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# B-plexins Regulate the Maturation of Neurons Through Microtubule Dynamics

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Declaration:

*Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.*

/Piret Laht/

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# B-pleksiinid mõjutavad mikrotorukeste liikumise kaudu närvirakkude küpsemist

PIRET LAHT

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KIRJASTUS

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# ORIGINAL PUBLICATIONS

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Plexin-B3 suppresses excitatory and promotes inhibitory synapse formation in rat hippocampal neurons.
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# INTRODUCTION

Development and functioning of the mammalian central nervous system (CNS) is a complicated multilevel task. A delicate balance has to be maintained between proliferation and apoptosis, migration and settling, neurite growth and pruning, attraction and repulsion, excitation and inhibition. It is like driving a car: you have to accelerate in order to get moving but you also need functional brakes to avoid crashing. Regulation of these processes is governed by a multitude of signalling molecules that are expressed by specific subsets of cells. Semaphorins and their receptors plexins and neuropilins are a group of guidance proteins that participate in all stages of CNS development: proliferation, migration, axon guidance, dendritic orientation, synaptogenesis, neuron survival and synaptic plasticity; and are thus important mediators of intercellular relations. Although class B plexins are widely expressed in the brain, certain aspects of their biology have remained poorly characterized.

The aim of this study was to identify novel protein-protein interactions of Plexin-B3 intracellular part and their functional significance. Since we found that Plexin-B1 and B3 can bind to microtubule end binding proteins (EB1, EB2 and EB3) we hypothesized that B-plexins regulate microtubule dynamics and through that also dendritogenesis and synaptogenesis.

Microtubule cytoskeleton is a key determinant in generating and maintaining neuronal morphology and function. Remodelling and reorganization of microtubules is required for the recognition of guidance cues, neurite growth as well as formation of synapses. EB proteins are central adaptors at microtubule tips that have been shown to form complexes with a variety of other proteins, but among these only a few transmembrane receptors have been described so far. To my best knowledge, no signalling pathways of class B-plexins have been described, that connect them directly to microtubule regulation. In this study, the role of all three B-type plexins was systematically analysed in the regulation of microtubule tip dynamics by following the behaviour of EB3-GFP reporter in live cells. Rat hippocampal neurons and different immunocytochemistry approaches were used to assess the importance of B-plexins in dendritic arbour development and synaptogenesis.

As a result we provide a novel insight into molecular mechanisms how semaphorin signals are transmitted to the cytoskeleton. Overall, our findings demonstrate that among their other functions B-plexins regulate microtubule growth, dendrite elongation and synapse formation, and hence could be connected to the pathogenesis of neurological disorders.

# ABBREVIATIONS

+TIP – microtubule plus-end binding protein
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC – adenomatosis polyposis coli protein
ARHGEF – Rho guanine nucleotide exchange factor
ASD – autism spectrum disorders
BDNF – brain-derived neurotrophic factor
BTBD3 – BTB (POZ) domain containing 3
CAP-Gly – cytoskeleton associated proteins Gly-rich domain
CH – calponin homology domain
CLASP – CLIP-associated protein
CNS – central nervous system
CRMP – collapsin response mediator protein
DCC – deleted in colorectal carcinoma, netrin 1 receptor
DIV – days in vitro
DLG – discs large homolog
DRG – dorsal root ganglion
EB – microtubule end binding protein
ERBB-2 – erb-b2 receptor tyrosine kinase 2 (HER-2)
F-actin – filamentous actin
FARP – FERM, RhoGEF (ARHGEF) and pleckstrin domain protein
FN – fibronectin repeat
GABA – γ-aminobutyric acid
GAD65 – glutamate decarboxylase 2, 65kDa
GAP – GTPase activating protein
GDI – guanine nucleotide dissociation inhibitors
GDNF – glial cell derived neurotrophic factor
GDP – guanosine diphosphate
GEF – guanine nucleotide exchange factors
GFP – green fluorescent protein
GnRH – gonadotropin-releasing hormone
GPI – glycosylphosphatidylinositol anchor
GRB2 – growth factor receptor bound protein 2
GST – glutathione S-transferase
GTP – guanosine triphosphate
GTPase – guanosine triphosphate hydrolase
GWAS – genome wide association study
HGF – hepatocyte growth factor
IC – intracellular domain
Ig – immunoglobulin
IP – immunoprecipitation
IPT – immunoglobulin-like fold shared by plexins and transcription factors
L1CAM – L1 cell adhesion molecule

LTD – long-term depression
LTP – long-term potentiation
MACF – microtubule actin crosslinking factor
MAP – microtubule associated protein
MAPRE – microtubule-associated protein, RP/EB family
MCAK – mitotic centromere associated kinesin (KIF2C - kinesin family member 2C)
MICAL – microtubule associated monooxygenase, calponin and LIM domain containing; molecule interacting with CasL
MTip – microtubule plus-end
NGF – neurotrophic growth factor
NMDA – N-Methyl-D-aspartic acid
NRP – neuropilin
NTS – N-terminal segment
OPC – oligodendrocyte precursor cell
PAK – p21 protein (Cdc42/Rac)-activated kinase
PDZ – postsynaptic density protein (PSD95), Drosophila discs large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PDZ-BM – PDZ domain binding motif
PI3K – phosphatidylinositol-3-kinase
PIP5K – phosphatidylinositol-4-phosphate 5-kinase
PKC – protein kinase C
PLC – phospholipase C
PLXN – plexin
PSD95 – post-synaptic density 95 kDa protein (DLG4)
PSI – protein domain found in plexins semaphorins and integrins
PTEN – phosphatase and tensin homolog
RanBP9 – Ran binding protein 9
RhoBD – Rho GTPase binding domain
ROCK – Rho-associated coiled-coil containing protein kinase
SEMA – semaphorin, Sema domain
TIRF – total internal reflection fluorescence
Trk – neurotrophic tyrosine kinase receptor
TSP – thrombospondin repeats;
VEGFR – vascular endothelial growth factor receptor
wt – wild type

# 1. REVIEW OF THE LITERATURE

## 1.1 History of Plexinology

First plexin was discovered when a Japanese group screened for molecules that govern specific neuronal recognition between retinal axons and optic tectum in *Xenopus* tadpoles (Takagi et al., 1987). One of the obtained monoclonal antibodies recognized a 220 kDa neuronal cell surface protein in plexiform layers (Ohta et al., 1992) and from that the plexin family got its name. Subsequently plexin cDNAs were characterized in humans (Maestrini et al., 1996) and mice (Kameyama et al., 1996a, Kameyama et al., 1996b). The human family was initially named SEX family as the first cDNA mapped on the sex chromosome X and the gene was named *SEX* (Maestrini et al., 1996) (now we know this gene as *PLXNA3*). Both groups noticed that plexins had a significant similarity in their extracellular portion with Met-like receptor tyrosine kinases especially in cysteine-rich PSI domains.

The science of semaphorins started in the beginning of the 1990s. Alex Kolodkin, Corey Goodman and others studied factors regulating axon guidance in grasshopper and *Drosophila* and they described both transmembrane (Sema1a) and secreted semaphorins (Sema2a) in insects, and the first human semaphorin (SEMA3A) (Kolodkin et al., 1992, Kolodkin et al., 1993). Concurrently a secreted semaphorin (Sema3A) was discovered from chick that was called collapsin at the time since it caused collapse of sensory ganglion growth cones (Luo et al., 1993). The first grasshopper semaphorin was initially called Fascilin IV as it regulated axon fasciculation. Soon the name "semaphorins" became widely used for all proteins of that family. The name comes from the Greek word "*semaphora*" – showing the way.

First evidence that plexins are functional receptors for semaphorins emerged from the studies of vaccinia virus semaphorin A39R that bound to a transmembrane receptor on human B lymphocytes that belonged to the plexin family (Comeau et al., 1998). It was named virus-encoded semaphorin protein receptor (VESPR) and is now better known as Plexin-C1. This finding was supported by evidence from *Drosophila* that Plexin-A is a neuronal receptor for class 1 semaphorins (Winberg et al., 1998). The role of neuropilins as mediators of secreted (class 3) semaphorin signals was known before (Kolodkin et al., 1997, He and Tessier-Lavigne, 1997). Later it was shown that physiologic Sema3A receptors consist of plexin and neuropilin stable complexes (Takahashi et al., 1999).

In 1999 the names and nomenclature of semaphorins and plexins were unified (Semaphorin nomenclature committee, 1999, Tamagnone et al., 1999). Semaphorins were divided into eight classes according to the organisms and structure. Class 1 – transmembrane invertebrate semaphorins; class 2 – secreted invertebrate semaphorins; class 3 secreted vertebrate semaphorins; class 4 – transmembrane vertebrate semaphorins with Ig-like domain; class 5 –

transmembrane semaphorins with thrombospondin repeats; class 6 – transmembrane semaphorins with larger cytoplasmic domain; class 7 – semaphorins with a GPI anchor; class V – semaphorins encoded by different viruses. Plexins were grouped to four types: A, B, C and D (Figure 1).

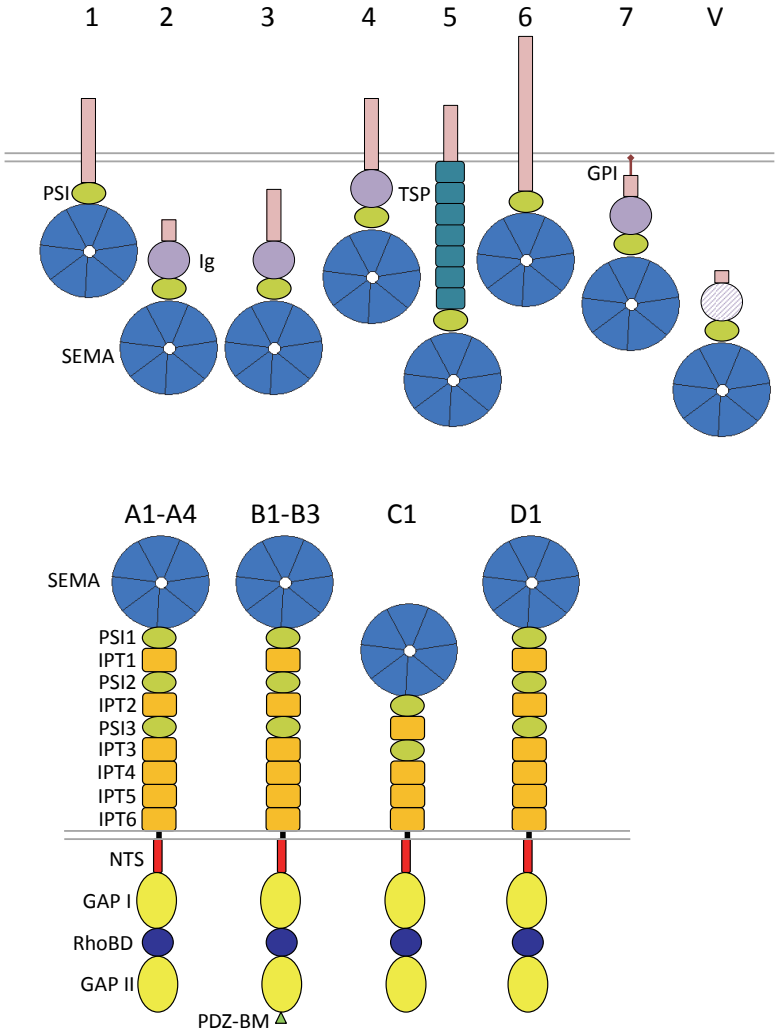


**Figure 1. Semaphorin and plexin families.** Protein domain names: SEMA – semaphorin; PSI – plexins semaphorins integrins; Ig – immunoglobulin; TSP – thrombospondin repeats; GPI – glycosylphosphatidylinositol anchor; IPT - immunoglobulin-like fold shared by plexins and transcription factors; NTS – N-terminal segment; GAP – GTPase activating protein; RhoBD – Rho GTPase binding domain; PDZ-BM – PDZ binding motif. Based on Tamagnone et al., 1999 and Perälä et al., 2012.

## 1.2 Ligands and co-receptors of plexins

The main ligands of plexins are semaphorins. Plexins can interact with semaphorins in *trans* or in *cis* and also reverse signalling is possible, where transmembrane semaphorins act as receptors and plexins as ligands (Toyofuku et al., 2004). Besides, plexins may interact homophilically in *trans* (Hartwig et al., 2005). Only recently it was found that plexins could use molecules without Sema domain as a ligand. Namely, Slit C-terminal fragment signals through Plexin-A1 in commissural axon guidance in the spinal cord (Delloye-Bourgeois et al., 2014). It has been known for longer that at different locations and development stages semaphorins may utilize different plexins and co-receptors, or some alternative receptors. Of greater interest are integrins, L1CAM, and receptor tyrosine kinases VEGFR, Met, Ron and ErbB-2 (reviewed in Perälä et al., 2012). Plexin-B2 interacts also with GDNF receptor Ret during kidney development (Perälä et al., 2011). In general class 3 and 6 semaphorins use class A or D plexins and class 4 semaphorins use B-plexins. Neuropilins are obligatory co-receptors of A-plexins for class 3 semaphorins. As neuropilins were identified first, they are often referred to as the primary receptors and plexins are considered to be co-receptors. In the light of structural studies (Janssen et al., 2012) I think that plexins are more important with their Sema domains and neuropilins act as co-receptors to stabilize the complex. For a detailed overview of different mammalian semaphorin receptors see Table 1.

**Table 1: Semaphorin receptors**

| Semaphorin | Receptors | References |
|---|---|---|
| Sema3A | Plexin-A1/Nrp1 or Nrp2 | (Takahashi et al., 1999) |
| | Plexin-A3/Nrp1 | (Cheng et al., 2001) |
| | Plexin-A4/Nrp1 | (Suto et al., 2005) |
| Sema3B | Plexin-A2/Nrp1 or Nrp2 | (Sabag et al., 2014) |
| | Plexin-A4/Nrp1 or Nrp2 | (Sabag et al., 2014) |
| Sema3C | Plexin-D1/Nrp1 | (Toyofuku et al., 2008) |
| Sema3D | Plexin-A2/Nrp1 | (Aghajanian et al., 2014) |
| Sema3E | Plexin-D1 | (Watakabe et al., 2006) |
| Sema3F | Plexin-A1/Nrp2 | (Claudepierre et al., 2008) |
| | Plexin-A2/Nrp1 | (Takahashi and Strittmatter, 2001) |
| | Plexin-A3/Nrp2 | (Cheng et al., 2001) |
| Sema4A | Plexin-B1 | (Yukawa et al., 2005) |
| | Plexin-B2 | (Yukawa et al., 2005) |
| | Plexin-B3 | (Yukawa et al., 2005) |
| | Plexin-D1 | (Toyofuku et al., 2007) |
| | Tim2 | (Kumanogoh et al., 2002) |
| Sema4B | ? | |
| Sema4C | Plexin-B2 | (Deng et al., 2007) |
| Sema4D | Plexin-B1 | (Tamagnone et al., 1999) |
| | Plexin-B2 | (Masuda et al., 2004) |
| | Plexin-B3 | (Maier et al., 2011) |
| | Plexin-C1 | (Chabbert-de Ponnat et al., 2005) |
| | CD72 | (Kumanogoh et al., 2000) |
| Sema4F | ? | |
| Sema4G | Plexin-B2 | (Maier et al., 2011) |
| Sema5A | Plexin-A1 | (Matsuoka et al., 2011a) |
| | Plexin-A2 | (Duan et al., 2014) |
| | Plexin-A3 | (Matsuoka et al., 2011a) |
| | Plexin-B3 | (Artigiani et al., 2004) |
| Sema5B | Plexin-A1 | (Matsuoka et al., 2011a) |
| | Plexin-A3 | (Matsuoka et al., 2011a) |
| Sema6A | Plexin-A2 | (Suto et al., 2005) |
| | Plexin-A4 | (Suto et al., 2005) |
| Sema6B | Plexin-A2 | (Tawarayama et al., 2010) |
| | Plexin-A4 | (Suto et al., 2005) |
| Sema6C | Plexin-A1 | (Yoshida et al., 2006) |
| Sema6D | Plexin-A1 | (Toyofuku et al., 2004) |
| Sema7A | Plexin-C1 | (Tamagnone et al., 1999) |
| | β1-integrin | (Pasterkamp et al., 2003) |

## 1.3 Plexins and semaphorins in the nervous system

### 1.3.1 Proliferation and migration of cells

Plexins mediate semaphorin signals in the nervous system on multiple levels and in all developmental stages. In embryogenesis and postnatally they participate in the proliferation and migration of neuronal progenitor cells as well as glial cells.

Class 3 semaphorins can have different functions depending on the cell type. New-born neurons are attracted by Sema3A, which is expressed in a descending gradient across the cortical layers, whereas its receptor Nrp1 is highly expressed in neurons during their radial migration in the cortex (Chen et al., 2008). Migrating interneurons lacking neuropilins go to the striatum (Marín et al., 2001). Sema3E is a natural negative regulator of the migration of Plexin-D1 positive Cajal-Retzius cells that are important coordinators of the growth of the neocortex (Bribián et al., 2014). Sema3F acts in the ventral tangential migration stream, confining the migrating neurons on the telencephalon surface by repelling from the deeper domain (Ito et al., 2008). In cerebellum development, Sema6A-Plexin-A2 control granule cell migration through nucleus-centrosome coupling. Sema6A is selectively expressed by postmitotic granule cells during their tangential migration, but not during their radial migration (Renaud et al., 2008, Renaud and Chédotal, 2014). Sema6A-deficient mice have granule cell migration defects in cerebellum, neocortex and hippocampus (Kerjan et al., 2005, Rünker et al., 2011).

While Plexin-B1 and B3 knockout mice are normal in the aspect of brain development (Deng et al., 2007, Worzfeld et al., 2009) in Plexin-B2 mutant mice proliferation and migration of neuron progenitors is compromised resulting in neural tube closure defects and exencephaly, which leads to neonatal lethality. The penetrance of this phenotype depends on genetic background (Deng et al., 2007, Friedel et al., 2007, Hirschberg et al., 2010). Excitatory cortical neurons are born in the ventricular zone of the telencephalon and Plexin-B2 is expressed in them. Sema4D is expressed next to the migration routes and enhances radial and tangential migration of developing neurons in a Plexin-B2-dependent manner. Sema4D enhances also migration of GABAergic neurons from the ganglionic eminence toward their correct destination zones in the cortical plate (Hirschberg et al., 2010). Cortical neuron migration is regulated by Plexin-B2 through RhoA activation and interaction with Rnd3 (Hirschberg et al., 2010, Azzarelli et al., 2014). Plexin-B2 is required also for normal cerebellar granule cell proliferation and migration. Plexin-B2$^{-/-}$ mice have smaller cerebellums with severely altered morphology in foliation as well as organization of cell layers (Friedel et al., 2007). The expression of Plexin-B2 ceases in cells that have completed migration into inner granular layer (Deng et al., 2007). In the cerebellum the ligands for Plexin-B2 are Sema4C and Sema4G (Maier et al., 2011). In the hippocampus migrating granule cell precursors

express Plexin-B2 and after they reach their target and differentiate Plexin-B2 is downregulated. In the hippocampus Sema4D may serve as a ligand (Deng et al., 2007). In addition, Plexin-B2 deficient mice have lamination defects in the olfactory bulbs that results from migration defects (Deng et al., 2007). Plexin-B2 regulates proliferation and migration of neuroblasts also postnatally in the forebrain subventricular zone (Saha et al., 2012). Taken together, Plexin-B2 is required for normal neuron migration in several parts of the brain.

Primary gonadotropin releasing hormone (GnRH-1) positive neurons co-express Plexin-B1 and Met receptors and Sema4D is used as an attractive signal along their migration route. PlexinB1-deficient mice exhibit a migratory defect of GnRH-1 neurons, resulting in reduction of this cell population in the adult brain (Giacobini et al., 2008). Also Sema7A promotes the migration of GnRH-1 positive neurons in the brain but does it mainly through β1-integrin receptor rather than Plexin-C1 (Messina et al., 2011). Still, Plexin-C1 is highly expressed in different subsets of migrating neurons as well as oligodendrocytes (Pasterkamp et al., 2007).

In the spinal cord boundary cap cells express Sema6A. That signal is recognized by migrating motor neurons that have Plexin-A2/Nrp2 complexes on their surface and this confines their bodies to the spinal cord (Bron et al., 2007). Plexin-A4 together with Sema6D act as gatekeepers only at the dorsal root entry site to organize the segregation of dorsal roots (Mauti et al., 2007). Sema3A-Plexin-A3 regulate the number of dorsal root ganglion (DRG) neurons by inducing apoptosis during development. Besides, Plexin-A3 and Plexin-A4 are essential for normal cell migration and axon guidance in sympathetic nervous system (Ben-Zvi et al., 2008).

Oligodendrocytes express all semaphorins and use them in migration (Cohen et al., 2003). Sema3A-Plexin-A4 act as repellents for oligodendrocyte precursor cells (OPCs) while Sema3F-Plexin-A3 signalling is attractive and also mitogenic (Spassky et al., 2002, Okada and Tomooka, 2012, Xiang et al., 2012). In Sema4D mutant mice the number of mature oligodendrocytes is increased in brain, thus Sema4D is used to limit the number of differentiated oligodendrocytes. Sema4D is expressed by OPCs and mature oligodendrocytes, while its receptors Plexin-B1 and CD72 are expressed by multiple cell types and are especially numerous on astrocytes (Taniguchi et al., 2009, Smith et al., 2015). Sema4F is expressed by OPCs and it regulates their migration in the developing brain and promotes oligodendrocyte differentiation (Armendáriz et al., 2012). In the peripheral nervous system Sema4F is expressed by both Schwann cells and the nerve fiber (axons) throughout development and is required for their normal contact. When Ras/Raf/MEK pathway is too active, Sema4F expression is downregulated or lost altogether and the contact between Schwann cells and axons loosens. This may lead to generation of neurofibromas (Parrinello et al., 2008).

### 1.3.2 Axon guidance and dendrite growth

Semaphorins are classical guidance molecules and one of their main functions is to ensure that axons find their proper targets. They also participate in dendrite arbour growth and orientation. Often it is complicated to discriminate the processes of neurite guidance and synaptogenesis. Different subsets of neurons utilize different molecular codes of ligand-receptor pairs to find their way. Surprisingly few studies deal with the role of B-plexins and class 4 semaphorins in the establishment of specific connections between neurons, especially when compared to A-plexins and class 3 and class 6 semaphorins. Here, I concentrate on class B plexins and their potential ligands. For other plexins and semaphorins I suggest a review by J. Pasterkamp dealing with neuronal connections (Pasterkamp, 2012).

Sema4A and Sema4D deficient mice have no obvious brain defects even in the double knock-out (Friedel et al., 2007). Sema4D is expressed throughout the CNS white matter by myelinating oligodendrocytes and inhibits strongly the growth of postnatal sensory and cerebellar granule cell axons (Moreau-Fauvarque et al., 2003). Besides, Sema4A and Sema4D induce growth cone collapse of hippocampal neurons by activating Plexin-B1 (Yukawa et al., 2005, Swiercz et al., 2002). In contrast, Sema4D stimulates axonal outgrowth of embryonic DRG neurons (Masuda et al., 2004) and cortical neurons (Worzfeld et al., 2004). Endogenously produced NGF and the activation of Trk receptor are required for Sema4D action on DRG neurons. These neurons express Sema4D as well as possible receptors Plexin-B1 and Plexin-B2 and this implies that Sema4D acts in autocrine or paracrine manner (Masuda et al., 2004).

Although Plexin-B2 deletion causes severe morphological defects in the cerebellum the specific connectivity between neuronal subtypes is maintained and these mice that survive have no obvious behavioural or motor deficits (Friedel et al., 2007). No defects of axon guidance nor behaviour have been reported for Plexin-B1 and Plexin-B3 knock-out mice either (Fazzari et al., 2007, Deng et al., 2007, Hirschberg et al., 2010, Worzfeld et al., 2009). Plexin-B3 and Plexin-B2 stimulate neurite (axon) outgrowth of cerebellar neurons in trans (Hartwig et al., 2005) suggesting a positive role in axon guidance.

Reports regarding the influence of Sema4D on dendritic arborisation have been contradictory. While Saito and colleagues observed Plexin-B1-mediated inhibitory effect of Sema4D on cortical neuron dendrite growth, Vodrazka et al reported that Sema4D potentiated the formation of higher order branches in hippocampal neurons (Saito et al., 2009, Vodrazka et al., 2009). The role of other class 4 semaphorins or B-plexins in dendritogenesis has not been studied.

### 1.3.3 Synaptogenesis

Neurons are linked in a functional network through synapses that may be electrical or chemical. Chemical synapses are usually classified on the basis of

neurotransmitters that they use and consequent electrophysiological effect. Altogether there are at least 100 different neurotransmitters. The majority of excitatory synapses are situated on dendritic spines and utilize glutamate or acetylcholine for transmission. Inhibitory synapses are assembled on pre-existing axon and dendrite or axon and cell soma cross points and use mainly GABA or glycine. Signal is transduced from the presynaptic axon terminal to postsynaptic cell dendrite, soma or axon (Purves et al., 2008).

Semaphorins and plexins were discovered as axon guidance molecules but a growing number of genetic as well as cellular and molecular studies have shown their role also in synaptogenesis. Semaphorins are usually negative regulators of dendritic spine and glutamatergic synapse formation. An elegant work in *C. elegans* showed that locally enriched PLX-1 inhibits synapse formation, and its subcellular localization is regulated by SMP-1 in *cis* (Mizumoto 2013). In adult mouse hippocampal neurons Sema3A application reduced the density of synapses (Bouzioukh et al., 2006). Sema3A and Sema7A mediate retrograde signals for elimination of climbing fiber to Purkinje cell synapses in the cerebellum (Uesaka et al., 2014). Sema3F-Plexin-A3 signalling is necessary for the elimination of inappropriate synapses during hippocampal development. Sema3F null mice have increased number of spines and are prone to epileptic seizures (Liu et al., 2005, Sahay et al., 2005, Tran et al., 2009). Sema5A regulates dendritic spine density and glutamatergic synapses in *cis* through Plexin-A2 RasGAP activity in a cell autonomous manner. Sema5A$^{-/-}$ and Plexin-A2$^{-/-}$ mice display an increase in hippocampal glutamatergic synapses and social behavior similar to autism spectrum disorders (Duan et al., 2014). Sema5B is a negative regulator of glutamatergic synapse formation in hippocampal neurons as well (O'Connor et al., 2009).

In contrast, semaphorins have positive effect on inhibitory synapse formation, and occasionally in the case of excitatory synapses. In animal and cellular models it has been shown that Sema3A plays a critical role in regulating the spine maturation of the layer V cortical neurons (Morita et al., 2006). Sema4D promotes the formation of GABAergic synapses through Plexin-B1 (Kuzirian et al., 2013). Both Sema4D and its receptor Plexin-B1 localize to postsynaptic membranes (Raissi et al., 2013, Lin et al., 2007). Sema4D does not use its intracellular domain to induce GABAergic synapse formation, but can signal as a membrane bound molecule. Therefore it is likely that Sema4D acts in *cis* through Plexin-B1 (Raissi et al., 2013). Also Sema4B colocalizes with postsynaptic markers and is involved in inhibitory as well as excitatory synapse formation (Burkhardt et al., 2005, Paradis et al., 2007), and Sema7A is required for normal inhibitory synapse maturation (Carcea et al., 2014).

Sema4B interacts with PSD95 through a PDZ-binding motif at the C-terminus. A similar motif can be found in Sema4C, 4G and 4F but not in Sema4A or 4D (Burkhardt et al., 2005, Inagaki et al., 2001). Sema4F is enriched in post-synaptic density fractions and colocalizes with PSD95 and synapsin1 at glutamatergic synapses (Schultze et al., 2001). It is theoretically

possible that those class 4 semaphorins that bind to PSD95 modulate glutamatergic synapses through reverse signalling mechanisms. Further studies are needed to clarify this question.

Taken together, different semaphorins and plexins are necessary for generation or elimination of a variety of synapses, but not all of them have been studied in that aspect.

### 1.3.4 Plexins and semaphorins in neuropsychiatric disorders

After birth precise connectivity between neurons is refined depending on their activity. Those neurons that are not connected to circuits die through apoptosis, supernumerary neurite branches and synapses are pruned and eliminated. Balanced synaptic plasticity is necessary for learning and memory formation. Dysregulated synaptogenesis underlies a variety of neuropsychiatric disorders, including schizophrenia, autism spectrum disorders (ASD) and mental retardation. Most neurodevelopmental disorders are characterized by loss of certain neurons or reduced connectivity between neurons. In contrast, ASD are a result of defective synapse elimination and consequently there are too many dendritic spines and excitatory synapses. Also the balance between excitatory and inhibitory signals is very important for normal functioning of the brain (Penzes et al., 2011, Bernardinelli et al., 2014). Very often no gross abnormalities are observed with only one mutated or dysregulated gene, because the functions are redundant and compensated by other proteins. But still sometimes things go wrong and the results vary from subtle behavioural abnormalities with no detectable morphological symptoms to severe defects in CNS morphology and mental retardation.

Certain genotypes or deregulation of Plexin-A2 and its ligands Sema3A and Sema3D have been linked with susceptibility to schizophrenia (Mah et al., 2006, Takeshita et al., 2008, Fujii et al., 2011). Blocking Sema4D with monoclonal antibodies led to cognitive improvement in Huntington disease mouse model (Southwell et al., 2015). Sema5A has been associated with autism (Melin et al., 2006, Weiss et al., 2009) and Parkinson's disease (Yu et al., 2014). Besides, Sema5A and one of its receptors Plexin-B3 have been associated with brain volume parameters and performance in intelligence tests (Zhu et al., 2013, Rujescu et al., 2007). *PLXNB2* gene is located at 22q13 and deletion of this region has been linked to autistic-like features and abnormalities in cerebellar structures (Hannachi et al., 2013, Aldinger et al., 2013). Sema6A and its receptor Plexin-A4 regulate neuronal connectivity and their depletion results in disorders that resemble schizophrenia and autism (Matsuoka et al., 2011a, Rünker et al., 2011, Suda et al., 2011). Microdeletions of chromosome 15q24 which include *SEMA7A* are associated with a syndrome characterized by autism, developmental delay, and abnormalities in somatosensation (McInnes et al., 2010). See also table 2.

**Table 2. Semaphorins and their receptors in neuropsychiatric disorders that are caused by defects in connectivity between neurons.**

| Gene | Connection to psychiatric disease | References |
|---|---|---|
| *SEMA3A* | Downregulated in schizophrenic cortex | (Arion et al., 2010) |
| *SEMA3A* | Upregulated in schizophrenic cerebellum | (Eastwood et al., 2003) |
| *SEMA3C* | Risk locus for autism spectrum disorders | (Walker and Scherer, 2013) |
| *SEMA3C* | Downregulated in schizophrenic cortex | (Arion et al., 2010) |
| *SEMA3D* | Susceptibility to schizophrenia in Japanese population | (Fujii et al., 2011) |
| *SEMA3E* | Downregulated in schizophrenic cortex | (Arion et al., 2010) |
| *SEMA3F* | Downregulated in schizophrenic cortex | (Arion et al., 2010) |
| *Sema3F* | Altered dendritic spine density (mouse model) | (Demyanenko et al., 2014) |
| *SEMA4D* | Downregulated in schizophrenic cortex | (Arion et al., 2010) |
| *SEMA4D* | Autism GWAS | (Hussman et al., 2011) |
| *Sema4D* | Huntington disease (mouse model) | (Southwell et al., 2015) |
| *SEMA5A* | Downregulated in autism | (Melin et al., 2006), (Weiss et al., 2009) |
| *SEMA5A* | Autism GWAS | (Hussman et al., 2011) |
| *SEMA5A* | Parkinson's disease | (Yu et al., 2014) |
| *Sema5A* | Autism (mouse model) | (Duan et al., 2014) |
| *SEMA6A* | Autism GWAS | (Hussman et al., 2011) |
| *Sema6A* | Psychiatric disorders resembling schizophrenia and autism (mouse model) | (Rünker et al., 2011) |
| *SEMA6D* | Downregulated in schizophrenic cortex | (Arion et al., 2010) |
| *SEMA7A* | Autism and other mental disorders | (McInnes et al., 2010) |
| *PLXNA2* | Susceptibility to schizophrenia in Japanese and American population | (Mah et al., 2006), (Takeshita et al., 2008) |
| *PlxnA2* | Autism (mouse model) | (Duan et al., 2014) |
| *PLXNA4* | Downregulated in autism | (Suda et al., 2011) |
| *PLXNB2* | Cerebellum abnormal structure | (Aldinger et al., 2013) |
| *PLXNB2* | Behavioral abnormalities and autistic-like features | (Hannachi et al., 2013) |
| *PLXNC1* | Autism GWAS | (Hussman et al., 2011) |
| *Nrp2* | Autism and epilepsy (mouse model) | (Gant et al., 2009) |

## 1.4 Plexins in different organs

Plexins were initially identified in the nervous system but piling evidence shows that they have important functions also in other organs.

In cardiovascular development secreted class 3 semaphorins are important inhibitory regulators of angiogenesis. Together with class 6 semaphorins and plexins A1, A2, A4 and D1 they also guide the formation of heart chambers and tracts. Conversely, class 4 semaphorins and B-plexins promote angiogenesis through activation of Rho GTPases (reviewed in Epstein et al., 2015).

Expression of semaphorins and plexins is characteristic for organs that comprise epithelial tubular structures like kidney, lung and liver. Plexin-B1 and B2 have antagonistic functions in kidney development Sema4D-Plexin-B1 pathway inhibits branching (Korostylev et al., 2008) but Sema4C-Plexin-B2 signalling promotes it (Perälä et al., 2011). Sema3A is secreted by podocytes (Villegas and Tufro, 2002) and is required for normal patterning of the ureteric bud tree and glomerular filtration barrier development (Reidy et al., 2013). During lung development Sema3A-Plexin-A1/Nrp1 pathway is crucial for normal alveolarization (Becker et al., 2011). Class 4 semaphorins and B-plexins are expressed in bronchial epithelial and smooth muscle cells (Smith et al., 2011, Korostylev et al., 2008, Zielonka et al., 2010). Several semaphorins are connected to various pulmonary diseases including asthma and cancer. Plexin-B1 is strongly expressed in the developing liver but its levels decrease in adulthood. Plexin-B2 expression is retained in adult liver sinusoids (Zielonka et al., 2010), and Sema3A has been associated with liver regeneration (Fu et al., 2012).

Recently, significant advances have been made in understanding of the roles of semaphorins in bone homeostasis and remodelling. Plexin-A1 is required for normal bone and cartilage development. It can use Sema3A or Sema6D as ligands. Sema4D-Plexin-B1 are negative regulators of bone mass formation and this is regulated by ovarian hormones (reviewed in Kang and Kumanogoh, 2013). Many semaphorins and plexins are involved also in tooth development (Lillesaar and Fried, 2004).

In the context of immune system Sema7A, Plexin-C1 and Plexin-D1 come first to my mind. Sema4A and Sema4D (CD100) and their receptor Plexin-B1 have also been addressed extensively in that aspect. Plexins and semaphorins mediate many cell processes critical for the immune system including cell-cell contact, migration, and cytokine secretion. For reviews see (Roney et al., 2013, Takamatsu and Kumanogoh, 2012).

## 1.5 Plexin structure

Plexins are type I transmembrane proteins (Figure 2). Human full-length plexins range in length from 1541 to 2108 aa. They are synthesized as precursor proteins containing a signal peptide, a Sema domain, three cysteine rich PSI domains (plexins, semaphorins, integrins), six glycine and proline rich IPT domains (immunoglobulin-like fold shared by plexins and transcription factors), a transmembrane domain (TM), a NTS (N-terminal segment), a bipartite RasGAP domain (Ras GTPase acticvating protein) that is split by a RhoBD (Rho GTPase binding domain) (Siebold and Jones, 2013). B-plexins have PDZ-binding motif at their C-terminus. Plexin-C1 lacks the domains that correspond to IPT2, PSI3 and IPT3 rendering it shorter than other plexins (see Figure 1).

The structure of full-length plexin has not been resolved but available are crystal structures of partial extracellular domains (Janssen et al., 2010, Nogi et al., 2010, Liu et al., 2010) and intracellular domains (He et al., 2009, Tong et al., 2009, Wang et al., 2013).

The *Sema domain* comprises approximately 500 residues and on the level of primary structure does not have significant conserved regions. Besides plexins, Sema domain can be found in semaphorins and Met and Ron receptor tyrosine kinases (Tamagnone et al., 1999). Crystal structures of different Sema domains have revealed that structurally it is a 7-blade beta propeller with extrusions in the first and fifth blade. Like most known propeller structures, Sema domain uses "loop and hook" system to close the circle between the first and the last blades. The structure is further stabilized by disulfide bridges (Antipenko et al., 2003). For semaphorin binding most important regions are within blade 1 and 3 and the extrusion in the fifth blade. They vary between different plexins enabling specific recognition of semaphorins (Janssen et al., 2010, Nogi et al., 2010). Unlike semaphorin Sema domains, plexin Sema domains do not form stable dimers (Siebold and Jones, 2013).

*PSI domains* are short 50-60 aa motifs that are characterized by conserved cysteine residues that form three disulfide bonds and its overall structure is a cysteine knot. PSI1 makes contacts with the Sema domain and stabilizes its orientation (Kozlov et al., 2004). PSI domains have also been called MRS motifs (Met related sequences) (Maestrini et al., 1996).

Little is known about the function of *IPT domains*. They are about 90 residues long and have immunoglobulin-like fold with two β-sheets. They are rich in glycine and proline and are therefore sometimes referred to as GP-rich domains. Together with PSI domains IPT domains form a flexible stalk between Sema domain and the cell membrane. According to Yvonne Jones IPT6 interacts with the Sema domain and the extracellular part of plexins assembles in a ring-like structure (Y. Jones Semaphorin meeting 2013 personal communication) (Figure 2A).

Plexins have one *transmembrane* helix and this region may contribute to specific interactions within the plexin dimer or with co-receptors like

neuropilins (Sawma et al., 2014). Plexin-B1 and B3 TM helixes are regular and tend to form dimers whereas other plexins have residues in their TM region that distort association. Modelling showed that Plexin-B1 TM and juxtamembrane region can form trimers as well (Zhang et al., 2015).

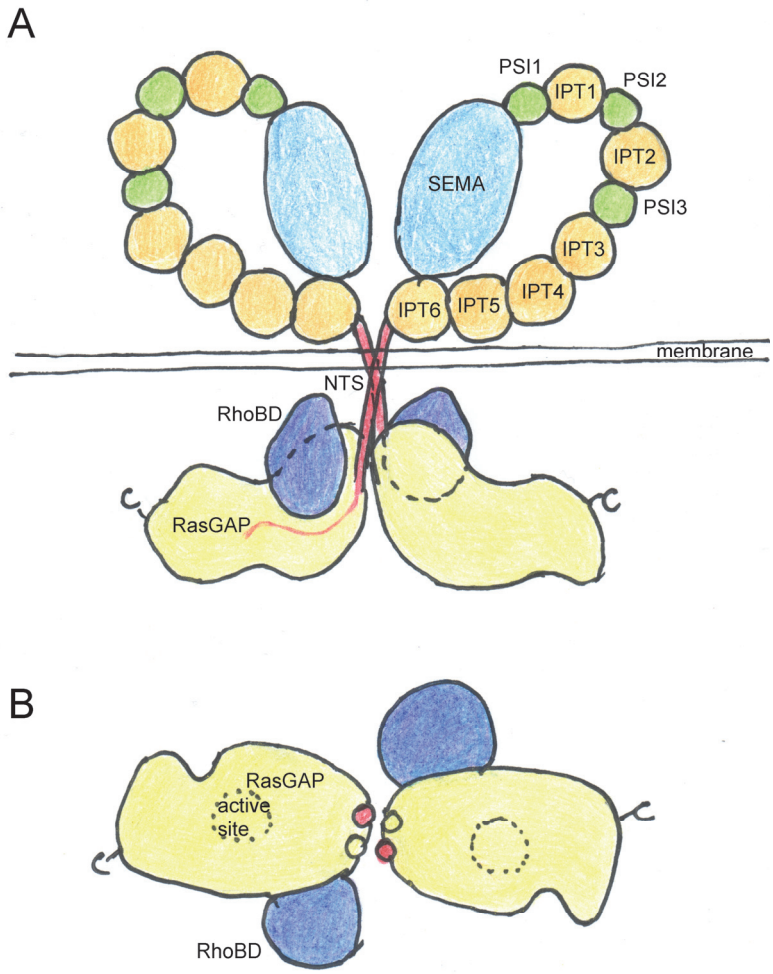


**Figure 2. Structure of the plexin dimer. (**A) Side view of a putative plexin structure. Extracellular part is based on Yvonne Jones personal communication and intracellular part is based on (Wang et al., 2013). See text for protein domains. (B) Top view of intracellular part.

The intracellular portion of plexins starts with the *NTS* that is also called juxtamembrane segment. It is about 70 residues in length and structurally consists of one longer and one shorter α-helix that are separated by a loop. In

monomeric crystal structures NTS adopts a kinked conformation with both the N-terminal and C-terminal halves interacting with the RasGAP domain (He et al., 2009, Wang et al., 2012). In dimeric structure the juxtamembrane helixes form a bundle with helixes 11 and 11' from the RasGAP domains and this bundle in essential for active dimer conformation (Wang et al., 2013). Conserved hydrophobic residues in the juxtamembrane helix stabilize the dimer (Barton et al., 2015).

Plexins have a characteristic bipartite *RasGAP domain* that is split by the RhoBD (He et al., 2009, Tong et al., 2009). Structurally the GAP domain of plexins is similar to canonical RasGAPs (such as p120GAP) and dual-activity GAPs (SynGAP and GAP1 family) that can inactivate Ras subfamily small GTPases (Wang et al., 2012). In primary structure the RasGAP domain of plexins is interrupted by the RhoBD, but it forms a separate module and minimally interferes with the GAP domain in tertiary structure (Wang et al., 2012). RasGAP domain consists of 18 α-helixes, and the overall shape resembles a bowl with the active site on the bottom of it, containing two conserved arginines that are essential for GAP activity (Tong et al., 2009).

The *RhoBD* extends out of the RasGAP domain and adopts an independent ubiquitin-like fold (Tong et al., 2007, He et al., 2009, Tong et al., 2009). Switch I and II regions of Rho GTPases interact with the hydrophobic patch on the plexin RhoBD that is distal to RasGAP domain and this does not cause any major conformational changes nor directly influences GAP activity (Wang et al., 2012).

The C-termini of B-plexins contain *PDZ-binding motif* TDL. It specifically interacts with PDZ domains of RhoGEFs (Swiercz et al., 2002, Perrot et al., 2002), but it does not bind PSD95 or other DLG family PDZ domains (Hirotani et al., 2002). This motif is essential for targeting plexins to the cell membrane and ligand induced cytoskeleton collapse (Artigiani et al., 2003).

Plexins are posttranslationally modified by different enzymes. Extracellular domains are glycosylated (Maestrini et al., 1996) and processed proteolytically. This cleavage by proteases converts plexins into heterodimeric receptors. Such receptors have increased semaphorin binding and functional response (Artigiani et al., 2003). Several intracellular tyrosine and serine/threonine residues are phosphorylated by different kinases regulating protein-protein interactions (Franco and Tamagnone, 2008, Yang and Terman, 2012).

Extracellular Sema domains of plexins are monomeric and are brought together by semaphorin dimers (Siebold and Jones, 2013). Deletion of the Sema domain is sufficient to shift the conformation towards the active state and induce cell or growth cone collapse. Soluble Sema domain can interact with the rest of the ectodomain and inhibit it (Takahashi and Strittmatter, 2001). So Sema domains keep plexins in inactivated state. Plexins are generally thought to form dimers mainly through their NTS helixes. Dimer conformation changes upon ligand binding enabling the activation of the GAP domain (Wang et al., 2013). There have also been suggestions that the intracellular domains of

Plexin-B1 could form trimers that are stabilized by Rac GTPase molecules that bind between RhoBD of one monomer and C-terminal half of the RasGAP domain of the next monomer (Bell et al., 2011). According to this model each semaphorin dimer would bind two separate plexin trimers inducing clustering of receptors.

## 1.6 Plexin signalling

In order to regulate cell migration, axon guidance and synaptogenesis plexins have to transmit extracellular signals to the machinery of cytoskeleton dynamics and membrane trafficking. Regulation of the activity of small GTPases is central in plexin signalling. Different plexins bind with different affinities to GTPases leading to specialized functional outcomes. In addition many other signalling pathways have been identified, but here I would be describing only those that are connected to GTPases, membranes and cytoskeleton (Figure 3).

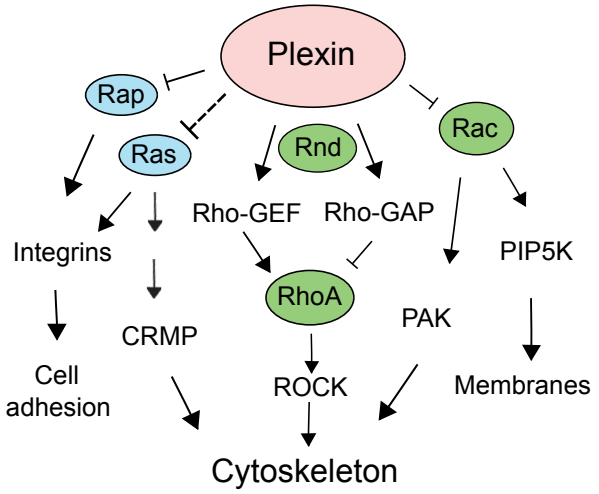


**Figure 3. Main signalling pathways of plexins.**

### 1.6.1 The superfamily of small GTPases

Small GTPases of the Ras superfamily act as molecular switches in cellular signalling. They cycle between an inactive GDP-bound state and an active GTP-bound form (Figure 4). The GDP/GTP cycle is promoted by the activity of two classes of molecules, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs induce the dissociation of GDP and assembly of the GTP-bound form, which is able to interact with downstream effectors. GAPs promote the hydrolysing activity of small GTPases, tilting the balance

toward GDP-bound inactive form. In addition, small GTPases can bind to proteins known as guanine nucleotide dissociation inhibitors (GDIs). GDIs sequester GTPases in their inactive state and protect them from degradation. The activity of GTPases is regulated also on transcriptional level and by posttranslational lipidation and phosphorylation that determines their subcellular localization (Rojas et al., 2012, Azzarelli et al., 2015).

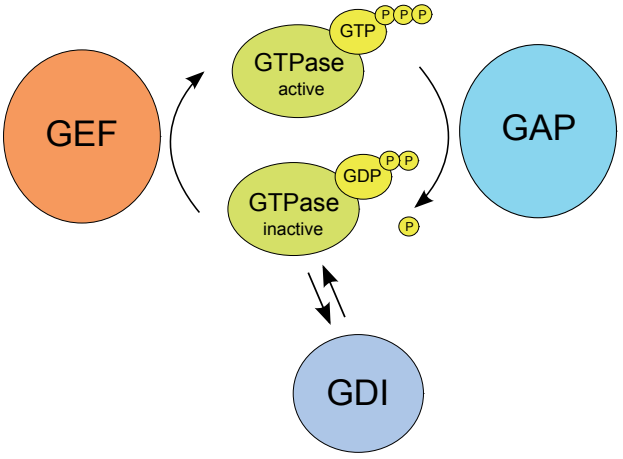


**Figure 4: Small GTPases cycle between active GTP-bound form and inactive GDP-bound form.** This cycle is regulated by different factors: GEF - guanine nucleotide exchange factor, GAP - GTPase activating protein, GDI - guanine nucleotide dissociation inhibitor. Modified from (Bento et al., 2013).

The Ras superfamily is divided into five major families: Ras, Rho, Arf, Ran, and Rab (Figure 5). Members of the Ras family act as regulators between receptors at the plasma membrane and effectors in the cytosol and they are missing in plants. Plants lack plexins and semaphorins as well. In general Ras GTPases regulate cell proliferation, differentiation, morphology, and apoptosis. The Rho family is involved in signalling networks that regulate cytoskeleton dynamics and cell polarity. The Rab family is by far the largest family of the Ras superfamily due to gene duplication. Rab family proteins regulate intracellular vesicular transport and the trafficking of proteins between different organelles via endocytotic and secretory pathways. In contrast, only one member of the Ran family is found in all eukaryotic lineages, with the exception of plants. Ran GTPase is ubiquitous in the cell and is involved in nuclear transport. Finally, the Arf family of proteins comprises the most divergent proteins, which, like the Rab family proteins, are involved in vesicle trafficking (Wennerberg et al., 2005, Iwashita and Song, 2008, Rojas et al., 2012).

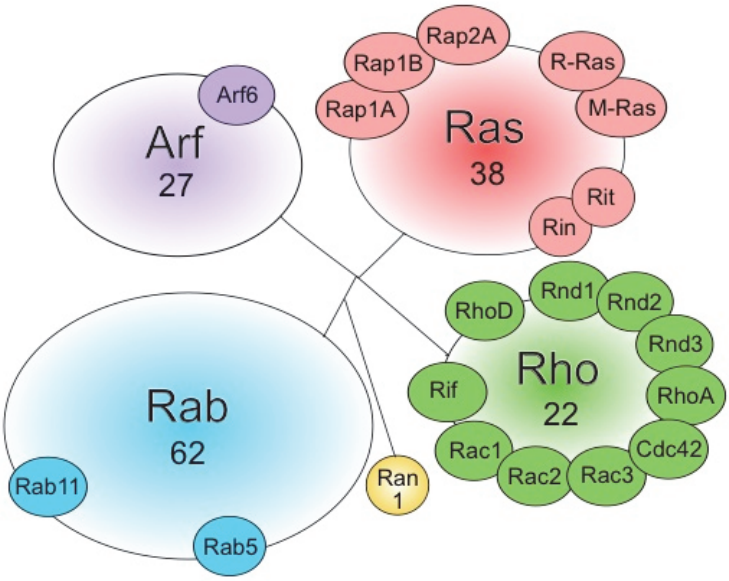


**Figure 5: The superfamily of human small GTPases** based on (Rojas et al., 2012). The number of members in each family is below the name. GTPases that have been implicated in plexin signalling are highlighted in separate bubbles.

### 1.6.2 RasGAP activity

Ras family proteins have different roles in regulating cytoskeleton dynamics, cell adhesion, migration, neuron polarization and neurite growth. There are 38 members in Ras family in humans (Rojas et al., 2012).

RasGAP domain of plexins constitutes the largest part of the intracellular domain and is the key signalling module during development. Transgenic mice with mutated GAP domains of Plexin-B2 or Plexin-D1 recapitulate the phenotypes of the respective null mutants in the developing nervous, vascular, and skeletal system (Worzfeld et al., 2014). Plexin RasGAP activity is required for semaphorin-induced cytoskeleton collapse (Oinuma et al., 2004), and regulation of dendritic spine density by Sema5A-Plexin-A2 (Duan et al., 2014). In *C. elegans* it has been shown that PLX-1 inhibits presynaptic formation via its RasGAP activity but it is dispensable for subcellular localization of PLX-1 (Mizumoto and Shen, 2013).

Two conserved catalytic arginine residues (Rohm et al., 2000) and semaphorin-induced dimerization (Wang et al., 2012) are essential for plexin GAP activity. In monomers RasGAP domain is in a closed conformation and inaccessible to small GTPases. Plexin dimerization induces relocation of the activation segment (GAP domain helixes 15-17) that in the resting state blocks the active site. This results in an open conformation with the active site oriented

towards the plasma membrane leaving enough space for membrane-bound small GTPases to fit in between (Wang et al., 2013) (Figure 6).

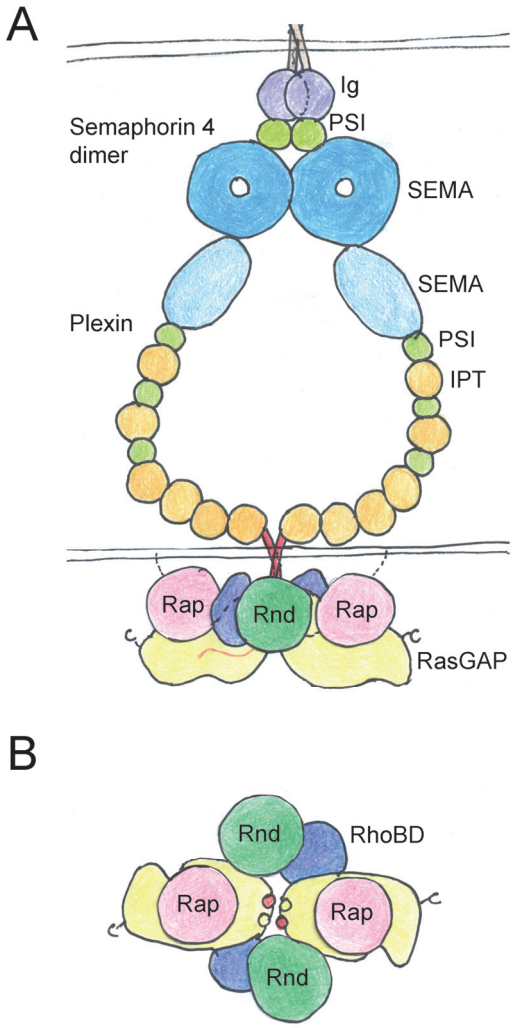


**Figure 6. Activated plexins bind Rap and Rnd GTPases.** (A) side view, (B) intracellular part from top. Based on (Wang et al., 2013).

At first it was suggested that plexins might be GAPs for Rho family GTPase Rnd (Rohm et al., 2000). Following biochemical and cellular studies by Oinuma and colleagues showed that plexins form complexes with R-Ras and M-Ras and act as GAPs for them decreasing the GTP-bound forms (Oinuma et al., 2004, Saito et al., 2009). R-Ras is important for axon growth (Oinuma et al., 2004) whereas M-Ras is in dendrites (Saito et al., 2009). However, later studies by other groups have failed to confirm direct GAP activity towards R-Ras and M-

Ras (Sakurai et al., 2010, Wang et al., 2012, Worzfeld et al., 2014). We have not been able to detect any GAP activity towards Ras family GTPases Rit and Rin (Rit2) either, although plexins interact with them (personal unpublished observations). Rit and Rin are atypical members of the Ras family with extended N- and C-termini and they have been implicated in neurite growth and neuron survival (Shi et al., 2013). Instead, plexins act directly as GAPs for the Rap branch of the Ras family. Plexin-B1 has GAP activity towards Rap1A and Rap1B but not Rap2A (Wang et al., 2012). Rap GTPases often act antagonistically to other Ras proteins (Ye and Carew, 2010). The initial confusion occurred because plexins inactivate R-Ras and M-Ras through an indirect mechanism. Active Rap inhibits p120 RasGAP and inactivation of Rap by plexins activates p120 RasGAP that in turn leads to enhanced GTP hydrolysis of R-Ras and M-Ras (Okada et al., 2015).

Recruitment of Rnd promotes GAP activity of most plexins in cells, only Plexin-C1 can do without them (Zanata et al., 2002, Oinuma et al., 2004, Uesugi et al., 2009, Okada et al., 2015). Controversially, in solution Rac1 addition had no positive effect on GAP activity (Wang et al., 2012) and mutating Rac1 binding residues (that greatly overlap with Rnd binding amino acids) did not abolish plexin GAP activity (Bell et al., 2011). Structurally Rac1 binds exclusively to the RhoBD and does not form any contacts with the GAP domain, nor does it induce notable conformational changes (Bell et al., 2011, Wang et al., 2012). Thus it is likely that Rnd or Rac binding to the RhoBD is required only in the cellular context for targeting plexin to the right location to enhance the possibility to meet Rap GTPases.

Furthermore, the GAP activity of plexins can be regulated by PKA phosphorylation of a serine residue in a close proximity to the second catalytic arginine. This generates a binding site for 14-3-3ε that prevents plexin interactions with Ras and Rap (Yang and Terman, 2012). Another RasGAP neurofibromin (NF1) is inhibited by a similar mechanism (Feng et al., 2004). In addition, 14-3-3 proteins are able to bind to Rnd and Rap1A C-terminal parts and inhibit them by translocating them from the plasma membrane (Riou et al., 2013).

Taken together, the RasGAP domain of plexins regulates directly the activity of Rap GTPases and indirectly R-Ras and M-Ras.

### 1.6.3 Rho GTPases

Mammalian Rho GTPases comprise a family of 22 proteins that regulate actin and microtubule components of the cytoskeleton. The best characterized members are RhoA, Rac1 and Cdc42. Different Rho GTPases are required for cell migration and protrusion formation. RhoA has a role in the initial events of protrusion and is activated at the cell edge. Rac1 and Cdc42 are activated behind the edge and with a temporal delay. They operate antagonistically to RhoA and activate pathways implicated in reinforcement and stabilization of newly

expanded protrusions (Machacek et al., 2009). In dendritic spine formation the situation is similar, RhoA-ROCK pathway is needed for initial spine growth and Cdc42-PAK pathway is required for sustained spine stabilization (Murakoshi et al., 2011).

Rnd1, Rnd2 and Rnd3 form a branch of atypical Rho GTPases. In contrast to classical GTPases, Rnd proteins do not show any intrinsic or stimulated GTPase activity. Rnd activity is controlled through gene expression, protein post-transcriptional modifications and subcellular localization. Interestingly, Rnd proteins evolved relatively recently and they are present only in vertebrates, indicating that they might be involved in more specialized neuronal functions than the other Rho GTPases (reviewed in Azzarelli et al., 2015). Rnd1 is involved in activity-dependent dendrite growth and dendritic spine formation (Ishikawa et al., 2006, Ishikawa et al., 2003).

Plexins are linked to Rho GTPases in multiple ways. The RhoBD of plexins interacts with certain members of the Rho family directly. Besides, plexins can recruit RhoGEFs, GAPs and GDIs.

***The role of RhoBD***

Several evidences indicate that preferential interactions occur between certain members of the plexin and the Rho families. Rac1 specifically interacts with the cytosolic domain of Plexin-B1 and B2, but not with that of Plexin-A1, A3, C1 or D1 (Vikis et al., 2000, Rohm et al., 2000, Driessens et al., 2001). In addition, the RhoBD of plexins can interact with other Rho family GTPases: Rnd1 binds to Plexin-A1, Plexin-B1 and Plexin-B3; Rnd2 with Plexin-B2, Plexin-B3 and Plexin-D1; Rnd3 with Plexin-B2; and RhoD with Plexin-A1 and Plexin-B1 (Vikis et al., 2000, Zanata et al., 2002, Oinuma et al., 2003, Uesugi et al., 2009, Fansa et al., 2013, Azzarelli et al., 2014, and personal unpublished observations).

Rac or Rnd binding to the RhoBD of plexins helps to target plexins to the plasma membrane and stabilize them there (Vikis et al., 2000, Swiercz et al., 2004). Rnd binding enhances plexin RapGAP activity in cells and also interaction with RhoGEFs and activation of RhoA. Besides, active Rac1 enhances Plexin-B1 binding to its ligand Sema4D. Sema4D in turn stimulates the interaction between plexin-B1 and active Rac1 (Vikis et al., 2000). On the other hand plexins compete with other proteins for Rnd and Rac binding and thus antagonize several signalling pathways. For example, Plexin-B1 competes with Rac1 binding with PAK (p21 activated kinase) that has a role in mediating cytoskeletal changes and axon guidance (Vikis et al., 2002), and Plexin-B2 inhibits Rac1-mediated cell motility (Roney et al., 2011).

***Plexins and Rho GEFs, GAPs and GDIs.***

Besides direct interaction with Rho family GTPases plexins can interact with different Rho regulators. B-plexins have a PDZ-binding motif TDL at their C-terminus that was shown by five different groups in 2002 to interact with PDZ-

domains of PDZ-RhoGEF (ARHGEF11) and LARG (leukemia-associated RhoGEF, ARHGEF12) (Swiercz et al., 2002, Perrot et al., 2002, Aurandt et al., 2002, Hirotani et al., 2002, Driessens et al., 2002). B-plexins are necessary to recruit RhoGEFs to the plasma membrane and activation of plexins with semaphorins leads to localized RhoA activation, cytoskeleton remodelling, and promotion of cell migration. A-plexins interact with FARP1 and FARP2, GEFs that activate Rac1, that are important for neurite remodelling (Toyofuku et al., 2005, Cheadle and Biederer, 2014, Mlechkovich et al., 2014).

RasGAP activity and regulation of RhoA function independently of each other (Oinuma et al., 2004, Sun et al., 2012). Rnd binding to the RhoBD promotes the interaction between plexins and RhoGEFs and thereby stimulates RhoA activity (Oinuma et al., 2003, Azzarelli et al., 2014). Another component of the RhoA regulation system is receptor tyrosine kinases. ErbB-2 of the EGF receptor family forms a complex with Plexin-B1 extracellular part (Plexin-B2 and B3 as well) and phosphorylates conserved tyrosine residues in the intracellular domain, generating binding sites for PLCγ. PLCγ facilitates interaction between B-plexins and RhoGEFs and activates RhoGEFs independently of its lipase activity. (Swiercz et al., 2004, Swiercz et al., 2009). In contrast, when Plexin-B1 is phosphorylated by HGF receptor Met, an adaptor protein Grb2 is bound and it recruits p190 RhoGAP to the complex leading to RhoA inactivation and inhibition of cell migration (Sun et al., 2012). Interestingly, tyrosine residues that are used to generate binding sites for PLCγ and Grb2 are conserved in all human plexins except for Plexin-B3. That statement is true for other organisms as well. This suggests that Plexin-B3 utilizes different mechanisms for regulation of Rho GTPases. Indeed, Sema5A activated Plexin-B3 interacts directly with RhoGDIα and promotes Rac1 binding to RhoGDIα thereby preventing Rac1 activation (Li and Lee, 2010).

A different mechanism for RhoA activation has been described for Plexin-B2 that competes with p190 RhoGAP for Rnd3 binding and fine-tunes the level of RhoA activity. p190 RhoGAP is recruited to the plasma membrane by Rnd3 and RhoA activity is inhibited. When Rnd3 is sequestered by Plexin-B2 it can no longer bind p190 RhoGAP and RhoA remains active (Azzarelli et al., 2014).

### 1.6.4 Membranes, endocytosis and receptor trafficking

Plexins are transmembrane receptors that can complex with various co-receptors. They regulate the activity of small GTPases that in general are associated with membranes. Very often the existence of the membrane is forgotten and its part in the assembly of protein complexes and as a source of lipid second messengers is discarded. The balance between membrane addition and removal dictates bidirectional axon guidance. Growth cone attraction involves asymmetric exocytosis while locally applied repellents like Sema3A facilitate endocytosis at that side only (Tojima et al., 2010). Sema3A induces a coordinated rearrangement of its receptors, Rac1 and F-actin during growth

cone collapse (Fournier et al., 2000). In response to negative guidance molecules, the function of Rac1 changes from promoting actin polymerization associated with axon growth to driving endocytosis of the plasma membrane, resulting in growth cone collapse (Jurney et al., 2002).

Upon Sema3E activation Plexin-D1 recruits PIP5K and Arf6 GEF GEP100/Brag2a facilitating the activation of Arf6 and subsequent retraction of cell protrusions (Sakurai et al., 2011). Arf6 participates in clathrin-independent endocytosis of different receptors including glutamate AMPA receptors and integrins (Vidal-Quadras et al., 2011, Scholz et al., 2010, Eva et al., 2012).

Plexin-A1 associates with Rab5, a small GTPase protein that regulates the motility and fusion of early endosomes. The interaction is mediated by the membrane fusion protein Rabaptin-5. Sema3A application stimulates Rab5 activation and endocytosis from axon growth cones (Wu et al., 2014).

Additionally, semaphorins regulate lipid kinases such as phosphatidylinositol-3-kinases (PI3Ks) (Basile et al., 2005) and phosphatidylinositol-4-phosphate 5-kinases (PIP5Ks) (Halstead et al., 2010, Binmadi et al., 2011). These kinases phosphorylate phosphatidylinositol in cellular membranes increasing locally negative charge (Goldenberg and Steinberg, 2010) that is important for targeting positively charged small GTPases to the plasma membrane (Heo et al., 2006). The action of lipid kinases is balanced by the lipid phosphatase PTEN (phosphatase and tensin homolog). Plexin-B1 activates PTEN and this promotes hippocampal neuron growth cone collapse (Oinuma et al., 2010).

### 1.6.5 Cytoskeleton

One of the main functions of plexins is to translate extracellular signals to cytoskeleton dynamics within the cell. Mammalian cytoskeleton consists of three main components: microfilaments of actin, intermediate filaments and microtubules consisting of tubulins. During cell motility different components of cytoskeleton are well co-ordinated. Activation of plexins, upon binding with their ligands semaphorins or by overexpressing some of the key components of the downstream signalling cascade can result in the collapse of the cytoskeleton or in contrast, promote cell adhesion or migration.

Plexins influence actin cytoskeleton largely through regulation of small GTPases. Integrin-based focal adhesive structures are disassembled within minutes upon plexin activation; this is followed by actin depolymerisation and, eventually, by cellular collapse (Barberis et al., 2004). Apart from signalling pathways of small GTPases some other mechanisms for actin regulation have been described. Plexin-B3 interacts with Fascin-1 an actin binding and bundling protein. Sema5A stimulation induces Fascin-1 phosphorylation by PKC, redistribution of Fascin-1 from the cytosol to protrusions, the disassembly of F-actin stress fibers and alterations in cell morphology (Li et al., 2012).

The cytoplasmic part of A-plexins associates with MICAL family enzymes. MICAL binds F-actin and oxidizes the methionine 44 residue within the D-loop of actin. This leads to disassembly of actin filaments as well as bundles (Hung et al., 2010, Hung et al., 2011). MICAL-induced actin remodelling is involved in the targeting of secretory vesicles containing immunoglobulin superfamily cell adhesion molecules (IgCAMs) to the neuronal growth cone membrane (Van Battum et al., 2014). Semaphorin activation promotes the formation of Plexin/MICAL/CRMP complex that releases MICAL enzymatic autoinhibition. In addition to actin regulation MICAL influences CRMP (collapsin response mediating protein) activity (Schmidt et al., 2008). CRMPs are well-known downstream components of semaphorin-plexin signalling that regulate microtubule cytoskeleton dynamics. Plexins do not interact directly with CRMPs, but rather plexins modulate small GTPases, which in turn activate kinases that phosphorylate CRMPs (Uchida et al., 2005, Ito et al., 2006). The effects of CRMP4 on axon growth and growth cone morphology are dependent on microtubule assembly, whereas filopodial extension relies on actin bundling (Khazaei et al., 2014). So MICALs and CRMPs have a dual role regulating both microtubules and actin.

A multi-domain scaffolding protein RanBP9 (Ran binding protein 9) interacts with Plexin-A1 intracellular domain and with α-tubulin (Togashi et al., 2006, Salemi et al., 2015). This complex may be involved in regulating microtubule dynamics. In addition, RanBP9 overexpressing transgenic mice have decreased levels of phosphorylated cofilin, an actin binding protein, and this correlates with reduced numbers of dendritic branches and dendritic spines (Wang et al., 2014).

## 1.7 Microtubule dynamics and neurons

Modulation of the cytoskeleton is essential for a variety of cellular processes. In post-mitotic neurons microtubules participate in migration, axon guidance, growth of dendrites and formation of synapses. In neuronal migration the leading edge microtubule plus-ends are captured at the actin cortex through calcium-dependent mechanisms and promote the translocation of the nucleus (Hutchins and Wray, 2014). One of the most important functions of microtubules is to establish a trafficking route for cellular cargos to sites where resources are needed the most, like growth cones of neurons. Microtubules in the growth cone are oriented with their plus-ends towards the periphery and switch between phases of growth and shortening. Asymmetric stabilization or destabilization of microtubules induces growth cone turning (Liu and Dwyer, 2014). Direct link between guidance cues and microtubules have recently been demonstrated for Netrin-1 receptor DCC that binds to neuron-specific β-tubulin isotype III (TUBB3) during axon attraction (Qu et al., 2013). Extracellular guidance cues modulate microtubule dynamics in axon growth cone steering also more indirectly influencing microtubule-associated proteins (MAPs) and

microtubule plus-end binding proteins +TIPs (reviewed in Liu and Dwyer, 2014).

In recent years, more attention has been paid to the role of microtubules in the formation and maintenance of connections between neurons. Microtubules are essential for dendritic spine maturation and synaptic plasticity, which are prerequisites for learning and memory formation (Gu et al., 2008). The morphology of dendritic spines depends on bidirectional interplay between actin and microtubule dynamics. Microtubule tips enter activated spines and promote their enlargement accompanied by increased excitatory synapse strength. Spine invasion by dynamic microtubules is relatively infrequent and transient (Jaworski et al., 2009). Microtubules enter spines from highly localized sites at the base of spines in response to synapse-specific calcium transients. Local actin polymerization and drebrin, a protein known to mediate interactions between F-actin and microtubules, act as positive regulators of microtubule entry into spines (Merriam et al., 2013). BDNF activation of its TrkB receptors prolonged the average dwell time of microtubules in dendritic spines and increased the amount of postsynaptic PSD95. Accumulation of PSD95 is associated with LTP (Hu et al., 2011). Synaptic NMDA receptor activation and subsequent LTP promoted microtubule spine invasions and lasting increases in spine size (Merriam et al., 2011). Conversely, NMDA-dependent LTD suppresses microtubule entry in dendritic spines and induces spine shrinkage (Kapitein et al., 2011).

## 1.8 Microtubule end binding EB/MAPRE family of proteins

End binding proteins (EBs) are highly conserved core components of microtubule plus-end tracking protein network. Mammalian cells express three members of the EB/MAPRE family—EB1, EB2, and EB3. EBs are relatively small proteins: EB1 268 aa, EB2 327 aa, EB3 281 aa. Overall their amino acid sequence is similar. EB2 has additional 43 aa at its N-terminus that may be omitted due to inefficient translation starting at the first methionine. An in-frame alternative splice variant of EB3 lacks codons 142-156 resulting in a 15 aa deletion in calponin homology domain (Juwana et al., 1999, Su and Qi, 2001). EB1 and EB2 are ubiquitously expressed, yet EB3 is found predominantly in the CNS and muscle (Nakagawa et al., 2000).

EBs consist of N-terminal calponin homology (CH) domain, responsible for the interaction with microtubules, a proline-rich linker region, and a C-terminal coiled coil domain (EB homology domain) that extends into a four-helix bundle, required for dimer formation and binding to various partners. It forms a hydrophobic pocket where +TIPs with the conserved SxIP motif bind. In the extreme C-terminus EB-s have a EEY motif that resembles alfa-tubulin C-terminus and interacts with CAP-Gly domain containing proteins (Akhmanova and Steinmetz, 2008, Slep, 2010). The overall shape of EB dimer resembles a asymmetric golf club (Buey et al., 2011) (Figure 7).

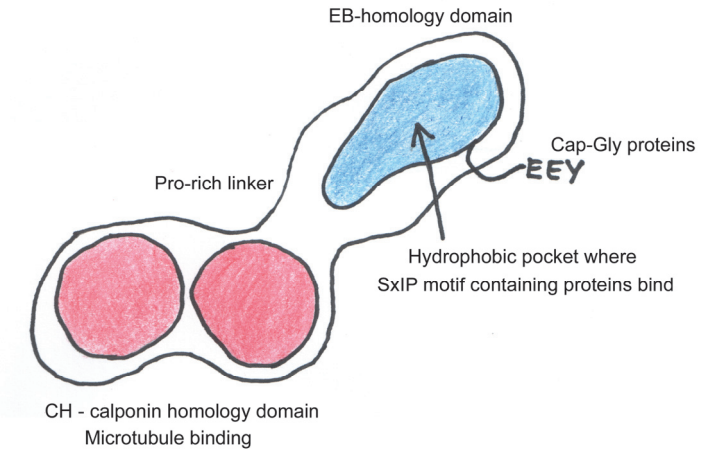


**Figure 7: Schematic structure of EB dimer** based on Akhmanova and Steinmetz, 2008 and Buey et al., 2011.

EBs interact with most other known +TIPs and recruit many of them to the growing microtubule ends (Bu and Su, 2003, Akhmanova and Steinmetz, 2008). EB1 and EB3, but not EB2, promote persistent microtubule growth by suppressing catastrophes. While dimerization is not essential for microtubule tip tracking by EBs it is a prerequisite for +TIP partner binding and anti-catastrophe activity in cells (Sen et al., 2013, Komarova et al., 2009).

### 1.8.1 Role of EBs in neurons

In the CNS EB1 and EB3 are important for neuron maturation. EB1 protein levels increase during axon formation (Morrison et al., 2002) and EB1 accumulates at the axonal endings in cultured rat hippocampal neurons (Gu et al., 2006). While EB1 is more abundant in young neurons and glial cells, EB3 characteristically concentrates at the microtubule tips in the dendrites and dendritic spines of mature neurons (Jaworski et al., 2009, Stepanova et al., 2003). *In vivo* EB3 expression levels are higher in the adult hippocampus and cortex than during embryonic and postnatal stages, and EB3 is required for normal dendritic spine maturation and plasticity (Gu et al., 2008, Jaworski et al., 2009).

EB1 and EB3 associate with members of the spectraplakin family, conserved scaffolding proteins that link different cytoskeleton components, and this is needed for vesicular transport and axon growth (Poliakova et al., 2014, Alves-Silva et al., 2012). Besides, EB3 interacts with different proteins that regulate the actin cytoskeleton, such as drebrin in advancing growth cones (Geraldo et al., 2008) and p140Cap in dendritic spines (Jaworski et al., 2009). EB3 interacts

also with PSD95, contributing to the regulation of microtubule dynamics during dendrite formation (Sweet et al., 2011).

Tau, MAP1B, and MAP2 are classical microtubule associating proteins (MAPs) that promote microtubule nucleation, polymerization, dynamics and stabilization in neurons. While MAP1B and Tau mainly localize to axons, MAP2 is present in dendrites (Sayas and Avila, 2014). It has been shown that EBs interact directly with all three classical neuronal MAPs. Tau is required for the proper accumulation of EBs at stretches of microtubule bundles at the medial and distal regions of the axon (Sayas et al., 2015). MAP1B inhibits EB binding to microtubule plus-ends (Tortosa et al., 2013) maintaining direct microtubule growth that suppresses axon branching and enhances axon elongation (Tymanskyj et al., 2012). The interaction of EB3 with MAP2 in adult neurons is modulated by neuronal activity. Under basal conditions, EB3 and MAP2 are both abundantly present in dendrites but they interact with different populations of microtubules; EB3 binds to the plus-ends of dynamic microtubules and MAP2 decorates stable microtubules. Induction of LTD by NMDA leads to the rapid removal of EB3 from microtubule plus-ends in dendritic spines leading to their shrinkage. EB3 is recruited along microtubule bundles in the dendritic shaft through direct interaction with MAP2 (Kapitein et al., 2011).

# 2. AIMS OF THE STUDY

In 2003 when I started my PhD studies very little was known about Plexin-B3. The original aim of this study was to characterize downstream signalling pathways of Plexin-B3 and their functional significance, concentrating on its previously identified interaction with a small GTPase Rin (Rit2). Later my focus shifted to microtubule end binding proteins (EBs) that came up in a yeast two-hybrid screen as potential interactors of Plexin-B3.

The aims of the current study were to:

1) verify the interaction between Plexin-B3 and microtubule end binding proteins EB1, EB2 and EB3;
2) study how B-plexins regulate microtubule dynamics;
3) describe subcellular localization of B-plexins in neurons;
4) characterize the influence of B-plexins on dendrite growth in rat hippocampal neurons;
5) analyse the role of B-plexins in synaptogenesis.

While concentrating on my main tasks I got interested also in the aspects of plexin and semaphorin phylogeny. An overview of this is provided as a bonus.

# 3. MATERIALS AND METHODS

Following methods were used in this study.

- Standard molecular cloning procedures to generate necessary plasmids for expression of different proteins (publication I, II)
- Site-directed mutagenesis with PCR (publication I and III)
- Cell culture and transfections (publications I, II, III)
- Bacterial expression and purification of proteins (publication I)
- GST pull-down assay (publication I)
- Co-immunoprecipitation (publication I)
- Western blot (publication I, III)
- Bioinformatic analysis (publication I and unpublished)
- Neurite outgrowth assay with neuroblastoma cell line Neuro2A (publication I)
- Recombinant Sema4D-Fc production in 293FT cell line (publication II)
- RNA extraction and quantitative PCR (publication II)
- Immunocytochemistry and confocal microscopy (publication II, III)
- Live cell imaging with total internal reflection (TIRF) microscopy (publication II)
- Analysis of microtubule tip dynamics (publication II)
- Analysis of dendrite growth including Scholl analysis (publication II)
- Analysis of synapse volume (publication III)
- Statistical analysis (publication I, II, III)

# 4. RESULTS AND DISCUSSION

## 4.1 Semaphorins and plexins in different organisms

In addition to my main objectives I studied the phylogeny of semaphorins and plexins (Figure 8) as no thorough descriptions on this subject have been published, and recently several full genomes of interesting organisms have become available. For reference protein sequences I used NCBI gene database (http://www.ncbi.nlm.nih.gov/gene) and for searching and comparing protein sequences of semaphorins and plexins of different organisms I used NCBI protein blast (http://blast.ncbi.nlm.nih.gov).

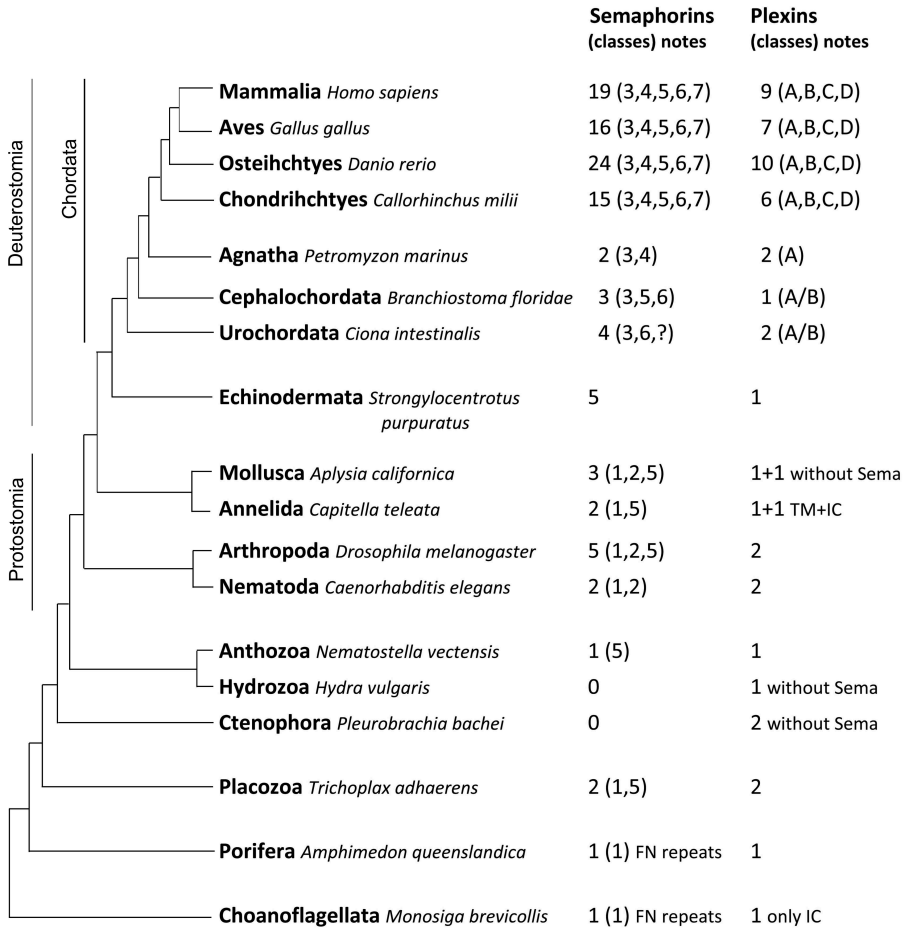


**Figure 8: Semaphorins and plexins in different phylogenetic groups.**
TM – transmembrane domain, IC – intracellular domain, FN – fibronectin.

Semaphorins and plexins are characteristic for multicellular animals as would be expected for proteins that mediate cell-cell communication. No plexins and semaphorins are known from prokaryotes, unicellular organisms, plants or fungi. Exceptional is a single cell choanoflaggellate *Monosiga brevicollis*, who forms colonial structures and in many aspects is similar to higher organisms. This little creature has a 441 aa protein XP_001744227 that is remarkably similar to the intracellular domain of plexins. It has a RasGAP domain that is split by a Rho-binding domain. *Monosiga* has also Sema and PSI domains containing 1764 aa transmembrane protein XP_001747329 that can be the ancestor of semaphorins and extracellular domains of plexins. In sponge *Amphimedon queenslandica* there is a protein similar to full-length plexins XP_003382745 and also one transmembrane semaphorin XP_003384929 that is similar to *Monosiga* protein as it contains fibronectin repeats. No semaphorins could be detected from ctenophores and hydras, and they have plexins without the Sema domain. Similar plexins devoid of the Sema domain are present in the genomes of annelids and snails as well. This implies that plexins could function as receptors through a mechanism that does not involve semaphorins. Nematode *C. elegans* has two transmembrane semaphorins whereas *Drosophila* has five semaphorins (1a, 1b, 2a, 2b and 5c), and both have two plexins. It appears that transmembrane semaphorins (most similar to class 6 vertebrate semaphorins) are more ancient than secreted ones. Also class 5 semaphorins, characterized by thrombospondin repeats, have emerged quite early in evolution being present in placozoas, corals and most other invertebrate groups.

Primitive chordates resemble invertebrates in the aspect of semaphorin and plexin gene numbers. Their plexins are most similar to vertebrate A-plexins but at the same time, share multiple characteristic features with B-plexins. In sea lamprey *Petromyzon marinus,* who occupies a key position close to the root of the vertebrate phylogenetic tree, two semaphorins (Sema3 and Sema4) and two plexins (similar to PlxnA1 and PlxnA4) were identified (Shifman and Selzer, 2006). The gene duplication event that gave rise to different semaphorin and plexin subfamilies must have occurred after the divergence of jawed vertebrates from jawless fish. Invertebrates and lamprey do not have any neuropilins, they are used as co-receptors for A-plexins only in vertebrates (Fujisawa, 2004, Shifman and Selzer, 2006). Vertebrates have usually nine plexins. Fish have more due to an additional duplication in their genomes and birds have only seven as they lack the X-chromosome that harbours *PlxnA3* and *PlxnB3* (Mauti et al., 2006). Humans (and other mammals) have nine plexins A1-A4, B1-B3, C1 and D1, and 19 semaphorins 3A-3F, 4A-4G (mammals lack Sema4E), 5A and 5B, 6A-6D and 7A.

## 4.2 Plexin-B3 interacts with EB-family proteins

### 4.2.1 Yeast two-hybrid screen (publication I)

In order to find novel interaction partners for Plexin-B3 intracellular part a yeast two hybrid screen was performed by Kaie Pill. Cytotrap system (Stratagene) was selected as it screens protein-protein interactions at the plasma membrane versus a conventional GAL4 system that is localized to the nucleus. With a GAL4-based yeast two-hybrid screen Nakayama and colleagues had identified seven potential interactors of Plexin-B3 intracellular domain: PDZ domain containing RhoGEF ARHGEF11 (a well-known binding partner of Plexin-B1), ubiquitin ligase complex proteins cullin 7 and cullin 9, scaffolding molecules MAGI2 and MAGI3 that are associated with cell adhesion sites, BTBD3 that controls dendrite orientation (Matsui et al., 2013), and extracellular matrix protein SPOCK2 (Nakayama et al., 2002, http://www.ncbi.nlm.nih.gov/gene).
Since Plexin-B3 is prominently expressed in the brain (Hartwig et al., 2005), we performed the yeast two-hybrid screen using human foetal brain cDNA library and Plexin-B3IC (amino acids 1274-1909) as a bait. 14 putative positive clones were isolated. Sequencing revealed that only three of those were in frame and encoded C-terminal domains of microtubule end binding proteins EB1 and EB3, and small GTPase Rin. Interaction between Plexin-B3 and Rin has been described (Hartwig et al., 2005). The clones containing EB1 and EB3 were further analysed in the yeast system to verify the interaction. Control experiments were performed also with EB2, which showed weaker binding affinity towards Plexin-B3IC. Plexin-B2IC construct failed to give positive results with any of the EB-family proteins in the yeast two-hybrid assay, indicating the specificity of the interaction.

Cytotrap is based on Ras activation pathway and this may be one of the reasons why we got relatively few positive clones. Plexins influence Ras pathway and could somehow interfere with the screening system. However, we obtained two interesting candidates for Plexin-B3 binding – microtubule end binding proteins EB1 and EB3.

### 4.2.2 EB homology domain binds to the SxIP motif in the NTS of plexins (publication I and unpublished)

To verify the interaction between EBs and Plexin-B3 we used GST pull-down assay. GST-tagged EB1, EB2 and EB3 were all able to form complexes with overexpressed 3xFLAG-Plexin-B3IC as well as endogenous full-length Plexin-B3 from rat cerebellum lysate. Co-immunoprecipitation experiments of endogenous proteins were not successful due to the fact that neither EB3 nor Plexin-B3 antibodies that we used work in IP. Deletion of the N-terminal

calponin homology domain of EBs had no adverse effects, thus the C-terminal EB homology domain is the one interacting with Plexin-B3.

To map the EB-binding site on Plexin-B3IC a series of deletion constructs were generated and their ability to bind to EBs was assessed. I tried several Plexin-B3 mutants with little success to map the EB-binding site. However, my luck turned in 2009 when EB-binding consensus sequence SxIP was described by Honnappa and colleagues (Honnappa et al., 2009). Notably, the NTS of Plexin-B3IC (amino acids 1283-1364) contains this motif. Mutations affecting RasGAP domain or RhoBD had little or no effect on EB binding, whereas deletion of the NTS of Plexin-B3IC abrogated the interaction. Later a point mutation was introduced in the SxIP motif, where residues Ile1328 and Pro1329 were replaced by Asn and Ala respectively. GST-EB3 only weakly interacted with this mutant (Figure 9) confirming our hypothesis that SxIP motif residing between Plexin-B3IC NTS helixes 1 and 2 is responsible for the interaction with EBs.

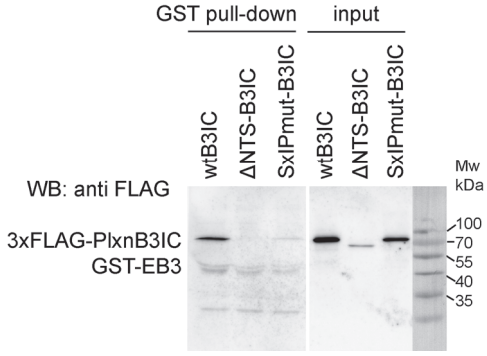


**Figure 9. GST-EB3 interacts with wild type (wt) Plexin-B3IC but not ΔNTS and weakly with SxIP mutant.** Plexin-B3IC constructs in p3xFLAG were overexpressed in 293FT cells and subjected to GST pull-down with affinity purified GST-EB3. Plexin-B3IC proteins were detected with anti FLAG.

Comparison of Plexin-B3 protein sequences of different mammals indicated that the EB-binding motif and the surrounding region are well conserved. The same applies for Plexin-B1. EB-binding motif is also present in some plexins of lower vertebrates and *Drosophila* Plexin-B, strongly suggesting that this interaction may be evolutionarily conserved. The EB-binding motif corresponding to the consensus sequence SxIP was found in human Plexin-A2, B1 and B3 but not others. GST pull-down with different plexins showed that EB1 formed complexes with Plexin-A2, B1 and B3. At the same time Plexin-B2 failed to interact with any of the EB-family proteins. This could be explained by the Ser>Ala substitution in the EB-binding motif in Plexin-B2 (Figure 10), and

is consistent with yeast two-hybrid results. Sequences that bind EBs have been thoroughly analysed (Buey et al., 2012, Leśniewska et al., 2014), and it has become clear that critical for EB binding are the SxIP motif, one to two preceding amino acids, and four to seven that follow. Within the SxIP motif serine can be substituted for threonine, isoleucine for leucine, and proline is irreplaceable. In the position of x positively charged Lys and Arg residues are preferred, but other amino acids are also tolerated. In plexins there is glycine that by Buey et al is considered to be unfavourable (Buey et al., 2012), but other studies show that SGIP sequence effectively binds to EBs (Jiang et al., 2012, Leśniewska et al., 2014). In general negatively charged Asp and Glu in the surroundings of the SxIP motif diminish EB binding and hydrophobic or positively charged ones favour it. The composition of Plexin-B1 and B3 EB-binding regions suggests that they bind with moderate affinity to EBs and Plexin-B1 should bind better as in -2 position it has a hydrophobic Leu and Plexin-B3 has a negatively charged Glu. EB-binding can be abolished by phosphorylation that adds negative charge (Buey et al., 2012). In Plexin-B3 EB-binding region there are two Ser, one Thr and two Tyr residues that potentially might be targets of protein kinases, but their phosphorylation analysis awaits further investigation.


**Figure 10. EB-binding motifs and their surroundings in human B-plexins.** Residues that bind EBs with high affinity are in black, weakly binding residues in grey and residues that abrogate interaction with EBs in red.

Single SxIP motifs bind relatively weakly to EBs, but this can be significantly enhanced by forming protein oligomers (Buey et al., 2012), and plexins may form dimers (Wang et al., 2013).

Other proteins with the SxIP motif (APC, MACF2, CLASP2, MCAK etc.) bind to the highly conserved hydrophobic cleft in the C-terminal domain of EBs. The EB-binding region is relatively unstructured in described proteins (Honnappa et al., 2009). Similar situation is in Plexin-B1, and presumably also in Plexin-B3, as the SxIP motif resides in a loop between two NTS helixes at the bottom of the RasGAP domain (Tong et al., 2009, Wang et al., 2013), and should be accessible for other proteins. It is very likely that EB-plexin

interaction is conformation dependent and the availability of EB-binding motif is dynamically regulated by semaphorins.

In conclusion, the interaction between EBs and plexins takes place between the EB homology domain and the SxIP motif in the NTS, and is restricted to certain plexins.

## 4.3 Plexins influence microtubule dynamics (publication II)

### 4.3.1. B-plexins control movement of microtubule tips

What is the functional meaning of EB-plexin interaction? EB1 and EB3 are important for microtubule growth in neurons and also in other cells. They stabilize MTips and promote microtubule polymerization (Akhmanova and Steinmetz, 2008). Plexins are guidance molecules that influence cytoskeleton dynamics so that the growth cone of an axon or a dendrite turns and follows the right path. They also govern fasciculation and branching (Pasterkamp, 2012). Localized changes in microtubule dynamics are an important component of the growth cone response to extracellular signals. Positive cues promote microtubule growth but repellents reduce microtubule polymerization rate (Kelly et al., 2010). In epithelial cells HGF induces reorganization of cytoskeletal structures and increases microtubule growth rate EB1 dependently (Gierke and Wittmann, 2012). In Myoblasts EB3 is necessary for the regulation of microtubule dynamics and microtubule capture at the cell cortex, which in turn regulates the formation of polarized membrane protrusions and cell fusion (Straube and Merdes, 2007). It is possible that plexin activation by semaphorins alters microtubule dynamics to generate cellular response. Another option is that vesicles containing plexins are transported to the plasma membrane during exocytosis and afterwards recycled to endosomes in a manner that is mediated by EBs and microtubules.

So we decided to investigate how plexins influence microtubule dynamics and whether the interaction with EBs makes any difference. To follow microtubule tips in live NIH3T3 cells we used EB3-GFP that had been used in previous studies as a reliable microtubule plus end marker (Stepanova et al., 2003). Single cells were imaged with TIRF microscopy with a 0.5 second interval. Ten longest EB3-GFP tracks per cell were manually selected and converted to kymographs. Microtubule growth rate (velocity) and characteristics of MTip dynamics (the number of rescues, catastrophes and pauses) were measured.

Plexin-B1IC treatment generated characteristic jagged kymographs with short phases of growth interrupted by catastrophes or pauses indicating increased dynamic instability of microtubule tips. Plexin-B2IC and Plexin-B3IC slightly increased the number of rescues, but to a lesser extent than Plexin-B1IC. As Plexin-B3 should bind to EBs with lower affinity compared to Plexin-

B1, it is reasonable that it had a milder effect. Plexin-B2 influence on microtubule tips may be indirect or mediated by other yet unidentified factors. Recently it was shown that Plexin-B2 regulates the orientation of the mitotic spindle (a structure based on microtubules) in kidney epithelium through its RasGAP activity and Cdc42 (Xia et al., 2015). When the interaction between Plexin-B3 and EBs was disturbed using ΔNTS-B3IC, we observed a reduction of the effect of Plexin-B3IC on microtubule tip behaviour.

When we analysed the growth rate of microtubules, then it appeared that B-plexins that are unable to bind to EB-s (B2IC and ΔNTS-B3IC) accelerated MTips, whereas Plexin-B1IC and wild type B3IC were similar to the control group. Thus plexins can promote microtubule growth via pathways that are not dependent on direct EB interaction, indicating that additional factors are involved. Possible candidates are certainly RhoA (Azzarelli et al., 2014), Rnd1 (Li et al., 2009) or other Rho-family GTPases that function as regulators of cytoskeleton.

It appears that plexins can affect microtubule dynamics in different ways. B-plexins can increase the velocity of microtubules through EB-independent pathways, but when they are able to bind to EBs plexins induce more catastrophes and pauses at microtubule tips thereby slowing them down. This demonstrates that plexins regulate the balance between microtubule growth and dynamic instability. We propose a model that upon semaphorin binding the conformation of plexin changes, enabling it to transiently sequester EB1 or EB3 from microtubule tips resulting in destabilization and retraction of microtubules (Figure 11).

### 4.3.2 Sema4D increases microtubule plus end dynamics in neurons

To further verify the role of B-plexins in regulating microtubule dynamics, rat hippocampal neurons were treated with the ligand of B-plexins – Sema4D. EB3-GFP was imaged and tracked in axons as well as dendrites. Consistent with earlier studies (Stepanova et al., 2003) the growth rate of EB3-GFP comets was twofold smaller in neurons than in fibroblasts. At the same time microtubule tips were more dynamic, having more catastrophes, pauses and rescues. This could be explained by the more confined space in neurites than in the fibroblast cell body. Sema4D treatment had a similar effect as Plexin-B1IC overexpression – the number of dynamic events increased and as a consequence microtubule velocity declined. It is very likely that Sema4D influences microtubule tips in neurons through Plexin-B1, as it is the high-affinity receptor for Sema4D (Tamagnone et al., 1999).

Taken together, Sema4D modulates the dynamic behaviour of microtubule tips both in axons and dendrites.

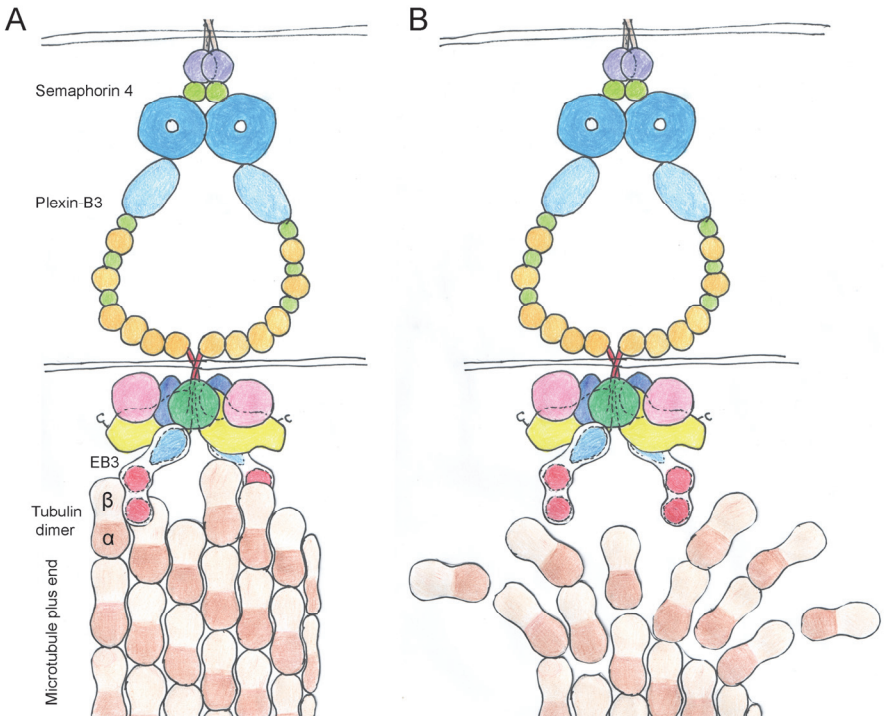


**Figure 11. Plexin-EB interaction induces depolymerization of microtubule plus ends. (**A) EBs are captured by activated Plexin-B3. (B) EBs can no longer bind to microtubules and the plus end of a microtubule is disassembled.

## 4.4 Plexin-B2 and B3 localization in neurons (publication III and unpublished)

In order to better understand the function of B-plexins in neurons we wanted to clarify the issue of their subcellular localization. Plexin-B1 localization in rat hippocampal neurons has been described previously (Lin et al., 2007). Plexin-B1 immunoreactivity was seen in neuronal cell bodies and along dendritic shafts as punctate structures that co-localized with PSD95 indicating its presence in excitatory synapses. Plexin-B2 is expressed widely throughout the nervous system and also in other organs (Worzfeld et al., 2004, Zielonka et al., 2010). Plexin-B3 is prominently expressed in CNS neurons (Hartwig et al., 2005) and oligodendrocytes (Worzfeld et al., 2004), and in contrast to other B-plexins its levels are low prenatally and rise after birth during the period of neurite growth and synaptogenesis. Detailed subcellular localization of Plexin-B2 and B3 in neurons has not been described in previous publications.

Our attempts to confirm the results regarding Plexin-B1 failed, as the purchased polyclonal antibody was unable to recognize Plexin-B1 in any of the

methods we tested. Suzanne Paradis has encountered similar problems (personal communication) and this sheds some doubt on the original publication of Plexin-B1 localization (Lin et al., 2007). To visualize Plexin-B2 and B3 we performed immunocytochemistry of rat hippocampal neurons with antibodies against different neuronal markers and plexins. We followed Plexin-B2 and B3 expression patterns through the development of neurons in culture at 7 DIV, 14 DIV and 21 DIV. Plexin-B2 and B3 expression levels varied in different neurons from very weak to moderate. Both were also visible in different MAP2 negative non-neuronal cells. Plexin antibody signals were strongest in neuronal cell bodies, diffuse in dendrites and occasionally they were detected also along axons. Plexin-B2 signal was stronger in younger cultures concentrating in the cell body membranes and at branching points of dendrites. Later in development Plexin-B2 levels in dendrites declined. In contrast to Plexin-B2, Plexin-B3 intensity in dendrites increased during maturation, which is consistent with the rise of mRNA levels in brain after birth. These results suggest that Plexin-B2 and B3 may regulate dendrite branching.

At 21 DIV we monitored co-localization of plexins with different synaptic markers. This time point was selected as most synapses have matured by then. Immunoreactivity of both plexins exhibited a punctuate distribution along dendrites partially co-localizing with the dendrite microtubule marker MAP2, but surrounding it rather than directly associating with it. Generally strong signals of plexins and synaptic proteins excluded each other, on rare occasions co-localization with presynaptic markers Synaptophysin1 and GAD65 could be observed. In conclusion, Plexin-B2 and B3 proteins appear in dendrites as puncta along the dendritic shaft, but there is no enrichment in synaptic compartments. Such extrasynaptic localization in dendrites has also been observed in the case of Plexin-A2, Plexin-A3, Sema5A (Duan et al., 2014) and Sema5B (O'Connor et al., 2009).

## 4.5 B-plexins in dendrite growth (publication II)

As B-plexins localize to dendrites and influenced microtubule dynamics that is an important mechanism behind dendrite growth, their role in dendritogenesis was examined. At 6 DIV rat hippocampal neurons were transfected with siRNAs targeting all three B-plexins, and dendritic arbours were analysed at 9 DIV. Depletion of B-plexins individually suppressed dendrite growth decreasing total dendritic length by 15-20%. Influence of B-plexin siRNAs on dendrite arborisation correlated with the ability of corresponding plexins to promote microtubule growth – Plexin-B1 did not accelerate microtubule tips and its knockdown had relatively mild consequences, whereas Plexin-B2 that increased microtubule velocity turned out to be most important of B-plexins for dendritogenesis. Plexin-B3 had an intermediate effect. As different plexins are likely to compensate for each other, we assessed the influence of double and

triple knockdowns as well. Indeed, the combinations of siRNAs had an additive effect, reaching the reduction of dendritic length by one third when the expression of all three B-plexins was suppressed. From these results it can concluded that all B-plexins positively regulate dendrite growth in a cooperative manner.

Plexin-B1, that in the microtubule tip speed aspect was indistinguishable from the control, was not very important for dendrite elongation. Our results are different from previous observations regarding the role of Plexin-B1 in dendritogenesis, where Plexin-B1 was described as a negative regulator (Saito et al., 2009). In our system overexpression of intracellular domains of B-plexins did not alter dendrite length or branching. Previous reports regarding the influence of Plexin-B1 ligand Sema4D on dendritic arborisation have also been contradictory (Saito et al., 2009, Vodrazka et al., 2009). While the first group observed Plexin-B1 mediated inhibitory effect of Sema4D on dendrite growth, the latter reported that Sema4D potentiated the formation of higher order branches. Actually both are in agreement with our observation that activated Plexin-B1 increases microtubule dynamic instability. More dynamic microtubule tips promote branch formation but at the same time increased number of catastrophes and pauses slows elongation.

Plexin-B2 overexpression increased microtubule velocity and its depletion individually had the most adverse effect on dendrite growth. Lack of Plexin-B2 has been associated with a small decrease in neurite length of olfactory bulb neurons (Saha et al., 2012). However, dendrite morphology was not examined in detail in that study. Still, it can be concluded that Plexin-B2 is a positive regulator of dendrite growth. Besides, Plexin-B2 and B3 promoted neurite growth of murine cerebellar neurons *in trans* (Hartwig et al., 2005) that can be explained by the homophilic interaction between plexins or they interfere with the signalling by class 4 semaphorins. Plexin-B3 can influence dendrites also via interaction with BTBD3 (Nakayama et al., 2002), a protein that regulates dendrite orientation in the neocortex in response to neuronal activity (Matsui et al., 2013), but this aspect has not been studied.

## 4.6 B-plexins and synapses (publication III)

### 4.6.1 B-plexins are negative regulators of excitatory synapses

Although Plexin-B2 and B3 localization did not overlap with synaptic markers in hippocampal neurons, we still decided to study whether they influence formation of different synapses. Previous works have shown that Plexin-B1 (Kuzirian et al., 2013) and several other plexins and semaphorins play a part in normal synaptogenesis. We chose the strategy of overexpressing the intracellular domains of B-plexins that are known to functionally mimic ligand-activated full-length plexins. Rat hippocampal neurons were transfected

at 16 DIV and analysed at 21 DIV. First, we assessed the impact of B-plexins on excitatory synapses that were visualized by staining presynaptic Synaptophysin1 or postsynaptic PSD95. All three B-plexins significantly reduced the amount of excitatory synapses. We further studied which domain of the intracellular part of Plexin-B3 is responsible for it. We found that mutants ΔNTS and SxIPmut, that are defective for EB3 binding, lacked the negative effect on glutamatergic synapse volume. EB3 is required for normal dendritic spine and synapse formation (Jaworski et al., 2009), and PSD95 is able to interact with EB3 (Sweet et al., 2011). It could be suggested that Plexin-B3 sequesters EB3 from its other interaction partners and thus interferes with its positive role in stabilizing postsynaptic structures in dendritic spines. That in turn leads to disassembly of the synapse and dissociation of presynaptic compartment as well.

Overall, our results indicate that activated B-plexins reduce the number of glutamatergic synapses and in the case of Plexin-B3 regulation of microtubule dynamics is involved in this process. In general our results agree with the study performed by Kuzirian and colleagues (Kuzirian et al., 2013), who described a transient decrease of glutamatergic synapses in response to Sema4D. The negative effect of B-plexins on glutamatergic synapses corroborates with multiple studies where the addition of semaphorins has been shown to reduce the number of synapses, or depletion of semaphorins or plexins has led to excessive formation of dendritic spines or excitatory synapses. Such observations have been made in case of class 3 semaphorins whose signals are mediated by A or D class plexins (Tran et al., 2009, Demyanenko et al., 2014, Ding et al., 2012), and Sema5A and Sema5B that use A-plexins as receptors (Duan et al., 2014, O'Connor et al., 2009, Matsuoka et al., 2011b).

### 4.6.2 Plexin-B1 and B3 promote the formation of inhibitory synapses

Next we determined how different B-plexins influence GABAergic synapse formation. As technically it is easier to count and measure the synapses around the cell soma, we focused on neuron bodies, based on the fact that Sema4D-induced inhibitory synapse formation is not dependent on subcellular localization (Kuzirian et al., 2013). Our results showed that Plexin-B1IC and Plexin-B3IC, but not Plexin-B2IC, acted in a positive manner on inhibitory synapses. In contrast to excitatory synapse formation, EB-binding does not seem to be of crucial importance for Plexin-B3IC as the SxIP mutant was still able to increase the volume of inhibitory synapses. Instead, activation of plexin RasGAP domain and Rho GTPases are necessary for the process. Taken together, Plexin-B1 and B3 positively regulate GABAergic synapse density and downstream signalling cascades involve Rap GTPase as well as Rho GTPase activity regulation.

It has been shown that Plexin-B1 is absolutely necessary for mediating the positive Sema4D signals in inhibitory synapse assembly (Kuzirian et al., 2013).

Thus it was anticipated that Plexin-B1IC over-expression would enhance the formation of GABAergic synapses. As Plexin-B3 resembles Plexin-B1 in many aspects it did not come as a surprise that it acted in a similar manner.

Plexin-B2IC overexpression did not influence inhibitory synapse assembly and it could not be explained by the difference in binding to EB proteins. Largest variability between our B-plexin constructs lies in the transmembrane and Rho binding domains and the explanation should be there. As the transmembrane domain of Plexin-B2 contains residues that do not favour dimer formation it has been suggested that its activation mechanisms are different from Plexin-B1 and B3 (Zhang et al., 2015). Positive effect of Plexin-B3 on inhibitory synapses required RasGAP activity that is dependent on plexin dimerization. Plexin-B2 is not able to form dimers spontaneously and therefore requires additional factors for RasGAP activation that were missing in our system.

Besides, Rnd1 may be the reason behind the contradictory role of Plexin-B2 in regulating inhibitory synapses. Rnd1 is highly expressed during synaptogenic period in the brain (Ishikawa et al., 2003) and may participate in inhibitory synapse formation. The Rnd1-binding loop in the RhoBD of Plexin-B2 is substantially different from other B-plexins and thus likely does not interact with Rnd1 unlike Plexin-B1 and B3 (Oinuma et al., 2004 and personal unpublished observations).

Taken together, Plexin-B1 and B3 are positive regulators of inhibitory synapse assembly while Plexin-B2 is not involved in this process.

## 4.7 Concluding remarks

The pattern of connections between neurons determines the functionality of the brain. Increasing evidence has established the importance of semaphorins and plexins in various aspects of CNS development including dendritogenesis and formation of synapses. This study significantly elaborates the knowledge of the role of Plexin-B3 and other B-plexins in those processes.

The results presented here indicate that Plexin-B1 and B3 interact with EB proteins and this makes microtubules more dynamic enabling the dendrites to find the correct path. On the other hand, plexins promote microtubule growth and dendrite elongation through alternative signalling pathways. Besides, our current results show that Plexin-B2 and B3 are located in dendrites of hippocampal neurons mostly extrasynaptically and mediate inhibitory signals of semaphorins to avoid excitatory synapse formation in wrong places. At the same time Plexin-B1 and B3 promote the formation of inhibitory synapses further regulating the balance of excitation and inhibition. We conclude that B-plexins play an important role in directly mediating semaphorin signals to the microtubule cytoskeleton thereby modulating dendrite growth and formation of synapses, and hence are participating in guiding and connecting the neurons in the brain.

*So what?*

My work is not going to bring peace to the world nor provide a cure for all diseases. Scientists have tried to figure out how the human body works for a long time, but there are still gaps in our knowledge. Every cell is a nanoscale four-dimensional puzzle. I have enjoyed solving puzzles since I was a child, only the nature of them has changed over the years. During the years of my PhD studies I have managed to find some missing pieces in the puzzle of human body and put them together. I know that it is not much but in the end every little bit counts. Contemporary methods of all kinds of omics enable to produce masses of datasets, but in order to understand all that big data little people like me, who study single proteins, are also needed.

# CONCLUSIONS

1. Plexin-B3 intracellular portion interacts with microtubule end binding proteins EB1, EB2 and EB3.
2. Plexin-B3IC N-terminal segment contains the conserved EB-binding motif SxIP and it is essential for plexin-EB interaction. The same motif can be found in Plexin-A2 and B1 as well.
3. B-plexins alter microtubule tip dynamics in two ways:
    - Plexin-B1IC and B3IC that can bind to EBs induce more catastrophes and pauses at microtubule tips thereby slowing them down.
    - Plexin-B2IC and ΔNTS-B3IC that do not bind to EBs increase microtubule growth rate.
4. Plexin-B1 high-affinity ligand Semaphorin 4D increases microtubule plus end dynamics in dendrites of rat hippocampal neurons.
5. Plexin-B2 and B3 proteins are present in neuronal cell bodies and dendrites, but do not colocalize with synapses. Also glial cells are positive for Plexin-B2 and B3.
6. Depletion of B-plexins inhibits dendrite growth.
    - The impact on dendrite length correlates with the ability of plexins to promote microtubule growth.
    - B-plexins promote dendrite growth in a cooperative manner.
7. B-plexins are negative regulators of excitatory synapses.
    - Plexin-B3 interaction with EBs participates in the elimination of excitatory synapses.
8. Plexin-B3 promotes inhibitory synapse assembly by regulating activity of Ras and Rho GTPases.

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# PUBLICATION I

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# Plexin-B3 interacts with EB-family proteins through a conserved motif

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ABSTRACT

*Background:* Plexins are transmembrane receptors that are highly expressed in the central nervous system. They participate in the patterning of neural connections and regulation of cell adhesion and motility in many cell types. The aim of this study was to characterize novel protein–protein interactions of plexin-B3 intracellular portion.
*Methods:* To identify new interactors of plexin-B3 yeast two-hybrid screen was performed. We used GST pull-down and co-immunoprecipitation to verify those results. Deletion mutants were used to map the interacting regions. The physiological relevance of this interaction was assessed with neurite outgrowth assay in Neuro2A cell line.
*Results:* We show that the N-terminal segment of intracellular domain of plexin-B3 interacts with microtubule plus end-binding proteins EB1, EB2 and EB3. The corresponding region in human plexin-A2, B1 and B3 contains the conserved EB-binding motif SxIP and these plexins also associate with EBs indicating the specificity of plexin-EB binding. As to the EB proteins, their N-terminal microtubule-binding domain is dispensable for plexin interaction. Plexin-EB interaction is involved in neurite growth as the synthetic peptide corresponding to the EB-binding region of plexin-B1 increases significantly the number of neurite tips in Neuro2A cells.
*Conclusions:* Microtubule end-binding proteins EB1, EB2 and EB3 interact with plexin-A2, B1 and B3 through a conserved EB-binding motif, which is located in their intracellular domain N-terminal segment.
*General significance:* The observed interaction between plexin intracellular domain and EBs suggests a novel function for plexins in regulating EB-mediated changes in microtubule dynamics and neurite growth.


## 1. Introduction

Development and functioning of the nervous system requires complex orchestration of numerous processes. It is generally believed that structural changes in neural network are based on the remodelling of actin cytoskeleton. Multiple signalling pathways, particularly those involving small GTPases of the Rho and Ras family, are known to control actin organization (for a review see [1]). In recent years, more attention has been paid to the role of microtubules in the formation and maintenance of connections between neurons. Microtubules are essential for dendritic spine maturation and synaptic plasticity that are considered to constitute the cellular bases of learning and memory [2].

Extracellular proteins, such as axon guidance molecules and neurotrophic factors, influence cytoskeleton dynamics via different signalling pathways. In the developing nervous system semaphorins and their transmembrane receptors plexins have received wide recognition as critical regulators of axonal navigation, neuronal polarity, migration and apoptosis [3,4]. There are nine different plexins in mammals: plexin-A1–A4, plexin-B1–B3, plexin-C1 and plexin-D1 [5]. Crystal structures of plexin-A3 and plexin-B1 intracellular domains have been resolved recently [6,7]. The cytoplasmic part of plexins consists of the N-terminal segment (NTS), bipartite RasGAP domain, which is split by Rho GTPase binding domain (RBD), and the C-terminal PDZ-binding motif (present only in B-plexins). Plexin-B1 functions as a GTPase activating protein for R-Ras [8] and M-Ras [9]. Since the RasGAP domain in plexins is conserved, it is likely that also other Ras-family GTPases are influenced by the activity of plexins. We have previously shown that plexin-B3 interacts with Rin, a neuron-specific Ras GTPase [10]. Moreover, plexins associate with Rho GTPases directly through their Rho-binding domain or indirectly by recruiting RhoGEFs [4] or RhoGDI [11].

End-binding proteins (EBs) are highly conserved core components of microtubule plus-end tracking protein network. Mammalian cells express three members of the EB/MAPRE family—EB1, EB2, and EB3 [12]. They are relatively small (268, 327 and 281 amino acids respectively) dimeric proteins containing an N-terminal calponin homology (CH) domain responsible for the interaction with microtubules, a proline-rich linker region, and a C-terminal coiled coil domain

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extending into a four-helix bundle that is required for dimer formation and binding to various partners. EBs interact with most other known microtubule plus end-binding proteins and recruit them to the growing end of microtubules [13,14]. An EB-binding motif has been characterized by Honnappa and colleagues [15] with a consensus sequence Ser-x-Ile-Pro, where serine can be conservatively replaced by threonine.

In this report, we demonstrate that EB-family proteins directly associate with plexin-B3 intracellular domain N-terminal segment. We also show that this domain contains a conserved EB-binding motif SxIP that is also present in other members of the plexin family, and that corresponding peptide is sufficient to modulate neurite growth.

## 2. Materials and methods

### 2.1. Plasmids

EB1, EB2 and EB3 in pEGFP-N1 (Clontech) were provided by N. Galjart [16]. For production of GST-fused proteins, EB coding sequences were introduced into EcoRI/NotI of pET42a (Novagen). N-terminally truncated EB1 and EB3 sequences were subcloned from yeast two-hybrid pMyr clones using EcoRI and SalI sites. Plexin-B3IC sequence (a.a. 1247–1909) was amplified using pIRES/plexin-B3 as a template [10] and subcloned into NotI/EcoRI p3xFLAG-CMV-9 (Sigma). Deletion mutants were constructed using PCR or NheI endonuclease (mutant B). Plexin-B3 deletion mutants G and H were cloned into NotI/BamHI of pFLAG-CMV-4 (Sigma) and EcoRI/BamHI of pIRESneo (Clontech), respectively. The difference in the last amino acid derives from different MCS sequences. Plexin-B1IC (a.a. 1476–2135) was amplified from pcDNA-VSV/plexin-B1 (a generous gift of L. Tamagnone) and plexin-B2IC (a.a. 1196–1838) from KIAA0315 (obtained from Kazusa DNA Research Institute) and were then inserted into NotI/EcoRV sites of p3xFLAG-CMV-9.

### 2.2. Antibodies

The antibody recognizing the Sema domain of plexin-B3 has been described in [10]. Anti-FLAG M2 antibody was purchased from Sigma. A rabbit anti-EGFP serum was provided by A. Merits.

### 2.3. Yeast two-hybrid library screening

Yeast two-hybrid analysis was performed with Cytotrap system (Stratagene) as described previously in ref. [10]. Briefly, plexin-B3IC/pSos bait, pMyr human foetal brain cDNA library and mGap plasmid cotransformant colonies were selected at permissive temperature, then candidate interactors were identified by transferring 800 positive clones to 37 °C. Candidates were tested by two rounds for ability of galactose-dependent growth at 37 °C and resulting positive clones were subjected to plasmid isolation and sequencing.

### 2.4. Cell culture and transfection

Human 293FT cells (Invitrogen) were grown in DMEM supplemented with 10% foetal bovine serum (PAA). The cells were transfected with plasmids using polyethylenimine (non-branched PEI, Sigma).

### 2.5. GST pull-down assay

*Escherichia coli* BL21(DE3) was used to express GST fusion proteins from pET42a constructs. The expressed proteins were purified using Glutathione Sepharose 4 Fast Flow (Amersham Biosciences) according to guidelines provided by the manufacturer. 293FT cells were lysed 1 day post transfection in a buffer containing 20 mM Tris–HCl pH 8.0, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA and Complete protease inhibitors (Roche). Rat cerebellum lysate was prepared using the same lysis buffer. The extracts were clarified by centrifugation at 13,000g for 10 min and incubated with GST-proteins immobilized on glutathione sepharose beads for 2 hours or overnight at 4 °C. Samples were washed once with lysis buffer and four times with TBS. Bound proteins were subjected to 10% SDS-PAGE and Western blot analysis. For GST fusion protein visualization PVDF membranes were stained with PageBlue protein staining solution (Fermentas).

### 2.6. Co-immunoprecipitation

293FT cells were cotransfected with pEGFP-N1/EB3 and pFLAG/plexin-B3 (a.a. 1–1329) or pIRES/plexin-B3 (a.a. 1–1328). Cell extracts were prepared as described above. Lysates were incubated with anti-EGFP antibody for 1 h, adsorbed to protein-A agarose (Sigma) beads at 4 °C for 16 h. The beads were washed three times with lysis buffer and two times with TBS, and boiled in SDS sample buffer for 5 min. After separating the samples using 7% SDS-PAGE, the proteins were detected by Western blotting with appropriate antibodies.

### 2.7. Neurite outgrowth assay

Neuro2A cells were seeded on 24-well plates at a density of $10^4$ cells per well in DMEM (PAA) containing 0.5% FBS (PAA), retinoic acid 10 μg/ml (Sigma) and BDNF 20 ng/ml (generous gift of H. Paves) to induce neurite growth. Peptides corresponding to putative EB-binding regions of B-plexins (11 amino acids) and MACF2 (12 amino acids [15]) were ordered from Genecust and were solubilized in PBS containing 10% DMSO. Each peptide was added to the medium at a final concentration of 1 μM. Cells were then incubated at 37 °C for 3 days. Two fields per well were photographed using Zeiss Axiovert phase contrast microscope (total number of fields $n = 21$). Cells with neurites and number of neurite tips were counted for each field with ImageJ software. Cells not bearing neurites and clumps of more than three cells were excluded from the analysis. The results are the means ± SEM of four independent experiments. Statistical significance was determined using ANOVA and students *t*-test with Bonferroni correction.

## 3. Results

To get a better insight into the mechanisms how signal transduction originating from plexin-B3 is converted to cellular processes, we performed a screen to identify additional binding partners for its intracellular domain. Since plexin-B3 is prominently expressed in the brain [10], we performed yeast two-hybrid screen using human foetal brain cDNA library and plexin-B3IC (amino acids 1274–1909) as a bait. As a result 14 putative positive clones were isolated. Sequencing revealed that only three of those were in frame and encoded C-terminal domains of microtubule end-binding proteins EB1 and EB3, and small GTPase Rin. The latter has been described previously by Hartwig et al. [10]. The clones containing EB1 and EB3 were chosen for further analysis to verify the interaction (Table 1). Control experiments were performed also with EB2, which showed weaker binding affinity towards plexin-B3IC. Plexin-B2 intracellular domain construct failed to give positive results with any of the EB-family proteins in the yeast two-hybrid assay, indicating the specificity of the interaction.

We next examined the direct binding of plexin-B3IC to EBs in vitro using GST pull-down assay. This analysis showed that all three EB proteins form complexes with plexin-B3IC (Fig. 1A). The microtubule-binding CH domain was dispensable for this interaction as ΔNEB1 (amino acids 81–268) and ΔNEB3 (amino acids 96–281) bound plexin-B3IC as effectively as the full-length EBs (Fig. 1B).

**Table 1**
Verification of the specificity of the interaction between plxnB3IC bait and EB-target proteins in yeast.

| Plasmids transformed | SD glucose (−UL) 25 °C | SD (−UL) 37 °C (after patching) | |
|---|---|---|---|
| | | Glucose | Galactose |
| plxnB3IC/pSos + EB1/pMyr | +++ | − | +++ |
| plxnB3IC/pSos + EB2/pMyr | +++ | − | + |
| plxnB3IC/pSos + EB3/pMyr | +++ | − | +++ |
| plxnB3IC/pSos + pMyr* | +++ | − | − |
| plxnB3IC/pSos + SB/pMyr** | +++ | − | +++ |
| plxnB2IC/pSos + EB1/pMyr | +++ | − | − |
| plxnB2IC/pSos + EB3/pMyr | +++ | − | − |
| plxnB2IC/pSos + pMyr* | +++ | − | − |
| plxnB2IC/pSos + SB/pMyr** | +++ | − | +++ |
| Coll/pSos + EB1/pMyr* | +++ | − | − |
| Coll/pSos + EB2/pMyr* | +++ | − | − |
| Coll/pSos + EB3/pMyr* | +++ | − | − |
| Coll/pSos + MAFB/pMyr* | +++ | − | − |
| MAFB/pSos + MAFB/pMyr** | +++ | − | +++ |

* Negative control experiments.
** Positive control experiments.

To ascertain an interaction between endogenous plexin-B3 and EBs, we performed GST pull-downs with rat cerebellum lysate (Fig. 1C). We found that full-length plexin-B3 also associated with all three EB-family proteins.

To map the EB-binding site on plexin-B3IC we generated a series of deletion constructs and assessed their ability to bind to EBs. Mutations affecting RasGAP domain or RBD had little or no effect on EB binding, whereas deletion of the N-terminal segment (amino acids 1283–1364, mutant A) of plexin-B3IC abrogated the interaction (Fig. 2B). Notably, the NTS of plexin-B3 contains the conserved EB-binding motif SxIP [15]. To confirm the interaction, we performed co-immunoprecipitations with EB3-GFP and constructs encoding plexin-B3 extracellular domain, transmembrane domain and NTS of the intracellular part (Fig. 2C). In one case the EB-binding SxIP motif was intact (mutant G), in the other construct the invariable proline was substituted for histidine (mutant H), and the latter was unable to bind to EB3-GFP. Taken together, these results demonstrate that the interaction takes place between the NTS of plexin-B3 and the EB-homology domain of EBs.

We next investigated whether the conserved EB-binding motif SxIP is present in intracellular domains of other human plexins (Fig. 3A). This motif was found to reside between NTS region helixes 1 and 2 in plexin-A2, B1 and B3. Comparison of plexin-B3 protein sequences of different mammals indicated that the EB-binding motif and the surrounding region are well conserved. The same applies for plexin-B1. EB-binding motif is also present in some plexins of lower vertebrates and *Drosophila* plexin-B, strongly suggesting that this interaction may be evolutionarily conserved.

To confirm the specificity of EB interaction with different plexins, GST pull-down was carried out with FLAG-tagged intracellular constructs (including transmembrane domains) of plexin-A2, B1, B2 and B3 (Fig. 3B). This experiment showed that while EB1 formed complexes with plexin-A2, B1 and B3, plexin-B2 did not. In fact, plexin-B2 failed to interact with any of the EB-family proteins (data not shown). This could be explained by the incomplete EB-binding motif in plexin-B2, and is consistent with yeast two-hybrid results. Taken together, the interaction between EBs and plexins is restricted to certain plexins and correlates with the presence or absence of the SxIP motif.

As plexins and EBs both have a role in neuritogenesis we decided to find out whether their interaction influences this process. Neuro2A cells were simultaneously treated with BDNF and RA, and peptides spanning the region of EB-binding motif of B-plexins (Fig. 3A, underlined). MACF2 peptide served as a positive control since it has been shown to bind to EB1 [15]. Plexin-B1 peptide significantly increased the number of neurite tips per cell as did MACF2 peptide, compared to plexin-B2 peptide and buffer treated control (Fig. 3C). Plexin-B3 peptide also elevated the number of neurite tips, but it did not reach statistical significance. Since plexin-B1 peptide influenced neurite growth similarly with MACF2 peptide, it is a strong indication that it forms a complex with EBs.

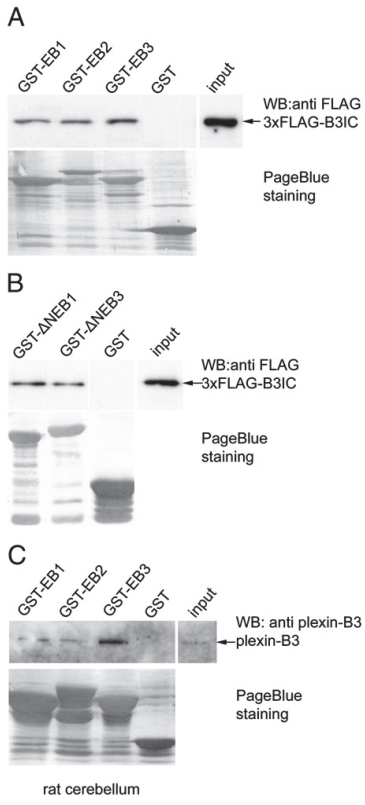


**Fig. 1.** Plexin-B3 interacts with EB-family proteins. (A) Plexin-B3IC complexes with all EB-family proteins. GST pull-down with GST-EB1, GST-EB2, GST-EB3, or GST (negative control) and transiently expressed 3×FLAG-plexin-B3IC (probed with anti-FLAG). (B) EB proteins do not need N-terminus to bind to plexin-B3IC. GST pull-down with GST-ΔNEB1 and GST-ΔNEB3. (C) Endogenous plexin-B3 from rat cerebellum lysate interacts with GST- EBs. Plexin-B3 was detected using anti plexin-B3 antibody.

## 4. Discussion

Regulating the reorganization of different components of cytoskeleton during cell migration and neurite extension is a sophisticated process where large multicomponent assemblies are under constant rearrangement. One of the main functions of plexins is to translate extracellular signals to cytoskeleton dynamics within the cell, integrating regulation of microtubules and actin filaments. The mechanisms, how plexins influence actin cytoskeleton, have been characterized in several studies. Semaphorin–plexin signalling regulates microtubules via CRMP-family proteins (for a review see [17]). Activation of plexins affects also kinases PI3K, Akt and GSK3β [4] that in turn regulate microtubule growth and shrinkage among their other functions. Interaction between plexins and EBs, presented in this study, adds another piece to the puzzle of cytoskeleton dynamics.

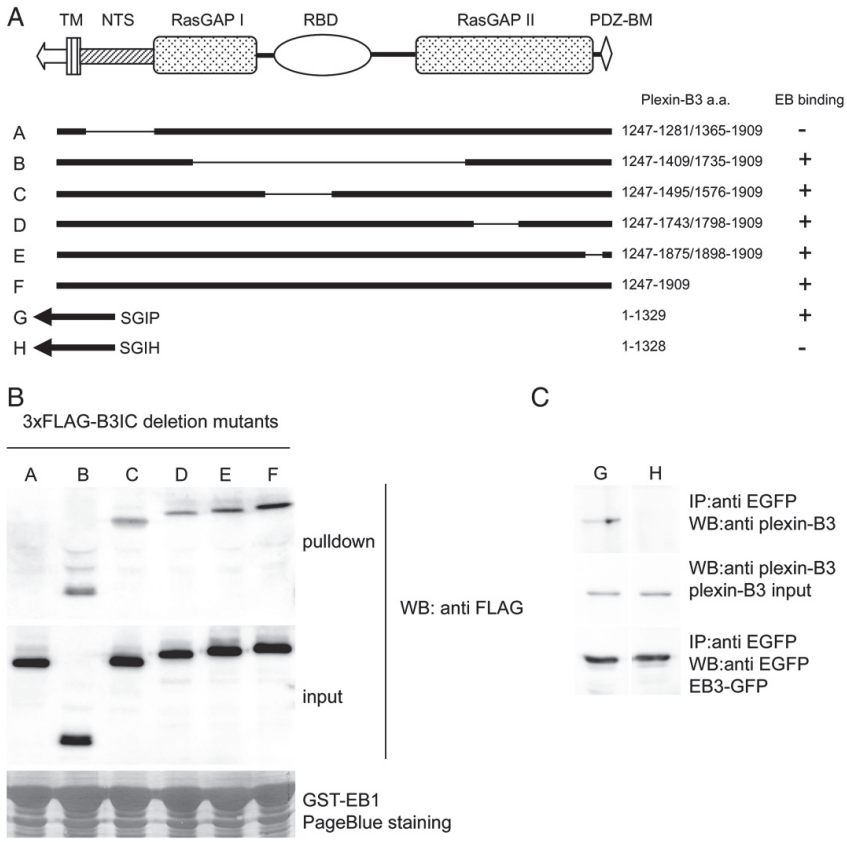


**Fig. 2.** Mapping the EB-interacting domains of plexin-B3IC. (A) Schematic representation of plexin-B3IC deletion mutants (A–H). TM: transmembrane region, NTS: N-terminal segment, RasGAP: Ras GTPase activating protein, RBD: Rho-binding domain, PDZ-BM: PDZ-domain binding motif. Extracellular region is indicated by arrows. (B) FLAG-tagged deletion mutants of plexin-B3IC were expressed in 293FT cells and subjected to pull down assay with GST-EB1 prebound to gluthathione sepharose. Plexin-B3IC was revealed by western blotting using anti-FLAG antibodies. (C) Intact EB-binding motif SxIP is essential for plexin-EB interaction. EB3-GFP and either plexin-B3 mutant G (SGIP) or mutant H (SGIH) were coexpressed in 293FT cells. Lysates were subjected to immunoprecipitation with antibodies against EGFP and plexin-B3 was detected using anti plexin-B3 antibody.

We have shown that EBs associate with N-terminal segments of plexin-A2, B1 and B3 via the conserved EB-binding motif SxIP. Other proteins with this motif (APC, MACF2, CLASP2, MCAK etc.) bind to the highly conserved hydrophobic cleft in the C-terminal domain of EB1. The EB-binding region is relatively unstructured in described proteins, and only 12-amino acid peptide is sufficient for protein–protein interaction [15]. In plexin-B1, and presumably also in plexin-B3, the SxIP motif resides in a loop between two helixes [7] and hence is similar to the situation described for other EB-interacting proteins. The surroundings of EB-binding motifs in plexins do not contain as many basic amino acid residues as in proteins described so far. Based on the overall structure of plexin intracellular domain [7,18] it is very likely that EB–plexin interaction is conformation dependent and the availability of EB-binding motif is dynamically regulated by semaphorins.

During development expression patterns of B-plexins and EBs (especially EB3) largely overlap in the brain. Plexin-B3 has been associated with promotion of neurite growth [10], verbal performance and white matter volume [19]. Both EB3 and plexin-B1 localize in dendritic spines, and are involved in regulation of neurite extension and dendritic spine density [2,20–22]. Overexpression of plexin-B1 reduces neurite length and branching [9,20]. On the other hand, Sema4D, the ligand of plexin-B1, stimulates branching and formation of dendritic spines in hippocampal neurons [22,23]. Our experiments demonstrated that plexin-B1 EB-binding motif peptide modified neurite outgrowth in Neuro2A cells, indicating that plexin-EB interaction is involved in this process. Thus it is plausible that EB proteins and plexins interact in the central nervous system in vivo and participate in microtubule reorganization. Activation of another neuronal receptor, namely NMDA receptor in rat hippocampal neurons, attenuates microtubule dynamics by removing EB3 from the growing microtubule plus-ends in dendrites resulting in dendritic spine shrinkage and long term depression (LTD) [24]. Whether activation of plexins has an effect on EB localization awaits further investigation.

In this study, we have described the association of plexin-A2, B1 and B3 with EB-family proteins. This interaction relies on the C-terminal EB-homology domain of EBs and the N-terminal segment of intracellular portion of plexins that contains the conserved EB-binding motif SxIP. To our best knowledge this is the first report describing a direct interaction between transmembrane receptors and EB-family proteins in multicellular organisms. The interaction of plexins and EBs is likely to play an important role in regulating neurite growth and synaptic plasticity and hence could be connected to memory formation as well as neurological disorders.

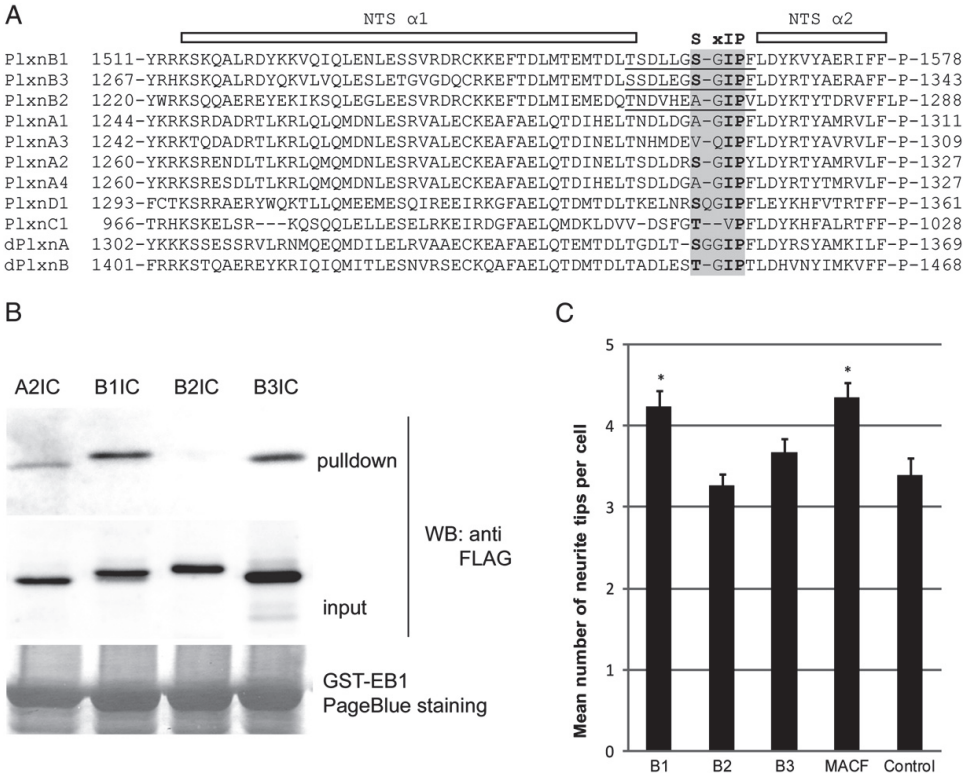


**Fig. 3.** (A) Alignment of NTS regions of human and *Drosophila* plexins. EB-binding motif is shadowed and amino acids fitting the consensus are in bold. Secondary structure elements (based on plexin-B1 crystal structure [7]) are shown on top. Sequences of peptides used in neurite outgrowth assay are underlined. (B) EB1 forms complexes with plexin-A2, B1 and B3, but not with plexin-B2. FLAG-tagged intracellular domains of plexins were expressed in 293FT cells and incubated with GST-EB1. Immunoblots of pull-downs were incubated with anti-FLAG. (C) EB-binding peptides increase the number of neurite tips. Neuro2A cells were treated for 3 days with peptides corresponding to the EB-binding regions of plexins B1, B2 and B3, and MACF2 as a positive control. The results are the means ± SEM of four independent experiments, $^{*}p<0.02$.

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# PUBLICATION II

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Research Article

# B-plexins control microtubule dynamics and dendrite morphology of hippocampal neurons

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ABSTRACT

Semaphorins and their receptors plexins are implicated in various processes in the nervous system, but how B-plexins regulate the growth of dendrites remains poorly characterized. We had previously observed that Plexin-B1 and B3 interact with microtubule end-binding proteins (EBs) that are central adapters at growing microtubule tips, and this interaction is involved in neurite growth. Therefore, we hypothesized that plexins regulate microtubule dynamics and through that also dendritogenesis. The role of all three B-plexins was systematically examined in these processes. B-plexins and their ligand Semaphorin-4D influence the dynamics of microtubule tips both EB-dependently and independently. EB3 as well as Plexin-B1, B2 and B3 turned out to have a significant role in the development of dendritic arbor of rat hippocampal neurons. Our results clearly indicate that semaphorin-plexin-EB pathway is one molecular mechanism how extracellular guidance cues are translated into intracellular mechanics. Taken together, Semaphorin-4D and B-plexins modulate the dynamic behavior of microtubule tips, and are therefore important in neurite growth.



## Introduction

Plexins are a family of transmembrane receptors that mediate semaphorin signalling mainly in the central nervous system [1,2]. Semaphorins regulate many processes requiring precisely coordinated and balanced modifications of cytoskeleton and membranes. Mammals have nine plexins of which three belong to the B-subfamily [3]. The same plexins can have diverse functions at different stages of development and in different cell types. Increasing evidence has established the importance of semaphorins and plexins in the proliferation and migration of neurons, guidance of growth cones and defining neural connectivity, as well as neurite arbor remodelling and synaptic plasticity at later stages of development (reviewed in Ref. [4]). Plexins can modulate cytoskeleton dynamics in various ways of which regulating the activity of small GTPases stands out. Although semaphorins are known regulators of processes that require microtubule (MT) dynamics, direct links between plexins and MTs are not known. MICALs (molecules interacting with CasL) could provide a bridge between A-plexins and MTs [5], but the effects induced by semaphorin-plexin signalling

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0014-4827/

on MTs and cell adhesion were MICAL independent [6]. RanBP9 (Ran binding protein 9) interacts with Plexin-A1 intracellular domain and may be involved in regulating MT dynamics [7], still RanBP9 binding to MTs awaits further verification. Semaphorins affect MT cytoskeleton through regulation of CRMPs (collapsin response mediating proteins) [8,9], but this does not happen via direct interaction, but rather plexins modulate small GTPases which in turn activate downstream kinases that phosphorylate CRMPs. We have previously shown that plexins A2, B1 and B3 interact with microtubule end binding proteins (EBs, also known as MAPREs) [10] that are central adapters at the ends of microtubule tips mediating interactions with a variety of other proteins and regulate MT tip dynamics [11].

All B-plexins are expressed in the central nervous system [12]. Plexin-B2 expression appears first during embryogenesis, peaks around birth, and continues to be expressed from there onwards. It is required for normal proliferation and migration of neurons during brain development [14,15]. Plexin-B1 expression follows, gradually declining after birth. The expression levels of Plexin-B3 are lower than B1 and B2 levels. It emerges around birth and is expressed steadily to adulthood [12,13]. Depletion of Plexin-B1 or B3 does not bring about any noticeable developmental defects [14,16] possibly due to redundant functions between different B-plexins. Double homozygous deletion of Plexin-B1 and B2 results in embryonic lethality [17] supporting this notion.

Semaphorin 4D is a high-affinity ligand for Plexin-B1 and a low-affinity ligand for B2 and B3 [13]. Sema4D was first described as a repellent for hippocampal axons [18], but in dorsal root ganglion neurons it stimulates axon outgrowth instead of retraction [19]. Several downstream effectors have been linked to Sema4D/Plexin-B1 pathway, mostly leading to regulation of small GTPases, activation of kinases or regulation of cytoskeleton. Plexin-B1 functions as a GTPase activating protein (GAP) for R-Ras in axons [20] and M-Ras in dendrites [21] leading to repulsion. In hippocampal neurons Plexin-B1 localizes to dendrites and dendritic spines and Sema4D stimulation increases spine density through Plexin-B1/RhoA/ROCK pathway [22]. There is considerably less information about the roles of Plexin-B2 and B3 in neurite growth and synaptogenesis. Since both of them are expressed during periods of dendrite outgrowth, spine formation and maintenance by different subsets of neurons [12,15,23], they are likely to participate in the formation of connections between neurons. Plexin-B2 and B3 *in trans* position promote axon growth of cerebellar neurons [23], and certain Plexin-B3 genotypes have been associated with larger white matter volumes in human brains [24]. Despite obvious phenotype of Plexin-B2 knockout mice, its effects on cellular level have remained largely unexplored.

Since PlexinB1 and B3 are able to bind to microtubule end binding proteins [10], local receptor-mediated destabilization of MTs facilitates protrusion formation [25] and plexins A3, A4 and D1 have been shown to play a role in defining neural connectivity [26,27], we hypothesized that B-plexins influence the dynamic behavior of MT tips and thus dendritogenesis. In this study we sought to determine whether B-plexins alter microtubule dynamic behavior and dendrite arbor formation of rat hippocampal neurons. We show that Sema4D and its receptors Plexin-B1, and to a lesser extent B2 and B3, modulate the dynamics of microtubule tips. We also characterize dendrite morphology of rat hippocampal neurons in response to depletion of EB1, EB3 and all three B-class plexins.

## Experimental procedures

### DNA constructs and siRNAs

Expression constructs of human B-plexins encoding transmembrane and intracellular domains (B1IC, B2IC, B3IC and ΔNTS-B3IC) were in p3xFLAG and have been described previously [10]. EB3 in pEGFP-N1 was provided by N. Galjart. Sema4D extracellular region was amplified from human cortex cDNA and inserted into pCMV-Fc-GFP.

Ambion Silencer® Select siRNA duplexes targeting rat mRNAs were following:

EB1 (GGACAAUUUUGAAUUCGUUtt),
EB3 (GGACUUGAAGCUGACCGUAtt),
Plexin-B1 (GUAUAUCAACAAGUACUAUtt),
Plexin-B2 (GCAAGUCCUUCCUUAUCAAtt),
Plexin-B3 (CCACAUCCACAGGUAUUAUtt),
Silencer Select Negative Control #1 siRNA.

### Cell culture and transfection

All animal procedures were performed in compliance with the local ethics committee. Primary rat hippocampal neurons were dissected from Sprague Dawley rat embryos (both male and female) at E20 and cultured on cover slips as described in [28]. Briefly, the hippocampi were dissected and cells were dissociated with 0.25% trypsin (Invitrogen), treated with 0.05% DNase I (Roche), and the cell suspension was plated on poly-L-lysine-coated cover slips in Neurobasal A medium (Invitrogen) with B27 supplement (Invitrogen), penicillin (PAA Laboratories, 100 U/ml), streptomycin (PAA Laboratories, 0,1 mg/ml), and 1 mM L-glutamine (PAA Laboratories). Mitotic inhibitor 5-fluoro-2′-deoxyuridine (Sigma) was added to the medium (10 μM) at 2 days *in vitro* (DIV). Neurons were transfected using Lipofectamine 2000 (Invitrogen) at 6 DIV for dendrite growth and branching measurements. siRNAs were used in 50 times molar excess compared to pEGFP to ensure that GFP positive neurons are cotransfected with siRNAs. NIH3T3 and 293FT cells were grown in DMEM supplemented with 10% FBS (PAA). The cells were transfected with plasmids using polyethylenimine (non-branched PEI, Sigma).

### Recombinant Sema4D production

293FT cells were transfected with Sema4D-Fc or empty Fc expression plasmids. After 20 h medium was replaced with serum-free DMEM and supernatants were collected 4 days after transfection, centrifuged and used straight away. Sema4D stimulation was carried out by replacing part of the culture medium with conditioned medium (Sema4D-Fc final concentration approximately 1 μg/ml).

### Analysis of siRNA effects on mRNA levels in cultured neurons

Rat hippocampal neurons were cultured in 12-well plates and co-transfected with pEGFP and siRNAs at 6 DIV. At 9 DIV RNA was

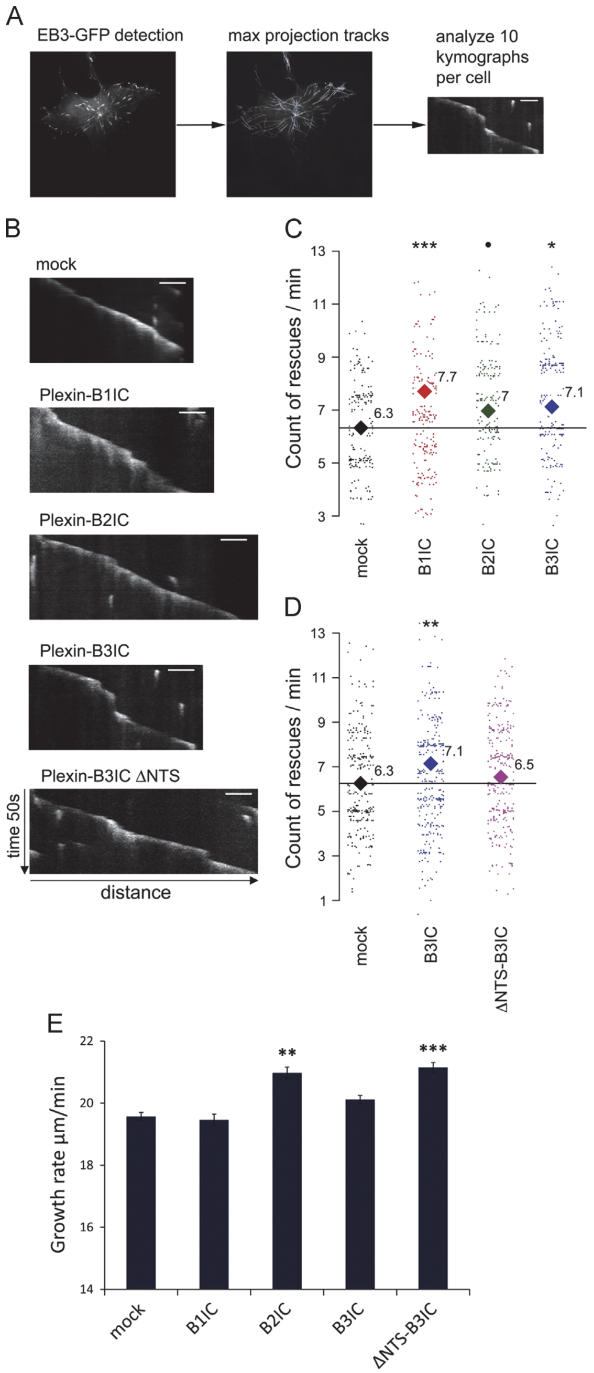


**Fig. 1 – MT tip dynamic behavior is regulated by B-plexins in NIH3T3 cells. The cells were co-transfected with EB3-GFP to visualize MT tip behavior (A). 100 images were acquired every 0.5 s with TIRFM. Tracks were drawn according to maximum projection image and kymograph function was used to visualize different phases of MT instability. (B) Representative images of kymographs, scale bars 2 µm. (C) Overexpression of intracellular domains (IC) of B-plexins increases the number of MT tip rescues and deletion of EB binding site (ΔNTS-B3IC) abolishes this effect (D). Small dots represent single MT tips and the means are marked with diamonds. (E) The influence of B-plexIC overexpression on microtubule growth rate. The results are the means ± s.e.m. of three independent experiments. For cell and MT tip numbers see Table 1. • $p = 0.1–0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.**

isolated using RNAWiz reagent (Ambion). Quantitative RT-PCR was performed with gene specific primers. Ppia was used as a reference. The siRNAs effectively reduced the amount of corresponding endogenous mRNA levels compared to the mock siRNA treated neurons (Fig. S1). Protein levels could not be assessed due to the lack of suitable antibodies.

### Immunostaining and image analysis

For immunocytochemistry the cultured neurons were fixed at 9 DIV in freshly prepared 4% paraformaldehyde, 4% sucrose in PBS at room temperature for 15 min. This was followed by permeabilization with 0.5% Triton X-100 in PBS and blocking in 3% BSA in PBS. Antibody incubations were carried out at room temperature for one hour. Primary antibody was rabbit anti-EGFP (1:2000, from A. Merits) and secondary antibody was goat anti rabbit Alexa488 (1:2000, Molecular Probes). Prolong Gold antifade reagent (Invitrogen) was used for mounting. A Zeiss LSM510 confocal microscope system was used to scan stacks of fixed neurons. Dendrites were imaged with a Plan Apochromat 20 × 0.8 NA objective (Zeiss). For neuron morphology maximum projections were generated. Branching patterns and length of dendrites of individual stained neurons were analyzed with the Filament Tracer module of the Imaris 6.4.2 software (Bitplane). Scholl analysis, that counts the number of dendrite intersections in a series of concentric circles, was performed with a step of 10 μm. At least 40 cells were analyzed from three independent culture preparations per treatment group.

### Live cell imaging and measurement of microtubule tip dynamics

NIH3T3 cells were grown on 40 mm cover slips, co-transfected with EB3-GFP and B-plexin-encoding plasmids, and imaged one day post-transfection. Rat hippocampal neurons were grown in 35 mm dishes with 10 mm glass microwell (MatTek) and transfected with EB3-GFP at 6 DIV. For Sema4D-Fc treatment freshly prepared conditioned medium was added and images were acquired within 1.5 h. Similarly prepared medium from cells transfected with empty Fc plasmid was used as a negative control. Timelapse images were collected at 37 °C on the TILL iMIC TIRF microscope equipped with Zeiss TIRF 100 × 1.46 NA objective and iXon888 EMCCD camera (Andor). GFP laser (488 nm) and filter set was used. 100 frame stacks of 16-bit images 1024 × 1024 pixels were collected with 50 ms exposure at two frames per second. MT dynamics parameters were measured manually using ImageJ. Maximum projection images were used to draw tracks. From neurons all suitable tracks in a frame were measured whereas ten longest tracks per cell were selected from NIH3T3. Kymographs were generated using Kymograph plugin (by J. Rietdorf and A. Seitz). Obtained coordinates were transferred to MS Excel where MT tip growth rate, number of rescues (transition from pause or shrinkage to growth), catastrophes (transition from growth or pause to regression) and pauses were calculated. Pause was defined as an event with a growth or shrinkage rate less than 0.02 μm/s.

## Statistical analysis

All experiments were repeated minimum three times using independent cell culture preparations. In case of microtubule measurements, the effects of different treatments were statistically tested using linear mixed effect models in R packages nlme and multcomp.

Values were corrected relative to the mean mock value of the same date. Significance of the differences was assessed using ANOVA and Tukey *post-hoc* test. For dendrite experiments statistical significance was determined using ANOVA and Tukey *post-hoc* test in R package stats. For Scholl analysis, the difference was evaluated point by point with unpaired two-tailed Student's *t*-test in MS Excel.

## Results

### B-plexins alter microtubule tip dynamics

Since Plexin-B1 and B3 interact with microtubule end binding proteins and Plexin-B2 does not, we asked whether they modulate MT tip dynamics differentially. To monitor their direct influence on MT tip behavior, NIH3T3 mouse fibroblasts were co-transfected with B-plexin constructs and EB3-GFP, a marker for growing MT tips [29]. We overexpressed the intracellular parts of B-plexins with transmembrane domains because they mimic full-length plexins activated by their ligands semaphorins [30]. Images were collected with TIRF microscopy and EB3-GFP comets were tracked for 50 s at two frames per second (Fig. 1A). Such settings enabled us to follow MT tracks from the centrosome to the cell membrane and detect also minor cytoskeleton dynamic events. MT growth rate (velocity) and characteristics of MT tip dynamics

**Table 1 – Microtubule dynamics in NIH3T3 cells. NIH3T3 cells were transiently cotransfected with EB3-GFP and indicated plasmid constructs. Live cells were imaged one day post-transfection. MT tip growth rate, number of rescues, catastrophes and pauses were calculated. At least three independent experiments were made. Results are presented as mean values ± SD for the group of MT tips.**

| Treatment | Number of MTs/cells | Growth rate (μm/min) | Rescues per minute | Catastrophes per minute | Pauses per minute |
|---|---|---|---|---|---|
| Control | 500/50 | 19.6 ± 3.0 | 6.3 ± 2.3 | 4.6 ± 2.2 | 1.7 ± 1.5 |
| Plexin-B1IC | 300/30 | 19.5 ± 3.2 | 7.7 ± 2.5 | 5.7 ± 2.2 | 2.0 ± 1.6 |
| Plexin-B2IC | 300/30 | 21.0 ± 3.2 | 7.0 ± 2.1 | 5.3 ± 2.0 | 1.7 ± 1.4 |
| Plexin-B3IC | 500/50 | 20.1 ± 2.9 | 7.1 ± 2.4 | 5.2 ± 2.3 | 1.9 ± 1.7 |
| Plexin-B3IC-ΔNTS | 300/30 | 21.1 ± 2.8 | 6.5 ± 2.3 | 4.8 ± 2.3 | 1.7 ± 1.5 |

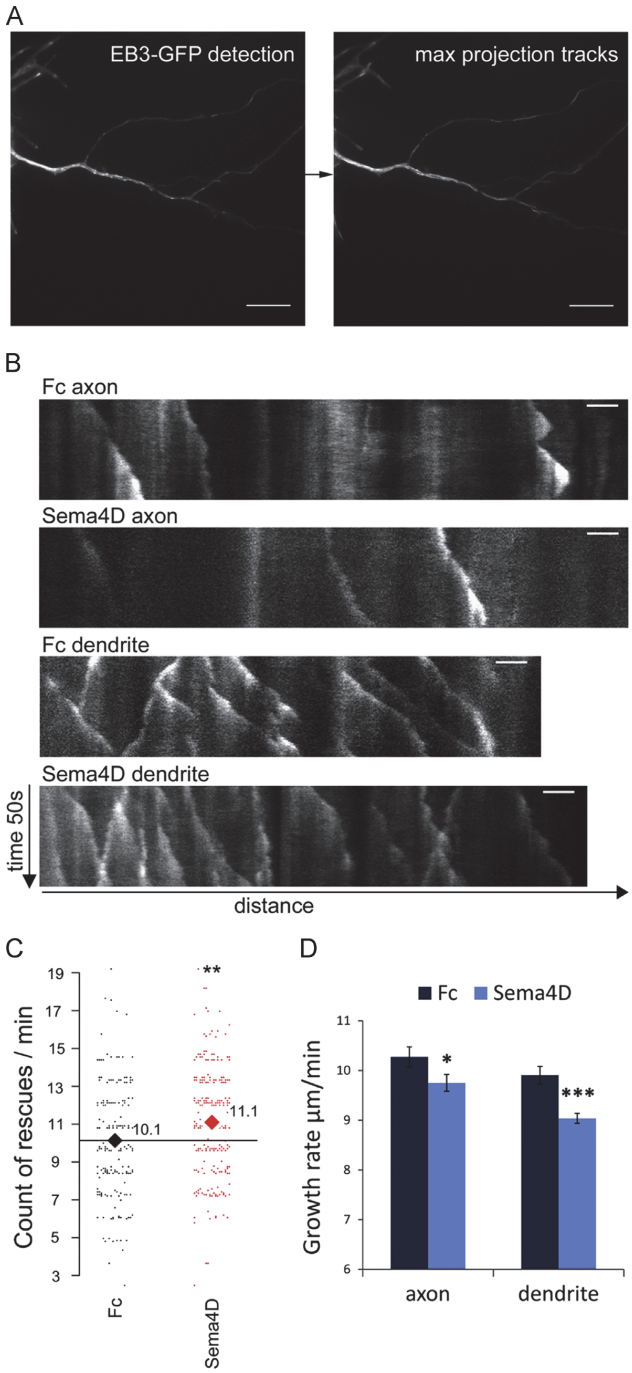


**Fig. 2 – MT tip dynamic behavior is regulated by Sema4D in hippocampal neurons. Neurons were transfected with EB3-GFP at 6 DIV to visualize MT tip behavior (A). At 7 DIV neurons were treated with mock medium (Fc) or Sema4D conditioned medium 10–90 min. Representative inverted kymographs are shown in (B), scale bars 2 µm. (C) Sema4D treatment increased the number of MT tip rescues. Small dots represent single MT tips and the means are marked with diamonds. (D) Sema4D treatment decreases MT growth rate. The results are the means ± s.e.m. of three independent experiments. For MT tip numbers see Table 2. *$p<0.05$, **$p<0.01$, ***$p<0.001$.**

(the number of rescues, catastrophes and pauses) were measured manually from kymographs, and these parameters (Table 1) were compatible with previous reports [29,31]. B1IC treatment generated characteristic jagged kymographs with short phases of growth interrupted by small catastrophes or pauses indicating increased dynamic instability of MT tips (Fig. 1B and C). The number of rescues is depicted on graphs, as it is the sum of catastrophes and pauses, and therefore a general parameter of MT tip dynamic instability. Plexin-B2IC and Plexin-B3IC slightly increased the number of rescues, but to a lesser extent than Plexin-B1IC (Fig. 1B and C). As the surroundings of EB-binding SxIP motif in Plexin-B3 contain one extra negatively charged amino acid residue compared to B1, which theoretically should diminish its binding affinity to EBs [32], it is reasonable that B3IC did not create as many MT tip rescues as B1IC. When the interaction between Plexin-B3 and EBs was disturbed using ΔNTS-B3IC (Plexin-B3 mutant devoid of EB-binding region), we observed a reduction of the effect of Plexin-B3IC on MT tip behavior (Fig. 1D), indicating the importance of the crosstalk of these proteins. When we analyzed the growth rate of MTips (Fig. 1E), then it appeared that B-plexins that are unable to bind to EB-s (B2IC and ΔNTS-B3IC) accelerated MTips, whereas B1IC and B3IC were similar to the control group. From that we conclude that plexins can affect MT dynamics in different ways. B-plexins can increase the velocity of MT tips through pathways that do not require direct interaction with EBs, but when they are able to interact with EBs (B1IC and B3IC) they induce more catastrophes and pauses, which counteracts the promotion of growth.

## Dendritic microtubule +TIP dynamics increases with Sema4D treatment

To further verify the role of B-plexins in regulating microtubule dynamics, rat hippocampal neurons that had been transfected with EB3-GFP at 6 DIV, were treated with the ligand of B-plexins—Sema4D. EB3-GFP was imaged and tracked as described above (Fig. 2A). Consistent with earlier studies [29] the growth rate of EB3-GFP comets was twofold smaller in neurons than in fibroblasts (Table 2). At the same time MT tips were more dynamic in neurons than in fibroblasts, having more catastrophes, pauses and rescues. This could be explained by the more confined space in neurites than in the fibroblast cell body. Sema4D treatment had a similar effect as B1IC overexpression in fibroblasts, the number of dynamic events increased and as a consequence MT velocity declined compared to the control (Fig. 2C and D). Although Sema4D influence in dendrites was slightly more pronounced than in axons, this difference did not reach statistical significance and thus data of rescues was pooled and presented as one graph. We conclude that Sema4D is able to influence MT tips in neurons likely through Plexin-B1.

## Dendrite growth is impaired by depletion of EB3

We examined whether EB1 and EB3 are required for regulation of dendrite remodelling in rat primary hippocampal neurons. It has been suggested that EB1 is necessary for axon but not dendrite development [33]. Indeed, EB1 mutant Drosophila neurons display defects in axon growth due to disorganized microtubules [34], but dendrites were not addressed in that study. EB3 is known to regulate maturation of dendritic spines [35–37] and its association with PSD95 alters dendrite growth [38], but the effect of EB3 depletion on dendrite growth and branching has not been described earlier. We have previously shown that specific plexins are able to bind to EB proteins and this interaction is involved in neurite growth [10]. Rat hippocampal neurons were co-transfected with siRNAs targeting EB1 or EB3 and a plasmid encoding EGFP for visualization at 6 DIV and analyzed at 9 DIV (Fig. 3). We used Imaris software filament tool to characterize dendritic arbor of EGFP positive neurons that were cotransfected with siRNAs. Scholl analysis was performed (Fig. 3A), total dendrite length (Fig. 3B) and number of dendrite tips (Fig. 3C) was calculated. EB3 siRNA impaired dendrite growth, whereas EB1 knockdown had a much milder effect. This is somewhat surprising as EB3 expression in cultured hippocampal neurons begins to rise only after 7 DIV and EB1 levels in neurons at that time point are higher than EB3 levels [39]. Our results support the observation that EB1 is involved in axon growth and to a lesser extent in dendrite growth, which is regulated mainly by EB3.

## B-plexins are positive regulators of dendrite growth

To systematically reveal the roles of all three mammalian B-plexins in dendrite development, at 6 DIV we targeted them with siRNAs, and analyzed at 9 DIV as described above. Depletion of B-plexins individually suppressed dendrite growth (Fig. 4A–C) decreasing total dendritic length by 15–20%. The impact on dendrite length correlates with the ability of plexins to promote microtubule growth (see also Fig. 1E)—Plexin-B1 does not accelerate MT tips and its knockdown had relatively mild consequences, whereas Plexin-B2 that increases MT velocity turned out to be most important of B-plexins for dendritogenesis. Plexin-B3 had an intermediate effect. As different plexins are likely to

**Table 2 – Microtubule dynamics in rat hippocampal neurons. Rat primary hippocampal neurons were transiently transfected at 6 DIV with EB3-GFP and at 7 DIV treated with mock Fc medium or Sema4D-Fc conditioned medium. Live cells were imaged for 50 s at a 0.5 s interval with TIRFM. MT tip growth rate, number of rescues, catastrophes and pauses were calculated. At least three independent experiments were made. Results are presented as mean values ± SD for the group of MT tips.**

| Treatment | Number of MTs | Growth rate (μm/min) | Rescues per minute | Catastrophes per minute | Pauses per minute |
|---|---|---|---|---|---|
| Fc axon | 66 | 10.3 ± 1.6 | 10.2 ± 3.1 | 8.3 ± 3.0 | 1.9 ± 1.9 |
| Sema4D axon | 83 | 9.8 ± 1.5 | 11.1 ± 2.6 | 9.6 ± 2.8 | 1.5 ± 1.4 |
| Fc dendrite | 140 | 9.9 ± 2.1 | 10.0 ± 2.7 | 8.4 ± 2.7 | 1.6 ± 1.5 |
| Sema4D dendrite | 187 | 9.0 ± 1.4 | 11.1 ± 2.8 | 9.7 ± 2.9 | 1.4 ± 1.5 |

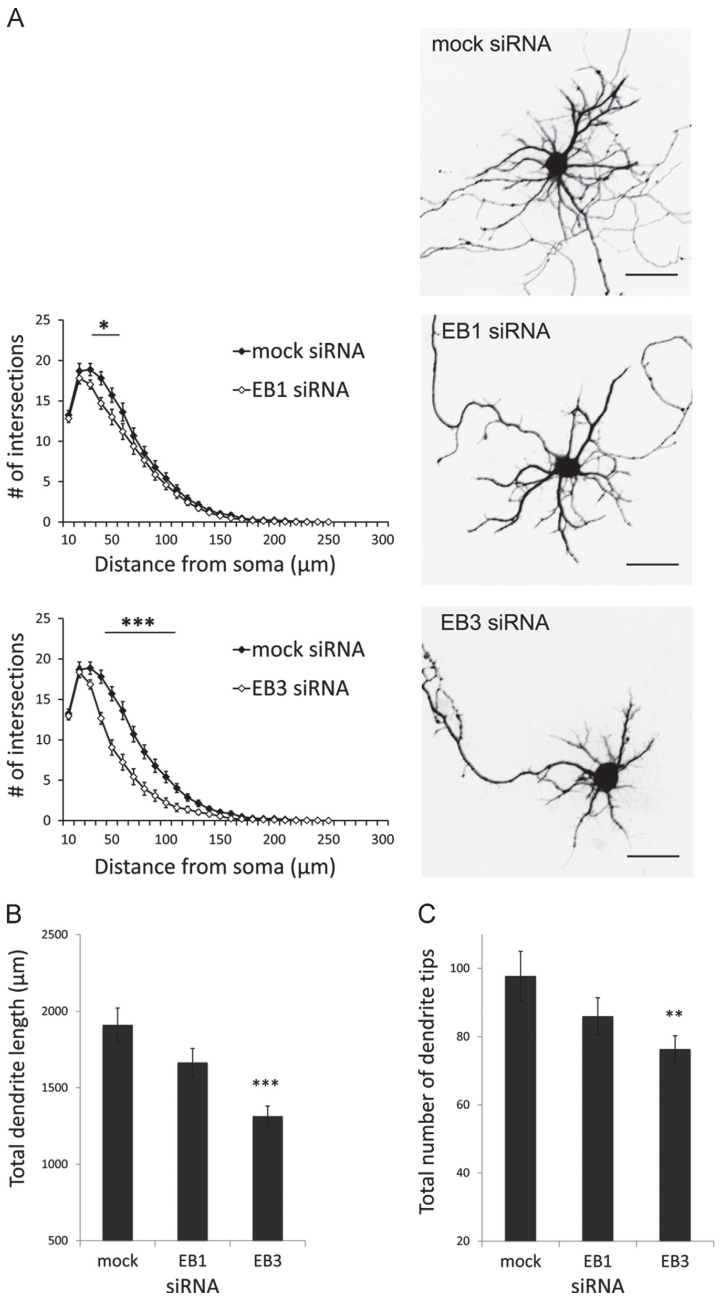


**Fig. 3 – EB3 depletion decreases dendrite growth. (A) EB3 siRNA impairs dendrite arborization. On the right are representative GFP images of hippocampal neurons co-transfected with pEGFP and mock siRNA, EB1 siRNA or EB3 siRNA at 6DIV and fixed at 9 DIV. Scale bars 50 μm. On the left are comparative Scholl analyses. Total length of dendrites (B) and total number of dendritic tips (C) were measured. The results are the means ± s.e.m. of three independent experiments ($n > 50$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.**

compensate for each other, we assessed the influence of double and triple knockdowns as well. Indeed, the combinations of siRNAs had an additive effect, reaching the reduction of dendritic length by one third when the expression of all three B-plexins was suppressed. From these results we can conclude that all B-plexins are involved in dendrite growth in a cooperative manner.

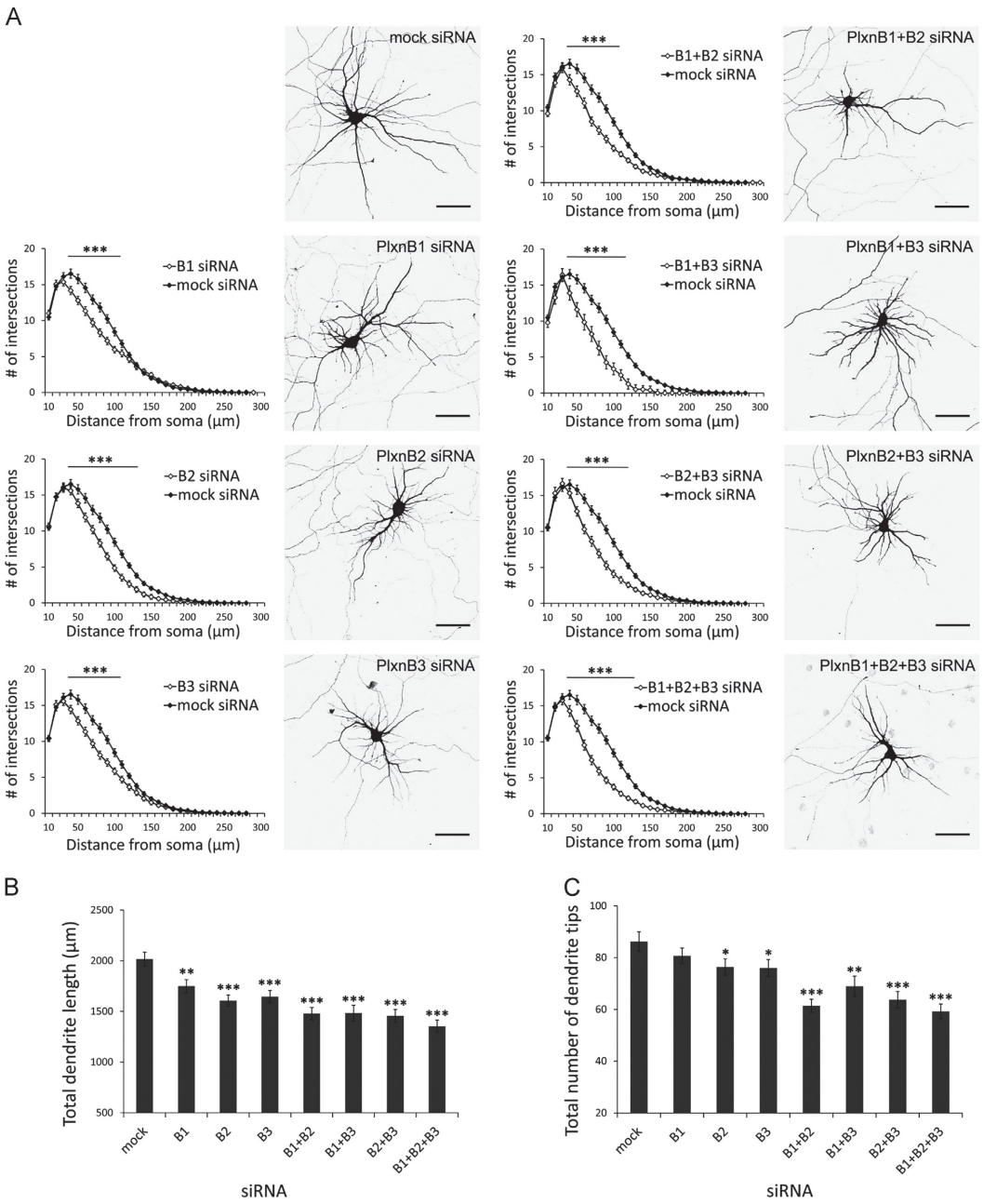


**Fig. 4 – B-plexins in dendrite development. (A) B-plexin knockdown interferes with dendrite growth. Hippocampal neurons were co-transfected with pEGFP and indicated siRNAs. Neurons were transfected at 6 DIV and fixed at 9 DIV. Representative images are on the right. Scale bars 50 μm. On the left are comparative Scholl analyses. Total length of dendrites (B) and total number of dendritic tips (C) were measured. The results are the means ± s.e.m. of three independent experiments ($n > 40$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.**

## Discussion

The microtubule cytoskeleton is a key determinant in generating and maintaining neuronal morphology and function. The remodelling and reorganization of microtubules in the growth cone are required for persistent growth cone advance as well as for the recognition of guidance cues [40]. EB proteins are central adaptor proteins at growing MT tips that have been shown to form complexes with a variety of other proteins [11,41], but among these only a few transmembrane receptors have been described so far [42,43]. In this study, the role of all three B-type plexins was systematically analyzed in the regulation of MT tip dynamics and dendrite growth. As a result we provide a novel insight into molecular mechanisms how semaphorin signals are transmitted to the cytoskeleton.

It appears that there is an important role in EB binding to plexins. We would like to emphasize that overexpression of B-plexins had a consistent and statistically significant effect on MT tip dynamics that correlated with their ability to interact with EB proteins (Fig. 1). Although at first sight the differences may seem small, one should bear in mind that both microtubule and dendrite growths are regulated by multiple factors and pathways. Therefore manipulating only one of them does not necessarily have an obvious outcome. Plexin-B1 and B3 that can bind to EBs induced more catastrophes and pauses at MT tips thereby slowing them down. Deletion of EB-binding region in B3IC resulted in a situation where it was still able to promote growth rate, but there were less catastrophes and pauses. This demonstrates that there is a balance between MT growth and dynamics. Plexins can promote MT growth via pathways that are not dependent on direct EB interaction, indicating that additional factors are involved. Possible candidates are certainly RhoA [44], Rnd1 [45] or other Rho-family GTPases that can interact with the Rho binding domain (RBD) of plexins [46].

Influence of B-plexin depletion on dendrite arborization (Fig. 4) correlated with induction of microtubule growth rate. As double and triple knockdowns of B-plexins had an additive effect, we conclude that they act in a cooperative manner. Plexin-B2 overexpression increased MT velocity and its depletion individually had the most adverse effect on dendrite growth. Lack of Plexin-B2 has been associated with a small decrease in neurite length of olfactory bulb neurons [47], however, dendrite morphology was not examined in detail in that study. Still, it can be concluded that Plexin-B2 is a positive regulator of dendrite growth. Plexin-B1, that in the MT tip speed aspect was indistinguishable from the control, was not very important for dendrite elongation. Our results are different from previous observations regarding the role of Plexin-B1 in dendritogenesis, where Plexin-B1 was described as a negative regulator [21]. In our system overexpression of intracellular domains of B-plexins did not alter dendrite length or branching (data not shown). Previous reports regarding the influence of Sema4D (high-affinity ligand of Plexin-B1) on dendritic arborization have also been contradictory [21,13]. While the first group observed Plexin-B1 mediated inhibitory effect of Sema4D on dendrite growth, the latter reported that Sema4D potentiated the formation of higher order branches. The discrepancy may be based on the different experimental setups as the outcome of plexin signalling varies significantly depending on the cellular context. Actually both are in agreement with our observation that activated Plexin-B1 increases microtubule dynamic instability. More dynamic MT tips promote branch formation but at the same time increased number of catastrophes and pauses slows elongation.

Our hypothesis that semaphorins and plexins regulate dendritogenesis via microtubule end binding proteins was further verified by the fact that Sema4D treatment increased the number of EB3-GFP dynamic events in live neurons (Fig. 2) that is appropriate for a guidance cue. Localized changes in microtubule dynamics are an important component of the growth cone response to extracellular signals. Positive cues such as NGF promote MT growth but repellents such as CSPGs reduce microtubule polymerization rate [48]. In epithelial cells HGF induces reorganization of cytoskeletal structures and increases MT growth rate EB1 dependently [49]. In Myoblasts EB3 is necessary for the regulation of microtubule dynamics and microtubule capture at the cell cortex, which in turn regulates the formation of polarized membrane protrusions and cell fusion [50]. In line with this data depletion of EB3 strongly interfered with dendrite growth (Fig. 3). Surprisingly, EB1 depletion had only mild consequences.

Taken together, plexins are multifunctional regulators of MT tips. When they interact with EB-s (Plexin-B1 and B3) they make microtubules more dynamic enabling the dendrites to find the correct path. We propose a model that upon semaphorin binding conformation of plexin intracellular domain changes, allowing it to transiently capture EB3, forcing the MT tip to pause or retract and reinitiate its growth, possibly recruiting also actin remodelling proteins to the complex. On the other hand, when plexins do not interact with EB-s (Plexin-B2) they accelerate microtubules and based on that also dendrite elongation. We conclude that B-plexins play an important role in directly mediating semaphorin signals to the microtubule cytoskeleton thereby guiding dendrite growth, and hence could be connected to the pathogenesis of neurological disorders.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2014.06.005.

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**Supplementary Information**

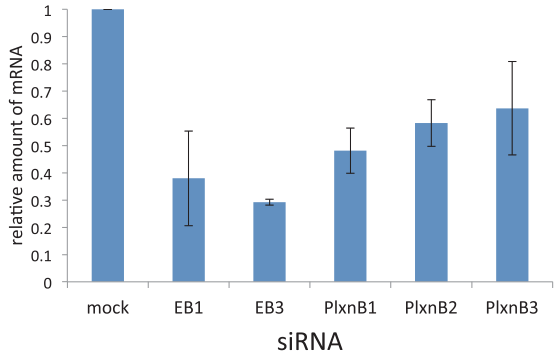


**Fig. S1 - Knockdown efficiency of EB1, EB3, Plexin-B1, Plexin-B2 and Plexin-B3 siRNAs. Rat hippocampal neurons were co-transfected with siRNAs and a vector encoding EGFP at 6 DIV. At 9 DIV RNA was extracted from cells and the mRNA levels were analyzed with quantitative RT-PCR. Ppia was used as a reference. The results are the means ± s.e.m. of three independent experiments. Relatively small reduction in mRNA levels is due to the poor transfection efficiency of neurons.**

# PUBLICATION III

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Research Article

# Plexin-B3 suppresses excitatory and promotes inhibitory synapse formation in rat hippocampal neurons

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ABSTRACT

Molecular mechanisms underlying synaptogenesis and synaptic plasticity have become one of the main topics in neurobiology. Increasing evidence suggests that axon guidance molecules including semaphorins and plexins participate in synapse formation and elimination. Although class B plexins are widely expressed in the brain, their role in the nervous system remains poorly characterized. We previously identified that B-plexins modulate microtubule dynamics and through this impact dendrite growth in rat hippocampal neurons. Here, we demonstrate that Plexin-B2 and Plexin-B3 are present in dendrites, but do not localize in synapses. We find that overexpression of all B-plexins leads to decreased volume of excitatory synapses, and at the same time Plexin-B1 and Plexin-B3 promote inhibitory synapse assembly. Plexin-B3 mutants revealed that these processes use different downstream pathways. While elimination of excitatory synapses is the result of Plexin-B3 binding to microtubule end binding proteins EB1 and EB3, the increase in inhibitory synapses is mediated by regulation of Ras and Rho GTPases. Overall, our findings demonstrate that Plexin-B3 contributes to regulating synapse formation.



## Introduction

Development and functioning of the mammalian central nervous system (CNS) is a complicated multilevel task. The importance of semaphorins in defining specific neuronal connections has been known since the discovery of this protein family in the 1990s [1,2]. Semaphorins and their receptor complexes consisting of plexins and neuropilins participate in all stages of CNS development: proliferation, migration, axon guidance, dendritic orientation, synaptogenesis, neuron survival and synaptic plasticity (reviewed in [3]).

Most neurodevelopmental disorders are characterized by loss of certain neurons or reduced connectivity between neurons. In contrast, autism spectrum disorders (ASD) are characterized by an excess of dendritic spines and synapses that result from defective synapse elimination [4]. The balance between excitatory and

Abbreviations: DIV, days *in vitro*; GABA, gamma-aminobutyric acid; GAD65, glutamate decarboxylase 65 kDa; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; glm, generalized linear model; IC, intracellular domain; MAP2, microtubule associated protein 2; Plxn, plexin; PSD95, postsynaptic density protein 95; Sema, semaphorin

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inhibitory signals is crucial for normal functioning of the brain [5]. Semaphorins are usually functioning as negative regulators of dendritic spine and glutamatergic synapse formation [6–11]. It is known that several semaphorins and plexins are mutated or aberrantly expressed in ASD where the elimination of inappropriate synapses is disrupted [12–16].

Surprisingly few studies deal with the role of B-plexins and their ligands class 4 semaphorins in the establishment of connections between neurons and in modulating synaptic plasticity. Sema4B is involved in glutamatergic as well as GABAergic synapse formation [17]. Sema4B colocalizes with postsynaptic markers in hippocampal neurons and interacts with PSD95 through a PDZ-binding motif at the C-terminus. A similar motif can be found in Sema4C, 4G and 4F but not in Sema4A or 4D [18,19]. The role of this interaction in synapse assembly has not been elucidated. Sema4D facilitates the formation of GABAergic synapses and transiently decreases the number of glutamatergic synapses [17,20]. It has been demonstrated that Sema4D effects on synaptogenesis are mediated by Plexin-B1 [20]. Plexin-B1 and Plexin-B3 knockout mice do not have clearly recognizable abnormalities in brain structures nor behaviour [21,24,25,26]. However, mild autistic behaviour is not always evident in conventional anxiety tests and might have been overlooked [11]. Plexin-B2 deficient mice exhibit defects in granule cell migration and brain morphology [23,24], but the effect of Plexin-B2 on synaptogenesis has not been studied in detail.

Studies regarding Sema4D [20,21], Sema5A [11] and Sema5B [8] have shown that the Sema domain of semaphorins is crucial in regulating synaptogenesis whereas the intracellular domain is dispensable. From that it can be concluded that the signal is transduced in a forward manner from the semaphorin to the plexin receptor. Semaphorins can be functional bound to membranes *in trans* or *in cis,* or in soluble forms. Both Sema4D and its receptor Plexin-B1 are present in postsynaptic membranes [21,22], favouring the *cis* interaction model.

Plexins are multitask proteins containing a signal peptide, a Sema domain, three cysteine rich PSI domains (plexins, semaphorins, integrins), six glycine and proline rich IPT domains (immunoglobulin-like fold shared by plexins and transcription factors), a transmembrane domain (TM), a juxtamembrane NTS (N-terminal segment), a bipartite RasGAP domain (Ras GTPase activating protein) that is split by a RhoBD (Rho GTPase binding domain). B-plexins have a PDZ-binding motif at their C-terminus that interacts with RhoGEFs [27]. Semaphorins facilitate plexin dimer formation, stabilization, GAP domain activation and downstream regulation of Rap GTPases [28], whose role in synaptogenesis has been proven [29]. The RapGAP pathway directly participates in Sema5A-Plexin-A2-mediated regulation of dendritic spine density [11], and PLX-1-guided establishment of tiling border of presynaptic terminals between different axons in *Caenorhabditis elegans* [30]. In addition, plexins regulate the activity of Rho GTPases. Sema4D activates RhoA-ROCK pathway through Plexin-B1-RhoGEF interaction and induces the formation of protrusions and dendritic spines [31,22].

In our previous study we showed that B-plexins participate in the regulation of dendrite growth and morphology [32]. To clarify the function of plexins at later stages of hippocampal neuron development, we analysed the role of B-plexins in synaptogenesis. Here, we demonstrate that Plexin-B2 and B3 proteins are present in neuronal cell bodies and dendrites, but do not colocalize with synaptic markers. We also provide evidence that B-plexins are negative regulators of glutamatergic synapses and in the case of Plexin-B3 this process involves interaction with microtubule end binding proteins. In contrast, overexpression of Plexin-B3 stimulates the formation of GABAergic synapses through regulation of Ras and Rho GTPases.

## Experimental procedures

### DNA constructs

Expression constructs of human B-plexins encoding transmembrane and intracellular domains (PlxnB1IC, PlxnB2IC, PlxnB3IC and mutated Plexin-B3IC constructs) were in p3xFLAG and have been described previously [33]. Additional Plexin-B3IC mutant was generated using site-directed mutagenesis to replace residues Ile1328 and Pro1329 in the EB-binding SxIP motif with Asn and Ala respectively. Expression of all plexin constructs was verified with immunocytochemistry and western blot (Supplementary Fig. 2). For Plexin-B3IC mutants see also Fig. 3C. pEGFP originated from Clontech.

### Cell culture and transfection

All animal procedures were performed in compliance with the local ethics committee. Primary rat hippocampal neurons were dissected from Sprague Dawley rat embryos (male and female) at E20. Briefly, the hippocampi were dissected and cells were dissociated with 0.25% trypsin (Invitrogen), treated with 0.05% DNase I (Roche), and the cell suspension was plated on poly-L-lysine-coated cover slips in 24-well plates in Neurobasal A medium (Invitrogen) with B27 supplement (Invitrogen), penicillin (PAA Laboratories, 100 U/ml), streptomycin (PAA Laboratories, 0,1 mg/ml), and 1 mM L-glutamine (PAA Laboratories). Mitotic inhibitor was omitted from the medium to allow glial cell growth for better neuron survival. Neurons were cultured for three weeks. Such cultures contained approximately 10% GAD65 positive GABAergic neurons, the rest were granular and pyramidal neurons. For synapse measurements neurons were cotransfected with pEGFP and different plexin constructs using Lipofectamine 2000 (Invitrogen) at 16 DIV. Empty pFLAG vector was used as a negative control.

### Immunostaining and microscopy

For immunocytochemistry the cultured neurons were fixed at 21 DIV in freshly prepared 4% paraformaldehyde, 4% sucrose in PBS (phosphate buffered saline, pH 7.4) at room temperature for 10 min and postfixed with cold methanol for 10 min. This was followed by permeabilization with 0.2% Triton X-100 in PBS and blocking in 3% BSA in PBS. Antibody incubations were carried out at room temperature for one hour and cells were washed with PBS-Tween. Primary antibodies used were: mouse anti-FLAG M2 (1:500, Sigma), rabbit anti-EGFP (1:2000, from A. Merits); chicken anti-MAP2 (1:5000, Abcam), guinea pig anti-Synaptophysin1 (1:2000, Synaptic Systems), mouse anti-PSD95 [6G6–1C9] (1:50, Abcam), guinea pig anti-GAD65 (1:500, Synaptic Systems), mouse anti-gephyrin (1:100, Synaptic Systems), rabbit anti-Plexin-B1 (1:100, Abcam, did not work) Armenian hamster anti-Plexin-B2 [3E7] (1:100, Abcam), rabbit anti-Plexin-B3 Sema domain (1:500, custom made [34]). Anti-Plexin-B3 affinity purified polyclonal antibody was obtained from rabbit immunized with a peptide

corresponding to human Plexin-B3 residues 354–369 (TSRCVTLPLDSPESYP). This peptide is conserved among Plexin-B3 proteins of different species but at the same time does not resemble sequences of other plexin family members. Its specificity was verified in immunocytochemistry (Supplementary Fig. 1). Secondary antibodies were: goat anti-rabbit IgG Alexa488 (1:2000, Molecular Probes), goat anti-mouse IgG Alexa568 (1:2000, Molecular Probes), goat anti-guinea pig IgG Alexa568 (1:2000, Molecular Probes), goat anti-chicken Alexa568 (1:2000, Molecular Probes), goat anti-Armenian hamster IgG DyLight488 (1:1000, Nordic BioSite). Prolong Gold antifade reagent (Invitrogen) was used for mounting.

Zeiss LSM510 confocal microscope system was used to scan stacks of fixed neurons. Neurons were imaged with Plan Apochromat 63 × 1.4 NA or Plan Apochromat 100 × 1.4 NA oil immersion objective (Zeiss). 5 to 8 0.8 μm optical sections with a step size of 0.41 μm and a resolution of 2048 × 2048 pixels were scanned. Within each experiment, images were acquired with identical settings for 561 nm laser that was used for synaptic markers. 488 nm laser intensity was kept constant while detector gain was adjusted according to the relative expression level of GFP in each cell.

### Image analysis and statistics

Synapse volume and number was quantified in a blinded manner as the overlap of GFP (marking the transfected neuron) and synaptic marker antibody. For PSD95 analysis three dendrite sections of 20–40 μm per cell were manually cropped, for GAD65 analysis cell bodies were selected, Synaptophysin1 images were acquired so that GFP positive axons of only a single glutamatergic cell (the type of neurons was assessed based on morphology) were in the field and were analysed as whole images. For synapse quantification Imaris 6.4.2 software (Bitplane) surfaces module was used. The threshold was kept identical for both channels within one experiment. GFP channel was used to create a mask that enabled to eliminate synapses of neighbouring cells. To calculate relative synapse volume and number, obtained values were divided by GFP volume. At least 40 cells were analysed from three independent culture preparations per treatment group. Synapse parameters within each experiment were normalized to the mean value of the control group transfected with pEGFP and pFLAG to account for the variation of neuron cultures and antibody staining intensity between experiments. Significance of the differences was assessed using glm test in R package stats. Bar plots were generated with MS Excel and images were assembled in Adobe Photoshop. LSM Image browser (Zeiss) and ImageJ (NIH) were also used to prepare the images.

## Results and discussion

### Localization of Plexin-B2 and Plexin-B3 in cultured rat hippocampal neurons

Despite the fact that the original discovery of plexins was based on a monoclonal antibody that recognized *Xenopus* plexin [35], the availability of specific and good antibodies for plexins and semaphorins has been poor. Often commercial and custom made antibodies fail to recognize plexins altogether or work only in western blot. Therefore, studies regarding expression patterns of plexins have been mainly done by mRNA in situ hybridization and thus do not give any information about protein localization.

Plexin-B1 subcellular localization in rat hippocampal neurons has been described previously [26]. Plexin-B1 immunoreactivity was seen in neuronal cell bodies and along dendritic shafts as punctate structures that colocalized with PSD95 indicating its presence in excitatory synapses. Our attempts to confirm those results failed, as the purchased polyclonal antibody did not recognize Plexin-B1 in any of the methods we tested (data not shown). Plexin-B2 is expressed widely throughout the nervous system and also in other organs [36,37]. Plexin-B3 is prominently expressed in CNS neurons [34] and oligodendrocytes [36] and in contrast to other B-plexins its levels are low prenatally and rise after birth during the period of neurite growth and synaptogenesis. As the subcellular localization of Plexin-B2 and B3 in neurons has not been published, then we performed immunocytochemistry of 21 DIV rat hippocampal neurons with different neuronal markers. This time point was selected as most synapses have matured by then. Plexin-B2 and B3 expression levels varied in different neurons from very weak to moderate. Both were also visible in different MAP2 negative non-neuronal cells consistent with previous Plexin-B3 studies [34,36]. Plexin-B2 expression in glial cells has not been described earlier. Plexin antibody signals were strongest in neuronal cell bodies, diffuse in dendrites and occasionally they were detected also along axons (Fig. 1A). Plexin-B2 was more prominent in the cell soma than Plexin-B3. Immunoreactivity of both plexins exhibited a punctuate distribution along dendrites partially colocalizing with MAP2 (Fig. 1A–C), but surrounding it rather than directly associating with it. When we compared the localization of these puncta with synaptic markers, no significant overlap was detected (Fig. 1B and C). Generally strong signals of plexins and synaptic proteins excluded each other, on rare occasions colocalization with presynaptic markers Synaptophysin1 and GAD65 could be observed. In conclusion, Plexin-B2 and B3 proteins appear in dendrites as puncta along the dendritic shaft, but there is no enrichment in synaptic compartments. Such extrasynaptic localization in dendrites has also been observed in the case of Plexin-A2, Plexin-A3, Sema5A [11] and Sema5B [8]. In *C. elegans* neurons PLX-1 is localized to dendrites as well as to the proximal and distal axons, but is largely absent from the synaptic domain [30]. Plexin-A1, Plexin-B1 and Sema4D have a different distribution, being enriched in the postsynaptic density fraction [11,22,21], and Sema7A colocalizes with inhibitory synapses [38]. However, many of these results are based on subcellular fractionation studies and the precise localization of proteins with immunocytochemistry has not been determined.

### B-plexins are negative regulators of excitatory synapses

Axon targeting and synaptic specificity are regulated by a combination of different molecular signals that can compensate each other. Loss of function experiments often fail to show any effects on synaptogenesis whereas experiments using gain of function are frequently more informative [39]. Therefore, we chose the strategy of overexpressing the intracellular domains of B-plexins, which is known to functionally mimic ligand-activated full-length plexins [40]. Rat hippocampal neurons were transfected at 16 DIV and analysed at 21 DIV. pEGFP was cotransfected to detect plexin overexpressing single neurons. All checked GFP-expressing neurons were also positive for B-plexin staining indicating efficient coexpression. Also

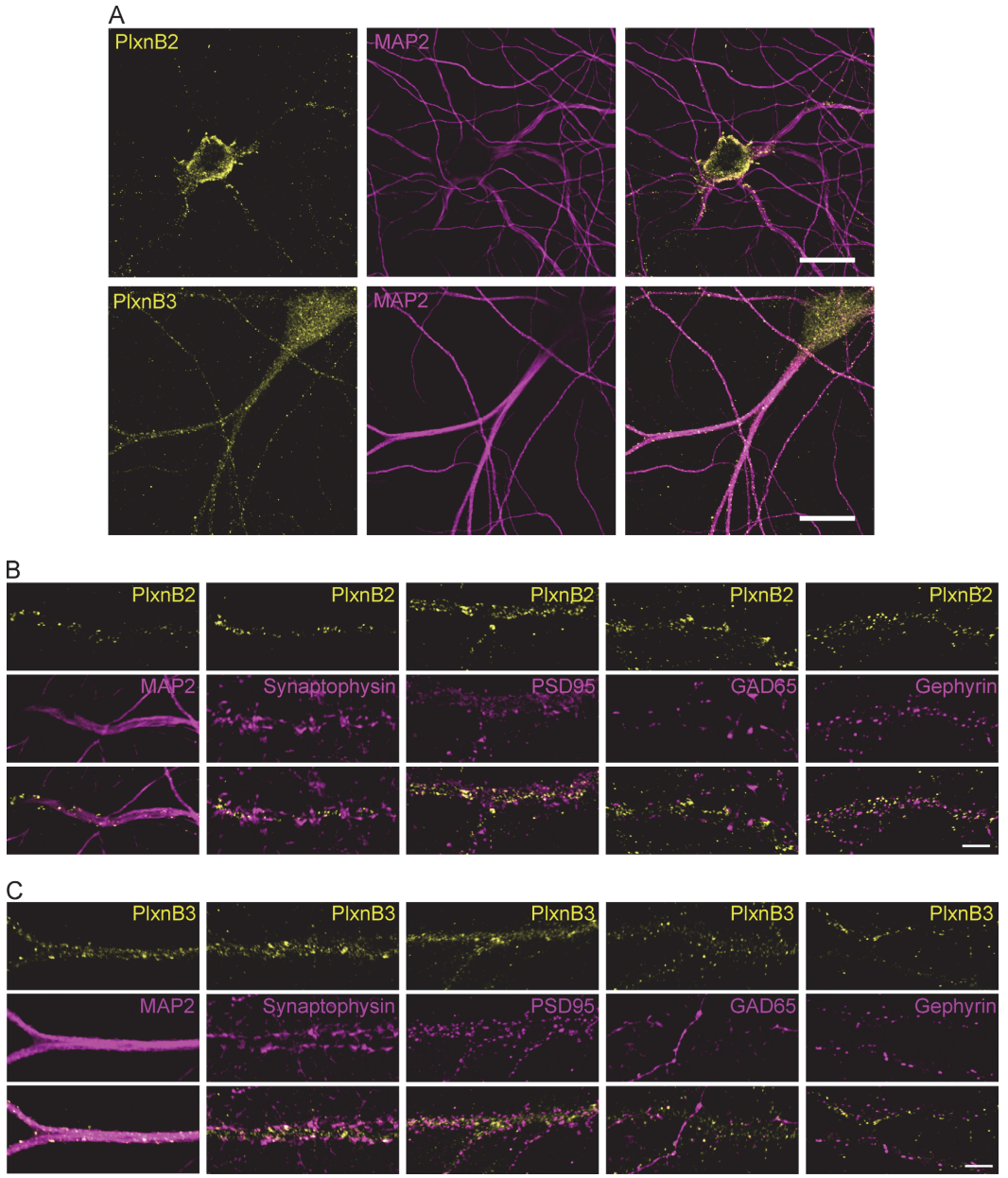


**Fig. 1 – Plexin-B2 and B3 expression in hippocampal neurons. Pseudocoloured confocal images of 21 DIV rat hippocampal cultures labelled with anti-Plexin-B2 or anti-Plexin-B3 antibody (yellow) plus the specified marker (magenta). (A) Plexin-B2 and B3 localize to neuron cell bodies and puncta along the dendrites labelled with MAP2. Maximum intensity projections of confocal images, scale bars 20 μm. (B) Co-immunolabelling with Plexin-B2 and synaptic markers: Synaptophysin1 (presynaptic), PSD95 (excitatory postsynaptic), GAD65 (inhibitory presynaptic) or gephyrin (inhibitory postsynaptic) demonstrated minimal colocalization with Plexin-B2. Single confocal slices of 0.8 μm are presented, scale bar 5 μm. (C) Same as (B), but with Plexin-B3.**

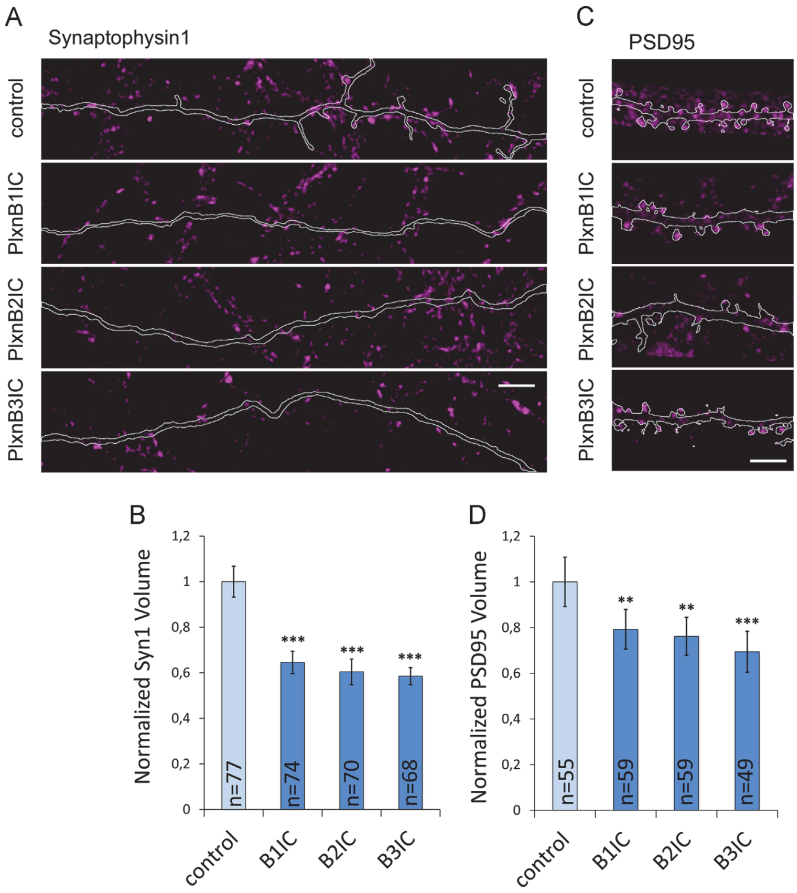


**Fig. 2 – B-plexins inhibit the formation of excitatory synapses. (A) Representative images of rat hippocampal axon sections transfected with different B-plexin intracellular domain (IC) constructs at 16 DIV and fixed at 21 DIV. Pseudocoloured maximum projections of confocal images labelled with anti-Synaptophysin1 antibody (magenta) to visualize the presynaptic side of synapses. Anti-EGFP signal was used to mark axon boundaries, scale bar 5 μm. (C) Same as in A, but with anti-PSD95 and dendrite segments are presented. (B,D) Quantification of Synaptophysin1 volume from A and PSD95 volume from C normalized to the mean value of the control group. The results are the means ± s.e.m. of three independent experiments. Cell numbers analysed are denoted on the bars. $^{**}p < 0.01$, $^{***}p < 0.001$, glm.**

the expression levels of different plexins and B3IC mutants were comparable (Supplementary Fig. 2). First, we assessed the impact of B-plexins on excitatory synapses. For that we analysed axons of glutamatergic neurons stained with presynaptic marker Synaptophysin1 or dendrites stained with postsynaptic marker PSD95. All three B-plexins significantly reduced the amount of excitatory synapses (Fig. 2). Overall our results agree with the study performed by Kuzirian and colleagues [20], who described a transient decrease of glutamatergic synapses in response to Sema4D. The reasons for the difference in the duration of the negative effect may lie in experimental strategy and time frame. Treatment with Sema4D activates plexins for a certain period, while our plexin constructs are constantly active. Our results do not agree with that of Lin and colleagues [26], who observed an increase in dendritic spine density, that generally is considered to reflect the number of excitatory synapses, upon Plexin-B1 activation with Sema4D. Given the naturally occurring large variation in hippocampal neuron morphology and spine density, these results should be handled with caution, as the number of analysed neurons in their study was very small. The observed inhibitory effect of plexins on glutamatergic synapses is in agreement with multiple studies where the addition of semaphorins has been shown to reduce the number of synapses, or depletion of semaphorins or plexins has led to excessive formation of dendritic spines or excitatory synapses. Such observations have been made in case of class 3 semaphorins whose signals are mediated by A or D class plexins [7,10,9], and Sema5A and Sema5B that use A-plexins as receptors [11,8,41].

We further studied which domain of the intracellular part of Plexin-B3 is responsible for the reduction of glutamatergic synapses (Fig. 3). Neither RasGAP domain mutation (ΔRasGAPII

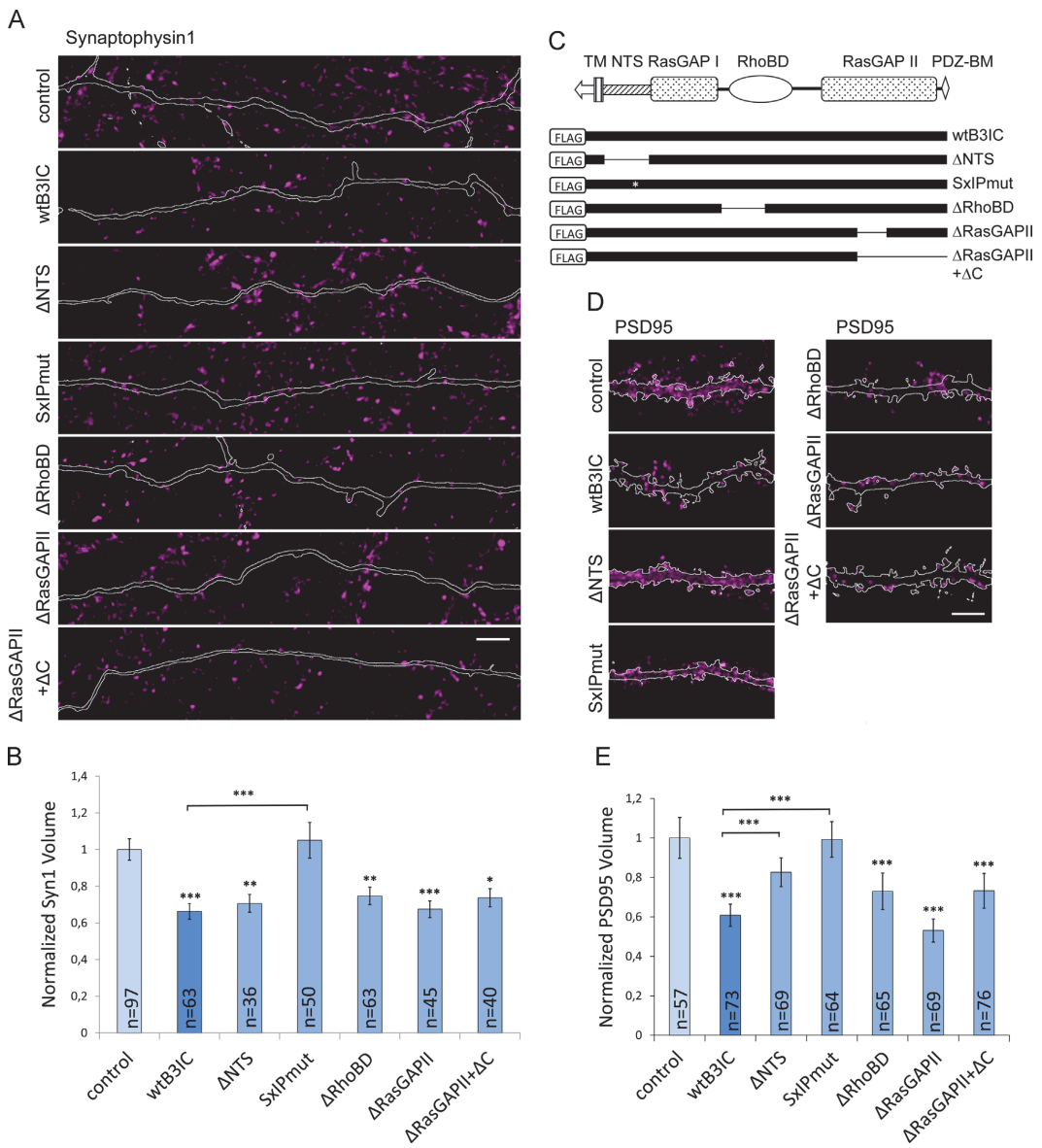


**Fig. 3 – Interaction with microtubule end binding proteins is required for Plexin-B3 to inhibit the formation of excitatory synapses. (A) Representative images of rat hippocampal axon sections transfected with different Plexin-B3 intracellular domain mutants at 16 DIV and fixed at 21 DIV. Pseudocoloured maximum projections of confocal images labelled with anti-Synaptophysin1 antibody (magenta) to visualize synapses. Anti-EGFP signal was used to mark axon boundaries, scale bar 5 µm. (D) Same as in A, but anti-PSD95 was used to mark postsynaptic densities in dendrites. (B,E) Quantification of synapse volume from A and D normalized to the mean value of the control group. The results are the means ± s.e.m. of three independent experiments. Analysed cell numbers are denoted on the bars. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, glm. (C) Diagram illustrating Plexin-B3 intracellular region deletion and point mutation constructs with N-terminal 3xFLAGtag. Relevant protein domains are indicated on top. TM - transmembrane domain, NTS - N-terminal segment, RasGAP - Ras GTPase activating protein, RhoBD - Rho GTPase binding domain, PDZ-BM—PDZ-binding motif.**

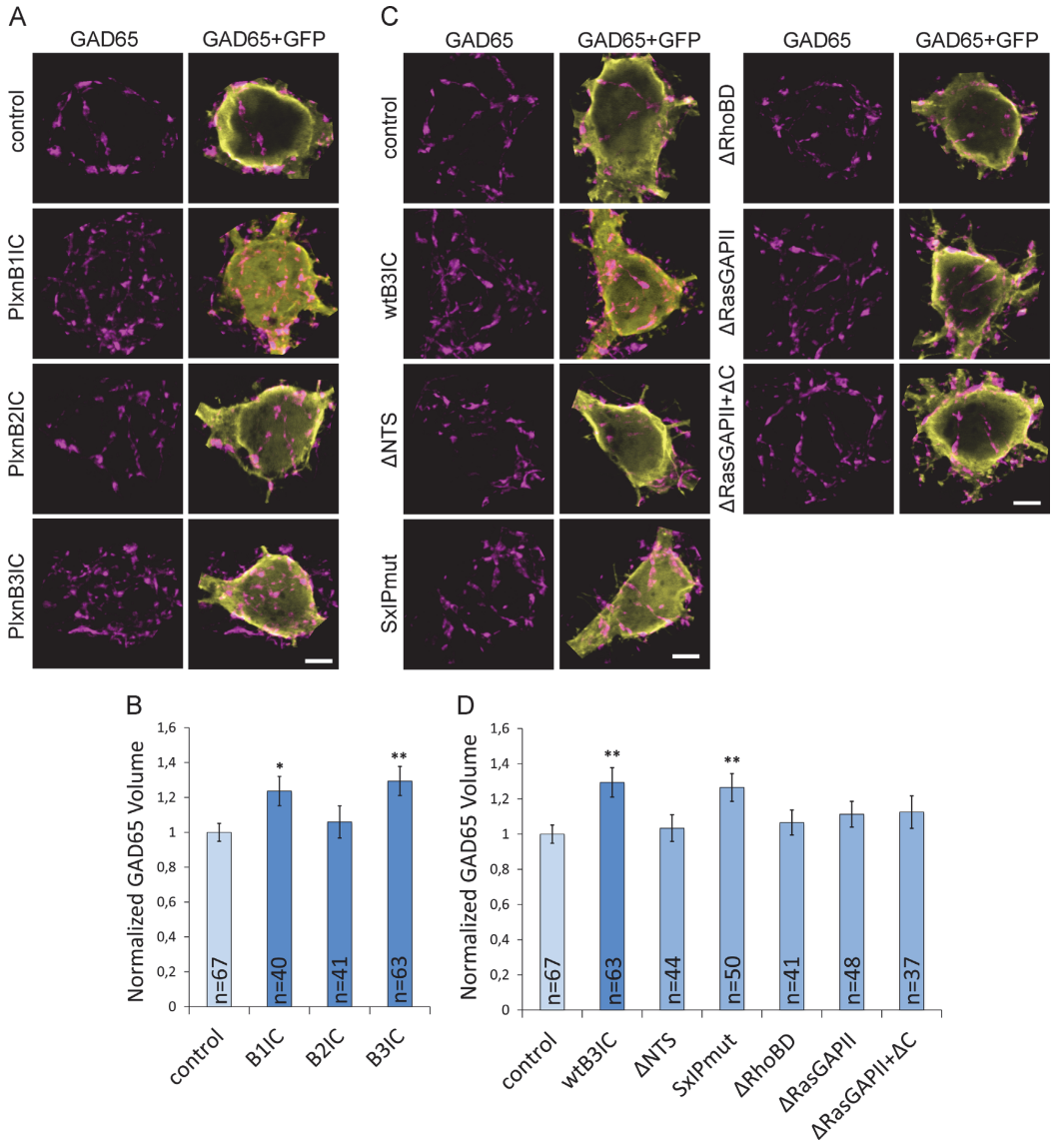


**Fig. 4 – Plexin-B1 and B3 facilitate inhibitory synapse formation. (A) Representative images of rat hippocampal neuron bodies transfected with different B-plexin intracellular domain (IC) constructs at 16 DIV and fixed at 21 DIV. Pseudocoloured maximum projections of confocal images labelled with anti-GAD65 antibody (magenta) to visualize inhibitory synapses and anti-EGFP (yellow), scale bar 5 µm. (C) Same as in (A), but with Plexin-B3IC mutants. (B,D) Quantification of synapse volume from A and C normalized to the mean value of the control group. The results are the means ± s.e.m. of three independent experiments. Analysed cell numbers are denoted on the bars. *$p<0.05$, **$p<0.01$, glm.**

and ΔRasGAPII+ΔC) nor the mutations interfering with Rho GTPase regulation (ΔRhoBD and ΔRasGAPII+ΔC) affected synapse elimination. Independence of RasGAP domain was surprising as its requirement for dendritic spine regulation has been shown in case of Plexin-A2 [11] and for synaptic tiling by *C. elegans* PLX-1 [30]. We found that Plexin-B3 mutants ΔNTS and SxIPmut, that are defective for microtubule end protein EB3 binding, were unable to reduce the volume of PSD95 (Fig. 3E), and SxIP mutant also the volume of Synaptophysin1 (Fig. 3B). Previously, we have shown that Plexin-B3 regulates microtubule dynamics and

dendrite growth through EB-proteins [32]. EB3 is required for normal dendritic spine and synapse formation [42], and PSD95 is able to interact with EB3 [43]. It could be suggested that Plexin-B3 sequesters EB3 from its other interaction partners and thus interferes with its positive role in stabilizing postsynaptic structures in dendritic spines. That in turn leads to disassembly of the synapse and dissociation of presynaptic compartment as well. As Plexin-B2 does not interact with EB3 then it has to use alternative signalling pathways. Overall, our results indicate that activated B-plexins reduce the number of glutamatergic synapses and in the case of Plexin-B3 regulation of microtubule dynamics is involved in this process.

### Intracellular domains of Plexin-B1 and B3 promote the formation of inhibitory synapses

To determine how different B-plexins influence GABAergic synapse formation, we over-expressed the intracellular domains of Plexin-B1, B2 and B3 in hippocampal neurons as described above. It has been shown that Plexin-B1 is absolutely necessary for mediating the positive Sema4D signals in inhibitory synapse assembly [20]. Sema4D caused an increase in GABAergic synapse density onto both the soma and dendrites of glutamatergic neurons and the absence of Plexin-B1 abrogated this phenomenon. As technically it is easier to count and measure the synapses around the cell soma, we focused on neuron bodies, based on the fact that Sema4D-induced inhibitory synapse formation is not dependent on subcellular localization [20]. The constructs used in this work mimic semaphorin-activated plexins and therefore we expected that Plexin-B1IC over-expression would enhance the formation of GABAergic synapses. That was indeed the case (Fig. 4A and B). Our results show that Plexin-B1IC and Plexin-B3IC, but not Plexin-B2IC, acted in a positive manner on inhibitory synapses.

In contrast to excitatory synapse formation, EB-binding does not seem to be of crucial importance for Plexin-B3IC as the SxIP mutant was still able to increase the volume of inhibitory synapses (Fig. 4C and D). Activation of plexin RasGAP domain and Rho GTPases are necessary for the process. RasGAP activity is probably inhibited in ΔNTS mutant due to impaired dimerization of Plexin-B3IC. Mutants ΔRasGAPII and ΔRasGAPII+ΔC do not have functional RasGAP domains either. Rho GTPase regulation is perturbed in ΔRhoBD and ΔRasGAPII+ΔC. Behaviour of these mutants did not differ significantly from negative control. Taken together, Plexin-B3 positively regulates GABAergic synapse density and downstream signalling cascades involve Rap GTPase as well as Rho GTPase activity regulation.

Surprisingly Plexin-B2 did not influence inhibitory synapse assembly and it could not be explained by the difference in binding to EB proteins. In contrast to Plexin-B1IC and B3IC, Plexin-B2IC has an effect on cell morphology inducing the growth of elongated protrusions in fibroblasts as well as hippocampal neurons, and it participates in cell migration [21]. This indicates that functionally B-plexins differ from each other. We do not know whether Plexin-B2 has different specificity towards Ras subfamily GTPases as direct RasGAP activity has been measured for all plexins but B2 and B3 [28]. On the level of primary structure the GAP domain of Plexin-B2 is similar to Plexin-B1 with some substitutions, which may lead to differences in binding to various Ras family GTPases. Largest variability between our B-plexin constructs lies in the transmembrane and Rho binding domains. As the transmembrane domain of Plexin-B2 contains residues that do not favour dimer formation it has been suggested that its activation mechanisms are different from Plexin-B1 and B3 [44]. Besides, Rnd1 may be the reason behind the contradictory role of Plexin-B2 in regulating inhibitory synapses. Plexin-B1 and B3 interact with Rnd1 [40, and personal unpublished observations]. The Rnd1-binding loop in the RhoBD of Plexin-B2 is substantially different from other B-plexins and thus likely does not interact with Rnd1. Rnd1 is highly expressed during synaptogenic period in the brain and localizes to dendritic membranes as distinct puncta [45], and may participate in inhibitory synapse formation.

The pattern of connections between neurons determines the functionality of the brain. Where exactly synapses form, is regulated by a multitude of molecular pathways, many of which remain to be characterized. The role of axon guidance proteins, including semaphorins and plexins in synaptogenesis is an emerging theme in molecular neurobiology. In summary, we have provided a detailed description of Plexin-B2 and B3 localization in hippocampal neurons that favours the *in cis* semaphorin-plexin signalling model in regulating synapse formation and disassembly. Based on literature and our current results it can be concluded that plexins are located in dendrites mostly extrasynaptically and mediate inhibitory signals of semaphorins to avoid excitatory synapse formation in wrong places. At the same time Plexin-B1 and B3 promote the formation of inhibitory synapses further regulating the balance of excitation and inhibition. Future experiments will be required to clarify the *in vivo* importance of B-plexins in establishing and maintaining synaptic balance.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2015.05.007.

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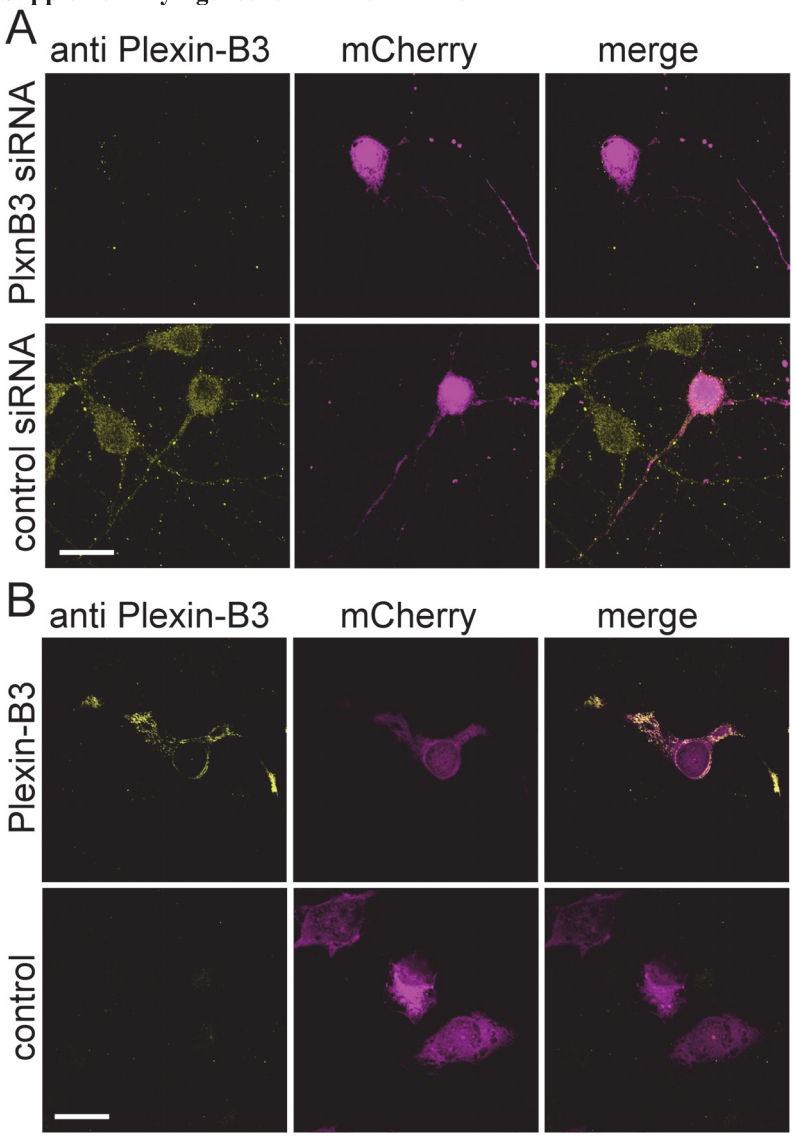
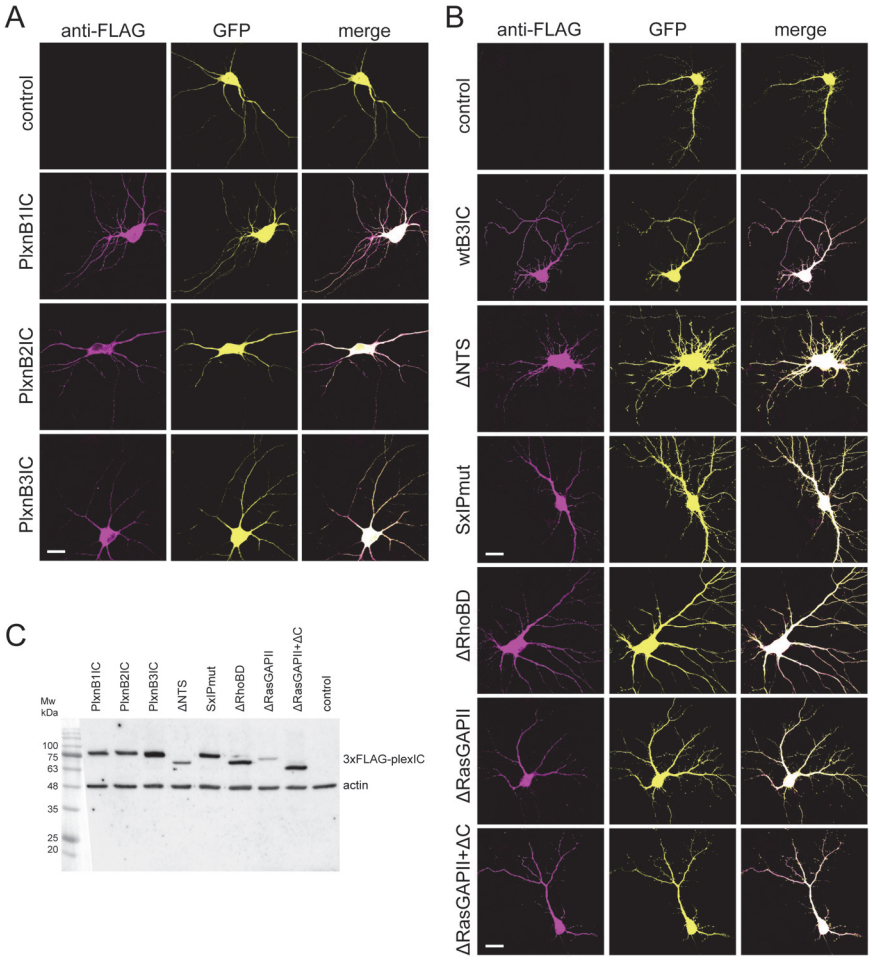
**Supplementary figures for Laht et al 2015**




**Supplementary figure 1** – Plexin-B3 antibody specificity in immunocytochemistry (ICC). Pseudocoloured single slices of confocal images of cells labelled with anti-Plexin-B3 antibody (yellow) plus cotransfected mCherry (magenta). Scale bars 20 µm. (A) Full-length human Plexin-B3 was overexpressed in 293FT fibroblasts. Cells were fixed with PFA and methanol and stained with anti-Plexin-B3 1:500 as described in Materials and Methods. Images were acquired with 100x oil immersion objective. Specific signal could be observed in the cytoplasm and membranes of Plexin-B3 overexpressing cells. (B) Rat hippocampal neurons were cotransfected with Plexin-B3 siRNA or control siRNA and pmCherry at 3 DIV and fixed at 9 DIV. ICC was performed as described. Plexin-B3 signal is significantly weaker in neurons treated with Plexin-B3 siRNA when compared to control siRNA treated neurons.




**Supplementary figure 2** – Expression control of different plexin intracellular domain constructs. (A) ICC of overexpressed intracellular domains of different B-plexins and (B) Plexin-B3IC mutants in rat hippocampal neurons. Pseudocoloured single slices of confocal images of neurons labelled with anti-FLAG antibody (magenta) and GFP (yellow). Scale bars 20 µm. Neurons were cotransfected with pEGFP and indicated plexin constructs in p3xFLAG at 13 DIV and fixed at 15 DIV. Images were acquired with 63x oil immersion objective. The expression levels of different plexins and subcellular localization were similar to each other. (C) 293FT fibroblasts were transfected with indicated plexin intracellular domain constructs in p3xFLAG. Cells were lysed one day post transfection and subjected to western blot with anti-FLAG antibody. Anti-actin was used in parallel as loading control.

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Heiti Paves showed me the way to the wonderful world of fluorescence microscopy. When you see something in live cells with your own eyes, it definitely gives a totally new perspective on molecular biology. A great part of my work relies on microscopy and image analysis, and it could not have been done without Heiti and his advice. He also urged me to take part in microscopy courses that have proven to be extremely beneficial for me.

I would like to thank Indrek Koppel for introducing me the art of neuron culture and providing assistance in that aspect whenever I needed.

The field of biostatistics has obtained a meaning for me thanks to Pille Pata, my sister Silja and Jaanus.

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I want to thank all my ancestors for providing me such good genes and all my teachers through the years who have shown me that everything can be fascinating if you go deep enough. I would like to highlight my first supervisors Ann Kilk and Lilian Järvekülg.

Last but not least, my greatest gratitude goes to my family who have enabled me a long and happy childhood and a supportive home.

# ABSTRACT

Development and functioning of the central nervous system is a complicated multilevel task where different processes have to be well co-ordinated. Plexins are a family of transmembrane receptors of semaphorins that in mammals have nine members: A1-A4, B1-B3, C1 and D1. They were discovered as axon guidance molecules, but it has become evident that plexins participate in all stages of brain development.

The general aim of my thesis was to find new interaction partners and signalling pathways for Plexin-B3, as its functions remained obscure. Yeast two-hybrid screen revealed that the intracellular part of Plexin-B3 forms complexes with microtubule end binding proteins EB1 and EB3. Further studies confirmed that the EB-homology domain interacts with the N-terminal segment (NTS) of Plexin-B3 intracellular domain that contains the conserved EB-binding motif SxIP. As EBs hold a central position in regulating microtubule plus-end dynamics we hypothesized that plexins influence microtubule growth. For that microtubule tips were labelled with EB3-GFP and monitored in live cells. Overexpression of Plexin-B1 and B3 that can bind to EBs induced more catastrophes and pauses. The ligand of Plexin-B1, Semaphorin 4D acted in a similar manner. In contrast, Plexin-B2 and Plexin-B3 ΔNTS mutant that do not bind EBs accelerated microtubule growth rate. Balance between dynamics and elongation of microtubules is important for dendrite growth as well as synaptogenesis. The role of B-plexins was systematically assessed in those processes in rat hippocampal neurons. With immunocytochemistry we determined that Plexin-B2 and B3 proteins localize in neurons mainly to the cell body and dendrites but are not especially enriched in synapses. It was found out that all three B-plexins are needed for normal dendrite growth and they act in a co-operative manner. In synaptogenesis overexpression of B-plexins had a negative effect on the formation of glutamatergic excitatory synapses, and Plexin-B1 and B3 promoted the formation of GABAergic inhibitory synapses. In the case of Plexin-B3 binding to EBs was involved in reducing excitatory synapses and regulation of Ras and Rho GTPases was of importance for the increase in inhibitory synapse volumes.

Taken together, these results show that Plexin-B3 binds to EB-family proteins through the SxIP motif, B-plexins influence microtubule tip dynamics, and are connected to dendrite growth and formation of synapses. This study significantly elaborates the knowledge of the role of Plexin-B3 and other B-plexins in neurons.

# KOKKUVÕTE

Inimese aju on imeline elund. Selle arenguks ja toimimiseks on vajalik erinevate molekulaarsete signaaliradade koostöö. Pleksiinid on rakupinna retseptorvalkude perekond, mille ligandid on semaforiinid ning nad osalevad närvisüsteemi arengu kõigis etappides. Juba pikka aega on teada, et semaforiinide lisamine põhjustab närvirakkude tsütoskeleti ehk rakutoese muutusi, kuid millised on selle nähtuse molekulaarsed tagamaad, on paljuski ebaselge. Rakutoes on pidevas liikumises, et võimaldada rakkudel paljuneda, sihipäraselt rännata ja kasvatada jätkeid.

Minu erilise tähelepanu pälvisid B-pleksiinid, iseäranis pleksiin-B3. Käesoleva doktoritöö eesmärgiks oli välja selgitada pleksiin-B3-e rakusisese osaga seonduvaid uusi valke ja nende interaktsioonide mõju närvirakkude arengule. Pärmi kaksikhübriidsüsteemi abil tuvastasime kaks seni teadmata seondujat – mikrotorukeste otsaga haakuvad valgud EB1 ja EB3. Alternatiivsete meetoditega tõestasime, et EB-d seovad veel pleksiin-A2-e ja pleksiin-B1-e, kuid mitte pleksiin-B2-e. Järjestuste võrdlemise ja mutantide abil ilmnes, et EBd kinnituvad pleksiin-B3-e rakusisese osa alguses olevale SxIP motiivile, mis on iseloomulik paljudele EB-dega seonduvatele valkudele.

Kuna EB valgud on olulised mikrotorukeste kasvu mõjutavad tegurid, siis jälgisime elus rakkudes EB3-GFP liikumist erinevate pleksiinide juuresolekul. EB-dega seonduvad pleksiin-B1 ja B3 põhjustasid mikrotorukeste otste sagedasemat peatumist ja kahanemist. Sarnane mõju oli ka pleksiin-B1 ligandi Semaforiin4D lisamisel roti neuronitele. Kui pleksiin-B3-l oli EB-dega ühenduv ala eemaldatud, siis ta kiirendas mikrotorukeste kasvu nagu pleksiin-B2.

Mikrotorukeste liikuvus on oluline närvirakkude arengus nii jätkete kasvatamisel kui ka hiljem rakkudevaheliste ühenduste, sünapsite, küpsemisel. Pleksiin-B2 ja B3 valgud paiknevad roti hippokampuse rakkudes põhiliselt närvirakkude kehas. Märkimisväärne osa pleksiinidest on koondunud põõsasjätketessse (dendriitidesse), kuid ei kattu sünapsitega. B-pleksiinide valgutasemete alandamine RNA vaigistamise teel põhjustas roti hippokampuse närvirakkude põõsasjätkete lühenemist. Nende ületootmine vanemates rakkudes mõjutas sünapsite teket. Kõik B-pleksiinid kahandasid glutamaadi vahendusel toimivate erutussünapsite mahtu, kuid samas pleksiin-B1 ja B3 suurendasid GABA-t tootvate pidurdussünapsite hulka. Pleksiin-B3 puhul seostus erutussünapsite vähenemine EB valkudega, aga pidurdussünapsite tekkel osalesid hoopis Ras ja Rho GTPaase mõjutavad pleksiini osad.

Kokkuvõtvalt võib öelda, et pleksiin-B3 seondub mikrotorukeste otsas olevate EB valkudega. Kõik B-pleksiinid mõjutavad mikrotorukeste dünaamikat ja sellest tulenevalt närvirakkude jätkete kasvu ja sünapsite teket.

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