CLASS 5 SEMAPHORINS MEDIATE SYNAPSE ELIMINATION AND ACTIVITY-DEPENDENT SYNAPTIC PLASTICITY IN HIPPOCAMPAL NEURONS

by

Wei Xiao

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Abstract

Although autism spectrum disorders (ASDs) have long been known to have a strong heritability, the genetic basis of these disorders has remained largely elusive. Hundreds of genes have been linked to ASDs, but most of them only contribute a small increase in risk. In 2009, a genome-wide association study identified Semaphorin 5A (*SEMA5A*) as a novel autism susceptibility gene. Sema5A is a member of the Semaphorin family, a large family consisting of secreted and membrane-associated proteins characterized by the Sema domain. Although initially identified as axon guidance cues, Semaphorins have been found to play numerous key roles in the development and function of the nervous system.

Here, I provide evidence that Sema5A, along with Sema5B, regulates dendritic morphology and excitatory synaptic elimination in hippocampal neurons. The overexpression of Sema5A/Sema5B negatively impacted dendrite complexity and reduced excitatory synapse density without affecting inhibitory synapses, in contrast the knockdown of Sema5A/Sema5B increased excitatory synapse density.

I also investigated the relationship between Sema5A/Sema5B and activity-dependent plasticity including long-term potentiation (LTP) and long-term depression (LTD), which are cellular models of learning and memory. It was demonstrated that the overexpression of Sema5A/Sema5B attenuated the LTP-mediated increase of synapse density, whereas the knockdown of Sema5A/Sema5B blocked the LTD-mediated decrease of synapse density. Furthermore, soluble Sema5A treatment altered the surface expression of the AMPA receptor subunit GluA1 with total level of GluA1 unchanged.

Finally, I examined the signaling mechanisms of Sema5A-mediated synapse elimination and plasticity. I found that *in vitro* Sema5A signalled through two members (Plexin A1 and Plexin A2) of the Plexin family, which are known as the neuronal receptors for the Semaphorin family. Moreover, TAG-1, a cell adhesion molecule also known as Contactin-2, was necessary for the function of Sema5A and Sema5B. Lastly I found that ALLN, an inhibitor of protease calpain, significantly rescued Sema5A-mediated synapse elimination, suggesting that calpain was downstream of Sema5A signaling in hippocampal neurons.

Thus, my data revealed a new role for class 5 Semaphorins in synapse density and plasticity, and may therefore provide insights into the critical roles of Sema5A in the general mechanisms of circuit formation and the specific etiology of ASDs.

Preface

The work presented here is largely the product of my own efforts. I wrote the entire thesis, including research chapters, with editorial comments from my supervisor, Dr. Tim O'Connor, and my supervisory committee.

In Chapter 1, all figures are used with permission from applicable sources.

Elements of Chapter 2 are currently in preparation for submission for publication. Sema5A and Sema5B in pDisplay constructs were cloned by our previous lab member Kristen Browne. The shRNA constructs against Sema5A were designed by myself and cloned by Kristen Browne. The pEx.Fc/Sema5A-Fc and the PSD-95-RFP constructs were kind gifts from Dr. David Sretavan (University of California, San Francisco, USA) and Dr. David Bredt (Johnson & Johnson Co., San Diego, USA) respectively. Hippocampal neuron cultures were provided by Dr. Shernaz Bamji lab (University of British Columbia, Vancouver, Canada). Experiments were designed by myself with guidance from Dr. Tim O'Connor and Dr. Bamji. All experiments and data analysis in this chapter was conducted by myself, with following exceptions: Inhibitory synapse density experiment and data analysis were done with assistance from Riki Dingwall (Figure 2.7). The experiment in Figure 2.11, panel A and C was done by Rachel Gomm, Bamji lab, UBC. All the figures were prepared by myself under the supervision of Dr. Tim O'Connor.

In Chapter 3, the Plexin A2 construct were a generous gift from Dr. Luca Tamagnone (University of Torino Medical School, Candiolo, Italy) and Plexin A1/ Plexin A3/Plexin B1/Plexin B2/Plexin B3 constructs were kind gifts from Dr. Matthieu Vermeren (The University of Edinburgh, Edinburgh, UK). Experiments were designed by myself with

guidance from Dr. Tim O'Connor. All experiments were performed and analyzed by myself. All the figures were prepared by myself under the supervision of Dr. Tim O'Connor.

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List of Abbreviations

AchR	acetylcholine receptor
ADF	actin depolymerizing factor
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ASD	autism spectrum disorder
BDNF	brain-derived neurotrophic factor
C1q	complement component 1q
C3	complement component 3
CAM	cell adhesion molecule
Calcineurin/PP2B	calcium–calmodulin dependent protein phosphatase 2B
CaMKII	calcium–calmodulin dependent protein kinase II
Cdk5	cyclin-dependent kinase 5
CNS	central nervous system
CSPG	chondroitin sulfate proteoglycans
DG	dentate gyrus
DGK	Diacylglycerol kinase
DRG	dorsal root ganglia
DSCAM	Down Syndrome Cell Adhesion Molecule
EC	entorhinal cortex
ECM	Extracellular matrix
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
F-actin	filamentous actin
FARP1	FERM, RhoGEF and pleckstrin domain-containing protein 1
GAP	GTPase-activating protein
GFP	green fluorescence protein
GKAP	Guanylate-kinase-associated protein
GPI	glycosylphophatidylinosito
GRIP	glutamate receptor interacting protein
GSK3	glycogen synthase kinase-3
HDAC	histone deacetylases
HSPG	heparan sulfate proteoglycans
IP3R	1,4,5-trisphosphate receptor
IRSp53	insulin receptor substrate p53
LIMK-1	LIM kinase 1
LTD	long-term depression
LTP	long-term potentiation
MAGUK	membrane-associated guanylate kinase
MAPK	mitogen-activated protein kinase
MEF2	myocyte enhancer factor 2
mGluR	metabotrobic glutamate receptor
MHC	Major histocompatibility complex

NCS	neuronal calcium sensor
N-cadherin	neural cadherin
NMDA	*N*-Methyl-D-aspartate
NMDAR	*N*-Methyl-D-aspartate receptor
NMJ	neuromuscular junction
OPC	oligodendrocyte precursor cell
p38MAPK	P38 mitogen-activated protein kinase
PAK1	p21-activated kinase 1
PICK1	protein interacting with C-kinase 1
PKA	protein kinase A
PKC	protein kinase C
PP1	protein phosphatase 1
PSD	postsynaptic density
PSD-95	postsynaptic density protein 95
PSI	plexin-Semaphorin-integrin
PTV	piccolo transport vesicle
RFP	red fluorescent protein
RIM	Rab3-interacting molecule
ROCK	Rho-associated protein kinase
Robo	Roundabout
SAP97	Synapse-associated protein 97
SAP-102	Synapse-associated protein 102
SAPAP	Synapse-associated protein 90/postsynaptic density-95-associated protein
SC	subicular complex
Sema	Semaphorin
SNAP	synaptosomal-associated protein
SNAP-25	synaptosomal-associated protein-25
SNARE	synaptosomal-associated protein (SNAP) receptor
SNP	single-nucleotide polymorphism
STV	synaptic vesicle protein transport vesicle
SV	synaptic vesicle
SynGTP	synaptic GTPase-activating protein
t-SNARE	target membrane SNARE
TSR	thrombospondin repeat
VAMP	vesicle-associated membrane protein
VDCC	voltage-dependent calcium channels
VGAT	vesicular GABA transporter
vGlut	vesicular glutamate transporter
WNT	wingless type

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Chapter 1: Introduction

1.1 Excitatory Synapses of the Central Nervous System

The mammalian nervous system consists of two components: the central nervous system (CNS) and the peripheral nervous system. The central nervous system contains the brain and the spinal cord and acts as the processing station to integrate information and sends commands. The peripheral nervous system connects the CNS to every other part of the body and controls directly all responses we make. The fundamental cellular units constituting the nervous system are cells specialized for the storage and propagation of electrochemical impulses known as neurons or nerve cells. Their structure allows them to transmit information in the form of electrical signals. The human nervous system consists of more than 100 billion neurons interconnected into a highly organized neural network (Squire 2008). The capacity of neurons to communicate and function within this network resides in specialized junctions called synapses. During development and throughout life, synapses are continuously formed, stabilized and eliminated. Inappropriate synaptic connections may lead to the disruption of neuronal communication and lead to various neurological disorders. Because synapses are of such central importance to neurobiology, our knowledge of synaptic development and function are critical to understand the normal and abnormal functions of the nervous system.

1.1.1 Excitatory Synapses Structure

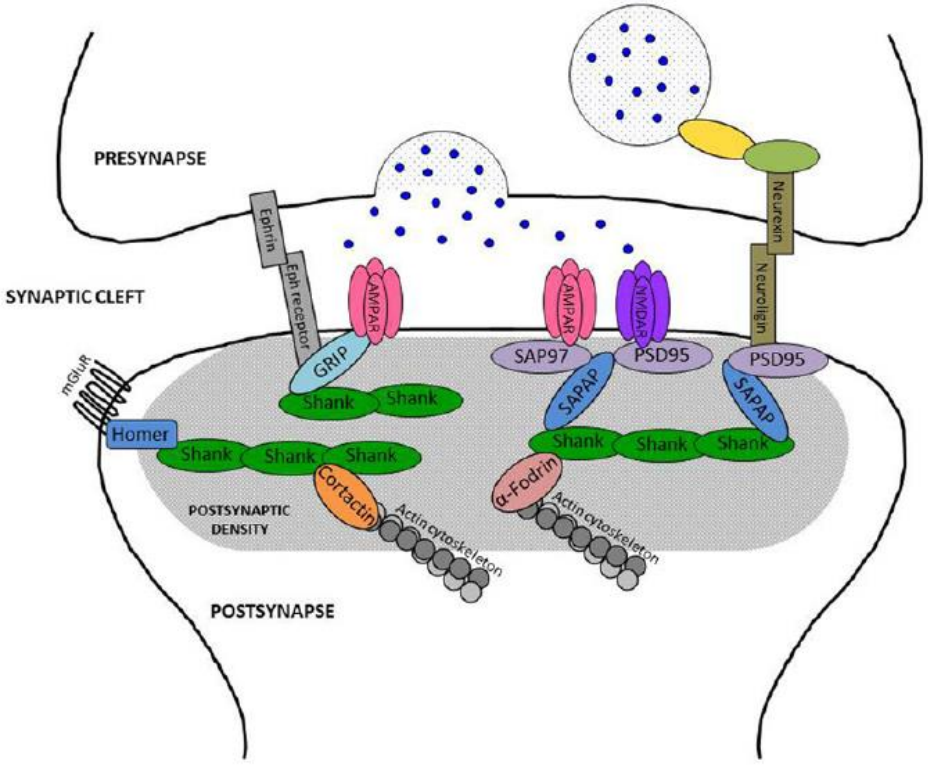
Although there is a high degree of morphological and molecular variability, all chemical synapses share three main structural elements: presynaptic specialization, postsynaptic sites

and a 20-40 nm space separating them named the synaptic cleft. The presynaptic specializations, also referred as the terminal boutons, are specialized regions in the presynaptic cell with abundant synaptic vesicles (SVs) containing neurotransmitters. Close to the presynaptic cell membrane lies the active zone, which is the site where SVs release their contents into the synaptic cleft through exocytosis. Mitochondria are enriched in presynaptic specializations, as the neurotransmitter release machinery requires a lot of energy. The postsynaptic site can be either on neurite shafts, the cell soma or on tiny protrusions called dendritic spines. The postsynaptic site is directly opposed to the presynaptic specializations and is densely packed with neurotransmitter receptors, ion channels, scaffolding proteins and intracellular signaling molecules. Once the neurotransmitters travel across the synaptic cleft and bind to the receptors on the postsynaptic membrane, they activate downstream signaling molecules, open ion channels and relay the nervous impulse to the postsynaptic neuron.

Chemical synapses can be classified as excitatory or inhibitory synapses by the types of neurotransmitter applied to transduce signals. Excitatory synapses are the synapses in which the release of neurotransmitter results in depolarization of the postsynaptic membranes and sometimes initiation of action potentials. In contrast, inhibitory synapses are characterized by hyperpolarization of the postsynaptic membrane, thus decreasing the probability of action potentials to be fired (Purves, Augustine et al.). In this dissertation, I will primarily concentrate on excitatory synapses that employ glutamate as the neurotransmitter.

Figure 1.1 Schematic structure of an excitatory CNS synapse.
An excitatory CNS Synapse contains presynaptic terminal, synaptic cleft and postsynaptic site. The presynaptic terminal contains synaptic vesicles that release neurotransmitters such as glutamate at the active zone. The presynaptic terminal is separated from the postsynaptic site by the synaptic cleft with a number of trans-synaptic adhesion molecules between them,

allowing for the communication between the pre-and postsynaptic membranes. The PSD is located directly opposite the presynaptic active zone where glutamate is released. Postsynaptic glutamatergic receptors such as AMPA and NMDA receptors are localized at the postsynaptic membrane to detect glutamate, which are stabilized by the scaffolding proteins in the postsynaptic density. The targeting, surface expression, localization and removal of glutamatergic receptors is regulated by interactions with PSD-95 and SAP97, and other multi-domain proteins such as GRIP. Shank proteins form multimeric sheets within the PSD where they interact with numerous PSD proteins and with the actin cytoskeleton. Shank interactions with the trans-synaptic bridge formed by neurexin/neuroligin also enables Shank-dependent regulation of presynaptic structure and function. Abbreviations: PSD-95, Postsynaptic density 95; SAPAP, Synapse-associated protein 90/postsynaptic density-95-associated protein; SAP97, Synapse-associated protein 97; GRIP, glutamate receptor interacting protein; mGluR, metabotropic glutamate receptor (Vyas and Montgomery, 2016).



1.1.1.1 Postsynaptic Density

Despite the overall structural similarities between excitatory and inhibitory synapses, there are several notable differences. For example, unlike inhibitory synapses which are found on dendritic shafts or the cell soma, the postsynaptic component of excitatory synapses is found

on the dendritic spines (Gray 1959). Excitatory synapses are characterized by the presence of the postsynaptic density (PSD), which is a protein dense region immediately beneath the postsynaptic membrane. Apposed directly to the active zone across the synaptic cleft, PSDs typically have a disclike shape and heterogeneous size varying between 200–800 nm in diameter and 30–60 nm in thickness (Collins, Husi et al. 2006). The primary function of the PSD is to provide scaffolding for the neurotransmitter receptors, such as glutamate receptors. In addition, it also regulates the trafficking and lateral diffusion of receptors. To achieve its function, the PSD consists of more than 1100 different proteins, including glutamate receptors, scaffolding molecules, cytoskeleton-associated proteins and signaling proteins (Figure 1.2) (Collins, Husi et al. 2006; Sheng and Hoogenraad 2007). Among them, the most abundant PSD-resident proteins includes calcium/calmodulin-dependent protein kinase II (CaMKII), postsynaptic density-95 (PSD-95), synaptic GTPase-activating protein (SynGAP), Shank and Homer (Figure 1.2) (Sheng and Hoogenraad 2007).

PSD-95 is a key PSD-resident protein as it can significantly impact the structure and function of the PSD (De Roo, Klauser et al. 2008). For example, PSD-95 overexpression in cultured hippocampal neurons increases glutamate receptor clustering, while PSD-95 knockdown increases spine turnover (El-Hussein, Schnell et al. 2000; Ehrlich, Klein et al. 2007). PSD-95 is more highly abundant in stable compared to transient spines and it is a member of the membrane-associated guanylate kinase (MAGUK) protein family (Kim and Sheng 2004; De Roo, Klauser et al. 2008). It contains three PDZ domains, an SH3 domain, and a guanylate kinase domain and also can multimerize to form an extended scaffold (Kistner, Garner et al. 1995; Kim and Sheng 2004). The variety of domains allows PSD-95 to associate with other

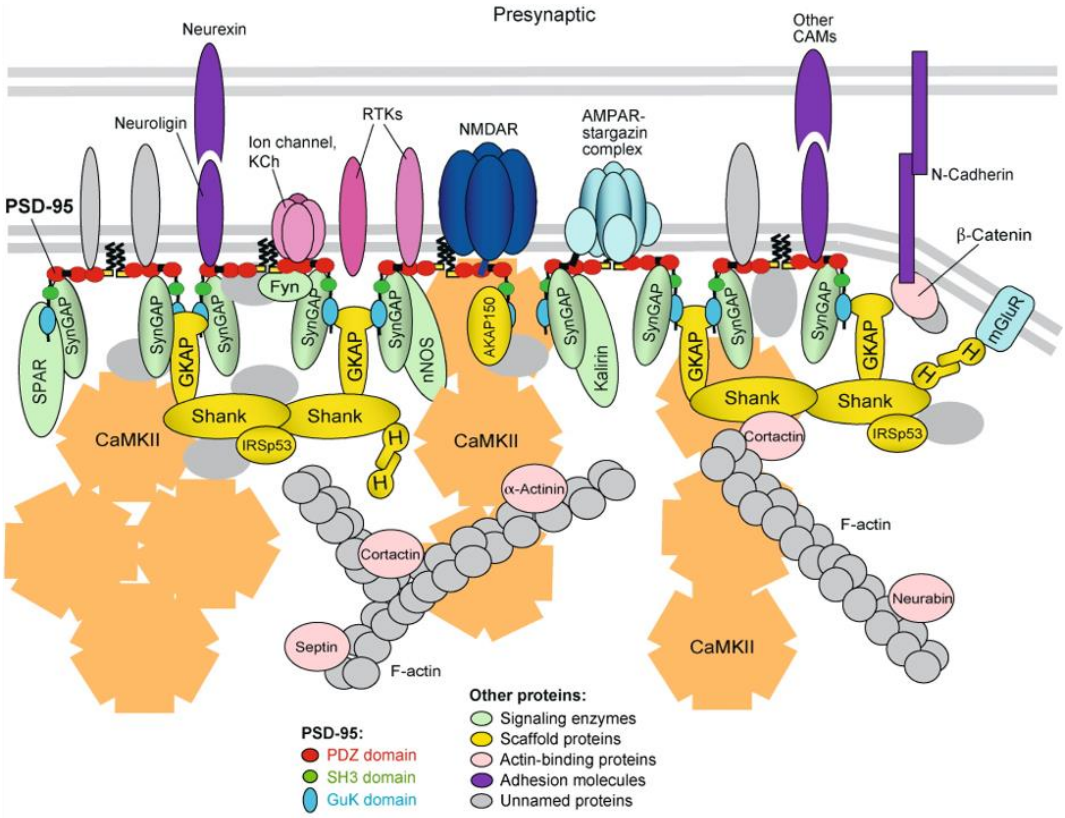
PSD proteins such as GKAP/SAPAP (guanylate kinase-associated protein/synapse-associated protein-associated protein), which further binds to Shank and Homer (Figure 1.2).

SynGAP is highly enriched at excitatory synapses and it interacts with a large complex consisting of PSD-95/SAP90, SAP102, and the NMDAR. It has been implicated in synapse plasticity as after LTP induction, it is rapidly dispersed from spines by CAMKII activation in hippocampal neurons (Chen, Rojas-Soto et al. 1998; Kim, Liao et al. 1998; Oh, Manzerra et al. 2004; Araki, Zeng et al. 2015). Moreover, *de novo* mutations in SynGAP have been identified in patients with ASDs and a range of conditions classified as neurodevelopmental disorders of variability, resulting in an inability to integrate multidimensional stimuli (Hamdan, Gauthier et al. 2009; Krepischi, Rosenberg et al. 2010; Hamdan, Daoud et al. 2011). Ultimately these proteins have a significant impact on Shank and Homer which are important scaffolding proteins that regulate the PSD structure and function, including the clustering of glutamate receptors.

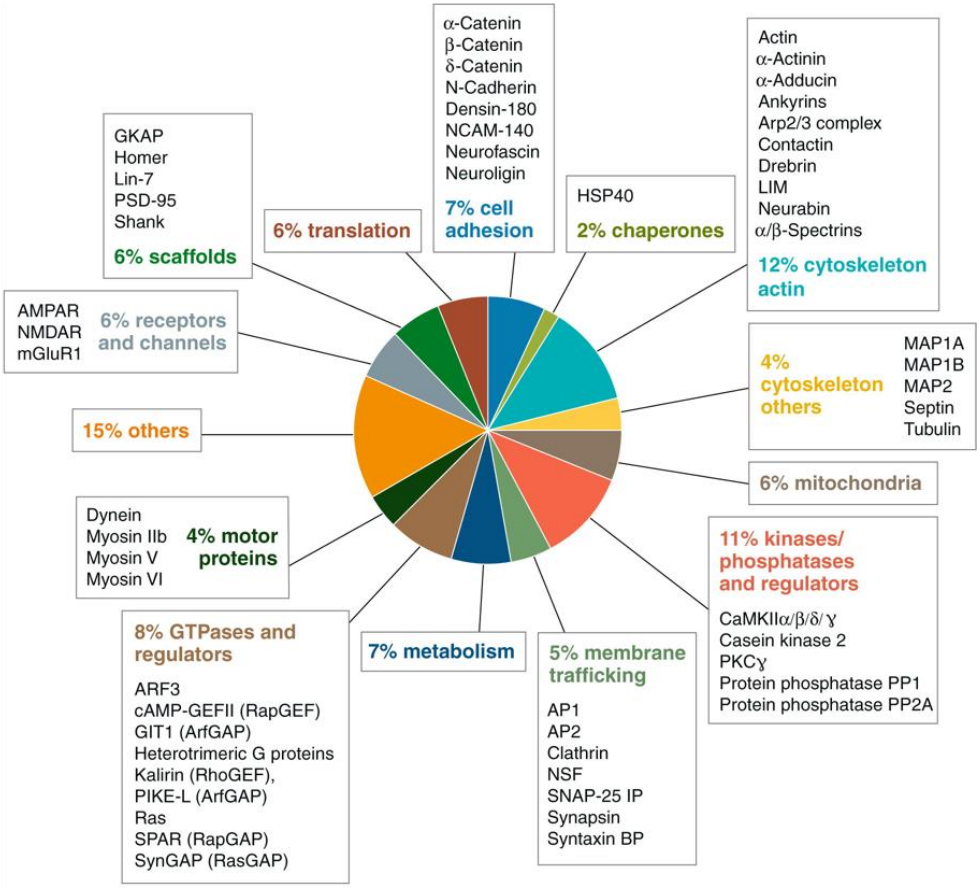
Figure 1.2 PSD structure and protein composition.
(A) Organization of proteins and protein-protein interactions in the PSD. Schematic diagram of the network of proteins in the PSD, with edge of PSD depicted at right. Only major families and certain classes of PSD proteins are shown [in approximate stoichiometric ratio and scaled to molecular size, if known (see text)]. Contacts between proteins indicate an established interaction between them. Domain structure is shown only for PSD-95 (PDZ domain, SH3 domain, GuK domain). Other scaffold proteins are colored yellow; signaling enzymes, green; actin binding proteins, pink. CaMKII (calcium/calmodulin-dependent protein kinase II) is depicted as dodecamer. Unnamed proteins signify the many other PSD proteins that are not illustrated in this diagram. Abbreviations: AKAP150, A-kinase anchoring protein 150 kDa; CAM, cell adhesion molecule; Fyn, a Src family tyrosine kinase; GKAP, guanylate kinase-associated protein; H, Homer; IRSp53, insulin receptor substrate 53 kDa; KCh, K^+ channel; mGluR, metabotropic glutamate receptor; nNOS, neuronal nitric oxide synthase; RTK, receptor tyrosine kinases (e.g., ErbB4, TrkB); SPAR, spine-associated RapGAP (Sheng and Hoogenraad, 2007).

(B) The variety of proteins in the PSD fraction. Pie chart showing the wide variety of proteins identified in the PSD fraction of the forebrain, categorized according to their cellular functions. Only small subsets of identified proteins are shown as examples (Sheng and Hoogenraad, 2007).

(A)


Presynaptic
Neurexin
Other CAMs
Neuroligin
RTKs
Ion channel, KCh
NMDAR
AMPAR-stargazin complex
N-Cadherin
PSD-95
β-Catenin
Fyn
SynGAP
GKAP
nNOS
AKAP150
Kalirin
SPAR
Shank
IRSp53
CaMKII
mGluR
Cortactin
α-Actinin
F-actin
Septin
Neurabin
Other proteins:
Signaling enzymes
Scaffold proteins
Actin-binding proteins
Adhesion molecules
Unnamed proteins
PSD-95:
PDZ domain
SH3 domain
GuK domain

(B)


GKAP
Homer
Lin-7
PSD-95
Shank
6% scaffolds
α-Catenin
β-Catenin
δ-Catenin
N-Cadherin
Densin-180
NCAM-140
Neurofascin
Neuroligin
7% cell adhesion
HSP40
2% chaperones
Actin
α-Actinin
α-Adducin
Ankyrins
Arp2/3 complex
Contactin
Drebrin
LIM
Neurabin
α/β-Spectrins
12% cytoskeleton actin
6% translation
AMPAR
NMDAR
mGluR1
6% receptors and channels
4% cytoskeleton others
MAP1A
MAP1B
MAP2
Septin
Tubulin
15% others
6% mitochondria
Dynein
Myosin IIb
Myosin V
Myosin VI
4% motor proteins
11% kinases/ phosphatases and regulators
CaMKIIα/β/δ/γ
Casein kinase 2
PKCγ
Protein phosphatase PP1
Protein phosphatase PP2A
8% GTPases and regulators
ARF3
cAMP-GEFII (RapGEF)
GIT1 (ArfGAP)
Heterotrimeric G proteins
Kalirin (RhoGEF),
PIKE-L (ArfGAP)
Ras
SPAR (RapGAP)
SynGAP (RasGAP)
7% metabolism
5% membrane trafficking
AP1
AP2
Clathrin
NSF
SNAP-25 IP
Synapsin
Syntaxin BP

1.1.1.2 AMPA and NMDA Receptors

AMPA receptors (AMPARs) mediate the majority of glutamatergic neurotransmission in the brain. They are heterotetrameric channels assembled by GluA1-A4 subunits (Traynelis, Wollmuth et al. 2010). Various combinations of these subunits govern the biophysical properties and functional properties of AMPARs. For instance, the presence of GluA2 subunits in AMPA receptors yields low calcium permeability (Hollmann, Hartley et al. 1991), and the redistribution of GluA1 subunits are important for the activity-dependent synaptic plasticity (Shi, Hayashi et al. 1999; Hayashi, Shi et al. 2000). In the hippocampus, the predominant combinations of subunits are GluA1/GluA2 and GluA2/GluA3 (Wenthold, Petralia et al. 1996; Lu, Shi et al. 2009).

The NMDA receptors (NMDARs) normally contribute less to the basal transmission, but rather play critical roles in synaptic plasticity. The NMDA receptor is activated when glutamate and glycine bind to it, and upon activation it allows positively charged ions such as Na^+ and K^+ to go through. Besides Na^+ and K^+, NMDA receptors are also highly permeable to Ca^{2+}. NMDARs contain two obligatory NR1 subunits plus two additional subunits amongst NR2A-D and/or NR3A-B subunits (Traynelis, Wollmuth et al. 2010). Interestingly, in postnatal development the composition of the NMDAR changes with NR2B being gradually replaced by NR2A (Watanabe, Inoue et al. 1992; Sheng, Cummings et al. 1994; Wenthold, Petralia et al. 1996).

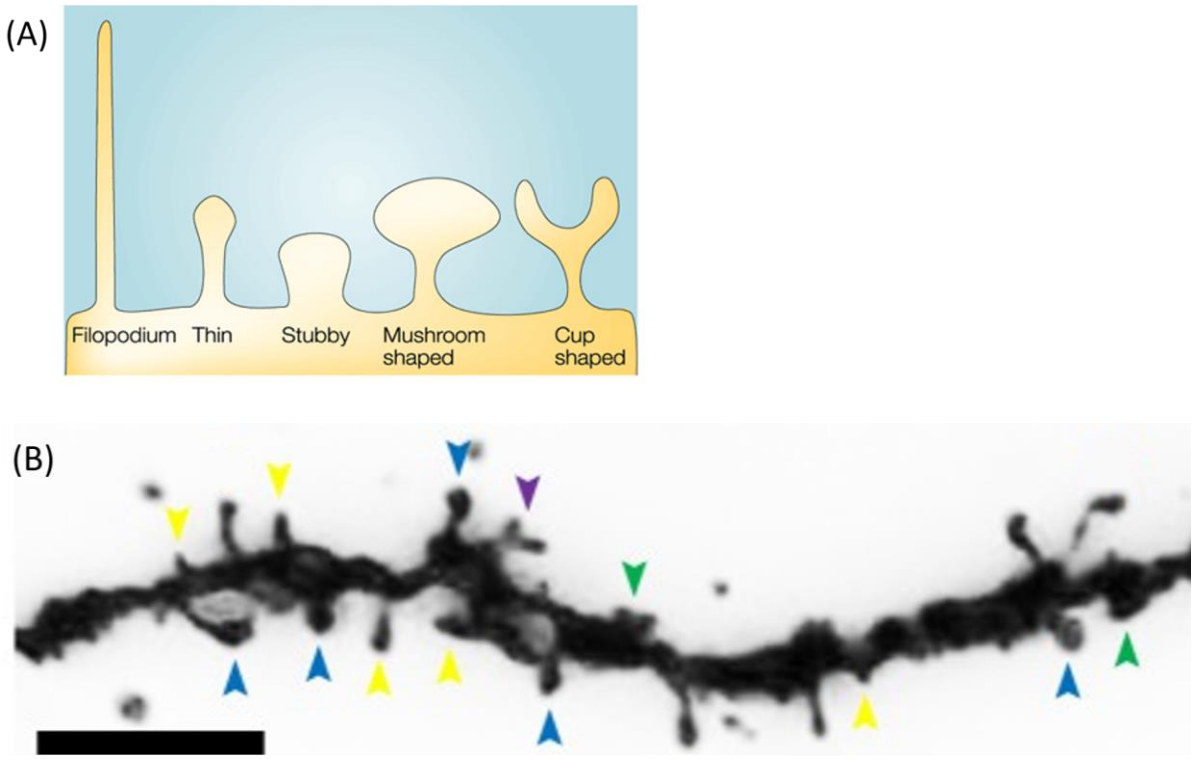
1.1.2 Dendritic Spines

As mentioned earlier, dendritic spines are small membranous protrusions along dendrites where the PSDs are found. In the mammalian CNS, the majority of the postsynaptic

compartments within glutamatergic synapses occur on dendritic spines (Hering and Sheng 2001; Alvarez and Sabatini 2007). The morphology of dendritic spines is highly diverse in size and shape, but a typical spine is 0.5-2 μm in length and contains a bulbous spine head connected to the dendritic shaft by a thin, constricted spine neck. Based on their shapes, dendritic spines are roughly classified into thin, stubby spines, mushroom spines and cup-shaped (Figure 1.3) (Peters and Kaiserman-Abramof 1970; Chang and Greenough 1984; Harris, Jensen et al. 1992). Another distinct type of dendritic protrusion, filopodia are abundant in young animals and considered as precursors of mature spines since they usually lack functional PSDs (Ziv and Smith 1996; Grutzendler, Kasthuri et al. 2002; Sekino, Kojima et al. 2007).

Figure 1.3 Structural classifications in spine shape.
(A) Schematic depiction of the common morphological classifications of spines relative to a filopodium (Hering and Sheng, 2001).

(B) Golgi-cox stained secondary dendritic branch of a Layer II/III pyramidal neuron in mouse primary visual cortex. Scale bar, 5 μm. Different spine types are indicated by arrowheads (yellow, thin spine; green, stubby; blue, mushroom; purple, cup-shaped). Adapted with permission from Risher et al., 2014.



Why do neurons choose to from excitatory synapses on dendritic spines rather than directly on the dendritic shafts for synaptic transmission? The prevailing opinion is that the dendritic spines do not only provide a physical bridge from the axon to the dendrite, but also serve as isolated compartments for postsynaptic biochemical responses. For instance, the spine neck has been shown to control the kinetics of postsynaptic calcium by preventing the calcium diffusing into the neighboring dendritic shaft, which is critical for glutamate receptors signaling (Sabatini, Maravall et al. 2001; Tonnesen, Katona et al. 2014).

Live imaging studies have revealed that spines are remarkably mobile and dynamic, whose shape, size and composition constantly change in response to synaptic activities during development (Ethell and Pasquale 2005; Tada and Sheng 2006). The morphology of the dendritic spines has long been proposed to be related to its function. For example, the spine size is highly correlated with the PSD size, and the latter is further correlated with changes in

synaptic strength (Kasai, Matsuzaki et al. 2003; Arellano, Benavides-Piccione et al. 2007). The glutamate sensitivity of a single spine can be modulated without affecting adjacent spines, indicating that individual synapse can function and be regulated independently (Matsuzaki, Honkura et al. 2004). Moreover, numerous studies have observed morphological remodeling of spines following synaptic activity, which is an essential process in synapse plasticity (Yuste and Bonhoeffer 2001). This will be discussed further in following discussion.

1.2 Formation and Refinement of Neural Circuits

The formation of neural circuits during development is a complex and poorly understood process. Appropriate neural connectivity requires the precise execution of multiple developmental events, including neuron differentiation and migration, axon guidance and dendritic growth, followed by synaptogenesis and subsequent refinement of synaptic connections (Juttner and Rathjen 2005; Waites, Craig et al. 2005). During the development of the vertebrate nervous system, these developmental processes occur with great precision, building a remarkably specific network of neural circuits that enable the brain to function properly. How this remarkable specificity is orchestrated during development has been one of the core questions in neuroscience research. In section 1.2, I will primarily concentrate my discussion on specific aspects of neural circuit formation including dendritic growth, synapse formation, maintenance and elimination. Activity-dependent synaptic mechanism will be discussed in section 1.3.

1.2.1 Dendritic Arborisation

Since Ramón y Cayal's influential studies, the diversity of dendritic arbor structures has been recognized and well characterized. Because most synaptic contacts are formed on dendrites, it is not surprising that the architecture of dendritic arbors determines the nature and number of synaptic inputs that a neuron receives and integrates (Hume and Purves 1981; Purves and Hume 1981; Purves, Hadley et al. 1986). Consequently, the results of abnormalities in dendrite development are profound, and often lead to circuit malformation accompanying severe neurodevelopmental disorders in humans (Purpura 1975; Kaufmann and Moser 2000). Thus, the proper morphological growth and arborisation of dendrites is an integral aspect of circuit formation. Although historically dendrites were believed to be intrinsically determined by gene expression patterns, it is increasing clear that dendrites are remarkably responsive to environmental signals such as neurotrophic factors, guidance cues, CAMs, cytoskeleton regulators and neuronal activity (McAllister 2000; Tada and Sheng 2006).

1.2.1.1 Intrinsic Genetic Factors

In low-density dissociated hippocampal neuronal cultures, neurons are isolated from their endogenous environment. With little influence of diffusible cues, inter-neuronal communication or neuronal activity, neurons form recognizable dendritic trees similar to what is observed *in situ*, indicating the presence of intrinsic mechanisms that regulate neuron type-specific dendritic arborisation. *Drosophila* has been used as a model organism over the years to identify genes that regulate neuronal dendritic morphology (Moore, Jan et al. 2002; Grueber, Jan et al. 2003; Li, Wang et al. 2004; Sugimura, Satoh et al. 2004; Kim, Burette et al. 2006; Parrish, Kim et al. 2006; Parrish, Emoto et al. 2007). For example, the ectopic

expression of the transcription factor Hamlet reduces dendritic growth and inhibits neurons from adopting complex dendrite morphology (Moore, Jan et al. 2002). A combination of gain- and loss-of-function experiments demonstrated that the level of branching complexity among the four different classes of larval dendritic arborisation neurons (class I, II, III and IV dendritic arborisation neurons) is determined by the combinatorial expression levels of three transcription factors include Cut, Abrupt, and Spineless (Grueber, Jan et al. 2003; Li, Wang et al. 2004; Sugimura, Satoh et al. 2004; Kim, Burette et al. 2006).

1.2.1.2 Extrinsic Factors

Although dendrites were believed to be wholly intrinsically determined historically, it has become increasingly clear that dendrites are remarkably responsive to environmental signals such as neurotrophic factors, guidance cues, CAMs, cytoskeleton regulators and neuronal activity (McAllister 2000; Tada and Sheng 2006).

A major class of extrinsic regulator of dendrite growth is the neurotrophin family that includes four members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). All of them and their receptors, the Trk family of proteins, are highly expressed in the developing nervous system and regulate dendritic growth and branching (Snider 1994; McAllister, Katz et al. 1999). Interestingly, the effect of the neurotrophins on dendritic morphology varies depending on the system, neuron type and the specific neurotrophin family member (McAllister, Lo et al. 1995; Horch and Katz 2002). Among them, BDNF is the most critical and well studied factor regulating dendrite outgrowth and branching. BDNF application increases dendritic complexity, number and length in developing cortical pyramidal neurons (McAllister, Lo et

al. 1995; Horch and Katz 2002), and these increases in dendrite branching act in a distance-dependent manner (Horch and Katz 2002). Consistently, BDNF overexpression destabilizes dendrites via increased basal dendrite sprouting and retracting of existing dendritic spines (Horch, Kruttgen et al. 1999). In the developing cerebellum, BDNF–/– mice exhibit stunted dendritic arbors of Purkinje cells. However at a later stage, BDNF application in cultured Purkinje neurons did not affect dendritic complexity, but instead dramatically increased dendritic spine density (Segal, Pomeroy et al. 1995; Schwartz, Borghesani et al. 1997; Morrison and Mason 1998; Shimada, Mason et al. 1998).

Many diffusible cues that influence axon orientation also regulate dendritic arborisation. In mammalian systems, Semaphorin 3A (Sema3A) secreted by cells in the marginal zone acts as a repellant for cortical axons but an attractant for the apical dendrites of cortical neurons (Polleux, Giger et al. 1998; Polleux, Morrow et al. 2000). Disruption of Sema3A signaling by blocking its receptor neuropilin1 resulted in disoriented apical dendrites (Polleux, Morrow et al. 2000). Another example of axon guidance protein involvement in dendritic arborisation is the Ephrins/Eph complex. Neurons from EphB1/EphB2/EphB3 triple knockout mice displayed a severe defect in dendrite morphogenesis (Hoogenraad, Milstein et al. 2005). Other axon guidance molecules such as Netrin (Kolodziej, Timpe et al. 1996; Hiramoto, Hiromi et al. 2000; Furrer, Kim et al. 2003) and Slit (Godenschwege, Simpson et al. 2002; Whitford, Marillat et al. 2002) have also been implicated in Drosophila dendrite morphogenesis. However, in those studies of axon guidance cues in dendritic arborisation, the underlying mechanisms were rarely tested.

CAMs are also good candidates for regulating dendrite morphogenesis. Indeed, L1 knockout mice exhibit abnormal morphogenesis of apical dendrites of pyramidal neurons

(Demyanenko, Tsai et al. 1999). Down Syndrome Cell Adhesion Molecule (DSCAM), a member of the Ig domain superfamily, is required for mediation of neuronal self-avoidance (Kolodziej, Timpe et al. 1996; Shelly, Cancedda et al. 2007). Cadherins/catenins also attracted much attention for their ability to mediate activity-dependent increases of dendrite growth.

1.2.2 Synapse Formation

When, where, and how synapses are formed plays a critical role in directing the precise connectivity of circuits that enables us to see, feel and think. In mammalian brains, synapse formation, namely synaptogenesis, occurs over a protracted period of time starting from embryonic development. In adult brains, synapse formation can still take place, and is proposed to provide the basis for learning, memory and cognition. The establishment of synapses is generally believed to begin with the physical contact mediated by filopodia between presynaptic axon and the postsynaptic dendrite, followed by the recruitment of pre- and postsynaptic proteins to these contact sites. These nascent synapses will grow bigger by increasing the spine size, the number of SVs and the area of the active zone and PSD (Scheiffele 2003; Waites, Craig et al. 2005). The formation of a mature synapse is marked by the presence of the synchronous action potential firing between presynaptic and postsynaptic sites (Waites, Craig et al. 2005).

Decades of work have led to the identification of hundreds of molecules involved in synaptogenesis. Presynaptically, numerous proteins are found to be involved in the neurotransmitter release machinery and to facilitate the SV trafficking (Phillips, Huang et al. 2001; Sudhof 2004). Postsynaptically, a large number of proteins have been determined to be

critical for receptor trafficking, synaptic transmission, and synaptic plasticity (Kennedy 2000; Kim and Sheng 2004; Montgomery, Zamorano et al. 2004). Additionally, between pre- and postsynaptic neurons, cell adhesion molecules (CAMs) form trans-synaptic contacts, which have also been implicated in regulating the initial stage of synapse formation as well as their plasticity afterwards (Scheiffele 2003; Waites, Craig et al. 2005; Craig, Graf et al. 2006; Garner, Waites et al. 2006). In this dissertation, I will first focus on the early steps of synapse formation and the underlying molecular mechanisms, while activity-dependent remodeling of synaptic connections will be discussed in greater detail later.

1.2.2.1 Presynaptic Assembly

The classical view of synaptogenesis suggests that the formation of presynaptic boutons begins when extracellular signals initiate synapse formation. Contact between an axon and a dendrite are established depending on a combined gradient of guidance cues. Upon contact, signaling through homophilic and heterophilic receptors induces the formation of synaptic connections between pre- and postsynaptic neurons (Scheiffele 2003). WNT family members such as WNT-7a and WNT-3 regulate presynaptic differentiation in the mammalian CNS (Hall, Lucas et al. 2000; Krylova, Herreros et al. 2002).

Recent evidence supports the hypothesis that many pre-and postsynaptic proteins are preassembled in neurons before contact takes place (Fletcher, Cameron et al. 1991; Ziv and Smith 1996; Rao, Kim et al. 1998). These proteins are localized to the small, heterogeneous clusters called transport packets (McAllister 2007). In young neurons, there are at least two types of transport packets: piccolo transport vesicles (PTVs) and SV protein transport vesicles (STVs).

PTVs are 80 nm dense-core vesicles containing many components of the presynaptic active zone, including scaffolding proteins (e.g. piccolo, bassoon and Rab3-interacting molecule (RIM)), adhesion molecules (e.g. N-cadherin), as well as SV regulatory proteins (e.g. syntaxin and SNAP-25) (Zhai, Vardinon-Friedman et al. 2001; Sabo, Gomes et al. 2006). PTVs are quite mobile, for they can move rapidly with an average rate greater than 0.35 μm/s (Shapira, Zhai et al. 2003). Moreover, they can move in both the anterograde and retrograde directions, split into smaller clusters, or coalesce into larger clusters (Shapira, Zhai et al. 2003). Some reports suggest that STVs docking sites have relative high probability to form stable neuron-neuron contacts (Sabo, Gomes et al. 2006).

Distinct from PTVs, STVs transport various SV-associated proteins and other proteins important for SV trafficking such as VAMP-2, synaptotagmin and VDCCs (Ahmari, Buchanan et al. 2000; Zhai, Vardinon-Friedman et al. 2001). The morphologies of STVs can be pleiomorphic, tubulo-vesicular organelles or SV clusters (Kraszewski, Mundigl et al. 1995; Ahmari, Buchanan et al. 2000). Similar to PTVs, STVs move rapidly and are capable of splitting or coalescing (Kraszewski, Mundigl et al. 1995; Dai and Peng 1996; Nakata, Terada et al. 1998; Ahmari, Buchanan et al. 2000). Both unidirectional and bidirectional transport of STVs have been observed, suggesting the involvement of multiple motor proteins (Kraszewski, Mundigl et al. 1995; Dai and Peng 1996; Nakata, Terada et al. 1998; Ahmari, Buchanan et al. 2000).

1.2.2.2 Postsynaptic Assembly

The structure of the postsynaptic specialization is more complex and heterogeneous than the presynaptic active zone, and the recruitment of postsynaptic proteins during synapse

formation is complicated. One of the earliest events of postsynaptic assembly is the recruitment of PSD-95 (Waites, Craig et al. 2005). It was generally believed that the clustering of PSD-95 occurred through a gradual accumulation (Friedman, Bresler et al. 2000; Bresler, Ramati et al. 2001; Okabe, Miwa et al. 2001). However, several studies also reported the presence of immobile clusters of PSD-95 in the predefined area along the dendrite prior to neuron-neuron contact (Washbourne, Bennett et al. 2002; Wenthold, Prybylowski et al. 2003). Similar to presynaptic proteins, NMDARs are not transported from long distance but rather clustered in discrete transport packets before synaptogenesis occurs (Washbourne, Bennett et al. 2002; Washbourne, Liu et al. 2004). Retrospective immunostaining shows that such transport packets possibly carry a scaffolding protein called synapse-associated protein 102 (SAP-102) and sometimes AMPARs as well, but not PSD-95 (Washbourne, Bennett et al. 2002; Sans, Prybylowski et al. 2003; Washbourne, Liu et al. 2004). AMPARs are recruited later to the sites of axon-dendrite contact than NMDARs (Friedman, Bresler et al. 2000). The non-overlap of AMPAR- and NMDAR-positive clusters and the different kinetics between them indicate independent mechanisms for recruitments of AMPARs and NMDARs to the synapses (Rao, Kim et al. 1998; Washbourne, Bennett et al. 2002). Before AMPARs recruited to the sites of synaptic contact, synapses are immature and postsynaptically silent.

1.2.2.3 Cell Adhesion Molecules in Synaptic Formation

CAMs have been found to promote the assembly of pre- and postsynaptic specifications. In the vertebrate NMJ, an extracellular-matrix protein called agrin is crucial for postsynaptic development (Glass, Bowen et al. 1996; Zhou, Glass et al. 1999). In central synapses between neurons, several classes of CAMs such as cadherins, ephrins, neuroligins and

neurexins are found to trigger initial trans-synaptic interaction and recruit synaptic components such as receptors, scaffolding proteins and synaptic vesicle proteins (Washbourne, Dityatev et al. 2004; McAllister 2007; Togashi, Sakisaka et al. 2009). For instance, N-cadherin is essential for the initial contact between pre- and postsynaptic membrane and the selective adhesion of specific pre-and postsynaptic cells (Benson and Tanaka 1998; Shapiro and Colman 1999; Togashi, Abe et al. 2002; Bozdagi, Valcin et al. 2004; Arikkath and Reichardt 2008).

1.2.3 Synapse Maintenance

Long-term confocal imaging in adult rodents *in vivo* has demonstrated that synapses are relatively stable in the mature brain and persist for several months, or even years (Trachtenberg, Chen et al. 2002; Holtmaat, Trachtenberg et al. 2005; Zuo, Lin et al. 2005; Majewska, Newton et al. 2006). This is the result of continuous maintenance by actin cytoskeleton and associated regulators, adhesion molecules and scaffolding proteins.

Notably, synapse maintenance in the CNS not only determines the passage of information through the brain, but also governs the elaboration of the dendritic arborisation, which further defines the structural architect of the brain (Wu and Cline 1998; Li, Hilgenberg et al. 1999; Rajan, Witte et al. 1999; Niell, Meyer et al. 2004; Hu, Nikolakopoulou et al. 2005; Ruthazer, Li et al. 2006; Chen, Tari et al. 2010). For example, synapses lacking maintenance tend to be unstable and dissembled, which lead to dendritic retraction, indicating that synaptic maturation and maintenance is vital for dendritic stability and preservation of neuronal networks (Clark 1957; Jones and Thomas 1962; Matthews and Powell 1962; Wiesel and Hubel 1963; Coleman and Riesen 1968; Vaughn 1989; Rajan, Witte et al. 1999; Wu, Zou et

al. 1999; Niell, Meyer et al. 2004; Cline and Haas 2008). Given the significance of synapse maintenance for human brain health, understanding how neurons achieve synaptic stability has been an important topic in neuroscience. Below I will briefly review the main cellular mechanisms mediating synaptic maintenance.

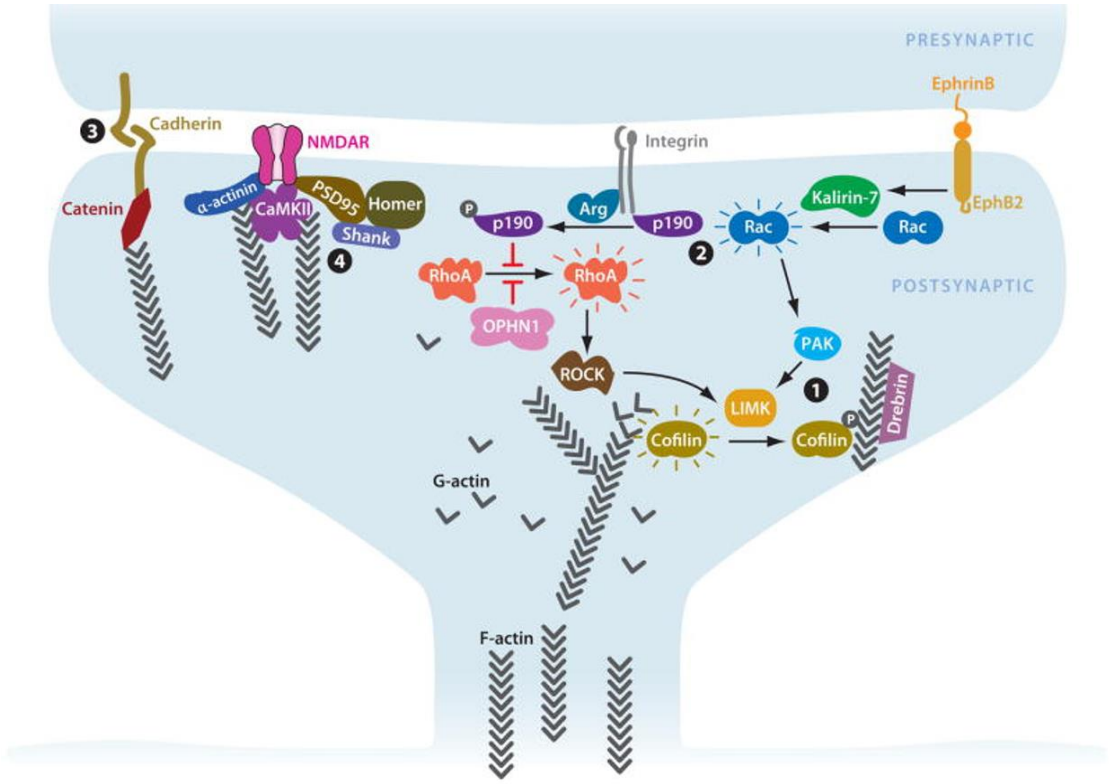
1.2.3.1 Actin Cytoskeleton and Its Regulators

The actin cytoskeleton and its regulators are critical for synapse maintenance. Essentially, the actin cytoskeleton serves as a structural support for presynaptic compartments and dendritic spines (Vaughn 1989). Presynaptic actin network acts as a platform to organize the neurotransmitter release machinery and facilitate vesicle trafficking and exocytosis (Vaughn 1989; Zhang and Benson 2001; Halpain 2003). At postsynaptic sites, filamentous actin (F-actin) provides integrity for dendritic spines and stability for numerous proteins within the PSD (Vaughn 1989; Schubert and Dotti 2007; Sekino, Kojima et al. 2007). For example, actin polymerization induced by jasplakinolide treatment causes spine enlargement, whereas disruption of actin polymerization by latrunculin-A or cytochalasin-D results in the disassembly of the scaffolding proteins PSD-Zip45, GKAP and Shank, which eventually leads to spine shrinkage or loss (Allison, Gelfand et al. 1998; Fischer, Kaech et al. 1998; Star, Kwiatkowski et al. 2002; Okamoto, Nagai et al. 2004; Kuriu, Inoue et al. 2006; Honkura, Matsuzaki et al. 2008).

The integrity of the actin cytoskeleton is controlled by a series of regulatory proteins (Figure 1.4). The Rho Family of GTPases, especially Rho and Rac, actively regulates the balance of actin polymerization and depolymerization within the spine (Nakayama, Harms et al. 2000; Tashiro, Minden et al. 2000; Fukata, Amano et al. 2001; Tashiro and Yuste 2004; Ethell and

Pasquale 2005). Key downstream effectors of Rho Family GTPases such as p21-activated kinase 1 (PAK1), insulin receptor substrate p53 (IRSp53), Rho-associated protein kinase (ROCK), LIM kinase 1 (LIMK-1) as well as actin depolymerizing factor (ADF)/Cofilin have also been highlighted in the regulation of synapse and dendritic spine stability in assorted studies (Maekawa, Ishizaki et al. 1999; Meng, Zhang et al. 2002; Meng, Zhang et al. 2003). Additionally, an F-actin binding protein independent of Rho GTPases signaling called Drebrin is predominantly expressed in early postnatal brain and correlates with alterations of spine size and shape (Shirao, Kojima et al. 1988; Ishikawa, Hayashi et al. 1994; Mammoto, Sasaki et al. 1998; Hayashi and Shirao 1999; Mizui, Takahashi et al. 2005; Kobayashi, Aoki et al. 2007; Biou, Brinkhaus et al. 2008; Ivanov, Esclapez et al. 2009).

Figure 1.4 Molecular mechanisms that regulate dendritic spine stability.
The actin cytoskeleton and its regulators, adhesion receptors, and scaffolding proteins provide physical support for long-term synaptic maintenance. Most signaling pathways regulate spine stability by either (1) directly or (2) indirectly (via RhoGTPase signaling pathways) regulating actin dynamics or its interactions with adhesion and scaffolding molecules. (3) Adhesion molecules mediate signaling from the presynaptic or extracellular compartments into dendritic spines to evoke changes in Rho GTPase and other signaling cascades that control F-actin structure and stability. (4) Scaffolding proteins both interact directly with F-actin to support cytoskeletal structure and organize signaling molecules (Lin and Koleske, 2010).



1.2.3.2 Scaffolding Proteins

As previously mentioned, the PSD area positively correlates with the spine head size, which further positively correlates with synaptic strength (Harris and Stevens 1989; Trachtenberg, Chen et al. 2002; Kasai, Matsuzaki et al. 2003; Holtmaat, Wilbrecht et al. 2006). Scaffolding

proteins are enriched in the PSD, where they deliver and anchor synaptic proteins and serve to link neurotransmitter receptors to their downstream signaling components (Figure 1.4).

PSD-95 is a major scaffolding protein abundantly enriched in the PSD at excitatory synapses. It has three PDZ domains, an SH3 domain, and a guanylate kinase-like domain. PSD-95 is of particularly importance for activity-dependent synaptic stabilization. Knockdown of PSD-95 in cultured neurons leads to reductions of spine density and size, as well as spine destabilization upon stimuli (Ehrlich, Klein et al. 2007). Additionally, DGKζ, a Diacylglycerol kinase (DGK) isoform necessary for the maintenance of spine density, is recruited to excitatory synapses by PSD-95 (Kim, Yang et al. 2009).

Similar to PSD-95, proteins of the Shank family are also core components of the PSD. Alternative splicing of Shank genes generates three isoforms of Shank proteins (Shank1, 2 and 3), all of which are expressed in the brain (Boeckers, Kreutz et al. 1999; Lim, Naisbitt et al. 1999). At the synapse, Shank and Homer cooperate to induce accumulation of Inositol 1,4,5-trisphosphate receptors (IP3Rs) to synapses. Tetramerization of Homer1b is required for the maintenance of spine structure and synaptic function (Hayashi, Tang et al. 2009). Shank1B overexpression promotes the stabilization by enlarging the spine heads (Xiao, Tu et al. 2000; Sala, Piech et al. 2001). In contrast, knockdown of Shank3 induces the formation of dendritic filopodia (Roussignol, Ango et al. 2005). Moreover, mutations of the *SHANK3* gene are associated with severe cognitive deficits including autism, which has been suggested to be caused by abnormalities of synapse formation and maintenance (Durand, Betancur et al. 2007).

1.2.3.3 Cell Adhesion Molecules

CAMs are also important regulators of synapse maintenance that promote trans-synaptic adhesion and stability, and provide a platform for bidirectional synaptic signaling between the pre- and postsynaptic compartments. Two major CAM complexes- cadherin/catenin complex and neurexin/neuroligin complex are highlighted in the mechanism of synapse maintenance. Neural cadherin (N-cadherin) is present at mature synapses and signals bidirectionally to coordinate stable adhesion by binding with catenin to stabilize the actin cytoskeleton (Perego, Vanoni et al. 2000; Togashi, Abe et al. 2002; Bamji, Shimazu et al. 2003; Abe, Chisaka et al. 2004; Bamji 2005; Bruses 2006; Kwiatkowski, Weis et al. 2007). Blockade of α-catenin function results in abnormally motile spine lacking presynaptic contact, which suggests that cadherin/catenin complex is a key regulator for the stability of synaptic contacts (Togashi, Abe et al. 2002; Abe, Chisaka et al. 2004). On the other hand, neuroligins are postsynaptic transmembrane proteins that bind to presynaptic neurexins to recruit key synaptic components. In neuron cultures, the neurexin/neuroligin complex not only functions alone, but also form complexes with PSD-95 to mediate bidirectional synaptic stability and maintenance (Graf, Zhang et al. 2004; Prange, Wong et al. 2004; Chih, Engelman et al. 2005; Varoqueaux, Aramuni et al. 2006; Craig and Kang 2007).

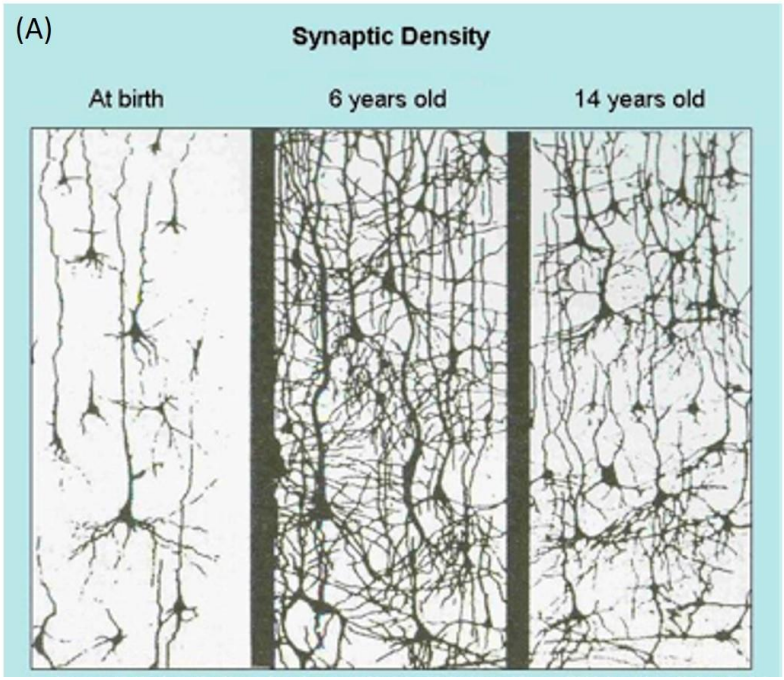
1.2.4 Synapse Elimination

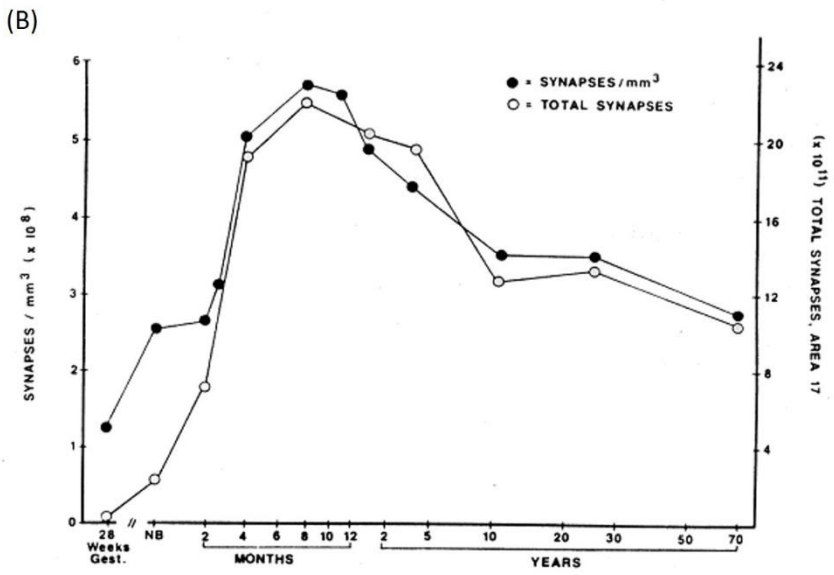
During early postnatal development, our brains undergo enormous changes. At birth, we already have almost the full complement of neurons we will ever have, and the number declines slightly as we age (Williams and Rakic 1988; Nowakowski 2006; Rakic 2006; Walloe, Pakkenberg et al. 2014). In contrast, synapses numbers increase at a dramatic rate

postnatally and peak at young childhood (Figure 1.5) (Huttenlocher and Dabholkar 1997; Liu, Somel et al. 2012). During this stage many exuberant synaptic connections are gradually eliminated (Figure 1.5) (Purves and Lichtman 1980; Hua and Smith 2004; Petanjek, Judas et al. 2011). Both immature and mature synapse elimination are affected by neuronal activity (Bastrikova, Gardner et al. 2008). Therefore, the selective elimination of excessive synapses is not only a critical process in shaping the initial neural circuits during development, but also an integral part of brain circuitry remodeling in response to experience and memory (Stevens, Allen et al. 2007).

Clear evidences of the elimination of synaptic connections have been found in the neuromuscular junction, autonomic ganglia, visual cortex and cerebellum (Purves and Lichtman 1980; Sanes and Lichtman 1999). However, compared to the large number of studies on synapse formation, our understanding of the details of synapse elimination remains vague. Recent advances in live-imaging techniques have allowed real-time monitoring of synapse morphology and quantitative measurement of the dynamics of synapse elimination *in vivo* (Feng, Mellor et al. 2000; Grutzendler, Kasthuri et al. 2002; Trachtenberg, Chen et al. 2002; Walsh and Lichtman 2003). For instance, in the primary visual cortex of young mice, synaptic connections were eliminated over one month, suggesting the prolonged significance of synapse elimination on functional synaptic connection synaptic connection (Grutzendler, Kasthuri et al. 2002; Gan, Kwon et al. 2003).

Figure 1.5 Overproduction of synapses and subsequent refinement of neural circuity. (A) Synapses are created with astonishing speed in the first three years of life. For the rest of the first decade, children's brains have twice as many synapses as adults' brains (Rima Shore, 1997). (B) Mean synaptic density in synapses/100 μm^3 (filled circles) and total synaptic density (open circles) in visual cortex at different ages after conception (Huttenlocher, 1990).





Notably, loss of a synapse is not likely a single event, but rather a result of dynamic balance between constant formation and elimination. This idea is supported by the observation that some lost postsynaptic inputs can be restored later on (Walsh and Lichtman 2003). Cellular processes and mechanisms involved in synapse elimination are not yet well characterized,

but it is increasingly apparent that several factors are contributing to synapse elimination. These factors include but are not limited to synaptic activity, competition for trophic factors, and distinct neuronal/non-neuronal signaling molecules.

1.2.4.1 Synapse Elimination Is an Activity-Dependent Competitive Process

Synapse elimination during development is driven by the process of neighboring axon inputs competing to innervate the same target cell. Such competition results in one or a few axons surviving to form synapses, while the others die back and are eliminated from the circuit. It is well documented that this competition is activity-dependent, since blockade of synaptic activity hinders the elimination (Thompson, Kuffler et al. 1979; Duxson 1982; Misgeld, Burgess et al. 2002). This has been further demonstrated by genetically inactivating neurotransmission of one among two inputs competing to innervate the same target cell, in which the strong input to a synaptic target cell destabilizes the weak one, thus differential efficacy affects the result of competitive elimination of supernumerary synapses (Buffelli, Burgess et al. 2003). In the visual system, the process of synapse elimination is reversible, which is also mediated by neural activity (Antonini, Gillespie et al. 1998).

How are changes in correlated neural activity translated into molecular mechanisms directing synapse elimination? One possible mechanism is that the neural activity may regulate synaptic strength through up- or down-regulation of proteins that execute synapse elimination. One example is the transcription factor myocyte enhancer factor 2 (MEF2). In hippocampal neurons, the activity of MEF2 in response to activity results in the induction of PSD-95 ubiquitination and degradation as well as spine loss (Flavell, Cowan et al. 2006; Shalizi, Gaudilliere et al. 2006; Barbosa, Kim et al. 2008; Tian, Kai et al. 2010; Tsai,

Wilkerson et al. 2012). In granule neurons of the cerebellar cortex, activity-dependent calcium signaling induces dephosphorylation of MEF2, which is required for the morphology of specialized postsynaptic structures termed dendritic claws (Shalizi, Gaudilliere et al. 2006). Furthermore, a genome-level analysis in hippocampal neurons identified a number of the genes that are activated by MEF2 (Flavell, Kim et al. 2008). Among these targets, Homer1 and BNDF stand out for their functions in regulating activity-dependent synaptic functions, indicating that MEF2 regulates the expression of a broad range of genes in restricting excitatory synaptic connectivity (Huang, Kirkwood et al. 1999; Poo 2001; Sala, Futai et al. 2003; Hong, McCord et al. 2008). At the molecular level, the phosphorylation state of MEF2 is regulated by the activation of calcineurin which is dependent on Ca2+ influx through L-type Ca2+ channels (Flavell, Cowan et al. 2006).

1.2.4.2 Molecules Involved in Synapse Elimination

Compared to the considerable body of knowledge on the mechanisms underlying synapse formation, relatively less is known about the molecular mechanisms that regulate synapse elimination. Recent studies have begun to recognize the role of the classical complement cascade in the process of synapse elimination (Balice-Gordon and Lichtman 1994; Stevens, Allen et al. 2007; Chu, Jin et al. 2010; Paolicelli, Bolasco et al. 2011; Schafer, Lehrman et al. 2012). The complement proteins, complement component 1q (C1q) and complement component 3 (C3), may initiate synapse elimination based on the observation that mice deficient in C1q and C3 show signs of disrupted synapse elimination such as failure to refine retinogeniculate connections and an excess of axonal boutons (Stevens, Allen et al. 2007; Chu, Jin et al. 2010). Another immune protein named Major histocompatibility complex class I also negatively regulates the density and function of cortical synapses and modulates

activity-dependent refinement and plasticity (Glynn, Elmer et al. 2011). Further indirect evidence for immune molecules functioning in synapse elimination is provided by the observation that microglia is necessary for the synaptic pruning in hippocampal neurons (Paolicelli, Bolasco et al. 2011), which was corroborated by the subsequent finding that microglia engulf and remodel developing synapses depending on activity and C3 receptor stimulation (Schafer, Lehrman et al. 2012).

Beside immune proteins, repulsive signaling molecules such as Semaphorins and Ephrins have also been implicated in synapse elimination (Bagri, Cheng et al. 2003; Liu, Low et al. 2005; Tada and Sheng 2006; Fu, Chen et al. 2007; O'Connor, Cockburn et al. 2009). The function of Semaphorins in synapse elimination will be further discussed in section 1.4.

1.2.4.3 Glial Cells in Synapse Elimination

Though the specific molecular mechanisms that drive synapse elimination are not fully known, new findings in the past decade support the importance of glial cells in synapse elimination in the developing brains. In mammals and *Drosophila*, synapses are removed actively through engulfment and phagocytosis regulated by glial cells (Eroglu and Barres 2010). For example, in the mammalian peripheral nervous system, microglia (a resident population of phagocytic glial cells) and Schwann cells (glial cells ensheathing peripheral axons) at the NMJ have been suggested to clear neural debris and eliminate synapses (Bishop, Misgeld et al. 2004). If phagocytosis is inhibited, axon pruning process is severely delayed, strongly implicating an active role for glia cells in triggering input elimination (Awasaki and Ito 2004).

The role of glial cells in synapse elimination is demonstrated in the CNS as well. Using electron microscopy and two-photon *in vivo* imaging in the mouse visual cortex, it was observed that microglia are preferentially localized to small and growing spines, and form close contacts with synaptic-associated elements including synaptic clefts (Tremblay, Lowery et al. 2010). Moreover, light deprivation induced microglia to become less motile and their morphology changed, suggesting that microglia may actively contribute to the experience-dependent synapse elimination in juvenile brains (Tremblay, Lowery et al. 2010). Microglia also actively engulf synapses in the mouse hippocampus (Paolicelli, Bolasco et al. 2011). Two significant synaptic proteins, PSD-95 and SNAP25, were found in the microglial processes, suggesting that microglia engulf synaptic material in the postnatal brain (Fuhrmann, Bittner et al. 2010). In mice lacking *Cx3cr1*, a chemokine fractalkine receptor exclusively expressed by microglia, synaptic elimination was delayed, resulting in increased density of dendritic spines and immature synapses (Fuhrmann, Bittner et al. 2010).

1.3 Synaptic Plasticity

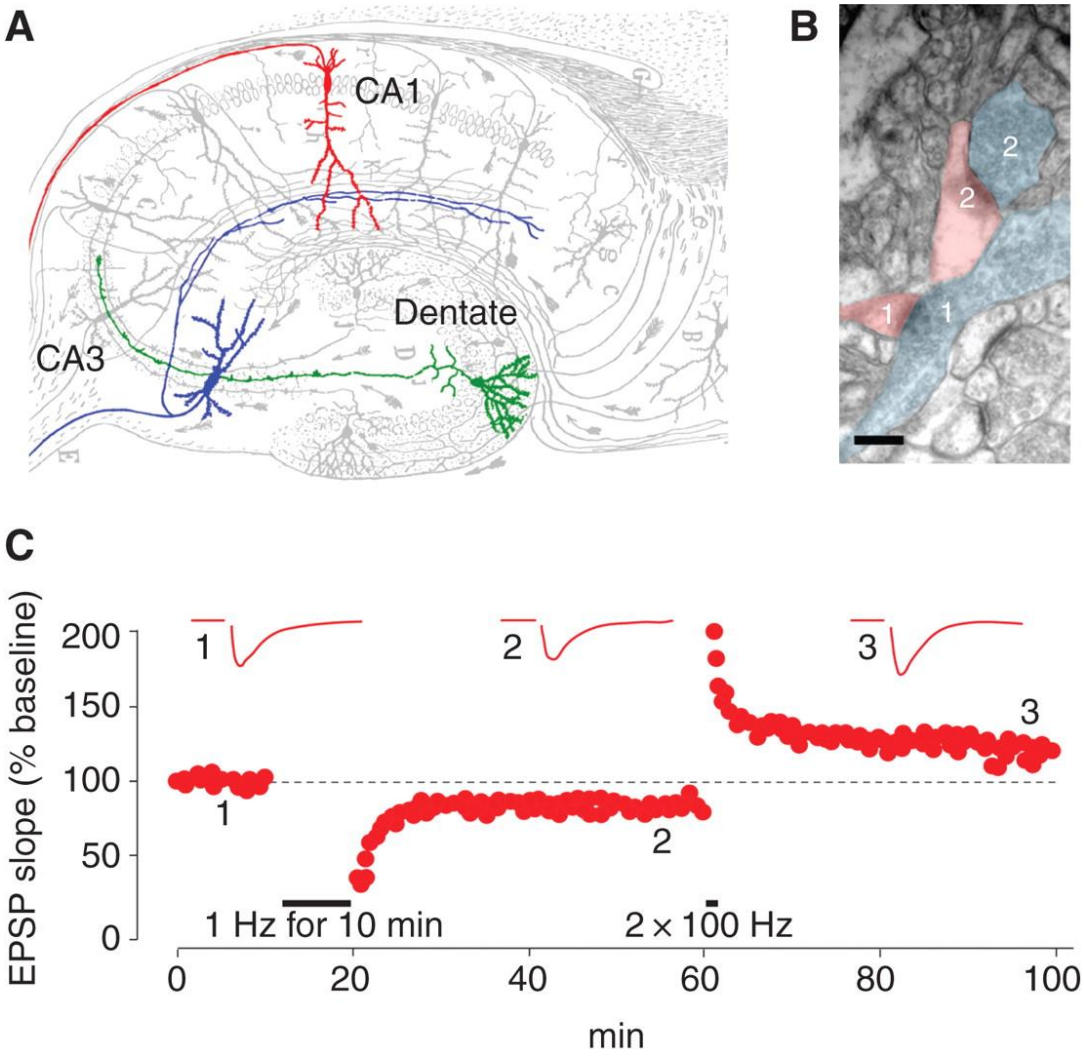
One of the remarkable features of the mammalian nervous system is its ability to constantly change synaptic morphology and strength in response to neural activity, which is referred to as synaptic plasticity. A long-standing hypothesis in neuroscience posits that, by modifying synaptic structures and functions, synaptic plasticity serves as the key molecular basis of learning and memory formation (Malinow and Malenka 2002; Holtmaat, Wilbrecht et al. 2006). Therefore, understanding the molecular underpinnings of synaptic plasticity will help us to uncover the neural mechanisms of learning and memory. Two of the most studied forms of activity-dependent synaptic modifications are LTP, a sustained increase in synaptic

strength resulting from synchronous stimulation, and LTD, which is manifested by a long-lasting reduction in the synaptic efficacy (Figure 1.6) (Bliss and Lomo 1973; Dudek and Bear 1992; Mulkey and Malenka 1992).

Figure 1.6 Long lasting synaptic plasticity in hippocampus.
(A) Historical drawing by Ramon y Cajal (1909) of the trisynaptic pathway in the hippocampus. LTP and LTD are induced by activation of NMDARs at synapses between CA3 and CA1 pyramidal neurons (blue and red). In contrast, LTP at mossy fiber synapses onto CA3 neurons (green on blue) is NMDAR-independent.

(B) This electron microscopy image shows the densely packed neuropil in the CA1 region of the hippocampus and highlights two asymmetric CA3-CA1 synapses. Note the typical "bouton en passant" configuration of synapse 1 and the prominent spine in synapse 2. The postsynaptic densities (PSDs) are visible. Scale bar, 200 nm. (Image kindly provided by Rafael Luján, Universitad de Castilla-La Mancha.)

(C) Bidirectional change in CA3-CA1 synaptic efficacy by LTD and LTP in the same synapses monitored by extracellular field recordings in an acute slice preparation of the hippocampus. (Lüscher and Malenka, 2012).



1.3.1 Hippocampus as A Model to Study Synaptic Plasticity

The hippocampus is located in the medial temporal lobe and is part of the limbic system. Classically, the hippocampal formation consists of four distinct structures that form unique unidirectional projections, namely the dentate gyrus (DG), the *Cornu Ammonis* (CA), the subicular complex (SC) and the entorhinal cortex (EC) (Andersen 2007). Because of its well-known anatomy and networks, together with its strict laminar organization, the hippocampus is frequently used for electrophysiological recordings *in vitro* and *in vivo* to study synaptic plasticity (Berger, Rinaldi et al. 1983).

On the organismic level, a large amount of literatures has indicated an essential role of the hippocampus in memory and learning, especially memory involved in spatial navigation (Milner and Penfield 1955; Milner 1972; Moser, Moser et al. 1993; Kesner and Hopkins 2006; Rogers and Kesner 2006). On the cellular level, the hippocampus has been shown to be a brain region of high synaptic plasticity throughout development and even adulthood (Bliss and Gardner-Medwin 1973; Bliss and Lomo 1973; Andersen 2007). These features make the hippocampus an ideal model system to study synaptic plasticity.

1.3.2 Long-Term Potentiation

In 1973, Bliss and Lomo first described that a train of high-frequency stimulation caused a sustained increase in synaptic efficiency in the rabbit hippocampus (Bliss and Lomo 1973). A series of subsequent reports during the 1970s confirmed that LTP was characterized by cooperativity, associativity and input specificity (Malinow and Malenka 2002; Malenka and Bear 2004; Caporale and Dan 2008). Since then, LTP has been viewed as the most compelling model of memory formation and has been intensely investigated. Over the last

few decades, a number of key mechanisms have been examined for the induction and expression of LTP, and hundreds of molecules associated with LTP have been identified in this process (Malenka 1994; Malenka and Bear 2004).

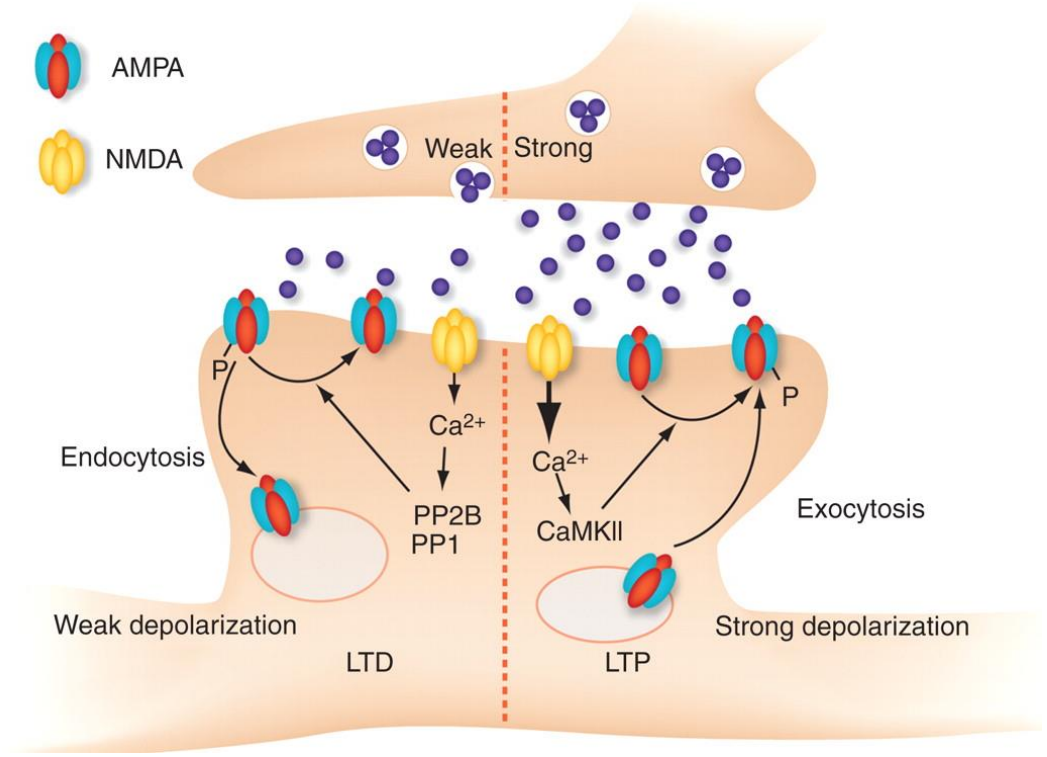
Different forms of LTP have been observed in a variety of regions of the brain, including the hippocampus, cerebral cortex, cerebellum and amygdala (Clugnet and LeDoux 1990; Tsumoto 1990; Maren 1996; Grasselli and Hansel 2014). In the following discussion regarding the mechanisms of LTP induction and expression, I will exclusively focus on of NMDAR-dependent LTP performed on excitatory synapses in the CA1 region of the hippocampus, which is the most studied form of LTP, and also most relevant for the research carried out for this dissertation.

1.3.2.1 Mechanisms of LTP Induction

It is well accepted that the NMDAR-dependent LTP is initiated by the Ca^{2+} influx observed after the activation of NMDARs, which allows Ca^{2+} to enter the dendritic spine (Bliss and Collingridge 1993; Malenka and Nicoll 1999). In support of the pivotal role of NMDARs in LTP induction, several reports have shown that inhibition of NMDAR activation with AP5 and MK801 blocked LTP and attenuated performance in spatial learning tasks (Collingridge, Kehl et al. 1983; Morris, Anderson et al. 1986; Coan and Collingridge 1987; Errington, Lynch et al. 1987). In particular, NR2A and NR2B subunits of NMDAR are critical for NMDAR function during LTP induction, since the disruptions of NR2A and NR2B led to attenuated LTP and impaired spatial learning (Sakimura, Kutsuwada et al. 1995; Kutsuwada, Sakimura et al. 1996; Kiyama, Manabe et al. 1998). Conversely, overexpression of the NR2B

subunit promoted LTP and enhanced learning and memory (Figure 1.7) (Tang, Shimizu et al. 1999).

Figure 1.7 Postsynaptic expression mechanisms of LTP and LTD.
Weak activity of the presynaptic neuron leads to modest depolarization and calcium influx through NMDA receptors. This preferentially activates phosphatases that dephosphorylate AMPA receptors, thus promoting receptor endocytosis. Strong activity paired with strong depolarization triggers LTP in part via CaMKII, receptor phosphorylation, and exocytosis (Lüscher and Malenka, 2012).



1.3.2.2 Signaling Cascades for LTP

What intracellular cascades are activated by Ca^{2+} and translate the Ca^{2+} signal into an increase in synaptic strength during LTP? Related reports have generated an enormous list of signal transduction molecules. But most of them have the ability to affect LTP but are not

absolutely required for LTP. Here I will discuss a few molecules that are key and essential for LTP.

Upon entry into the postsynaptic neurons, Ca^{2+} leads to the activation of CaMKII. Overwhelming evidence indicates that CaMKII is a critical factor in the induction of LTP (Malenka and Nicoll 1999; Lisman, Schulman et al. 2002). Both inhibition of CaMKII activity and genetic deletion of the *CaMKII* gene were shown to block LTP (Malenka, Kauer et al. 1989; Malinow, Schulman et al. 1989; Silva, Stevens et al. 1992). On the other hand, increasing the concentrations of active CaMKII in CA1 neurons is sufficient to induce LTP (Pettit, Perlman et al. 1994; Lledo, Hjelmstad et al. 1995). An important property of CaMKII is its autophosphorylation at Thr-286, which allows it to be constitutively active long after induction of LTP (Kennedy, Bennett et al. 1990). The autophosphorylation of CaMKII is necessary for LTP, because the replacement of endogenous CaMKII with a non-phosphorylatable CaMKII mutant at Thr-286 impaired LTP and spatial learning (Fukunaga, Muller et al. 1995; Giese, Fedorov et al. 1998). Finally, CaMKII can directly phosphorylate GluA1 at Ser-831, which has been proposed to contribute to the LTP-associated increase of AMPAR conductance (Davies, Lester et al. 1989).

Several other protein kinases have also been implicated to play key roles in LTP, although evidence supporting their necessities is considerably weaker than that of CaMKII (Lynch 2004). For many years, protein kinase C (PKC) has been suggested to be important for LTP. Analogous to CaMKII, PKC inhibitors have been reported to block LTP, whereas increasing postsynaptic PKC activity can enhance LTP (Hu, Hvalby et al. 1987; Linden and Routtenberg 1989; Malinow, Schulman et al. 1989; Bliss and Collingridge 1993; Malenka and Nicoll 1999). Protein kinase A (PKA**)** has been implicated in LTP during early

development, and later in development it may boost CaMKII activity indirectly via phosphorylation of inhibitor 1, an endogenous protein phosphatase inhibitor (Blitzer, Connor et al. 1998; Brown, Blitzer et al. 2000). More recently, the mitogen-activated protein kinase (MAPK) cascade that activates extracellular signal-regulated kinases (ERKs) has also been shown to be required for LTP induction (Sweatt 2004; Thomas and Huganir 2004).

1.3.2.3 Mechanisms of LTP Expression

It is widely believed that the modulation in AMPAR properties and number is the major mechanism for the expression of LTP (Malenka and Nicoll 1999; Malinow and Malenka 2002; Song and Huganir 2002; Bredt and Nicoll 2003). Early experiments demonstrated the increase in AMPAR currents following LTP stimulation (Zamanillo, Sprengel et al. 1999; Hayashi, Shi et al. 2000). Subsequent studies revealed that GluA1 is critical for changes in AMPAR properties. In response to LTP induction, Ca^{2+} influx activates CAMKII, which in turn phosphorylates GluA1 at Ser-831 and increases the conductance of AMPARs (Derkach, Barria et al. 1999; Kristensen, Jenkins et al. 2011).

The increase of synaptic AMPARs appears to be mediated by SNARE-dependent exocytosis to perisynaptic locations, and subsequently to synaptic sites via lateral diffusion (Mulkey and Malenka 1992; Luscher, Xia et al. 1999; Carroll, Beattie et al. 2001; Kennedy and Ehlers 2011). Notably, GluA2-containing AMPARs are replaced by the inserted AMPARs during LTP expression stage, which are primarily heteromeric GluA1/GluA2 or homomeric GluA1 with a higher conductance (Adesnik and Nicoll 2007; Liu and Zukin 2007).

Consistent with the increase in synapse strength, the expression of LTP is also accompanied by enlarged PSDs and spines, as well as the formation of new spines that lasts for hours

(Engert and Bonhoeffer 1999; Toni, Buchs et al. 2001; Matsuzaki, Honkura et al. 2004). However, such structural changes were not observed when LTP stimulation is blocked by AP5 (Kopec, Li et al. 2006). These observations raise the question whether mechanisms underlying the functional and structural plasticity during LTP are independent or not, which is an active area of investigation nowadays.

1.3.2.4 Chemical LTP

Because electrically-induced LTP is applicable only to a fraction of synapses in cultured neurons or brain slices, other methods including bath application and pressure pulse ejection of reagents such as glycine and TEA have been developed for inducing LTP in a large population of synapses. Among them, bath application has become the favorable method because it is easy to manipulate and globally strengthens synaptic transmission. Glycine, a NMDAR coagonist, has been one of the major chemical used to induce chemical LTP (Shahi and Baudry 1993; Shahi, Marvizon et al. 1993). Glycine-induced LTP specifically stimulates NMDARs only at synapses receiving spontaneous release of glutamate and subsequently causes Ca^{2+} influx, which leads to an increase in postsynaptic Ca^{2+} concentration. This increase in intracellular calcium activates calcium-dependent kinases such as CaMKII and PKC and result in an increase in mEPSC amplitude (Shahi and Baudry 1993; Oh and Derkach 2005). Furthermore, like electrically-induced LTP, the glycine-induced LTP is also associated with NMDAR-dependent spine enlargement and synapse formation (Park, Salgado et al. 2006; Korkotian and Segal 2007; Ovtscharoff, Segal et al. 2008).

1.3.3 Long-Term Depression

After LTP was discovered in the early 1970s, it took a few years before the predicted activity-dependent decrease of synaptic transmission termed LTD was experimentally demonstrated in the hippocampus (Lynch, Dunwiddie et al. 1977). Since then, various forms of LTD have been identified in various brain regions, supporting its physiological significance (Kirkwood and Bear 1994; Kemp and Bashir 1997; Feldman, Nicoll et al. 1998; Cho, Kemp et al. 2000; Kemp, McQueen et al. 2000). These various forms of LTD can be found in different brain regions, but can also be found in the same region depending on the stimulus protocol, specific experimental conditions and developmental stage (Oliet, Malenka et al. 1997; Kemp, McQueen et al. 2000; Pavlov, Riekki et al. 2004; Nosyreva and Huber 2006). Although they seem identical, they use distinct molecular mechanisms and possibly have different functions in the nervous system. For example, LTD can be described as either homosynaptic or heterosynaptic, depending on whether the postsynaptic activity is driven by the presynaptic activity of the same synapse (Bear and Abraham 1996; Li and Burrell 2009). In the following discussion about mechanisms of LTD induction and expression, I will exclusively focus on the NMDAR-dependent LTD, which is relevant for the type of LTD explored in this dissertation.

1.3.3.1 Mechanisms of LTD Induction

To date, multiple stimulation protocols, from prolonged low-frequency stimulation to application of receptor agonists, have been established to stimulate LTD (Collingridge, Peineau et al. 2010). The mechanisms of LTD may vary by different forms of LTD, but similar to LTP, the induction of LTD typically starts from NMDAR-dependent Ca^{2+} influx

and relies on activations of glutamate receptors and downstream serine/threonine phosphatase cascades (Figure 1.8) (Mulkey and Malenka 1992; Mulkey, Herron et al. 1993; Mulkey, Endo et al. 1994).

In NMDAR-dependent LTD, the elevation of postsynaptic Ca^{2+} concentration is mediated by NMDAR activation, which has been observed in a variety of brain regions including the hippocampus and cortex (Mulkey and Malenka 1992; Mulkey, Herron et al. 1993; Kemp and Bashir 2001). Ca^{2+} influx is required for both LTP and LTD during their induction, and the particular properties of the Ca^{2+} signal is the key determinant dictating whether LTP or LTD is evoked (Bienenstock, Cooper et al. 1982; Lisman 1989). It is now well accepted that compared to the significant but short-lasting Ca^{2+} influx caused by robust receptor activation during LTP induction, LTD is induced by a moderate but long-lasting Ca^{2+} influx produced by a modest NMDAR activation (Lisman 1989; Malenka 1994; Collingridge, Peineau et al. 2010).

1.3.3.2 Signaling Cascades for LTD

How does the increase in postsynaptic Ca^{2+} lead to the decrease of synaptic transmission? Using enzyme inhibitors, studies on LTD reveals a sequence of events, in which the binding of Ca^{2+} to calmodulin forms a calcium–calmodulin complex, and subsequently activates both CaMKII and calcium–calmodulin dependent protein phosphatase 2B (calcineurin or PP2B) (Mulkey, Herron et al. 1993; Mulkey, Endo et al. 1994; Lisman, Schulman et al. 2002). This results in the dephosphorylation and inactivation of inhibitor 1, which in turn activates protein phosphatase 1 (PP1). The activation of PP1 subsequently leads to the dephosphorylation and endocytosis of AMPARs (Lee, Kameyama et al. 1998; Carroll, Lissin

et al. 1999; Lee, Barbarosie et al. 2000; Man, Lin et al. 2000). In support of this model, the induction of LTD *in vivo* has been demonstrated to be followed by an increase in PP1 activity (Thiels, Norman et al. 1998), whereas PP1 and PP2 inhibitors have been reported to block LTD or even reverse established LTD (Mulkey, Herron et al. 1993; Mulkey, Endo et al. 1994).

In addition to this classic calcium-dependent cascade, numerous different proteins have been reported to be involved in the clathrin-dependent AMPAR endocytosis. AP2, a clathrin adaptor protein, binds to the GluA2 subunit and transports GluA2-containing AMPARs to clathrin (Lee, Liu et al. 2002). Hippocalcin, a member of the neuronal calcium sensor (NCS) family, forms a complex with GluA2/AP2 and mediates AMPAR endocytosis upon sensing small rises in postsynaptic Ca^{2+} (Palmer, Lim et al. 2005). Moreover, various serine/threonine protein kinases have been implicated in LTD intracellular signaling cascades, which includes PKA, PKC, Protein interacting with C-kinase 1 (PICK1), cyclin-dependent kinase 5 (cdk5), P38 mitogen-activated protein kinase (p38MAPK) and glycogen synthase kinase-3 (GSK3) (Brandon, Zhuo et al. 1995; Collingridge, Isaac et al. 2004; Ohshima, Ogura et al. 2005; Zhu, Pak et al. 2005; Peineau, Taghibiglou et al. 2007; Peineau, Nicolas et al. 2009).

1.3.3.3 Mechanisms of LTD Expression

While the exact mechanisms linking the NMDAR mediated Ca^{2+} influx to decreased synaptic strength is incompletely explored, a large body of evidence suggests that LTD is predominantly expressed through changes in AMPAR properties. One of such changes is the removal of AMPARs from the postsynaptic membrane through clathrin-mediated

endocytosis, which is believed to be critical in the expression of LTD (Carroll, Lissin et al. 1999; Man, Lin et al. 2000; Wang and Linden 2000; Carroll, Beattie et al. 2001; Luscher and Frerking 2001). This internalization of surface AMPARs during LTD is specifically associated with the phosphorylation state of GluA2 subunit (Ahmadian, Ju et al. 2004; Hayashi and Huganir 2004; Fox, Russell et al. 2007). In addition, regulation of the AMPAR function through modulation of the phosphorylation state of AMPAR subunits is another attractive mechanism of LTD expression. For example, dephosphorylation of GluA1 subunit at Ser-845 controls the open probability of GluA1-containing AMPARs, and is postulated to reduce AMPAR responses (Roche, O'Brien et al. 1996; Banke, Bowie et al. 2000; Song and Huganir 2002).

During LTP, the functional modifications of AMPARs are accompanied by increased spine volume and density. Therefore, LTD expression that results in the internalization of synaptic AMPARs may also result in shrinkage and loss of pre- and postsynaptic elements. Indeed, a few studies have confirmed spine shrinkage and loss in the hippocampus after LTD (Nagerl, Eberhorn et al. 2004; Zhou, Homma et al. 2004; Wang, Yang et al. 2007; Becker, Wierenga et al. 2008). However, such LTD-associated changes in spine morphology and density do not share the same signaling pathway as the decrease in synaptic responses (Zhou, Homma et al. 2004). Therefore, the mechanisms underlying the decrease in synaptic strength versus spine elimination induced by LTD needs to be further clarified.

1.3.3.4 Chemical LTD

Similar to electrically-induced LTP, electrically-induced LTD only affects a fraction of the synapses in cultured hippocampal neurons, therefore LTD induced by chemical stimulation

has been a useful tool to globally depress synaptic strength. Specifically, NMDAR-dependent chemical LTD can be induced by applying NMDA to hippocampal slices, which nonselectively activates both synaptic and extrasynaptic NMDARs (Lee, Kameyama et al. 1998; Kamal, Ramakers et al. 1999; Li, Dozmorov et al. 2004).This NMDA-induced LTD mimics some features of electrically-induced LTD such as a persistent decrease of amplitude of mEPSP and dephosphorylation of GluA1 on Ser-845 (Kameyama, Lee et al. 1998; Lee, Kameyama et al. 1998; Lu, Man et al. 2001). Furthermore, chemical LTD induction also causes spine head shrinkage without affecting the spine density (He, Lee et al. 2011).

1.3.4 Synaptic Plasticity in Learning and Memory

Since the proposal of the Hebbian rule in 1949 (Hebb 1949), synaptic plasticity has become a key cellular mechanisms of memory formation. However, the rule was not formally validated until the discovery of LTP by Bliss and Lømo in 1973 (Bliss and Lomo 1973). Since then, hippocampal LTP has become the primary experimental model for examining the synaptic mechanisms of learning and memory. This is partly due to the properties that LTP shares with memory formation (Bliss and Collingridge 1993; Bennett 2000; Martin, Grimwood et al. 2000). For example, LTP can be provoked quickly and be persistent from minutes or months (Bliss and Lomo 1970; Bliss and Lomo 1973). Secondly, LTP is input specific, which means that LTP at one synapse does not spread to adjacent synapses (Bliss and Collingridge 1993). Furthermore, LTP also exhibits associativity, that is, a weak stimulation of one pathway coupled with simultaneous strong stimulation of another pathway can induce LTP of the weak input (Collingridge, Kehl et al. 1983). Additional evidence supporting the involvement of LTP in learning and memory was provided by studies on the rat hippocampus, where LTP was observed during learning *in vivo* (Whitlock, Heynen et al.

2006). In contrast, pharmacologically blocking LTP (Morris, Anderson et al. 1986; Davis, Butcher et al. 1992) or impairing LTP by knocking out some protein kinases interfered with the acquisition of learning (Abeliovich, Chen et al. 1993).

LTD is another classic form of experience-dependent synaptic plasticity (Kemp and Bashir 2001). The role of LTD in learning and memory is more elusive compared to LTP, although there are studies that support the importance of LTD for maintaining the flexibility in our information storage system (Dong, Bai et al. 2013; Connor and Wang 2016). For instance, the perirhinal cortex is necessary for single-exposure visual recognition learning, and such learning is associated with long-term reductions in neuronal responsiveness, indicating that LTD may underlie perirhinal cortex-dependent learning (Zhu, McCabe et al. 1996; Brown and Xiang 1998; Xiang and Brown 1998; Brown and Aggleton 2001; Warburton, Koder et al. 2003; Massey, Phythian et al. 2008; Tamagnini, Burattini et al. 2012).

The cellular and molecular mechanisms of LTD are not as well understood as LTP, however, it is increasingly clear that LTD is not a simple reverse of LTP (Braunewell and Manahan-Vaughan 2001; Nabavi, Fox et al. 2014). More likely, LTP and LTD provide distinct but complimentary mechanisms to adjust synaptic strength, which underlie memory acquisition and inactivation respectively (Braunewell and Manahan-Vaughan 2001; Yang, Huang et al. 2005).

Last, it is worth noting that although LTP and LTD are attractive candidate mechanisms for brain information storage, there is still little empirical evidence directly linking synaptic plasticity to cognition and memory (Barnes 1995; Goda and Stevens 1996; Holscher 1999).

1.4. Semaphorins

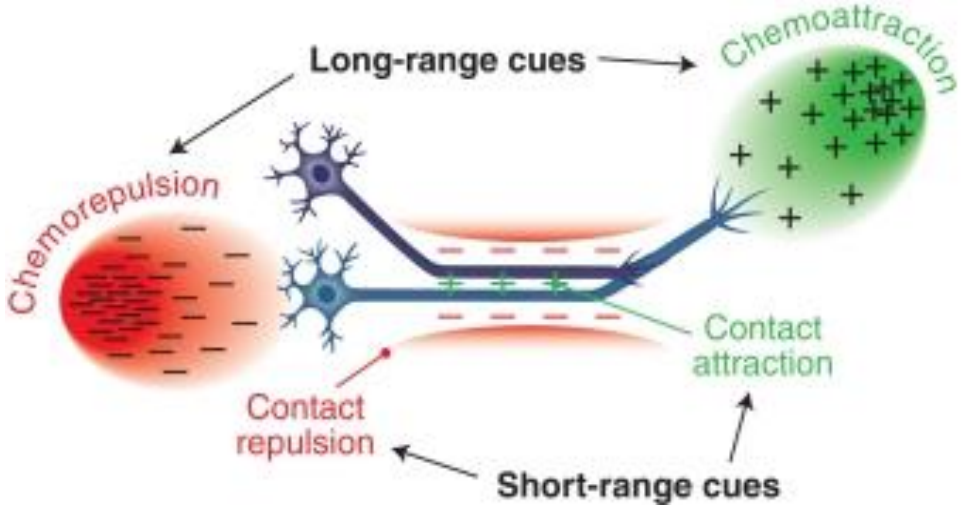
1.4.1 Axon Guidance Cues

1.4.1.1 Axon Guidance in The Mammalian Central Nervous System

One of the most important mechanisms involved in the formation of neural networks is the guidance of the growing axons to locate and recognize their specific targets (Dodd and Jessell 1988). This process is accomplished by a highly mobile, actin enriched structure located at the terminal of the axon called the growth cone. In response to extracellular axon guidance cues, the growth cones undergo classic processes such as turning, fasciculation, collapse, retraction and stalling (Lance-Jones and Landmesser 1981; Harris 1986; Guthrie and Lumsden 1992; Suter and Forscher 2000). Thus, the growth cone serves as a transfer station transducing extracellular signals into changes in axonal orientation.

Axon guidance cues can be either attractive or repulsive, working in either short-range or long-range. Attractive cues attract growth cones toward the source of the cue, while repulsive cues repel growth cones away from the source of the cue (Tessier-Lavigne and Goodman 1996). Short-range cues are often membrane-bound molecules anchored to stationary cells or extracellular matrix (ECM), while long-range cues are usually diffusible molecules that establish concentration gradients and function in a distance (Kolodkin and Tessier-Lavigne 2011). By interacting with each other and producing a gradient of environmental signals, these axon guidance cues enable extending axons to find their appropriate synaptic partners (Figure 1.8).

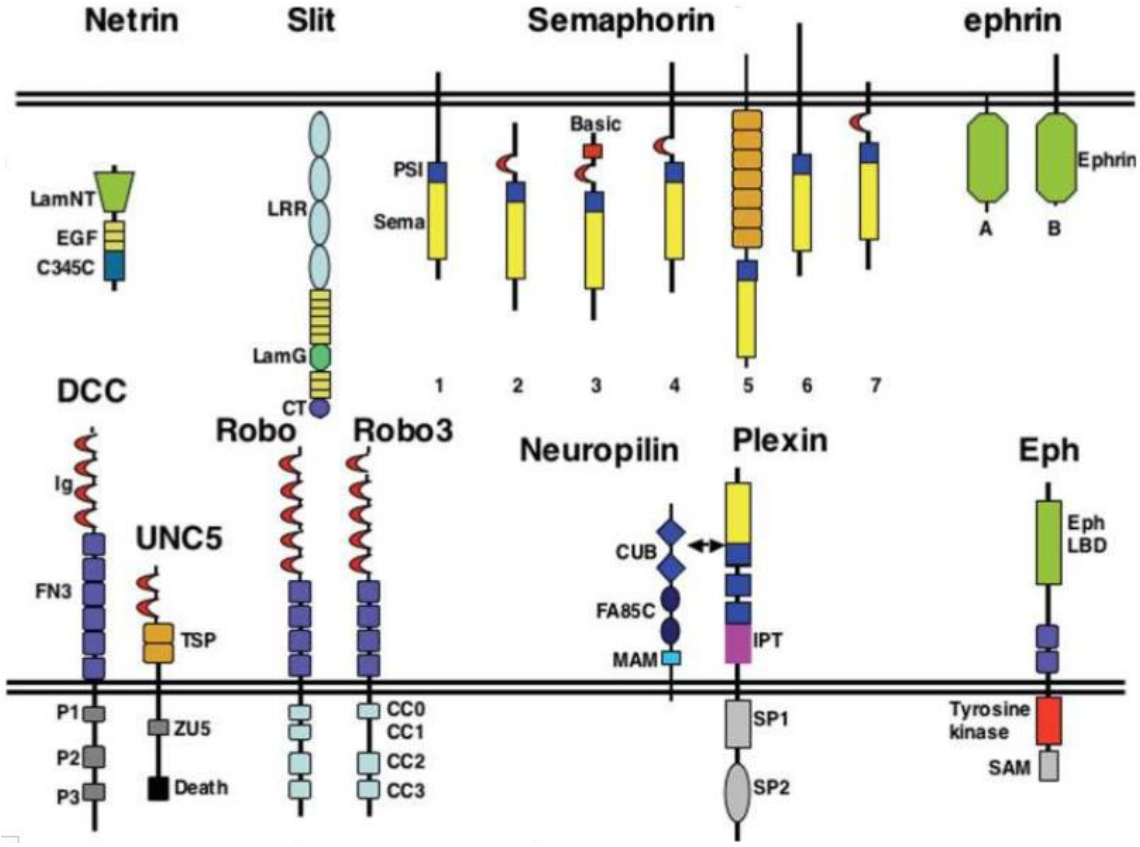
Figure 1.8 The diversity of neuronal guidance mechanisms.
Neuronal processes are guided by cues that can function at long- and short-distances to mediate either attractive or repulsive guidance (Kolodkin and Tessier-Lavigne, 2011).



1.4.1.2 Classic Axon Guidance Cue Families

Studies in the past several decades has advanced our understanding of numerous guidance cues and their receptors, as well as their molecular signaling cascades (Yu and Bargmann 2001; Dickson 2002; Huber, Kolodkin et al. 2003). Through various biochemical, genetic and tissue culture approaches in both invertebrates and vertebrates, four classic families of guidance molecules have been identified, namely netrins, Slits, ephrins and Semaphorins (Figure 1.9) (Kolodkin and Tessier-Lavigne 2011). Each individual family will be briefly introduced below, except that the Semaphorin family will be described in more detail later.

Figure 1.9 Summary of the four families of instructive guidance cues and receptors. P1 to P3, CC0 to CC3, and SP1 and SP2 marked conserved regions in DCC, Robo, and Plexin. The divergent member of the Robo family Rig-1/Robo 3 lacks the CC1 region (Yaron and Zheng, 2006).



Netrins are a small family of laminin-related glycoproteins, characterized with an amino-terminal globular domain and three epidermal growth factor (EGF)-like repeats (Kolodkin and Tessier-Lavigne 2011). In *C. elegans*, netrin was initially found to mediate circumferential axon guidance (Yu and Bargmann 2001). Subsequent studies then identified vertebrate netrins as chemoattractant factors for spinal commissural axons in the ventral midline floor plate (Tessier-Lavigne, Placzek et al. 1988; Hedgecock, Culotti et al. 1990; Placzek, Tessier-Lavigne et al. 1990; Kennedy, Serafini et al. 1994). Shortly after, it was

demonstrated that the guidance signals triggered by netrins could be either attractive or repulsive (Kennedy, Serafini et al. 1994; Colamarino and Tessier-Lavigne 1995; Varela-Echavarria, Tucker et al. 1997). Netrins mediate their bifunctional effects through DCC and the UNC5 families of receptors. Specifically, the UNC-5 protein is mainly involved in repulsive signaling, whereas netrin attraction is mediated through UNC-40/DCC (Leung-Hagesteijn, Spence et al. 1992; Chan, Zheng et al. 1996; Keino-Masu, Masu et al. 1996; Kolodziej, Timpe et al. 1996; Tessier-Lavigne and Goodman 1996; Leonardo, Hinck et al. 1997).

Slits are secreted proteins, involved in spinal commissural axonal guidance through their receptor Roundabout (Robo) (Whitford, Marillat et al. 2002; Ma and Tessier-Lavigne 2007). In Drosophila and mice, Slits confer inhibitory signals by preventing axons from re-crossing the midline, since in Slit knockout animals the axons linger at the midline and re-cross it sometimes (Seeger, Tear et al. 1993; Kidd, Bland et al. 1999; Long, Sabatier et al. 2004), In addition, Slit also acts as a repellent cue on retinal axons and olfactory bulb axons (Li, Chen et al. 1999; Wu, Wong et al. 1999; Plump, Erskine et al. 2002).

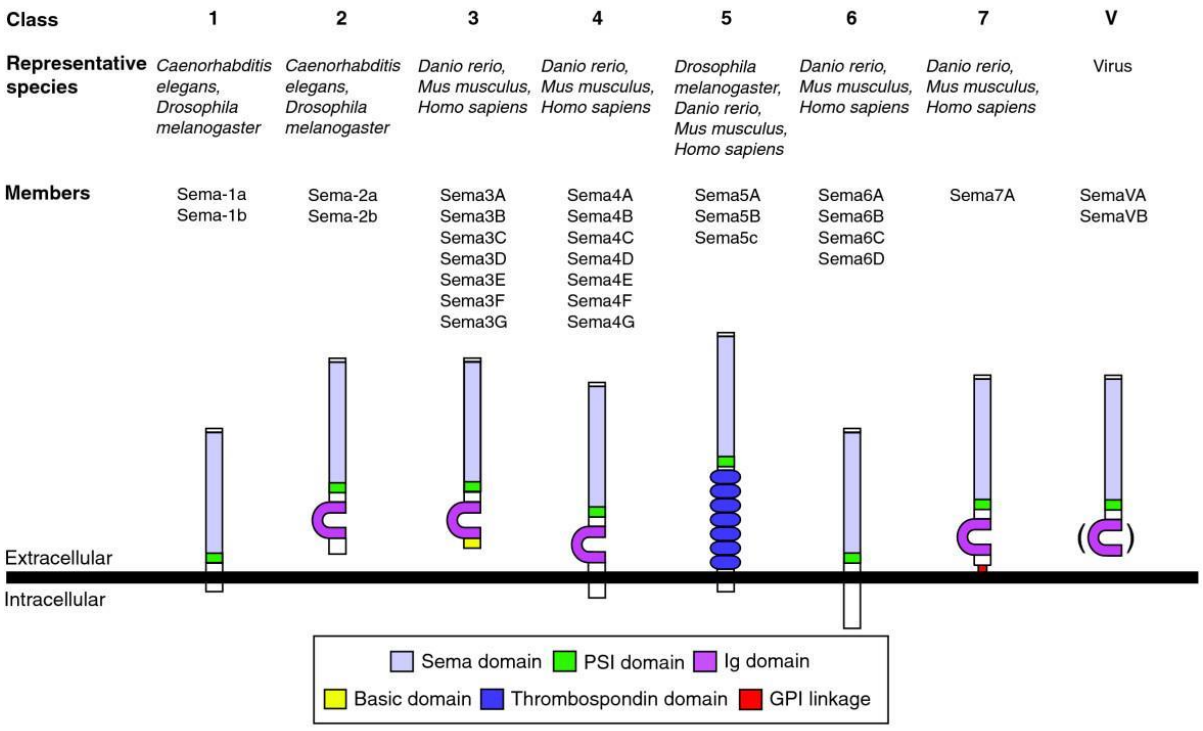
Ephrins are all membrane-associated molecules signaling through their receptors Ephs (Klein 2012). There are two types of ephrins: class A and class B ephrins. Class A ephrins are membrane anchored through a glycosylphophatidylinositol (GPI) linkage and bind to Eph A receptors, meanwhile class B ephrins contain a transmembrane domain and bind to Eph B receptors. It is notable that the ephrin signaling can be rather versatile, as ephrins not only serve as repellent cues regulating axon fasciculation and topographic map formation in the visual system but also mediate attractive signaling along the dorso-ventral axis (Wilkinson 2001; Hindges, McLaughlin et al. 2002; Mann, Peuckert et al. 2002). Moreover, ephrin/Eph

signaling can occur in the reverse direction, that is, from Eph to ephrin (Holland, Gale et al. 1996).

1.4.2 Semaphorin Family

The Semaphorins are the largest family of classic axon guidance cues consisting of more than 25 distinct genes characterized in diverse animal species. They are secreted transmembrane proteins characterized by a conserved 500-amino-acid Semaphorin (Sema) domain (Figure 1.10) (Kolodkin, Matthes et al. 1993; Kolodkin 1996; Tessier-Lavigne and Goodman 1996). Besides the Sema domain, individual Semaphorin may contain additional sequence motifs. Based on their structural homology and amino acid sequence similarity, Semaphorins are grouped into eight classes. Class 1 and 2 (and a single class 5) Semaphorins are found in invertebrates, class 3 to 7 Semaphorins are in vertebrate and class V is encoded by viruses (Figure 1.10) (Dickson 2002; de Wit and Verhaagen 2003). The expression of Semaphorins was best described in the nervous system, but they are also present in most, or perhaps all, other tissues (Tran, Kolodkin et al. 2007). The principle receptors of Semaphorins are Plexins and neuropilins (Roth, Koncina et al. 2009; Pasterkamp 2012; Giacobini and Prevot 2013). In certain circumstances, Semaphorins themselves can serve as receptors (Cafferty, Yu et al. 2006; Sweeney, Chou et al. 2011). Although Semaphorins are implicated mainly as axonal repellents, some of them are known to be attractive or bifunctional (Wong, Yu et al. 1997; Bagnard, Lohrum et al. 1998; Wong, Wong et al. 1999; Polleux, Morrow et al. 2000; Fenstermaker, Chen et al. 2004; Kantor, Chivatakarn et al. 2004).

Figure 1.10 Primary structures of members of the Semaphorin family.
All proteins are shown with their amino termini to the top. Class 1 Semaphorins are invertebrate transmembrane proteins and are structurally very similar to the class 6 Semaphorins of vertebrates. Class 2 Semaphorins (also from invertebrates) are secreted; they are structurally similar to vertebrate class 3 Semaphorins, which have a stretch of highly basic amino acids in their carboxy-terminal region. Class 4, 6, and 7 Semaphorins have been identified only in vertebrates. Class 4-6 Semaphorins are transmembrane proteins. Class 5 Semaphorins are present in both vertebrates (Sema5A, Sema5B) and invertebrates (Sema5c) and contain seven canonical type 1 thrombospondin repeats (TSRs). Class 6 Semaphorins contain variable, alternatively spliced cytoplasmic portions. The lone class 7 Sema (Sema7A) contains a membrane-associated GPI moiety at its carboxy terminus. Class V Semaphorins are highly similar to class 7 Semaphorins and are found in DNA viruses, including vaccinia (a close relative to the cowpox virus), human smallpox (variola virus), fowlpox, mousepox (ectromelia virus), and alcelaphine herpesvirus type 1 virus (AHV). Some class V Semaphorins (the SemaVA proteins) do not contain an Ig domain, whereas others do (SemaVB proteins). Sema, Semaphorin; PSI, plexin-Semaphorin-integrin; Ig, immunoglobulin-like; GPI, glycosylphosphatidylinositol (Yazdani and Terman, 2006).



Over the last decade it has been found that Semaphorins not only guide axonal extension, but also play significant roles in many different aspects of neural circuit development (de Wit and Verhaagen 2003; Mann, Chauvet et al. 2007). Dysfunctions of Semaphorins have been

linked to several neurodevelopemtnal disorders such as epilepsy, retinal degeneration, Alzheimer's disease, motor neuron degeneration, schizophrenia, and Parkinson's disease (Adams and Tucker 2000; de Wit and Verhaagen 2003; Pasterkamp and Kolodkin 2003; Rice, Huang et al. 2004).

1.4.2.1 Axon Pathfinding

As mentioned above, the majority of Semaphorins have been indentified as repulsive cues to axon growth and extension. Among them, the secreted class 3 Semaphorins are certainly the most studied. In chicken DRG culture, addition of Sema3A induces a dramatic growth cone collapse and retraction through the redistribution and disassembly of F-actin (Fan, Mansfield et al. 1993; Luo, Raible et al. 1993). Other neuronal cells such as sensory, sympathetic and hippocampal neurons have also been reported to respond to repulsive signals of secreted class 3 Semaphorins (Koppel, Feiner et al. 1997; Varela-Echavarria, Tucker et al. 1997; Chedotal, Del Rio et al. 1998; Raper 2000). However, Semaphorins can also permissively influence axon outgrowth. This is the case when Sema3C and Sema3F promote the growth of cortical axons and olfactory bulb axons respectively (Bagnard, Lohrum et al. 1998; Takahashi, Nakamura et al. 1998; de Castro, Hu et al. 1999). Subsequent studies showed that the determination of attraction and repulsive functions of Semaphorins relies on the temporal regulation of Semaphorins and differential expression of their receptors (Renzi, Wexler et al. 2000; Wolman, Liu et al. 2004; Huber, Kania et al. 2005).

1.4.2.2 Synaptic Development and Plasticity

Early evidence that invertebrate Semaphorins control synaptic development came from work on the Drosophila giant fiber, which showed that giant fiber axons in Sema1A loss-of-

function mutants were either mistargeted or reached their target interneurons without forming synapses (Godenschwege, Hu et al. 2002).

In the cerebral cortex and hippocampus, Sema3F constrains the spine size and density on apical dendrites but not basal dendrites (Tran, Rubio et al. 2009). Bath application of Sema3F increases synaptic transmission in both CA1 pyramidal and dentate granule neurons through a postsynaptic mechanism (Sahay et al., 2005). On the other hand, Sema3A mediates synapse maintenance in cerebellum (Uesaka, Uchigashima et al. 2014). Consistently, acute application of soluble Sema3A induces rapid synaptic depression in CA1 hippocampal neurons (Bouzioukh, Daoudal et al. 2006). In addition, mRNA levels of some Semaphorins and their receptors are regulated by experience and activity (Shimakawa, Suzuki et al. 2002; Barnes, Puranam et al. 2003; Holtmaat, Gorter et al. 2003; O'Donnell, Stemmelin et al. 2003), suggesting the interesting possibility that regulation of these Semaphorins may play a role in synapse plasticity.

Several class 4 Semaphorins have been shown to interact with PSD-95 and induce the formation of selective subsets of synapses *in vitro* (Inagaki, Ohoka et al. 2001; Schultze, Eulenburg et al. 2001; Burkhardt, Muller et al. 2005). Specifically, in cultured hippocampal neurons knockdown of Sema4B reduces both excitatory and inhibitory synapse density, whereas knockdown of Sema4D selectively decreases inhibitory synapse density (Paradis, Harrar et al. 2007).

Class 5 Semaphorins also appear to regulate synaptic development. Studies on hippocampal cultures demonstrated that Sema5A inhibits synaptogenesis while Sema5B promotes synapse elimination (O'Connor, Cockburn et al. 2009; Duan, Wang et al. 2014).

Taken together, these studies suggest that although initially discovered as chemorepellents in axon guidance, Semaphorins also function in the formation, maintenance and elimination of synaptic connections in invertebrates and vertebrates.

1.4.3 Receptors and Signaling of Semaphorin Family

The principal receptors for Semaphorins are Plexins, which are all large transmembrane proteins that are divided into four classes (Plexins A1-A4, B1-B3, C1, D1), in addition to the two Plexins (Plexin A, Plexin B) found in invertebrate species (Fujisawa 2004; Hota and Buck 2012). For example, Sema4D bind to Plexin B2 to mediate axon outgrowth (Masuda, Furuyama et al. 2004), whereas Sema6D binds to Plexin A1 to inhibit endocardial cell migration (Kimura, Taniguchi et al. 2007). All Plexins contain a Sema domain for ligand binding, and a GTPase-activating protein (GAP) important domain for downstream signaling. Most Semaphorins bind directly to Plexins, while class 3 Semaphorins require neuropilins as co-receptors to carry out their functions (Huber, Kolodkin et al. 2003; Fujisawa 2004). In vertebrates, there are two neuropilins: neuropilin 1 and neuropilin 2. Although the neuropilins alone are not sufficient to stimulate signaling downstream of class 3 Semaphorins (Chen, Chedotal et al. 1997; Takahashi, Nakamura et al. 1998), they are essential ligand binding components for Semaphorin signaling in diverse biological systems (Koncina, Roth et al. 2007; Roth, Koncina et al. 2009).

The signaling pathways that act downstream of Semaphorin/Plexin have been well investigated within the context of axon guidance and cell migration. A number of key signaling events have been identified, including Plexin GAP domain activation, actin cytoskeleton remodeling, and the control of cell adhesion (Yazdani and Terman 2006).

The activation of Plexin GAP domain activity upon Semaphorin binding is one of the initial intracellular molecular events that occur in all Semaphorin/Plexin pathways (Tran, Kolodkin et al. 2007; Zhou, Gunput et al. 2008; Cagnoni and Tamagnone 2014). This domain regulates the activity of small GTPases such as Rho, Rac, and Cdc42, which provide a critical link between Semaphorin/Plexin complexes and the actin cytoskeleton (Huber, Kolodkin et al. 2003; Kolodkin and Tessier-Lavigne 2011). Besides ligand binding, the ability of Plexins to activate small GTPases is also affected by intrinsic RhoGTPase like Rnd1, which is necessary for Semaphorin functions in cell adhesion and neurite outgrowth (Negishi, Oinuma et al. 2005; Pascoe, Wang et al. 2015).

The major role of small GTPases in Semaphorin signaling is to regulate the organization and remodeling of the actin cytoskeleton at several points, including filament nucleation and branching (Arp2/3 complex), filament extension (capping protein), retrograde flow (myosin) and actin recycling (cofilin) (Dickson 2001; Giniger 2002). For example, the growth cone collapse induced by Sema3A in mouse DRG neurons is correlated with a rapid increase in the phosphorylation state of cofilin, an actin binding protein which destabilizes actin filaments when phosphorylated (Aizawa, Wakatsuki et al. 2001); MICAL, a redox enzyme that binds to the cytosolic part of Plexin A in Drosophila, mediates Sema1A/Plexin A-induced repulsive axon guidance through actin disassembly (Hung, Yazdani et al. 2010; Zhou, Gunput et al. 2011)

Semaphorin/Plexin signaling also controls cell adhesion, mostly through the interaction with integrin. Multiple Plexins have been reported to decrease cell adhesion by inactivating Ras GTPases to antagonize integrin-mediated adhesion (Kinbara, Goldfinger et al. 2003; Negishi, Oinuma et al. 2005; Oinuma, Katoh et al. 2006). In addition, stimulation with Semaphorins

can increase integrin-dependent adhesion via classical adhesion-associated signaling proteins such as focal adhesion kinases and MAP kinase (Pasterkamp, Peschon et al. 2003; Choi, Duke-Cohan et al. 2014).

1.4.4 Class 5 Semaphorins

Among all the Semaphorins, two vertebrate class 5 Semaphorins, Sema5A and Sema5B are particularly unique in that their ectodomains contain seven type 1 and type 1-like TSRs, which have been characterized to be permissive for attachment to extracellular matrix substrates and axon growth (Figure 1.10) (Krutzsch, Choe et al. 1999; Adams and Tucker 2000; Li, Ivanoff et al. 2002). Sema5A and Sema5B proteins share 72% homology and 58% identity in amino acid sequence. From N- to C-terminus, they both have a Sema domain, a Plexin-Semaphorin-integrin (PSI) domain, TSRs, a transmembrane domain and a short cytoplasmic tail (Figure 1.10) (Adams, Betz et al. 1996).

Sema5A has been found in various organs of various model organisms, including muscle, heart, lung, spleen, and brain. In the nervous system, the first report of Sema5A expression is in embryonic day 14-15 mouse brains (Adams, Betz et al. 1996). After that, further examination of Sema5A distribution showed that Sema5A is also expressed in the optic nerve, hippocampus and telencephalon (Oster, Bodeker et al. 2003; Medina, Legaz et al. 2004; Pineda, Garcia et al. 2005). On the other hand, in the brain Sema5B is mainly expressed in the telencephalon, as well as cortex and amygdala (Skaliora, Singer et al. 1998; Lett, Wang et al. 2009). Much less is known about the expression of Sema5A and Sema5B in glial cells, however it has clearly been shown by RT-PCR that oligodendrocyte and

oligodendrocyte precursor cells (OPCs) express both Sema5A and Sema5B while astrocytes do not.

1.4.4.1 Functions of Class 5 Semaphorins

Both Sema5A and Sema5B were initially identified as repulsive axon guidance cues for their abilities to retract and collapse growth cones (Goldberg, Vargas et al. 2004; To, Church et al. 2007; Lett, Wang et al. 2009; Liu, Wang et al. 2014). However, multiple studies have demonstrated that Sema5A can exert both attractive and inhibitory effects on developing axons of the fasciculus retroflexus, through the interactions with heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs) (Kantor, Chivatakarn et al. 2004). In non-neuronal cells, Sema5A is also involved in tumor cell migration and vasculature patterning (Fiore, Rahim et al. 2005; Li and Lee 2010; Sadanandam, Varney et al. 2010; Li, Law et al. 2012).

The majority of work on Sema5B has also been focused on its function in axon guidance (To, Church et al. 2007; Lett, Wang et al. 2009; Liu, Wang et al. 2014). However, it has also been demonstrated that Sema5B can mediated synapse elimination in hippocampal cultures (O'Connor, Cockburn et al. 2009). In addition, Sema5A and Sema5B constrain retinal neurite outgrowth of retinal neurons both *in vitro* and *in vivo* (Matsuoka, Chivatakarn et al. 2011).

1.4.4.2 Receptors and Binding Partners of Class 5 Semaphorins

Multiple studies have resulted in inconsistent conclusions regarding Sema5A's receptor(s). In non-neuronal cells, it was found that Sema5A activates Plexin B3 to regulate the collapse of NIH3T3 cells, the motility of glioma cells and the migration of endothelial cells (Artigiani,

Conrotto et al. 2004; Li and Lee 2010; Li, Law et al. 2012). During the development of fasciculus retroflexus, Sema5A interacts with CSPGs and HSPGs to exert bifunctional effects on growing axons by (Kantor, Chivatakarn et al. 2004). A more recent study demonstrated that Sema5A binds to PleixnA1 and Plexin A2 with high affinity *in vitro* and that the function of Sema5A to inhibit synaptogenesis of dentate granule cells is mediated through Plexin A2 *in vivo* (Duan, Wang et al. 2014). The inconsistency of Sema5A receptors may be resulted from the different cell types used in these studies, and reflect the differential regulation of Sema5A effects contributed by various Plexins.

As to Sema5B, it was demonstrated that Sema5A and Sema5B together inhibited neurite outgrowth through Plexin A1 and Plexin A3 in rodent retinal neurons (Matsuoka, Chivatakarn et al. 2011), while the receptor only for Sema5B has not been claimed yet. However, previously we have shown that Sema5B functions in part through TAG-1, an immunoglobulin superfamily cell adhesion molecule (IgSF-CAM), to inhibit sensory axon growth in the ventral spinal cord, implicating TAG-1 as a component of the Sema5B/Plexin complex (Liu, Wang et al. 2014)

1.4.5 Sema5A and Autism

Autism is a broadly defined behavioral disorder characterized by impaired social interactions, repetitive behaviors and communication deficits, and collectively referred to “autism spectrum disorders” (ASDs) (Klauck 2006; Miles 2011; Lai, Lombardo et al. 2014). Although highly heritable, its genetic basis is heterogeneous involving a large number of susceptibility genes (Maestrini, Paul et al. 2000). Accumulative literatures has highlighted the importance of synaptic proteins such as neuroligin, shank3 and CNTNAP2 in the

pathology of autism, raising the possibility that impaired neural circuitry caused by dysfunction of synaptic proteins contributes to ASDs (Peca, Feliciano et al. 2011; Penagarikano, Abrahams et al. 2011; Tsai, Wilkerson et al. 2012; Uchino and Waga 2013; Rothwell, Fuccillo et al. 2014; Duffney, Zhong et al. 2015). Consistently, emerging evidences of surplus synaptic connectivity in autistic brains or mice models suggest that the regulation of synapse formation and elimination may be a key factor in the disorder (Hutsler and Zhang 2010; Tang, Gudsnuk et al. 2014). In 2009, a genome wide association study found that Sema5A is genetically linked to ASDs (Weiss, Arking et al. 2009). After mapping over 500,000 single nucleotide polymorphisms (SNP) on more than one thousand families, one SNP that was significantly correlated with ASDs was identified to be upstream of the Sema5A gene. Significantly, the expression of Sema5A is reduced in the brains of autistic patients (Melin, Carlsson et al. 2006; Weiss, Arking et al. 2009). Therefore, understanding how Sema5A impacts the connectivity of developing circuits may shed light on the molecular basis and potential pathological mechanisms underlying ASDs.

1.5 Overall Objective and Hypothesis

Though the Semaphorin family was initially identified as molecules important for directing outgrowth of axons, they also play numerous key roles in the proper development and function of the CNS (de Wit and Verhaagen 2003; Pasterkamp 2012). Disruptions in the function of Semaphorins or their downstream signaling components have been implicated in various psychiatric and neurodegenerative disorders such as epilepsy and schizophrenia. Compared with other Semaphorins, we are still far from having a coherent understanding of Sema5A. In a genome wide association study, Sema5A has been found genetically linked to

ASDs and Sema5A expression level is decreased in autistic brains (Melin, Carlsson et al. 2006; Weiss, Arking et al. 2009; Mosca-Boidron, Gueneau et al. 2016). Since ASD is a neurodevelopmental disease caused by abnormal neural connectivity, the overall objective of this thesis is to understand the function of Sema5A in synaptic formation and development. Since the hippocampus is pivotal in memory encoding and modulating memory consolidation, any impairment in the hippocampus can remarkably affect an individual's ability to process and retain memories. Indeed, it has been postulated that the impaired neural activities in hippocampal regions might have be relevant to autism (DeLong 1992). Therefore in this dissertation, I will mostly use hippocampal neurons as a model to study the functions of Sema5A and Sema5B in the CNS.

Synapses are highly plastic structures as the morphology and strength of synapses are dramatically changing in response to neural activity. Synapse formation and elimination through activity-dependent processes are critical for the refinement of neuronal circuits during development. Thus, it is important to examine whether Sema5A and Sema5B are associated with activity-dependent synaptic plasticity.

My hypotheses are that 1) Sema5A and Sema5B negatively regulate dendritic morphology and synapse elimination during development, and 2) Sema5A and Sema5B suppress the activity-dependent increase in synapse density underlying synaptic plasticity. In chapter 2, I investigated the roles of Sema5A and Sema5B in shaping dendritic morphology as well as synapse density under basal conditions and in response to changes in activity (LTP and LTD). In chapter 3, I identified the receptor(s) responsible for Sema5A function in hippocampal neurons by manipulating the expression or activity of candidate molecules together with exogenous Sema5A treatment or Sema5A overexpression. Furthermore, I

examined the potential signaling pathway by which Sema5A activity translates to changes in synaptic density.

Chapter 2: Class 5 Semaphorins Mediates Dendritic Morphology and Activity-Dependent Synaptic Elimination

2.1 Introduction

One of the remarkable features of the mammalian nervous system is the ability to modify synaptic strength in an experience-dependent manner. It has been postulated for decades that the brain uses long-lasting modifications of synaptic strength to accomplish this function. Two main types of such activity-dependent modifications are LTP, a sustained increase in synaptic strength resulting from synchronous stimulation, and its opposite form, LTD. It is widely accepted that hippocampal neurons are capable of undergoing bidirectional synaptic modifications, such as synapse formation and elimination in response to changes in activity (Dudek and Bear 1993; Huerta and Lisman 1995; Bear 2003). However, the molecular mechanisms underlying the synapse assembly and disassembly in response to neural activity has not been well described.

Semaphorins, characterized by a conserved, cysteine-rich domain called the Sema domain, comprise the largest family of axon guidance cues and includes more than 25 distinct genes characterized in diverse animal species (Kolodkin, Matthes et al. 1993; Kolodkin 1996; Tessier-Lavigne and Goodman 1996; Bagnard, Lohrum et al. 1998; Miyazaki, Furuyama et al. 1999). Though originally identified as cues to guide the axon pathfinding, it is increasing clear that Semaphorins are persistently expressed after the stage of axon guidance and play roles in multiple neural processes including synapse formation and function (de Wit and Verhaagen 2003; Mann, Chauvet et al. 2007; Tran, Kolodkin et al. 2007).

Among all the Semaphorins encoded by mammalian genes, two class 5 Semaphorins, Sema5A and Sema5B, are particularly unique for their TSR domains. Though first considered as an inhibitory axon guidance cue based on its ability to repel and collapse growth cones (Oster, Bodeker et al. 2003), Sema5A has been shown to act bi-functionally to exert both attractive and inhibitory effects on developing axons of the fasciculus retroflexus (Kantor, Chivatakarn et al. 2004). Previously Sema5B has been shown to reduce synapse size and eliminate synapse in hippocampal neurons (O'Connor, Cockburn et al. 2009). Given the homology between Sema5A and Sema5B, it is highly likely that Sema5A may also regulate synapse disassembly and therefore be critical in the refinement of synaptic connections.

In a recent genome wide association study, *Sema5A* has been identified as a susceptibility gene to autism, which is broadly defined behavioral disorder characterized by impaired social interactions, repetitive behaviors and communication deficits (Weiss, Arking et al. 2009). Moreover, the expression of Sema5A is reduced in the brains of autistic patients (Melin, Carlsson et al. 2006). Therefore, understanding how Sema5A impacts the connectivity of developing circuits may help to recover the genetic basis and molecular mechanisms underlying ASDs.

Here we show that two class 5 Semaphorins (Sema5A and Sema5B) negatively control dendritic growth and complexity, as well as synaptic density in rodent hippocampal neurons. Moreover, Sema5A and Sema5B mediate the changes in synapse density induced by LTP and LTD, implicating the role of Class 5 Semaphorins in activity-dependent synaptic plasticity. Finally, I show that Fc.5A treatment induced the withdrawal of surface GluA1 subunits without changing the total GluA1 level, which is possibly involved in the mechanism of Sema5A-mediated activity-dependent synaptic plasticity. Our studies provide

a novel role of class 5 Semaphorins in the refinement of synaptic connections in mammalian brains.

2.2 Materials and Methods

2.2.1 Recombinant DNA Constructs and Antibodies

Full-length rat or human Sema5A and mouse Sema5B were subcloned into the *Xma* I/*Sac* II sites of the haemagglutinin (HA) epitope tagged pDisplay expression vector (Invitrogen). The empty pEx.Fc and Sema5A-Fc constructs were kind gifts from Dr. David Sretavan (University of California, San Francisco). The PSD-95-red fluorescent protein (RFP) construct was a kind gift from Dr. David Bredt (Johnson & Johnson Co., San Diego, USA).

The dilution and sources of the primary antibodies used were: anti-PSD-95 (1:500, Abcam ab2723), anti-vGlut1 (1:750, Millipore AB5905), anti-gephrin (1:500, Synaptic Systems 147011), VGAT (1: 750, Synaptic Systems 131004), anti-haemagglutinin (HA) (1: 1,000, Cell Signaling Technology C29F4) and anti-γ-Tubulin (1: 10,000, Sigma T6557). The dilution and sources of the secondary antibodies used were: IgG-horseradish peroxidase (HRP; mouse 1: 1,000, BioRad 170-6516 and rabbit 1: 3,000, Jackson ImmunoResearch 711-035-152), Alexa-Fluor 568 goat anti-mouse (1: 500; Life Technologies A-11031), and Cy5 goat anti-guinea pig (1: 1,000, Abcam ab6567).

2.2.2 *In situ* Hybridisation

In situ hybridization was performed on brain cryostat sections according to standard methods. Briefly, brains of nine postnatal day 21 (P21) Sprague–Dawley male rats (Charles River Laboratories) were dissected and fixed with 4% (v/v) paraformaldehyde (PFA, Sigma)

at 4°C overnight followed by washing in PBS. The brains were then cryoprotected in 30% sucrose and embedded in Tissue-Tek (Sakura), and cut at 30 μm thickness with a cryostat. Digoxigenin-labeled riboprobe was transcribed from the coding sequences of Sema5A (3353–3860 bp) in PCR2.1 according to the manufacturer's recommendations (Roche). Sense probes generated with reverse sequence from the same region were used as a control. The tissue sections were treated with proteinase K at room temperature for 15 min and then re-fixed with 4% PFA to ensure the firm attachment of the sections to the microscope slides. Sections were pre-hybridized in hybridization buffer without DIG-labeled probe for 1 hr at 72°C and then incubated with probes (~ 2 g/ml) overnight at 72°C. Hybridization buffer is 50% formamide, 5X standard saline citrate, 1 mg/ml yeast tRNA, 1% SDS, 10% salmon sperm (Thermo Scientific). After hybridization, three washes were carried out for 15 min each at 72°C with buffer I (50% formamide, 5X SSC, 1% SDS) followed by three washes at 65°C with buffer III (50% formamide, 2X SSC). The results of section *in situ* hybridization were photographed on an upright microscope (Nikon TE 400) with a digital camera.

2.2.3 Cell Culture and Transfection

2.2.3.1 Primary Hippocampal Neurons

Cultures of hippocampal neurons were prepared from embryonic day 18 (E18) Sprague–Dawley rats (Charles River Laboratories) of either sex following a previously described protocol (Xie, Markesbery et al. 2000). Briefly, neurons were plated at a density of 200 cells per mm^2 on poly-L-lysine coated coverslips. Cultures were maintained in Neurobasal media with 10% fetal bovine serum (FBS, Invitrogen), 0.5 mM L-glutamate (Gibco), Penicillin/Streptomycin (50 U/ml; 50 μg/ml, Gibco), NeuroCult SM1 (StemCell, instead of

B27 supplement). Neurons were transfected with Lipofectamine 2000 (Invitrogen) at 9-10 days *in vitro* (DIV) according to the manufacturer's recommendations, and used for experiments at 12-14 DIV.

2.2.3.2 HEK Cells

HEK293 cells were cultured and transfected using polyethylenimine (PEI; Sigma) as previously described (Browne, Wang et al. 2012), in a 3:1 ratio with the total vector DNA to be transfected per condition. HEK293 cells were incubated for 48 h before being used in an experiment. Stable cell lines expressing HA-Sema5A or HA-Sema5B were selected and maintained with 500 μg/ml G418 (Gibco) in an incubator at 37°C with 5% CO_2.

2.2.4 Preparation and Validation of shRNA Vectors

Sequences for RNAi targeting were analysed using pSico Oligomaker v1.5 software (Jacks Lab, Massachusetts Institute of Technology, USA) and the oligo duplex palindromes designed for hairpin loop formation were generated by Integrated DNA Technologies (IDT). The Sema5A shRNA sequence was generated to target sequences: 5′-CACCCCGTCGTCTCCTACA. Annealed oligo duplex palindromes were cloned into the *Xho* I/*Hpa* I restriction sites of the pLentilox 3.7 (pLL3.7) expression vector, which contains an enhanced green fluorescent protein (EGFP) sequence driven by a CMV promoter. The shRNA vectors targeting Sema5B was generated and validated as previously described (O'Connor, Cockburn et al. 2009). The specificity of the Sema5A shRNA was verified for its ability to knockdown rat Sema5A expression and the lack of knockdown effect on human Sema5A or mouse Sema5B expression.

2.2.5 Western Blot Analysis

HEK293 cells were rinsed in cold PBS, and homogenized in ice-cold Radio immuno-precipitation assay buffer (RIPA buffer, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl pH 8.0, 0.1% SDS, 150 mM NaCl) supplemented with a protease inhibitor cocktail (Roche). Cell lysates harvested by using plastic scrapers were sonicated using Branson Sonifier 250 and cleared by centrifugation for 30 min at 4 °C. Total protein concentration of the supernatant was measured using BCA protein assay (Pierce). Proteins were separated by SDS-poly-acrylamide gel electrophoresis (SDS-PAGE), analysed by immunoblotting with the indicated antibodies and visualized using enhanced chemiluminescence (ECL, Pierce) on a Versadoc 4000 (Bio-Rad) following standard protocol.

2.2.6 Immunocytochemistry

Rat hippocampal neuron cultures were fixed in pre-warmed 4% PFA/sucrose solution for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 15 min, followed by blocking with 10% goat serum in PBS for 1 h at room temperature. Following blocking, primary antibodies were applied to the cells in antibody solution (PBS, 1% goat serum) overnight at 4°C. The cells were then rinsed in PBS three times for 10 min each, followed by incubation with secondary antibodies diluted in antibody solution for 1 h at room temperature. Then the cells were rinsed again in PBS three times for 10 min each and mounted on microscope slides (Globe Scientific) in Prolong Gold (Molecular Probes).

2.2.7 Generation of Sema5A-Fc Protein and Bath Application

The method of generating Sema5A-Fc protein recombinant protein has been described before (Oster, Bodeker et al. 2003). Briefly, HEK293 stable cell lines expressing an IgG-Fc domain fused full-length soluble Sema5A protein (Fc-Sema5A) or an IgG-Fc domain alone (Fc) were generated. When stable, these cells were cultured in normal medium for three days, and then switched to serum-free medium. Three days after, cell culture supernatant was collected, spun down to pellet cell debris and concentrated through AMICON 50MWCO centrifugal filters (Millipore). Fc-Sema5A or Fc protein was isolated by Protein-A/G Dynabeads (Invitrogen), and protein concentrations and purity were determined by SDS-PAGE followed by Coomassie staining and comparison with bovine serum albumin (BSA) standards. For bath application, transfected neurons were incubated with 25nM Fc-5A or Fc protein diluted in culture media just prior to addition to the cells.

2.2.8 Neuronal Activation

Neuronal activity was modified using previously described chemical LTP (cLTP) or chemical LTD (cLTD) protocols (Lu, Man et al. 2001; Brigidi, Santyr et al. 2015). Briefly, maintenance media was replaced with an Mg^{2+}-free extracellular solution (140 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 25 mM HEPES, 33 mM D-glucose, pH 7.35, 320 $mosmol^{-1}$) and supplemented with 0.5 μM tetrodotoxin (TTX, Alamone Labs), 20 μM Bicuculline methiodide (Fluka BioChemika) for 20 min. To induce cLTP, extracellular solution was supplemented with 200 μM glycine for 3 min. To induce cLTD, the media was supplemented with 20 μM NMDA (Abcam) plus 10 μM glycine (Roche) for 3 min. Neurons were then

washed once with fresh extracellular solution (containing 0 mM $MgCl_2$ for LTP or 2 mM $MgCl_2$ for LTD) and continually maintained at 37 °C for the duration of activity stimulation.

2.2.9 Confocal Imaging

All the cells were imaged using an Olympus Fluoview 1000 inverted confocal microscope (× 60/1.4 Oil Plan-Apochromat). When imaging fixed cells, identical acquisition parameters were used for all cells across all separate cultures within an experiment. Approximately 10-20 images were acquired and analyzed for each condition within an experiment for at least three repeats. For time-lapse imaging, a baseline image of the transfected neurons was acquired before bath application. Acquisition parameters were set on this basis and maintained across bath application. The representative confocal images of PSD-95/vGlut1/gephrin/VGAT channels shown in the figures were subjected to a radius of 0.4-pixel Gaussian blur. The levels and contrast of representative confocal images might be moderately adjusted in Photoshop CS software (Adobe Systems, Inc.) using scientifically accepted procedures, and no information was obscured or eliminated from the original images (Rossner and Yamada 2004).

2.2.10 Biotinylation Assay

Biotinylation of all surface proteins was performed as previously described (Diering, Mills et al. 2011). Briefly, cells were rinsed in ice-cold PBS-CM solution (PBS containing 1 mM $MgCl_2$ and 0.1 mM CaCl2) and then incubated in PBS-CM supplemented with 0.5 mg/ml of Sulfo-NHS-SS-biotin (Thermo Scientific) for 30 min at 4 °C to label surface proteins. Neurons were then washed once with PBS-CM, and twice with PBS-CM containing 20 mM glycine for 7 min each at 4 °C to quench excessive biotinylation. Neurons were then lysed in

lysis buffer of PBS-CM containing 1mM Phenylmethanesulfonyl fluoride (PMSF, Sigma), 1% IGEPAL CA-630 detergent (Sigma) and protease inhibitor cocktail (Roche) on ice. The concentration of total protein was measured by BCA assay (Pierce). Equal amounts (10 μg) of cell lysates from each condition were then immobilized on Neutravidin agarose beads (Thermo Scientific) overnight at 4 °C, washed seven times in lysis buffer, and boiled at 80 °C in SDS sample buffer containing 100 mM dithiothreitol (Thermo Fisher) for 10 min. The level of GluA1 was detected by western blot using GluA1 antibody. Equal amounts of whole-cell lysates (10 μg) were boiled at 95 °C for 5 min and loaded as input control. γ-Tubulin was also labelled as loading control.

2.2.11 Image Analysis and Quantification

2.2.11.1 Analysis of Dendrite Morphology

Images of GFP-labeled neurons were acquired using a 20x objective. Dendritic branching point was determined manually. Dendritic length was measured by manual tracing dendritic shaft with pencil tool of ImageJ software. Sholl analysis was performed using the ImageJ Sholl Analysis Plugin (http://www. biology.ucsd.edu/labs/ghosh/software/). Background dendrites extending into the image view from neighboring neurons were manually deleted. The origin of the concentric radii was set at the midpoint of cell soma. Analysis parameters were as follows: starting radius, 1μm; ending radius, 320 μm; radius step size, 2 μm.

2.2.11.2 Spine Density Analysis

Dendritic spine density was determined by manually tracing GFP-labeled protrusions emanating from the dendritic shaft. Spines were defined as any protrusion between 0.5μm to

5 μm in length and with a length: width ratio smaller than 4. To calculate spine density, dendritic spine numbers were divided by total dendritic length as described above.

2.2.11.3 Synapse Density Analysis

To focus our analysis, we only assayed synapse density on transfected neurons. For all experiments neurons were co-transfected with GFP and a mask was generated in Photoshop CS that outlined the GFP fluorescence in spines and dendritic shafts. The cell soma and background neurites were removed from the GFP channel, and then the GFP channel overlaid onto vGlut1 and PSD-95 channels in excitatory synapse density analysis, or VGAT and gephyrin channels in inhibitory synapse density analysis (as shown in Fig 2.5B). Confocal images for a particular experiment were subjectively thresholded using ImageJ software and the same threshold was used across all images obtained in one single experiment throughout the experimental analysis.

2.2.11.4 Analysis for Time-Lapse Imaging Experiments

A mask of the GFP channel without the cell soma was generated by Photoshop CS to outline the area of analysis. A threshold was determined on the baseline image and applied to all the image acquired at each time point of the treatment.

Puncta were defined as a thresholded fluorescence cluster with an area between 0.05 and 3 μm^2. Puncta area and IntDen (the product of area and mean grey value) were determined by ImageJ. An Image J co-localization plugin was used to calculate points of co-localization between different channels (http://rsb.info.nih.gov/ij/plugins/colocalization.html). Synapse density was defined as overlapping areas greater than 4 pixels in size between vGlut1 and

PSD-95 channels (or VGAT and gephyrin channels), when intensity ratio of the two channels was greater than 50%.

2.2.12 Statistical Analysis

All measurements are presented as mean ± SEM. For all imaging experiments, 'N' refers to the number of cells used per condition, over at least three separate cultures, with the exception of the analysis performed in time-lapse imaging, where 'N' refers to the total number of imaged live neurons per condition and is specified in the figure legends. Data collection and analysis were not performed blind to the conditions of the experiment and were not acquired with any specific randomization procedure. However, cells were assigned to experimental groups and analysis was performed with absolutely no bias, and by different experimenters. All data were analysed in Prism software (GraphPad Software, Inc.). Statistical significance was determined by Student's t-test, one-way analysis of variance or repeated-measures one-way analysis of variance with post-hoc tests as indicated in figure legends. All figures were generated using Illustrator CS software (Adobe Systems, Inc.).

2.3 Results

2.3.1 Class 5 Semaphorins Regulate Dendritic Morphology

Previous studies have shown robust expression of Sema5A and Sema5B in both developing and adult rodent hippocampus (Adams, Betz et al. 1996; O'Connor, Cockburn et al. 2009; Duan, Wang et al. 2014). We first examined the expression pattern of Sema5A in juvenile rat brains. Using *in situ* hybridization, strong expression of Sema5A was observed in the CA1-CA3 pyramidal cell layer and dentate gyrus, which is essentially identical to that of Sema5B

mRNA (Figure 2.1) (Duan, Wang et al. 2014). The sense RNA probes gave no significant hybridization signals.

Next we asked whether manipulating expression levels of Sema5A and Sema5B impacts dendritic morphological development of hippocampal neurons *in vitro*. Dissociated hippocampal neurons at DIV10 were transfected with a GFP vector to visualize neurite morphology, together with the pDisplay vector as a control or full length Sema5A and/or Sema5B. Only healthy neurons were selected and subjected to imaging and analysis, as defined by the absence of dendritic beading, intact preservation of their membranes, and a full, uninterrupted dendritic arbor. The dendritic morphology was analyzed by assessing total dendritic length, dendritic branching points and dendritic complexity. Sema5A or Sema5B overexpression significantly decreased total dendritic length and total number of branching points (Figure 2.2B, C). Double overexpression of Sema5A and Sema5B did not produce further decrease compared to single overexpression (Figure 2.2B, C). Moreover, Sholl analysis showed that there was a specific reduction in the number of dendritic intersections at middle arbor regions (40-240 μm) in Sema5A and Sema5B overexpressing neurons, indicating lower dendritic complexity (Figure 2.2D). Again, compared to overexpression of Sema5A or Sema5B, there was no further decrease of intersection numbers in neurons with Sema5A/Sema5B double overexpression.

2.3.2 Class 5 Semaphorins Regulate Synapse Elimination

Given the results that indicated that Sema5A and Sema5B interferes with proper dendritic arbor formation, I next considered whether Sema5A and Sema5B also regulate dendritic spine density. Overexpression of Sema5A and/or Sema5B induced a spine loss in transfected

neurons, as shown by a significant decrease in spine density (Figure 2.3 A, B), whereas no significant difference was observed in the spine head width between control neurons and Sema5A and/or Sema5B overexpressing neurons (Figure 2.3C).

Sema5B has previously been demonstrated to mediate the maintenance and elimination of synapses in hippocampal neurons (O'Connor, Cockburn et al. 2009). Therefore, I asked whether Sema5A had a similar function as Sema5B. To investigate the role of Sema5A at synapses, three constructs encoding short hairpin RNA (shRNA) targeting rat Sema5A gene were generated. The backbone vector (pLentilox 3.7, pLL3.7) contained an enhanced GFP sequence driven by a CMV promoter, which allowed me to positively identify transfected cells. The region of the Sema5A gene where these shRNAs were targeted is shown in Figure 2.4A. In order to validate the ability of these shRNA vectors to knockdown Sema5A, they were transiently transfected into HEK293 cell cultures that stably express HA-tagged rat Sema5A or mouse Sema5B. Introduction of the shRNA vectors against Sema5A resulted in a substantial reduction of Sema5A expression without affecting Sema5B expression in the HEK293 cells, as tested by both western blot analysis and immunostaining (Figure 2.4B, C, results obtained with the single shRNA vector with best knockdown effects are shown). The shRNA vector against Sema5B which was described previously (O'Connor, Cockburn et al. 2009), was also transfected alone or together with Sema5A shRNA vector to knockdown Sema5B.

To determine the function of Sema5A on hippocampal synapses, we performed both loss of function and gain of function experiments. Hippocampal neurons were co-transfected with a GFP vector in combination with the indicated constructs at DIV10, and subjected to immunostaining for the pre- and postsynaptic markers vesicular glutamate transporter 1

(vGlut1) and postsynaptic density protein 95 (PSD-95) at DIV13 (Figure 2.5A). A mask made of the GFP channel without the cell soma and background neurites was overlaid onto the vGlut1 and PSD-95 channels. Excitatory synapses were identified by the colocalized clusters of the vGlut1 and PSD-95 puncta within the mask (Figure 2.5B). I found significantly lower synapse density in the neurons expressing Sema5A and/or Sema5B relative to the control neurons (Figure 2.6A, C), whereas neurons with double overexpression of Sema5A and Sema5B did not show a further decrease of the synapse density (Figure 2.6A, C). In contrast, Sema5A or Sema5B knockdown led to significantly increased synapse density, indicating that Sema5A functions as a negative regulator of synapse in hippocampal neurons, just like Sema5B has been previously shown (Figure 2.6B, D). Again, no further increase of synapse density was found in neurons with Sema5A/Sema5B double knockdown (Figure 2.6B, D).

Based on the observation that Sema5A overexpression resulted in the reduction in synapse density, I investigated whether the soluble Sema5A protein would have a similar effect on synapses density. To answer this, I bath applied various concentration of soluble Fc-Sema5A protein (Fc-5A is the ectodomain of mouse Sema5A conjugated to the Fc region of human IgG) in cultured hippocampal neurons. Within two hours, Fc-5A treatment resulted in a concentration-dependent reduction of synapse density without impacting gross neurite morphology (Figure 2.6E), which is consistent with observations of soluble Sema5B protein (Figure 2.6F).

To determine if Sema5A and Sema5B also affect the density of inhibitory synapses, neurons were transfected with Sema5A and/or Sema5B and subjected to immunochemistry. Inhibitory synapses were defined by colocalized clusters of the pre- and postsynaptic markers

vesicular GABA transporter (VGAT) and gephyrin. I found that in neurons overexpressing Sema5A or Sema5B, similar levels of inhibitory synapse density were observed as control neurons, therefore the manipulation of Sema5A and/or Sema5B expression level in hippocampal neuron cultures influences only excitatory synaptic density but had no effect on inhibitory synapses (Figure 2.7).

Although it is evident that Sema5A negatively regulates synaptic density, it remains unclear whether the net loss of synapse number is due to the elimination of present synapses or the suppression of synaptogenesis. To address this question, I examined whether the number of PSD-95 puncta, which are reliably associated with synapses, changes after Sema5A addition. Neurons expressing GFP and PSD-95-RFP to visualize the postsynaptic densities were treated with 25nM Fc-5A and subsequently live-imaged every 10 minutes for two hours. Fc-5A treatment resulted in a reduction of PSD-95 puncta as early as 20min post bath application of Fc-5A, and at the end of two hours I observed a 40% decrease of the PSD-95-FRP puncta density and a 50% decrease of the PSD-95-FRP puncta integrated density, indicating a quick and significant loss of synapses (Figure 2.8). In contrast, in neurons treated with just the Fc fragment, there were only minimal fluctuations of the PSD-95-RFP puncta density or integrated density over time (Figure 2.8). Therefore, our findings support the conclusion that Sema5A acts as a synapse eliminator rather than a repressor of synaptogenesis.

2.3.3 Class 5 Semaphorins Are Involved in Activity-Dependent Synaptic Plasticity

It has been well established that once synapses of hippocampal neurons are formed during development, they are subsequently refined in an activity-dependent manner. Indeed,

activity-dependent synaptic refinement is essential for maintenance and elimination of synaptic connections in brain development (Grutzendler, Kasthuri et al. 2002; Matsuzaki, Honkura et al. 2004; De Roo, Klauser et al. 2008). It is possible that activity-induced changes of synapse density may be correlated with alteration of expression of eliminating factors such as Sema5A and Sema5B. Using RT-PCR, my collaborators (Mathew Piva and Rachel Gomm, UBC) showed that mRNA levels of Sema5A and Sema5B were regulated by LTP and LTD (Appendices Figure 1). Furthermore, it was also found that Sema5A quickly inserted into the cell membrane shortly following LTD induction, confirming that synaptic activity regulates the expression and subcellular location of class 5 Semaphorins in young hippocampal cultures (Appendices Figure 2).

Because neural activity regulates Sema5A and Sema5B levels and distribution, and Sema5A and Sema5B regulate glutamatergic synapse density, I hypothesized that synaptic activity may regulate glutamatergic synapse density through Sema5A and Sema5B in the neuronal development.

To test this hypothesis, I acutely induced chemical LTP in neurons that expressed high levels of Sema5A and/or Sema5B. Similar to our previous results (Figure 2.6A, C), Sema5A and/or Sema5B overexpression resulted in a significant decrease in synapse density. In contrast, LTP induction significantly increased excitatory synaptic density, but this increase was abrogated by Sema5A overexpression (Figure 2.9). Neurons with Sema5B overexpression or Sema5A/Sema5B double overexpression also resulted in a loss of the increase in synapse density normally observed after LTP (Figure 2.9). Thus, Sema5A and Sema5B counteracted the synapse formation mediated by LTP, suggesting that Sema5A and Sema5B may be involved in activity-dependent synaptic refinement. However, it is possible that

Sema5A/Sema5B mediated synapse elimination and LTP-induced synapse formation are independent events that cancel each other.

To address this possibility, we asked whether they act independently when we induced LTP and reduced the expression of Sema5A/Sema5B. I predicted that if Sema5A/Sema5B expression affects synapse density independently from the mechanism of LTP, then I should observe a further increase of synapse density in neurons with a reduced expression of Sema5A/Sema5B after LTP induction. However, such further increase was not observed (Figure 2.10). Therefore, our data suggest that Sema5A/Sema5B shared some overlapping mechanisms with neural activity to influence synapse density, rather than affecting synapse density independently from neural activity-dependent synaptic plasticity.

To further examine the function of Sema5A/Sema5B in activity-mediated refinement, I tested whether they are critical factors that stimulate the synapse elimination associated with LTD. To test this, I manipulated the expression of Sema5A and/or Sema5B and induced chemical LTD in neurons. Induction of LTD in control neurons resulted in a significant decrease in synapse density within 5 hours after induction (Figure 2.11). Consistent with my hypothesis, in neurons where I knocked down the expression of Sema5A, the LTD-mediated synapse loss was partly reduced (Figure 2.11B). Furthermore, LTD-mediated decrease of synapse density was completely lost by Sema5A and Sema5B double knockdown, confirming that both of them are critical in mediating the synapse elimination associated with LTD (Figure 2.11C).

2.3.4 Fc-5A Treatment Decrease the Surface Level of GluA1 Subunit

While I have demonstrated that Sema5A can eliminate synapses in hippocampal neurons, it is unclear whether Sema5A may impact activity-dependent synaptic plasticity through other

mechanisms. To address this, I examined the levels of GluA1 subunit to determine whether Sema5A mediates activity-dependent synaptic plasticity affects the expression levels and distribution of AMPAR subunits. DIV13 hippocampal neurons were treated with 50 nM Fc-5A or control Fc for one hour and then all surface proteins were examined using a well characterized biotinylation assay (Figure 2.12). Biotinylated neurons were lysed and pulled down using streptavidin agarose. The changes in total and surface amount of AMPAR subunit GluA1 were analysed by western blotting. Quantification of the intensity of GluA1 bands revealed that there was no significant difference in the amount of total GluA1 expression between Fc-5A treated neurons and control neurons; however, the levels of biotinylated surface GluA1 subunits was significantly lower following Fc-5A bath application (Figure 2.12B). These results suggest that Sema5A can regulate the subcellular distribution of GluA1 in hippocampal neurons.

Figure 2.1 Sema5A is enriched in rodent hippocampus.
(A) Schematic diagram of the RNA probe used for detecting Sema5A mRNA.

(B) Representative images showing *in situ* hybridization analysis of Sema5A mRNA using sense (Left) or antisense (Right) probe in juvenile rat coronal sections. Strong Sema5A expression was detected in the hippocampus, including the dentate gyrus (DG), CA1 and CA3.

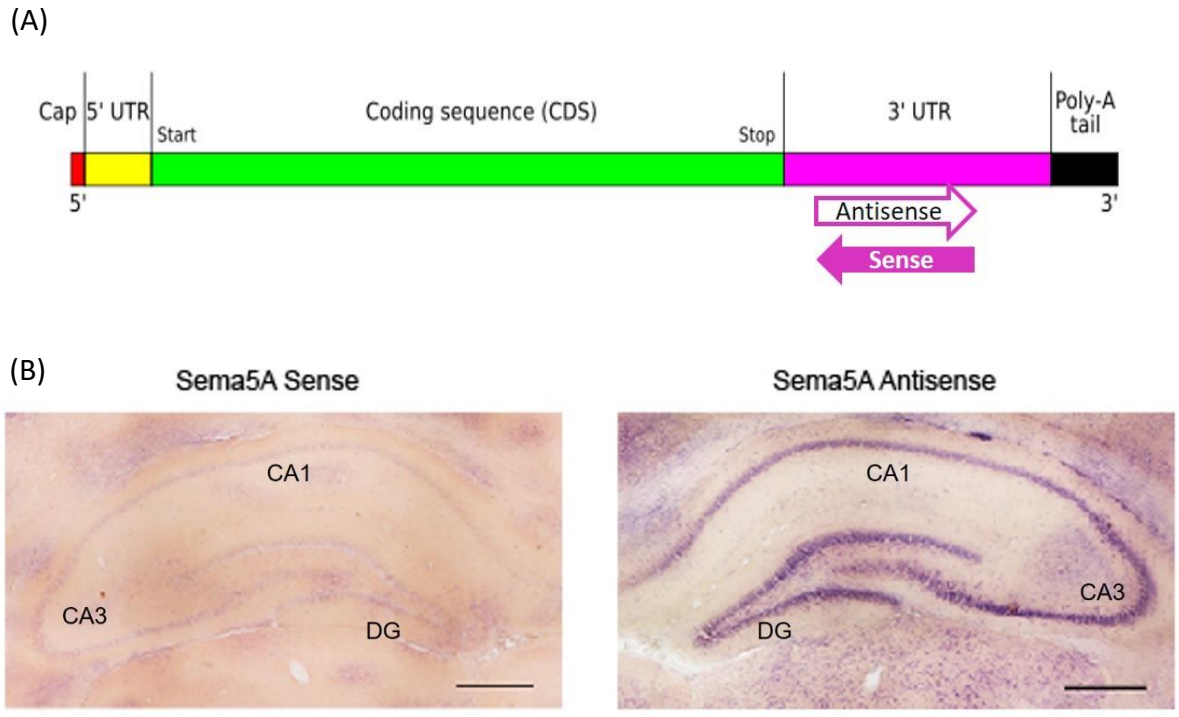


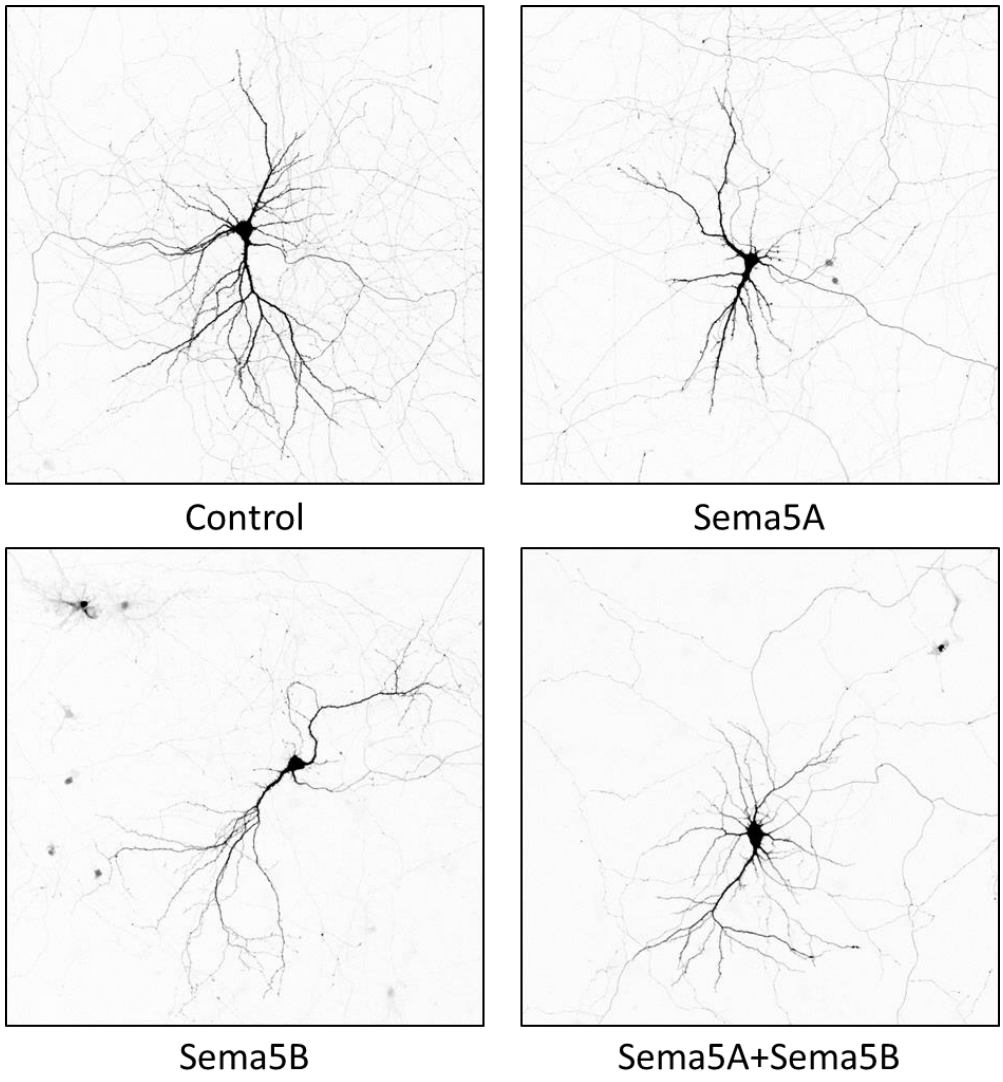
Figure 2.2 Altered expression levels of Sema5A and Sema5B negatively affect dendritic morphological development of hippocampal neurons *in vitro*.
(A) Representative confocal images of hippocampal neurons at DIV13 expressing GFP plus either empty pDisplay vector (Control) or indicated class 5 Semaphorin constructs.

(B, C) Sema5A and/or Sema5B overexpression significantly decreases total dendritic length in panel B and the number of dendritic branching points in panel C. **p < 0.01, ***p < 0.001, one-way ANOVA with Tukey's test post hoc.

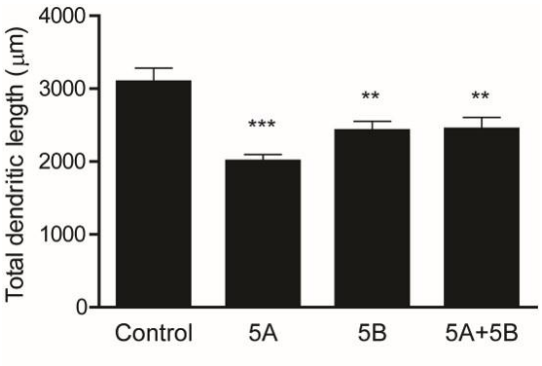
(D) Sholl analysis of hippocampal neurons demonstrates a decrease of dendritic intersections of the dendritic tree in neurons expressing Sema5A and/or Sema5B.

N=38-53 neurons per condition from ≥3 separate cultures.

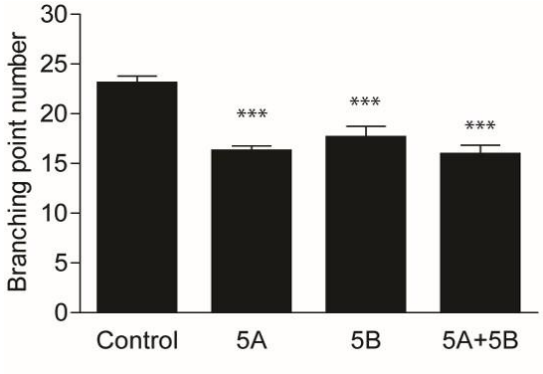
(A)



(B)


Total dendritic length (μm)
4000
3000
2000
1000
0
***
**
**
Control
5A
5B
5A+5B

(C)


Branching point number
30
25
20
15
10
5
0
***
***
***
Control
5A
5B
5A+5B

(D)

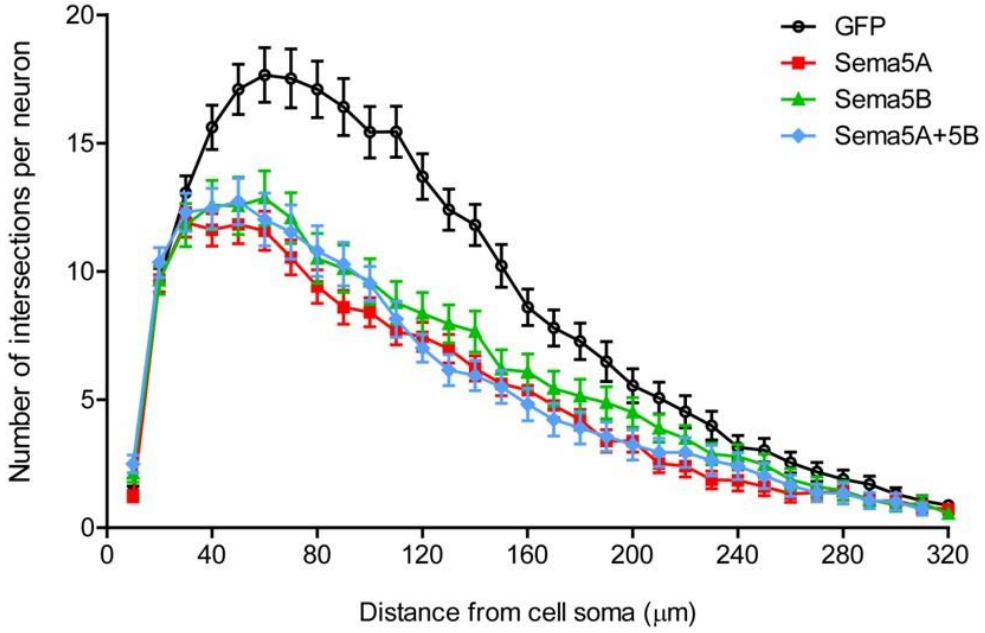

Number of intersections per neuron
20
15
10
5
0
0
40
80
120
160
200
240
280
320
Distance from cell soma (μm)
GFP
Sema5A
Sema5B
Sema5A+5B

Figure 2.3 Altered expression levels of Sema5A and Sema5B negatively affect dendritic spine density but not spine head width in hippocampal neurons *in vitro*.
(A) Representative dendritic segments of hippocampal neurons at DIV13 expressing GFP plus either empty pDisplay vector (Control) or class 5 Semaphorin constructs.

Scale bar represents 5µm.

(B) Sema5A and/or Sema5B overexpression significantly decreases dendritic spine density.

(C) There is no different in spine head width among different groups.

N=38-53 neurons per condition from ≥3 separate cultures. ***p < 0.001, n.s., non-significant, one-way ANOVA with Tukey's test post hoc.

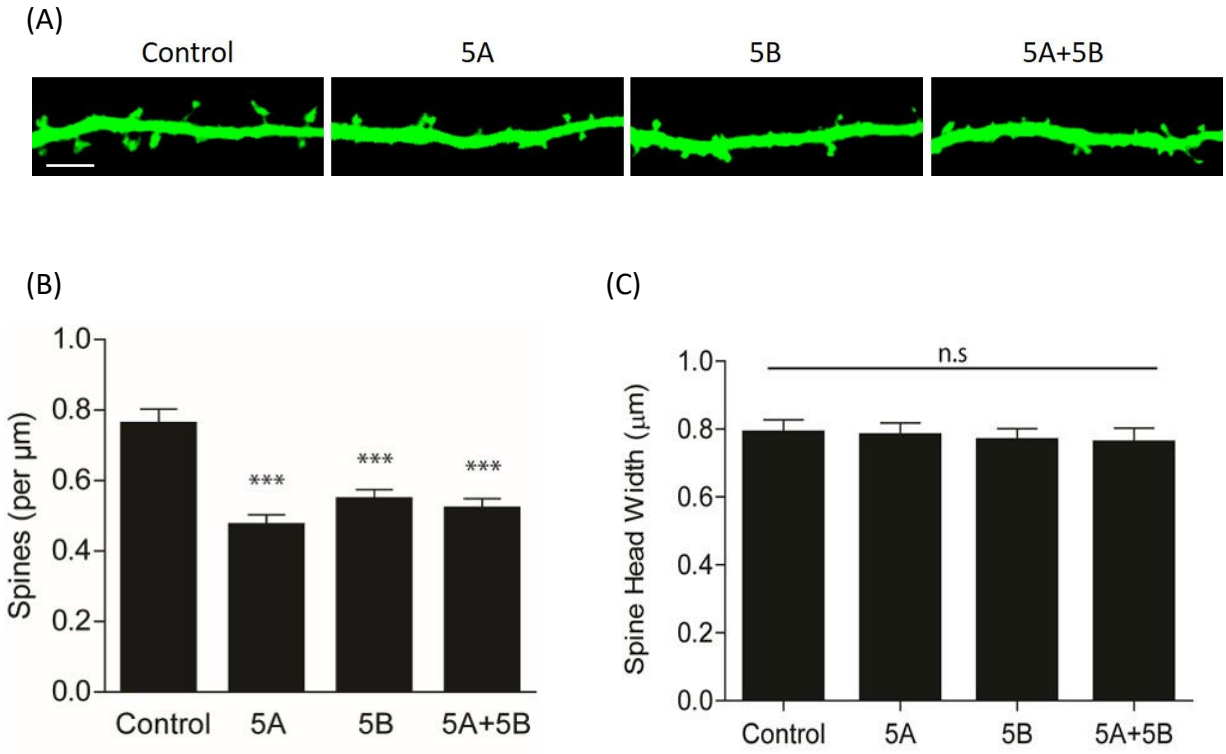


Figure 2.4 Validation of Sema5A knockdown constructs.
(A) Three shRNA constructs have been designed against rat HA-Sema5A.

(B) (Upper) Immunoblot of cellular lysates of HEK293 stable cell lines expressing rat HA-Sema5A or mouse HA-Sema5B which were transfected with empty pLL3.7 vector or Sema5A shRNA construct, probed with HA antibody and γ-tubulin. Sema5A shRNA construct effectively reduces level of Sema5A but not Sema5B protein. (Lower) Immunoblot of cellular lysates of HEK293 stable cell line expressing human HA-Sema5A which was transfected with empty pLL3.7 vector or Sema5A shRNA construct, probed with HA antibody and γ-tubulin. The Sema5A shRNA construct specifically knockdowns rat Sema5A protein but not human Sema5A protein. γ-tubulin was used as a loading control.

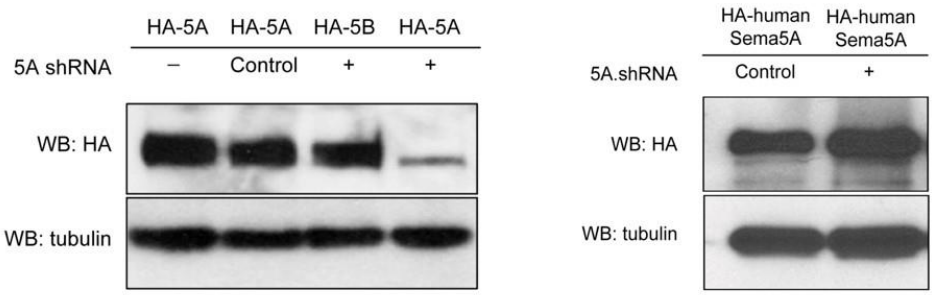
N=3 separate cultures.

(C) HEK293 stable cell lines expressing HA-rat Sema5A/HA-Sema5B/HA-human Sema5A were transfected with empty pLL3.7 vector or Sema5A shRNA vector (green) and subjected to immunocytochemistry. Two days post transfection, the knockdown effect of Sema5A shRNA construct was estimated based on the intensity of the HA immunofluorescence signal (red) in transfected cells.

(A)

shRNA1: 102-121bp shRNA2: 168-186bp shRNA3: 3050-3068bp

(B)



(C)

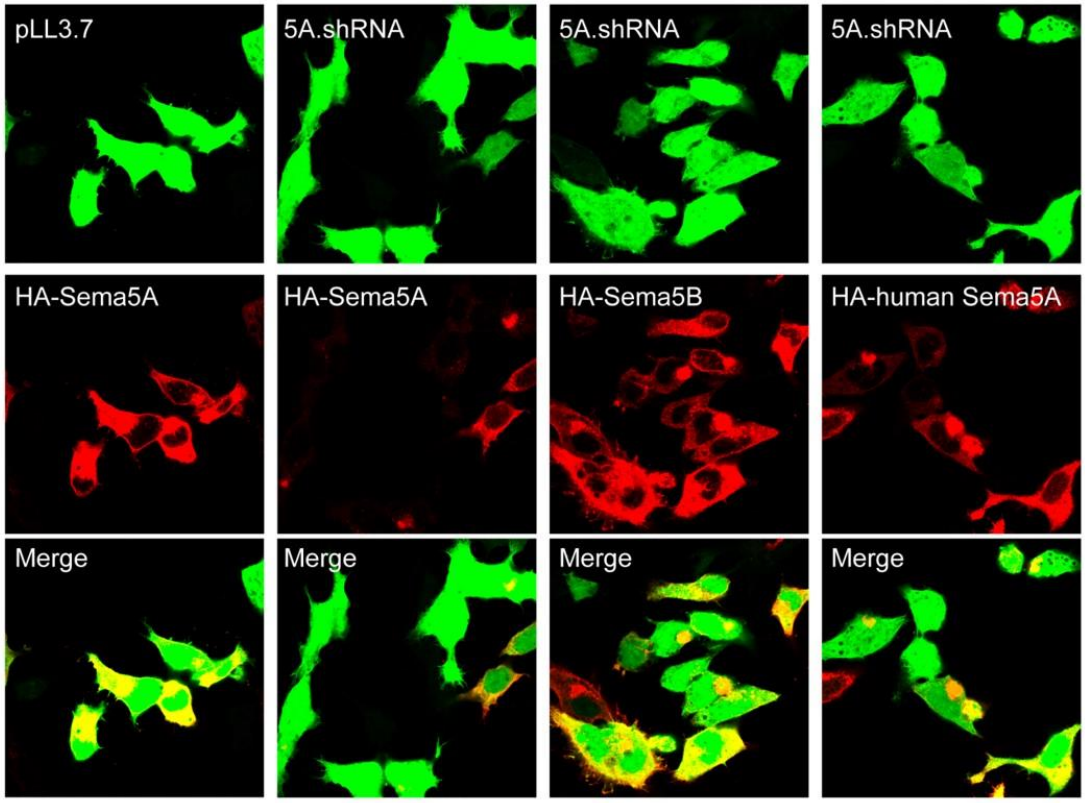


Figure 2.5 Timeline and method to analyze synapse density.
(A) Schematic demonstrating the experimental timeline. Neurons dissected from E18 hippocampus were transfected at DIV10 and assessed 3 days later.

(B) Example of synapse density analysis. To measure excitatory synapse density, neurons were transfected with GFP vector or other vectors carrying GFP expression along with interested constructs, fixed and immunolabeled for PSD-95 (cyan) and vGlut1 (magenta). A mask of GFP channel made by removing cell soma and background neurites was applied to PSD-95 and vGlut1 channels. The density of excitatory synapses, defined by the density of colocalized PSD-95 and vGlut1 clusters along dendrites, was analyzed for each neuron.

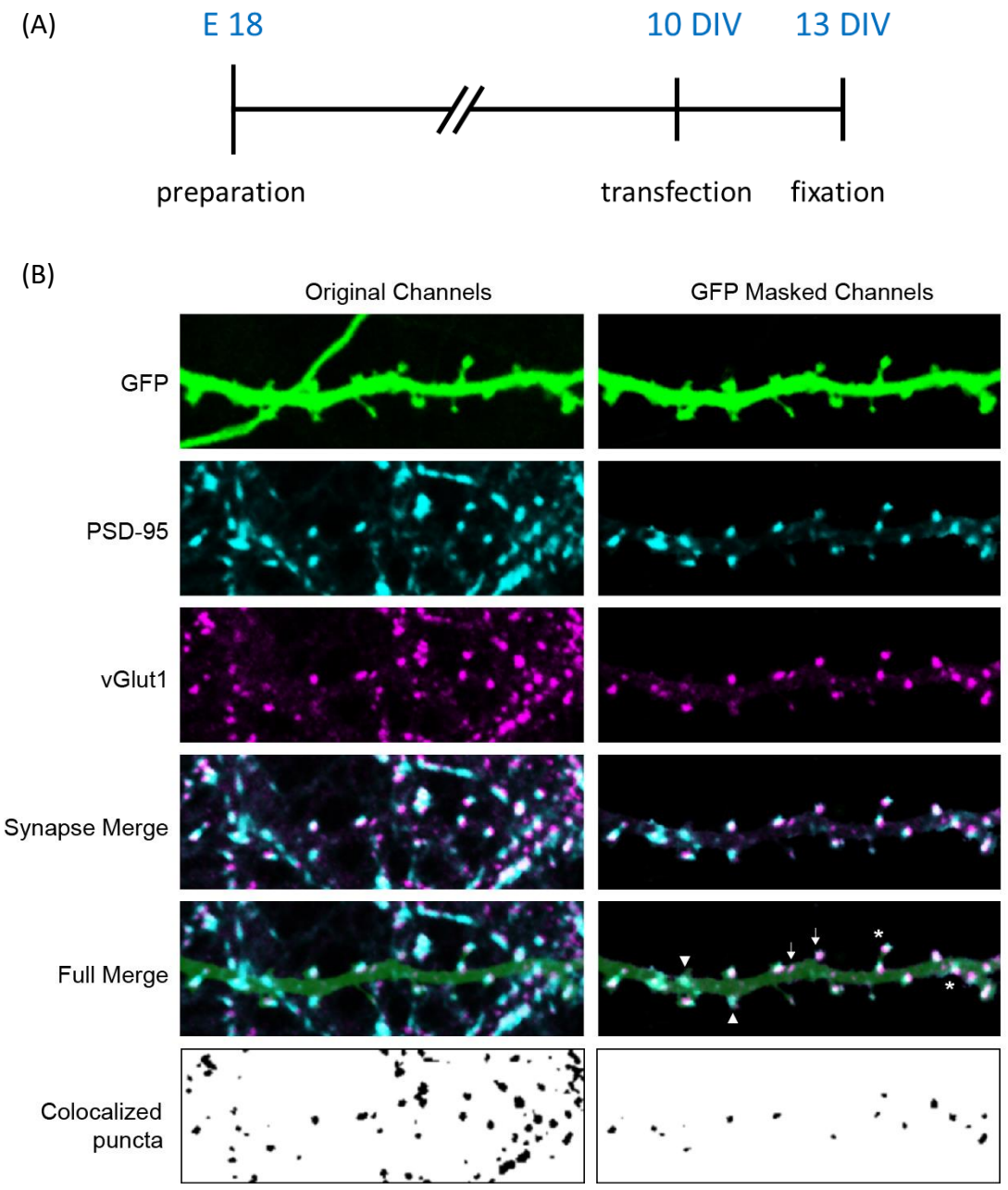


Figure 2.6 Class 5 Semaphorins negatively regulate excitatory synapse density.
(A) Representative dendritic segments of hippocampal neurons expressing GFP plus either pDisplay vector (Control) or class 5 Semaphorins and stained for PSD-95 (cyan) and vGlut1 (magenta).

(B) Representative dendritic segments of hippocampal neurons expressing empty pLL3.7 vector (Control) or indicated knockdown vectors and stained for PSD-95 (cyan) and vGlut1 (magenta). Black Puncta in colocalized mask images indicate excitatory synapses.

(C) Quantification of synapse density of neurons shown in panel A. Sema5A and/or Sema5B overexpression significantly decreases synapse density.

N=35-45 neurons per condition from 3 separate cultures.

(D) Quantification of synapse density of neurons shown in panel B. Knockdowns of class 5 Semaphorins significantly increase synapse density.

N=35-45 neurons per condition from 3 separate cultures.

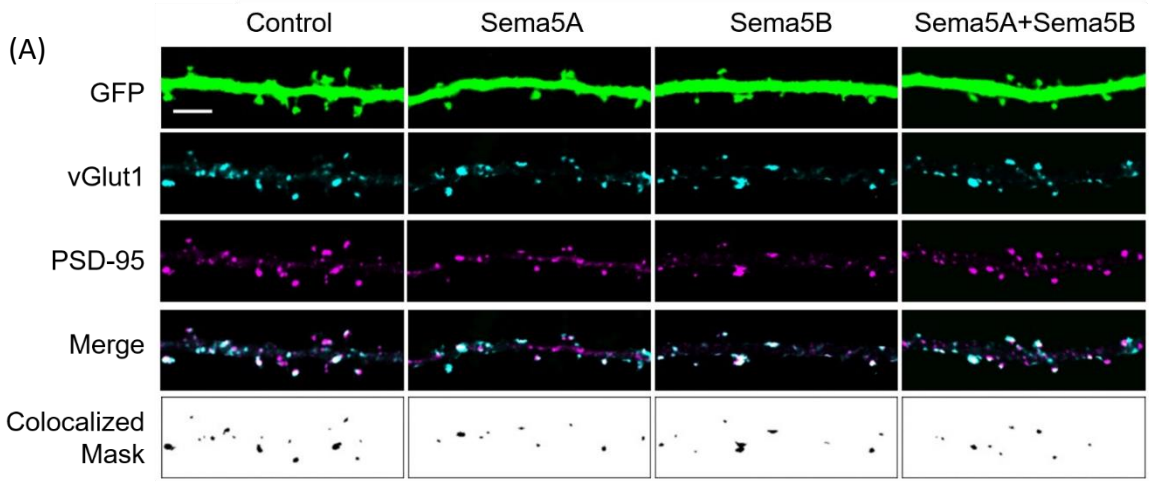
(E) Quantification of synapse density of neurons expressing GFP and treated with different concentration of Fc-5A for 2h at DIV13. Bath application of Fc-5A caused concentration-dependent reduction of synapse density.

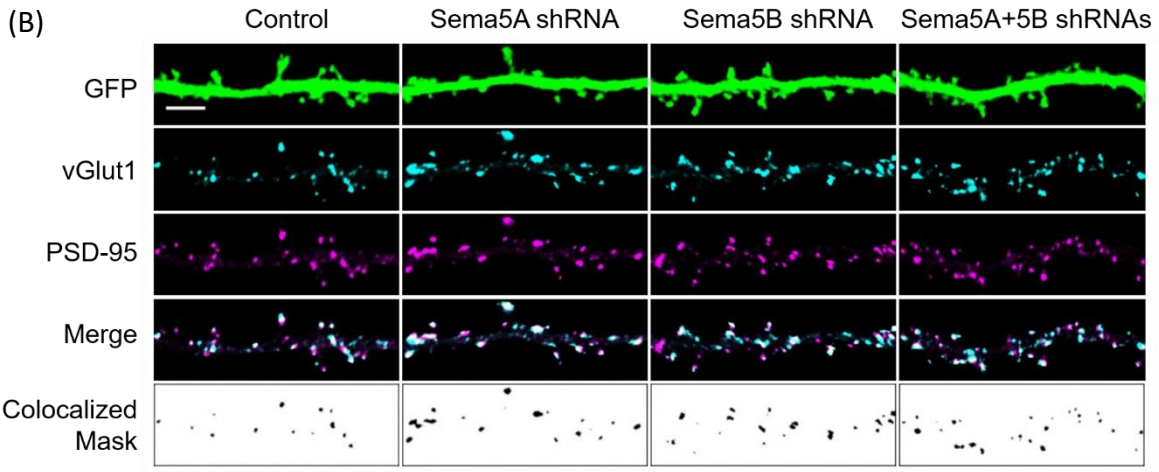
N=25-30 neurons per condition from 3 separate cultures.

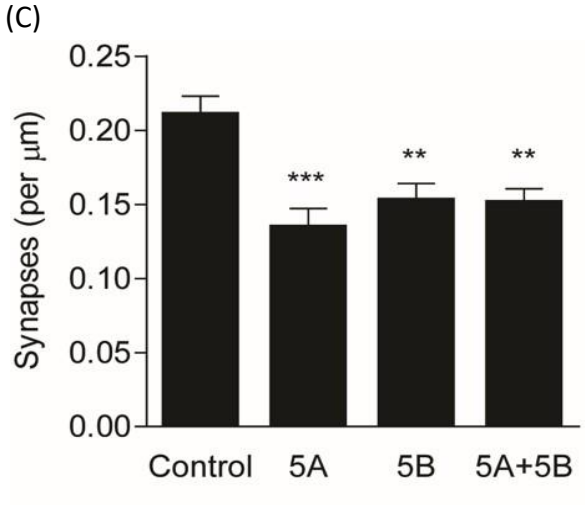
(F) Quantification of spine and synapse density of neurons expressing GFP and treated with different concentration of 6XHis tagged Sema5B for 2h at DIV13. Bath application of 6XHis Sema5B caused concentration-dependent reduction of synapse density. Spine and synapse density were normalized to the control neuron without treatment.

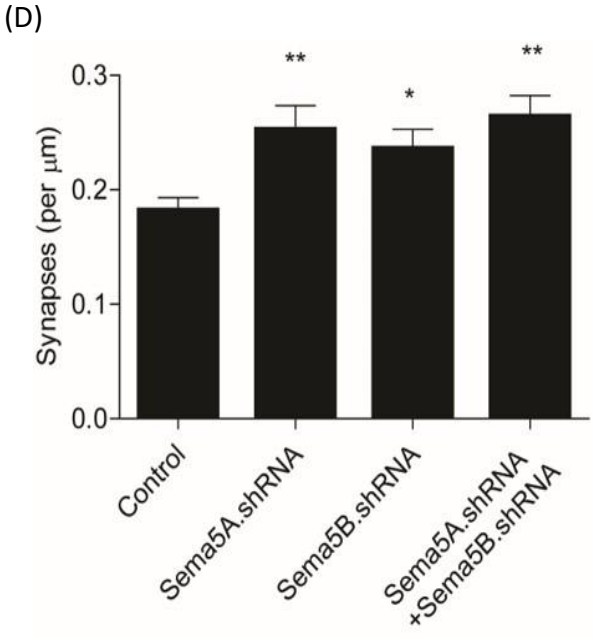
N=20-40 neurons per condition from ≥3 separate cultures.

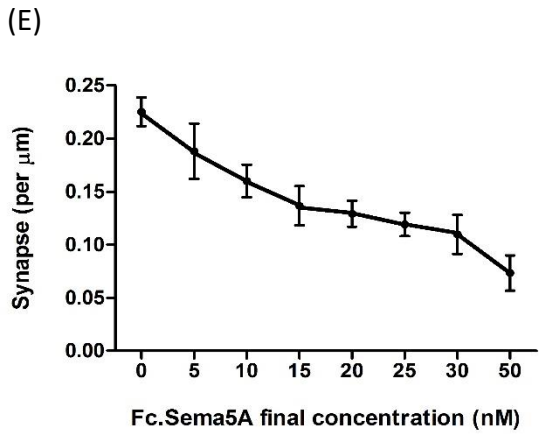
*p < 0.05, **p < 0.01, one-way ANOVA with Tukey's test post hoc. Scale bars in panel A and B represent 5 μm.




(B)
Control
Sema5A shRNA
Sema5B shRNA
Sema5A+5B shRNAs
GFP
vGlut1
PSD-95
Merge
Colocalized Mask


(C)
Synapses (per µm)
0.25
0.20
0.15
0.10
0.05
0.00
***
**
**
Control
5A
5B
5A+5B


(D)
Synapses (per µm)
0.3
0.2
0.1
0.0
**
*
**
Control
Sema5A.shRNA
Sema5B.shRNA
Sema5A.shRNA +Sema5B.shRNA


(E)
Synapse (per µm)
0.25
0.20
0.15
0.10
0.05
0.00
0
5
10
15
20
25
30
50
Fc.Sema5A final concentration (nM)

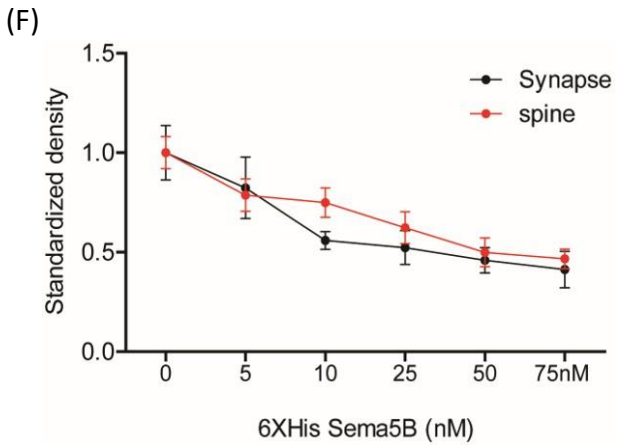

(F)
Standardized density
1.5
1.0
0.5
0.0
Synapse
spine
0
5
10
25
50
75nM
6XHis Sema5B (nM)

Figure 2.7 Class 5 Semaphorins do not impact inhibitory synapse density.
(A) Representative dendritic segments of hippocampal neurons expressing GFP plus pDisplay vector (Control) or class 5 Semaphorin constructs and stained for gephrin (magenta) and VGAT (cyan).

Scale bar represents 5 μm.

(B) Quantification of inhibitory synapse density of neurons shown in panel A. Sema5A or Sema5B overexpression did not affect inhibitory synaptic density.

N=34-37 neurons per condition from ≥3 separate cultures. n.s., nonsignificant, paired student's test.

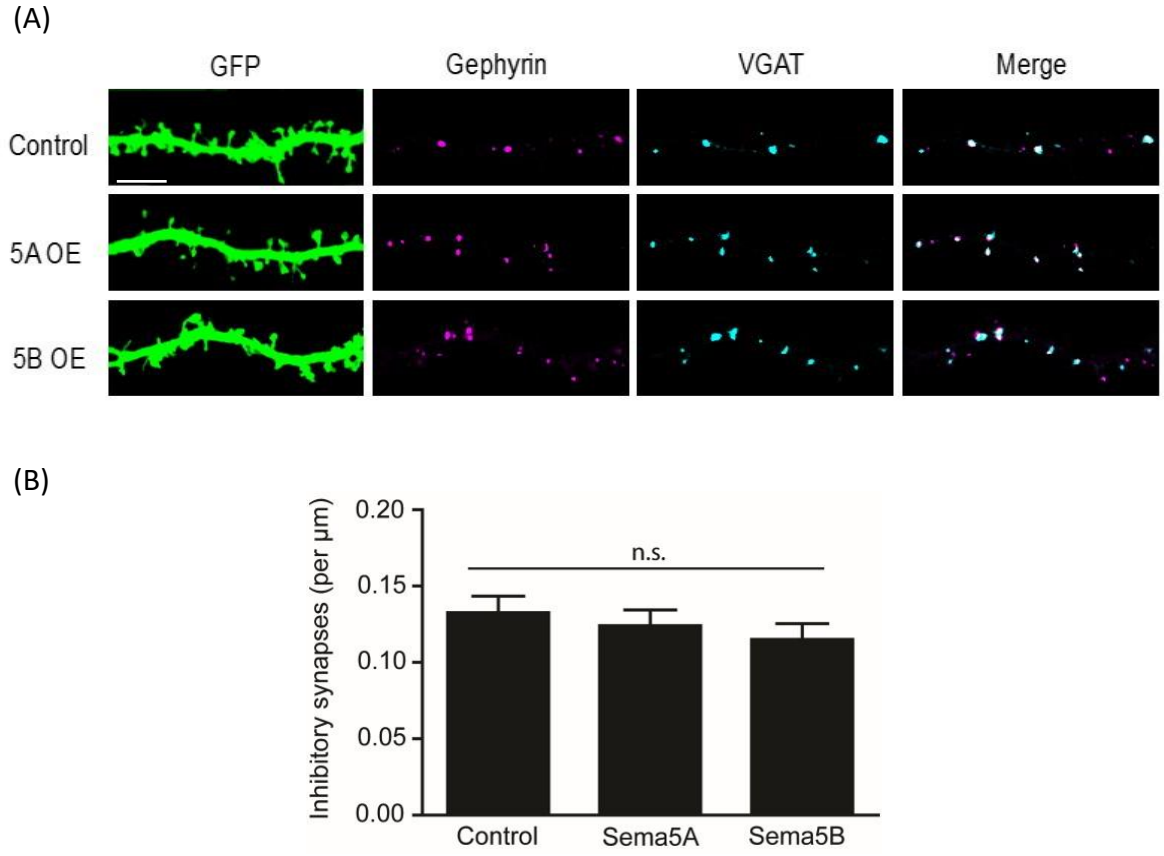


Figure 2.8 Soluble Sema5A protein eliminates synapse density.
(A) Representative dendritic segments of hippocampal neurons expressing GFP plus PSD-95-RFP and exposed to human IgG (Control) or Fc-5A for 120 min. Confocal images were acquired every 20 min for 120 min following bath application. Scale bar represents 5 μm.

(B, C) Quantification of density and integrated density of PSD-95 puncta shown in panel A. Treatment with Fc-5A for 120 min resulted in an elimination of approximately 40% of PSD-95-RFP puncta density and 50% of PSD-95-RFP puncta integrated density, whereas in control neurons only slight fluctuations were observed over time.

N=6 neurons for Fc treatment and 13 neurons for Fc-5A treatment. *p < 0.05, **p < 0.01, one-way ANOVA with Tukey's test post hoc.

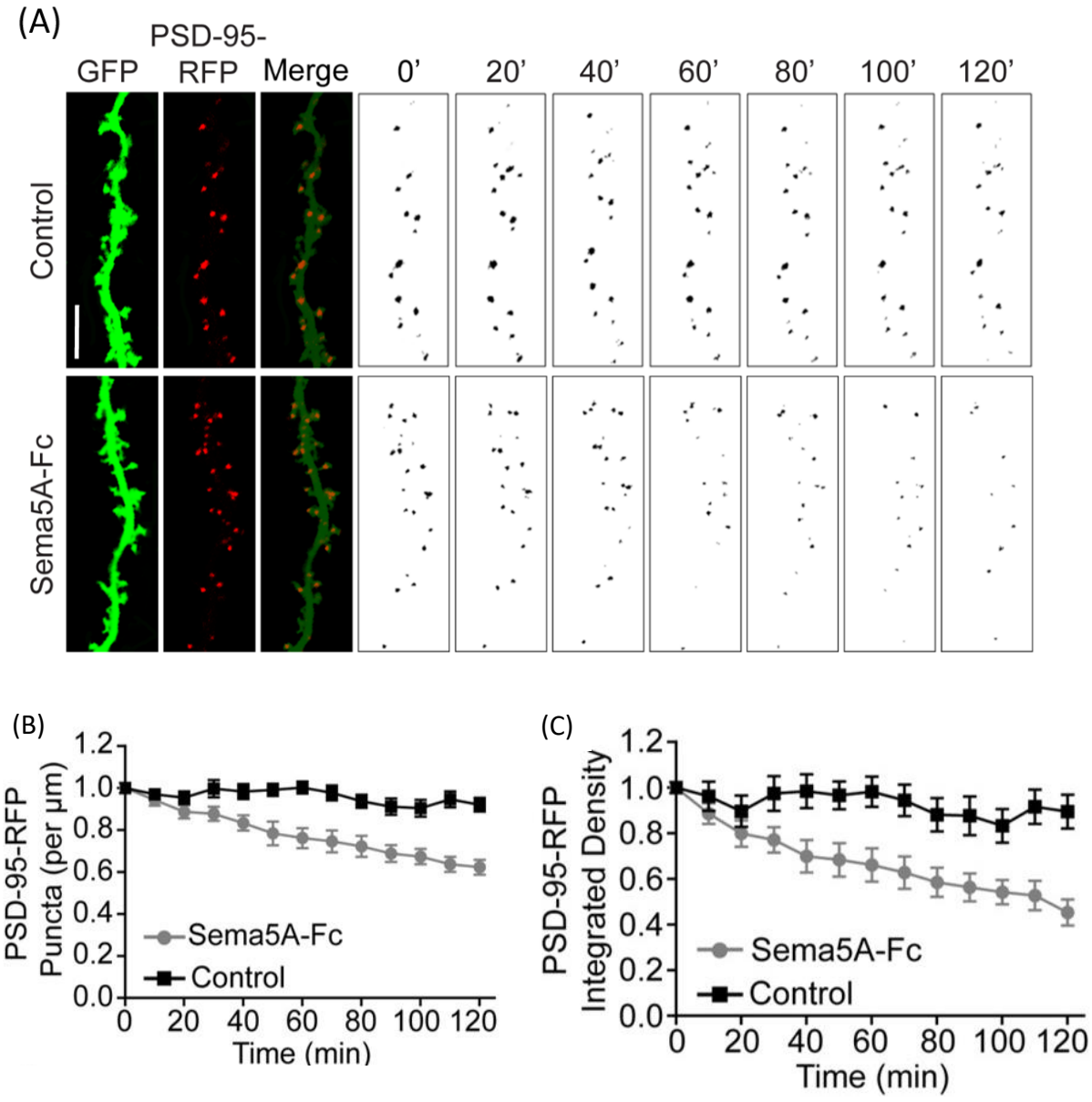
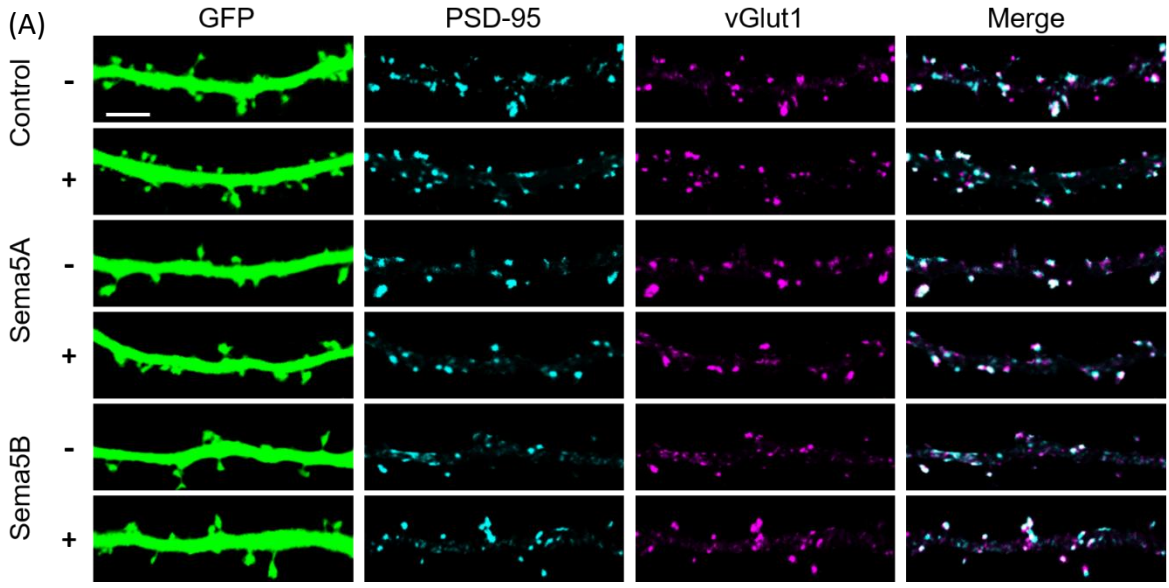


Figure 2.9 Overexpression of class 5 Semaphorins counteracts effect of chemical LTP to increase synapse density.

(A) Representative dendritic segments of hippocampal neurons expressing GFP plus either pDisplay vector (Control) or Sema5A or Sema5B constructs. At DIV13, cultures were fixed 5h after glycine treatment or control treatment and stained for PSD-95 (cyan) and vGlut1 (magenta). Scale bar represents 5 µm.

(B) Quantification of synapse density of neurons shown in panel A.

N=39-45 neurons per condition from ≥3 separate cultures. **p < 0.01, ***p < 0.001, n.s., non-significant, one-way ANOVA with Bonferroni's test post hoc.



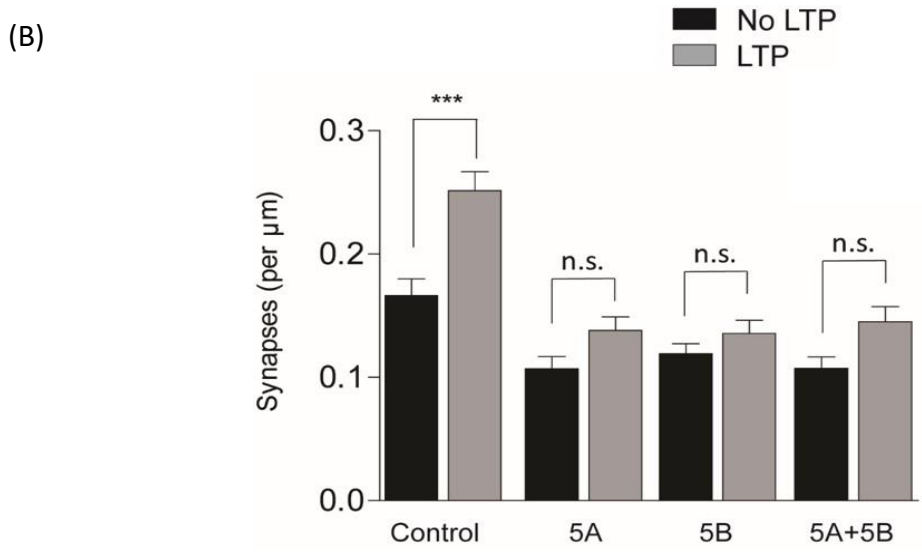


Figure 2.10 Knockdown of class 5 Semaphorins does not promote LTP-mediated increase of synapse density.

(A) Representative dendritic segments of hippocampal neurons expressing pLL3.7 vector (Control) or shRNAs construct targeting Sema5A or Sema5A/Sema5B. At DIV13, cultures were fixed 5h after glycine treatment or control treatment and stained for PSD-95 (cyan) and vGlut1 (magenta). Scale bar represents 5 μm.

(B, C) Quantification of synapse density of neurons shown in panel A.

N=23-61 neurons per condition from ≥3 separate cultures. **p < 0.01, ***p < 0.001, n.s., non-significant, one-way ANOVA with Bonferroni's test post hoc.

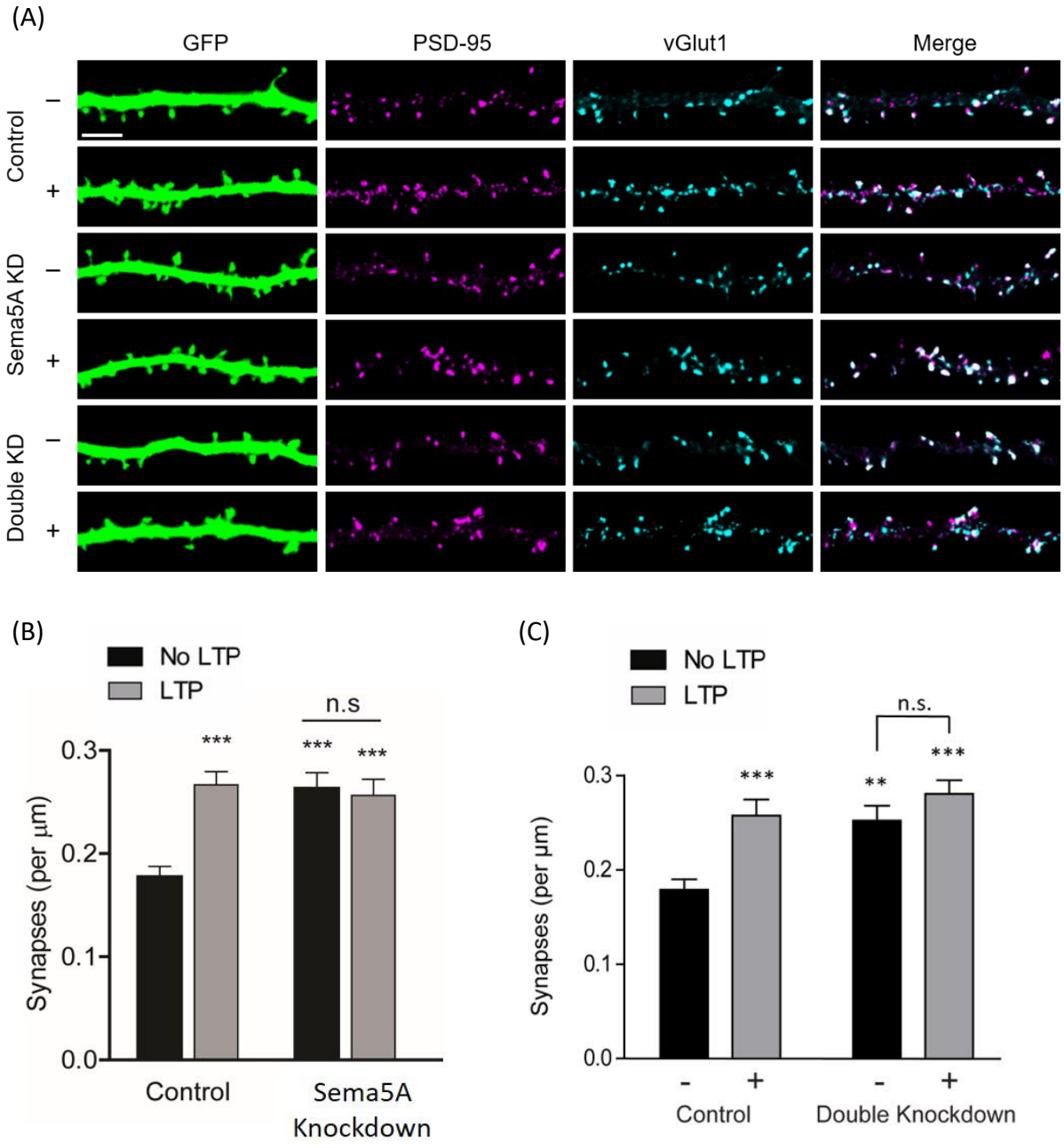
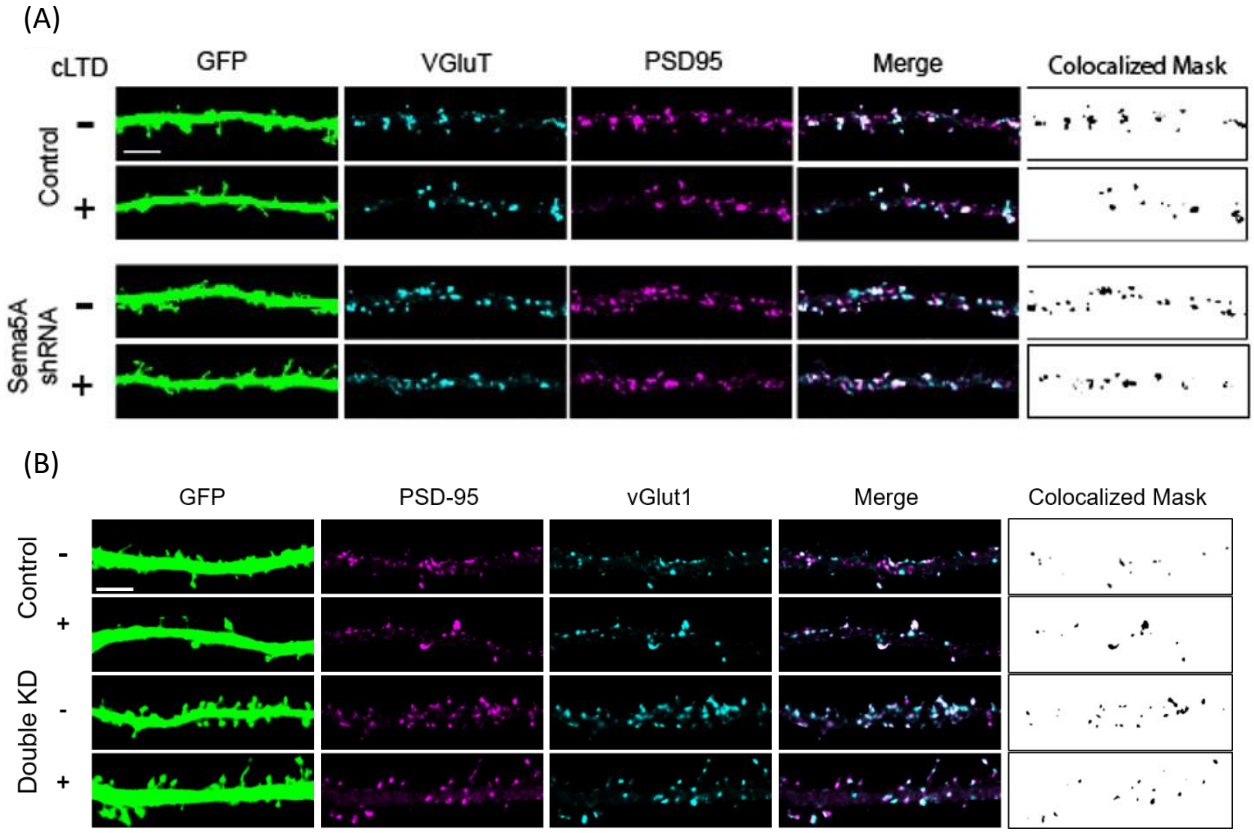


Figure 2.11 Knockdown of class 5 Semaphorins blocks effect of chemical LTD to decrease synapse density.

(A, B) Representative dendritic segments of hippocampal neurons expressing pLL3.7 vector or shRNAs against Sema5A or Sema5A and Sema5B. At DIV13, cultures were fixed 5h after NMDA treatment or control treatment and stained for PSD-95 (cyan) and vGlut1 (magenta). Black Puncta in colocalized mask images indicate excitatory synapses. Scales bars represent 5 μm.

(C) Quantification of synapse density of neurons shown in panel A and B. Sema5A knockdown partly blocked the effect on LTD-mediated decrease of synapse density, whereas Sema5A and Sema5B double knockdown totally blocked the effect on LTD-mediated decrease of synapse density.

N= 20-81 neurons per condition from ≥3 separate cultures. ***$p < 0.001$, n.s., non-significant, one-way ANOVA with Bonferroni's test post hoc.



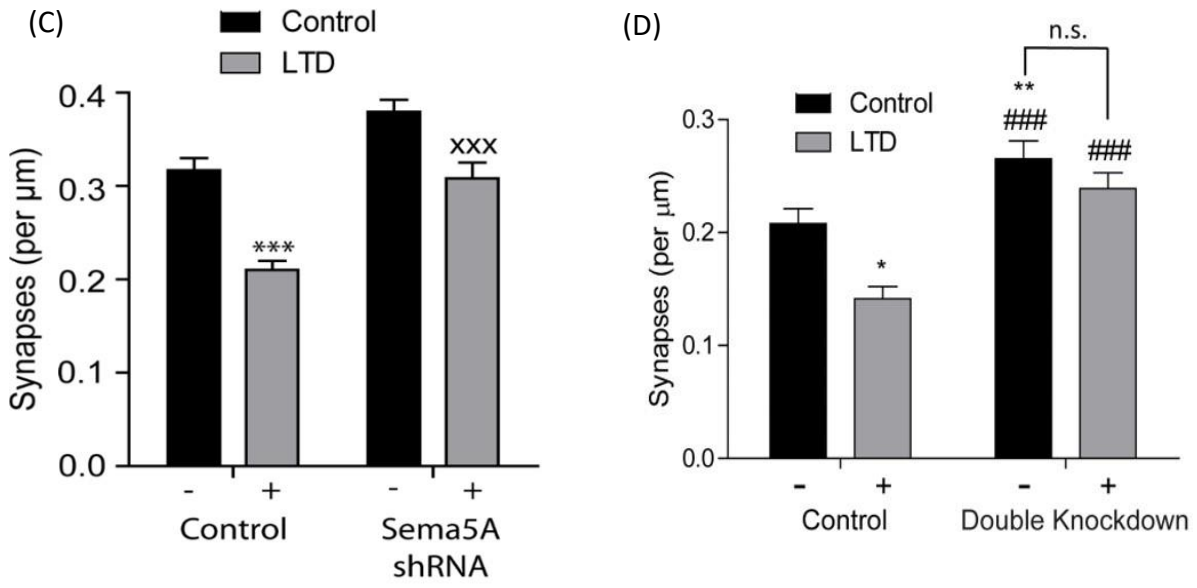
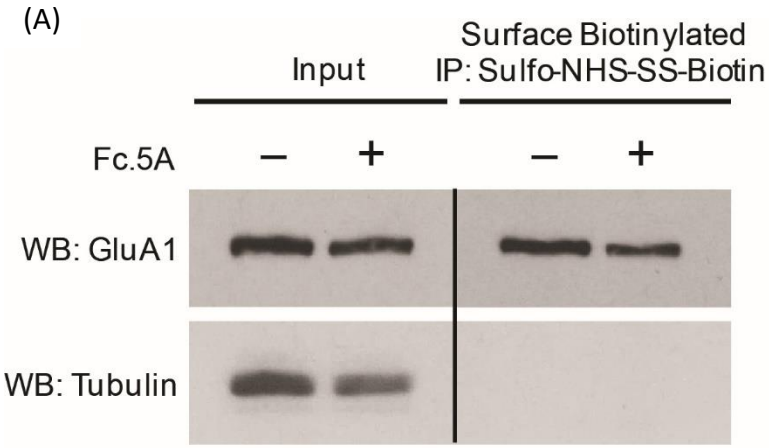

(C)
Control
LTD
Synapses (per µm)
0.4
0.3
0.2
0.1
0.0
***
xxx
-
+
-
+
Control
Sema5A
shRNA
(D)
n.s.
**
###
###
Control
LTD
Synapses (per µm)
0.3
0.2
0.1
0.0
*
-
+
-
+
Control
Double Knockdown

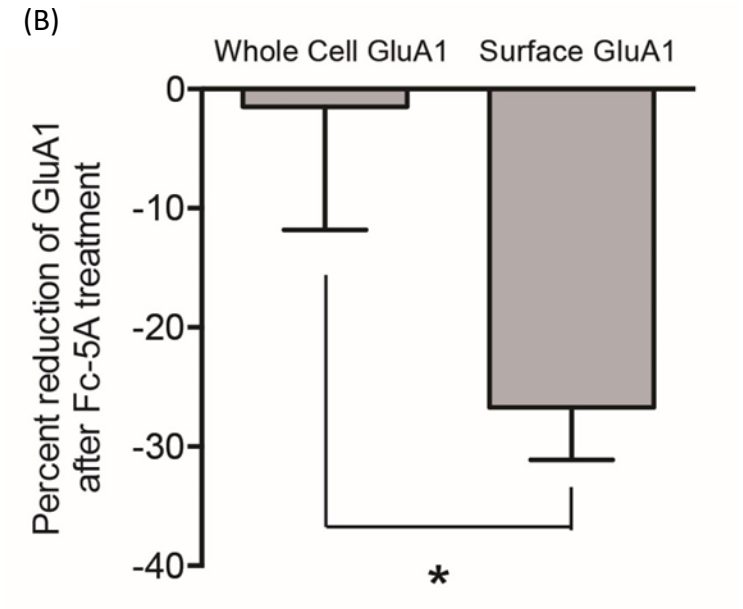
Figure 2.12 Fc.5A treatment decreases GluA1 cell surface levels but not its whole cell levels in hippocampal neurons.
(A) Hippocampal neurons at DIV13 were treated for 1h with Fc-5A (50 nM), followed by surface biotinylation and western blot.

(B) Quantification of total levels of GluA1. Values were normalized to loading control γ-Tubulin.

(C) Quantification of cell surface levels of GluA1. Values were normalized to whole cell input.

N=3 independent experiments. *p<0.05, student t-test.





2.4 Discussion

Research in the last few decades has unveiled the crucial role for axon guidance cues in the process of axonal targeting during the development of the nervous system. Among the four canonical families of guidance cues, the Semaphorin family is the largest and has received increasing attention due to its implication in a series of neural developmental processes other than axon targeting. In this chapter, I demonstrated that two class 5 Semaphorins, Sema5A and Sema5B reduced the dendritic complexity in hippocampal neurons *in vitro*. I also found that overexpression of Sema5A, as shown previously for Sema5B, resulted in a decrease in spine density and excitatory synapse density, but had no effect on inhibitory synapse density. Using time-lapse imaging, I ascertained that the reduction of excitatory synapse density was driven by active elimination of mature synapses.

To examine their physiological role further, I determined that Sema5A and Sema5B are involved in activity-dependent synaptic refinement associated with LTP and LTD. Overexpression of Sema5A and/or Sema5B counteracted the LTP-mediated increase of synapse density, whereas Sema5A and Sema5B knockdown blocked the LTD-mediated decrease of synapse density. Taken together, our results suggest that Sema5A and Sema5B are instrumental in limiting the dendritic morphology and the establishment of hippocampal connections, and that they may play a key role in activity-dependent synaptic refinement in hippocampal neurons. Finally, I demonstrated that the level of surface GluA1 is decreased upon Fc-5A treatment, which is possibly involved in Sema5A-mediated synaptic plasticity.

2.4.1 Sema5A and Sema5B Inhibit Dendritic Growth and Dendritic Branching

Like axons, dendritic morphology includes growth, extension and branching, all of which must be precisely regulated during nervous system development (Jan and Jan 2003). Previous studies in the developing brain have demonstrated a role for Semaphorins in dendritic growth and arborisation (Polleux, Morrow et al. 2000; Fenstermaker, Chen et al. 2004; Zhuang, Su et al. 2009), whereas the specific functions of class 5 Semaphorins remained to be investigated. Neurons with Sema5A and/or Sema5B overexpression exhibit less branching and have smaller dendritic trees than control neurons, suggesting the basal levels of Sema5A and Sema5B are necessary for proper dendritic morphology and complexity of hippocampal neurons. Our findings broaden our understanding of the function of class 5 Semaphorins in nervous system development. Considering the importance of Sema5A and Sema5B in normal dendrite elaboration, alterations in the levels of Sema5A and Sema5B during development may hinder neurons from sustaining appropriate dendritic complexity required for healthy neural circuitry.

2.4.2 Sema5A Mediates Synapse Elimination of Mature Synapses

Beside being inhibitory axon guidance cues, Sema5A and Sema5B have received increased attention for their roles in synapse formation and development. Previous work has demonstrated that Sema5B is a robust stimulator of synapse elimination in hippocampal neurons (O'Connor, Cockburn et al. 2009). Moreover, Sema5A has been shown to inhibit synaptogenesis in hippocampal dentate granule cells (Duan, Wang et al. 2014). Consistently, I found that either overexpression or protein treatment of Sema5A or Sema5B decreased spine and synapse density, whereas knockdown of Sema5A and/or Sema5B led to an increase

in synapse density of cultured hippocampal neurons. Together with previous studies, our finding extends the functions of Sema5A and Sema5B beyond axon guidance to constrain spine density and synapse density. Using time-lapse imaging of hippocampal neurons treated with soluble Sema5A protein, I monitored the dynamic changes of present synapses and observed a remarkable synapse loss after bath application of Fc-5A. The elimination caused by Fc-5A treatment is rapid and efficient, as PSD-95-RFP puncta started to disassemble within 20 mins following Fc-5A treatment and lost approximately 30% of the synaptic integrated density by 40 mins compared to control neurons (Figure 2.8). Such rapid changes suggested that the Sema5A-mediated reduction of synapse density is an active process of elimination of excitatory synapses, rather than an inhibition of ongoing synaptogenesis. The mechanisms and signaling pathway of Sema5A-induced synapse elimination will be further tested in the next chapter.

2.4.3 Sema5A and Sema5B Regulate Activity-Dependent Synaptic Refinement

Though the initial formation of connections in the mammalian CNS does not require neural activity, those connections are refined in an activity-dependent manner later (Craig, Blackstone et al. 1994; Verhage, Maia et al. 2000; Varoqueaux, Sigler et al. 2002; Harms and Craig 2005). In our experiments, the overexpression of Sema5A and Sema5B decreased the degree of LTP-mediated increase in synapse density. However, such LTP-mediated increase was not promoted by Sema5A knockdown and Sema5A/Sema5B double knockdown, which rules out the possibility that Sema5A was a simple synapse eliminator independent from the neural activity. This conclusion is further supported by the LTD data. Neurons lacking Sema5A did not fully exhibit the LTD-mediated decrease in synapse density, suggesting that neural activity requires Sema5A to regulate LTD-mediated synapse elimination. Moreover,

double knockdown of Sema5A and Sema5B completely blocked the effect of LTD, making Sema5A and Sema5B both necessary for the synapse elimination observed after LTD. Thus, Sema5A and Sema5B mediate the effect of activity-dependent synaptic plasticity on synapse density.

2.4.4 Fc-5A Treatment Decreases Surface Expression of GluA1

It is well demonstrated that LTP and LTD induction are facilitated by AMPAR trafficking via PKA-mediated phosphorylation of the GluA1 subunit, and redistribution of GluA1 subunits at synaptic sites is important for activity-dependent synaptic plasticity (Roche, O'Brien et al. 1996; Banke, Bowie et al. 2000; Shepherd and Huganir 2007; Kessels and Malinow 2009). Sema5A associates with HSPGs and CSPGs (Kantor, Chivatakarn et al. 2004), and proteoglycans have been found to promote glutamatergic synaptic transmission and synaptogenesis via GluA1 distribution (Allen, Bennett et al. 2012). Consistent with these studies, here I report that upon Fc-5A treatment, there is a 25% decrease of the surface GluA1 level, while no change is observed in the total GluA1 level. Because the total amount of GluA1 does not change, the decrease of surface GluA1 level is most likely caused by AMPAR trafficking, rather than modification of protein expression or degradation. Given the crucial role of AMPAR subunits distribution and AMPAR trafficking in the modulation of synaptic plasticity (Terashima, Cotton et al. 2004; Kessels and Malinow 2009; Mejias, Adamczyk et al. 2011; Lu, Khatri et al. 2014), subcellular redistribution of AMPARs may be part of the mechanism of Sema5A-mediated synaptic refinement. Together with the fact that Sema5A is inserted into the cell surface after LTD induction, our data suggests that neural activity regulates excitatory synapse plasticity at least in part through its effects on Sema5A relocation to the plasma membrane, which further mediates disassembly of synaptic proteins

such as PSD-95 and redistributes AMPARs to reduce synaptic strength and induce synapse elimination.

Chapter 3: Exploring the Receptor and Signaling Pathway of Sema5A

3.1 Introduction

Semaphorins play a variety of roles at neuronal developmental stages such as of neural circuit establishment and neuronal differentiation, axon guidance and synaptogenesis (Yoshida 2012). To exert their effects, Semaphorins bind to their receptors on the cell surface and initiate an extensive range of intracellular signaling events (Roth, Koncina et al. 2009; Pasterkamp 2012; Giacobini and Prevot 2013). The principle receptors for Semaphorins are the proteins of Plexin family, which consists of nine large transmembrane proteins grouped into four groups (Tamagnone, Artigiani et al. 1999; Fujisawa 2004; Hota and Buck 2012; Perala, Sariola et al. 2012). In addition, two neuropilins (neuropilin 1 and neuropilin 2), which are only found in vertebrates, can serve as co-receptors for the class 3 Semaphorins (Tamagnone, Artigiani et al. 1999). Over the last two decades, many of the receptors for specific Semaphorins have been investigated in a variety of different cell types and organisms (Tamagnone, Artigiani et al. 1999; Fujisawa 2004).

Various receptors and binding partners for Sema5A have been proposed. In human glioma, Plexin B3 expression upon stimulation by Sema5A was shown to inhibit cell migration and prevent invasive growth (Li and Lee 2010; Li, Law et al. 2012). The inhibition of neurite outgrowth mediated by Sema5A and Sema5B is abolished in retinal neurons of Plexin A1/Plexin A3 double knockout mice (Matsuoka, Chivatakarn et al. 2011). A more recent study identified a high-affinity interaction between Sema5A and Plexin A1 or Plexin A2 on neurons *in vitro* and revealed that Sema5A can inhibit synaptogenesis of dentate granule cells

through Plexin A2 *in vivo* (Duan, Wang et al. 2014). Except the report that Sema5B works together with Sema5A through Plexin A1 and Plexin A3 in retinal neurons, presently no Plexin has been reported to be the receptor only for Sema5B. However, our previous work has shown that TAG-1 is required for Sema5B to function as a chemorepellent cue in the ventral spinal cord (Liu, Wang et al. 2014). This was not the first time that TAG-1 was identified as being a component of a Semaphorin receptor complex. Indeed, TAG-1 interacts directly with neuropilin 1 and modulates the endocytosis of the L1/neuropilin 1 in Sema3A receptor complex (Law, Kirby et al. 2008). Thus, it is possible that TAG-1 is involved in Sema5A signaling to mediate the function of Sema5A in the hippocampus.

Both Sema3A (Gallo 2006; Qin, Liao et al. 2010; Kaczmarek, Riccio et al. 2012) and Sema5B (To, Church et al. 2007) have been shown to stimulate growth cone collapse by inducing the disassembly of the actin meshwork in growth cones, and this disassembly was due to the activation of calpain (To, Church et al. 2007; Qin, Liao et al. 2010; Kaczmarek, Riccio et al. 2012). Calpains are a family of calcium-dependent proteases known to proteolytically cleave a number of actin cytoskeleton regulators including cortactin, cadherin, and β-catenin (Goll, Thompson et al. 2003; Franco and Huttenlocher 2005). In addition, calpains are known to regulate synaptic efficacy and structure (Lynch and Baudry 1987; Vinade, Petersen et al. 2001; Abe and Takeichi 2007). In immature (DIV10) hippocampal neuronal cultures, it has been shown that inhibition of calpains results in an increase in the presynaptic puncta density with the synapse density unchanged (Sun and Bamji 2011). Whether calpains are major effectors of Sema5A-mediated synapse elimination is unknown.

In this chapter, I first demonstrate that Plexin A1 and Plexin A2 mediate the function of Sema5A in synapse elimination *in vitro*. Next, I demonstrate that TAG-1 is required for the

function of Sema5A by showing that blocking TAG-1 completely rescued the decrease of the synapse density caused by Sema5A and/or Sema5B overexpression. Then, I show that global inhibition of calpain activity totally blocked the effect of Fc-5A treatment or Sema5A overexpression, thus calpain is likely signaling downstream of the Sema5A/Plexin complex. Together, our data identify the potential receptors and shed light on the molecular pathway through which activity of Sema5A can translate into the synapse elimination and synaptic plasticity in hippocampal neurons.

3.2 Materials and Methods

3.2.1 Recombinant DNA Constructs and Antibodies

Full-length rat or human Sema5A was subcloned into the *Xma* I/*Sac* II sites of the haemagglutinin (HA) epitope tagged pDisplay expression vector (Invitrogen). The empty pEx.Fc and Sema5A-Fc constructs were kind gifts from Dr. David Sretavan (University of California, San Francisco). Full-length TAG-1 was inserted into pcDNA 3.1 construct. The Plexin A2 construct was a generous gift from Dr. Luca Tamagnone (University of Torino Medical School, Candiolo, Italy) and Plexin A1/ Plexin A3/Plexin B1/Plexin B2/Plexin B3 constructs were kind gifts from Dr. Matthieu Vermeren (The University of Edinburgh, Edinburgh, UK).

The dilution and sources of the primary antibodies used were: anti-PSD-95 (1:500, Abcam ab2723), anti-vGlut1 (1:750, Millipore AB5905), anti-Glutamate Receptor 1 (AMPA subtype) (1:300, Abcam ab31232) and anti-γ-Tubulin (1: 10,000, Sigma T6557). The dilution and sources of the secondary antibodies used were: IgG-horseradish peroxidase (HRP; rabbit

1: 3,000, Jackson ImmunoResearch 711-035-152), Alexa-Fluor 568 goat anti-mouse (1: 500; Life Technologies A-11031), and Cy5 goat anti-guinea pig (1: 1,000, Abcam ab6567).

3.2.2 Cell Culture and Transfection

Cultures of hippocampal neurons were prepared from embryonic day 18 (E18) Sprague–Dawley rats (Charles River Laboratories) of either sex following a previously described protocol (Xie, Markesbery et al. 2000). Briefly, neurons were plated at a density of 200 cells per mm^2 on poly-L-lysine coated coverslips. Cultures were maintained in Neurobasal media with 10% fetal bovine serum (FBS, Invitrogen), 0.5 mM L-glutamate (Gibco), Penicillin/Streptomycin (50 U/ml; 50 µg/ml, Gibco), NeuroCult SM1 (StemCell, instead of B27 supplement). Neurons were transfected with Lipofectamine 2000 (Invitrogen) at DIV9-10 according to the manufacturer's recommendations, and used for experiments at 12-14 DIV.

For the ALLN bath application, neurons were treated with 20 µM ALLN (Calbiochem, San Diego, CA) for 24 h at DIV13.

For TAG-1 blocking experiment, neurons were incubated in either culture media alone or culture media containing 25 µg/ml mouse anti-TAG-1 antibody (23.4-5, Developmental Studies Hybridoma Bank, Iowa City, IW, USA) for 24-48 hours.

3.2.3 Western Blot Analysis

HEK293 cells were rinsed in cold PBS, and homogenized in ice-cold Radio immuno-precipitation assay buffer (RIPA buffer, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mM Tris-HCl pH 8.0, 0.1% SDS, 150 mM NaCl) supplemented with a protease inhibitor

cocktail (Roche). Cell lysates harvested by plastic scrapers were sonicated using Branson Sonifier 250 and cleared by centrifugation for 30 min at 4 °C. Total protein concentration of the supernatant was measured using BCA protein assay (Pierce). Proteins were separated by SDS-poly acrylamide gel electrophoresis (SDS-PAGE), analysed by immunoblotting with the indicated antibodies and visualized using enhanced chemiluminescence (ECL, Pierce) on a Versadoc 4000 (Bio-Rad) following standard protocol. Band intensities on western blot image were measured by densitometry using Image J, and normalized to that of the γ-tubulin band.

3.2.4 Immunocytochemistry

Rat hippocampal neuron cultures were fixed in pre-warmed 4% PFA/sucrose solution for 15 min, permeabilized with 0.1% Triton X-100 in PBS for 15 min, followed by blocking with 10% goat serum in PBS for 1 h at room temperature. Following blocking, primary antibodies were applied to the cells in antibody solution (PBS, 1% goat serum) overnight at 4°C. The cells were then rinsed in PBS three times for 10 min each, followed by incubation with secondary antibodies diluted in antibody solution for 1 h at room temperature. Then the cells were rinsed again in PBS three times for 10 min each and mounted on microscope slides (Globe Scientific) in Prolong Gold (Molecular Probes).

3.2.5 Generation of Sema5A-Fc Protein and Bath Application

The method of generating Sema5A-Fc protein recombinant protein has been described before (Oster, Bodeker et al. 2003). Briefly, HEK293 stable cell lines expressing an IgG-Fc domain fused full-length soluble Sema5A protein (Fc-Sema5A) or an IgG-Fc domain alone (Fc) were generated. When stable, these cells were cultured in normal medium for three days, and then

switched to serum-free medium. Three days after, cell culture supernatant was collected, spun down to pellet cell debris and concentrated through AMICON 50MWCO centrifugal filters (Millipore). Fc-Sema5A or Fc protein was isolated by Protein-A/G Dynabeads (Invitrogen), and protein concentrations and purity were determined by SDS-PAGE followed by Coomassie staining and comparison with bovine serum albumin (BSA) standards. For bath application, transfected neurons were incubated with 25nM Fc-5A or Fc protein diluted in culture media just prior to addition to the cells.

3.2.6 Confocal Imaging

All the cells were imaged using an Olympus Fluoview 1000 inverted confocal microscope (× 60/1.4 Oil Plan-Apochromat). When imaging fixed cells, identical acquisition parameters were used for all cells across all separate cultures within an experiment. Approximately 10-20 images were acquired and analyzed for each condition within an experiment for at least three repeats. For time-lapse imaging, a baseline image of the transfected neurons was acquired before bath application. Acquisition parameters were set on this basis and maintained across bath application. The representative confocal images of PSD-95/vGlut1 channels shown in the figures were subjected to a radius of 0.4-pixel Gaussian blur. The levels and contrast of representative confocal images were also moderately adjusted in Photoshop CS software (Adobe Systems, Inc.) using scientifically accepted procedures.

3.2.7 Image Analysis and Quantification

To focus our analysis, I only assayed synapse density on transfected neurons. For all experiments neurons were co-transfected with GFP and a mask was generated in Photoshop CS that outlined the GFP fluorescence in spines and dendritic shafts. The cell soma and

background neurites were removed from the GFP channel, and then the GFP channel overlaid onto the vGlut1 and PSD-95 channels in excitatory synapse density analysis. Confocal images for a particular experiment were subjectively thresholded using ImageJ software and the same threshold was used across all images obtained in one single experiment throughout the experimental analysis.

3.2.8 Statistical Analysis

Statistical analysis was performed in Prism software. All measurements are presented as mean ± SEM. For all imaging experiments, 'N' refers to the number of cells used per condition, over at least three separate cultures, with the exception of the analysis performed in time-lapse imaging, where 'N' refers to the total number of imaged live neurons per condition and is specified in the figure legends. Data collection and analysis were not performed blind to the conditions of the experiment and were not acquired with any specific randomization procedure. However, cells were assigned to experimental groups and analysis was performed with no bias, and by different experimenters. All data were analysed in Prism software (GraphPad Software, Inc.). Statistical significance was determined by Student's t-test, one-way analysis of variance or repeated-measures one-way analysis of variance with post-hoc tests as indicated in figure legends. All figures were generated using Illustrator CS software (Adobe Systems, Inc.).

3.3 Results

3.3.1 Neurons Expressing Plexin A1 or Plexin A2 Exhibit Enhanced Synapse Elimination in Response to Sema5A

Understanding how Sema5A signals will be significantly advanced by the identification of its receptor(s). To achieve this goal, I combined overexpression of candidate Semaphorin receptors (Plexin A1-A3, Plexin B1-B3) with Fc-5A treatment or Sema5A co-transfection to test whether the expression of any of the candidate receptors enhanced synapse disassembly. First, hippocampal neurons were co-transfected with GFP and individual candidate receptors at DIV10. At DIV13, neurons were bathed in 25 nM Fc-5A or control Fc for two hours, followed by synapse density analysis. Without the overexpression of Plexins, Fc-5A treatment alone reduced the synapse density approximately by 25% ± SEM (Figure 3.1A, B). In contrast, the overexpression of Plexin A1 and Plexin A2 enhanced the response of neurons to Fc-5A treatment, almost doubling the percentage of synapses compared to non Fc-5A treatment (Figure 3.1A, B).

Next, individual candidate Plexin receptors were co-expressed with GFP plus Sema5A and synapse density was examined. I found that the overexpression of Plexin A1 or Plexin A2 enhanced the degree of synapse loss mediated by Sema5A overexpression, judging from the percentage of synapse loss compared to neurons without any Plexin overexpression (Figure 3.1C, D). Therefore, Plexin A1 and Plexin A2 enhance the synapse disassembly mediated by Sema5A, raising the possibility that Plexin A1 and Plexin A2 are the key Sema5A receptor(s) responsible for synapse elimination in hippocampal neurons. Whether they are the functional

receptor for Sema5A will needed to be confirmed by loss-of-function experiments of Plexin A1 and Plexin A2.

3.3.2 Sema5A and Sema5B Require TAG-1 to Eliminate Synapses

Our previous work has shown that the functions of Sma5B is mediated in part through TAG-1, since blocking the function of TAG-1 largely reduces the effect of Sema5B on growth cone collapse and neurite outgrowth (Liu, Wang et al. 2014). In this study, I tested the possibility that Sema5A also functions through TAG-1. I co-transfected neurons with GFP plus either control pDisplay or Sema5A and/or Sema5B constructs, and added an anti-TAG-1 antibody to the cultures as described previously to inhibit the function of TAG-1 (Law, Kirby et al. 2008; Liu, Wang et al. 2014). Consistent with our previous result (Figure 2.6C), Sema5A and/or Sema5B overexpression induced a significant loss of synapse density. Surprisingly, in the presence of the anti-TAG-1 antibody, the reduction caused by class 5 Semaphorins was completed rescued (Figure 3.2A, B), while cultured neurons bathed with the anti-TAG-1 antibodies alone did not impact synapse density. This suggests that the function of Sema5A and Sema5B in synapse elimination may be mediated in part through TAG-1.

It is interesting to note that TAG-1 overexpression did not further promote the effect of Sema5A on synapse elimination (Figure 3.2 C-H). To clarify the function of TAG-1 in Sema5A elimination of synapses, neurons were transfected with TAG-1 and treated with Fc-5A. TAG-1 overexpression alone did not significantly affect the synapse density, and the percentage of synapse loss between control neurons and TAG-1 overexpressing neurons was not significantly different (Figure 3.2 C-E). This conclusion was further supported by the

Sema5A and TAG-1 co-overexpression, in which TAG-1 overexpression neither changed the synapse density, nor the percentage of synapse loss between control neurons and Sema5A overexpressing neurons (Figure 3.2 F-H). Together, our data show that TAG-1 is necessary for the function of Sema5A to induce synapse elimination, whereas overexpression of TAG-1 is not sufficient to enhance the effect of Sema5A on synapse density.

3.3.3 Calpain Activity Functions Downstream of Sema5A to Mediate Synapse Elimination

To block the activity of the protease calpain in hippocampal cultures, neurons expressing GFP were pre-treated with 20 μM ALLN for 24 hours before Fc-5A treatment. Then I analyzed the synapse density to assess the effect of ALLN treatment on Sema5A function (Figure 3.3). Consistent with our previous results (Figure 2.6E), 25 nM Fc-5A treatment alone for 2 hours led to a ~30% reduction in synapse density (and an apparent reduction of dendritic spines, data not shown) (Figure 3.3). Interestingly, though ALLN treatment alone did not have a significant effect on synapse density, the synapse loss caused by Fc-5A treatment was completely rescued by the addition of ALLN (Figure 3.3). Thus, these data show that Sema5A-mediated synapse elimination requires the activity of calpain, suggesting that calpain as a signaling element downstream of Sema5A.

Figure 3.1 Overexpression of Plexin A1 or Plexin A2 enhances the degree of synapse loss mediated by Sema5A.

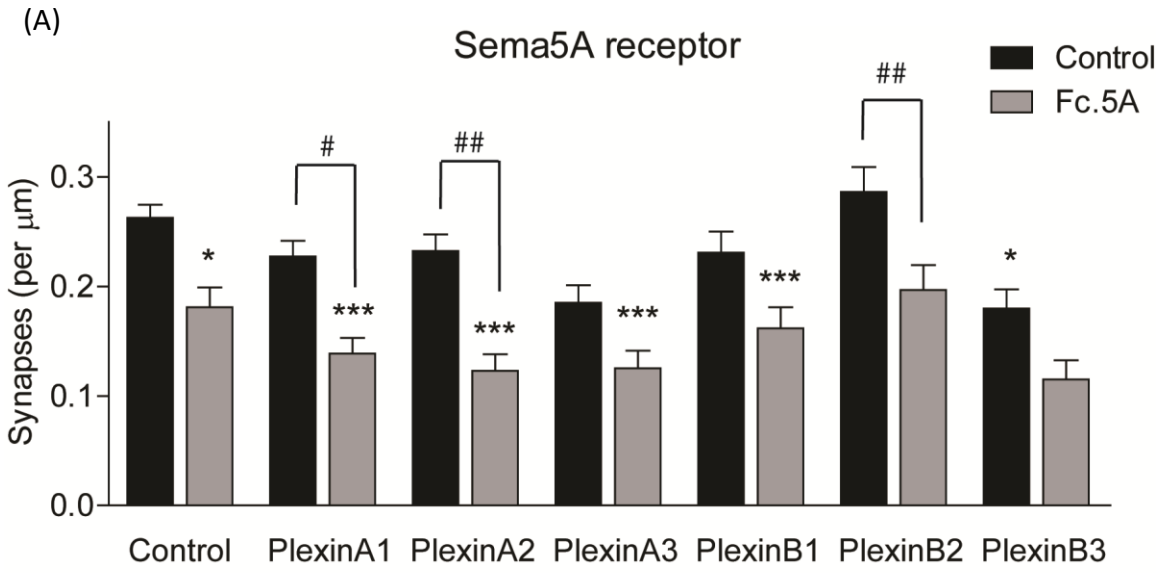
(A) Quantification of synapse density of neurons transfected with indicated Plexins at DIV10 and treated with 25 nM Fc-5A for 2h at DIV13. Asterisks denote significance relative to control neurons without Fc-5A treatment, and hashtags denote significance within groups with or without Fc-5A treatment.

(B) Quantification of percentage of change in synapse density relative to the same condition without Fc-5A treatment shown in panel A. Overexpression of Plexin A1 or Plexin A2 significantly enhances the effect of Fc-5A treatment to decrease the synapse density.

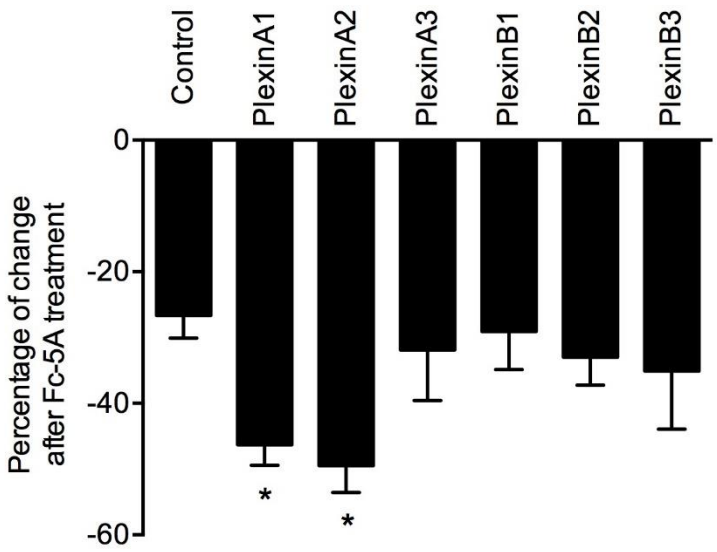
(C) Quantification of synapse density of neurons transfected with individual Plexin and GFP and Sema5A. Asterisks denote significance relative to control neurons without Sema5A overexpression, and hashtags denote significance within groups with or without Sema5A overexpression.

(D) Quantification of percentage of change in synapse density relative to the same condition without Sema5A overexpression shown in panel C. Overexpression of Plexin A1 or Plexin A2 significantly enhances the effect of Sema5A overexpression to decrease the synapse density.

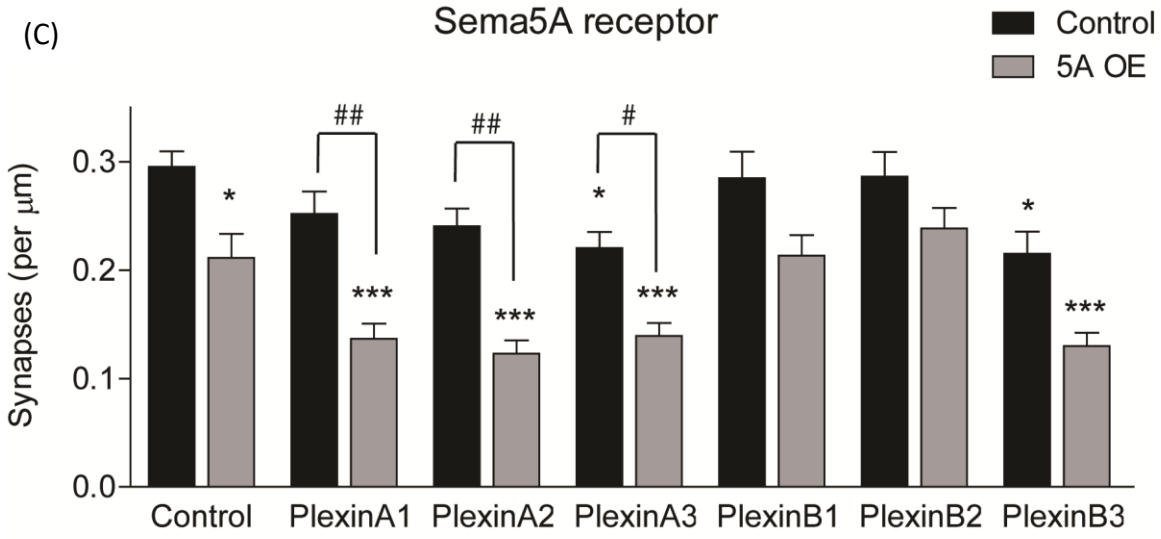
N≥26 neurons per condition from 3 separate cultures. *, $^{\#}$ p<0.05, **, $^{\#\#}$ p<0.01, ***p<0.001, one way Anova with Bonferroni's post hoc test.



(B)


Control
PlexinA1
PlexinA2
PlexinA3
PlexinB1
PlexinB2
PlexinB3
0
-20
-40
-60
Percentage of change after Fc-5A treatment
*
*

(C)


Sema5A receptor
Control
5A OE
Synapses (per μm)
0.3
0.2
0.1
0.0
##
##
#
*
***
Control
PlexinA1
PlexinA2
PlexinA3
PlexinB1
PlexinB2
PlexinB3

(D)

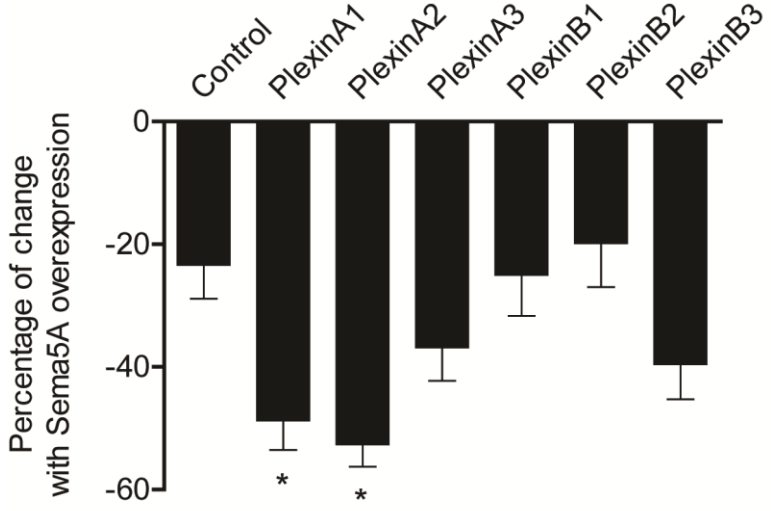

Control
PlexinA1
PlexinA2
PlexinA3
PlexinB1
PlexinB2
PlexinB3
0
-20
-40
-60
Percentage of change with Sema5A overexpression
*
*

Figure 3.2 TAG-1 is necessary for Sema5A-mediated synapse elimination, whereas its overexpression does not enhance the effect of Sema5A on decreasing synapse density. (A) Representative dendritic segments of hippocampal neurons expressing GFP plus pDisplay vector (Control) or Sema5A and/or Sema5B constructs and treated with TAG-1 antibody. Neurons were stained for PSD-95 (cyan) and vGlut1 (magenta). Black Puncta in colocalized mask images indicate excitatory synapses.

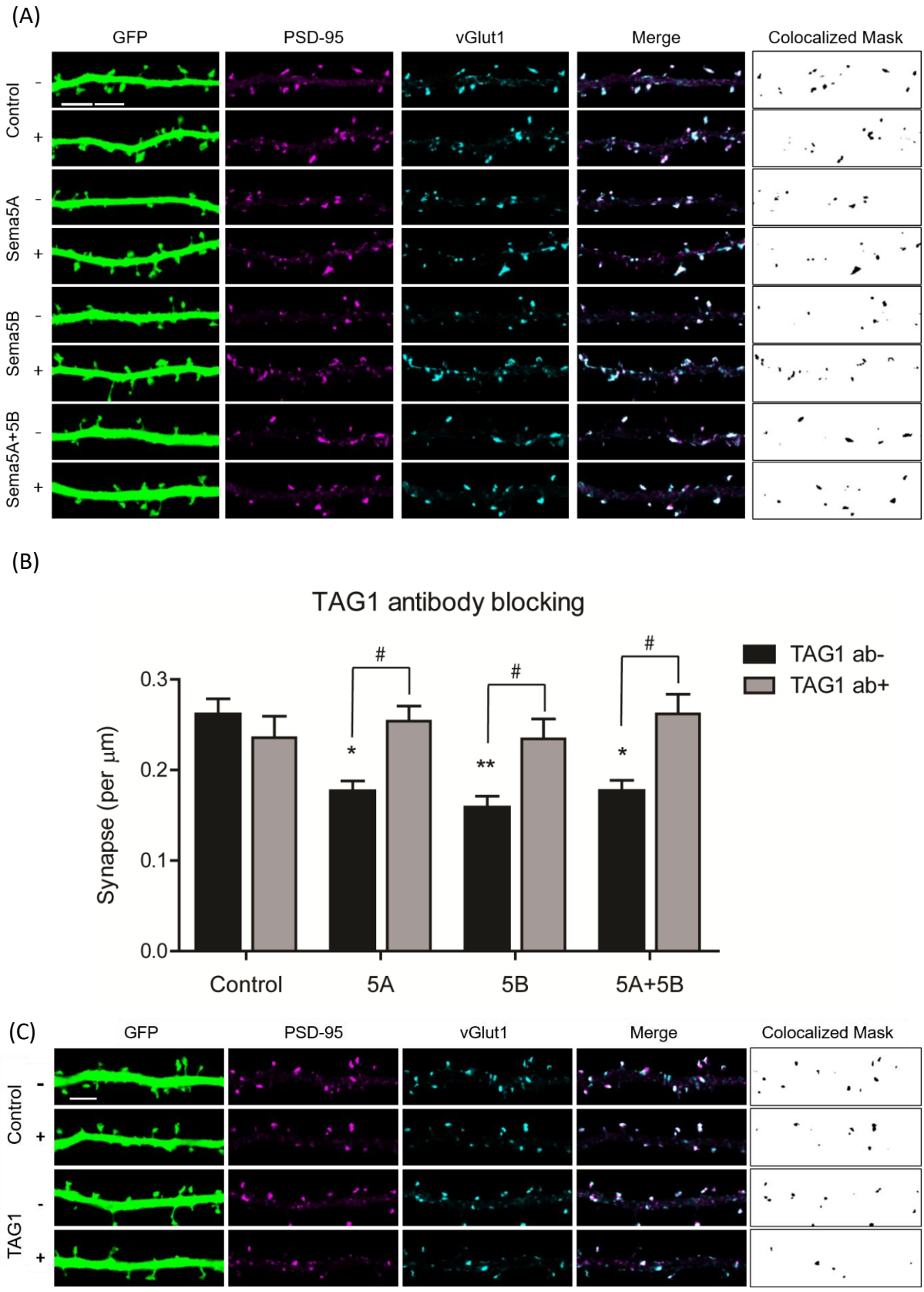
(B) Quantification of synapse density of neurons shown in panel A. Overexpression of Sema5A and/or Sema5B results in a significant reduction of synapses, which is completely rescued by the blocking of TAG-1 activity. Asterisks denote significance relative to control neurons without TAG-1 antibody treatment, and hashtags denote significance within groups with or without TAG-1 antibody treatment.

(C, F) Representative dendritic segments of hippocampal neurons expressing GFP with/without TAG-1 and treated with 25 nM Fc-5A for 2h (C), or neurons co-expressing GFP, TAG-1 and Sema5A (F). Neurons were stained for PSD-95 (cyan) and vGlut1 (magenta). Black Puncta in colocalized mask images indicate excitatory synapses.

(D, G) Quantification of synapse density of neurons shown in panel C and F. Overexpression of TAG-1 does not enhance the effect of Fc-5A treatment (D) or Sema5A overexpression (G) to decrease the synapse density. Asterisks denote significance relative to control neurons without TAG-1 antibody treatment, and hashtags denote significance within groups with or without TAG-1 antibody treatment.

(E, H) Quantification of percentage of change in synapse density relative to the same condition without TAG-1 antibody treatment shown in panel D and G.

Scale bars in panel A, C and F represent 5 μm. $N \geq 23$ neurons per condition from 3 separate cultures. *, $^{\#}p<0.05$, **, $^{\#\#}p<0.01$, ***$p<0.001$, n.s., nonsignificant, one way Anova with Tukey's post hoc test.


(A)
GFP
PSD-95
vGlut1
Merge
Colocalized Mask
Control
Sema5A
Sema5B
Sema5A+5B
-
+
(B)
TAG1 antibody blocking
TAG1 ab-
TAG1 ab+
Synapse (per μm)
0.3
0.2
0.1
0.0
#
*
**
Control
5A
5B
5A+5B
(C)
GFP
PSD-95
vGlut1
Merge
Colocalized Mask
Control
TAG1
-
+

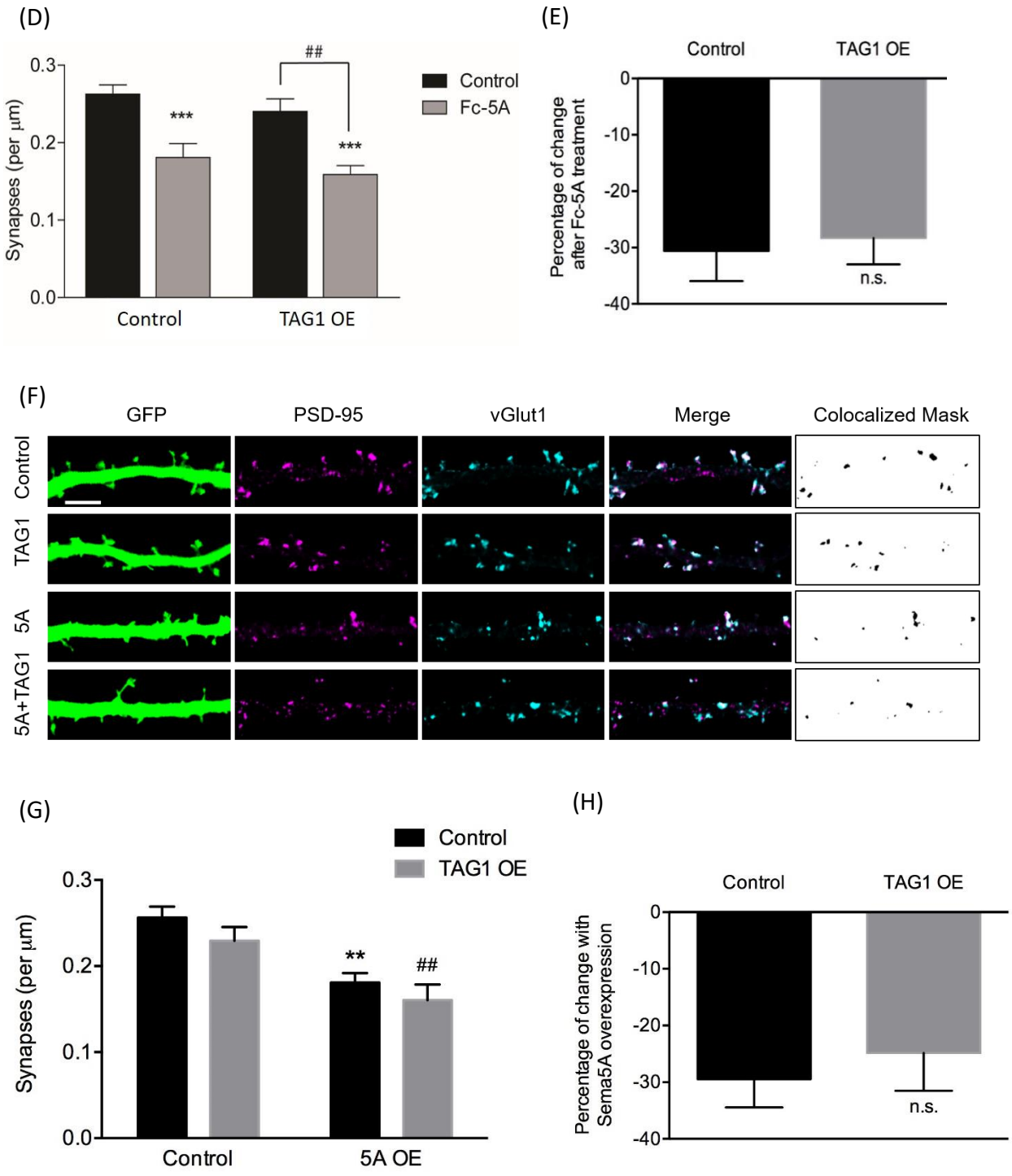
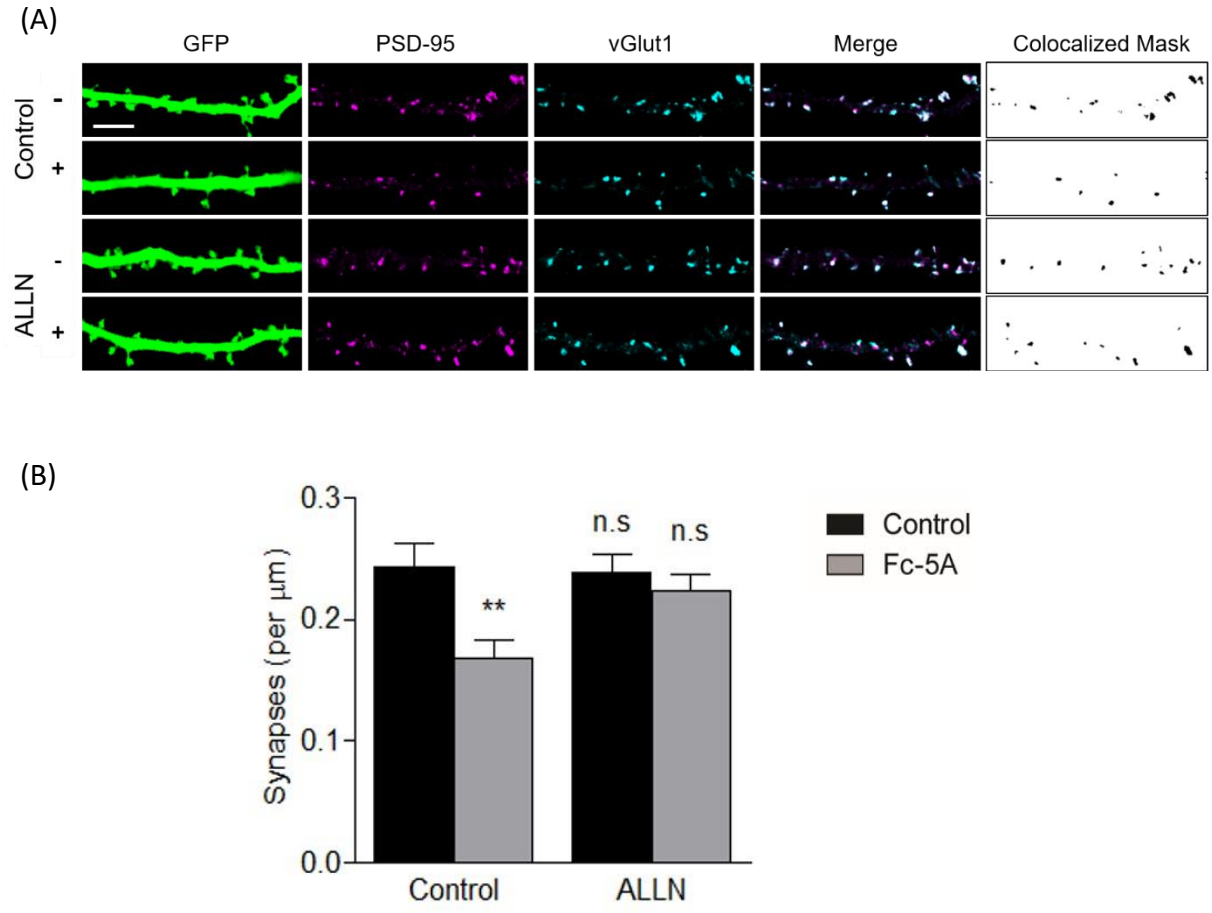

(D)
Synapses (per μm)
0.3
0.2
0.1
0.0
***
##
***
Control
Fc-5A
Control
TAG1 OE
(E)
Control
TAG1 OE
Percentage of change after Fc-5A treatment
0
-10
-20
-30
-40
n.s.
(F)
GFP
PSD-95
vGlut1
Merge
Colocalized Mask
Control
TAG1
5A
5A+TAG1
(G)
Control
TAG1 OE
Synapses (per μm)
0.3
0.2
0.1
0.0
**
##
Control
5A OE
(H)
Control
TAG1 OE
Percentage of change with Sema5A overexpression
0
-10
-20
-30
-40
n.s.

Figure 3.3 Inhibition of calpain rescues the elimination of synapses by Sema5A.
(A) Representative dendritic segments of hippocampal neurons expressing GFP pre-treated with 20 μM ALLN for 24h, and then bath applied with 25 nM Fc-5A for 2h. Neurons were stained for PSD-95 (cyan) and vGlut1 (magenta). Black Puncta in colocalized mask images indicate excitatory synapses.

(B) Quantification of synapse density of neurons shown in panel A. Fc-5A treatment resulted in a 30% reduction of synapses, which was completely rescued by the addition of ALLN.

Scale bar represents 5 μm. N≥30 neurons per condition from 3 separate cultures. *p<0.05, n.s., nonsignificant to control neurons without ALLN treatment, one way Anova with Tukey's post hoc test.



3.4 Discussion

In the previous chapter, I presented evidence that Sema5A may be responsible in part for the synapse elimination observed after cLTD in hippocampal neurons. To understand the molecular mechanisms of Sema5A signaling during synapse elimination and synaptic plasticity, I attempted to identify its receptor(s) and signaling pathway using multiple analyses. By overexpressing candidate receptors *in vitro*, I showed that Sema5A mediated synapse elimination can be enhanced by overexpressing Plexin A1 and Plexin A2. Subsequently, I found that blocking the adhesion molecule TAG-1 resulted in an abrogation of Sema5A-mediated synapse elimination. Furthermore, I found that inhibiting calpain activity prevented the synapse elimination mediated by Sema5A, suggesting calpain is an integral component of Sema5A signaling.

3.4.1 Plexin A1 and Plexin A2 Are Potential Receptors for Sema5A

Several Plexins have been identified as the receptor for Sema5A in multiple types of non-neuronal cells and neuronal cells (Oster, Bodeker et al. 2003; Artigiani, Conrotto et al. 2004; Goldberg, Vargas et al. 2004; Li and Lee 2010; Matsuoka, Chivatakarn et al. 2011; Li, Law et al. 2012; Duan, Wang et al. 2014). Here, I show that synapse elimination after Fc-5A treatment or Sema5A overexpression is enhanced by increased expression of either PlexinA1 or PlexinA2, suggesting that they are potential receptors for Sema5A and can signal synapse elimination. It is worth noting that our results are obtained from gain-of-function experiments and need to be confirmed by loss-of-function experiments. In addition, although currently there is no single Plexin candidate that I found to affect synapse density, it will still be worth

confirming whether overexpression of Plexin constructs alone can change hippocampal synapse density or not.

3.4.2 Sema5A Functions Through TAG-1

In 2014, Liu et al. examined the role of TAG-1 in regulating sensory afferents projecting into the developing spinal cord (Liu, Wang et al. 2014). Consistent with the observations in chick, I found bath application of an anti-TAG-1 antibody abrogated the Sema5A-induced decrease of synapse density, suggesting that TAG-1 was required for Sema5A to mediate its function in synapse elimination. It will be of interest to examine whether anti-TAG-1 antibody of other species such as human has the same effect on blocking the synapse elimination induced by Sema5A overexpression. In dorsal root ganglia (DRG) neurons, TAG-1 interacts directly with neuropilin 1, a co-receptor for Sema3A and regulates Sema3A signaling by differential endocytotic trafficking (Dang, Smythe et al. 2012). Whether it functions in a similar manner for Sema5A signaling remains to be tested.

Strikingly, when TAG-1 is overexpressed, neurons did not show an enhanced degree of synapse elimination in response to Fc-5A treatment or Sema5A overexpression. Thus, TAG-1 does not transduce Sema5A signals in a concentration-dependent manner. Together, our data support the idea that TAG-1 is a component of the Sema5A receptor complex and serve as a "switch" to Sema5A signaling, in which a certain level of TAG-1 activity is necessary to activate signaling pathways downstream of the Sema5A/Plexin complex, whereas addition of excessive TAG-1 does not promote signaling.

3.4.3 Calpain is Downstream of Sema5A Signaling

Calpains are calcium-dependent cysteine proteases highly expressed in the CNS and regulate numerous cellular processes. For example, by selective cleavage of components of adhesion complexes, calpains play critical roles in the regulation of cell motility (Carragher and Frame 2002). In this dissertation, by showing that Sema5A-mediated synapse elimination is lost after inhibition of calpain activity, I provided evidence that calpain is required for the function of Sema5A. This is not the first time calpain has been suggested to be part of the signaling pathway of Semaphorins. Previous studies have shown Sema3A and Sema5B induce growth cone collapse by stimulating calpain-dependent disassembly of actin meshwork (To, Church et al. 2007; Qin, Liao et al. 2010; Kaczmarek, Riccio et al. 2012). Additionally, the response of precrossing commissural axons to Sema3B through Plexin A1 is mediated by calpain-1 activity, which is suppressed by floor plate signals (Nawabi, Briancon-Marjollet et al. 2010). The molecular mechanism transuding the signals from Semaphorins to calpains is unknown, however, the MAPK/ERK pathway is a strong candidate to mediate Semaphorin-induced calpain activity. On one hand, MAPK/ERK activation has been observed in the axon turning response and axon outgrowth downstream of Sema3A, Sema4D and Sema7A (Campbell and Holt 2003; Pasterkamp, Peschon et al. 2003; Aurandt, Li et al. 2006; Ito, Morita et al. 2014);while on the other hand, phosphorylation of calpain-2 mediated by MAPK/ERK stimulates its catalytic activity to degrade cytoskeletal proteins, which is required for adhesion turnover and cell migration (Glading, Chang et al. 2000; Cuevas, Abell et al. 2003; Zhang, Liu et al. 2011).

It appears that calpains also function in regulating synaptic efficacy and structure. A number of calpain substrates are proteins that are localized in various components of the synapse,

which include but are not limited to PSD-95, cortactin, cadherin, β-catenin and NMDAR (Bi, Bi et al. 1998; Lu, Rong et al. 2000; Perrin, Amann et al. 2006; Abe and Takeichi 2007; Jang, Jung et al. 2009). Though the substrate(s) of calpain in Sema5A signaling pathway remains to be examined, this list implicates a possible direct mechanism for Sema5A regulation of calpain-dependent synaptic elimination in hippocampal neurons (Lynch and Baudry 1987; Vinade, Petersen et al. 2001; Wu and Lynch 2006; Abe and Takeichi 2007; Liu, Liu et al. 2008).

Chapter 4: Discussion

Accumulating studies have begun to develop the idea that, in addition to axon guidance, Semaphorins and other axon guidance molecules also regulate numerous other processes during development to establish proper neural circuitry (Shen and Cowan 2010; Yoshida 2012). In this dissertation, I demonstrate that two class 5 Semaphorins, Sema5A and Sema5B, regulate dendritic elaboration by inhibiting the dendritic growth and arborisation. Moreover, Sema5A and Sema5B negatively regulate the density of excitatory synapses by driving active elimination of existing synapses and therefore contribute to the regulation of activity-dependent synaptic plasticity. Finally, I examined potential Sema5A's receptor(s) and signaling pathway that transduce Sema5A activity into synapse elimination. Taken together, these results suggest that Sema5A and Sema5B refine the organization of neural circuits through the coordinated inhibition of dendritic growth and synaptic connections in rodent hippocampal neurons.

4.1 Sema5A and Sema5B Negatively Regulate Dendritic Tree Development

Dendritic development is crucial for the establishment of precise neural networks. Previous data has shown that Sema3A mediates dendritic growth and orientation of cortical pyramidal neurons (Polleux, Morrow et al. 2000; Fenstermaker, Chen et al. 2004). Moreover, Sema6A and its receptor PlexA4 control dendritic growth of motor neurons through the action of FERM, RhoGEF and pleckstrin domain-containing protein 1 (FARP1) (Zhuang, Su et al. 2009). Consistent with these studies, I found that Sema5A and/or Sema5B overexpression decreased the total dendritic length, branching number and dendritic complexity of neurons

(Figure 2.2), suggesting a role for Sema5A and Sema5B in constraining dendritic growth and arborisation.

Currently the mechanisms underlying the functions of Sema5A and Sema5B in dendritic branching is unknown. However, it has been found that calpain, which is downstream of Sema5A signaling in synapse elimination, is also activated by compartmentalized calcium transients to trigger dendrite pruning in *Drosophila* sensory neurons (Kanamori, Kanai et al. 2013). Moreover, calpain is required for the dendritic remodeling after neuronal injury (Faddis, Hasbani et al. 1997). Considering the wide distribution of calpain in the CNS, it is likely that calpain is also involved in the signaling of Sema5A to negatively regulate dendritic growth and arborisation. On the other hand, duplication of Methyl-CpG binding protein 2 (MECP2) gene, which cause autistic behaviors, has been found to cause severe defects of dendritic arborisation and spine dynamics (Jiang, Ash et al. 2013). Given the fact that MECP2 regulates sensory function through Sema5B and Robo2 (Leong, Lim et al. 2015), it is worthing testing whether MECP2 is also involved in Sema5A signaling to regulate dendritic arborisation.

Since our results are based on gain-of-function experiments employing cultured hippocampal neurons, it would be interesting to examine whether Sema5A and/or Sema5B knockdown evokes the opposite effects on dendritic arbors. In addition, to further understand the importance of Sema5A/Sema5B in circuit function, all of these experiments should be replicated *in vivo*.

4.2 Sema5A and Sema5B Mediate Excitatory Synapse Elimination

During development, an excess of neural connections is formed between different regions of the brain. The neural circuitry is subsequently refined as the brain matures, maintaining necessary synapses and eliminating surplus ones so that synapses are formed at the right time and place. Failure in this process is thought to underlie several neurological disorders including mental retardation, autism and schizophrenia (Eastwood 2004; Bassell and Warren 2008; Sudhof 2008). Therefore, the mechanisms directing synapse elimination are fundamental to neural development and plasticity.

Recent work has identified critical roles of several axon guidance cues in regulating synapse formation and function (Shen and Cowan 2010; Yoshida 2012), while only a few studies have focused on axon guidance molecules as being important regulators of synapse elimination. It has been demonstrated that both myocyte enhancer factor 2 (MEF2) transcription factors and ephexin5 suppress excitatory synapse numbers in hippocampus (Flavell, Cowan et al. 2006; Margolis, Salogiannis et al. 2010), and RSY-1 antagonizes presynaptic assembly in *C. elegans* (Patel and Shen 2009). As to the Semaphorin family, Sema5B was previously showed to mediate synapse elimination (O'Connor, Cockburn et al. 2009). Application of soluble Sema3A induces rapid synaptic depression in adult CA1 hippocampal neurons (Bouzioukh, Daoudal et al. 2006). Another class 3 Semaphorin, Sema3F, negatively regulates spine development and synaptic structure by governing the spatial distribution and morphogenesis of dendritic spines (Sahay, Kim et al. 2005). These finding demonstrate that the signaling of Semaphorins refines complex circuit organization in the CNS by negatively regulating synapse homeostasis.

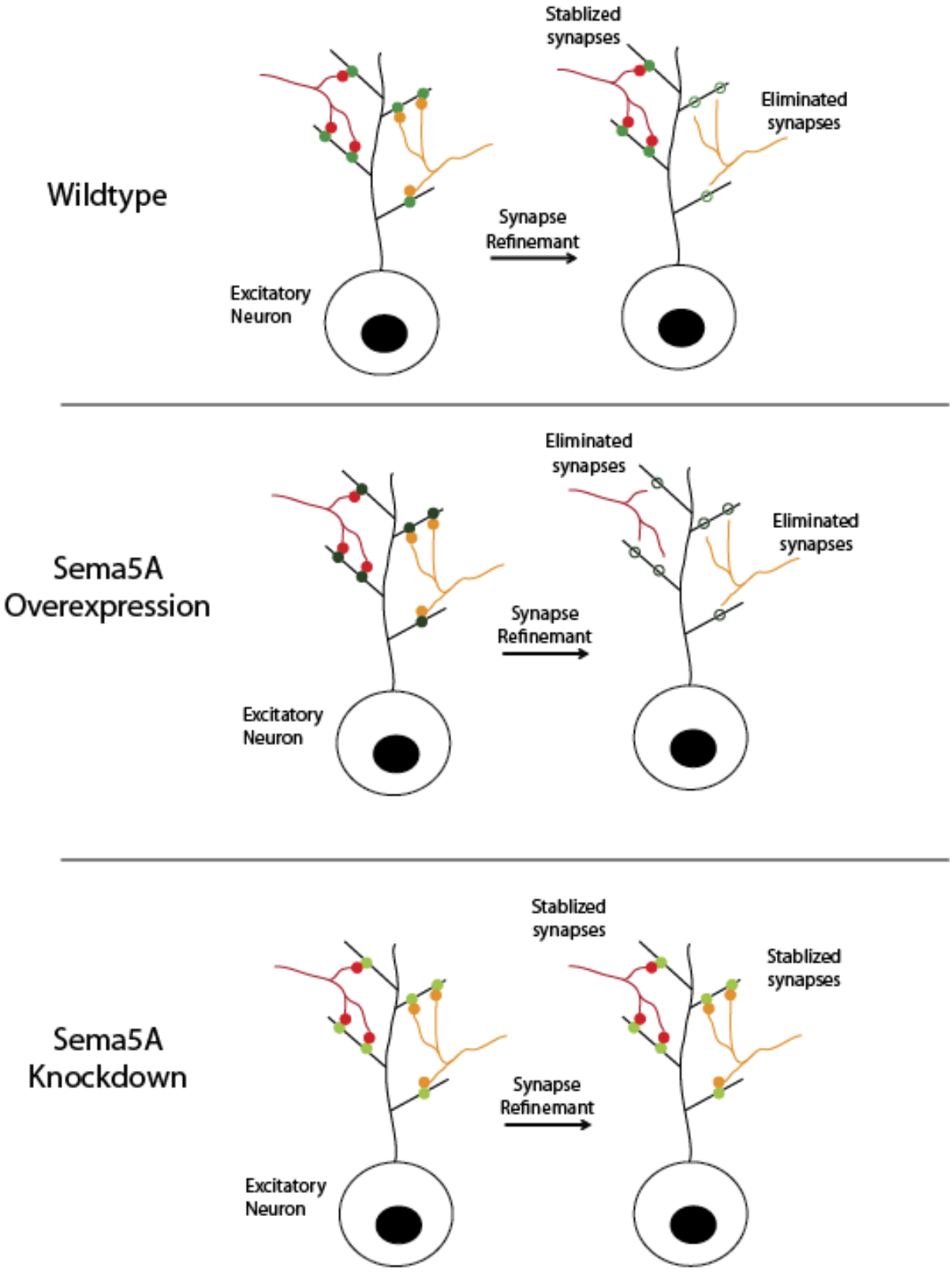
Using time-lapse imaging, I found that Sema5A is an active eliminator of existing synapses but not a suppressor of synaptogenesis (Figure 2.8). This elimination specifically targets excitatory synapses, since the density of inhibitory synapses was unaffected. Together with the previous results on Sema5B (O'Connor, Cockburn et al. 2009), my data demonstrate that both Sema5A and Sema5B eliminate excitatory synapses to control the balance between excitatory and inhibitory connections, thereby ensuring the exquisite balance of activity in neural circuits (Figure 4.1).

Figure 4.1 Schematic illustration of Sema5A function in synapse elimination.
During the establishment of neural circuit, the refinement of synaptic connections is constantly undertaken by stabilizing necessary synapses (filled circles) and eliminating surplus synapses (open circles) in response to environmental signals.

(Upper panel) In wild-type neurons, the synapse density is maintained at a proper level.

(Middle panel) In neurons with Sema5A overexpression, extra synapses are eliminated by Sema5A thus the synapse density decreases.

(Bottom panel) In neurons lacking Sema5A expression, less synapses are eliminated by Sema5A thus the synapse density increases.


Wildtype
Stablized synapses
Eliminated synapses
Synapse Refinemant
Excitatory Neuron
Sema5A Overexpression
Eliminated synapses
Eliminated synapses
Synapse Refinemant
Excitatory Neuron
Sema5A Knockdown
Stablized synapses
Stablized synapses
Synapse Refinemant
Excitatory Neuron

4.3 Sema5A and Sema5B Negatively Regulate Activity-Dependent Synaptic Plasticity

A number of studies have shown that the levels of some Semaphorins and neuropilins (Sema3F/neuropilin 2 and Sema3A/neuropilin 1/Plexin A complex) are affected by neural activity (Shimakawa, Suzuki et al. 2002; Barnes, Puranam et al. 2003; Holtmaat, Gorter et al. 2003; O'Donnell, Stemmelin et al. 2003; Lee, Kim et al. 2012), however there has not been a demonstration that they are involved in regulating the synapse structure in response to activity. To the best of my knowledge, this study is the first work to show that Semaphorins can directly regulate activity-dependent synaptic refinement in the nervous system.

Synaptic competition is the primary mechanism for activity-dependent refinement of the nervous system during development. This mechanism claims that competitive interactions within neighboring synapses are associated with synaptic structural plasticity. Specifically, high-frequency glutamatergic stimulation at individual spines, which leads to input-specific synaptic potentiation and eventually the shrinkage and weakening of nearby inactive synapses (Oh, Parajuli et al. 2015). The competition resulted in a substantial number of synapses being removed, while the survivors become stabilized and mature. Based on this theory, I propose a model which posits that, to refine neural circuity, synapses are continuously disassembled and reformed in an activity-dependent manner, and Sema5A can control both of these processes and change the balance between excitation and inhibition. By up- or down-regulating Sema5A surface levels, LTP and LTD activity control overall synapse density and neuronal excitability. This is supported by the observation that levels of Sema5A at the neuronal surface increase following LTD (Appendices Figure 2), presumably

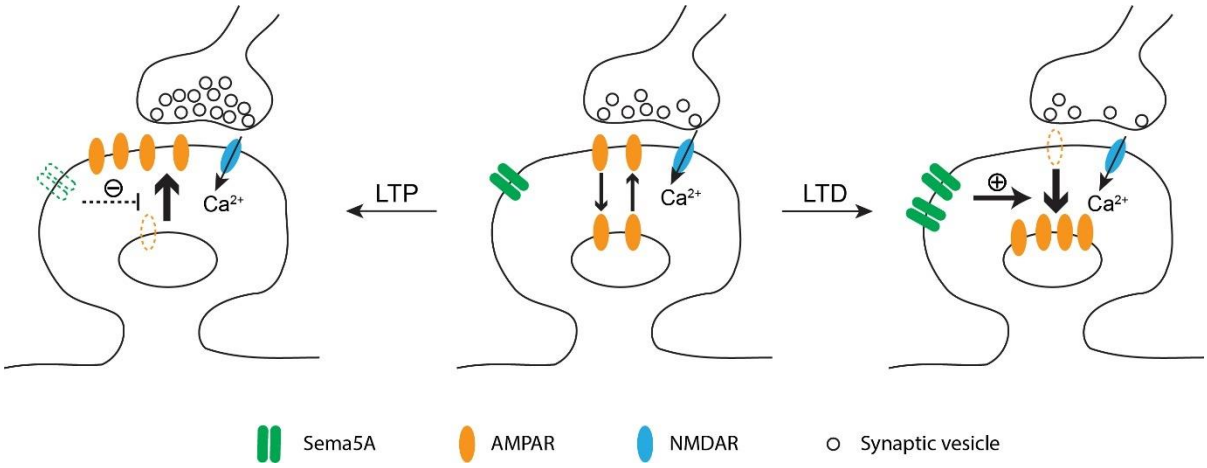
to interact with Sema5A receptor(s) and stimulate synapse elimination. Thus, the overexpression of Sema5A counteracts LTP-mediated increase in synapse density, and the knockdown of Sema5A blocks LTD-mediated decrease in synapse density. However, it is puzzling that Sema5A knockdown doesn't further improve LTP-mediated increase in synapse density, which I postulate is due to synaptic scaling to compensate for the changes in synapse number and maintain stable synaptic strength. To further validate this model, it will be important to determine whether Sema5A levels decrease at the cell surface in response to LTP.

How exactly do the changes in surface Sema5A level translate into the changes in synapse density? Our finding that Fc-5A treatment reduced surface GluA1 expression may help to fill this gap. Biotinylation experiment showed that bath application of Fc-5A for 1h caused a 25% reduction of surface GluA1 without changing the total level of GluA1. Because GluA1 is an indicator of AMPAR recruitment (Hayashi and Shirao 1999; Hayashi, Shi et al. 2000), Sema5A may regulate activity-dependent synaptic plasticity through the endocytosis of AMPAR and subsequent destabilization of presynaptic inputs and loss of glutamatergic synapses (Ripley, Otto et al. 2011). Therefore, it is tempting to speculate that by redistributing GluA1 from synapses, Fc.5A treatment destabilizes presynaptic inputs and leads to structural elimination of excitatory synapses (Figure 4.2). According to our model, the level of surface AMPARs is maintained by a proper balance between endocytosis and exocytosis. Under LTP conditions, Sema5A is internalized from the cell membrane, thus exhibiting less inhibitory effect on the AMPARs exocytosis. While under LTD conditions, Sema5A is inserted into the cell membrane, and promotes the endocytosis of AMPARs.

Figure 4.2 A hypothetical model of Sema5A regulating synaptic plasticity through restricting the synaptic delivery of AMPA receptors.
(Middle Panel) Under basal condition, AMPARs are undergoing continuous exocytosis and endocytosis, and a constant level of AMPARs on the cell surface is maintained by balancing these two opposite trafficking processes. Cell surface Sema5A mediates the redistribution of GluA1 from synapses.

(Left Panel) LTP stimulus induces AMPAR insertion at the cell surface. LTP-induced Sema5A internalization promotes the exocytosis of AMPARs in the spines.

(Right Panel) LTD stimulus induces AMPAR internalization from the cell surface. LTD-induced Sema5A withdrawal promotes the endocytosis of AMPARs in the spines.



4.4 Sema5A Signaling in Synapse Elimination and Synaptic Plasticity

Interaction between transmembrane Semaphorins and Plexins can be either *in cis* or *in trans*, leading to different downstream signaling events (Haklai-Topper, Mlechkovich et al. 2010; Tawarayama, Yoshida et al. 2010). Considering that the soluble Sema5A eliminates synapses to a similar extent as when Sema5A is overexpressed (Figure 2.6), it seems likely that Sema5A functions cell autonomously through an appropriate receptor complex. A previous works on Sema5A in dentate granule cells also support this hypothesis (Duan, Wang et al. 2014). This *in cis* interaction may activate Plexin signaling and lead to the regulation of synaptic circuits in the same way transmembrane Semaphorin SMP-1 activates the class A

Plexin homologue PLX-1 in *C. elegans* motoneuron axons, regulating its subcellular localization and leading to the inhibition of synapse formation (Mizumoto and Shen 2013).

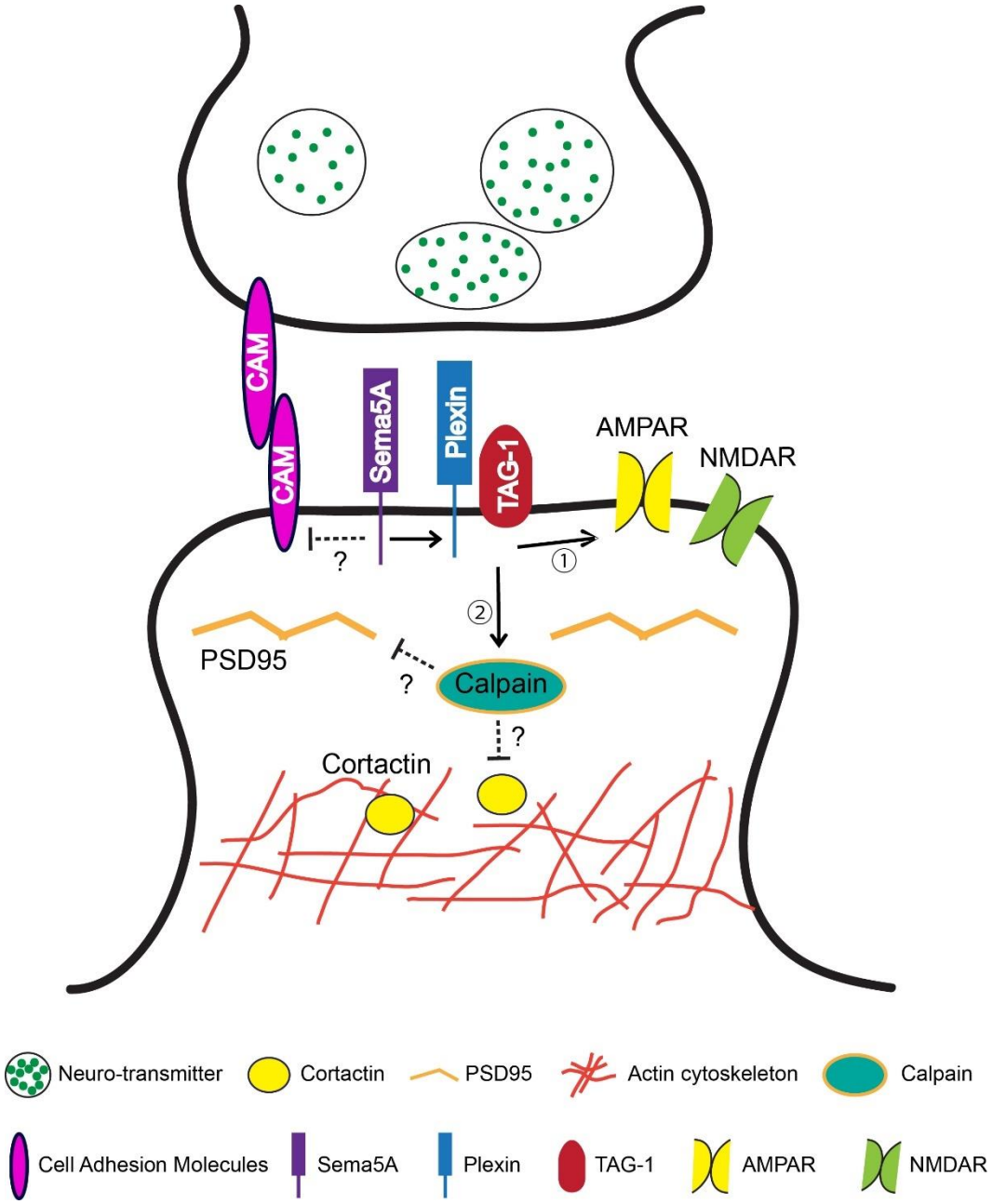
Our data suggests that both Plexin A1 and Plexin A2 are capable of facilitating the function of Sema5A in hippocampal neurons *in vitro*. However, whether they are the actual functional receptors of Sema5A has to be further tested by loss-of-function and *in vivo* experiments. The downstream signaling of Sem5A after receptor binding is even less understood. The initial intracellular molecular event that occurs following Sema5A binding to Plexin A1/Plexin A2 is possibly the activation of the GTPase domain of Plexins to control the level of small GTPase. For instance, in glioma cells Rac1 is inactivated by Sema5A to suppress cell motility (Li and Lee 2010; Li, Law et al. 2012). Whether the same signaling event is involved during synapse remodeling by Sema5A is unknown. HSPGs and CSPGs have been shown to bind to Sema5A and alter its function (Kantor, Chivatakarn et al. 2004). Multiple research groups have found that HSPGs are required for distinct aspects of synapse development such as synaptogenesis, synaptic plasticity and neural activity modulation (Lauri, Kaukinen et al. 1999; Dityatev, Dityateva et al. 2004; Johnson, Tenney et al. 2006; Allen, Bennett et al. 2012; Siddiqui, Tari et al. 2013; Song and Kim 2013; Korotchenko, Cingolani et al. 2014). It is tempting to speculate that HSPGs/CSPGs may be important co-factors of Sema5A that regulate synapse elimination in the hippocampus.

Though the signaling pathways of Sema5A/Sema5B regulating dendritic growth and synapse density may not be identical, the actin cytoskeleton has been highlighted as the pivotal target in both processes (Zhang and Benson 2001; Etienne-Manneville and Hall 2002; Okamoto, Nagai et al. 2004; Ethell and Pasquale 2005), and is a common target of several Semaphorin signaling pathways (Bielenberg, Shimizu et al. 2008; Brown and Bridgman 2009; Hung and

Terman 2011; Li, Law et al. 2012). Therefore, it is not surprising that the structural elimination of hippocampal synapses mediated by Sema5A is accomplished through actin destabilization and disassembly.

Figure 4.3 summarizes a proposed mechanism underlying Sema5A-meidated synapse refinement. Sema5A is expressed on the postsynaptic membrane and activates its Plexin receptor(s). In the presence of TAG-1, the interaction between Sema5A and Plexin stimulates both short-term and long-term effects on synaptic plasticity. In particular, Sema5A can evoke rapid response of AMPARs to be recruited from cell surface, which leads to the decrease in synaptic strength and the destabilization of presynaptic inputs. Meanwhile, Sema5A also stimulates a relatively slow pathway through calpain, which presumably targets scaffolding proteins such as PSD-95 and regulators of actin cytoskeleton such as cortactin to induce the actin disassembly, eventually leading to structural elimination of synaptic components. In addition, a huge number of studies have emphasized the importance of cell adhesion molecules in the function and plasticity of synaptic connections. Whether Sema5A also signals through cell adhesion molecules to mediate synaptic refinement is an intriguing question that awaits further study.

Figure 4.3 A proposed model describing Sema5A-induced signaling cascades involved in synaptic refinement.
Sema5A and Plexin(s) are both located at postsynaptic membrane, and their *in cis* interaction induces both (1) the fast response to redistribute AMPARs, and (2) the slow response by activating calpain to cleave its postsynaptic substrates such as PSD-95 and cortactin. Given the importance of cell adhesion molecules in synaptic function and plasticity, Sema5A also possibly interacts with some cell adhesion molecules to mediate synaptic refinement.



4.5 Sema5A and Autism Spectrum Disorders

After the initial phase of neuronal outgrowth and growth cone navigation, the expression level of most axon guidance molecules diminishes, whereas Sema5A and Sema5B retain their expressions throughout life into adulthood (Figure 2.1) (Duan, Wang et al. 2014).

Therefore, it is interesting to speculate that Sema5A and Sema5B continue to function as regulators of synapse structural plasticity. If so, the abnormalities of their regulation or function may contribute to some neurodevelopmental disorders. Indeed, Sema5A has been implicated in ASDs (Weiss, Arking et al. 2009), which is a behavioral syndrome characterized by impaired social interactions. The role of *SEMA5A* as an ASD susceptibility gene has drawn increasing attention by the mounting evidence from genome-wide association studies (GWAS), cDNA microarray assay and RNA profiling of brains (Melin, Carlsson et al. 2006; Prandini, Pasquali et al. 2012; Cheng, Quinn et al. 2013; Mosca-Boidron, Gueneau et al. 2016). Results presented here are consistent with the hypothesis that the disruption of the Sema5A function to prune superfluous or incorrect connections during hippocampal development can contribute to the improper development of neural circuitry, a pathology characteristic of ASDs. Our study on Sema5A function may provide insights into the general mechanisms of circuit formation and the specific etiology of ASDs.

4.6 Perspective and Future Experiments

In Chapter 3, I explored the mechanisms underlying Sema5A-induced synapse elimination. However, to more fully understand how Sema5A eliminates synapses, a number of additional experiments will be required. The following is a brief summary of the experiments that address some of the weaknesses that remain:

1) PlexinA1 and PlexinA2 have previously been shown to be possible receptors for Sema5A. To determine whether PlexinA1 and/or PlexinA2 are necessary for Sema5A to eliminate synapses, shRNA constructs against PlexinA1 and PlexinA2 should be generated and transfected into hippocampal neurons combined with Sema5A co-overexpression or Fc-5A

treatment, and examine whether PlexinA1 and/or PlexinA2 knockdown impacts Sema5A-induced synapse elimination. I expect that PlexinA1 and PlexinA2 knockdown will totally abrogate Sema5A-induced synapse elimination.

2) To identify the small GTPase that is activated upon Sema5A signaling in hippocampal neurons, I will measure the levels of active Rac1/Cdc42 and RhoA with a GST-PBD pulldown assay or Rhotekin G-LISA assay at various time points after Fc-5A administration. I hypothesize that Sema5A mediates synapse elimination through Rac1 inactivation and this will be observed using the pulldown assay. To further test whether the inactivation of Rac1 contributes to Sema5A-induced synapse elimination, hippocampal neurons can be transfected with a constitutively active Rac1 construct and treated with Fc-5A, and the synapse density will be analyzed to investigate whether Sema5A-mediated synapse elimination is suppressed after constitutive Rac1 activity.

3) To address whether the decreased level of cell surface GluA1 in response to Fc-5A treatment is due to increased GluA1 internalization or an inhibition of GluA1 insertion into the plasma membrane, hippocampal neurons can be surface-biotinylated at 4°C and then transferred back to 37°C and treated with Fc-5A. After treatment, biotinylated receptors on the cell surface can be stripped away and any internalized biotinylated AMPARs can be isolated with streptavidin and measured by western blot with GluA1 antibodies. I hypothesize that Fc-5A treatment will lower the level of GluA1 on the cell surface by promoting AMPAR internalization.

4) To investigate whether Sema5A-induced AMPAR internalization is a key step in the observed synapse elimination, Fc-5A can be bath applied to hippocampal neurons together

with GluA23Y peptide that blocks GluA2 internalization (Hardt, Nader et al. 2014) and the synapse density will be analyzed. This peptide has been used successfully by the Wang laboratory to prevent Clathrin-mediated GluA2-dependent AMPAR endocytosis. Since the vast majority of AMPARs are GluA1/GluA2 or GluA2/GluA3 heteromers, GluA23Y peptide will largely block the endocytosis of synaptic AMPARs. I hypothesize that Fc-5A will fail to eliminate synapses when AMPAR endocytosis is blocked.

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Appendices: Supplementary Data

Figure S1 Neural activity regulates transcription levels of Sema5A and Sema5B.
(a) cLTP treatment significantly decreased Sema5A and Sema5B mRNA expression 30 and 120 minutes following stimulation when compared to untreated cells.

(b) cLTD treatment significantly increased Sema5A and Sema5B mRNA expression 120 minutes following stimulation when compared to untreated cells.

One Way ANOVA with Bonferroni's *post hoc* test, ***p<0.001; n = 12 (qPCR reaction repeated in triplicate from 4 separate cultures).

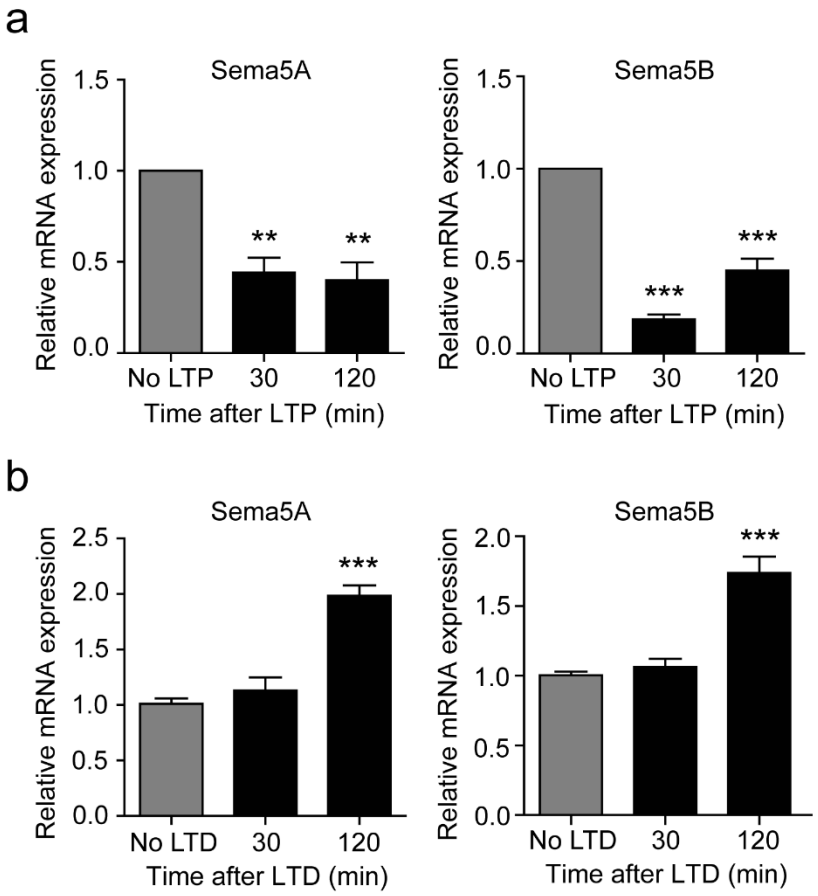
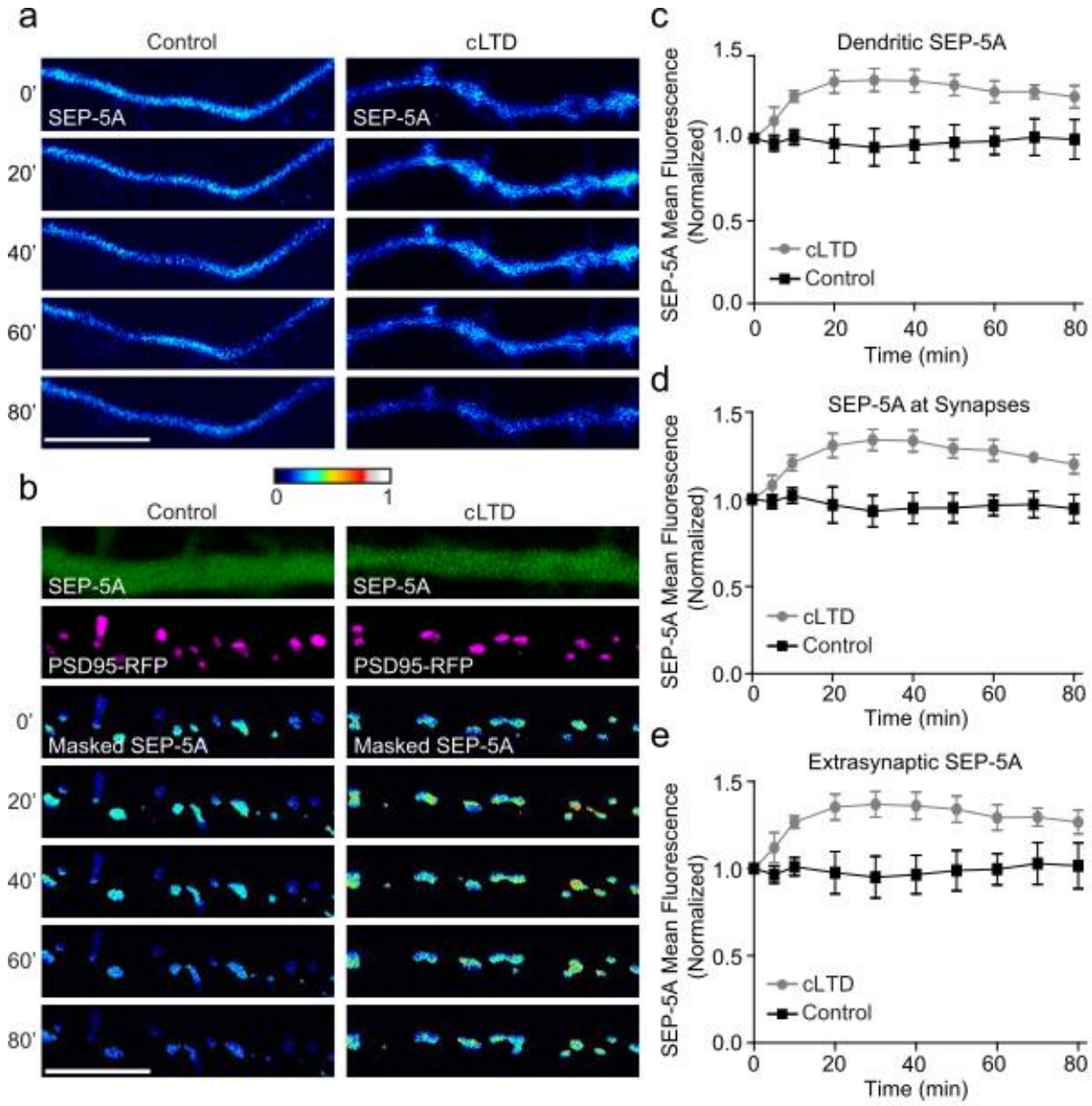


Figure S2 Sema5A is inserted into the plasma membrane following LTD but not specifically to the synaptic membrane.
(a-b) Confocal images of DIV13 hippocampal neurons co-transfected with Sema5A- SEP, PSD-95-RFP and BFP immediately following and 20, 40, 60, 80 minutes after control treatment or cLTD stimulation with 20μM NMDA and 10 μM glycine. Sema5A-SEP fluorescence is diffuse throughout the dendrite and increases after cLTD stimulation. Sema5A-SEP fluorescence is pseudo-coloured to illustrate fluorescence intensity scale from 0 (black) to 1 (white). Scale bars = 3.5μm. (a) Dendritic Sema5A-SEP fluorescence (b) PSD-95-RFP mask of Sema5A-SEP fluorescence to determine fluorescence changes at synapses.

(c) Quantification of dendritic Sema5A-SEP fluorescence overtime compared to fluorescence at 0 min in control cells and following cLTD stimulation (n=4 cells in 3 separate cultures; repeated measures ANOVA $p<0.05$, with post' hoc Bonferroni's test: 20, 30, 40, 50 minutes were significantly different at $p<0.05$).

(d) Quantification of Sema5A-SEP fluorescence at PSD-95-RFP puncta overtime compared to fluorescence at 0 min in control cells and following cLTD stimulation (n=169 and 163 puncta, respectively in 4 cells over 3 separate cultures; repeated measures ANOVA $p<0.0001$, with post'hoc Bonferroni's test: 20, 30, 40, 50, 60, 70, 80 minutes were significantly different at $p<0.01$).

(e) Quantification of Sema5A-SEP fluorescence outside of PSD-95-RFP puncta overtime compared to fluorescence at 0 min in control cells and following cLTD stimulation (n=4 cells in 3 separate cultures; repeated measures ANOVA $p<0.05$, with post' hoc Bonferroni's test: 20, 30, 40 minutes were significantly different at $p<0.05$). (f) Sema5A-SEP fluorescence normalized for photobleaching ("detrended"). (c-f) All graphs show mean ± SEM.


a
Control
cLTD
0'
20'
40'
60'
80'
SEP-5A
b
0
1
PSD95-RFP
Masked SEP-5A
c
Dendritic SEP-5A
SEP-5A Mean Fluorescence (Normalized)
cLTD
Control
Time (min)
d
SEP-5A at Synapses
e
Extrasynaptic SEP-5A