EPHA/EPHRIN-A SIGNALING IN EXCITATORY SYNAPSE DEVELOPMENT

by

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ABSTRACT OF THE DISSERTATION

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Interactions between EphA receptors and ephrin-A ligands have been implicated in the regulation of postsynaptic spine density, astrocytic glutamate transport, and synaptic plasticity. Nonetheless, many aspects of the bidirectional EphA/ephrin-A signaling remain elusive. The goal of this dissertation was to characterize expression of EphA and ephrin-A proteins in hippocampal cultures and to examine effects of ephrin-A activation and EphA inhibition on the development of excitatory synapses in vitro. Affinity probe method utilizing EphA-Fc and ephrin-A-Fc chimeric protein constructs was used to detect endogenous ephrin-A and EphA proteins, respectively, on the surface of living hippocampal cells in culture. Additionally, IIIA4 anti-EphA3 antibody, in combination with ephrin-A3-Fc affinity probe, was employed to characterize expression of surface EphA3 receptor and its ligand-binding ability. Finally, to test how ephrin-A activation and EphA inhibition affect excitatory synapse development, a combination of chronic treatments with clustered and unclustered EphA3-Fc chimeras was utilized along with quantitative immunofluorescence microscopy. Data presented in this dissertation showed that EphA and ephrin-A proteins were present on the surface of

hippocampal astrocytes and in peridendritic areas of hippocampal neurons. In addition, EphA3 receptor, expressed on the surface of hippocampal cells, displayed differential ability for ligand binding. Lastly, activation of ephrin-A reverse signaling increased the expression of postsynaptic protein PSD-95 and counteracted the repulsive effects of EphA receptor inhibition on synapse formation. These results indicate that EphA/ephrin-A signaling plays attractive and repulsive roles in the development of excitatory synapses and might be regulated by the EphA receptor differential ability for ligand binding.

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TERMINOLOGY

Ephs : receptor protein tyrosine kinases; named after their expression in **e**rythropoietin-**p**roducing human **h**epatocellular carcinoma cell line; Eph receptors are divided into two classes: EphA and EphB

Ephrins : ligands for Eph receptors; named after a short form for **Eph** family **r**eceptor **in**teracting proteins; ephrins are divided into two classes: ephrin-A and ephrin-B

EphA : refers to all receptors in EphA class (EphA1-EphA8, and EphA10); EphA receptors preferentially bind ephrin-A ligands

Ephrin-A : refers to all ligands in ephrin-A class (ephrin-A1-ephrin-A5); ephrin-A ligands preferentially bind EphA receptors

EphA3 : EphA3 receptor tyrosine kinase; other names: HEK/HEK4 (human), Tyro4 (rat), Mek4 (mouse), cek4 (chicken)

EphA3-Fc: recombinant mouse EphA3-Fc protein chimera consisting of the extracellular domain of EphA3 receptor fused to the Fc fragment of human IgG1 (R&D Systems)

Ephrin-A3-Fc: recombinant mouse ephrin-A3-Fc protein chimera consisting of the ephrin-A3 ligand protein fused to the Fc fragment of human IgG1 (R&D Systems)

MAP2: microtubule-associated protein 2; expressed in neuronal cell bodies and dendrites

GFAP: glial fibrillary acidic protein; astrocytic marker

PSD-95: postsynaptic density protein 95; membrane-associated guanylate kinase (MAGUK); scaffold protein expressed at the postsynaptic terminals of excitatory synapses

VGLUT1: vesicular glutamate transporter 1; protein associated with the synaptic vesicles at the presynaptic terminals of excitatory synapses

CHAPTER 1 INTRODUCTION

Synapse number and function are perturbed in many neurological disorders including Autism Spectrum Disorders, Rett syndrome, Schizophrenia, and Alzheimer's disease (Della Sala and Pizzorusso, 2014; Penzes et al., 2011). Understanding of the factors regulating synapse development and function is needed to gain insight into the pathology of neurological disorders and for design of successful treatments. Eph receptor tyrosine kinases and their ligands ephrins have been implicated in regulation of synapse formation and function (Hruska and Dalva, 2012) and accumulating evidence links disruptions in Eph/ephrin signaling system with a variety of neurological disorders (Calo et al., 2006; Chen et al., 2012; Sheffler-Collins and Dalva, 2012; Wurzman et al., 2015). The main goal of this dissertation is to examine expression of EphA and ephrin-A proteins in the hippocampal network in vitro and to analyze the effects of EphA/ephrin-A signaling on the process of excitatory synapse development.

1.1 Hippocampal Culture as Model System to Study Eph/Ephrin Function in Synaptic Development

The hippocampus is a brain structure implicated in learning and memory, and its neuronal network is classically characterized as a trisynaptic circuit (Amaral and Witter, 1989; Neves et al., 2008). Figure 1.1A shows the three main connections in the hippocampus (Yassa and Stark, 2011). The first type of a synaptic connection, in the hippocampal trisynaptic circuit, is formed between entorhinal cortex axons (perforant

path, pp) and granule cell dendrites in the molecular layer of dentate gyrus (DG). The second type of a synapse occurs between granule cell axons (mossy fibers; mf) and the proximal dendrites of CA3 pyramidal neurons in the stratum lucidum. The third type of a synapse is between axon collaterals of CA3 pyramidal neurons (Schaffer collaterals; Sc) and dendrites of CA1 pyramidal neurons. Figure 1.1B presents a transverse hippocampal section from a postnatal day 14 (P14) mouse that was immunolabeled with markers of different hippocampal cell types according to an immunohistochemical procedure described in the methods section. Calbindin is expressed in a subset of granule cells in DG and labels their axons (mossy fibers) along with their dendrites (Scharfman et al., 2007). Anti-calbindin staining delineates stratum lucidum, were granule cells form synapses with proximal dendrites of CA3 neurons. Additionally, most of the granule cells and CA1 pyramidal neurons express in their nuclei CTIP2 (Williams et al., 2011), which marks the location of DG and CA1 area.

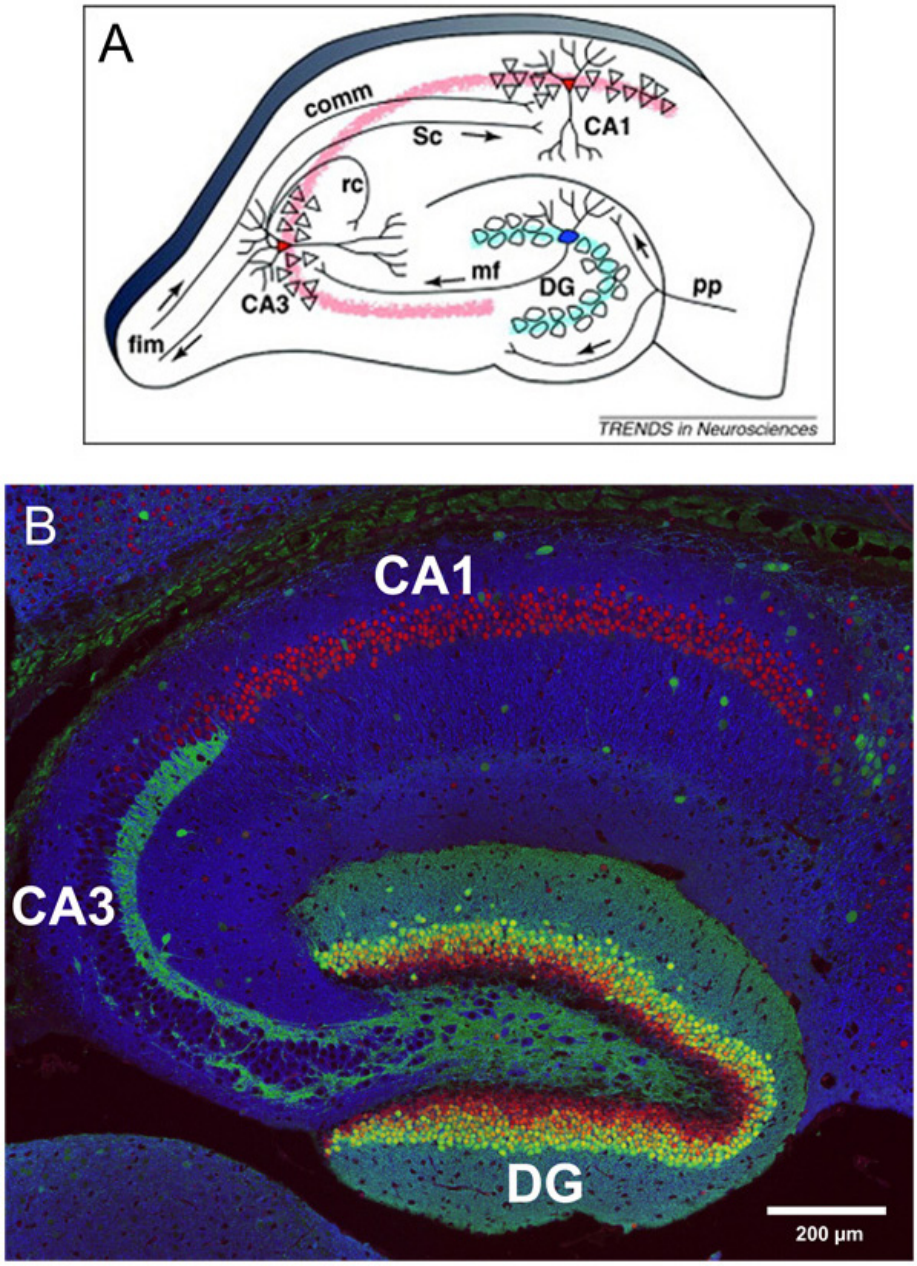

Figure 1.1 Trisynaptic circuit of the hippocampus. A. Diagram showing main connections in the hippocampus (Yassa and Stark, 2011). Synapse type #1 occurs between entorhinal cortex axons (perforant path, pp) and dendrites of granule cells in dentate gyrus (DG). Synapse type #2 is formed between granule cell axons (mossy fibers; mf) and proximal dendrites of CA3 pyramidal neurons. Synapse type #3 is between axon collaterals of CA3 pyramidal neurons (Schaffer collaterals; Sc) and dendrites of CA1 pyramidal neurons. **B.** Transverse section of P14 mouse hippocampus that was immunolabeled with antibodies to calbindin (green), CTIP2 (red), and MAP2 (blue) showing hippocampal cell types and overall architecture.

Mostly feedforward hippocampal network is a well described and useful model to study different types of synaptic plasticity, including long-term potentiation (LTP), while using both in vivo and in vitro methods (Neves et al., 2008). Additionally, primary hippocampal culture became one of the most popular culture systems to study various aspects of neurobiology, including many features of synapse formation and function (Grabrucker et al., 2009). Primary hippocampal cultures have been shown to maintain specificity of synaptic connections (Williams et al., 2011) and they have been successfully employed to study Eph/ephrin function in synapse formation, postsynaptic spine dynamics, and homeostatic synaptic plasticity (Fu et al., 2011; Fu et al., 2007; Margolis et al., 2010). In this dissertation, neonatal/postnatal hippocampal cultures (Figure 1.2) were used to investigate the role of EphA/eprhrin-A signaling in excitatory synapse development.

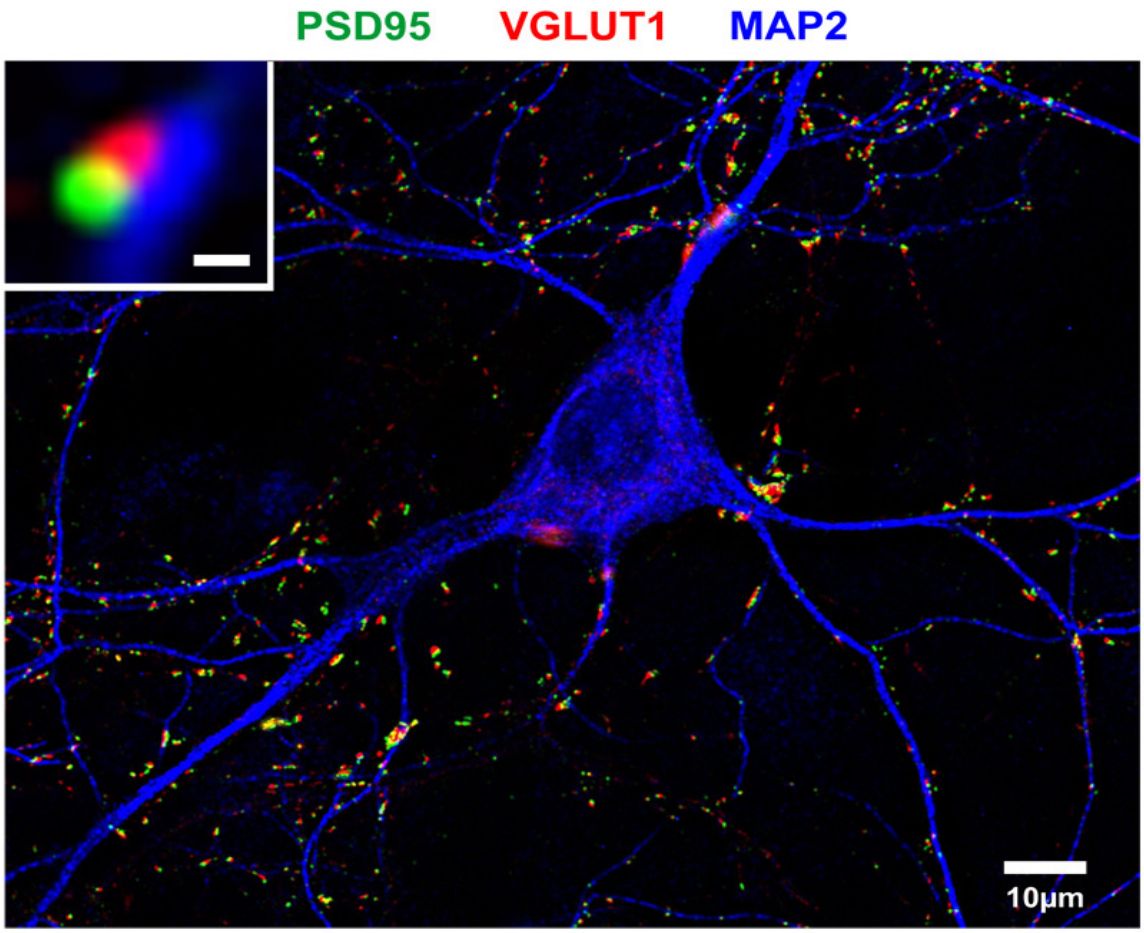

Figure 1.2 Pyramidal neuron in culture at 13 div expressing pre- and postsynaptic markers of excitatory synapses. P1 hippocampal culture at 13 div was immunolabeled with an antibody detecting postsynaptic protein PSD-95 (green), presynaptic protein VGLUT1 (red), and neuronal marker MAP2 (blue). Inset: excitatory synapse with pre- and postsynaptic terminals apposing each other, scale bar 0.3 µm.

1.2 Eph Receptor and Ephrin Ligand Structure and Modes of Signaling

Eph receptors and their binding partners, ephrin ligands, are membrane-bound proteins that can initiate bidirectional signaling in a cell contact-mediated manner. Ephs and ephrins are expressed in specific brain regions, including the hippocampus, and perform important functions in the developing and adult brain. Eph/ephrin signaling has

been shown to regulate cell migration, axon guidance, generation of topographic maps, as well as synaptogenesis and synaptic plasticity (Martinez and Soriano, 2005). Intercellular interactions mediated by Eph receptors and ephrin ligands can lead to repulsion or attraction, depending on the molecular context, and many aspects of these processes are not yet well understood (Hruska and Dalva, 2012; Lisabeth et al., 2013).

Eph receptors belong to the largest subfamily of receptor tyrosine kinases (RTKs). Depending on their structure and binding affinities, Eph receptors and their ligands, ephrins, are categorized into two groups, A and B. In mammals, group EphA consists of nine receptors (EphA1-EphA8, and EphA10) and group EphB includes five receptors (EphB1-EphB4, and EphB6). With respect to the ligands, mammals have five ephrin-A ligands (ephrin-A1-ephrin-A5) and three ephrin-B ligands (ephrin-B1-ephrin-B3) (Egea and Klein, 2007). While EphA receptors preferentially interact with ephrin-A ligands, EphB receptors have highest affinity for ephrin-B ligands. However, there are exceptions to the rule, and they include EphA4 that interacts with both ephrin-A and ephrin-B, and EphB2 that binds ephrin-A5 (Pasquale, 2004) .

Eph receptors are transmembrane proteins with the extracellular portion containing highly conserved ligand-binding domain at the N-terminus (Figure 1.3A). In addition, the extracellular region contains a cysteine-rich segment and two fibronectin type III repeats. In the cytoplasmic portion of Eph receptors, juxtamembrane region links the plasma membrane with the tyrosine kinase domain. Finally, the kinase domain is succeeded by a sterile-α-motif (SAM) and PDZ-binding site at the C-terminus. Ephrin ligands contain N-terminal domain that interacts with Eph receptors (Figure 1.3A).

Ephrin-A ligands are bound to the plasma membrane by the glycosylphosphatidylinositol anchor (GPI anchor), and ephrin-B ligands are transmembrane proteins with a cytoplasmic domain containing PDZ-binding motif (Martinez and Soriano, 2005; Pasquale, 2005).

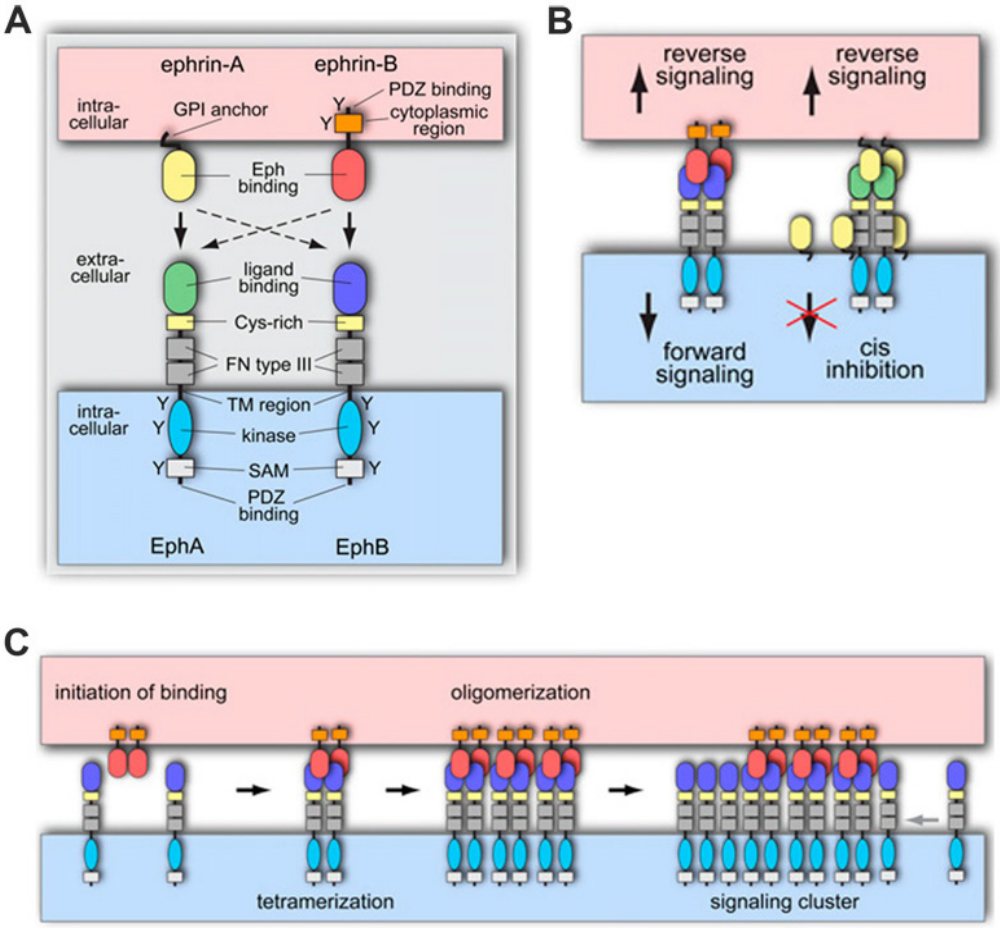

Figure 1.3 Structure of Eph receptors and ephrin ligands. Characteristics of Eph/ephrin bidirectional signaling. A. Eph receptors and ephrin ligands are proteins bound to the cell membrane and initiation of their signaling is cell contact-dependent. In general, EphA class of receptors interacts with ephrin-A ligands, and EphB class of receptors interacts with ephrin-B ligands (with a few exceptions). **B.** Eph receptors bind to ephrin ligands in trans, which can activate receptor-mediated forward signaling and ligand-mediated reverse signaling. In addition, interactions between ephrin-A ligands and EphA receptors in cis can inhibit receptor forward signaling. **C.** Formation of Eph/ephrin heterotetramers starts the bidirectional signaling and is followed by oligomerization of the receptor/ligand complexes. Additionally, ephrin-independent receptor clustering can occur that might result in signal amplification (modified from Pitulescu & Adams, 2010).

Eph/ephrin signaling is bidirectional, which means that both Eph receptor and ephrin ligand upon binding can initiate signaling in their respective cells. Activation of Eph receptors induces forward signaling and activation of ephrin ligands elicits reverse signaling (Figure 1.3B). Besides binding of Ephs and ephrins in trans, when they are expressed on opposite cells, Eph/ephrin interactions can also occur in cis, when they are present on the same cell. Cis binding leads to Eph receptor inhibition (Figure 1.3B). During cell-to-cell contact, heterotetramerization between two Eph receptors and two ephrin ligands bound in trans induces Eph/ephrin signaling and is followed by formation of larger signaling clusters (Figure 1.3C) (Pitulescu and Adams, 2010).

Forward signaling is mediated by activated Eph receptors that interact with other signaling proteins, including proteins containing SH2 domain e.g. Nck and PDZ domain e.g. Rho and Ras small GTPases. Reverse signaling by transmembrane ephrin-B ligands occurs through interactions with kinases e.g. Src that phosphorylate ephrin-B cytoplasmic domain and SH2 or PDZ-containing proteins. Additionally, reverse signaling by ephrin-A ligands that are attached to the membrane by a GPI anchor can proceed by interactions with transmembrane proteins like TrkB and p75 neurotrophin receptors (Lisabeth et al., 2013; Pasquale, 2008).

Eph/ephrin interactions may result either in repulsion or attraction. Initial adhesion upon binding of Eph receptors and ephrin ligands can be transformed into repulsion by means of trans-endocytosis or proteolytic cleavage. Attraction or adhesion can occur due to low level of Eph receptor kinase activity, which can happen with co-expression of kinase-inactive Eph receptors (Lisabeth et al., 2013; Pasquale, 2005).

Eph/ephrin bidirectional interactions are complex and often full of contradictions, but a wide array of biological processes that they affect underscores the need to better understand their signaling (Lisabeth et al., 2013; Pasquale, 2005).

1.3 Eph Receptor and Ephrin Ligand Signaling in Synapse Formation and Function

Eph/ephrin signaling regulates many aspects of synapse development and function and both classes, EphA/ephrin-A and EphB/ephrin-B, have been implicated in these processes (Hruska and Dalva, 2012).

EphB receptors and ephrin-B ligands are expressed at hippocampal synapses and their signaling is associated with attractive or synaptogenic effects (Hruska and Dalva, 2012). For example, EphB2 receptor expressed on non-neuronal cells induced presynaptic terminals on the contacting axons (Kayser et al., 2006). In turn, ephrin-B1 and ephrin-B2, present on the axons, were found to be responsible for the synaptogenic effect (McClelland et al., 2009). Additionally, in the triple EphB1-B3 KO mouse synapse density is reduced, and EphB2 receptor was shown to bind and induce NMDA receptor phosphorylation as well as regulate LTP in a kinase-independent manner (Klein, 2009).

EphA receptors and ephrin-A ligands are also expressed in the hippocampus (Galimberti et al., 2010; Murai et al., 2003; Nestor et al., 2007; Otal et al., 2006; Wang et al., 2003; Yue et al., 2002). EphA/ephrin-A signaling has been shown to regulate certain aspects of synaptic development and function, but their role in these processes is less understood (Hruska and Dalva, 2012). Interestingly, synapse density is reduced in the hippocampus of ephrin-A5 KO mice (Otal et al., 2006) and BDNF-induced synapse

formation was decreased in ephrin-A5 KO hippocampal cultures (Marler et al., 2008). Moreover, perfusion of hippocampal slices with a soluble ephrin-A5-Fc protein construct that activates EphA receptors was shown to enhance CA1 synaptic activity which resembled LTP (Gao et al., 1998b). Acute application of ephrin-A5-Fc to hippocampal cultures prevented BDNF-dependent increase in miniature postsynaptic current (mPSC) frequency (Bi et al., 2011). Additionally, astrocytic ephrin-A3 ligand along with the postsynaptic EphA4 receptor have been shown to control spine morphology, astrocyte-mediated glutamate transport, and hippocampal LTP (Carmona et al., 2009; Filosa et al., 2009; Murai et al., 2003).

Eph/ephrin signaling is clearly involved in various aspects of synapse formation and function. While there is strong evidence for EphB/ephrin-B attractive (synaptogenic) effects, still little is known about EphA/ephrin-A signaling in synapse formation. Therefore, this dissertation will focus on EphA/ephrin-A signaling system in excitatory synapse development.

1.4 Aims of the Dissertation

First, expression of EphA receptors and ephrin-A ligands on the surface of cultured hippocampal cells was characterized to provide an accurate context for the following functional analysis of EphA/ephrin-A signaling during synapse development in vitro.

Data discussed in this dissertation was organized into two chapters that describe two aims of this work:

Chapter 3 (Aim 1): Characterize EphA and Ephrin-A Protein Expression in Hippocampal Cultures

Chapter 4 (Aim 2): Examine Effects of Ephrin-A Activation and EphA Inhibition on Excitatory Synapse Development

CHAPTER 2 EXPERIMENTAL METHODS

2.1 Neonatal/Postnatal Hippocampal Cell Culture

All experiments were performed according to Rutgers University regulations for the care and use of laboratory animals. Hippocampi from C57BL/6 P0-P1 pups were dissected in ice-cold Hanks' balanced salt solution (HBSS) under sterile conditions. After dissection, hippocampal tissue was chopped into small pieces in ice-cold modified B-medium (Opitz et al., 2007) that contained DMEM/F12, 2% B27 supplement, 100 µg/ml human apo-transferrin, 5 µg/ml human insulin, 25 ng/ml progesterone, 5 ng/ml sodium selenite, and 16 µg/ml putrescine. The dissected tissue was digested in 0.625 mg/ml trypsin/B-medium solution in the 5% CO_2 incubator at 37 C° for 1 hour. The digested tissue was washed with warm HBSS to remove trypsin, transferred to B-medium containing 0.5% fetal bovine serum (FBS), and allowed to recover from digestion at 37 C° for 1 hour. Then, the digested tissue was dissociated by trituration with a fire-polished glass pipette and plated at density of ~180 cells/mm^2 on poly-D-lysine coated dishes. The plating medium was removed after 1 hour incubation at 37 C°, and 1.5 ml of fresh B-medium with 0.5% FBS was added to the culture. At approximately 24 hours after plating (1 div), 750 µl of the culture medium was removed and replaced with 1250 µl of Neurobasal medium supplemented with 0.5% FBS, 2% B27, and 0.5 mM L-glutamine. On 3 div, 1/3 of the culture medium was exchanged for fresh Neurobasal medium with 2% B27 and 0.5 mM L-glutamine. From that point, cultures were fed in this way every 3-4 days.

2.2 EphA-Fc Chimera Clustering and Treatment of Hippocampal Cultures

Recombinant mouse EphA3-Fc chimera (R&D Systems) was used for treatments of hippocampal cultures. EphA3-Fc chimera is a homodimer of the extracellular domain of EphA3 receptor linked to the Fc portion of human immunoglobulin G1 (IgG1). Human Fc fragment (R&D Systems) was used for control treatments. EphA3-Fc and Fc fragment were clustered at 1:5 weight ratio using donkey anti-human Fc antibody (Jackson ImmunoResearch Laboratories) for 1 hour at room temperature (Fu et al., 2011). Cultures at 11 div were treated for 24 hours with 5 µg/ml of either clustered EphA3-Fc or unclustered EphA3-Fc that was added to conditioned medium. Clustered and unclustered Fc fragments were applied at 5 µg/ml to the respective control cultures.

2.3 Detection of Surface EphA Receptors and Ephrin-A Ligands in Live Cultures

The affinity probe method was used for detection of surface EphA receptors and ephrin-A ligands (Flanagan et al., 2000; Gale et al., 1996). To label surface EphA receptors, 2 µg/ml of a dimeric (unclustered) ephrin-A3-Fc chimera (R&D Systems) was added to conditioned medium in cultures, and the cells were returned to a CO_2 incubator for 5 min. Similarly, to label surface ephrin-A ligands, 2 µg/ml of clustered EphA3-Fc chimera was incubated with live cells for 5 min. Unclustered and clustered Fc fragments were used at 2 µg/ml in the respective control cultures. After the incubation with chimeras, cells were washed quickly several times with prewarmed (HBSS) and fixed with ice-cold 100% methanol for 5 min at -20 C°. After fixation, cultures were washed and rehydrated with phosphate buffered saline (PBS) and blocked for 20 min

with a blocking buffer consisting of 5% donkey serum and 1% bovine serum albumin (BSA) diluted in PBS. Next, cells were incubated for 40 min at room temperature with donkey anti-human Fc antibody conjugated to Alexa Fluor 488 (1:1000; Jackson ImmunoResearch Laboratories). After washing with PBS and blocking for additional 20 min, respective primary antibodies in several different combinations were applied to the cultures for 1 hour at room temperature. Dilutions of the primary antibodies were as follows: chicken anti-GFAP (1:4000, Millipore), guinea pig anti-MAP2 (1:2000; Synaptic Systems), guinea pig anti-VGLUT1 (1:20,000; Synaptic Systems), chicken anti-MAP2 (1:16,000; Abcam), and mouse anti-PSD-95 (1:400; Thermo Fisher Scientific). After the primary antibody application, cells were washed with PBS and incubated for 40 min with the respective secondary antibodies that included donkey anti-guinea pig TRITC , donkey anti-chicken Alexa Fluor 647 (1:1000; Jackson ImmunoResearch Laboratories) , and donkey anti-mouse Alexa Fluor 555 (1:1000; Thermo Fisher Scientific). Finally, cells were washed and ProLong Gold antifadent (Thermo Fisher Scientific) was used as mounting medium before applying a coverslip (#1.5 thickness). All antibody working solutions were made in the blocking buffer and all steps of the procedure were performed in a humidified chamber in the dark.

2.4 Labeling of Surface EphA3 Receptors in Live Cultures with the IIIA4 Anti-EphA3 Monoclonal Antibody

A mouse monoclonal antibody IIIA4 against EphA3 was generously donated by Dr. Andrew Boyd, and was used to label surface EphA3 receptors in live hippocampal

cultures. 4 µg/ml of the IIIA4 antibody along with 2 µg/ml of ephrin-A3-Fc chimera were added to cells for 5 min to detect specifically EphA3 receptors and all members of EphA family, respectively. After the incubation, cultures were quickly washed several times with prewarmed HBSS and immediately fixed with ice-cold 100% methanol for 5 min at -20 C°. Next, cells were washed and rehydrated with PBS, blocked for 20 min, and incubated with donkey anti-mouse Alexa Fluor 555 antibody (1:1000; Thermo Fisher Scientific) for 40 min at room temperature. Then, cultures were washed again with PBS and donkey anti-human Fc Alexa Fluor 488 antibody (1:1000; Jackson ImmunoResearch Laboratories) was applied for 40 min. Subsequently, cultures were washed, blocked for 20 min, and incubated with chicken anti-MAP2 antibody (1:16,000; Abcam) for 1 hour. After the primary antibody incubation and washing, donkey anti-chicken Alexa Fluor 647 secondary antibody (1:1000; Jackson ImmunoResearch Laboratories) was added to cultures for 40 min. Lastly, cells were washed and ProLong Gold (Thermo Fisher Scientific) mounting medium was applied along with a coverslip. All antibodies, except IIIA4, were diluted in the blocking buffer containing 5% donkey serum and 1% BSA diluted in PBS.

2.5 Immunostaining of Cultures Treated with Protein Chimeras

At the end of the 24-hour treatment, hippocampal cultures were washed several times with prewarmed PBS and fixed with ice-cold 100% methanol for 5 min at -20 C°. After fixation, cells were washed with PBS, rehydrated, and blocked for 30 min at room temperature with buffer containing 5% donkey serum and 1% BSA in PBS. Mouse

monoclonal anti-PSD95 antibody (1:400, Thermo Fisher Scientific) was applied to cultures in the blocking buffer either overnight at 4 C° or for 1 hour at room temperature. All other primary and secondary antibodies were diluted in an antibody buffer containing 2% donkey serum and 1% BSA in PBS. After the primary antibody incubation, cells were washed with PBS and incubated with donkey anti-mouse Alexa Fluor 555 secondary antibody (1:1000; Thermo Fisher Scientific) for 1 hour at room temperature. Then, cultures were washed again and blocked for 30 min before incubation with guinea pig anti-VGLUT1 (1:12,000; Synaptic Systems) and chicken anti-MAP2 (1:8000; Abcam) primary antibodies for 1 hour at room temperature. Next, cells were washed and donkey anti-guinea pig Alexa Fluor 488 and donkey anti-chicken Alexa Fluor 647 secondary antibodies (1:1000; Jackson ImmunoResearch Laboratories) were applied to cultures for 1 hour. Subsequently, cells were washed and DAPI solution (300 nM, Thermo Fisher Scientific) was added to cultures for 15 min to counterstain the nuclei. Finally, ProLong Gold (Thermo Fisher Scientific) mounting medium and a coverslip were applied at the end of the staining procedure.

2.6 Tissue Processing and Immunohistochemistry

Hippocampal tissue from postnatal day 14 (P14) C57BL/6 mouse was dissected in cold PBS on ice and fixed by immersion for 60 hours at 4 C° with 2% paraformaldehyde (PFA) and 4% sucrose dissolved in PBS. After fixation, tissue was rinsed with PBS, dehydrated in a series of ethanol solutions, and cleared in Histochoice (Amreco). Paraplast X-tra (Leica Biosystems) was used for tissue infiltration, which was performed at 56-58 C°

under 7 inHg negative pressure in a vacuum oven. Finally, tissue was embedded in Paraplast X-tra, and 5 µm transverse hippocampal sections were cut on AO Spencer 820 microtome.

To prepare paraffin sections for immunohistochemistry, they were first deparaffinized in Histochoice, rehydrated in a series of ethanol solutions, and rinsed first in dH_2O and then in Tris-buffered saline (TBS). Heat-induced epitope retrieval (HIER) was carried out at 100 °C for 12 min in Tris-EDTA buffer consisting of 10 mM Tris, 1 mM EDTA, 0.05% Tween-20 (pH 9.0). Slides were cooled down in Coplin jars on a bench top for 30 min using a step-wise procedure, where twice 1/3 of the hot Tris-EDTA buffer was exchanged for fresh room temperature Tris-EDTA buffer. This exchange was done 10 and 20 min after the end of the epitope retrieval, and after that step slides were rinsed in dH_2O and washed in TBS.

Sections were blocked for 1 hour at room temperature with blocking buffer containing 10% donkey serum, 1% BSA, 0.1% Triton X-100, and 0.3 M glycine in TBS. Then, primary antibodies including rabbit anti-calbindin (1:1000; Swant), rat monoclonal anti-CTIP2 (1:200; Abcam), and chicken anti-MAP2 (1:1600; Abcam) were applied for 3 hours in an antibody diluent consisting of 5% donkey serum, 1% BSA, and 0.1% Triton X-100 in TBS. Next, sections were washed in a wash buffer containing 0.3% donkey serum, 1% BSA in TBS and incubated for 40 min in the dark with secondary antibody solutions made in the antibody diluent. Secondary antibodies included donkey anti-rabbit Alexa Fluor 488 (1:800; Thermo Fisher Scientific), donkey anti-rat Alexa Fluor 546 (1:800; Thermo Fisher Scientific), and donkey anti-chicken Alexa Fluor 647 (1:800; Jackson

ImmunoResearch Laboratories). Subsequently, slides were washed first with the wash buffer and then with TBS. Finally, ProLong Gold antifadent (Thermo Fisher Scientific) and a coverslip were applied to the slides. All steps of the immunostaining procedure were carried out with gentle agitation on a horizontal shaker. Additionally, blocking and antibody incubations were performed in a humidified chamber and washing steps in Coplin jars.

2.7 Image Acquisition and Processing

Images were collected on Olympus IX81 wide field fluorescence microscope equipped with QImaging Retiga EXi CCD camera and IPLab imaging software (BD Biosciences). Z-stacks containing 12 frames at 0.15 µm z-step were captured using 60x oil-immersion objective (NA 1.4). Before image quantification, 3D-blind deconvolution, correction of color misregistration, maximum intensity projection, and brightness/contrast adjustments were performed in IPLab software. Figure 2.1 shows an example of raw and deconvolved image from this study. This type of deconvolution preserves the relative differences in intensity values and can be used for quantitative image analysis (Wallace et al., 2001). Figure 2.2 explains how color misregistration was corrected to accurately assess labeling in merged multichannel images.

In experiments, where cultures underwent specific treatments, the acquisition parameters and processing procedures were the same for all images within an experiment. Additionally, all imaging in this type of experiments was performed blind to the treatment conditions.

An image of the immunolabeled hippocampal section as seen in Figure 1.1 was constructed from 10x photographs assembled into a mosaic using MosaicJ plug-in (Thevenaz and Unser, 2007).

For display purposes only, images were resized in Adobe Photoshop either without resampling, where the original resolution and pixels are visible (indicated in the figure description) or with resampling. Additionally, some pixels in the images were saturated to improve visualization of the data in the figures.

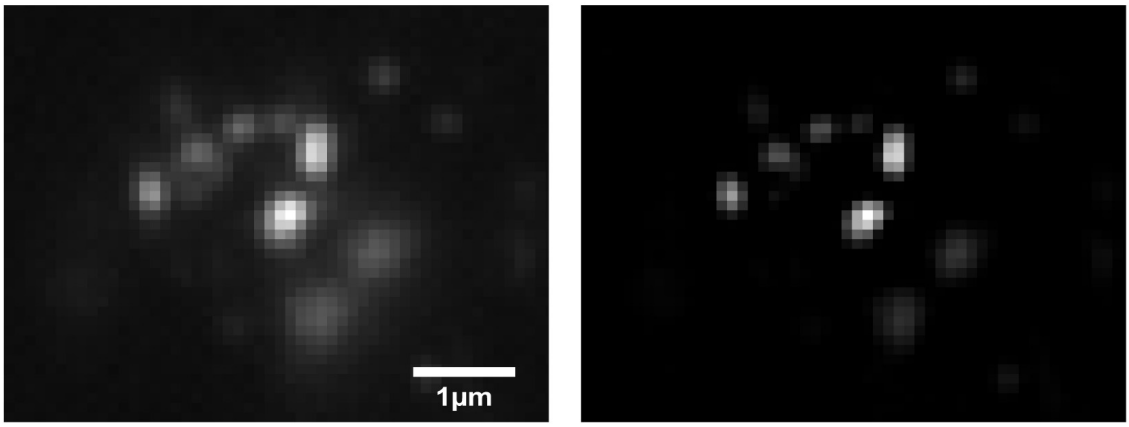

Figure 2.1 Image deconvolution. Left: raw image. Right: deconvolved image. 3D-blind deconvolution was performed in IPLab software to deblur images and enhance signal to noise ratio. Images in this figure were resized without resampling and original pixels are shown.

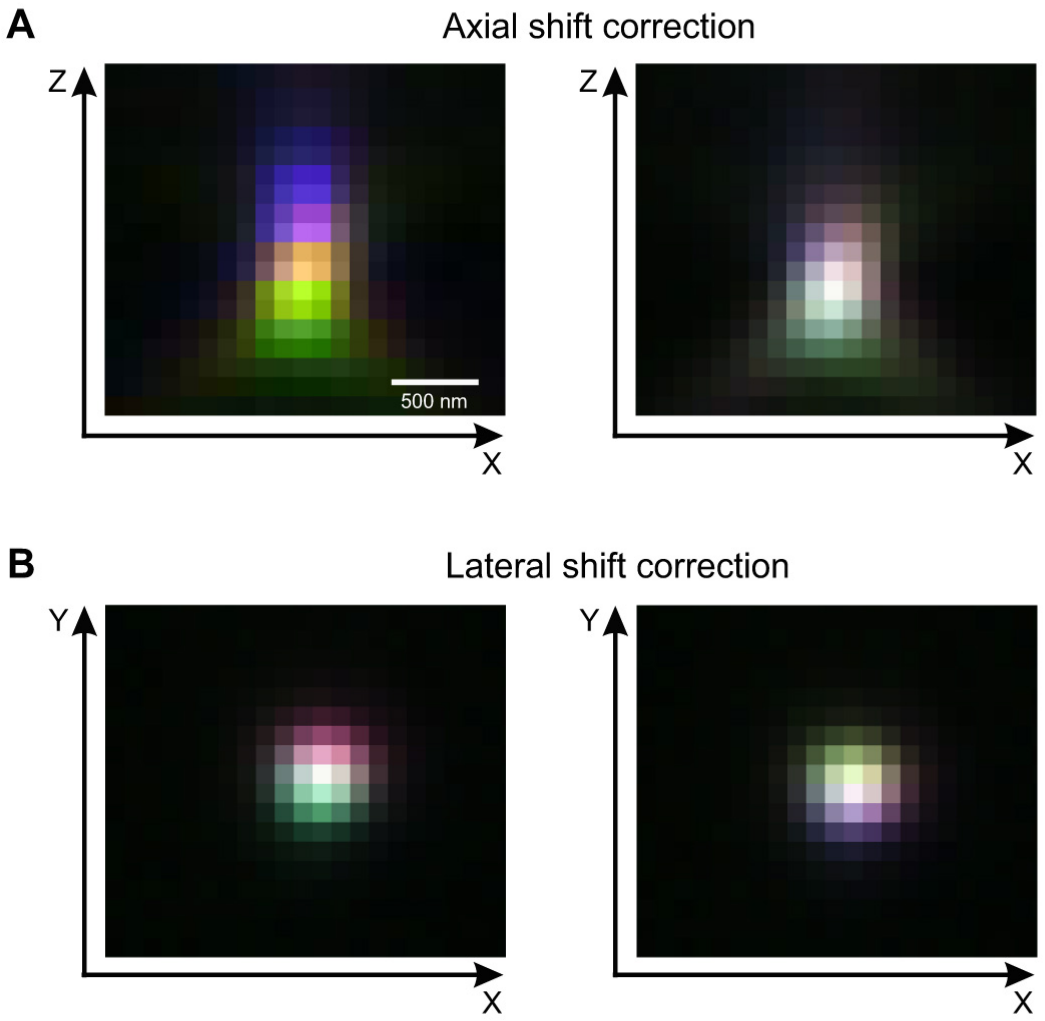

Figure 2.2 Correction of color misregistration between three color channels using multicolor fluorescent beads. Z-stacks of 6 µm in diameter multicolor fluorescent beads (FocalCheck™ fluorescence microscope test slide; Thermo Fisher Scientific) were collected on Olympus IX81 wide field fluorescence microscope with 60x oil-immersion objective. Extent of the shift between green, red, and far-red color channels in axial and lateral directions was calculated from a merged z-stack image. Correction of color misregistration was performed by shifting position of the frames in a z-stack in axial and lateral directions in order to align all three color channels. Such color alignment is critical for proper image analysis and quantification and it was applied to all images in this study. **A.** Merged three-channel image of a multicolor fluorescent bead shown from the axial direction before correction of color misregistration (left) and after correction was performed (right). Three color channels are shown: green, red, and far-red (pseudocolored blue). **B.** On the left, color misregistration in the lateral direction shown in the merged three-channel image of the same multicolor fluorescent bead as in (A). On the right, corrected color misregistration in the lateral direction. Original pixels in this image are shown.

2.8 Data Quantification and Statistical Analysis

SynPAnal software (Danielson and Lee, 2014) was used to measure density of EphA and ephrin-A-positive puncta in locations negative for both GFAP and MAP2 labeling, in GFAP-positive astrocytes, and MAP2-positve dendrites in hippocampal cultures at 11 div. EphA and ephrin-A density was quantified using segmentation in merged 60x images consisting of three color channels (green: either EphA or ephrin-A, red: GFAP, and blue: MAP2). Images with EphA and ephrin-A staining were segmented at 4 and 5 times the highest background value, respectively, where the background was measured in ten different locations within each image. Three and six merged images were analyzed for EphA and ephrin-A, respectively. Mean measurement area for GFAP-/MAP2- locations was 92 μm^2 (14-53 measurements per image), for GFAP+ astrocytes it was 71 μm^2 (18-49 measurements per image), and for MAP2+ dendrites it was 16 μm^2 (21 dendritic segments that were 20 µm in length per image). These locations were manually outlined in SynPAnal software. Statistical significance was assessed by one-way independent-samples ANOVA with post hoc Tukey test and n represented number of analyzed images. Error bars display standard error of the mean ±SEM.

After specific treatments, cultures were triple-labeled with anti-MAP2, anti-PSD-95, and anti-VGLUT1 antibodies, and quantitative puncta analysis was performed in SynPAnal software. Anti-MAP2 staining was carried out to identify neurons with pyramidal morphology and to label their dendrites. PSD-95-positive puncta (red channel) and VGLUT1-positive puncta (green channel) were segmented at a threshold

that was 4.5 times the mean background value measured in the dendritic areas. Fluorescent puncta were quantified in four 20 µm long dendritic segments per neuron. Dendritic segments were manually outlined at least 40 µm away from the cell body to avoid quantification of the giant mossy fiber synapses (Lee et al., 2013). Only puncta greater than or equal to 4 pixels in size were included in the measurements. Average signal intensity of the fluorescent puncta within each 20 µm dendritic segment was measured by SynPAnal software. These intensity values were then averaged for each neuron and the population mean was calculated from 5-11 neurons (20-44 dendritic segments) per treatment condition (Lee et al., 2013) in an experiment. Average background intensity measured on the dendrites for each treatment was subtracted from each respective population mean. Three independent replicate experiments (three different cell culture platings) were analyzed to assess effects of the specific treatments. In addition to immunofluorescence signal intensity, number of synaptic and nonsynaptic PSD-95 and VGLUT1-positive puncta was counted manually in the outlined 20 µm long dendritic segments. PSD-95 and VGLUT1-positive puncta apposing each other were defined as synaptic. PSD-95-positive puncta without a VGLUT1-labeled presynaptic partner or VGLUT1-positive puncta without a PSD-95-labeled postsynaptic partner were defined as nonsynaptic. All quantitative analysis was performed blind to the experimental conditions. One neuron out of eighty-nine quantified was excluded from the analysis because of a very high dendritic synapse density and presence of excitatory synapses on its cell body, which suggested that it might have been a particular type of an interneuron (Gulyas et al., 1999). Data representing mean intensity values were

normalized to controls (Valcu and Valcu, 2011) for clarity. The one-way correlated-samples analysis of variance (ANOVA) with a post hoc Tukey test was used to assess significant differences between more than two treatment conditions. Statistical analysis was done on three independent experiments and error bars represent standard error of the mean ± SEM. SEM values for a correlated-samples design were calculated according to Cousineau and O'Brien.

CHAPTER 3 EPHA AND EPHRIN-A PROTEIN EXPRESSION

Multiple EphA receptors and ephrin-A ligands are expressed in developing and adult hippocampus in vivo (Cooper et al., 2009; Murai et al., 2003; Otal et al., 2006; Tremblay et al., 2007; Wang et al., 2003; Yue et al., 2002). To investigate the role of EphA/ephrin-A signaling in the excitatory synapse development in vitro, expression of these proteins was first characterized in the hippocampal cultures at 11 div with respect to neuronal, astrocytic, and synaptic markers.

Several commercially available antibodies to EphA receptors and ephrin-A ligands were examined and proved not to be specific (data not shown). Therefore, the affinity probe method was employed to characterize expression of these proteins (Flanagan et al., 2000; Gale et al., 1996) in hippocampal cultures. This technique utilizes chimeric protein constructs composed of the extracellular portion of either Eph receptor or ephrin ligand, in a dimer form, fused to a human Fc IgG tag. To detect surface EphA receptors, dimeric (unclustered) ephrin-A3-Fc chimera was added to living hippocampal cultures for 5 min. Because a dimeric EphA3-Fc chimera did not detect ephrin-A ligands beyond the background level (data not shown), clustered EphA3-Fc was added to living cultures for 5 min to label the ligands effectively. After washing and fixation, bound affinity probes were detected with anti-human Fc antibody conjugated to a fluorophore. Promiscuity of binding between different EphA receptors and ephrin-A ligands allows to detect all members of the EphA class by ephrin-A3-Fc and all members of the ephrin-A class by clustered EphA3-Fc (Pasquale, 2004). Additionally, the choice of EphA3-Fc and ephrin-A3-Fc, as affinity probes, limited the detection to ephrin-A class and EphA class,

respectively. A reason for this specificity is that EphA3 doesn't cross-react with ephrin-B class of ligands and ephrin-A3 doesn't cross-react with EphB class of receptors, as it is observed for EphA4 that binds ephrin-B ligands and ephrin-A5 that binds EphB2 receptors (Himanen et al., 2004; Pasquale, 2004). Figure 3.1 summarizes the affinity probe procedure. In addition to affinity probe method, expression of a specific member of the EphA receptor family, EphA3, was examined with anti-EphA3 (IIIA4) antibody that binds to the extracellular domain of EphA3 protein in its native conformation (Boyd et al., 1992; Vearing et al., 2005). Labeling of EphA receptors, including EphA3, and ephrin-A ligands in living cultures has an advantage of detecting proteins that are displayed on the cell surface in their functional context.

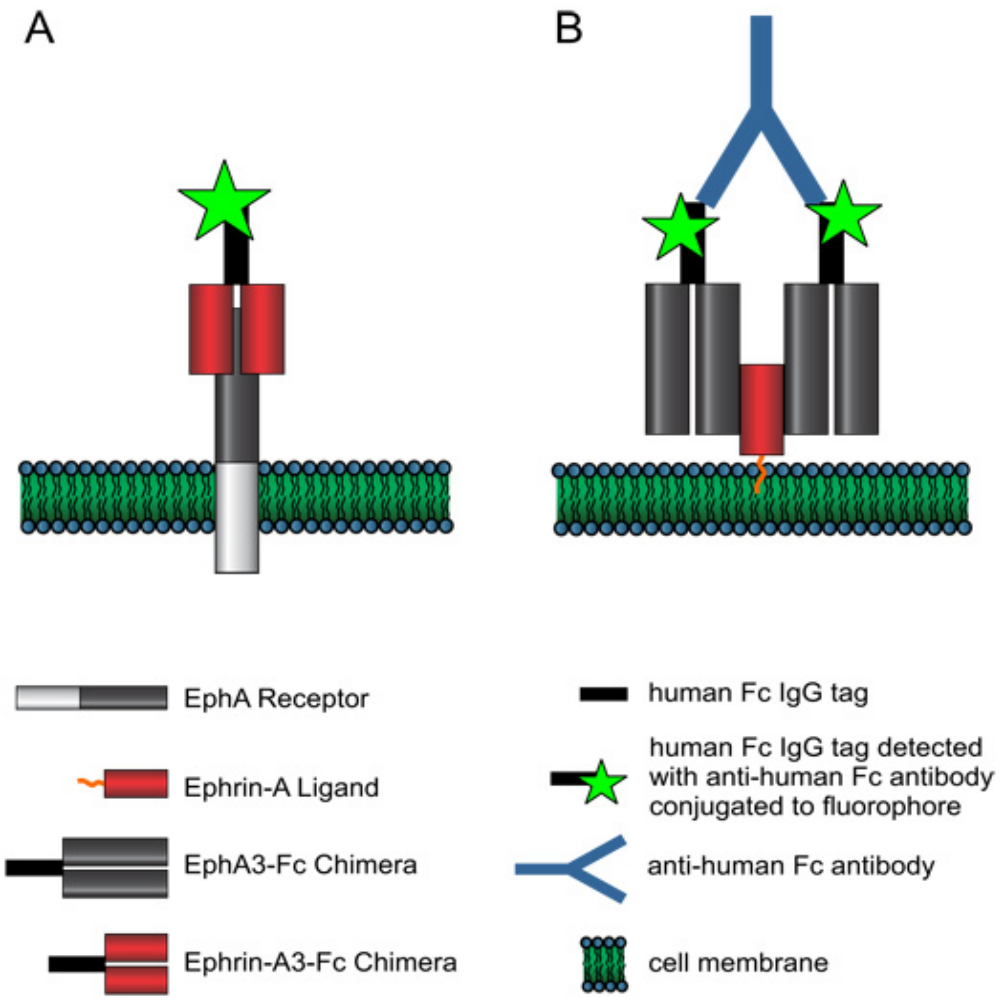

Figure 3.1 Detection of surface EphA receptors and ephrin-A ligands by affinity probe method. A. Endogenous EphA receptors were detected with chimeric constructs containing ephrin-A3 ligand fused to a human Fc IgG fragment. **B.** Endogenous ephrin-A ligands were detected by chimeric constructs consisting of the extracellular domain of the EphA3 receptor fused to a human Fc IgG fragment. To enhance labeling of the ephrin-A ligands, EphA3-Fc chimera was first clustered with anti-human Fc antibody. Endogenous receptors and ligands bound to their respective chimeric constructs were visualized with anti-human Fc antibodies conjugated to a fluorophore.

3.1 EphA Receptors and Ephrin-A Ligands Were Detected on the Surface of Cultured Hippocampal Neurons and Astrocytes

Characterization of EphA receptor and ephrin-A ligand localization was performed with respect to hippocampal neurons, with focus on the dendrites, and GFAP-positive astrocytes in vitro. Axons and other cell types present in hippocampal cultures (i.e. oligodendrocytes and microglia) will need further investigation. Figure 3.2 shows a hippocampal neuron at 11 div stained by anti-MAP2 antibody situated over a layer of astrocytes labeled by anti-GFAP antibody. Astrocytes showed varying levels of GFAP expression and most of them had protoplasmic morphology with few defined processes. These morphological characteristics are typical of hippocampal astrocytes in vitro labeled by anti-GFAP antibody (Pinto et al., 2000). Detection of GFAP along with MAP2 can help in localization of other proteins, and in this work, it was used to begin the characterization of EphA receptor and ephrin-A ligand distribution in hippocampal cultures.

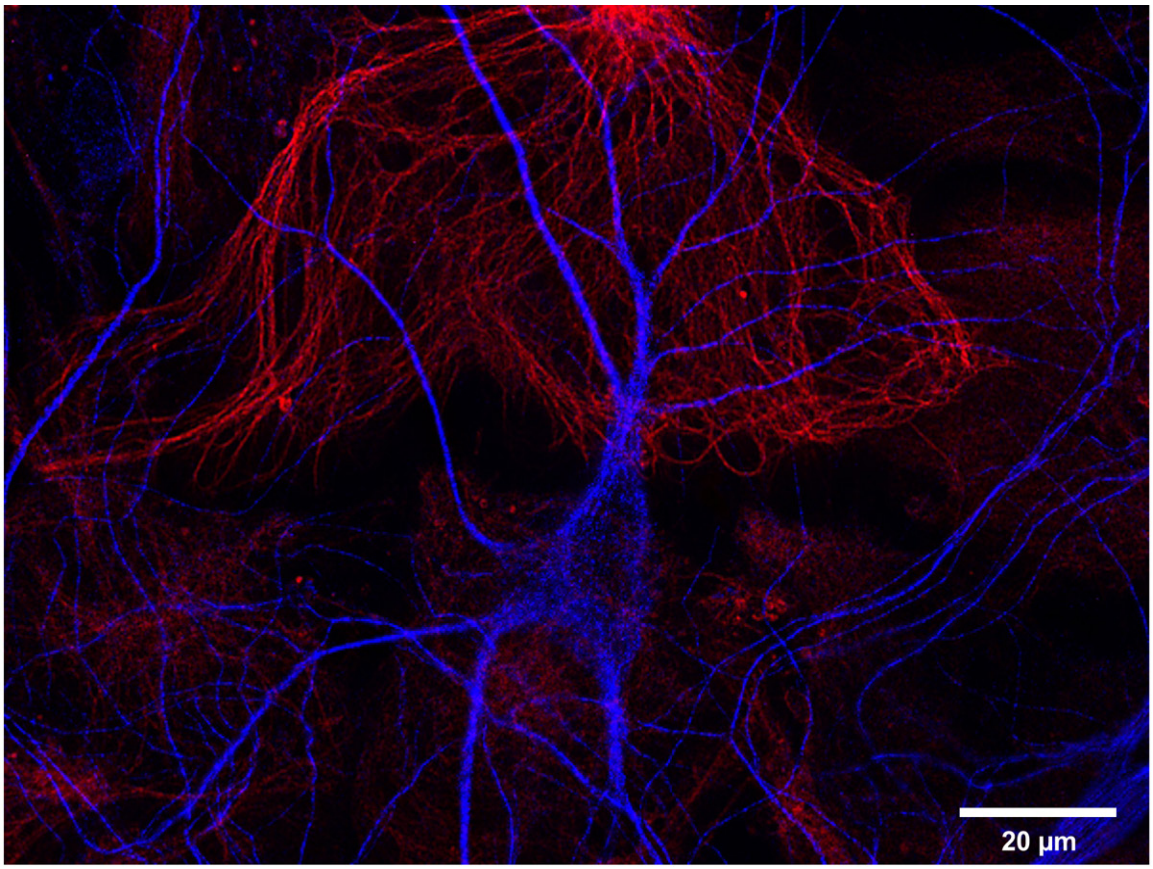

Figure 3.2 Hippocampal culture at 11 div immunolabeled with anti-GFAP and anti-MAP2 antibodies showing relationship between astrocytes and neurons in vitro. Hippocampal neurons (blue) grow on a layer of astrocytes expressing varying levels of GFAP (red). Neuronal dendrites are often in direct contact with astrocytes.

Using ephrin-A3-Fc as an affinity probe, EphA receptors were detected on the surface of hippocampal neurons and astrocytes that were later immunolabeled with anti-MAP2 and anti-GFAP antibody, respectively (Figure 3.3). EphA-positive puncta appeared to vary in fluorescent signal (Figure 3.3C), which might indicate different EphA receptor content depending on a location. EphA-positive puncta appeared to be on the dendrites of the hippocampal neurons or in close contact with them (Figure 3.3D). In addition, many labeled EphA receptors were found in the locations surrounding the dendrites. In these areas, astrocytes were detected with anti-GFAP antibody (Figure 3.3E). Specificity

of the affinity probe procedure and immunolabeling was examined with a control culture, which was processed in the same way as the experimental one, but the EphA3-Fc chimera was replaced with the Fc fragment, and primary antibodies were omitted. The control images that were acquired and processed in the same way as the experimental ones did now show labeling (Figure 3.3F).

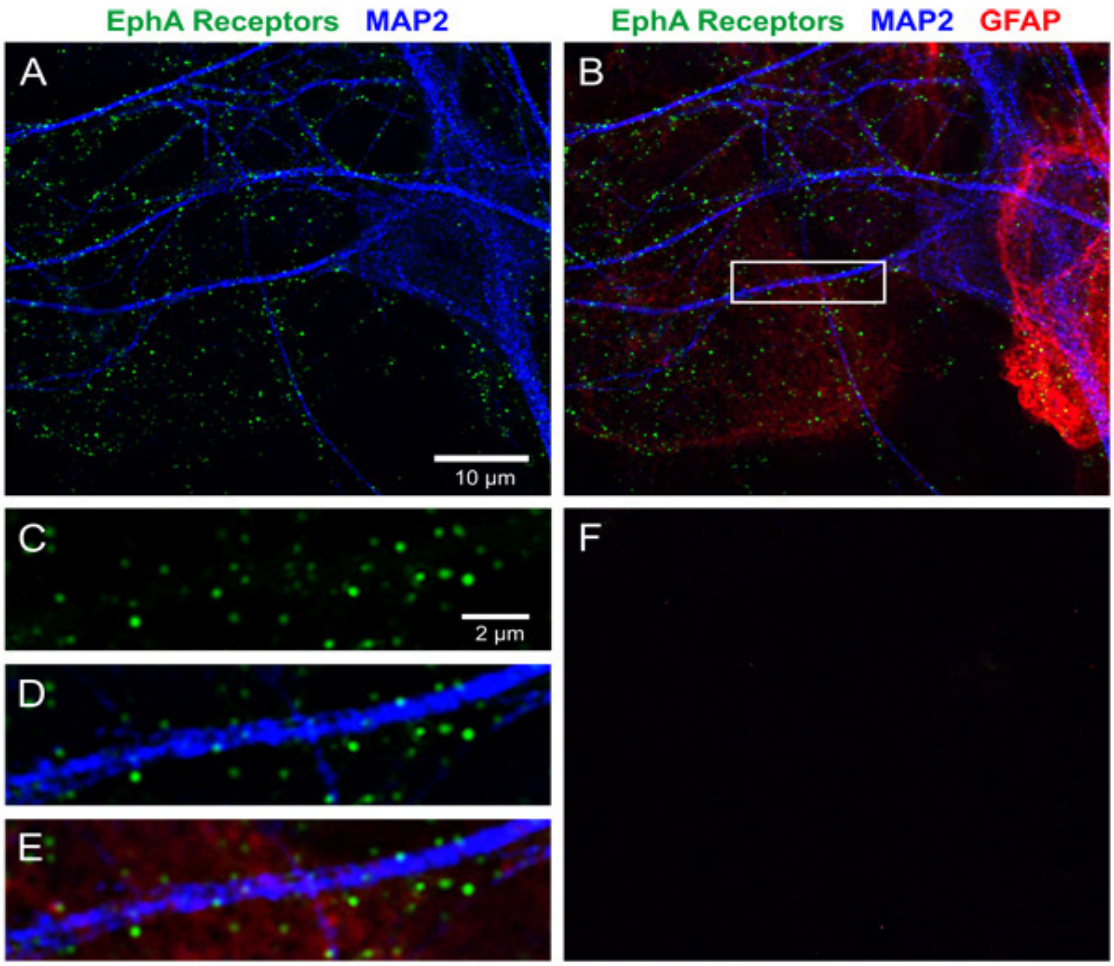

Figure 3.3 Expression of surface EphA receptors in the hippocampal cultures labeled with ephrin-A3-Fc. Surface EphA receptors (green) were labeled in living hippocampal cultures at 11 div with ephrin-A3-Fc chimera, then fixed and immunostained for neuronal marker MAP2 (blue) and astrocytic protein GFAP (red). **A-B.** Present the same region of the triple-labeled hippocampal culture and show the presence of EphA receptors on the surface of hippocampal cells in vitro. **A.** Two fluorescence channels are shown with EphA receptors (green) and MAP2 (blue). **B.** GFAP (red) is shown in addition to EphA receptors and MAP2. **C-E.** Display boxed area in (B). **C.** EphA receptors alone (green). Note that some EphA-positive puncta are brighter than

others, which might suggest various levels of EphA receptor protein in different locations. **D.** EphA receptors (green) and MAP2 (blue). Some EphA receptors are found in a close contact with the dendrites, and others are present in the neighboring areas. **E.** All three channels merged: EphA receptors (green), MAP2 (blue), and GFAP (red). GFAP-labeled astrocytes express EphA receptors on their surface and are in an intimate contact with the dendritic processes. **F.** Control image of a culture, where Fc fragment was added instead of ephrin-A3-Fc, and primary antibodies were omitted during the staining procedure. Scale bar in (A) is the same for (B and F), scale bar in (C) is the same for (D and E).

In sister hippocampal cultures, surface ephrin-A ligand expression was examined by applying clustered EphA3-Fc chimera as an affinity probe. As EphA receptors, ephrin-A ligands were also detected on the surface of hippocampal cells in vitro, but they appeared to be less abundant (Figure 3.4). Similarly to EphA receptors, ephrin-A-positive puncta varied in the level of the fluorescent signal (Figure 3.4C). Some ephrin-A ligand-positive puncta appeared to be located either on or right next to dendrites, and others were found in the surrounding areas (Figure 3.4D), where the GFAP-labeled astrocytic processes were detected (Figure 3.4E). The specificity control was performed in an analogous way as described for EphA receptor detection, and it did not show labeling (Figure 3.4F).

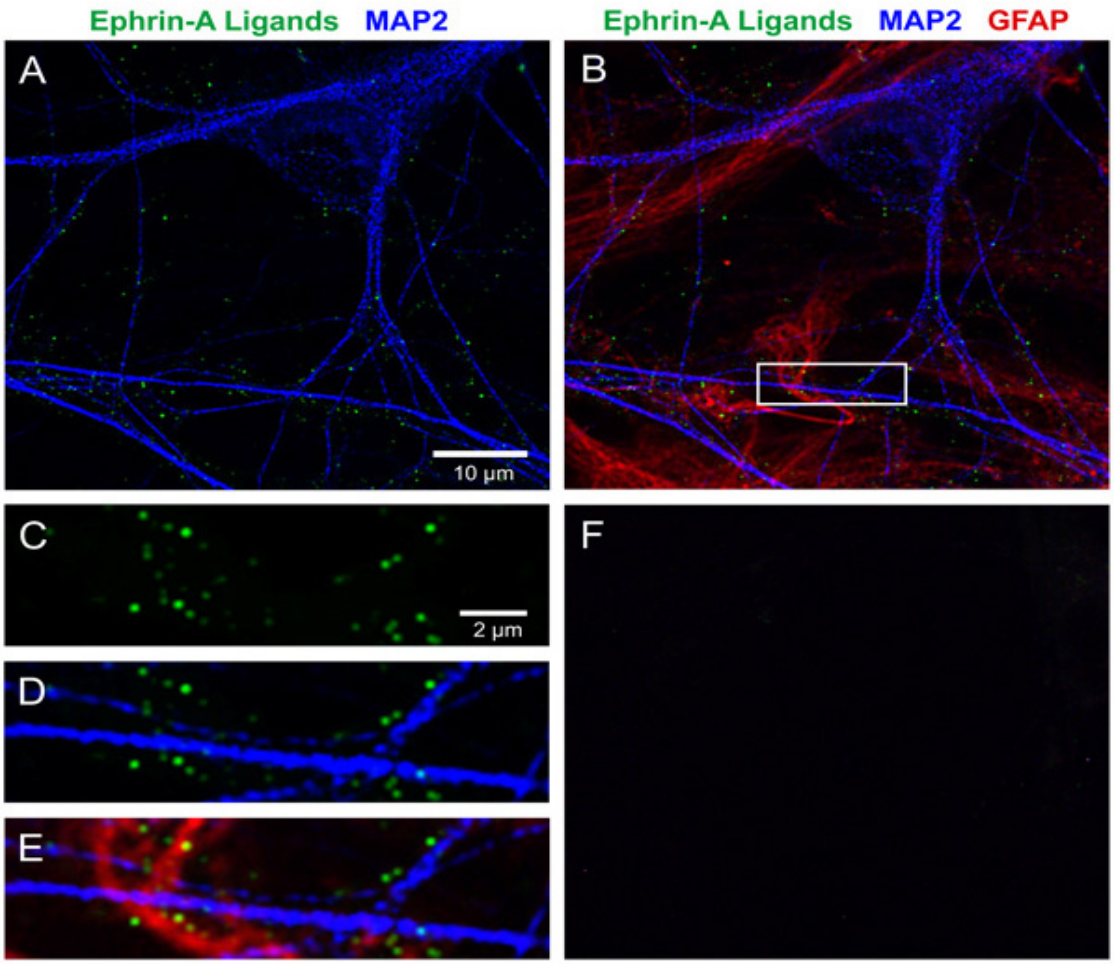

Figure 3.4 Expression of surface ephrin-A ligands in the hippocampal cultures labeled with EphA3-Fc. Surface ephrin-A ligands (green) were detected in living hippocampal cultures at 11 div with clustered EphA3-Fc chimera. After fixation, cultures were immunolabeled for neuronal marker MAP2 (blue) and astrocytic protein GFAP (red). **A-B.** Show the same area of the triple-labeled hippocampal culture and indicate expression of ephrin-A ligands on the surface of hippocampal cells in vitro. **A.** Two fluorescence channels are displayed with ephrin-A ligands (green) and MAP2 (blue). **B.** Includes all three channels, ephrin-A (green), MAP2 (blue), and GFAP (red). **C-E.** Show boxed area in B. **C.** Ephrin-A ligands alone (green). Note different signal levels of the puncta representing ephrin-A ligands. **D.** Ephrin-A ligands (green) and MAP2 (blue). Dendrites are surrounded by ephrin-A-positive puncta and a subset of the fluorescent puncta is in a close contact with the dendritic processes. **E.** All three channels merged: ephrin-A ligands (green), MAP2 (blue), and GFAP (red). Many of the labeled ephrin-A ligands seem to be expressed on the surface of the astrocytes. **F.** Control picture of hippocampal cells, where Fc fragment was added in place of clustered EphA3-Fc, and primary antibodies were omitted during staining procedure. Scale bar in (A) is the same for (B and F), scale bar in (C) is the same for (D and E).

In order to better understand EphA and ephrin-A protein distribution in hippocampal cells in vitro, mean densities of EphA and ephrin-A puncta were quantified with respect to GFAP and MAP2 labeling. The analysis was performed on the three categories of defined regions in the triple-labeled hippocampal cultures:

Category #1 (GFAP-/MAP2-): Regions negative for both GFAP and MAP2 staining that could potentially contain neuronal axons and other cell types present in hippocampal cultures i.e. oligodendrocytes and microglia. GFAP-negative astrocytes also might have been a part of this category (Bushong et al., 2004; Ogata and Kosaka, 2002; Walz and Lang, 1998).

Category #2 (GFAP+): Regions with GFAP-positive labeling only, which included GFAP+ astrocytic cell bodies and processes that were located in between neurons and in between dendrites. GFAP+ regions colocalizing with neuronal dendrites were excluded from this category to avoid quantification of EphA and ephrin-A puncta that might have been of dendritic origin. It should be noted that this category can also contain EphA and ephrin-A that might be expressed by axons and oligodendrocytes as well as microglia.

Category #3 (MAP2+/GFAP+-): Peridendritic regions along MAP2-positive dendrites of hippocampal neurons that included dendritic segments either colocalizing or not colocalizing with GFAP+ astrocytic processes. These two types of dendrites were analyzed together because it was impossible to make a clear distinction between them, which was caused by the uneven nature of the GFAP labeling that made evaluation of colocalization between the MAP2-positive and putative GFAP-negative areas

problematic. Therefore, this category represented EphA and ephrin-A puncta that might have been of dendritic and astrocytic origin.

Quantitative analysis of EphA and ephrin-A density in the three defined regions of hippocampal cultures is shown in Figure 3.5. EphA receptor distribution was strikingly different in the examined locations (Figure 3.5A). EphA receptor mean density was 8 times higher in the peridendritic areas (MAP2+/GFAP+-) than in the regions negative for both GFAP and MAP2 (GFAP-/MAP2-) ($P < 0.01$) and 2.7 times higher than in the regions positive for GFAP only (GFAP+) ($P < 0.01$). Additionally, EphA receptors appeared to be more enriched in the regions positive only for GFAP (GFAP+) than in the regions negative for both GFAP and MAP2 (GFAP-/MAP2-), but that difference did not reach statistical significance. Similarly to EphA receptors, ephrin-A ligands were differentially distributed in the three examined locations of hippocampal cultures (Figure 3.5B). Ephrin-A ligand mean density was 12 times higher in the peridendritic areas (MAP2+/GFAP+-) than in the locations negative for GFAP and MAP2 (GFAP-/MAP2-) ($P < 0.01$) and 3.7 times higher than in regions positive for GFAP only (GFAP+) ($P < 0.01$). Moreover, ephrin-A ligand mean density tended to be higher in the regions positive for GFAP only (GFAP+) than in the locations negative for both GFAP and MAP2 (GFAP-/MAP2-), but that distinction was not statistically significant.

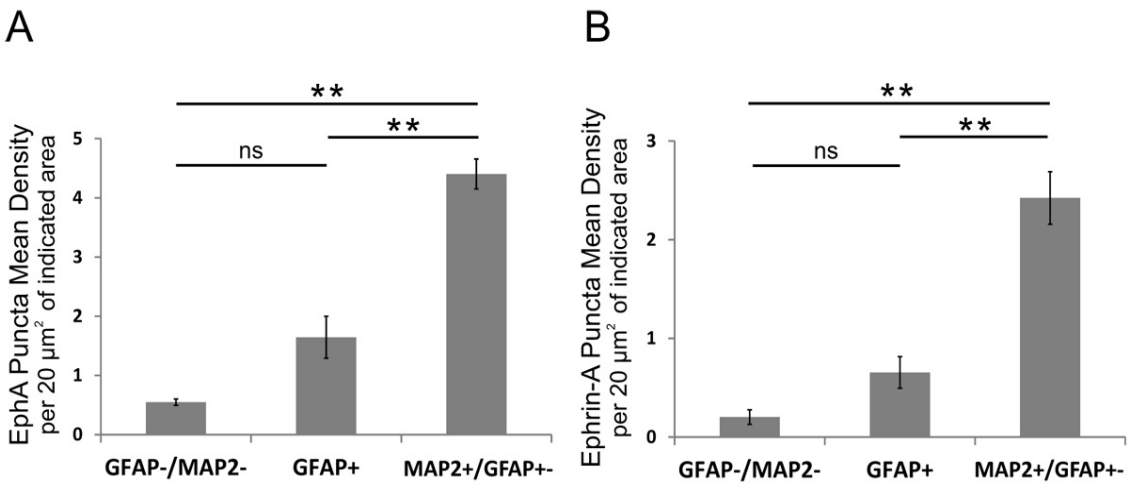

Figure 3.5 EphA and ephrin-A distribution in hippocampal cultures with respect to GFAP+ astrocytes and MAP2+ neuronal dendrites. Mean density of surface EphA and ephrin-A-positive puncta was measured in three different regions of hippocampal cultures: 1. Locations negative for both GFAP and MAP2 labeling (GFAP-/MAP2-), 2. Locations positive for GFAP only (GFAP+), and 3. Peridendritic regions containing MAP2-positive dendrites either with or without direct contact with GFAP-positive astrocytes (MAP2+/GFAP+-). **A.** EphA was found in all three examined locations in the hippocampal cultures, but density of peridendritic EphA (MAP2+/GFAP+-) was significantly higher than EphA density in GFAP-/MAP2- regions and regions positive only for GFAP (GFAP+). Density of EphA in the regions positive only for GFAP (GFAP+) tended to be higher than in GFAP-/MAP2- areas, but that difference was not statistically significant. **B.** Surface ephrin-A was found in all examined locations in hippocampal cultures, but density of ephrin-A in peridendritic regions (MAP2+/GFAP+-) was significantly higher than ephrin-A density in regions positive for GFAP only (GFAP+). Additionally, ephrin-A showed a trend for higher enrichment in the regions positive only for GFAP (GFAP+) than in GFAP-/MAP2- areas, but that difference did not reach statistical significance.
EphA: n = 3, ephrin-A: n = 6; n represents number of analyzed images; ns – nonsignificant ($P > 0.05$); ** $P < 0.01$ with one-way independent-samples ANOVA and post hoc Tukey test. Scale bars represent ±SEM.

These results indicate that, in the hippocampal cultures, EphA receptors and ephrin-A ligands had highest enrichment in the peridendritic areas, where EphA and ephrin-A proteins could have been expressed by the neuron and/or the astrocyte. The exact origin of EphA receptors and ephrin-A ligands in the peridenritic locations will need to be investigated in the future studies. In addition, both EphA and ephrin-A showed a

trend for higher enrichment on the surface of GFAP+ astrocytes than in the GFAP-/MAP2- regions suggesting that astroglia could have contributed to EphA and ephrin-A expression profiles in the hippocampal cultures.

3.2 EphA Receptors and Ephrin-A Ligands Were Localized Next to a Subset of Pre- and Postsynaptic Terminals of Excitatory Synapses

Localization of the surface EphA receptors and ephrin-A ligands was investigated with respect to pre- and postsynaptic markers of excitatory synapses in the hippocampal cultures at 11 div. Only a subset of pre- and postsynaptic terminals of excitatory synapses was in a direct contact with EphA receptors and ephrin-A ligands, while most of the terminals were not. Figure 3.6 shows examples of EphA-positive puncta that were found close to the presynaptic terminals labeled with anti-VGLUT1 antibody (Figure 3.6A) and next to the postsynaptic terminals stained with anti-PSD-95 antibody (Figure 3.6B). Figure 3.7 presents examples of the ephrin-A-positive puncta that were detected near a subset of the presynaptic (Figure 3.7A) and postsynaptic (Figure 3.7B) terminals of the excitatory synapses. There were fewer excitatory synapse contacts with ephrin-A-positive puncta nearby than synaptic contacts with adjacent EphA-positive puncta. In addition, neither EphA nor ephrin-A colocalized with either pre- or postsynaptic markers of the excitatory synapses at this developmental stage in vitro.

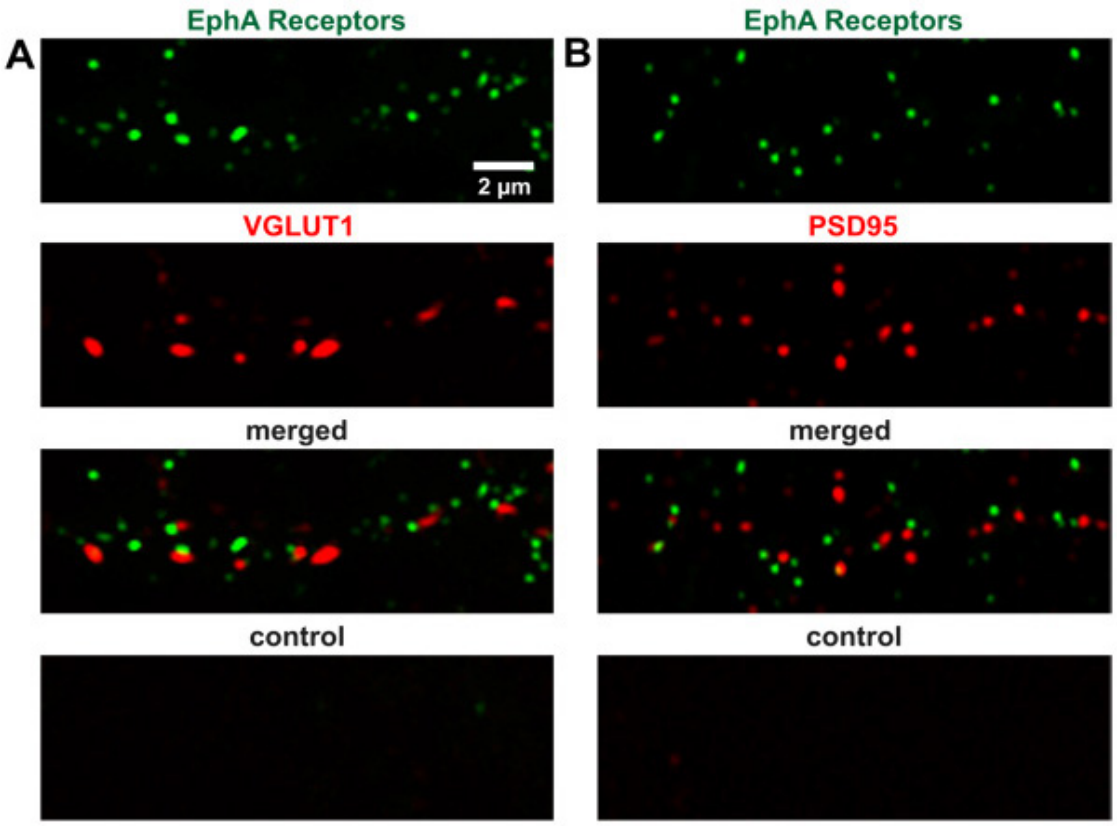

Figure 3.6 Localization of surface EphA receptors near a subset of presynaptic (VGLUT1) and postsynaptic (PSD-95) terminals of excitatory synapses. Surface EphA receptors were labeled with ephrin-A3-Fc (green) in living hippocampal cultures at 11 div. After fixation, cells were additionally colabeled either with anti-VGLUT1 antibody (red in A) or anti-PSD-95 antibody (red in B). **A.** Surface EphA receptors (green) and the presynaptic marker of excitatory synapses VGLUT1 (red). Merged image reveals that some of EphA-positive puncta are located close to a subset of the presynaptic terminals of excitatory synapses. Bottom panel shows a control image where the Fc fragment was used instead of ephrin-A3-Fc, and primary antibodies where not included in the staining procedure. **B.** Surface EphA receptors (green) and the postsynaptic marker of excitatory synapses PSD-95 (red). Some puncta representing EphA receptor protein are detected near a subset of the postsynaptic terminals of excitatory synapses (merged image). Control image is shown in the bottom panel. Scale bar in (A) is the same for all images in the figure.

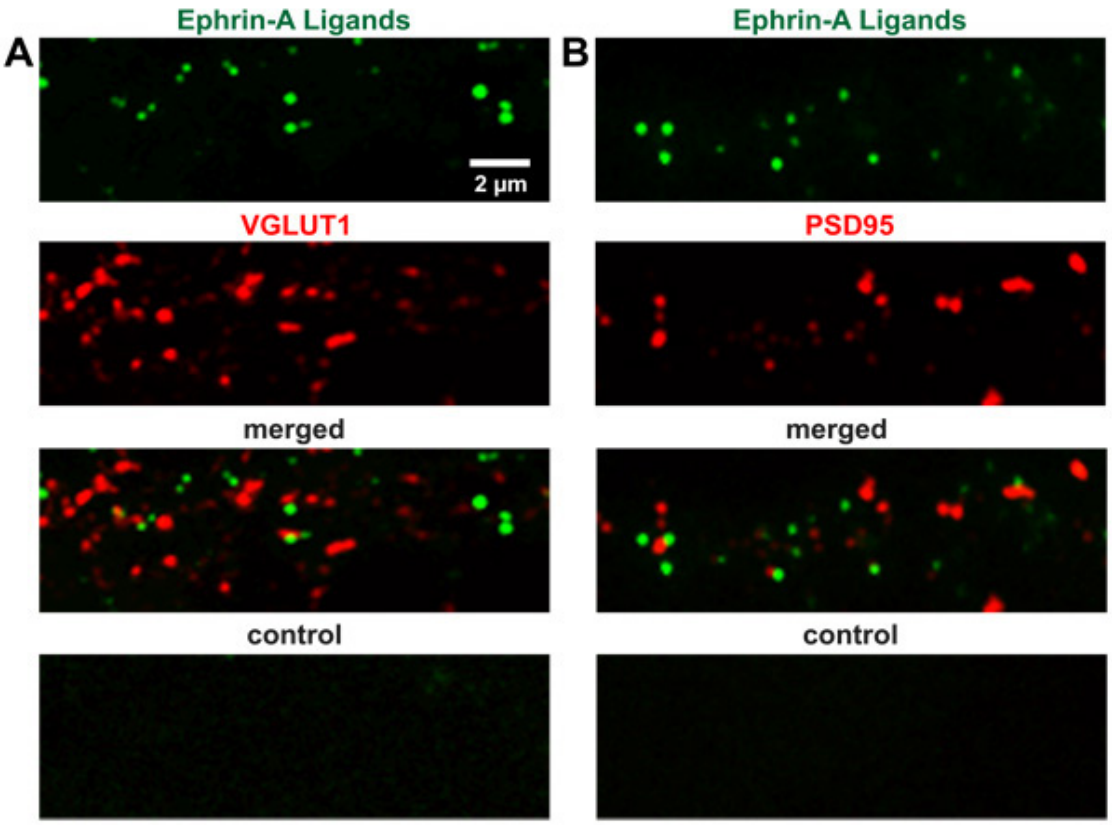

Figure 3.7 Localization of surface ephrin-A ligands near a subset of presynaptic (VGLUT1) and postsynaptic (PSD-95) terminals of excitatory synapses. Surface ephrin-A ligands were detected with clustered EphA3-Fc (green) in living hippocampal cultures at 11 div. Additionally, cultures were immunolabeled either with anti-VGLUT1 antibody (red in A) or anti-PSD-95 antibody (red in B). **A.** Surface ephrin-A ligands (green) and the presynaptic marker of excitatory synapses VGLUT1 (red). Some ephrin-A-positive puncta are located next to a subset of the presynaptic terminals of excitatory synapses (merged image). **B.** Surface ephrin-A ligands (green) and the postsynaptic marker of excitatory synapses PSD-95 (red). Some ephrin-A ligands are found close to a subset of the postsynaptic terminals of excitatory synapses (merged image). Bottom panels in A and B show control images for both types of staining. Scale bar in (A) is the same for all images in the figure.

3.3 Localization of EphA3 Receptor in Hippocampal Cultures and EphA3's Apparent Differential Ability to Bind Ephrin-A3-Fc

To examine expression of the EphA3 receptor on the surface of cultured hippocampal cells, a monoclonal antibody (IIIA4) against EphA3 was used. The IIIA4 antibody (a generous gift from Dr. Andrew Boyd) binds to the native form of the EphA3 receptor protein with a subnanomolar affinity (Boyd et al., 1992; Janes et al., 2011; Vearing et al., 2005). To compare the distribution of EphA3 receptor with respect to other EphA family members, IIIA4 antibody was added to living cultures for 5 minutes, along with the ephrin-A3-Fc affinity probe. Cells were then washed, fixed and colabeled with anti-MAP2 antibody. Surface EphA3 receptor protein, similarly to other EphA family members, was found on or near dendritic processes and in between the dendrites (Figures 3.8 and 3.9). Additionally, EphA3-positve fluorescent puncta varied in the signal intensity, which suggests differential EphA3 protein level between the puncta (Figure 3.9B). While the EphA3 receptors were detected with IIIA4 anti-EphA3 antibody, the affinity probe ephrin-A3-Fc should, theoretically, label all EphA receptor family members (including EphA3). Surprisingly, certain EphA3-positive puncta (stained by IIIA4 antibody) were not colabeled with the ephrin-A3-Fc affinity probe (Figure 3.9). This observation suggests that EphA3 receptors expressed on the surface of living hippocampal cultures display a differential ability to bind the exogenously applied ephrin-A3-Fc ligand.

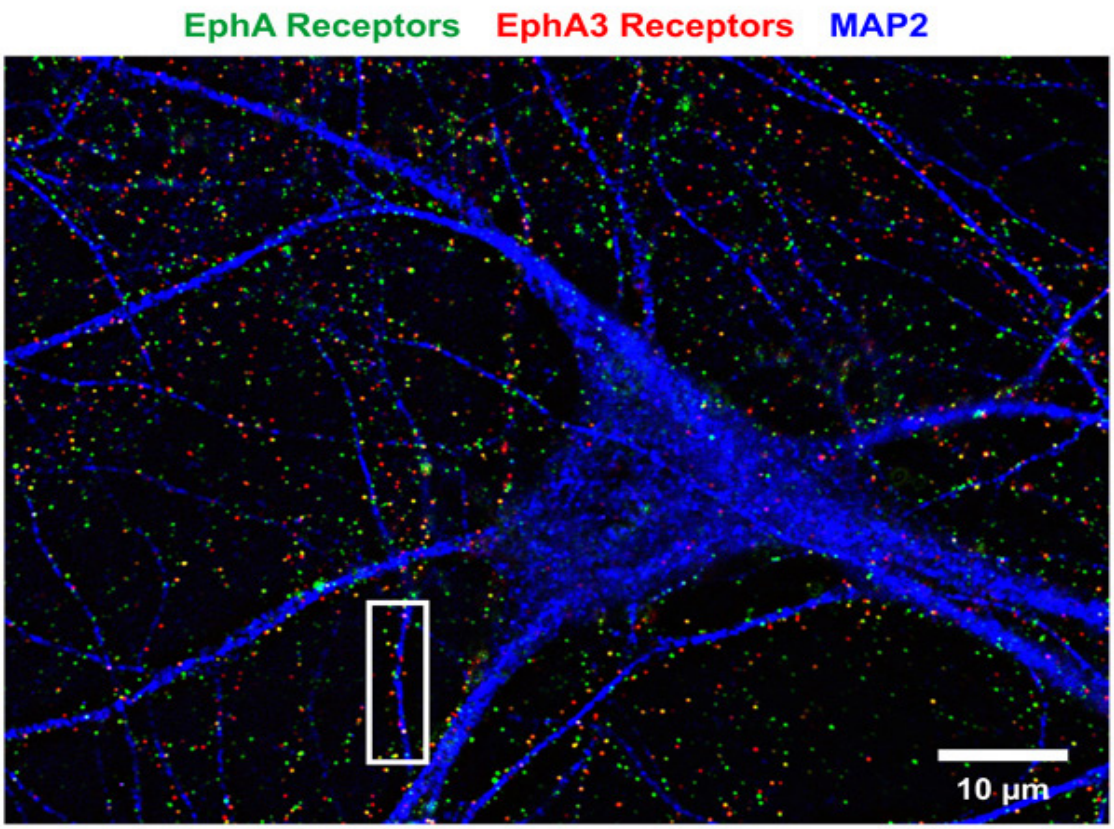

Figure 3.8 Localization of the EphA3 receptor protein labeled with anti-EphA3 (IIIA4) antibody in combination with the affinity probe method detecting EphA receptor family members with ephrin-A3-Fc. Surface EphA3 receptors were labeled with a monoclonal antibody (IIIA4) to EphA3 (red) and ephrin-A3-Fc was used as an affinity probe to detect all surface EphA receptor family members (green). Detection of the receptors was performed in live hippocampal cultures at 11 div, and after fixation, neurons were stained with anti-MAP2 antibody (blue). Surface EphA3 receptor protein, as other EphA family members, was found on or near the dendrites and in their surroundings. Boxed area with details of the labeling is shown in Figure 3.9.

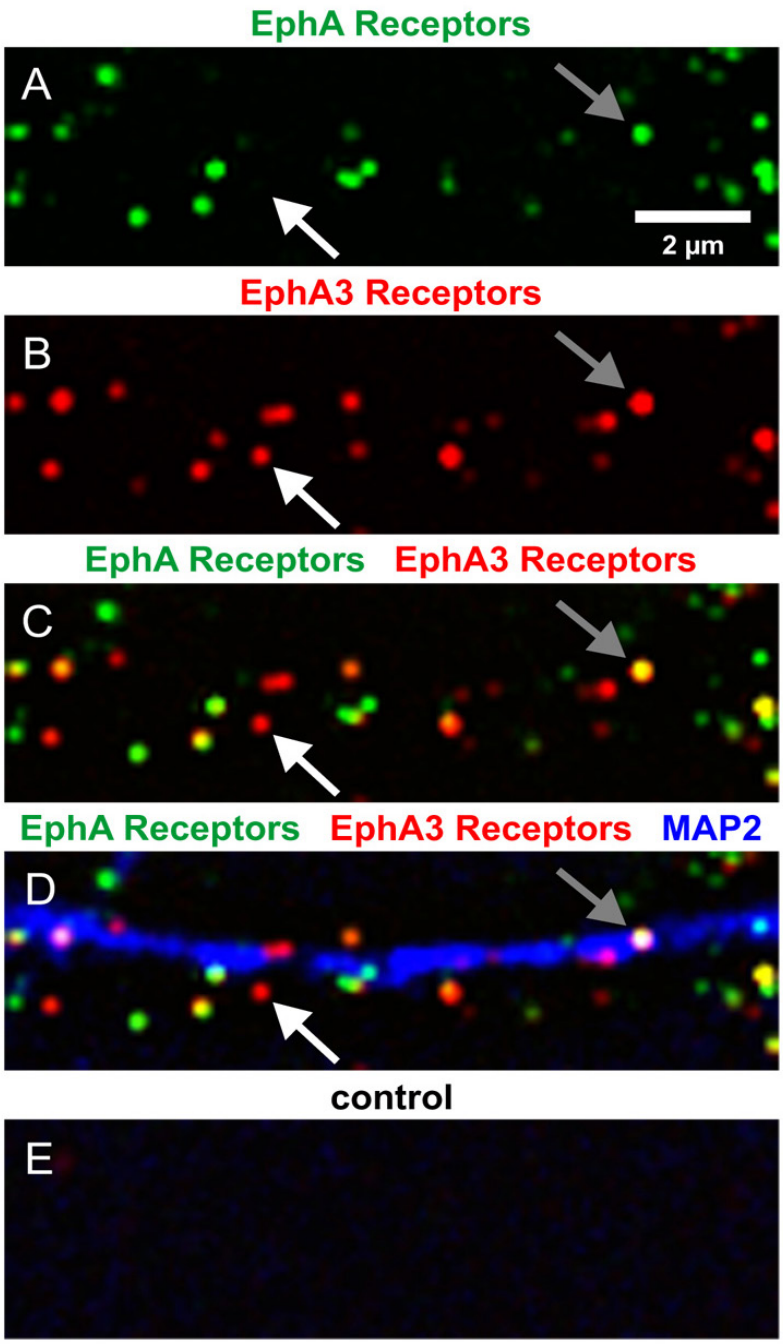

Figure 3.9 Differential ability of the EphA3 receptor to bind ephrin-A3-Fc. A-D. Boxed area from Figure 3.8. EphA receptor family members detected by the ephrin-A3-Fc affinity probe (green), EphA3 receptor labeled by the monoclonal antibody (IIIA4) to EphA3 (red), and MAP2 (blue). **A.** Single channel with the labeling of EphA receptor family members. **B.** Single channel with the labeling of EphA3 receptor. **C.** Merged two color channels with EphA family members (green) and EphA3 (red). Notice that while some EphA3 receptors were detected by both, the anti-EphA3 antibody and ephrin-A3-Fc affinity probe (grey arrows), others were only detected by the anti-EphA3 antibody and not by the ephrin-A3-Fc affinity probe (white arrows). **D.** Three

color channels merged with EphA family members (green), EphA3 (red), and MAP2 (blue). Similarly to other EphA receptors, EphA3 is found on or in close contact with the dendrites and in the neighboring areas. **E.** Control image, where the Fc fragment was added to the culture instead of ephrin-A3-Fc and primary antibodies were omitted.

CHAPTER 4 ATTRACTIVE ROLE OF EPHRIN-A ACTIVATION

4.1 Chronic Treatments with Clustered and Unclustered EphA3-Fc Had Differential Effects on PSD-95 Protein Level

4.1.1 EphA3-Fc Chimera Treatment Design to Distinguish Between Activation of Ephrin-A Reverse Signaling and Blockade of EphA Forward Signaling

Chimeric protein constructs containing Eph or ephrin extracellular domains fused to the Fc fragment of human IgG can be used to either activate or block Eph/ephrin signaling (Bourgin et al., 2007; Carmona et al., 2009; Fu et al., 2011; Murai et al., 2003). Formation of Eph/ephrin signaling clusters underlies signal initiation by these proteins (Egea and Klein, 2007; Himanen et al., 2010; Janes et al., 2012; Pitulescu and Adams, 2010). Eph receptor activation can be induced by clustered or membrane-attached forms of ephrin ligands, but not by monomeric soluble forms (Davis et al., 1994). Moreover, bidirectional nature of Eph/ephrin signaling (Aoto and Chen, 2007; Lisabeth et al., 2013; Pasquale, 2005) needs to be taken under consideration, when using chimeras of either Eph receptors or ephrin ligands to manipulate Eph/ephrin signal transduction.

In this study, to investigate the effects of ephrin-A ligand activation on development of excitatory synapses in vitro, clustered EphA3-Fc chimera was used. Treatment of hippocampal cultures with the clustered EphA3-Fc has potentially a dual effect on EphA/ephrin-A signaling. It can activate endogenous ephrin-A ligands (reverse signal activation), but it also can block endogenous EphA receptors (forward signal inhibition).

Endogenous EphA receptors are blocked in this type of treatment because they cannot interact with the endogenous ephrin-A ligands, which are bound to the clustered EphA3-Fc chimera (Figure 4.1A). To distinguish between these two effects, treatment with unclustered EphA3-Fc chimera was added to the experimental design. Similarly to clustered EphA3-Fc, the unclustered EphA3-Fc can block the endogenous EphA receptors (forward signal inhibition), but as a homodimer, it theoretically cannot activate ephrin-A ligands as strongly as the clustered form can (Klein, 2012) (Figure 4.1B). The two types of treatment, one with clustered and the other with unclustered EphA3-Fc chimera, differ in the potential to activate ephrin-A signaling. This difference was used to distinguish between the effects of ephrin-A activation and EphA inhibition on the development of excitatory synapses in vitro. Control treatments were performed with either clustered or unclustered Fc fragments (Figure 4.1C), and they were combined for data quantification.

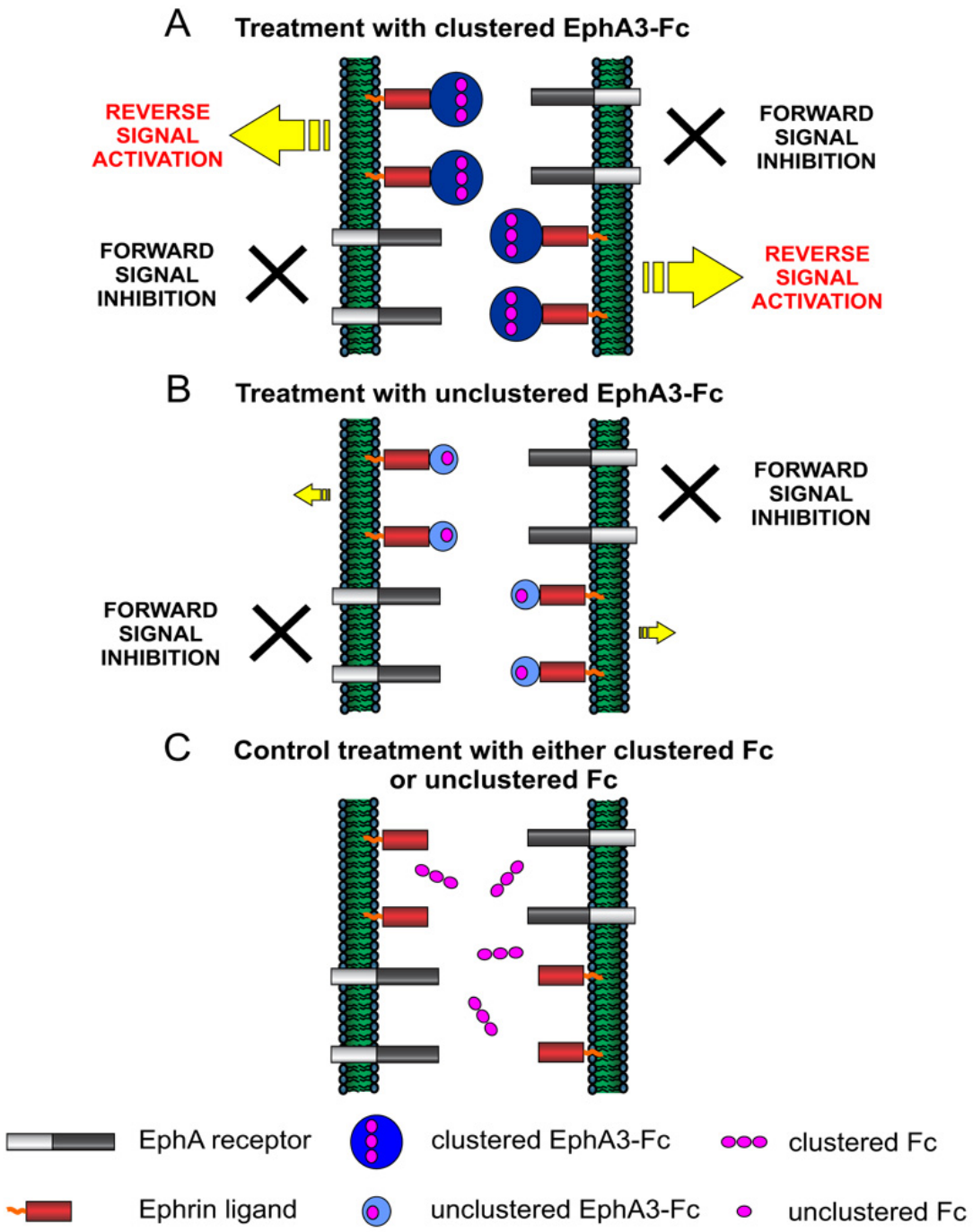

Figure 4.1 Comparison between treatments with clustered and unclustered EphA3-Fc chimeras. A. Treatment with clustered EphA3-Fc activates reverse signaling by ephrin-A ligands and blocks forward signaling by EphA receptors. **B.** Treatment with unclustered EphA3-Fc also blocks forward signaling by EphA receptors, but it doesn't activate ephrin-A reverse signaling as strongly as clustered EphA3-Fc. **C.** Control treatments with either clustered of unclustered Fc (only clustered Fc control is shown for simplicity).

4.1.2 Chronic Treatment with Clustered EphA3-Fc but Not with Unclustered EphA3-Fc Increased PSD-95 Protein Level

In order to examine the effects of EphA/ephrin-A signaling on the development of excitatory synapses in vitro, hippocampal cultures where treated with 5 µg/ml of either clustered or unclustered EphA3-Fc chimera for 24 hours from 11 to 12 div. 5 µg/ml of either clustered or unclustered Fc fragment was added for the same period of time to the control cultures. Treated cultures were fixed and triple labeled with antibodies to detect presynaptic protein VGLUT1, postsynaptic protein PSD-95, and neuronal marker MAP2. Three independent experiments were analyzed with respect to pre- and postsynaptic protein expression level, excitatory synapse density, and nonsynaptic puncta number using immunocytochemistry combined with deconvolution fluorescence microscopy.

First, expression level of the postsynaptic protein PSD-95 and the presynaptic protein VGLUT1 was examined in the three types of treatments. Mean fluorescence signal intensity for each labeled protein was measured in four 20 µm long dendritic segments per neuron and 23-33 neurons per experiment were analyzed. Dendritic segments were chosen at least 40 µm away from the cell body to exclude quantification of the giant mossy fiber synapses between granule cells and CA3 neurons (Lee et al., 2013). This allowed focusing the analysis on the connections between CA3-CA1 and CA3-CA3 pyramidal neurons. Figure 4.2 presents representative images of anti-PSD-95 (A, E, and I) and anti-VGLUT1 (B, F, and J) staining in the Fc-treated (A, B), clustered EphA3-Fc-treated (E, F), and unclustered EphA3-Fc-treated (I, J) cultures. Treatment with clustered

EphA3-Fc visibly increased the immunofluorescence signal level of PSD-95 protein (Figure 4.2E) as compared to the Fc control (Figure 4.2A) and treatment with unclustered EphA3-Fc (Figure 4.2I). Clustered EphA3-Fc increased mean PSD-95 signal intensity by 37% with respect to the Fc control ($P < 0.01$) and 31% with respect to the unclustered EphA3-Fc treatment ($P < 0.01$) (Figure 4.3 A). There was no significant difference in the mean PSD-95 signal intensity between Fc control cultures and cultures treated with the unclustered EphA3-Fc. With respect to the mean signal intensity of the presynaptic protein VGLUT1, there was some variability between the experiments observed and no clear trend or significant difference could be determined (Figure 4.3B).

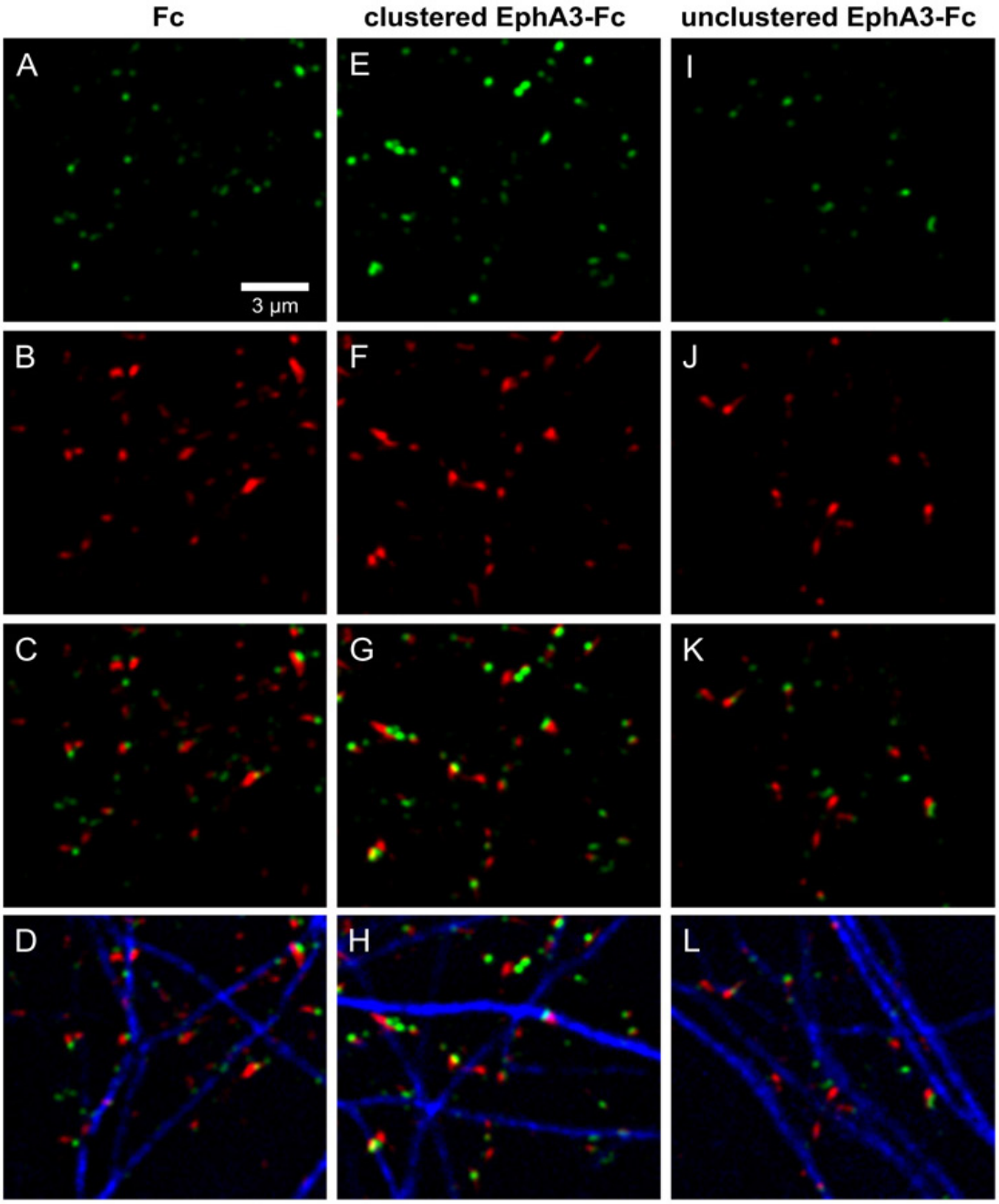

Figure 4.2 Effects of chronic treatments with clustered and unclustered EphA3-Fc chimeras on the properties of excitatory synapses in hippocampal cultures at 12 div. Representative images of excitatory synapses from three treatment conditions showing labeling of a postsynaptic protein PSD-95 (green), presynaptic protein VGLUT1 (red), and neuronal marker MAP2 (blue). Sister cultures were treated either with 5 µg/ml of Fc fragment (control), 5 µg/ml of clustered EphA3-Fc or 5 µg/ml unclustered EphA3-Fc for 24 hours from 11 to 12 div. After treatments, cells were fixed and triple labeled with anti-PSD-95, anti-VGLUT1, and anti-MAP2 antibodies. Image acquisition and processing were performed using the same parameters for all treatments within each experiment. **A**, **E**, and **I** PSD-95; **B**, **F**, and **J** VGLUT1; **C**, **G**, and **K** Merged two color

channels with PSD-95 (green) and VGLUT1 (red); **D**, **H**, and **L** Merged three color channels with PSD-95 (green), VGLUT1 (red), and MAP2 (blue). **A-D.** Treatment with Fc fragment. **E-H.** Treatment with clustered EphA3-Fc. Note the increase in the PSD-95 signal intensity (green, **E**). **I-L.** Treatment with unclustered EphA3-Fc. Note the decrease in excitatory synapse density in (K). Scale bar in (A) applies to all images.

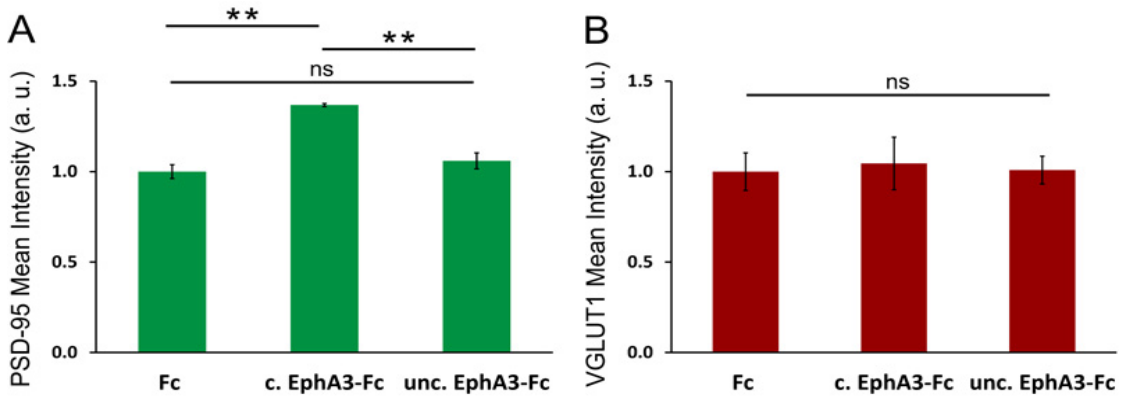

Figure 4.3 Chronic treatment with clustered EphA3-Fc increased PSD-95 protein level, but did not affect VGLUT1 in cultured hippocampal neurons. A. Normalized PSD-95 and VGLUT1 (**B**) mean signal intensity in cultures treated with Fc fragment (control), clustered EphA3-Fc, and unclustered EphA3-Fc for 24 hours from 11-12 div. n = 3 independent experiments; ns - nonsignificant ($P > 0.05$); ** $P < 0.01$ with one-way correlated-samples ANOVA and post hoc Tukey test. Scale bars represent ± SEM.

4.2 Unlike Chronic Treatment with Unclustered EphA3-Fc, Clustered EphA3-Fc Did Not Decrease Excitatory Synapse Density

Excitatory synapse density was also examined in the same cultures. Excitatory synaptic contacts were defined as structures, where VGLUT1- and PSD-95-positive puncta directly appose each other. Synapse number was counted in the same dendritic segments that were first analyzed for PSD-95 and VGLUT1 fluorescence signal intensity. Figure 4.2 shows representative images of excitatory synapses in Fc-treated control

cultures (C, D), clustered EphA3-Fc-treated cultures (G, H), and unclustered EphA3-Fc-treated cultures (K, L). Figure 4.4 presents results of the quantitative analysis from three independent experiments. Mean synapse density decreased 24% in cultures treated with unclustered EphA3-Fc as compared to Fc-treated control cultures (decrease in mean synapse density was 1.5 ±0.24 synapse/ 20 µm dendrite; P< 0.01 versus Fc and c. EphA3-Fc). In contrast, clustered EphA3-Fc treatment did not significantly change mean synapse density from the control values.

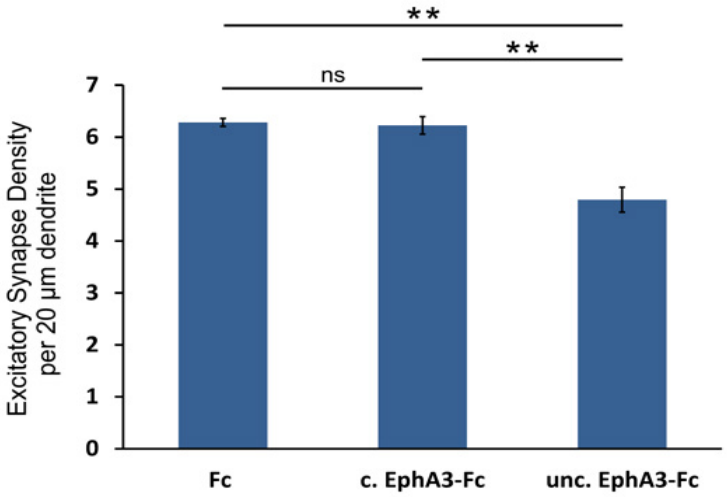

Figure 4.4 Chronic treatment with unclustered EphA3-Fc decreased excitatory synapse density in cultured hippocampal neurons. Quantification of the mean excitatory synapse density in hippocampal cultures after treatments with Fc fragment (control), clustered EphA3-Fc, and unclustered EphA3-Fc for 24 hours from 11-12 div. Treatment with unclustered EphA3-Fc decreased mean synapse density as compared to Fc control, but after clustered EphA3-Fc treatment mean synapse density stayed unchanged. n = 3 independent experiments; ns - nonsignificant (P > 0.05); ** P < 0.01 with one-way correlated-samples ANOVA and post hoc Tukey test. Scale bars represent ± SEM.

4.3 Chronic Treatments with either Clustered or Unclustered EphA3-Fc Didn't Affect Nonsynaptic PSD-95 and VGLUT1 Puncta Density

In addition to excitatory synapse density, number of the nonsynaptic VGLUT1- and PSD-95-positive puncta was quantified per dendrite length. VGLUT1 and PSD-95 fluorescent puncta were defined as nonsynaptic, when they did not have a directly apposed post- and presynaptic partner, respectively. In theory, nonsynaptic puncta population would include solitary VGLUT1- and PSD-95-positive puncta, but also developing synapses and synaptic contacts in the process of elimination. In the latter case, the apposing synaptic partners might not have been detected with the anti-VGLUT1 and anti-PSD-95 immunofluorescence. Quantitative analysis of the nonsynaptic VGLUT1 and PSD-95 puncta density showed that there were no significant differences between cultures treated with Fc, clustered EphA3-Fc, and unclustered EphA3-Fc (Figure 4.5). However, treatment with the unclustered EphA3-Fc showed a trend to decrease nonsynaptic PSD-95 puncta density with $P = 0.08$ (Figure 4.5A).

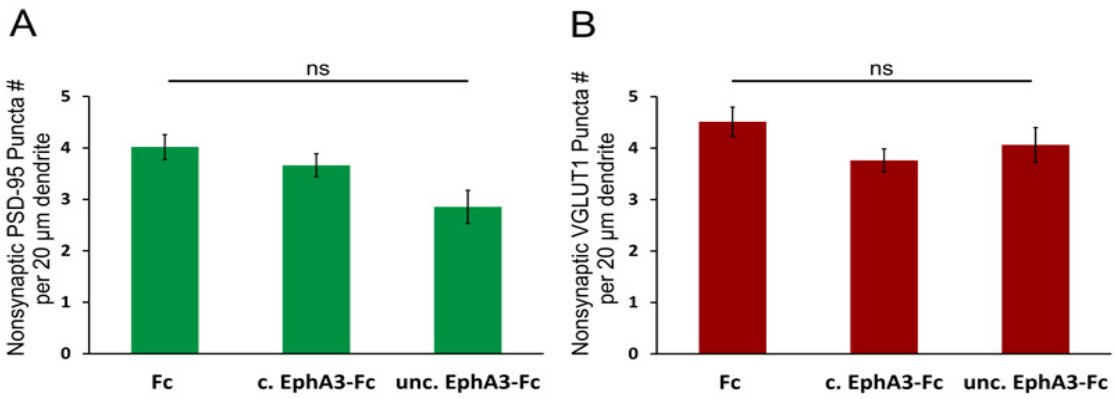

Figure 4.5 Chronic treatments with either clustered or unclustered EphA3-Fc had no effect on the nonsynaptic PSD-95 and VGLUT1 puncta density. A. Quantification of the nonsynaptic PSD-95 puncta density in hippocampal cultures treated with Fc fragment (control), clustered EphA3-Fc, and unclustered EphA3-Fc for 24 hours from 11-12 div. n = 3 independent experiments; ns - nonsignificant, where P = 0.08 with one-way correlated-samples ANOVA **B.** Quantification of the nonsynaptic VGLUT1 puncta density. No significant differences were observed. Scale bars represent ±SEM.

4.4 Synaptic Effects of the Treatments - Summary of the Results

Chronic (24-hour) treatments of young (11-12 div) hippocampal cultures with clustered EphA3-Fc and unclustered EphA3-Fc affected developing excitatory synapses in clearly different ways, which are summarized in Table 4.1. Clustered EphA3-Fc increased PSD-95 protein level, while unclustered EphA3-Fc decreased excitatory synapse density, as assessed by the quantitative immunofluorescence. Additionally, treatment with the unclustered EphA3-Fc showed a tendency to decrease nonsynaptic PSD-95-positive puncta density, but that trend did not reach statistical significance (P = 0.08).

Table 4.1 Summary of the treatment results.

Treatment Type	Effect on Eph/ephrin Signaling	PSD95 Protein Level	VGLUT1 Protein Level	Synapse Density	Non-synaptic PSD-95 Puncta Density	Non-synaptic VGLUT1 Puncta Density
Clustered EphA3-Fc	**1.** Activates ephrin-A reverse signaling **2.** Blocks EphA receptor forward signaling	+	0	0	0	0
Unclustered EphA3-Fc	**1.** Blocks EphA receptor forward signaling	0	0	−	0*	0

+ **significant increase**

− **significant decrease**

0 **no significant effect**

0* **no significant effect (decrease; p= 0.08)**

CHAPTER 5 DISCUSSION

There are three main findings of this dissertation. First, EphA receptors and ephrin-A ligands were found on the surface of astrocytes and in peridentritic areas in young (11 div) hippocampal cultures. Second, endogenous EphA3 receptors showed a differential ability to bind ephrin-A3-Fc chimera. Third, activation of ephrin-A reverse signaling increased PSD-95 expression level and protected excitatory synapses from the repulsive effects of EphA receptor inhibition. Discussed in this dissertation, expression of EphA receptors and ephrin-A ligands in hippocampal cultures is largely consistent with previous reports of EphA and ephrin-A expression in vivo (Galimberti et al., 2010; Hara et al., 2010; Murai et al., 2003; Nestor et al., 2007; Otal et al., 2006; Wang et al., 2003; Yue et al., 2002). Additionally, differential ability of EphA3 receptor to bind ephrin-A3-Fc might indicate the presence of regulatory mechanisms like cis inhibition in the hippocampus. Finally, results of the treatments with clustered and unclustered EphA3-Fc chimeras suggest that EphA/ephrin-A signaling can play an attractive/permissive role in the excitatory synapse development, in addition to well described repulsive effects of these proteins on developing neurites (Brownlee et al., 2000; Drescher et al., 1995; Frisén et al., 1998; Gao et al., 1998a; Lim et al., 2008; Nakamoto et al., 1996; Yue et al., 2008) and dendritic spines (Fu et al., 2007; Murai et al., 2003; Zhou et al., 2012).

5.1 Astrocytic and Peridendritic Expression of Surface EphA Receptors and Ephrin-A Ligands in Hippocampal Cultures

Distribution of EphA receptors and ephrin-A ligands on the surface of hippocampal cells at 11 div was investigated in this study with the affinity probe method. Both EphA and ephrin-A were found at the highest density in the peridendritic areas, where they might have been of neuronal and/or astrocytic origin. In addition, at a lower density EphA and ephrin-A were localized in the GFAP-positive locations that were not in direct contact with neuronal dendrites. Regions of the cultures that were negative for both GFAP and MAP2, which might have contained neuronal axons, oligodendrocytes, and microglia appeared to have the lowest mean densities of EphA and ephrin-A. Axons have been shown to co-express EphA and ephrin-A in developing visual system (Connor et al., 1998; Hornberger et al., 1999) and axonal EphA receptors play crucial role in generation of hippocamposeptal projection (Yue et al., 2002). It should be noted that neuronal axons that were not investigated in this study could have been an equal component of both GFAP+ and GFAP-/MAP2- regions of the cultures. Therefore, axonal expression of EphA and ephrin-A might have contributed to the mean density values in both regions. Interestingly, both EphA and ephrin-A showed a trend for higher enrichment in GFAP+ regions than in GFAP-/MAP2- regions of the cultures, suggesting expression on the surface of the astrocytes. This would be in agreement with previous studies indicating that astrocytes express EphA receptors (Nestor et al., 2007; Tremblay et al., 2007) and ephrin-A ligands (Afshari et al., 2010; Murai et al., 2003; Winslow et al., 1995). Specifically, mRNAs for multiple EphA receptors have been detected in acutely

isolated hippocampal astrocytes, cortical astrocytes in culture (Nestor et al., 2007), and EphA4 protein was found in a subset of astrocytic processes in adult hippocampus (Tremblay et al., 2007). With respect to ephrin-A ligands, mRNAs of all ephrinAs (ephrin-A1-ephrin-A5) have been reported in purified astrocytic cultures (Afshari et al., 2010) and astrocytic ephrin-A3 protein expression has been documented in the adult hippocampus (Murai et al., 2003). Moreover, in co-cultures of cortical neurons and astrocytes, ephrin-A5 mRNA was detected in astrocytes at a significantly higher level than in neurons (Winslow et al., 1995). This was supported by localization of ephrin-A ligands with EphA5-IgG affinity probe on astrocytes, but not on cortical neurons in the same study.

Highest enrichment of EphA receptors and ephrin-A ligands in peridendritic areas suggests that these proteins might play a role in hippocampal synapse development. In this study, it was not feasible to discern between dendritic and astrocytic origin of EphA and ephrin-A in peridendritic locations. However, it's possible that both dendrites and astrocyte processes were contributing to the peridenritic expression of these proteins. The following findings could support this interpretation. Multiple EphA receptors (EphA3-EphA7) are expressed by hippocampal neurons during development (Cooper et al., 2009; Yue et al., 2002) as well as ephrin-A3 (Galimberti et al., 2010; Stein et al., 1999) and ephrin-A5 ligands (Stein et al., 1999). Moreover, EphA4 has been shown to be expressed in a subset of astrocytic filopodia that encapsulate hippocampal synapses (Tremblay et al., 2007) and astrocytic processes expressing ephrin-A3 have been detected close to dendritic spines in adult hippocampus (Murai et al., 2003).

Characterization of the exact origin of EphA and ephrin-A in the peridenritic areas of hippocampal neurons would be very helpful in understanding their role in developmental processes. Superresolution imaging by stimulated emission depletion (STED) microscopy could be a useful technique to reach that goal (Panatier et al., 2014).

Presented in this dissertation, peridendritic expression of surface EphA receptors and ephrin-A ligands locates these proteins close to the sites of developing synapses. However, most of the excitatory synapses in young (11 div) hippocampal cultures were not in a direct contact with EphA and ephrin-A proteins, and no expression of EphA or ephrin-A was detected at the pre- and postsynaptic sites. Nevertheless, a subset of pre- and postsynaptic terminals with a clear direct contact with EphA or ephrin-A-positive puncta was observed. Small proportion of these direct contacts might still have an important impact on developing synapses. The data presented shows a snapshot of a hippocampal culture during a very dynamic process of synapse development (Ahmari et al., 2000; Cohen-Cory, 2002; Dailey and Smith, 1996; Zhu et al., 2016; Ziv and Smith, 1996). Therefore, it cannot show a full picture of all the interactions that EphA and ephrin-A proteins potentially might have with dendritic and synaptic structures over time. It is known that perisynaptic astrocytic processes can dynamically change their engagement with dendritic spines within minutes (Haber et al., 2006; Verbich et al., 2012) and astrocytic EphA/eprhin-A signaling might also be dynamic in nature. It’s been reported that disruption of EphA/ephrin-A signaling decreased the lifetime of newly formed dendritic filopodia that experienced dynamic astrocytic contacts, but did not affect the filopodia that were not in contact with astrocytic processes (Nishida and

Okabe, 2007). Therefore, it's possible that direct astrocytic EphA or ephrin-A contacts with the synapse could be initiated/terminated on a dynamic basis as checkpoints of synaptic development. Depending on the nature of EphA/ephrin-A signal (or lack of it), developing synapse status could be directed either towards elimination or stabilization.

Additionally, EphA and ephrin-A labeling consisted of fluorescent puncta with variable signal intensities suggesting differential EphA and ephrin-A protein abundance depending on a location, which would likely have functional consequences. An indication of the functional relevance for EphA or ephrin-A differential level of expression may come from research on the development of the visual system. During formation of a retinocollicular topographic map, graded EphA expression on retinal ganglion cells guides their axons to the right locations in the superior colliculus, where ephrin-A ligands are expressed in a complementary gradient (Feldheim and O'Leary, 2010). It's been shown that the retinocollicular topographic pattern doesn't depend on the absolute, but rather on the relative EphA expression level on retinal ganglion cells and that the relative difference in EphA expression determines the competition between retinal ganglion axons (Brown et al., 2000). Similarly, variable levels of EphA and ephrin-A proteins along the dendrite of a hippocampal neuron might be defining the competition between various dynamic processes that are undergoing during development, including synapse formation.

5.2 Not All Endogenous EphA3 Receptors Are Available for Ephrin-A3-Fc Binding

EphA3 receptor was localized in the hippocampal cultures at 11 div with the IIIA4 anti-EphA3 antibody. The IIIA4 antibody (a generous gift from Dr. Andrew Boyd) was originally employed to isolate human EphA3 receptor protein (HEK) from pre-B acute lymphoblastic leukemia cell line (Boyd et al., 1992). The IIIA4 antibody binds native form of the EphA3 receptor protein with a subnanomolar affinity (Boyd et al., 1992; Vearing et al., 2005). As presented in this dissertation, detection of EphA3 receptor in living hippocampal cells was combined with the detection of EphA family members by the ephrin-A3-Fc affinity probe. It was expected that ephrin-A3-Fc would bind all EphA receptor family members (including EphA3) because of the promiscuity within each class of Ephs and ephrins (Pasquale, 2004). Ephrin-A3 ligand binds EphA3 receptor with a strong affinity, as shown by enzyme-linked immunosorbent (ELISA) assays (Noberini et al., 2012). Therefore, it was unexpected to observe that not all endogenous EphA3 receptors bound ephrin-A3-Fc in living hippocampal cultures. In other words, IIIA4-labeled population of EphA3 receptors was comprised of EphA3 receptors that bound ephrin-A3-Fc ligand, and EphA3 receptors that did not bind ephrin-A3-Fc ligand. It's been suggested that in the affinity probe method ephrin-A-Fc might not detect all endogenous EphA receptors because some of the EphA receptors are bound to their endogenous ephrin-A ligands, and therefore they are masked (Sobieszczuk and Wilkinson, 1999). Binding between endogenous EphA receptors and ephrin-A ligands can take place in trans, when EphA and ephrin-A are expressed on different cells and in cis, when EphA and ephrin-A are co-expressed on the surface of the same cell (Carvalho

et al., 2006; Yin et al., 2004). Interestingly, cis binding between EphA and ephrin-A inhibits EphA receptor activation (Carvalho et al., 2006; Yin et al., 2004). Additionally, interactions in cis can occur either through ligand binding domain of the EphA receptor (Yin et al., 2004) or through other areas of EphA extracellular domain (Carvalho et al., 2006). These studies could help to explain data, presented in this dissertation, showing that certain EphA3 receptors expressed on the surface of hippocampal cells were not able to bind a high affinity ephrin-A3-Fc ligand. EphA3 receptors that were already bound to the endogenous ephrin-A ligands either in trans or in cis would not be able to bind the exogenous ephrin-A3-Fc affinity probe. Inhibition of EphA receptor signal activation by cis interactions with ephrin-A ligands have been shown to play important roles in axon guidance of retinal ganglion cells (Hornberger et al., 1999) and spinal motor neurons (Kao and Kania, 2011). If cis EphA/ephrin-A interactions occur in the hippocampus remains to be determined.

5.3 Divergent Effects of Ephrin-A Activation and EphA Inhibition on Excitatory Synapse Development

5.3.1 EphA/Ephrin-A Signaling Can Induce Repulsion or Attraction at the Synapse

Interplay between Eph forward signaling and ephrin reverse signaling makes Eph/ephrin interactions very complex, and they can involve repulsion or attraction (Pasquale, 2005).

EphA receptor activation (forward signaling) has been shown to have repulsive effects on postsynaptic spines (Lisabeth et al., 2013). Short-term (45 min) treatment of acutely isolated hippocampal slices from adult mice with unclustered ephrin-A3-Fc reduced spine length and density (Murai et al., 2003). Similarly, spine density was decreased after chronic application of clustered ephrin-A1-Fc to cultured postnatal hippocampal slices (24 hour treatment) and dissociated hippocampal cultures (treatment at 20 div for 5-24 hours)(Fu et al., 2007). In both cases, EphA4 receptor activation was involved in the repulsive effects on the postsynaptic spines (Fu et al., 2007; Murai et al., 2003). Moreover, activation of EphA4 was found to play a role in downregulation of AMPA receptor subunit GluA1 and decrease in the amplitude and frequency of miniature excitatory postsynaptic currents (mEPSCs) (Fu et al., 2011; Fu et al., 2007).

Activation of EphA forward signaling has also been shown to have attractive effects, instead of repulsive ones, on synapse formation (Akaneya et al., 2010). In that study, chronic activation of EphA receptors in hippocampal E18 cultures for 6 days or in P0 slice cultures for 4 days, with clustered ephrin-A5, showed synaptogenic effects, including increase in expression of NMDA receptor subunits and PSD-95. Additionally, in EphA5-functional knockout (EphA5$^{lacZ/lacZ}$) NMDA receptor and PSD-95 expression was decreased as compared to wild-type controls (Akaneya et al., 2010). Another study by Clifford et al. showed that EphA4 overexpression, in cortical neurons, promoted dendritic spine maturation and increased density of spines expressing PSD-95 at 14 div.

In addition to EphA receptor signaling, the role of ephrin-A ligands in synapse formation and function has also been investigated. In the adult hippocampus, ephrin-A3-enriched astrocytic processes surround excitatory synapses that express EphA4 on the postsynaptic spines (Murai et al., 2003). In the hippocampus of ephrin-A3 null mice, aberrant dendritic spines were observed, but spine density was not affected. In addition, knock-out of ephrin-A3 increased expression of astrocytic glutamate transporters (Carmona et al., 2009). Stimulation with EphA2-Fc (unclustered) chimera reduced glutamate transport in wild-type, but not in the ephrin-A3 null hippocampal slices, indicating that ephrin-A3 reverse signaling was responsible for the regulation of glial glutamate uptake (Carmona et al., 2009). Interestingly, the interactions between ephrin-A3 and EphA4 at the excitatory synapses have been shown to play a role in regulation of LTP (Filosa et al., 2009). In addition to ephrin-A3, ephrin-A5 affects excitatory synapses in the hippocampus (Otal et al., 2006). In P21 ephrin-A5 KO mice, synapse density was reduced in the hippocampal commissural areas, which suggests attractive/synaptogenic role for ephrin-A5 signaling (Otal et al., 2006).

Discussed here studies indicate the importance of EphA/ephrin-A signaling in synapse formation and function, but also show how intricate and elusive EphA/ephrin-A bidirectional interactions are. The main goal of this dissertation was to shed light on the interplay between EphA forward signaling and ephrin-A reverse signaling in excitatory synapse development, and interpretation of the collected data will be discussed in the next two sections.

5.3.2 Experimental Design Uncovered the Effects of Ephrin-A Reverse Signal Activation on Excitatory Synapse Development

When EphA receptors were chronically blocked in young (11-12 div) hippocampal cultures, excitatory synapse density decreased. In contrast, when ephrin-A ligands were chronically activated, at the same time when EphA receptors were blocked, synapse number did not change and increase in PSD-95 level was observed. Results of the chronic treatments that manipulated EphA/ephrin-A signaling are summarized in Table 1. Experiments with clustered EphA3-Fc and unclustered EphA3-Fc chimeras were designed to distinguish between effects of ephrin-A ligand activation and EphA receptor inhibition, as described in Figure 4.1. Different outcomes of the treatments that were obtained in this study suggested that the assumptions of the experimental design were valid. It was assumed that clustered EphA3-Fc (clusters of receptor dimers) would activate ephrin-A ligands significantly stronger than unclustered EphA3-Fc (receptor dimers). Different effects of the two types of treatments on excitatory synapse development indicated that indeed clustered EphA3-Fc substantially activated ephrin-A ligands over unclustered EhA3-Fc. This agrees with the current understanding of Eph/ephrin signaling, where higher order Eph and ephrin clusters have larger signaling potential than heterotetramers composed of two Eph receptors and two ephrin ligands (Klein, 2012).

5.3.3 Ephrin-A Activation Increased PSD-95 Expression Level and Protected Excitatory Synapses from Repulsive Effects of EphA Receptor Inhibition

Data presented in this dissertation indicate that chronic ephrin-A activation enhances expression of postsynaptic protein PSD-95 in young (11-12 div) hippocampal cultures, and it can counteract the repulsive effects of EphA receptor inhibition. EphA receptor inhibition with unclustered EphA3-Fc displayed repulsive effects because it reduced excitatory synapse density. Moreover, it displayed a trend ($P = 0.08$) to decrease the number of nonsynaptic PSD-95-positive puncta, which might represent the sites of actively forming synaptic contacts (Gerrow et al., 2006). On the other hand, synapse density was not changed when ephrin-A activation and EphA inhibition occurred simultaneously in the treatment with clustered EphA3-Fc. The net effect of the treatment with clustered EphA3-Fc suggests that ephrin-A activation displayed an attractive (synaptogenic) action in contrast to EphA inhibition that displayed a repulsive (anti-synaptogenic) action. Interestingly, attractive ephrin-A and repulsive EphA, appeared to act postsynaptically, which was reflected in the increase in PSD-95 protein expression due to ephrin-A activation and decrease in nonsynaptic PSD-95 puncta density due to EphA inhibition.

In addition to the above interpretation, based on complexity of bidirectional Eph/ephrin signaling, one can also speculate that the application of unclustered EphA3-Fc not only inhibited EphA receptor activation, but also inhibited ephrin-A activation. Unclustered EphA3-Fc, as a receptor dimer, might have a restricted potential to activate ephrin-A signaling (Klein, 2012) and because the endogenous ephrin-A ligands interact

with it, they cannot be activated by the endogenous EphA receptors. Therefore, inhibition of ephrin-A activation might have been responsible for the decrease in synapse density in cultures treated with unclustered EphA3-Fc. Importantly, in this second scenario, ephrin-A inhibition by unclustered EphA3-Fc would exert repulsive (anti-synaptogenic) role and ephrin-A activation by clustered EphA3-Fc would still exert attractive (synaptogenic) role.

Attractive or synaptogenic role of ephrin-A reverse signaling, presented in this dissertation, agrees with several findings that have been published up to date. In ephrin-A5 KO mice, synapse density was decreased in the hippocampus (Otal et al., 2006). Additionally, Marler et al. showed that ephrin-A5 interacts with TrkB, a tyrosine kinase receptor activated by BDNF and NT-4 neurotrophins (Barbacid, 1994). Interestingly, in hippocampal slice cultures from ephrin-A5 KO mice, the BDNF-mediated rise in synapse density was significantly reduced at 13 div as compared to the wild type suggesting a possible link between ephrin-A5 and BDNF in a synaptogenic pathway (Marler et al., 2008). Furthermore, BDNF was shown to promote trafficking of PSD-95 to dendrites, a process downstream from the NMDA receptor activation (Yoshii and Constantine-Paton, 2007) and NMDA receptor function could be modulated by activation of astrocytic ephrin-A3, which reduces glutamate uptake (Carmona et al., 2009). Importantly, PSD-95 overexpression was shown to increase stability of developing synapses (Taft and Turrigiano, 2014), which might explain the attractive/synaptogenic effect of ephrin-A5 activation presented in this dissertation.

Taken together, attractive role of ephrin-A reverse signaling in development of excitatory synaptic connections fits well into the myriad of possible mechanisms, which will need to be investigated in the future.

5.4 Conclusions and Future Directions

Gene mutations in EphA3 and EphA7 have been linked to neurodevelopmental diseases (Casey et al., 2012; Traylor et al., 2009). Additionally, ephrin-A2/ephrin-A3 double knockout mice display a phenotype associated with Autism Spectrum Disorders (Wurzman et al., 2015). The connection between EphA/ephrin-A signaling system and aberrant neurodevelopment makes it essential to better understand EphA/ephrin-A function in the brain. This dissertation characterized expression of EphA/ephrin-A proteins in hippocampal cultures and analyzed EphA/ephrin-A signaling in the context of excitatory synapse development. Importantly, this work extends the current understanding of ephrin-A reverse signaling by adding regulation of PSD-95 protein expression into the equation. Future experiments may explore possible connections between glutamate receptors, BDNF/TrkB signaling, and ephrin-A activation. Additionally, different ephrin-A knockout animals could be used to identify specific ephrin-A protein(s) responsible for the attractive function in the excitatory synapse development. Finally, the effects of ephrin-A activation on excitatory synapse function could be examined by electrophysiological recordings.

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