopic expression, undesirable effects caused by chronic expression of the gene, failure to express the transgene at physiologically relevant levels.
Inducible transgenesis	The expression of transgene is under the control of promoters sensitive to exogenously applied substances (e.g. tetracycline).	Side effects of the triggering substance, leakage transcription
Use of "reporter genes"	Easily detected proteins (e.g. GFP) are used as the selective markers of physiological activity or anatomic characteristics.	
Targeted modifications of endogenous genes		
Knock-out	Targeted gene deletion	Chronic absence of a gene may cause abnormal development, embryonic or early postnatal lethality, functional compensation. Functional redundancy (many proteins are present as multiple isoforms derived from different genes).
Conditional knock-out • region-specific • inducible	Only selected cells lack the gene, or the gene is switched "on" and "off" by applied substances.	
Knock-in	Targeted mutations of the gene of interest or introduction of a new gene in the selected locus (e.g. substitution of a gene by reporter gene)	

Cell-cell and cell-extracellular matrix interactions in hippocampal plasticity

Composition and function of the ECM in the brain tissue

ECM accounts for a relatively large volume of the nervous tissue. On average it has been estimated to occupy about 20% of the brain in adults and twice as much in newborn animals (Nicholson and Sykova, 1998). More than a century ago Camillo Golgi described reticular structure which surrounds cell bodies of neurons. This perineuronal net represents a complex of ECM molecules which together with the meshwork of glial processes form an envelope around nerve cells. Molecular composition of the perineuronal nets associated with different populations of neurons is unique and changes in the course of development suggesting functional significance of active dynamic regulation of perineuronal net elements (Celio et al., 1998; Fox and Caterson, 2002).

Table 2

Major ECM components of the central nervous system

ECM componets		Examples
Glycoproteins		Laminins, fibronectin, tenascin, thrombospondins
Proteoglycans		Syndecan, glypican, agrin, aggrecan, versican, phosphacan
• glycosaminoglycans	- chondroitin/dermatan sulfate - heparan sulfate/heparin - keratan sulfate - hyaluronan	
Secreted signalling molecules		bFGF, HB-GAM

The structure of ECM is highly organized and consists of several components. Major constituents in the ECM of the central nervous system (CNS) include glycoproteins: laminins, vitronectin, thrombospondins, tenascins; and various proteoglycans in which core protein is covalently bound to glycosaminoglycans (GAGs) (Table 2). ECM molecules provide adhesive substrate necessary for neuronal migration and morphogenesis during development. They also create molecular network to maintain mechanical support for the cells in the brain tissue. As an adhesive substrate for cell-surface molecules, such as integrins, the ECM is critical for the regulation of the cell shape and motility (Nikonenko et al., 2003; Suter et al., 1998). In the nervous system, the ECM is crucial for many developmental processes such as neuronal migration, neurite outgrowth, growth cone guidance and synapse formation and stabilization (Ruegg, 2001). In the adult brain, numerous studies have demonstrated the role of ECM in neuropathological conditions (Bruckner et al., 1999; Gutowski et al., 1999; Knott et al., 1998; Sobel and Ahmed, 2001) as well as in physiological processes like synaptic plasticity (reviewed by Dityatev and Schachner, 2003). Chondroitin sulfate proteoglycans (CSPGs; e.g. aggrecan, brevican, neurocan, phosphacan) and heparan sulfate proteoglycans (HSPGs; e.g. agrin, glypican, cerebroglycan, perlecan, and syndecans) form two major categories of proteoglycans present in the ECM (Bandtlow and Zimmermann, 2000; Hartmann and Maurer, 2001). Most of the functions of the proteoglycans are mediated by their glycosaminoglycan side chains, which bind to various signalling factors and cell-surface molecules. In addition to the integral components of the ECM, several secreted growth/differentiation factors, e.g. fibroblastic growth factors (FGF's) and HB-GAM are present in the extracellular space. The biological activity of these factors can be critically modulated by their interaction with the ECM components. For example, heparan sulfate is essential for the biological activities of the FGFs (Raman et al., 2003).

In the brain, the functional role of the ECM extends beyond the regulation of cellular morphology. The extracellular space serves as a low-resistance conducting media for the transmembrane currents created by neuronal activity. By its virtue, the ECM regulates the diffusion of ions, neurotransmitters and other neuroactive substances in the extracellular space (Nicholson and Sykova, 1998). For example, the main neurotransmitters GABA and glutamate bind not only to the postsynaptic receptors that mediate fast neurotransmission, but also to presynaptic auto- and heteroreceptors that regulate neurotransmitter release probability and thereby short-term dynamics of synaptic transmission. Activation of presynaptic and extrasynaptic receptors is dependent on neurotransmitter 'spillover', which is regulated by active uptake mechanisms but also by tortuousity of the extracellular space. Consequently, changes in the ECM composition can critically influence synaptic efficacy, neuronal excitability, synapse specificity and volume transduction in the brain (Kullmann et al., 1999; Min et al., 1998).

Dynamic remodeling of ECM in the nervous system

The ECM is no longer seen as a static embedding in which cells reside. The ECM composition is being constantly modified throughout the life both in the peripheral nervous system (Connor, 1997; Sanes et al., 1986) as well as in the CNS (e.g. Koppe et al., 1997; Yamaguchi, 1996). Given the number of neuronal functions influenced by the ECM, its remodelling during development, in response to physiological stimuli and under pathological conditions provides a powerful mechanism for structural and functional regulation of nervous tissue.

The physical parameters of the extracellular space in brain are altered in several pathological conditions and following neuronal trauma (reviewed by Sykova et al., 2000). For example, peripheral nerve axotomy causes an upregulation of F (floor plate)-spondin mRNA and protein level (Burstyn et al., 1998). HB-GAM, agrin, glypican, and syndecans accumulate in amyloid plaques in Alzheimer's disease (vanHorssen et al., 2002; Verbeek et al., 1999; Wisniewski et al., 1996). HSPGs were suggested to play an important role in the formation and persistence of senile plaques. A number of different CSPGs are increased in the nervous system at the region where the glial scar forms following the lesion. Up-regulation of these molecules is believed to restrict axonal regeneration at the site of injury (Morgenstern et al., 2002; Properzi et al., 2003; Zuo et al., 1998).

Further, regulation of ECM components in response to neuronal activity might provide a way for physiological regulation of neuronal excitability, plasticity and synchrony. In fact, expression of several ECM components is regulated in response to neural activity patterns. For example, Narp (synaptic pentraxin enriched at glutamatergic synapses on most aspiny but not spiny hippocampal and spinal cord neurons) was originally cloned as an immediate-early gene rapidly induced in neurons by HFS or repeated electroconvulsive seizures (Reti and Baraban, 2000; Tsui et al., 1996). Agrin expression in the CNS, particularly in hippocampal neurons *in vivo*, has been demonstrated to be regulated in an activity-dependent manner (Cohen et al., 1997; Lesuisse et al., 2000). Effects of activity blockade on agrin expression depend on the degree of synapse maturation. Action potential-dependent neurotransmission blockade at early and late phases of synapse maturation had contrasting effects on the level of agrin mRNA (Lesuisse et al., 2000). In addition, agrin has been demonstrated to activate the immediate early gene c-fos in cortical neurons through a Ca^{2+}-dependent mechanism (Hilgenberg et al., 2002). Among other ECM and cell adhesion molecules expressed in activity-dependent manner are HB-GAM (Lauri et al., 1996), tenascin C (Nakic et al., 1998), N-cadherin, neural cell adhesion molecule (NCAM) and L1 (Itoh et al., 1997).

In addition, fast activity-induced changes in the composition of ECM might be obtained by the activity of extracellular proteases. Matrix metalloproteinases (MMPs) are the group of ECM degrading enzymes that play a crucial role in neural migration, development, growth and repair by matrix remodelling (Shapiro, 1998). There is accumulating evidence that the balance of MMPs and their tissue inhibitors (TIMPs) play an important role in the brain function as they have been implicated in a number of neural diseases (reviewed by Skiles et al., 2001). Activity-dependent mechanisms of regulation have been demonstrated for both the activity of MMP (Jourquin et al., 2003) and tissue-type plasminogen activator (tPA) (Gualandris et al., 1996). Thus, under normal conditions changes in the activity of MMPs may contribute to the expression of synaptic plasticity and learning and memory (Wright et al., 2002). However, the physiological significance of these mechanisms is only beginning to be understood.

ECM and activity dependent synaptic plasticity

It is becoming increasingly evident that activity-induced synaptic plasticity in the brain involves changes in the neuronal morphology (Harris et al., 2003; Yuste and Bonhoeffer, 2001). Initially, structural alterations were proposed to be necessary for long-term maintenance of functional changes in the synaptic efficacy (Buchs and Muller, 1996; Ostroff et al., 2002; Toni et al., 1999), based on the findings that late but not early phases of LTP are dependent on protein synthesis and gene transcription. The first ECM receptors reported to be involved in the regulation of hippocampal LTP were the integrin type of cell adhesion molecules. Blockade of extracellular interactions of integrins inhibits expression of LTP 40 minutes after its induction (Bahr et al., 1997; Xiao et al., 1991). Inhibition of other cell-matrix receptors, including PSA-NCAM (Luthi et al., 1994; Muller et al., 1996), cadherins (Tang et al., 1998) and syndecans (Lauri et al., 1999) affects expression of LTP even faster, consistent with rapid remodelling of synaptic structures in response to neuronal activity (Bonhoeffer and Yuste, 2002; Dunaevsky and Mason, 2003).

Manipulations of ECM interactions do not seem to influence baseline synaptic transmission. This is consistent with a 'passive' role of ECM receptors as an inhibitory constraint for synaptic remodelling and/or growth in response to signals inducing synaptic plasticity (reviewed by Abel et al., 1998; Fields and Itoh, 1996). According to this view, downregulation of cell-adhesion is necessary for the HFS induced plastic changes in synaptic function and morphology. Proposed mechanisms for reduction of cell adhesion in synaptic plasticity include internalization or proteolytic cleavage of the cell-surface ECM receptors (Bukalo et al., 2001; Mayford et al., 1992; Nakagami et al., 2000), and calcium dependent downregulation of cadherin mediated adhesion (Tamura et al., 1998; Tang et al., 1998). Cleavage or shedding of HSPGs in

response to neuronal activity might represent a similar regulation mechanism (Asundi et al., 2003).

Instead of merely acting as a structural limit, an active role for ECM components and cell surface ECM receptors in regulation of synaptic transmission has been proposed. This more recent view is supported by several findings.

Narp selectively interacts with the AMPA receptor subunits GluR1-3 and directly affects receptor clustering (Brien et al., 2002; Xu et al., 2003), a mechanism proposed to be critical for expression of LTP (Malinow and Malenka, 2002). Also heparin has been reported to bind AMPA receptors and alter kinetic properties of single channel activity (Hall et al., 1996; Sinnarajah et al., 1999). Thus it is possible that extracellular matrix components can directly affect functional properties of AMPA receptors. On the other hand, tenascin-R and tenascin-C bind voltage-gated sodium channels and have been suggested to play an important role in modulation of their activity and localization in neurons (Srinivasan et al., 1998; Xiao et al., 1999). In addition, tenascin-C has been implicated in L-type voltage-dependent Ca^{2+} channel-mediated signalling (Evers et al., 2002). ECM molecules were also demonstrated to affect GABAergic transmission. Tenascin-R and its associated carbohydrate HNK-1 modulate perisomatic inhibition in hippocampus via regulation of GABA release in perisomatic synapses suppressing postsynaptic $GABA_B$ receptor activity (Saghatelyan et al., 2001; Saghatelyan et al., 2003).

In addition, transmembrane proteins, which bind ECM components, might act as independent signalling receptors to mediate activity-induced changes. HFS-induced changes in the interaction of the cytosolic domain of syndecan-3, a functional receptor of HB-GAM, with intracellular signalling molecules has been reported (Lauri et al., 1999). Already 10 minutes after induction of LTP in area CA1 in the hippocampus, association of syndecan-3 with tyrosine kinase fyn and an actin-binding protein cortactin was significantly increased, suggesting a role for this signalling complex in the mechanism of LTP expression. Also, specific signalling, which involves protein kinases Fnk and Snk, has been proposed for laminin-binding integrins during LTP induction (Kauselmann et al., 1999).

HB-GAM and TSR domain proteins in neuronal development and plasticity

Thrombospondin type 1 repeats (TSRs) are characteristic protein domains of thrombospondin-1 (TSP-1) and thrombospondin-2 (TSP-2), and they are important for cellular effects of thrombospondins. TSRs are ancient domains present in a variety of species from *C. elegans* to human and are characteristic for a number of extracellular and cell-surface proteins. TSR domains often bind to heparin and heparan sulfate (HS) and are defined by a conserved cysteine/tryptophan motif. The presence of these repeats probably determines biological functions and properties of the particular protein (reviewed by Naitza et al., 1998).

TSR superfamily proteins contain from one to seven TSR domains and specialize in cell surface and matrix binding. They are abundantly expressed in the developing nervous system and are involved in the cell attachment and motility. For example, F (floor plate)-spondin was initially identified as an axon growth and guidance factor (Klar et al., 1992). Among other neurite-promoting TSR domain proteins are HB-GAM, midkine (MK), UNC-5, semaphorins Sema5A and B, and TRAP (Adams and Tucker, 2000; Kilpelainen et al., 2000). It has been suggested that TSR domain provides a basic cell surface-binding protein module that is involved in neurite growth and guidance (Rauvala et al., 2000).

Structure of HB-GAM

One of the ECM proteins implicated both in the developmental formation of neuron-target contacts and in neuronal plasticity in the adult hippocampus is heparin-binding growth-associated molecule (HB-GAM). HB-GAM, also known as pleiotrophin (Ptn; Li et al., 1990), is a secreted 18 kDa protein which is associated with the HS-containing proteoglycans of the cell-surface and ECM (Rauvala, 1989). HB-GAM consists of 136 amino acids with a high proportion of cationic residues (24%) stabilized by 5 intrachain disulfide bridges. The HB-GAM sequence is highly conserved across vertebrate species. It folds into a structure containing two β-sheet domains connected by a flexible linker (fig. 4) (Kilpelainen et al., 2000; Iwasaki et al., 1997). Thus, HB-GAM domains are relatively independent and in solution move in respect to each other. Both domains consist of three antiparallel β-strands, and show significant homology to the TSR motif (Kilpelainen et al., 2000). The lysine-rich N- and C- terminal regions of HB-GAM form random coils in solution. HB-GAM binds with high affinity to heparin. Nuclear magnetic resonance studies showed that heparin binds to the β-sheet domains and induces structural changes in the HB-GAM molecule. In contrast the N- and C- tails apparently do not contribute to the heparin binding of HB-GAM.

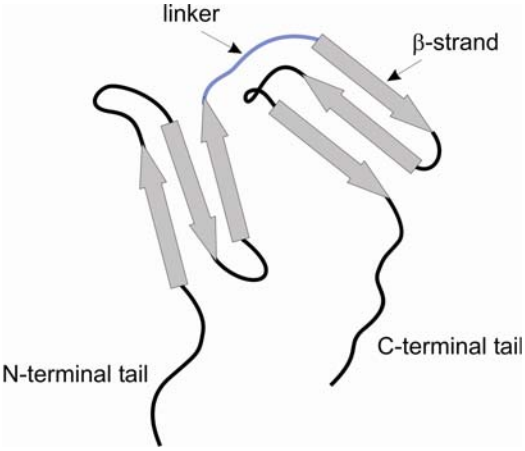

Figure 4. Two-domain structure of HB-GAM. TSR domains formed by three antiparallel β-sheet strands are connected via flexible linker region.

TSR containing proteins in the developing nervous system

HB-GAM is expressed in the nervous system in a distinctive spatiotemporal manner. It is abundant in the developing nervous system, where its expression peaks around 1-2 weeks postnatally coinciding with the differentiation of neurons and glia. In some cells in the distinct brain regions the expression of HB-GAM continues to adulthood (Rauvala et al., 1994). The overall pattern of expression as well as the *in vitro* functional results support a role for HB-GAM as a component of the extracellular matrix that regulates neuronal cell motility and differentiation (for reviews, see Rauvala and Peng, 1997; Bohlen and Kovesdi, 1991). Recombinant, matrix-bound HB-GAM promotes neurite outgrowth and can act as an axonal guidance factor in cell culture (Rauvala et al., 1994). Further, HB-GAM localizes to the developing fiber pathways as well as to embryonic basement membranes, suggesting a role for HB-GAM in the formation of neuron-target contacts. Indeed, HB-GAM can promote both pre- and postsynaptic differentiation in the neuromuscular junction (Dai and Peng, 1996; Peng et al., 1995). The effect of neuronal agrin isoform on AChR clustering in the neuromuscular junction was demonstrated to be strongly potentiated by HB--GAM. Thus, it has been proposed that HB-GAM acts as an integral component of agrin signalling mechanism (Daggett et al., 1996).

Other TSR domain containing proteins also play an important role in the development of nervous system. F-spondin and mindin are secreted adhesion proteins that share structural and biochemical similarities (Klar et al., 1992; Umemiya et al., 1997). Expression patterns of these molecules overlap in developing and adult rat cerebral cortex, particularly in pyramidal and granule cells of hippocampus (Feinstein et al., 1999). Both proteins promote adhesion and neurite outgrowth in embryonic hippocampal and sensory neurons. TSR domains of F-spondin have been demonstrated to be sufficient to promote neurite outgrowth (Feinstein et al., 1999). Similarly, TSR domains of TSP-1 are critical for the neurite outgrowth and cell attachment effects in hippocampal neurons (Osterhout et al., 1992).

However, none of individual TSR domains are indispensable for the development of the nervous system as indicated by mutant mice studies. Mice lacking HB-GAM are viable, breed normally and show no major histological defects in the nervous system (Amet et al., 2001). Similarly, no apparent morphological abnormalities were detected in the CNS of MK knockout mice (Nakamura et al., 1998), TSP-1 (Lawler et al., 1998) and TSP-2 deficient mice (Kyriakides et al., 1998). TSP-1/TSP-2 double knockout mice were generated recently and demonstrated delayed wound healing (Agah et al., 2002). Unfortunately, the study did not address regeneration in the nervous tissue. Very recently, however, TSP-1/TSP-2 deficient mice were reported to have decreased number of synapses in cortex (Washbourne et al., 2004). Modular organization of the ECM components may provide the structural basis to maintain a high level of functional redundancy of these proteins. Compensation between TSR proteins thus may account for the lack of a pronounced developmental phenotype in mutants without particular TSR-containing molecule.

Expression of HB-GAM and other TSR domain proteins in the adult brain

In adults, the expression of HB-GAM is limited to certain neuronal subpopulations, including the pyramidal neurons of the hippocampus (Wanaka et al., 1993). In addition to this basal level of expression, HB-GAM is induced by stimuli causing neuronal injury or seizures (Takeda et al., 1995; Wanaka et al., 1993). HB-GAM expression is increased in rat brain following ischemic injury. Sustained upregulation of HB-GAM expression was observed e.g. in astrocytes from 7 to 14 days after the injury (Yeh et al., 1998). Similarly, following kainic acid treatment, the expression of HB-GAM is downregulated in neurons (within 48 h), but induced in astrocytes 4 days after the injury. On the other hand, a rapid (30 min) neuronal induction of HB-GAM mRNA expression has been reported in the hippocampal area CA1 in response to pentylene-tetrazole induced seizures (Wanaka

et al., 1993) and in the forebrain in response to tetrahydrocannabinol, the major psychoactive component of cannabis (Mailleux et al., 1994). Interestingly, two active promoters have been described for HB-GAM in mice (Sato et al., 1997), suggesting that two distinct pathways may control HB-GAM expression.

Neuronal expression of HB-GAM mRNA is induced by high-frequency neuronal activation inducing LTP (Lauri et al., 1996). HFS-induced expression of HB-GAM was not completely blocked unless antagonists of both NMDA-receptors and voltage-gated calcium channels were used. Therefore, calcium influx via both of these routes contributes to the regulation of HB-GAM expression (Lauri et al., 1996). The activity-dependent enhancement in HB-GAM expression was the first finding suggesting involvement of endogenous HB-GAM in the regulation of synaptic plasticity in the hippocampus. Further studies indicated that application of recombinant HB-GAM into hippocampal slices inhibits HFS-induced LTP in area CA1, while single-pulse evoked synaptic responses are not affected (Lauri et al., 1998).

Though significant levels of expression of F-spondin and mindin in rat hippocampus persist during adulthood, the functional role of these proteins in adult brain remains unclear. Both proteins were suggested to be involved in activity-dependent neural plasticity and remodelling (reviewed by Scherer and Salzer, 1996). Modulation of F-spondin binding to the ECM by plasmin supports the possible involvement of this protein in activity-dependent processes (Tzarfaty et al., 2001). In addition, it has been suggested that during the activity-dependent synaptic plasticity in hippocampus F-spondin acts as a target for the serine protease tPA (Tzarfaty et al., 2001). Further studies are warranted to explore the involvement of other TSR domain proteins in the regulation of hippocampal LTP.

Receptor molecules for HB-GAM

Currently there are three transmembrane proteins identified as the receptor molecules for the HB-GAM: syndecan-3, receptor-like protein tyrosine phosphatase ζ/β (RPTPβ/ζ) and the orphan receptor tyrosine kinase anaplastic lymphoma kinase (ALK). Core proteins of syndecan-3 and RPTPβ/ζ carry GAG side chains which are necessary for the HB-GAM binding.

Syndecan family of HSPGs and their role in the nervous system. Syndecan-3

Expression patterns of syndecans in the nervous system

Syndecans and glycosylphosphoinositide (GPI)-linked proteins glypicans represent two families of cell-surface HSPGs. These two membrane-associated protein classes are the major carriers of heparin sulfates at the cell surface.

Syndecans are type I membrane-spanning proteins present on the cell surface of most cell types. They regulate a variety of biological processes including cell-extracellular matrix interactions, cell adhesion and motility (reviewed by Bandtlow and Zimmermann, 2000; Woods, 2001). There are 4 mammalian syndecans that are the products of different genes: syndecan-1, syndecan-2 (fibroglycan), syndecan-3 (N-syndecan) and syndecan-4 (ryodocan or amphiglycan) (fig. 5). Intracellular and transmembrane domains are highly conserved in all four syndecans. However, ectodomains of syndecans are structurally distinct. The extracellular side of the core proteins of different syndecans carry various numbers of GAG chains. Most of them are HS GAGs, but some are chondroitin/dermatan sulfate chains.

The expression of syndecans is tightly regulated. They are induced during development, after injury and following various physiological stimuli (Bernfield et al., 1999; Hsueh and Sheng, 1999; Lauri et al., 1999). In addition, different syndecans are expressed in a cell-specific manner. Each syndecan has a different distribution in the brain. The expression of syndecan-1 in the adult brain is restricted mainly to the cerebellum, while syndecan-2 and syndecan-3 are expressed in many brain regions including cerebellum, hippocampus, dentate gyrus, cerebral cortex, and thalamus. In contrast to syndecan-2 and -3, which are expressed predominantly by neurons, syndecan-4 is produced specifically in the glial cells and displays a diffuse distribution throughout the brain (Ethell and Yamaguchi, 1999). Syndecan-3 has also been demonstrated to be expressed by oligodendrocyte progenitors but not by terminally differentiated oligodendrocytes or by astrocytes (Winkler et al., 2002).

In all brain regions syndecan-2 is predominantly localized at the synaptic structures. Its spatial and temporal expression pattern matches the one of synaptic marker synaptophysin. Immunoelectron microscopy studies revealed both pre- and postsynaptic localization of syndecan-2. Though

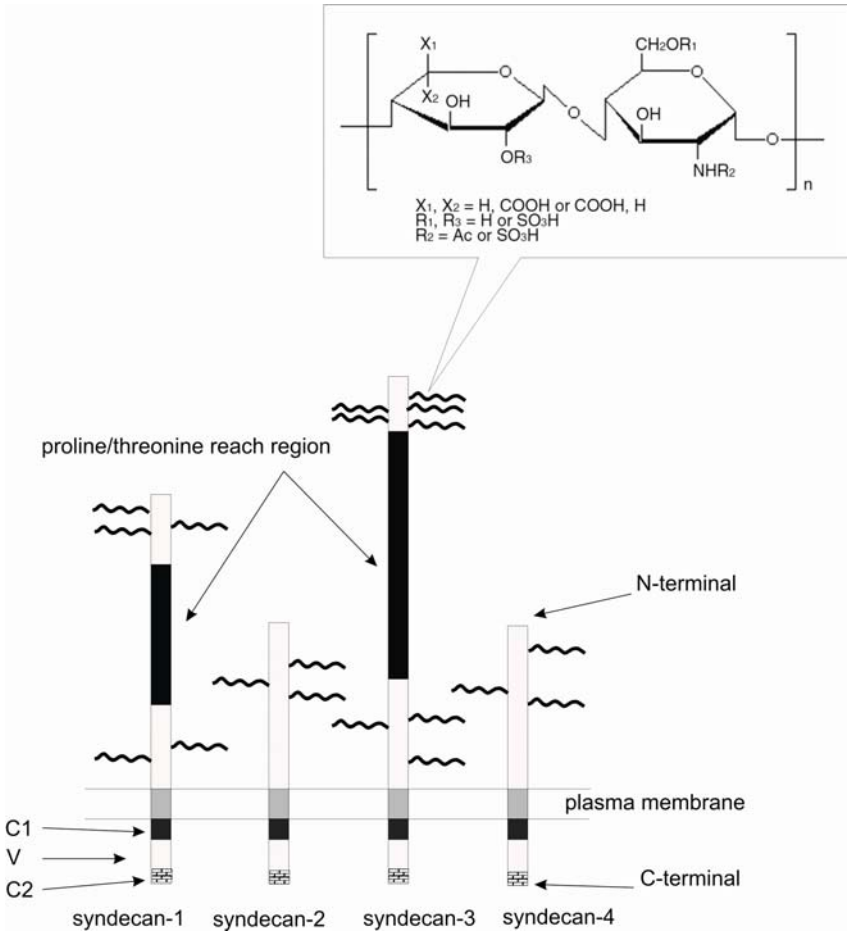

Figure 5. Syndecan family of transmembrane proteoglycans in mammalian tissues. Schematic representation of core proteins with GAG side chains (wavy lines). Inset shows the structure of HS GAG. Conserved cytoplasmic domains C1 and C2 as well as variable region (V) are marked at the intracellular side.

the staining was mostly associated with the synaptic membranes, some signal was also detected outside of the synapses. In synapses syndecan-2 binds to the postsynaptic density-95/disc large/zona occludens (PDZ) domain of calcium, calmodulin-associated serine/threonine kinase (CASK) (rat homolog of abnormal cell LINeage-2 [LIN-2]) via its COOH terminus (Hsueh et al., 1998).

Unlike syndecan-2, syndecan-3 staining in the brain is mainly associated with fiber tracts and axon pathways suggesting that this HSPG is concentrated in the axons (Hsueh and Sheng, 1999; Kinnunen et al., 1998a). However, immunoelectron microscopy also revealed perisynaptic localisation of syndecan-3 in the area CA1 of hippocampus following tetanic stimulation of Schaffer collaterals (Lauri et al., 1999). The expression level of syndecan-3 is more pronounced during the early stage of postnatal development than in the adult brain (Carey et al., 1997; Nolo et al., 1995). Syndecan-3 expression starts at the late stages of embryonic development and increases during early postnatal period reaching the maximum around postnatal day 7, after which it declines (Carey, 1997). Low levels of expression are maintained in some areas of the nervous system in adults. In addition, syndecan-3 expression is enhanced in hippocampus-derived neural stem cells following differentiation induced by retinoic acid (Inatani et al., 2001). Syndecan-3 expression has been recently demonstrated to be associated with the migrating neurons in developing nervous system, particularly in the migratory stream from the rat olfactory placode (Toba et al., 2002).

Structure of syndecan-3

Syndecan-3 is a 120 kDa HSPG originally found in rat Schwann cell membranes (Carey et al., 1992). The core protein of syndecan-3 consists of 442

amino acids and six structural domains. The extracellular part of syndecan-3 core protein contains an N-terminal signal peptide, two regions for GAG attachment separated by mucin homology domain enriched in proline and threonine. The GAG attachment domains consist of ser-gly dipeptides preceded and followed by acidic residues. The GAG attachment site near the N-terminus in all syndecans bears HS chains, whereas the other one, near the plasma membrane, in case of syndecan-1 and -4 may also carry chondroitin sulfate chains. The membrane proximal region of the cytoplasmic domain (C1) is similar in all four members of the syndecan family and displays a close sequence homology to some other transmembrane proteins containing PDZ-binding sites (e.g. neurexin I/III and glycophorin C) (fig. 5). The tetrapeptide EFYA (glutamic acid-phenylalanine-tyrosine-alanine), C2, at the end of the C-terminus is also conserved in all syndecans, suggesting that certain common mechanisms of protein-protein interactions are important for syndecan functions. Between the conserved C1 and C2 parts of the intracellular domain syndecans have a variable part (V). This region is conserved between species, but differs in syndecans 1-4. The transmembrane domain and the ectodomains play a role in oligomerization of syndecans (Asundi and Carey, 1995). Oligomerization of syndecans enhance their interaction and lateral association with other cell surface molecules (e.g. integrins, thus modulating cell adhesion) (Couchman and Woods, 1999).

In addition to the membrane-anchored form of syndecans they may be present in the ECM as the released molecules shed from the plasma membrane (Kim et al., 1994). Soluble fragments of syndecan-3 have been suggested to contribute to the structure of ECM (Akita et al., 2001). The ectodomains shed from the plasma membrane retain GAG chains and the ability to bind extracellular ligands. The binding activity of the shed syndecans is indistinguishable from that of the membrane-associated forms. Soluble syndecans are important in the storage and appropriate representation of the heparin-binding growth factors (e.g. FGF-2). Syndecans may also increase effective concentration of the growth factors at the plasma membrane and modulate their binding to the membrane receptors. Shedding of the syndecan extracellular domains is tightly regulated and requires the activity of the MMPs (Asundi et al., 2003; Fitzgerald et al., 2000). Certain other proteases and growth factors can also modulate shedding of syndecan ectodomains (Subramanian et al., 1997).

Extracellular ligands for syndecan-3

Syndecans bind to a number of extracellular adhesive molecules and growth factors, but the binding ability varies between the family members. In contrast to other syndecans, syndecan-3 does not bind to most insoluble ECM components (e.g. fibronectin, laminin, collagens) (Woods et al., 2000; Salmivirta et al., 1994; Suzuki et al., 2003), but it does bind to the FGF and heparin-binding growth/differentiation factors HB-GAM and MK (Chernousov and Carey, 1993; Nakanishi et al., 1997; Raulo et al., 1994).

In the developing central nervous system syndecan-3 is colocalised with HB-GAM on the cell membrane of growing axons (Kinnunen et al., 1998a). Syndecan-3 mediates HB-GAM-induced neurite outgrowth acting as the receptor molecule for HB-GAM (Kinnunen et al., 1996; Kinnunen et al., 1998b; Raulo et al., 1994).

Syndecans may also function as co-receptors for extracellular growth factors. For example, binding to syndecans and other HSPGs can significantly modify the ability of FGF to interact with its transmembrane tyrosine kinase receptor (FGFR) (Ornitz, 2000; Schlessinger et al., 2000). Biological activity of FGF is dependent on its binding to HSPGs. In particular, it is important whether it is bound to the membrane-anchored of released (shed) form of syndecan (Carey, 1997).

Possible interaction of syndecan-3 and NCAM has been recently suggested; however no direct evidence exists supporting this idea (Toba et al., 2002). In the peripheral nervous system syndecan-3 binding to the particular collagen type V protein mediates Schwann cell adhesion to the ECM and activates the Erk1/Erk2 protein kinases (Chernousov et al., 1996; Erdman et al., 2002).

Intracellular signalling mediated by syndecan-3

Syndecans are important for transduction of extracellular signals into the cells. Through their cytoplasmic domains syndecans are involved in regulation of cytoskeleton organization and thus regulate cell shape and motility (reviewed elsewhere Yoneda and Couchman, 2003). The C-terminal EFYA sequence highly conserved in all syndecans interacts with several PDZ domain-containing proteins: syntenin, CASK/LIN and synectin (Gao et al., 2000; Grootjans et al., 1997; Hsueh et al., 1998). In addition, one more binding partner, synbindin, has a region with limited homology to the PDZ domain (Ethell et al., 2000). The C1 domain in syndecan-3 interacts with c-src, c-fyn, cortactin and tubulin (Kinnunen et al., 1998a).

Since the C1 and C2 domains are highly homologous in syndecans it is likely that the same C1- and C2-dependent interactions are characteristic for all syndecans. Indeed, for example syntenin and CASK bind to all four syndecans. However, while syntenin interacts equally well with all syndecans, CASK binds more easily to syndecan-2 and -4 than to syndecan-1 and -3 (Grootjans et al., 2000). It is possible that the V domain can modulate the interactions of the C1 and C2 domains with intracellular partners of syndecans. Thus some signalling pathways may be unique for individual syndecans, e.g., little is known regarding the involvement of src-mediated molecular cascade in the signal transduction mechanisms employed by syndecans other than syndecan-3.

RPTPβ/ζ

RPTPβ/ζ is a receptor-like protein tyrosine phosphatase expressed specifically in the nervous system (Levy et al., 1993). Three isoforms of RPTPβ/ζ have been identified as the products of alternative RNA splicing: the short and long receptor forms, and the secreted form (also known as phosphacan) (fig. 6). The extracellular region of all three isoforms contains a carbonic anhydrase domain, fibronectin type III domain and a large cysteine-rich spacer domain. Phosphacan consists of the entire extracellular part of the molecule. The receptor forms of RPTPβ/ζ have also a single membrane-spanning domain and two cytoplasmic phosphatase domains. In addition, the secreted form and the long receptor form carry chondroitin sulfate side chains attached to the 860-residue insert in the cystein-rich region. Thus the long receptor form and phosphacan are chondroitin sulfate proteoglycans. RPTPβ/ζ bears structural and functional similarity to the cell adhesion molecules (reviewed by Peles et al., 1998). All three forms of RPTPβ/ζ are found in the developing and adult nervous system. RPTPβ/ζ is mainly expressed on the surface of the radial glia and astrocytes. However, its mRNA can also be detected in some neuronal populations (e.g. in hippocampal pyramidal cell, dentate granule cells, cerebellar Purkinje cells, neurons in striatum, neocortex, etc.) (Snyder et al., 1996). The receptor forms of RPTPβ/ζ and phosphacan display a different developmental profile of expression. The amount of the secreted form of RPTPβ/ζ increases progressively during development while the expression of the transmembrane forms does not change very much (Sakurai et al., 1996).

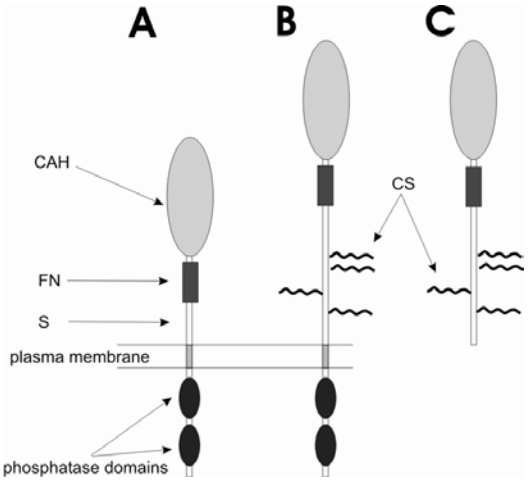

Figure 6. Schematic representation of the structure of three RPTPβ/ζ isoforms: short receptor form(A), long receptor form (B), and phosphacan (C). CAH, carbonic anhydrase domain; FN, fibronectin type III domain; S, cysteine-rich spacer domain (S); CS, chondroitin sulfate chains.

RPTPβ/ζ binds to a number of cell adhesion molecules and ECM components and thus is involved in a variety of functions mediated by the cell-cell and cell-matrix contacts. RPTPβ/ζ interacts with the cell surface proteins contactin (Peles et al., 1995), NCAM, neuron-glia cell adhesion molecule (Ng-CAM) (Milev et al., 1994), tenascins in the extracellular matrix (Barnea et al., 1994; Milev et al., 1998), and heparin-binding secreted proteins such as amphoterin (Milev et al., 1998), MK (Maeda et al., 1999) and HB-GAM (Maeda et al., 1996). During development RPTPβ/ζ is implicated in the neuronal migration, axon outgrowth and guidance by coupling extracellular cues to the signal transduction pathways in the cells (see Holland et al., 1998). The extracellular variant of RPTPβ/ζ, phosphacan, displays an overlapping localization pattern with HB-GAM during embryonic development and in postnatal period (Milev et al., 1998). High affinity binding of phosphacan to HB-GAM is mediated mainly by chondroitin sulfate chains and can be significantly diminished by chondroitinase treatment. Interestingly, binding to tenascin-R is dependent on the core protein of phosphacan but not its GAGs (Milev et al., 1998). Functional data that RPTPβ/ζ and its secreted form suppress HB-GAM-induced neuronal migration and neurite outgrowth of cortical neurons (Maeda et al., 1996; Maeda and Noda, 1998) also suggest that RPTPβ/ζ and HB-GAM comprise a ligand-receptor pair. Similarly to HB-GAM, the expression of RPTPβ/ζ is enhanced following CNS injury in the areas of axonal sprouting as well as in the regions of glial scarring (Snyder et al., 1996).

Anaplastic lymphoma kinase

ALK is a member of the insulin receptor subfamily of receptor tyrosine kinases with closest homology to leukocyte tyrosine kinase (LTK). It is a transmembrane protein (fig. 7) with the molecular mass of 200-220 kDa, and it has recently been shown to specifically bind HB-GAM in intact cells and in cell-free binding assays (Stoica et al., 2001). ALK was originally discovered in a chromosomal rearrangement associated with anaplastic large cells lymphomas (Morris et al., 1994). The receptor tyrosine kinase ALK is specifically expressed in both central and peripheral nervous system. *In situ* hybridization studies revealed that ALK expression begins late in embryonic development (not detected until embryonic day 11 in mice) and peaks during the neonatal period. Similarly to HB-GAM, ALK is downregulated in older animals. The level of expression reaches its minimum around three weeks after birth and persists in adults only in some cell populations (e.g. in thalamus, hypothalamic area, cerebellum, cerebral cortex). HB-GAM signalling through ALK has been demonstrated to be important in the growth of glioblastoma multiforme (Powers et al., 2002). HB-GAM interaction with the extracellular domain of ALK induces ALK phosphorylation and subsecuent intracellular signal transduction via the adaptor Src homology 2 domain-containing protein (Shc), insulin receptor substrate-1 (IRS-1), extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3 (PI-3) kinase (Stoica et al., 2001). In addition, HB-GAM leads to phosphorylation of phospholipase C gamma (PLC-γ), which is typically activated by BDNF, basic FGF (bFGF) and other growth factors (Schlessinger, 2000). However, no expression of ALK was found in the hippocampus and dentate gyrus (Iwahara et al., 1997; Morris et al., 1997). Thus, though HB-GAM/ALK interactions may be important for some of the HB-GAM biological effects it is unlikely that they play a role in the regulation of synaptic plasticity or hippocampus-dependent learning.

Figure 7. Schematic representation of anaplastic lymphoma kinase. LBS, ligand-binding sequence; MAM, meprin/A5/PTPmu domain; TM, transmembrane domain; TK, tyrosine kinase domain.

Summary

Previous studies demonstrated that in addition to its role in the regulation of neurite outgrowth and axonal guidance during development ECM-associated molecule HB-GAM is involved in the regulation of hippocampal LTP. HB-GAM binds with high affinity to syndecan-3. This cell-surface HSPG displays similar pattern of expression to that of HB-GAM during development and in response to LTP-inducing HFS. It also blocks LTP, which makes it a good candidate for the role of functional receptor mediating effects of HB-GAM on synaptic plasticity. The involvement of HB-GAM and syndecan-3 in the regulation of hippocampal LTP also suggests that these molecules may be implicated in the hippocampus-dependent learning and memory. However, the data on the role of endogenous HB-GAM as well as syndecan-3 in hippocampal functions and regulation of learning behavior is missing.

HB-GAM belongs to the diverse family of TSR domain containing proteins sharing a common property of binding to heparin-type glycans. TSR proteins are ECM-associated and cell-surface molecules mediating cell-cell and cell-matrix interactions and involved in the various biological functions. It is though unknown whether individual TSR domains may act as independent signalling units or higher order organization is required for their effects in the nervous system.

Aims of the study

Mutant mice were used to study the *in vivo* function of HB-GAM and its receptor syndecan-3 in hippocampal synaptic plasticity and hippocampus-related forms of behavior. The specific aims of this work were as follows:

1. To study whether genetic manipulations leading to either lack or overexpression of HB-GAM in mutant mice affect synaptic plasticity and/or learning and memory **(I)**.

2. To determine whether the effects of HB-GAM on hippocampal functions are mediated by syndecan-3 using mice lacking this heparan sulfate proteoglycan **(II)**.

3. To study cellular mechanisms underlying altered synaptic plasticity in the HB-GAM mutant mice **(III, unpublished data).**

4. To characterise structure/function relations of the HB-GAM protein **(IV)**.

Materials and methods

Here only a brief outline of the materials and methods used in the current study is given. More detailed descriptions of the experimental procedures are provided in the corresponding sections of the original publications (see Table 3 for the references).

Genetically modified mice were studied in the present work to characterise the role of HB-GAM and syndecan-3 in the synaptic plasticity and learning and memory. Three different mutants were generated: HB-GAM knockouts, HB-GAM overexpressing mice and syndecan-3 deficient mice. Previously described (Amet et al., 2001) chimeric male HB-GAM knockouts on the C57BL/6J × 129 hybrid background were mated to 129S2/SvHsd females in order to generate inbred strain of HB-GAM deficient mice. Transgenic mice overexpressing HB-GAM used in the studies were hemizygous animals on inbred FVB/NHsd or in F1 FVB/NHsd × 129S2/SvHsd hybrid backgrounds. Syndecan-3 deficient animals were 129SV × C57BL/6J hybrids.

In vitro electrophysiological experiments were done using transverse hippocampal slices. Recordings were made from the CA1 area of hippocampus. Field excitatory postsynaptic potentials (fEPSPs) from *stratum radiatum* were elicited by stimulation of Schaffer collaterals. The relationships between the presynaptic fiber volley amplitude and the slope of fEPSP at different stimulation intensities were used to evaluate basal synaptic transmission. Possible changes in short-term plasticity and presynaptic functions were checked by measuring paired-pulse facilitation ratio. High-frequency and low-frequency stimulation trains were applied to elicit long-term changes in the efficacy of synaptic transmission. Recordings from *stratum pyramidale* were conducted to measure population spike responses. Visualized whole-cell patch-clamp recordings from CA1 pyramidal cells were made using infrared microscopy. Recordings were made in voltage-clamp mode. To study the basic properties of $GABA_A$ receptor-mediated transmission GABAergic currents were pharmacologically isolated and miniature and spontaneous inhibitory postsynaptic currents (IPSC) were recorded. Paired-pulse depression of evoked IPSCs was used to assess functional properties of GABAergic neurotransmission.

Behavioral testing included evaluation of basic neurological functions, sensory and motor abilities (postural, righting and visual placing reflexes, pain sensitivity, rotarod, open field test), and tests assessing anxiety-like behavior and learning and memory. Morris water maze test was used to assess hippocampus-dependent spatial learning. Elevated plus maze test and light-dark exploration tests were conducted to measure general level on anxiety. In addition, contextual fear conditioning and cue learning tests were carried out to evaluate associative learning.

Histological methods included hematoxylin/eosin for general evaluation of gross morphology and estimation of cell densities; and Bielschowsky silver impregnation to visualize axonal projections. Golgi staining was used to visualize dendritic spines on the hippocampal pyramidal cells. Immunofluorescence of synaptophysin staining was measured using confocal microscopy to evaluate the density of presynaptic butons. Cell cultures were used for neurite outgrowth assays, transfilter migration assays and to study distribution of AMPA and NMDA glutamate receptors and syndecan-3 in hippocampal neurons by means of immunostaining.

Table 3.

Method	Publication
In vitro electrophysiology using hippocampal slices from rats and transgenic mice	
• Field potential recordings	I-IV
• Whole-cell patch-clamp recordings	III
Production of genetically modified mice	I, II
Behavioral testing of transgenic mice	I, II
Morphological analysis	
• Histological methods	I, II
• Immunofluorescence and confocal microscopy	II
In vitro cell cultures	II, IV

Results

Morphology of the HB-GAM and syndecan-3 mutant mice (I,II)

Three different mutant mice lines were used in electrophysiological and behavioral studies: HB-GAM overexpressing transgenic mice, HB-GAM knockouts (Amet et al., 2001) and syndecan-3 deficient mice. In the HB-GAM transgenic mice the coding region of HB-GAM was under the control of the human PDGF β-chain promoter, which produces preferential expression in neurons (Sasahara et al., 1991). Transgene-positive mice showed about two-fold overexpression of the HB-GAM protein in the hippocampus compared to the endogenously occurring HB-GAM. The mutant mice lacking HB-GAM and syndecan-3, as well as the mice overexpressing HB-GAM, were all born in expected Mendelian ratios, displayed normal life span, and were apparently healthy and fertile. None of the genetically manipulated mice lines have obvious anatomical or histological brain abnormalities.

Detailed morphological analysis of hippocampus and motor cortex of the HB-GAM transgenic mice using hematoxylin-eosin and Bielschowsky-silver impregnation method did not reveal any changes in the layer structure, cell density and major axonal projections in the mutant mice as compared to the wild-type controls. Similarly, the syndecan-3 knockout mice did not display any gross morphological changes in brain histology (I: fig. 1c; II: fig. 2a). Hippocampal neurons cultured *in vitro* from the syndecan-3 knockouts appeared normal in morphology (II: fig.1d).

Syndecans are involved in regulation of cell shape and motility during development through their interactions with cytoskeleton. In particular, syndecan-2 has been shown to induce maturation of dendritic spines in hippocampal neurons through signalling mechanism of EphB, the member of Eph receptor tyrosine kinase family (Ethell and Yamaguchi, 1999; Ethell et al., 2001). Syndecan-3 is also phosphorylated by EphB1 *in vitro* (Asundi and Carey, 1997). Thus, we used Golgi impregnation and DiI staining to visualize dendritic spines in the hippocampal neurons in order to see whether their morphology is affected by syndecan-3 deletion. No differences in overall appearance of the pyramidal neurons were detected between the syndecan-3 knockout mice and the wild-type controls. The shape of the dendritic spines, their length and density were similar in both genotypes (II: fig. 2b, c). To estimate synaptic density in the area CA1 of hippocampus we also used immunostaining with antibodies against the presynaptic marker synaptophysin. No differences between the syndecan-3 knockout mice and their wild-type controls were found in the level of synaptophysin immunofluorescence (II: fig 2b; fig. 8).

Synaptic distribution of AMPA and NMDA receptors in cultured hippocampal neurons as well as the AMPA/NMDA ratio, assessed by immunostaining, was indistinguishable in the syndecan-3 deficient and the wild-type mice.

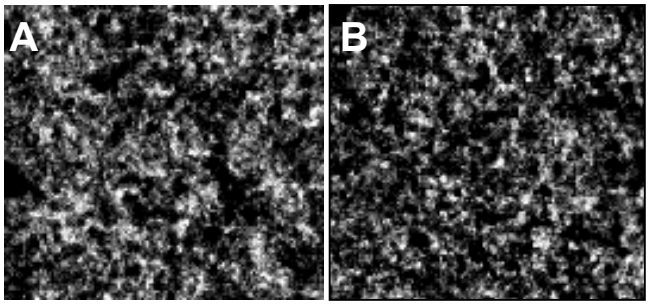

Figure 8. Synaptophysin immunofluorescence in the area CA1 of hippocampus is indistinguishable in wild-type (A) and syndecan-3 deficient (B) mice.

Synaptic plasticity in the HB-GAM knockout mice (I, unpublished)

Mice lacking HB-GAM were originally produced in the C57BL/6Jx129/Ola hybrid background (Amet et al., 2001). In that first report of the HB-GAM knockout mice the enhanced hippocampal plasticity in the mutants was only revealed when using sub-threshold protocol for LTP induction. Here in the follow-up study we used HB-GAM knockout mice after several back-crossings to 129S2/SvHsd strain in order to generate an inbred line and thus to reduce the possible variation of the phenotype caused by genetic background. The input-output curves of single-pulse evoked synaptic responses, which reflect the relationship between the presynaptic fiber volley amplitude and the fEPSP slope, were similar in the hippocampal slices from the knockout mice and wild-type controls (I: fig. 4a). Paired-pulse facilitation (PPF), a form of short-term synaptic plasticity, was also unaffected by the mutation in the interpulse interval range from 20 to 200 ms (I: fig. 4b).

LTP induced by high-frequency stimulation in the area CA1 of hippocampus, however, was substantially enhanced in the mice lacking endogenous HB-GAM compared to the control animals (fig. 9 a). We did not find any difference in synaptic responses evoked by high-frequency train stimulation between the knockout and wild-type mice. Slow NMDA receptor-mediated components of field recordings after tetanic stimulation were also indistinguishable in both experimental groups (I: fig. 4d, e). No differences between the genotypes were found in LTP induced by lower stimulation frequency trains (10Hz/1s) (fig. 9 b). PPF measured one hour after LTP induction was not affected either in the mutants or the wild-type mice (I: fig. 4f).

To check whether deficiency of HB-GAM in the mutant mice affects the properties of AMPA and NMDA receptor-mediated responses we performed whole-cell patch-clamp recordings from the pyramidal neurons of the CA1 area of hippocampus. The current-voltage relations of the pharmacologically isolated AMPA component of synaptic currents, obtained at the holding potentials between -80 and +20 mV, revealed normal responses in the mutant mice (Pavlov, Segerstråle, Rauvala and Taira, unpublished results; fig. 10 a). Both the wild-type and knockout mice exhibit similar I-V relationships of the NMDA receptor-mediated current (fig. 10 b). To estimate whether the AMPA and NMDA components are present in similar proportions in the mutant mice and the control animals, we plotted the current-voltage curves of the NMDA receptor-mediated component normalized to the AMPA component at -80 mV recorded from the same cells. Again no difference between the genotypes was detected, suggesting that the AMPA/NMDA ratio is not changed in the mutants (fig. 10 c).

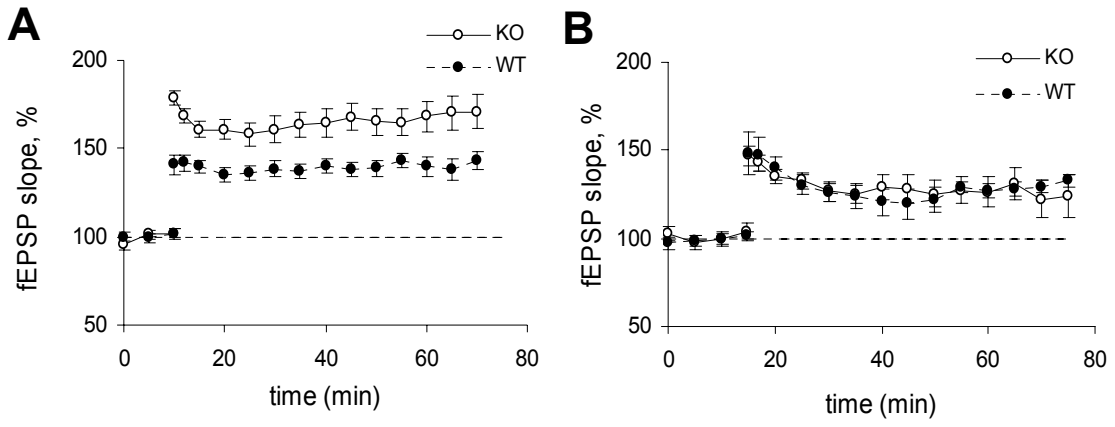

Figure 9. (A) LTP in the area CA1 of hippocampus induced by the 100 Hz high-frequency stimulation protocol is significantly higher in the HB-GAM knockout mice (n=6) that in the wild-type controls (n=6). (B) LTP induced by the 10 Hz stimulation protocol. The mutant mice (n=3) exhibit a similar level of potentiation as compared to the wild-type control animals (n=4). Data represent mean±SEM.

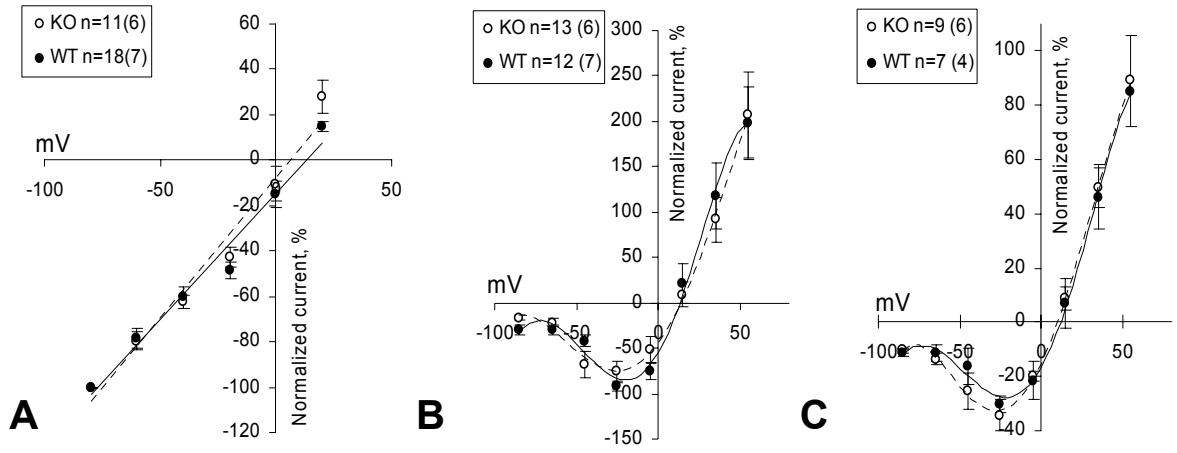

Figure 10. Similar current-voltage curves for isolated AMPA and NMDA EPSCs in the CA1 region of hippocampus of the HB-GAM knockouts and the wild-type mice (Pavlov, Segerstråle, Rauvala and Taira, unpublished results). Voltage dependence of the EPSC amplitude normalized to the maximal inward current for the AMPAR- (A) and NMDAR-mediated (B) responses. (C) Averaged amplitudes of NMDA receptor-mediated responses expressed as the percentage of the AMPA receptor-mediated current at -80 mV recorded from the same cells.

Behavioral phenotype of the HB-GAM knockout mice (I)

Mutant mice were examined in a number of behavioral tests and did not display any abnormalities in general health, neurological reflexes, motor functions or sensory abilities. Morris water maze test for spatial learning revealed that though the escape time decreased rapidly both in the wild-type and the knockout mice, the mice lacking HB-GAM show a slightly delayed escape time during the training period. The mutant mice also performed poorly in the first transfer test as compared to the control animals. However, in the second transfer test, after nine training blocks, both genotypes already had a similar preference to the trained quadrant and spent an equal time in the circle around the platform. The third transfer test was made after the mice had learned to find the platform moved to the opposite quadrant. In this task the knockout mice spent significantly less time in the previous target zone. In addition, the HB-GAM deficient mice spent a longer time in near the pool wall in the first and third transfer tests than the wild-types (I: fig. 5). Fear conditioning experiments revealed a lower context-dependent freezing in the knockout mice compared to the control group, while no changes were observed in the cued fear conditioning (I: fig. 6d). The HB-GAM knockout mice showed a higher anxiety-like behavior than control animals in the elevated plus maze test (I: fig. 6c).

Synaptic plasticity in the HB-GAM overexpressing mice (I)

Similarly to the HB-GAM knockout mice, animals with an enhanced level of HB-GAM expression did not have any changes in the basal properties of synaptic transmission in the area CA1 of hippocampus. Input-output curves and PPF ratios before and one hour after LTP induction were indistinguishable in the mice overexpressing HB-GAM and wild-type littermate controls. Nevertheless, in contrast to the mice lacking HB-GAM, the overexpressing mice displayed attenuated LTP induced by tetanic stimulation. Thus the effect of the enhanced expression of endogenous HB-GAM is in agreement with the previously reported suppressory action of recombinant HB-GAM on LTP induction (Lauri et al., 1998). Synaptic fatigue and the shape of consecutive responses during the HFS stimulation of Schaffer collaterals were similar in the HB-GAM transgenic and wild-type mice.

HB-GAM and GABAergic inhibition in hippocampus (III)

GABAergic inhibition plays an important role in the control of glutamatergic synaptic plasticity in the hippocampus. Blockade of GABAergic transmission is known to facilitate LTP while GABA agonists favour induction of LTD instead of LTP in a range of induction protocols (Steele and Mauk, 1999; Wigstrom and Gustafsson, 1983). Further experiments were designed to test the hypothesis that augmentation in GABAergic transmission underlies attenuated LTP in the HB-GAM transgenic mice. In support of that, the level of LTP induced after application of $GABA_A$ receptor blocker, picrotoxin, was similar in the HB-GAM overexpressing mice and the wild-type controls.

Field recordings from the CA1 *stratum pyramidale* demonstrated that picrotoxin wash-in was accompanied by a significantly increased facilitation of the population spike responses in the transgenic mice compared to the wild-type control animals, suggesting that a more powerful inhibitory control exists in the hippocampus of the mutants under normal conditions (III: fig. 2).

Whole-cell patch-clamp recordings from the pyramidal cells in the area CA1 of hippocampus were performed to investigate the basic properties of GABAergic transmission. While the kinetics and the mean amplitude of the spontaneous IPSCs (sIPSCs) were similar in both genotypes, the transgenic mice demonstrated an enhanced frequency of sIPSCs compared to wild-type littermate controls (III: fig. 3). These data provide further evidence for the enhanced GABAergic transmission in the hippocampus of the transgenic mice. GABA receptor-mediated synaptic currents are known to decrease in response to repetitive stimulation, thus functional inhibitory control diminishes during high-frequency stimulation (Davies et al., 1990). Paired-pulse depression of evoked IPSCs was studied in the range of interpulse intervals from 50 to 800 ms. In contrast to the wild-type mice, which displayed marked depression of the second IPSCs in the paired-pulse stimulation, the HB-GAM overexpressing mice demonstrated significantly reduced level of paired-pulse depression (III: fig. 5). The frequency of miniature IPSCs (mIPSCs), however, was similar in the HB-GAM overexpressing mice and in the control group. No effect of the mutation on the properties of single events was found.

Behavioral analysis of the mice overexpressing HB-GAM (I)

The expression of the HB-GAM transgene did not lead to any sensory or motor disabilities in the mutant mice. Though the escape latencies did not differ between the wild-type and the transgenic mice during the training period in the water maze task, significant differences were found between the genotypes in the first and the second transfer tests (I: fig. 3a, b, c). In both transfer tests the transgenic mice spent more time swimming in the platform quadrant than the wild-type mice. Subsequent training to learn the position of the platform moved to the opposite quadrant of the water maze and the following, third, transfer test showed similar results for the transgenic and control animals (I: fig. 3 d). Wild-type mice expressed more thigmotaxis during the first two transfer tests. However, the difference reached the level of significance only in the second transfer test (I: fig. 3 e). In the fear conditioning test, the mice overexpressing HB-GAM displayed less freezing to the CS tone than control mice, but the context dependent freezing was not different (I: fig. 6 b). In the elevated plus maze test the transgenic mice displayed reduced anxiety-like behavior. They made more entries into the open arms and stayed there longer than the wild-type animals. The number of closed arm entries was indistinguishable between the genotypes (I: fig. 6 a).

Synaptic plasticity in the syndecan-3 deficient mice (II)

The electrophysiological phenotype of the mice lacking syndecan-3 very much resembled that of the HB-GAM knockout mice. Deletion of the syndecan-3 gene had no effect on the baseline synaptic transmission or PPF (II: fig. 3). LTP in the area CA1 of hippocampus was strongly enhanced in the mice lacking syndecan-3 (II: fig. 4 a). A similar increase of LTP level was demonstrated for homo- and heterozygous mutants. Saturation of the LTP was reached following the 3rd train stimulus both in the wild-type and syndecan-3 deficient mice. However, the level of maximal potentiation was higher in the knockouts (II: fig. 4 c). No differences between the genotypes were revealed in response to the low-frequency stimulation (II: fig. 4 d). Since syndecan-3 is important for mediating neurite outgrowth effects of HB-GAM during development (Kinnunen et al., 1996) we tested whether it is also involved in modulation of LTP by HB-GAM. Indeed, whereas pressure injection of HB-GAM into the CA1 dendritic area attenuated LTP in the wild-type mice, it had no effect on the level of potentiation in the mice lacking syndecan-3 (II: fig. 5).

Behavioral analysis of the mice lacking syndecan-3 (II)

Like the HB-GAM mutant mice, the syndecan-3 knockouts were indistinguishable from the wild-type control animals in the tests for the basic neurological reflexes, sensory and motor functions. During the training period in the Morris water maze the escape latency was slightly higher in the knockout mice compared to the control group, though the effect was significant only in one training block (II: fig. 6 a). However, in contrast to the wild-type mice, the knockout animals did not show any spatial preference to the platform quadrant in the first transfer test. A better performance in the wild-type group was also retained in the second transfer test, when the mice deficient of syndecan-3 spent significantly more time swimming in the opposite quadrant (II: fig. 6 b-e). No differences were revealed in thigmotaxis between genotypes. Fear conditioning experiments also revealed spatial learning deficits in the syndecan-3 knockout mice. The mutants displayed reduced freezing in the context discrimination task as compared to the wild type mice (II: fig. 7 a). In

addition, no genotype-dependent changes were detected in the taste aversion test (II: fig. 7 c). Anxiety-like behavior of the syndecan-3 knockout mice in the elevated plus-maze and light-dark exploration tests was similar to that of wild-type mice.

Structure/function dissection of HB-GAM (IV, unpublished data)

Binding studies using plasmon resonance indicated that the lysine-rich tails had no effect on heparin binding properties of HB-GAM since the intact protein displayed the same affinity values as the di-TSR domain of HB-GAM. However, the individual N- and C-terminal domains of HB-GAM bound heparin considerably weaker than the di-TSR domain. Thus, though each TSR domain can interact with heparin, both domains are clearly required for high affinity binding. We next tested whether the binding properties of the purified TSR domains of HB-GAM correlate with their functional activity. Injection of the di-TSR fragment effectively inhibited LTP in the area CA1 of hippocampus, while the single N- and C-terminal domains displayed milder effects and did not abolish LTP (IV: fig. 7). Intriguingly, despite striking structural similarity with HB-GAM and similar heparin binding affinity (Kilpelainen et al., 2000; Tumova, personal communication) midkine (MK) application did not abolish hippocampal LTP induced by high-frequency stimulation, nor did the di-TSR domain of MK (fig. 11). Neurite outgrowth assays demonstrated that native HB-GAM as well as its di-TSR domain induced neurites from the primary cultured hippocampal neurons in matrix bound form. Both N- and C-terminal single TSR domains failed to induce neurites at coating concentrations tested. In addition, single TSR domains failed to inhibit neurite outgrowth induced by intact coated HB-GAM when applied to the assay medium, while the di-domain polypeptide inhibited neurite outgrowth at values close to the value of intact protein (IV: fig. 6). Consistently with heparin-binding experiments and neurite outgrowth assays the di-domain polypeptide of HB-GAM produced similar but slightly weaker inhibitory effect on neuronal migration as compared to native HB-GAM.

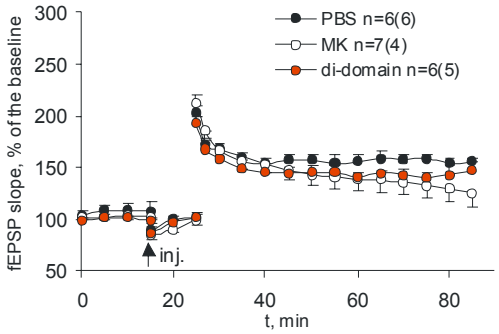

Figure 11. Effect of MK and its di-TSR domain polypeptide on LTP in the area CA1 of hippocampus (Pavlov, Taira and Rauvala, unpublished results). Pressure injection of the polypeptides into the *stratum radiatum* was made 10 minutes before HFS stimulation. Time of injection is marked by the arrow. Data represent mean±SEM. N numbers refer to the number of slices; the numbers of animals are given in parentheses.

Discussion and Conclusions

HB-GAM as a negative regulator of synaptic plasticity in hippocampus

The role of HB-GAM in hippocampal LTP and spatial learning and memory was examined by using two groups of mice in which the expression of the molecule is either enhanced or abolished. Opposite genetic manipulations in our study led to opposite electrophysiological and behavioral consequences: overexpression of HB-GAM resulted in attenuated LTP and better learning performance while loss of expression led to enhanced LTP and impaired learning and memory. Finding of opposite changes in LTP and spatial learning in the HB-GAM knockout and overexpressing mice speaks for a gene-specific effect rather than a general background gene effect. Due to the technical reasons transgenic and knockout mice were produced in different genetic backgrounds. This precluded the direct comparison of the mutant mice in our study. Instead, both mutant groups had their own controls, respective wild-type littermates, which allowed us to evaluate the direction of the changes in plasticity or behavior caused by the mutation.

HB-GAM and LTP

The first report implicating HB-GAM in the regulation of synaptic plasticity in the hippocampus demonstrated that its expression is enhanced following tetanic stimulation (Lauri et al., 1996). Application of recombinant HB-GAM into hippocampal slices was later shown to inhibit HFS-induced LTP in the area CA1 without affecting single-pulse evoked synaptic responses (Lauri et al., 1998). However, it was impossible to exclude the possibility that the suppressor effect on LTP was due to the inhibition of the endogenous HB-GAM by the injected protein (see also discussion in I). The use of mutant mice demonstrated that the HB-GAM transgenic mice that display a modest overexpression of the transgene in the brain have a clearly attenuated LTP whereas disruption of the HB-GAM gene enhances LTP in the area CA1 of hippocampus. Thus our data strongly support the idea that HB-GAM acts as an inducible inhibitor of synaptic plasticity in the hippocampus. The importance of negative regulatory mechanisms of long-term plasticity and memory storage is discussed elsewhere (Abel et al., 1998). Such mechanisms might be of particular importance in the course of maturation when LTP-like processes occur during synaptic development. The fact that LTP of the AMPA component of fEPSP induced in young animals tend to decay at the rate depending on the test stimulation evidences in favour of an activity-dependent mechanism compensating overall changes in synaptic efficacy during development (Xiao et al., 1996). In this respect an important question about the role of HB-GAM in the neonatal plasticity still remains open.

An important question in relation to the LTP phenotype of the mutant mice is whether HB-GAM suppresses NMDA receptor function (e.g. through syndecan-3/tyrosine kinase signalling), and deletion of HB-GAM facilitates NMDA receptor activation. It is also possible that HB-GAM directly affects AMPA receptor-mediated transmission or shifts the AMPA/NMDA current ratio. However, we did not find any supporting evidence for these suggestions. The slow NMDA receptor-mediated component of HFS trains in field recordings (c.f. Davies et al., 1991) was not affected either by HB-GAM deletion (see also Amet et al., 2001) or by HB-GAM overexpression. Further, distribution of AMPA and NMDA receptors in cultured hippocampal neurons from the syndecan-3 knockout mice was also unaffected by the mutation. Finally, there were no changes in either NMDA or AMPA receptor-mediated neurotransmission in the HB-GAM knockout mice compared to the wild-type control animals. Thus, our data together with previous results, demonstrating that local application of recombinant HB-GAM in the CA1 area of rat hippocampal slices does not alter pharmacologically isolated AMPA and NMDA receptor-mediated responses (Lauri et al., 1998), suggest that HB-GAM is not directly involved in the regulation of these glutamate receptors.

Interestingly, along with a rather slow effect of HB-GAM on LTP expression as demonstrated by the injection studies [(Lauri et al., 1998) II, IV], there may exist an additional mechanism involved in the immediate effect of the protein on the LTP induction, which would explain the difference in the post-tetanic potentiation in all mutants described here. It is possible that in addition to syndecan-3 (see discussion below) HB-GAM also interacts with other syndecans expressed in the nervous system in a developmentally regulated and region specific manner (e.g. Mitsiadis et al., 1995). Members of the syndecan family seem to substitute each other to some extend. For example, syndecan-1 overexpression in hypothalamus mimics a physiological effect of syndecan-3 on feeding behavior (Reizes et al., 2001). Another HSPG, syndecan-2, is concentrated in the synaptic junctions at the pre- and postsynaptic sites (Hsueh et al., 1998). Its expression pattern increases in

parallel with the synaptophysin suggesting its role in synaptogenesis, in particular at the late stages of synaptic development (Hsueh and Sheng, 1999). Indeed, syndecan-2 was demonstrated to play a critical role in spine development being phosphorylated by the EphB receptor tyrosine kinase (Ethell and Yamaguchi, 1999; Ethell et al., 2001). Further studies are warranted to envestigate whether there are any changes in the expression of other syndecans in the syndecan-3 defficient mice.

Another receptor that could mediate HB-GAM signalling is RPTPβ/ζ (Maeda and Noda, 1996). The interaction between HB-GAM and RPTPβ/ζ is important for HB-GAM-induced neuronal migration (Maeda and Noda, 1998) and morphogenesis of cell dendrites (Tanaka et al., 2003). RPTPβ/ζ is expressed by subsets of neurons and astrocytes in some brain areas including hippocampus (Shintani et al., 1998). It is worth to note that RPTPβ/ζ associates with sodium channels and affects sodium currents (Ratcliffe et al., 2000). Remarkably, the electrophysiological phenotype of the mice deficient in PTPδ, which belongs to the same receptor family as RPTPβ/ζ, resembles the phenotype of the HB-GAM and syndecan-3 knockouts in that these mutants display enhanced hippocampal LTP (Uetani et al., 2000). The RPTPζ knockout mice have been generated recently and were shown to have age-dependent enhancement of hippocampal LTP and deficient learning in Morris water maze. The effects of mutation are believed to be due to the changes in Rho-associated kinase pathway (Niisato et al., 2005). Modulation of phosphatase activity by HB-GAM may thus regulate excitability of the neurons and contribute to the expression on plasticity. Interaction of HB-GAM with RPTPβ/ζ has been suggested to inhibit its catalytic activity, which may lead to an increased level of phosphorylation of β-catenin (Meng et al., 2000). Depolarization upregulates association of β-catenin with E-cadherin and causes β-catenin redistribution into the spines (Murase et al., 2002). On the contrary, HB-GAM through β-catenin phosphorylation may disrupt β-catenin/E-cadherin association and weaken cell-cell adhesion, which in turn would affect activity-induced morphological changes and attenuate LTP.

TSR family proteins in brain development and plasticity

Apparently the native protein structure is required for the biological effects of HB-GAM. Heparin binding properties of the single HB-GAM TSR domains are significantly diminished as compared to the intact molecule. In line with these results functional data from the electrophysiological experiments and neurite outgrowth assays indicated that single TSR domains fail to induce neurite outgrowth and show much less inhibitory effect on LTP induction. Presumably some co-operative process which involves both TSR-domains of the molecule is essential for HB-GAM activity. However, several other biological effects of HB-GAM seem to rely on the different structural determinants of its functions. For instance, angiogenesis activity of HB-GAM functions independently of its transforming activity. Both effects rely on the specific amino-acid sequences and do not require the intact molecule (Deuel et al., 2002; Zhang et al., 1999). Thus, only certain functions of HB-GAM are determined by the higher order organization of its domains.

TSR containing proteins are important for neuronal development participating in cell migration, neurite outgrowth and axon pathfinding (Adams and Tucker, 2000). They also play a role in synaptogenesis and contribute to synaptic plasticity. TSP1 and TSP2 released from astrocytes induce synapse formation and are necessary for aligning and maintaining adhesion between pre- and postsynaptic sites (Washbourne et al., 2004). Modulation of cell-cell and cell-extracellular matrix interaction is also important for synaptic plasticity and regulation of synapse morphology in the adult brain.

TSR domains of the proteins belonging to the TSR superfamily are known to mediate functional interactions with ECM components through binding to GAG chains of proteoglycans. Thus HSPGs and CSPGs can modulate the biological effect of these proteins through binding to their TSR domains. Such mechanism has been demonstrated for semaphorin Sema5A. TSR domains of Sema5A are critical for regulatory interactions with sulfated proteoglycans that determine whether the protein would exert attractive or inhibitory effect on the developing axons (Kantor et al., 2004). Interestingly, Sema5A not only affects neurite outgrowth in the developing CNS but also contributes to axon inhibition after injury in the adult suggesting that similar mechanisms remain functional throughout the life span (Goldberg et al., 2004).

Plasmin-mediated cleavage of the TSR domains from F-spondin, another member of the TSR superfamily, has been recently shown to be important for the modulation of F-spondin attachment to the ECM (Tzarfaty et al., 2001). It is tempting to speculate that a similar mechanism involving proteolytic cleavage may regulate the activity of HB-GAM. However, it remains to be determined whether individual TSR fragments of

the TSR domain proteins have the signalling functions of their own. Given such complexity of structure/function relationship, it is not very surprising that although MK is highly homologous to HB-GAM it produces much weaker effects on LTP.

Implications for behavior

Modifications in the hippocampal synaptic plasticity in the mutant mice reported here were accompanied by alterations in the hippocampus-dependent learning. Though, changes in the behavioral phenotype of the HB-GAM mutants, as well as of the syndecan-3 knockout mice, were relatively mild compared to the wild-type control animals. Abnormalities in spatial learning and memory in the mutants were not severe and could be revealed only as deficits in some particular tasks. For example, the Morris water maze test showed normal acquisition of the hidden platform task and normal swimming and thigmotaxis in the pool, but a deficit in the probe trial during the transfer test in the syndecan-3 null mice. Thus, only some components of the spatial navigation seem to be affected by the mutation. Obviously, many parallel learning processes take place in the Morris water maze task. However, the transfer test for spatial search is considered to be the most specific for the hippocampus-dependent spatial memory (Wolfer et. al., 1992).

It is also possible that behavioral phenotypes could be partially masked by the genetic background. Synaptic plasticity and learning vary in different mouse strains routinely used for genetic manipulations (Bampton et al., 1999; Nguyen et al., 2000b; Nguyen et al., 2000a; Voikar et al., 2001; Wolfer et al., 2002). Previous studies, which utilized hybrid genetic background, revealed enhanced plasticity in the HB-GAM knockout mice using a sub-threshold protocol for LTP induction (Amet et al., 2001). However, as demonstrated here back-crossing of HB-GAM knockouts into the 129S2/SvHsd strain to generate an inbred line did show enhanced LTP induced by the conventional 100Hz/1s high-frequency protocol. The differences in the genetic background in which the mutation is studied may account not only for the slight variations in electrophysiological (e.g. the level of LTP) and behavioral phenotype of the control animals, but also qualitatively alter the manifestation of the mutation. Accordingly, in this study it was hard to differentiate whether manipulations with the level of HB-GAM expression have specific effects on the two forms of fear conditioning, or whether this was due to an effect of the genetic background. HB-GAM deficient mice reported here displayed decreased performance in both spatial learning tasks: Morris water maze and context-dependent conditioning test. However, in the transgenic mice, where overexpression of HB-GAM enhanced spatial learning in the water maze, the mutation had no effect on context fear conditioning, but affected cued fear conditioning. Similar to the HB-GAM knockouts, syndecan-3 deficient mice displayed compromised learning in water maze test and reduced contextual freezing with no apparent abnormalities found in a number of hippocampus-independent behavioral tests. However, the question whether HB-GAM/syndecan-3 actions are also important in brain structures other than hippocampus still remains opens. For example, it is currently unknown whether HB-GAM expression in amygdala undergoes activity-induced changes in expression as it does in the hippocampus.

Interestingly, anxiety-like behavior in the elevated plus maze task was affected in the HB-GAM mutants, but not in the syndecan-3 deficient mice. Decreased anxiety and improved performance in spatial learning tests in mice overexpressing HB-GAM is an intriguing finding, since the use of anxiolytic drugs usually severely impairs memory (e.g. Korneyev, 1997). Notably, similar to the HB-GAM knockouts, MK deficient mice also demonstrated memory impairments and increased anxiety (Nakamura et al., 1998).

Altered GABAergic transmission in the HB-GAM transgenic mice

In the present work (III) we demonstrated that enhanced expression of HB-GAM in transgenic mice is accompanied by alterations in GABAergic neurotransmission. Blockade of $GABA_A$ receptors by picrotoxin resulted in a more prominent increase of the CA1 population spike amplitude in the transgenics compared to the wild-type animals. This suggests that under normal conditions a more powerful inhibitory control exists in the hippocampus of the mutant mice. In line with field recordings whole-cell patch-clamp experiments showed that sIPSCs frequency is enhanced in the mice overexpressing HB-GAM, indicating that spontaneous GABAergic drive is accentuated in the mutants. We suggest that increased hippocampal $GABA_A$ receptor-mediated inhibition in the transgenic mice contributes to suppressed LTP in these animals. Indeed, under $GABA_A$ receptor blockade the magnitude of LTP was similar in the HB-GAM overexpressing mice and their wild-type controls. In addition, transgenic mice display decreased anxiety-like behavior which could also be the result of the increase in GABAergic inhibition in the hippocampus.

A potential link between HB-GAM and the GABAergic inhibition is bFGF. Recent results suggested that HB-GAM inhibits the activity of bFGF by competing for the binding the specific GAG epitopes at the cell surface (Hienola et al., 2004). It has been demonstrated that bFGF modulates GABAergic neurotransmission (Tanaka et al., 1996) and promotes hippocampal LTP induced by high-frequency stimulation (Terlau and Seifert, 1990). Thus, it may explain opposite effects of HB-GAM and bFGF on LTP induction (Ishiyama et al., 1991; Lauri et al., 1998). Activity of bFGF has been shown to be regulated upon binding to syndecan family of HSPGs. Notably both bFGF and HB-GAM have similar affinity to syndecan-3 and compete for binding with it *in vitro* (Kinnunen et al., 1996; Raulo et al., 1994), suggesting that interplay between bFGF and HB-GAM could be mediated by syndecan-3. Further, molecular mechanism underlying the effects of HB-GAM on GABAergic system may also involve HSPG agrin, the activity of which depend on its interaction with HB-GAM (Daggett et al., 1996). Agrin takes part in the maturation of pre- and postsynaptic elements in hippocampal neurons and recently has been shown to be critically involved in the clustering of GABA receptors (Ferreira, 1999), but see also (Li et al., 1999).

It is possible that changes in $GABA_A$ receptor-mediated signalling are also involved in the neurite outgrowth promoting effects of HB-GAM during perinatal development. Apart from being a primary inhibitory neurotransmitter in the CNS GABA is implicated in the neural migration and development stimulating neurite extension and maturation. Further studies are warranted to explore whether the GABAergic system is implicated in the HB-GAM mediated cell motility.

It is unknown whether the number of GABAergic neurons is changed in the hippocampus of the HB-GAM and syndecan-3 mutant mice compared to their wild-type controls. Such developmental alterations may be one of the possible cauces of the current findings. Unfortunately, one could not distinguish between developmental changes and regulation of GABAergic neurons in the adult using conventional knockout mice. Inducible knockout mice would be of a great advantage to clearly differentiate between the two possibilities.

Role of syndecan-3 in HB-GAM signalling

Biological effects of HB-GAM during development, such as HB-GAM-induced neurite outgrowth, are mediated by its interaction with the heparan sulfate chains of syndecan-3 (Raulo et al., 1994). Antibodies to syndecan-3 inhibit the neurite growth of embryonal forebrain neurons on HB-GAM coated matrix (Raulo et al., 1994). Syndecan-3 is a transmembrane proteoglycan strongly expressed in the developing nervous system, but it is also present in the adult brain (Carey, 1996). The expression pattern of syndecan-3 correlates spatially and temporally very well with the expression of HB-GAM in the nervous system (Nolo et al., 1995). Like HB-GAM, syndecan-3 is expressed in an activity-dependent manner in hippocampal pyramidal neurons so that the expression level of its mRNA is enhanced after induction of LTP by HFS (Lauri et al., 1999).

To study syndecan-3 function *in vivo* we produced mice lacking this HSPG. Knockout animals were viable and apparently healthy. No evident developmental or morphological abnormalities were detected. Nevertheless, the mutant mice displayed specific alterations in hippocampal synaptic plasticity and hippocampus-dependent learning and memory. The enhanced level of HFS-induced LTP in the area CA1 of hippocampus in the syndecan-3 null mutant mice resembled the one in the HB-GAM knockout mice. The similarity of the biological effects of syndecan-3 and HB-GAM is stressed by the fact that application of soluble syndecan-3 blocks HFS-induced LTP (Lauri et al., 1999) in the same way as HB-GAM does (Lauri et al., 1998). Thus, exogenous application of syndecan-3 and its lack in the mutant mice produce opposite effects on LTP. To confirm that syndecan-3 is involved in the HB-GAM regulation of hippocampal LTP we evaluated the effect of recombinant HB-GAM in the syndecan-3 mutant mice. HB-GAM administration did not affect post-tetanic potentiation in slices from the wild-type mice, but caused a rapid decay of LTP. However, this phenomenon was not observed in slices from the knockout mice, suggesting that syndecan-3 acts as a functional receptor for HB-GAM in the regulation of LTP. The relevant issue, however, not studied in the present work would be to examine the expression profiles of syndecan-3 and another HB-GAM receptor molecule RPTPβ/ζ in the HB-GAM transgenic mice.

Binding of HB-GAM to syndecan-3 results in the phosphorylation of a kinase-active protein complex containing src-family kinases c-Src and Fyn and the Src-substrate cortactin in neuronal cultures (Kinnunen et al., 1998b). Interestingly, assembly of this molecular complex is strongly upregulated following induction of LTP in the hippocampus, thus speaking for involvement of syndecan-3 mediated transmembrane signalling in LTP (Lauri et al., 1999). Cortactin in turn regulates actin polymerization, which may lead to the structural changes of the synaptic contact (Uruno et al.,

2001). On the other hand, there are several PDZ domain containing molecules (e.g. syntenin, CASK/Lin-2, synbindin and synectin) which interact with the intracellular domain of syndecan-3 and other syndecans (Ethell et al., 2000; Gao et al., 2000; Grootjans et al., 1997; Grootjans et al., 2000; Hsueh et al., 1998). All these molecules bind to the C-terminal EFYA sequence, fully conserved among syndecans. Notably, syntenin was recently shown to interact with glutamate receptor subunits GluR1-4 and mGluR7b (Hirbec et al., 2002), raising the possibility that syndecans are involved in the glutamate receptor targeting, trafficking or recycling.

Somewhat similar to the role in hippocampal plasticity and behavior, syndecan-3 was found to be an important modulator of hypothalamic feeding signaling pathways. Activity-induced oscillations in the level of syndecan-3 occur in hypothalamus and are regulated by feeding state. Food deprivation and refeeding entails several-fold changes in syndecan-3 level, suggesting that these fluctuations are involved in the regulation of feeding behavior (Reizes et al., 2001). Loss of syndecan-3 in the knockout mice results in reduced hyperphagia after food deprivation (Reizes et al., 2001). Additionally, altered feeding behavior and energy metabolism make syndecan-3 null mice resistant to the high-fat diet-induced obesity due to lowered food intake in male mice and an increase in energy expenditure in females (Strader et al., 2004). However, it remains to be determined if HB-GAM is involved in the regulation of feeding behavior by syndecan-3. Another intriguing question that warrants further studies is whether the same plasticity mechanisms involving syndecan-3 are utilized to provide sensitivity to external stimuli in the hypothalamus and hippocampus.

LTP and memory

Since the discovery of LTP in the hippocampus, the brain region known to be associated with learning and memory (Bliss and Lomo, 1973), this phenomenon is generally considered as a putative cellular mechanism of memory formation. LTP exhibits several features making it an attractive candidate for the cellular mechanism of learning and memory. Similar to the memory formation, LTP is triggered within seconds, and persists over a period of time. The induction of LTP follows the Hebbian rule (i.e. conjunctive pre- and postsynaptic activation is required for a change in the strength of a synaptic connection to occure), which is believed to be necessary for the mechanism of information storage as well. And finally, LTP is characterized by the properties of cooperativity, associativity, and input-specificity (Bliss and Collingridge, 1993). Many studies, indeed, clearly showed that pharmacological or genetic manipulations leading to the ablation of hippocampal LTP suppress hippocampus-dependent learning and memory (Roman et al., 1999). Moreover, several elegant experimental designs allowed to demonstrate an occlusion of memory formation by saturation of LTP, indicating that LTP-like changes are necessary for learning and memory *in vivo* (Moser et al., 1998; Castro et al., 1989; McNaughton et al., 1986). Molecular mechanisms involved seem to be rather similar in the case of LTP and certain forms of learning. Such signaling molecules as CaMKII, PKC and ERK. are implicated in both LTP and spatial learning. Further, expression of many molecules crucial for memory formation is modulated by the LTP-inducing stimuli (Lynch, 2004).

However, several controversies still exist. During the past several years it became evident that there is no straightforward relationship between LTP and memory (for critical reviews see Gerlai, 2002; Martin et al., 2000; Morris et al., 2003; Roman et al., 1999; Stevens, 1998). Although many studies using genetically modified mice showed that LTP and hippocampus-dependent learning and memory are correlated, there are an ever increasing number of examples in which these two phenomena dissociate. Several mutant mice lines were characterized in which enhanced LTP was accompanied by no changes in learning and memory (Jun et al., 1998; Manabe et al., 2000) or even impaired hippocampus-dependent learning (Cox et al., 2003; Gu et al., 2002; Migaud et al., 1998; Pineda et al., 2004). Additional examples include mice with normal LTP but improved learning (Collinson et al., 2002), impaired LTP and unaffected (Nosten-Bertrand et al., 1996; Zamanillo et al., 1999) or improved (Koponen et al., 2004) spatial learning and memory.

Changes in LTP induction and maintenance are not necessarily linked to changes in learning and memory. There may be several explanations why LTP and memory are not co-varying. The findings may imply the existence of multiple forms of LTP with specific relevance to certain forms of learning and memory. Also, even though in the current study the syndecan-3 deficient mice along with enhanced LTP displayed higher LTP saturation level following repetitive HFS trains the possibility that impaired learning in those mice as well as in the HB-GAM knockouts was due to the constant potentiation of synapses in the CA1 during the lifetime may not be excluded. In this case enhanced plasticity would preclude naturally occurring LTP-like changes and thus perturb memory formation (Moser et al., 1998; Brun et al., 2001). Finally, it is often the case that one form of

synaptic plasticity in a limited population of synaptic contacts from a particular brain structure (e.g. CA3-CA1 connections in the hippocampus) is compared to the learning of a special task that depends on a multitude of brain processes and neural networks. So it is not strange at all, that the correlation between measured LTP and learning at the behavioral level is often weak. Whatever the particular reason for the dissociation between LTP and memory is, we interpret our results to mean that manipulations, which interfere with the mechanism of synaptic plasticity in the hippocampus, would also affect spatial learning and memory.

ECM molecules in regulation of synaptic plasticity: possible mechanisms

The functions attributed to the extracellular matrix in the nervous system range from regulation of early development and differentiation of neuronal cells to modulation of activity-dependent plasticity in the adult. Currently, the TSR domain containing proteins, and in particular HB-GAM, are among the best characterized examples of ECM factors affecting both the development of neuron-target contacts in the developing system, and activity-dependent synaptic plasticity in the adult brain.

Three types of actions for the ECM components in the synaptic plasticity can be proposed (fig. 12). First (A), by regulating cellular motility and morphology, the ECM may contribute to structural alterations that are associated with the expression of synaptic plasticity. Their role might be 'passive' and restrict morphological alterations in synaptic plasticity; thus reorganization of ECM and cell-ECM interactions is required to allow expression of plasticity. Second (B), ECM components could coordinate transsynaptic signalling during plasticity. According to this scheme, ECM ligands would mediate signals related to expression of synaptic plasticity via their cell surface receptors. Third (C), the ECM defines the physical parameters of the extracellular space, which regulates diffusion of soluble signalling molecules in the extracellular space. These mechanisms, overlapping and acting in concert, provide powerful means for structural and functional regulation of the nervous system.

Conclusions

The results of this study indicate an important role of HB-GAM in brain function in adults. Genetic manipulations leading to either deletion or overexpression of HB-GAM in mutant mice specifically affected the expression of LTP in the area CA1 of hippocampus without changing the properties of basal synaptic transmission. These *in vitro* effects of mutations were paralleled by altered performance in hippocampus-dependent learning tasks *in vivo*. Remarkably, contrasting phenotypes were observed as the result of opposite genetic manipulations. Together with previous results our findings suggest that HB-GAM acts as an inducible factor restricting synaptic plasticity in the hippocampus. Further characterisation of the HB-GAM overexpressing mice revealed accentuated hippocampal $GABA_A$ receptor-mediated inhibition. The finding that may explain decreased level of LTP in these mice as the result of lower predisposition of glutamatergic synapses for LTP induction. It is however unclear whether acute effects of HB-GAM relay on the alteration of GABAergic transmission.

Present data also support the suggestion that syndecan-3 is involved in regulation of LTP by HB-GAM. Mice lacking syndecan-3 displayed electrophysiological and behavioral phenotype similar to that of HB-GAM knockout mice, namely enhanced level of LTP and impaired spatial learning and memory. Further, application of recombinant HB-GAM which suppresses LTP in wild-type animals had no effect in the syndecan-3 deficient mice.

HB-GAM consists of two β-sheet domains homologous to thrombospondin type I repeat and hens is a member of a larger superfamily of ECM-associated and cell surface molecules, TSR domain-containing proteins. Our results indicate that though individual domains of HB-GAM can bind heparan sulfate this binding is much weaker than binding of the di-TSR fragment. Accordingly only di-domain polypeptide produced biological effects similar to that of the native HB-GAM. The linker region between two TSR domains had no effect on protein function. Thus a co-operative process involving both domains is implicated in the biologically relevant interaction with cell surface heparan sulfates.

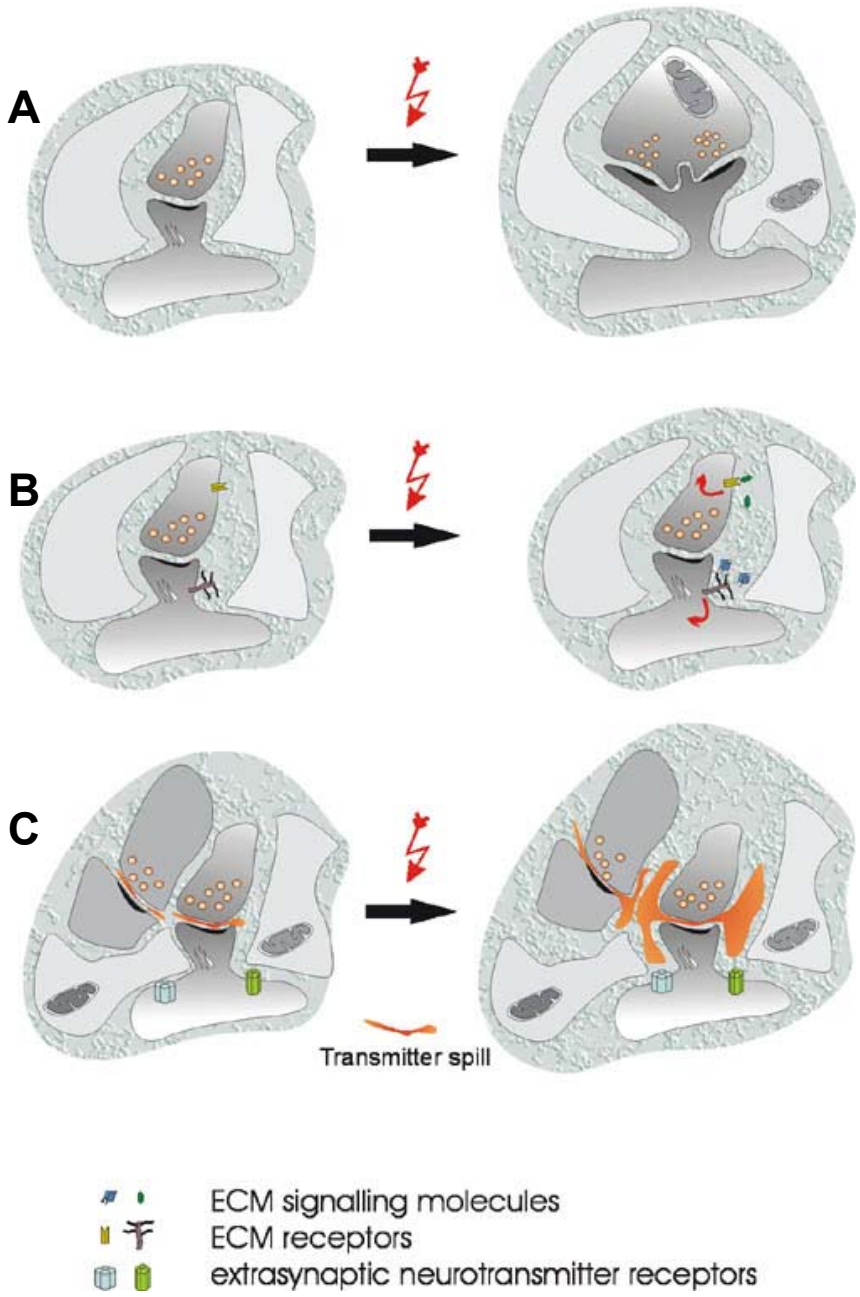

Figure 12. Proposed mechanisms by which activity-dependent changes in the extracellular matrix could affect neuronal activity and synaptic plasticity (see text for details).

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