INTERACTON BETWEEN EPHRIN/EPH AND BDNF IN MODULATING HIPPOCAMPAL SYNAPTIC TRANSMISSION AND SYNAPSE FORMATION

by

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ABSTRACT OF THE DISSERTATION

Interaction between Ephrin/Eph and BDNF in Modulating Hippocampal Synaptic Transmission and Synapse Formation

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The hippocampus is a brain structure known to be critical for learning and memory consolidation. Abnormal development or damage to this structure is known to play a role in developmental or degenerative neurological disorders such as autism and Alzheimer's disease. In this thesis, I argue that an interaction between ephrin/Eph and BDNF signaling pathways is critical for the development of the selective connection of CA3 neurons to CA1 neurons in hippocampus. This claim was evaluated on the basis of electrophysiology evidence about the ephrin/Eph – BDNF interaction in synaptic activity, and the effect of the interrupting Eph and BDNF signaling on the hippocampal projection specificity onto CA1 neurons by functional synapse identification via the combination of electrophysiology and immunocytochemistry.

First, I confirmed that the primary hippocampal neuronal culture can be a model for studying the specificity of synaptic connection within the hippocampal circuitry. Functional synapses were characterized by recording from pairs of cells which we subsequently identified with immunocytochemical labeling. Most connections were unidirectional, and I found that when one of the cells was a CA1 neuron (identified by labeling with the CA1 marker anti-SCIP) it was predominantly the postsynaptic member of the pair, a result consistent with *in vivo* connectivity. Second, ephrin-A/EphA signaling was shown to produce a transient increase in synaptic transmission and be able to inhibit the effect of subsequent BDNF application on synaptic activity. These electrophysiological experiments were suggestive of possible interaction between ephrin-A/EphA and BDNF in modulation synapse formation. Third, interruption of the endogenous EphA signaling by the kinase dominant negative EphA constructs significantly changed the natural synaptic connection selectivity in the hippocampal circuitry and dramatically increased the bi-directional connections in the culture as a consequence.

The empirical results presented in this thesis provide a valuable mechanism for hippocampal trisynaptic circuitry development and function through balancing opposite influences of various modulating factors at specific developmental phases.

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I reserve this last sentence for my family. I want to thank my mother, father and two lovely sisters for their love and support over so many years for my study, regardless of whatever I did. Here, I want to say "I love you!" to all of them.

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TERMINOLOGY

- Ephrin-A5: also known as AL-1, RAGS, LERK-7, and EFL-5, a member of the ephrin ligand family which binds members of the Eph receptor family
- EphA5: also known as Ehk1, Bsk, Cek7, Hek7, and Rek7, a member of the Eph receptor family which binds members of the ephrin ligand family
- EphA3: also known as Cek4, Mek4, Hek, Tyro4, and Hek4, a member of the Eph receptor family which binds members of the ephrin ligand family
- Ephrin-A5-Fc: Recombinant Ephrin-A5/Fc Chimera from R & D (the extracellular domain of ephrin-A5 was fused to the Fc region of IgG1)
- EphA5-Fc: Recombinant EphA5/Fc Chimera from R & D (the extracellular domain of EphA5 was fused to the Fc region of IgG1)
- EphA: refer to the general EphA receptor family
- Ephrin-A: refer to the general Ephrin-A ligand family
- EphA5(K-)-GFP: GFP-tagged kinase deficient EphA5 mutant DNA
- EphA3(K-)-GFP: GFP-tagged kinase deficient EphA3 mutant DNA
- EphA(K-)-GFP: refer to both EphA3(K-)-GFP and EphA3(K-)-GFP
- SCIP: also known as Oct-6 and Tst-1, a POU-III subfamily member that is a marker of hippocampal CA1 neurons

CHAPTER 1

INTRODUCTION

Abnormal neuronal connections have been found in variety of neurological diseases, such as Huntingtion's disease, Alzheimer's disease, schizophrenia and Autism (Dityatev and El-Husseini, 2006). So, it is of much significance to investigate the mechanisms underlying the brain circuitry development. It is well known that the formation of neural connectivity is influenced by a variety of attractive/repulsive guidance cues and neurite promoting/collapsing factors. For example, brain-derived neurotrophic factor (BDNF) has been extensively demonstrated to promote synapse formation and neuronal function while the ephrin/Eph family of ligands and receptors are prominent guidance molecules in pruning and synapse elimination. This thesis focuses on the question of how the BDNF and ephrin/Eph interaction affect the hippocampal synapse formation and function.

1.1 Hippocampal Tri-synaptic Circuitry

The hippocampus is part of the brain structures of the brain limbic system, and numerous studies have revealed its function in learning and memory consolidation (Scoville and Milner, 1957; Zola-Morgan etc., 1986; Bliss and Collingridge 1993; Parkin

1996; Izquierdo et al., 2008; Bird and Burgess 2008; Neves et al., 2008), although precise mechanisms remain elusive. The intrinsic connections in hippocampus comprise a "trisynaptic circuit" or loop when cut transverse to its longitudinal (septal-temporal) axis (Figure 1.1, Neves et al., 2008). The first synapse is made by cells from the surface layers of the entorhinal cortex onto granule cells of the dentate gyrus, through the perforant pathway. The second synapse is made by granule cells of the dentate gyrus onto pyramidal cells of Cornu Amonnis subfield 3 (CA3), via the mossy fiber system. The third synapse is made by axon collaterals of the CA3 pyramidal cells onto the pyramidal cells of the CA1 subfield, known as the Schaffer collateral system (Blackstad, 1956; Atoji and wild, 2006).

In addition to these ipsilateral connections, hippocampal neurons communicate with cells in the contralateral hippocampus via commissural pathways (Blackstad, 1956; Buzsàki and Eidelberg, 1981). For example, in the rodent brain, both CA3 and CA1 of the hippocampus receive topographically organized projection from the opposite CA3, and the dentate gyrus receives a major input from the cells of the contralateral polymorphic layer of the DG (dentate gyrus).

An apparently important feature of the intrinsic circuitry of the hippocampal proper is its unidirectional projections or connection selectivity. The dentate gyrus cells project to CA3 field whereas the CA3 cells do not generally project back to the dentate granule cells. Similarly, CA1 pyramidal cells do not project back to CA3 (Blackstad, 1956; Atoji and wild, 2006; Neves et al., 2008; Raisman G et al., 1966; Hjorth-Simonsen,

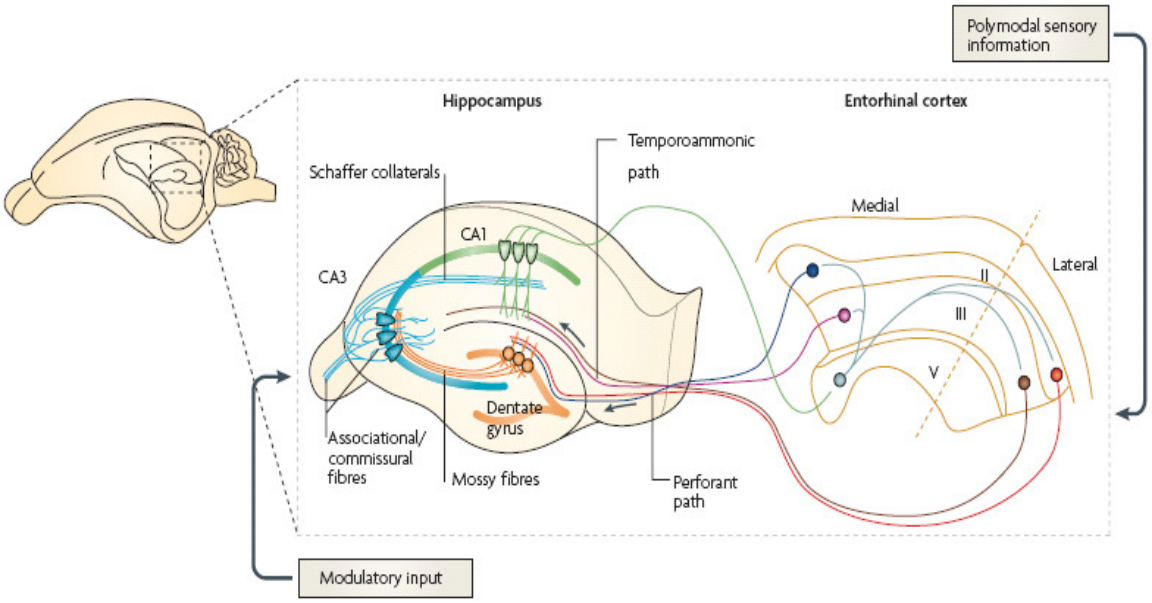

Figure 1.1 A diagram of the hippocampal trisynaptic circuit.

The major input to the hippocampus is from the perforant path, which conveys sensory information from neurons in layer II of the entorhinal cortex to the dentate gyrus. Perforant path axons make excitatory synaptic contacts with the dendrites of granule cells. Granule cells project to the proximal apical dendrites of CA3 pyramidal cells through the mossy fibres. CA3 pyramidal cells project to ipsilateral CA1 pyramidal cells through Schaffer collaterals and to contralateral CA3 and CA1 pyramidal cells through commissural connections.

In addition to the sequential trisynaptic circuit, there is also a dense associative network interconnecting CA3 cells on the same side. The distal apical dendrites of CA1 pyramidal neurons receive a direct input from layer III cells of the entorhinal cortex. There is also substantial modulatory input to hippocampal neurons. The three major subfields have an elegant laminar organization in which the cell bodies are tightly packed in an interlocking C-shaped arrangement, with afferent fibres terminating on selective regions of the dendritic tree. (Neves et al., 2008)

1973). Thus, information flow from the DG through the other sub-fields of hippocampus follows a largely serial path. To understand the nature of developmental disorders that involve the hippocampus, it is very important to clarify the mechanisms that produce this selectivity of connectivity, which is highly organized and presumably strictly regulated.

There are numerous mechanisms that shape synaptic connectivity during development, involving both synaptogenic and antisynaptogenic factors that promote and prevent synapse formation, respectively. A variety of factors (such as neurotrophins and neural guidance molecules etc.) are involved in this process. Among them, BDNF has been widely accepted as a promoting factor for synapse formation and neuronal function (Rose et al., 2004; Stoop and Poo, 1996). The ephrin/Eph family of ligands and receptors are guidance molecules well known for their repellant action in neuronal path-finding and effects on synapse formation (Martínez and Soriano, 2005). In the following sections, the function of BDNF and ephrin/Eph families in neuronal development will be described.

1.2 BDNF and Synapse development

As synaptic modulators, neurotrophins play important roles in the development and maintenance of synapses and the acute modification of synaptic structure and function. BDNF has been studied extensively and has been shown to modulate the

development of neural connections in various systems, ranging from the neuromuscular junction to the cortex (Lewin and Barde, 1996; Rose et al., 2004; Stoop and Poo, 1996; Tyler et al., 2002).

BDNF has been implicated in axonal path-finding during neuronal development. Both excess BDNF (Engelhardt et al., 2007; Galuske et al., 2000; Cabelli et al., 1995) and blockade of BDNF signaling (Cabelli et al., 1997; Mandolesi et., 2005) can lead to abnormal patterning of ocular dominance columns during a critical period of visual cortex development. In the hippocampal system, BDNF-TrkB signaling inhibition by K252a interfered with mossy fiber pathfinding during the development and regeneration (Tamura et al., 2006). It was also reported that BDNF gradient oriented axonal growth in cultured granule cells by causing a localized intracellular Ca2+ gradient around growth cones through TRPC (transient receptor potential canonical) channels (Li et al., 2005; Gomez, 2005).

Roles have been assigned for the dynamic elaboration of axon terminals and dendritic spines, indicating a direct and mostly promoting role for target-derived BDNF during synaptic patterning in the developing central nervous system. BDNF treatment for twenty-four hours can increase polarized retinal ganglion cell (RGC) neurite outgrowth (Bosco et al., 1999) and spine density in apical dendrites of CA1 neurons (Alonso et al., 2004; Tyler et al., 2003).

BDNF also has significant roles in shaping dendritic and axonal morphology (Horch and Katz, 2002; McAllister et al., 1997; Murphy et al., 1998; Tolwani et al., 2002; Cohen-Cory et al., 1995; Gallo et al., 1998). Over-expression of BDNF can increase basal

dendrite sprouting and regression of dendritic spines while decreasing higher order processes (Horch et al., 1999), implying that BDNF signaling is involved in both neuronal connectivity formation and pruning during development.

Actually, the effects of BDNF signaling on dendritic morphology are unexpectedly complex. For example, the presence of serum in tissue culture media can affect the cellular response to BDNF (Chapleau et al., 2008). For hippocampal slices cultured in serum-free media, BDNF increased the proportion of mature spines (stubby spines) in apical dendrites of CA1 pyramidal neurons, while BDNF had the opposite effect on spine morphology when slices were maintained in serum-containing media: it increased the proportion of immature spines (mushroom and thin spines) and decreased the proportion of mature spines (Chapleau et al., 2008).

Regarding synapse formation, there is considerable evidence for positive effects of BDNF. Overexpression of BDNF in mice increased the number of synapses in sympathetic ganglia in the developing visual cortex (Huang et al., 1999), while loss of function experiments with TrkB knockout in vivo at early developmental time reduced hippocampal Schaffer collateral synapse formation in mice (Luikart et al., 2005). Although there is some controversy about whether excitatory or inhibitory synapses were affected by BDNF, the majority view is that BDNF can promote the formation of both excitatory and inhibitory functional synapses (Vicario-abejon et al., 1998; Collin et al., 2001; Aguado et al., 2003; Seil et al., 2000; Mclean Bolton et al., 2000; Elmariah et al., 2004; Palizvan et al., 2004).

More specifically about synaptic proteins, it was demonstrated that exogenous BDNF accelerated the maturation of quantal size and synaptic localization of vesicle proteins, such as synapsin-1, synaptophysin and synaptobrevin (Wang et al., 1995; Liu et al., 1997; Tyler et al., 2001; Tartaglia et al., 2001), and increased NMDA receptor and GABA receptor cluster size, number, synaptic localization (Elmariah et al., 2004). Research on the mechanisms generally suggested a requirement for TrkB signaling (Rico et al., 2002; Martinez et al., 1998) at both presynaptic and postsynaptic sites (Luikart et al., 2005) during synapse formation. Consistently, BDNF was also transported to, and released from, both pre- and postsynaptic sites in an activity-dependent manner (Magby et al., 2006; von Bartheld et al., 1996; Haubensak et al., 1998; Hartmann et al., 2001; Kohara et al., 2001). Overall, BDNF/TrkB signaling appears necessary for synapse formation during activity-dependent refinement of synaptic connectivity.

1.3 BDNF and Synaptic Transmission

Related to its roles in neural development and synapse formation, BDNF modulates excitatory transmission in various systems and appears to be essential to molecular mechanisms of synaptic plasticity (Lu and Gottschalk, 2000).

BDNF has been demonstrated to be a modulator in synaptic transmission. The basal release of BDNF is necessary for maintaining the presynaptic transmitter release machinery and enabling sustained presynaptic transmission during high-frequency stimulation (HFS) (Figurov et al., 1996). Previous work in our lab has shown a

retrograde signaling effect on synaptic transmission where the depolarization induced postsynaptic BDNF release can signal back to the presynaptic cells to increase the transmitter release probability (Magby et al., 2006). In addition, BDNF and the ERK pathway has been shown to be involved in the enhancement of immature CA3 - CA1 connections by correlated network activity in the hippocampus (Mohajerani et al., 2007). Bath perfusion of hippocampal slices with BDNF has been shown to potentiate CA3–CA1 synapse transmission persistently (Kang and Schuman, 1995, 1996). From a different aspect, BDNF also attenuated synaptic fatigue at CA1 synapses induced by HFS (Gottschalk et al., 1999). Similar effects were also shown in vivo in the dentate gyrus, visual cortex, and insular cortex (Messaoudi et al., 1998; Jiang et al., 2001; Escobar et al., 2003). In some cases, loss-of-function experiments indicated the deficits in hippocampal basal synaptic transmission and LTP (long-term potentiation) in BDNF knock-out animal that was rescued by BDNF application, suggesting an acute role for BDNF in synaptic function (Patterson et al., 1996).

The response to exogenous BDNF application in the hippocampus can be very different for different preparations (cell culture, slice, and whole animal), different means and durations of BDNF delivery, and different concentrations of BDNF applied. For example, BDNF treatment of embryonic or early postnatal hippocampal neurons resulted in an acute and lasting potentiation in excitatory synaptic transmission (Alder et al., 2002; Alder et al., 2005; Levine et al., 1995a; Levine et al., 1998; Crozier et al., 1999; Lessmann et al., 1994; Li et al., 1998). However, in the adult hippocampus, a brief application of BDNF induced a calcium transient in dendrites and spines without

affecting synaptic efficacy while several minutes application of BDNF triggered a long-lasting increase in synaptic efficacy which was termed as BDNF-LTP (BDNF-induced long-term potentiation) (Kovalchuk et al., 2002).

Aside from excitatory transmission, BDNF was also shown to affect inhibitory (GABAergic) synaptic transmission, but in a different way. In general, BDNF has been shown to decrease the efficacy of inhibitory synaptic transmission by down-regulation of chloride transport as well as its presynaptic effects (Tanaka et al., 1997; Frerking et al., 1998; Wardle and Poo, 2003). Thus, BDNF appears to strengthen excitatory (glutamatergic) synapses and weaken inhibitory (GABAergic) synapses, resulting in increased neuronal activity.

1.4 BDNF and LTP (long-term potentiation)

A role of BDNF in synapic plasticity LTP has also been demonstrated (Lu et al., 2008; McAllister et al., 1999; Korte et al., 1995, 1996; Bramham and Messaoudi, 2005; Patterson et al., 1996; Kang, 1997). Inhibiting BDNF/TrkB signaling by TrkB inhibitors blocked LTP in hippocampal or visual cortical slices (Figurov et al., 1996). BDNF knockout animals also exhibited impaired LTP in hippocampal slice (Korte et al., 1995) which was restored by re-expression or treatment with BDNF (Korte et al., 1996; Patterson et al., 1996). Following the induction of hippocampal LTP, the spine density can increase possibly via generation of new spines (Trommald et al., 1996; Maletic-Savatic et al., 1999; Engert et al., 1999), or multiple spine synapses (Toni et al., 1999),

suggesting that neuronal activity can enhance local synthesis/secretion of BDNF, which would in turn regulate synaptic efficacy or growth. Promisingly, BDNF has been shown to be able to rescues LTP in Huntington's disease (HD) knock-in mice that had discretely disrupted processes required for both induction and stabilization of LTP, which suggested the potential of neurotrophin regulation in treating cognitive deficits (Lynch et al., 2007).

Different from other neurotrophins, although diffusible, BDNF works through a spatially restricted, synapse-specific and activity-dependent manner (Stent, 1973; Bliss and Collingridge 1993; Linden, 1994; Malenka and Nicoll 1999; Zhang et al., 2002). For example, it was reported that BDNF must be applied to the synapse (spatially close) but not cell body (further from synapse), to elicit the acute effect (Stoop and Poo, 1995) whereas focal application of NT3 to any part of motor neurons, without the spatial restriction, could elicit acute synaptic potentiation (Chang and Popov, 1999). The localized BDNF-induced synaptic potentiation was indicated to be related with the spatially restricted calcium influx (Zhang et al., 2002). As for the synapse specific function, BDNF was demonstrated to preferentially potentiate immature synapses with lower release probability without affecting nearby mature synapses in hippocampal cultures (Lessmann and Heumann 1998; Berninger et al. 1999). BDNF action is also activity-dependent regarding its selection of targets. When two sets of Schaffer collaterals-CA1 synapses in the same hippocampal slice are monitored simultaneously, BDNF treatment potentiates the tetanized pathway without affecting the untetanized pathway (Gottschalk et al. 1998). Interestingly, accordant to the activity-dependent

BDNF action, activity-dependent release of BDNF has also been demonstrated (Goodman et al., 1996; Balkowiec and Katz, 2000; Hartmann et al., 2001; Kohara et al., 2001; Gartner and Staiger, 2002). BDNF is unique in many different ways, though the mechanism has remained quite open.

Whether BDNF-induced synaptic potentiation occurs primarily by a presynaptic or postsynaptic action is intensely debated. Many studies have provided evidence for presynaptic mechanisms through enhancement of glutamate release (Xu et al., 2000; Tyler et al., 2002; Kafitz et al., 1999 ; Lessmann et al., 1994; Li et al., 1998; Jovanovic et al., 2000; Zakharenko et al., 2003). Enhancement of transmitter release by BDNF may be caused by a BDNF-induced increase in cytosolic calcium (Berninger et al., 1993; Stoop et al., 1996), eventually resulting in changes in the efficacy of synaptic vesicle exocytosis (Pozzo-Miller et al., 1999; Bradley et al., 1999). Evidence for postsynaptic actions has also been obtained, such as phosphorylation of neurotransmitter receptors and electrophysiological modulation (Black, 1999; Crozier et al., 1999; Levine et al., 1995; Levine et al., 1998; Thakker-Varia et al., 2001; lin et al., 1998; for review see Rose et al., 2004). Overall, both pre- and postsynaptic trkB signaling might significantly contribute to BDNF-induced synaptic potentiation (Drake et al., 1999). Supporting this idea, the pre- and postsynaptic BDNF action on synaptic transmission in hippocampal neurons can be dissected into two components, with the early and late phase of the BDNF response presumed to be presynaptic and postsynaptic mechanism respectively (Alder et al., 2005).

Combining with physiological consequences of BDNF signaling in the hippocampus, i.e. modulation of presynaptic function (Tyler et al., 2002b), BDNF-induced dendritic remodeling likely contributes to its role in the establishment and activity-dependent refinement of neuronal networks necessary and fundamental for synaptic plasticity and hippocampal-dependent learning and memory (Amaral et al., 2007; Amaral and Pozzo-Miller, 2007; Tyler et al., 2002a; Chapleau et al., 2008). In addition, BDNF has been implicated extensively in neurological disorders, such as Alzheimer's disease, Huntitington's disease and psychiatric disorders etc (Chao et al., 2006; Chen et al., 2006), which makes BDNF a very attracting candidate for this study.

1.5 Ephrin/Eph in Synapse formation

Ephrin ligands and Eph receptors are widely expressed in the developing and adult nervous system (Lieble et al., 2003; Gao et al., 1998; Stein et al., 1999) and divided into two subclasses, the A-family and the B-family, based on sequence conservation, binding affinities and the mode of ephrin membrane attachment (Figure 1.2). Ephrin-A ligands predominantly bind to EphA receptors while ephrin-B ligands bind to EphB receptors, but some cross binding has also been found, such as ephrin-A5 binding to EphB2 and ephrin-B3 binding to EphA4 (Martínez and Soriano, 2005). Unlike BDNF, ephrins/Ephs are not diffusible molecules but require cell-to-cell contact to activate signaling and have bi-directional signaling from both the receptor and the ligand (Aoto and Chen, 2007; Holland et al. 1996; Bruckner et al. 1997; Knoll & Drecher, 2002; Davy et

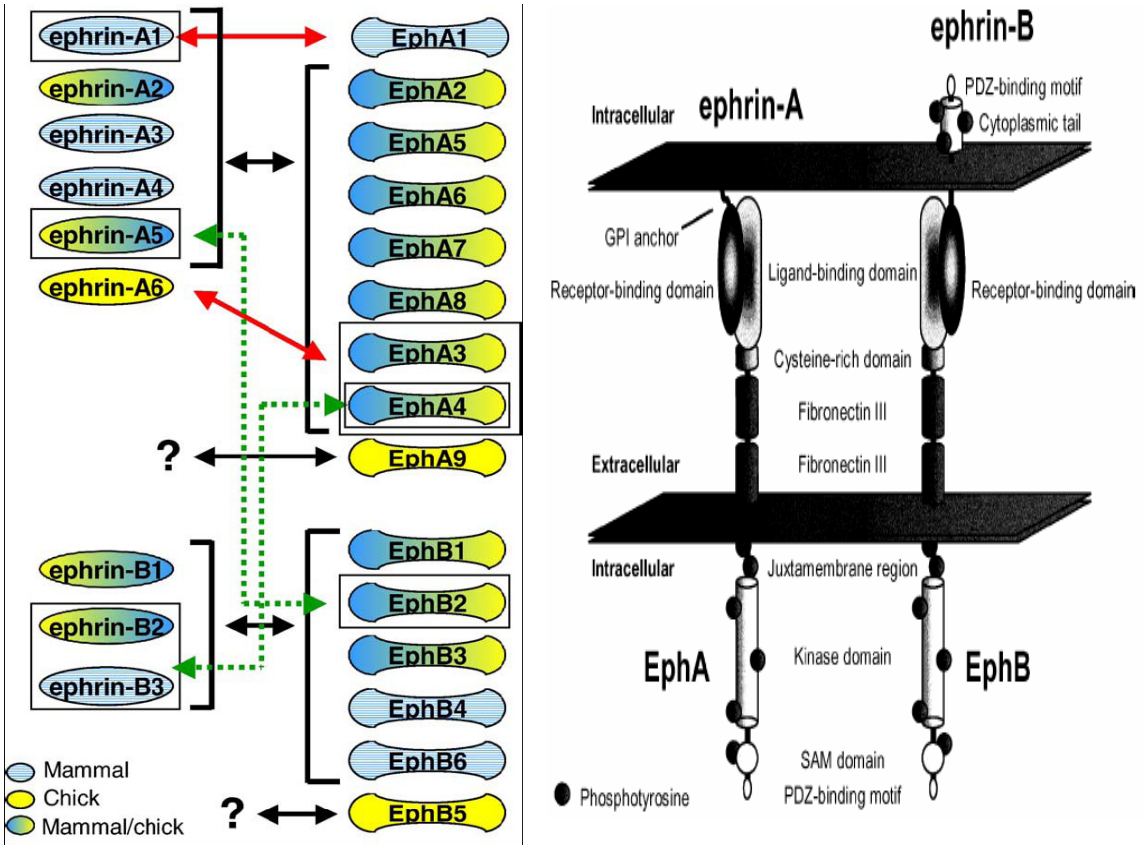

Figure 1.2 The ephrin/Eph family of receptor tyrosine kinases

This family is divided into two subclasses, the A-family and B-family, based on sequence conservation, binding affinities and the mode of ephrin membrane attachment. Ephrin-A1–A5 are tethered to the cell membrane by a glycosyl phosphatidylinositol (GPI) anchor, while ephrinB1–B3 have a transmembrane domain that is followed by a short cytoplasmic region. (Martínez and Soriano, 2005)

al., 2000; Knoll et al., 2001). Ephrin/Eph members are involved not only in axonal guidance and topographic mapping but also in subsequent synapse formation and maturation (Flanagan & Vanderhaeghen, 1998; Klein, 2004; Liebl et al., 2003; Knoll & Drescher, 2002; McLaughlin & O'Leary, 2005).

Regulating the formation of topographic maps is one of the major roles of ephrins/Ephs in various systems such as the visual system (Dickson, 2002; Rasband et al., 2003; Nakagawa et al., 2000; Williams et al., 2003; Sakurai et al., 2002), the olfactory system (Cutforth et al., 2003; Dufour et al., 2003), motor neuron innervations (Helmbacher et al., Eberhart et al., 2004) and the spinal cord neuronal circuit of central pattern generators (Kullander et al., 2003). This axon guidance action of ephrins/Ephs has generally been found to be based on repellant cues. During intercellular communication, activation of EphA by ephrin-A was found to induce spine retraction and reduce the number of neurites (Brownlee et al., 2000; Murai et al., 2003; Xu et al., 2003; Munoz et al., 2005; Weinl et al., 2003; Harbott et al., 2005 Yue et al., 1999).

Some ephrin-A members are also able to activate EphB2-mediated growth cone collapse and neurite retraction (Himanen et al., 2004). Ephrin-Bs have a similar repulsive action on neurite outgrowth (Benson et al., 2005; Hoogenraad et al. 2005; Vermeren et al., 2000). Mechanistically, the ephrin/Eph mediated process retraction may be due to protease mediated ephrin cleavage, internalization and axonal detachment (Hattori et al., 2000; Holmberg et al., 2000), or the inhibition of integrin-mediated adhesion by dephosphorylation of FAK and paxillin following EphA activation (Miao et al., 2000).

The actions of ephrins/Ephs are not exclusively repellent, however. In addition to the collapsing effect, branch-promoting activity during neuronal development has also observed for both ephrin-A and ephrin-B members (Mann et al., 2002; Zhou et al., 2001; Gao et al., 1999). It was reported that ephrin binding to Eph could activate different signaling cascades in subsets of neurons and exert distinct effects (Eberhart et al., 2004; Gao et al., 2000). It is also interesting that axonally localized EphA and ephrin-A induced opposite functions through the same integrin and MAP kinase signalling pathway but in different directions, with activation of EphA resulting in repulsion and ephrin-A leading to increased attraction and/or adhesion of growth cones (Davy et al., 1999; Miao et al., 2000; Miao et al., 2001; Huai & Drecher, 2001; Marquardt et al., 2005). The dual effects of ephrin/Eph were suggested to be functionally significant. Ephrin/Eph may regulate topographic map formation by stimulating axonal outgrowth and arborization early during pathway formation followed by pruning mistargeted axons later (Gao et al., 1999), an idea consistent with the progressive expression of inhibition on outgrowth of embryonic spinal motor neurites by ephrin-A5 (Wang et al., 2001).

Ephrin/Eph signaling is also involved in synapse formation and maturation. In vitro studies on cultured neurons implicated EphB receptor forward signaling as a positive signal in spine formation (Palmer & Klein, 2003). Triple knockout of EphB1, EphB2, and EphB3 in mice significantly impeded morphogenesis and synapse formation and disturbed clustering of both f-actin and PSD-95 in the dendritic protrusions (Kayser et al., 2006; Henkemeyer et al., 2003). In ephrinB3 knock-out mouse shaft synapse

formation was reduced in the hippocampus, which was rescued by overexpression of glutamate receptor-interacting protein 1 (GRIP1) (Aoto et al., 2007). EphA family signaling disruption by overexpressing EphA5-Fc reduced the density of synaptic terminals in the entorhinal and commissural termination layers in hippocampus (Martinez et al., 2005). Consistently, in vivo knock-out of ephrin-A5 decreased synapse density in those layers that receive commissural/associative axons (Otal et al., 2006), indicating the positive regulation of ephrin-A/EphA interactions in synapse development and neural connectivity of hippocampus. Furthermore, neuron-glial cross-talk through ephrin-A3/EphA4 signaling was demonstrated to be critical for modeling hippocampal spine morphology with either ephrin-A-Fc application or in EphA knockout mice (Murai et al., 2003).

Is there a direct association of ephrin/Eph with structural and signaling molecules at the synapse to support ephrin/Eph regulation of the formation or function of synapses? Specific interactions between the extracellular domains of the EphB2 receptor and the NR1 subunit of NMDA receptors have been found, which was triggered by ephrin-B ligand stimulation independent of EphB receptor signaling and resulted in an increase in the density of NMDA receptor synaptic clusters and the number of presynaptic release sites in culture (Dalva et al., 2000). This suggests that ephrin-B ligands in synaptic membranes can induce the maturation of glutamatergic synapses by promoting NMDA receptor aggregation. The specific EphB2/NMDA receptor association might link ephrin/Eph signaling to the synaptic functions with activity-driven mechanisms.

1.6 Ephrin/Eph and Synaptic Function

The extensive expression of Ephrin/Eph family members in developing and adult hippocampus (Gao et al., 1998; Murai et al., 2003; Otal et al., 2006; Lieble et al., 2003 Rodenas-Ruano et al., 2006; Grunwald et al., 2004; Stein et al., 1999) suggests the potential participation of ephrin/Eph in hippocampal plasticity beyond neuronal pathfinding and synapse formation. Biochemical inhibition of ephrin-A/EphA signaling caused significant impairments in LTP, LTD and behavioral performance (Gerlai, 2001; Gerlai & McNamara, 2000; Gao et al., 1998; Gerlai et al., 1999). Results from EphB2-deficient and ephrin-B3-deficient mice demonstrated the requirement for ephrin-B/EphB in hippocampal long-term synaptic changes (Grunwald et al. 2001; Henderson et al. 2001; Murai and Pasquale 2002; Armstrong et al., 2006). The importance of the ephrin-B ligand reverse signaling was also indicated by rescue of the defects in LTP by addition of the EphB2 receptor isoform suggested to play role at the synapse (Grunwald et al. 2001; Henderson et al. 2001; Grunwald et al., 2004; Rodenas-Ruano et al. 2006;), which might be supported by the much higher ephrinB2/B3 expression level in postsynaptic CA1 neurons than in presynaptic CA3 neurons for Schaffer collateral pathway (Grunwald et al. 2001; Liebl et al. 2003).

Regarding mechanisms of ephrin involvement in synaptic plasticity, there is evidence showing that it can be similar to the mechanism for BDNF modulation. For instance, in young cultured neurons, EphB2 receptor activation by ephrin-B1 stimulation recruited and activated Src-family kinases, which then phosphorylated certain subunits

of the NMDA receptor and enhanced NMDAR-mediated Ca2+ influx and phosphorylation of the transcription factor CREB and the following CREB-dependent transcriptional events (Grunwald et al. 2001; Takasu et al. 2002). This suggested that ephrin/Eph signaling might influence activity-dependent plasticity by modulating Ca2+ related gene expression during development of synaptic connections. The cross-over between BDNF modulation and Eph signaling mechanisms suggests the potential interaction between these two pathways, which might be critical for neuronal development.

Ephrin/Eph signaling may also influence protein trafficking and synaptic currents (Song & Huganir, 2002) through bi-directional internalization of Eph–ephrin complexes, which might therefore trigger internalization of other co-localized proteins, such as ion channels (Murai & Pasquale, 2003; Hattori et al., 2000). In addition to synaptic partners, extrasynaptic interaction also contributes to ephrin modulation of synaptic plasticity. There are data implicating ephrinB–EphB interactions in non-NMDA receptor-dependent forms of synaptic plasticity – Mossy fiber LTP, which requires clustering of postsynaptic EphB receptors via PDZ interactions (Contractor et al. 2002).

Ephrins also have crosstalk with other signaling pathways, such as the receptor tyrosine kinase Ryk (Trivier et al., 2002; Halford et al., 2000) and the multi-transmembrane protein ARMS (Ankyrin Repeated-rich Membrane Spanning). ARMS is a common substrate for Eph and Trk receptors (Kong et al., 2001), suggesting an interesting interaction between ephrins and neurotrophin signals during axonal growth, synaptogenesis and plasticity. So far, there have been few studies of neurotrophin -

ephrin interactions in synaptic function. The goal of this thesis is to shed light on this issue by investigating the interaction between BDNF and ephrin/Eph in synaptic transmission and synapse formation.

1.7 Aims of the Thesis

The central aim of this thesis is to argue that ephrin/Eph – BDNF interaction is critically involved in shaping the development of selective hippocampal synaptic connectivity, especially the CA3 → CA1 synapse, and to empirically test this claim with electrophysiological, immunocytochemical and molecular biological techniques.

The first step was to establish an in-vitro model to be able to study the selective synapse formation of CA3 pyramidal cells onto CA1 pyramidal cells. Although the in-vivo or acutely cut hippocampal slice preparation is best for keeping the hippocampal circuitry intact, it is not compatible with the single-cell analysis and molecular biological manipulations needed to understand mechanism. An alternative is the dissociated hippocampal neuron culture, which is much easier to manipulate experimentally. The most important question to ask, however, is whether hippocampal neurons in dissociated cultures maintain the unidirectional connectivity seen in the intact hippocampus. For the CA3 → CA1 synapse, I provide evidence that the answer is "yes" by characterizing functional synaptic connections with paired-cell patch clamp recording and identifying the CA1 neurons with immunocytochemistry using an antibody against SCIP, a CA1 pyramidal cell-specific transcription factor (for more detail,

see Figure 2.1). Having established this point, the way was clear to perform detailed experiments with dissociated hippocampal neuron cultures.

The second step was to investigate whether ephrin/Eph signaling interacts with BDNF signaling in hippocampal synaptic activity measured by electrophysiology. Soluble ephrin-A5 ligand (ephrin-A5-Fc) or soluble EphA5 receptor (EphA5-Fc) was applied to the culture during whole-cell patch recording of ongoing synaptic activity. BDNF was subsequently applied to determine whether the BDNF modulation was affected. These molecules were also applied in the reverse order, with BDNF perfusion preceding ephrin-A5-Fc or EphA5-Fc application to test the potential effect of BDNF signaling activation on the EphA/ephrin-A signaling. After the ephrin/Eph – BDNF interactions were described, the potential mechanisms were investigated in terms of pre- or postsynaptic action through the analysis of miniature excitatory postsynaptic currents. Furthermore, the involvement of EphA receptor signaling in the direct synaptic effect of Ephrin-A5-Fc was tested using the kinase domain negative EphA3 and EphA5 constructs to interrupt the endogenous EphA signaling.

The final part of the thesis was to study the involvement of ephrin-A/EphA – BDNF interaction in hippocmapal synapse formation, which is hypothesized on the basis of the above data. First of all, the ephrin-A and EphA distribution pattern during development was investigated to see if there is any correlation between their expression patterns and formation of the hippocampal circuitry. Then, the balance of ephrin/Eph and BDNF signaling was manipulated to study the role of their interaction in the specificity of CA3 → CA1 connection. An EphA3 dominant negative construct linked

with GFP (EphA3(K-)-GFP) and an EphA5 dominant negative construct linked with GFP (EphA5(K-)-GFP) were used to interfere with the endogenous EphA signaling, and anti-BDNF antibody was used for the functional blocking of BDNF signaling in the hippocampal cultures. Then electrophysiology and immunocytochemistry were combined to identify the types (CA1 or CA3) of the presynaptic and postsynaptic cells forming the functional synapses in order to see whether the connection specificity of the CA3 → CA1 synapse was changed. The hypothesis is that EphA signaling is involved in the establishing the unidirectional nature of the CA3 → CA1 connectivity, and that it is mediated through an interaction with BDNF.

The global significance of this research is to provide the groundwork necessary for deciphering the neurological diseases with abnormal synapse formation especially in the hippocampus system.

CHAPTER 2

EXPERIMENTAL METHODS

2.1 Primary Hippocampal Neuronal Cultures

Embryonic day 18 (E18) fetuses of time-mated pregnant Sprague-Dawley rats or embryonic day 16 (E16) fetuses of time-mated pregnant C57BL_6 mice were removed in accordance with institutional guidelines for the care and use of animals. Fetal hippocampi were dissected and dissociated by trypsinization followed by trituration through fire-polished Pasteur pipettes. Neurons were plated in poly-D-lysine coated 35-mm Nunc dishes (Nalge Nunc International, Roskilde, Denmark) at 4 x 105 cells/dish and maintained in serum-free medium (SFM). SFM was composed of a 1:1 mixture of Ham's F-12 and minimal essential medium containing GLUTAMAX and Earle's salts supplemented with 25 μg/mL insulin, 100 μlg/mL transferrin, 60 μM putrescine, 20 nM progesterone, 30 nM selenium, and 6 mg/mL glucose (Gibco, Grand Island, NY). Cells were grown at 37° C in 5% CO_2.

2.2 Electrophysiological Recordings

Whole-cell patch clamp recordings were performed after 13-16 (mostly 14) days in culture (DIV). The in house software WINCLAMP (developed by Dr. Mark R Plummer) was used. Currents were recorded with an Axoclamp 200 amplifier, digitized at 2.9 kHz and filtered at 5 kHz. Pyramidal-type cells were recorded in voltage clamp mode with a holding potential of -50mV. The external bath solution (NRS) was (in mM) 1.67 $CaCl_2$, 1 $MgCl_2$, 5.36 KCl, 137 NaCl, 17 glucose, 10 HEPES and 13.15 sucrose. The pipette solution contained (in mM) 105 Cs-methanesulfonate, 17.5 CsCl, 10 HEPES, 0.2 EGTA, 8 NaCl, 2 Mg-ATP, 2 Na-ATP, 0.3 Na-GTP, and 20 phosphocreatine. The pH of the internal solution was set to 7.3 with CsOH. Pipette resistance ranged from 3-5 MΩ. Cell capacitance was typically 10-20 pF and access resistance was 7-20 MΩ.

To record ongoing synaptic activity, cells were held at -50 mV. To isolate spontaneous miniature postsynaptic currents (mEPSCs) in synaptic activity experiments, 1 μM tetrodotoxin (TTX) was added to the perfusing solution (NRS) and NRS supplemented with other test compounds. To block NMDA currents, 50 μM APV or 20 μM MK801 was added to the perfusing NRS.

For paired recording, two pipettes were used to record from a pair of neurons. The modified internal solution contained (in mM) 105 K-methanesulfonate, 17.5 KCl, 10 HEPES, 0.2 EGTA, 8 NaCl, 2 Mg-ATP, 2 Na-ATP, 0.3 Na-GTP, and 20 phosphocreatine. The pH of the internal solution was set to 7.3 with KOH. One neuron (presynaptic) was injected with 30 ~ 80 mA of depolarizing current to fire a single action potential in current-clamp mode at a frequency of 0.2 Hz, while another neuron (postsynaptic) was voltage clamped at -50 mV to record evoked postsynaptic currents. For some

experiments where the cells were labeled while being recorded, 1 mM pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid; Sigma) was added to the electrode solution.

20ng/ml BDNF (Peprotech, Rocky Hill, NJ), 2ug/ml ephrin-A5-Fc, 2ug/ml EphA5-Fc, or 2ug/ml Fc (R&D systems, Minneapolis, MN) solutions for perfusion were diluted in NRS. Vehicle control was the 10^{-6} BSA/PBS (BSA, Sigma) solution, which was also used to prepare BDNF and Ephrin-A5-Fc/EphA5-Fc aliquots.

2.3 Data Analysis

The data were analyzed by integrating the synaptic currents for each sweep (synaptic charge) or summing the number of miniature events per sweep and then grouping into one minute bins with the in house software WINDISP (developed by Dr. Mark R. Plummer). Baseline is considered the average value during the 2 min prior to switching the perfusion system (from NRS to the first experimental condition). Percent increases were determined by dividing the binned data during experimental exposure by the baseline value. Student's t-test was used for statistical comparisons ($P < 0.05$ indicating significance).

For the miniature events frequency analysis, the currents were picked up by WINDISP by setting a certain threshold that can best mimic the actual events frequency and amplitude. The frequency and average amplitude of these events during a certain period of time were given by the program.

For paired recordings, the peak amplitude of evoked EPSC under each experimental condition was analyzed by averaging 10 sweeps.

2.4 Immunocytochemistry

Ephrin-A5-Fc and EphA5-Fc (R&D system) were used to examine the cellular distribution of EphA/ Ephrin-A. DIV 14 hippocampal cultures were incubated with 2μg/ml Ephrin-A5-Fc or EphA5-Fc in conditioned medium for 30 min at 37 degrees. Dishes were rinsed twice with cold PBS (Sigma), Fixed in 100% cold methanol for 5 minutes at -20 degrees, rehydrated with PBS for 10 minutes at room temperature, and blocked for 30 minutes in 5% normal goat serum (NGS) diluted in 5% bovine serum albumin (BSA, Sigma) in PBS. Secondary antibody (Goat anti Human Fc-FITC, Sigma) was applied at 1:100 in 5% NGS/PBS for 1 hour. In order to determine the synaptic localization of EphA/Ephrin-A, the cultures were then double labeled with rabbit anti-synaptophysin (1:200, Sigma) and mouse anti-MAP2 (1:200, Chemicon) together for 2 hours. Dishes were washed with PBS, incubated with goat anti-rabbit A546 (1:500, Molecular Probes), and goat anti-mouse A350 (1:200, Molecular Probes), in 5% NGS for 1 hour, rinsed in PBS, mounted by DABCO, and cover-slipped.

The primary antibodies to EphA5 (1:100) and EphA3 (1:100) (R&D system) were also used to determine the distribution pattern of the EphA5 or EphA3 receptor in hippocampal neurons. Dishes were fixed in 4% Para-Formaldehyde (PFA) for 7 minutes

at room temperature and then 100% cold methanol for 3 minutes at -20 degrees, blocked for 30 minutes in 10% donkey serum (DS) in PBS, and incubated with the antibodies to EphA5 or EphA3. Secondary antibody (donkey anti-goat alexa 546, Molecular Probe) was applied at 1:400 in 10% NGS/PBS for 1 hour. In order to determine the localization of EphA5 or EphA3 in CA1 or CA3 neurons, the cultures were then blocked for 30 minutes in 5% NGS, double labeled with rabbit anti-SCIP (1:200, a generous gift from Dr. Dies Meijer, Ilia et al., 2002) and mouse anti-MAP2 (1:200, Chemicon) together for 2 hours in 5% NGS. Dishes were then incubated with goat anti-rabbit A488 (1:100, Molecular Probes), and goat anti-mouse A647 (1:700, Molecular Probes) for 1 hour.

Images were taken with an Olympus 1X81 fluorescence microscope and analyzed with 3D-blind deconvolution using IPLab (Scanalytics, Fairfax, VA) V3.62 imaging software. The average intensity quantification in certain regions of interest for each color channel was done consistently after background subtraction with IPLab. All images were processed in the same way. To count the average density of the presynaptic or postsynaptic ephrin-A or EphA, the images were segmented as puncta based on an intensity threshold for the individual channels to be analyzed, and the number, size and intensity were quantified with IPLab. For example, in order to analyze the colocalization of ephrin-A or EphA (green channel) with synaptophysin (red channel), the yellow puncta were quantified.

2.5 Mapping the Type of Recorded Cells

In order to identify the origin (hippocampal CA3 or CA1) of the cells that form a functional synapse determined by electrophysiology (Figure 2.1a), the fluorescent dye pyranine was injected to the cells during paired recording. After recording, photographs were taken at 40X with both regular light (Hoffman modulation optics) (Figure 2.1b) and fluorescence (Figure 2.1c) illumination with the field centered on the recorded cells. The images were then used as a map to identify the presynaptic and postsynaptic cells in the whole dish after the subsequent immunocytochemical staining. Once immunolabeling with anti-SCIP was finished, fluorescence pictures (Figure 2.1e, f and g) were taken again. By comparing those pictures, I was able to tell whether the presynaptic or postsynaptic cell was SCIP positive versus SCIP negative. The SCIP positive cells were considered to be CA1 pyramidal cells, whereas the SCIP negative cells that made excitatory synapses were most probably CA3 pyramidal cells (usually the pyramidal-like cells were selected to do recording) but might also be dentate gyrus cells since the source of the cells in the culture includes all parts of the hippocampus: dentate gyrus, CA3 and CA1 regions. Please see Figure 2.1 for the better illustration of this method.

2.6 Immunohistochemistry for Slice

Hippocampal slices obtained from animals of various ages were used for the immunohistochemistry with the anti-SCIP antibody. The slice was fixed in 4% Para-

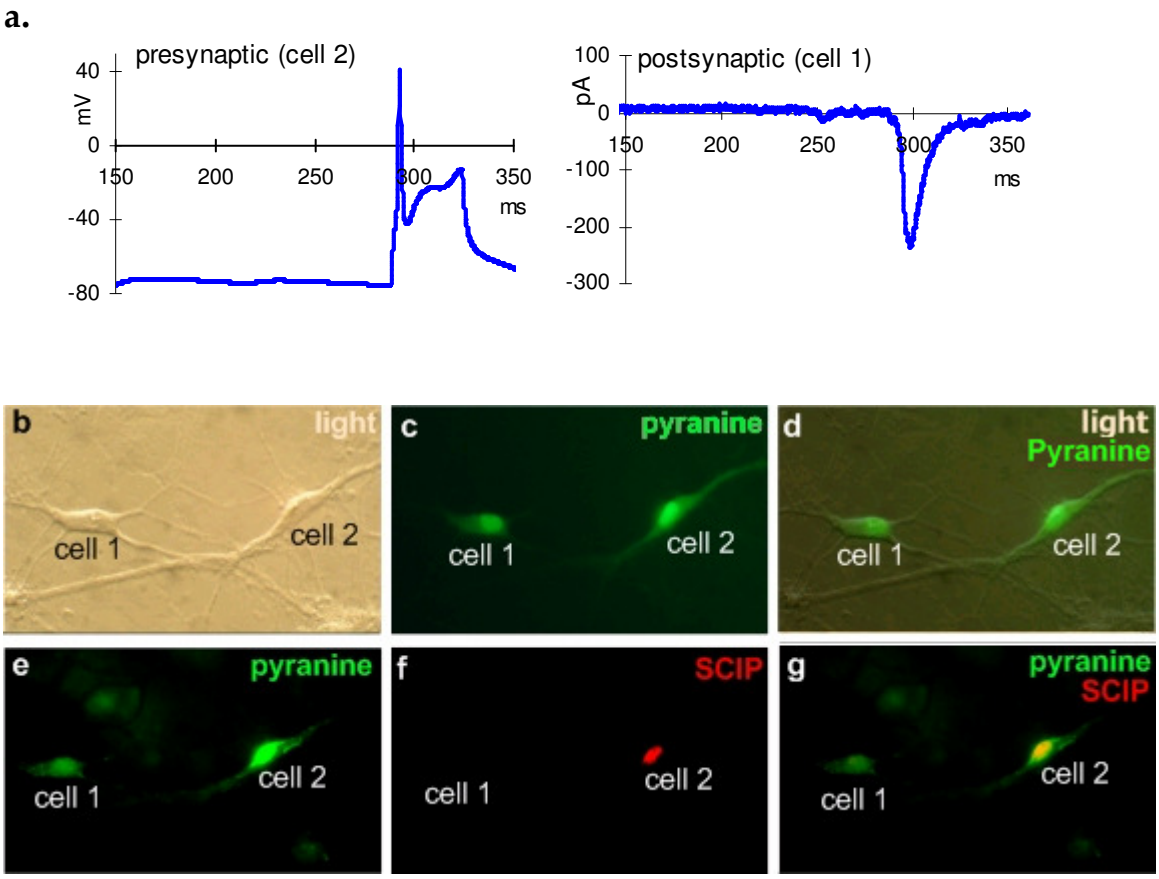

Figure 2.1 Method for identifying the recorded cells

a. Paired recording from two pyramidal cells in culture. Left: action potential recorded by current clamp recording from cell 2; right: postsynaptic current recorded by voltage clamp recording from cell 1 in picture b. **b**. Regular light Hoffman picture shows the morphology and location in the context of the recorded cells. **c**. Fluorescence picture for the same field as panel b shows that the recorded cells were successfully labeled with pyranine. **d**. merged picture of b and c. **e**. The recorded cells were found based on the pyranine labeling. According to the location and morphology in pictures b and c, cell 1 and cell 2 were identified. **f**. Cell 1 but not cell 2 was labeled by anti-SCIP antibody (red). **g**. merged picture of e and f. Switching the recording orientation revealed that cell 1 did not form a functional synapse onto cell 2. Thus, in this pair of cells, the synaptic connection is unidirectional with the postsynaptic cell being a CA1 pyramidal cell (SCIP positive) and the presynaptic cell presumed to be a CA3 pyramidal cell since it is pyramidal-like and SCIP negative.

Formaldehyde (PFA) for 20 minutes at room temperature and then 100% cold methanol for 7 minutes at -20 degrees, blocked for 60 minutes in 10% donkey serum (DS) in PBS, and incubated with the antibody to SCIP (1:200) for 2 hours. After washing for 45 minutes, the secondary antibody (goat anti-rabbit alexa 546, Molecular Probe) was then applied at 1:400 in 10% NGS/PBS for 2 hours. The slice was then counter stained with DAPI (1:100), washed for 45 minutes, and cover-slipped.

2.7 Biochemistry

The Dissociated hippocampal neurons were treated with 10ng/ml BDNF with or without 2g/ml ephrin-A5-Fc for varying time periods. Western blot analysis was used to assess the extent of p42/44 MAPK phosphorylation using a phosphorylation-specific antibody. The blots were then reprobed for total p42/44 MAPK to evaluate relative protein levels. Intensity was quantified with ImageJ. (This work was done in collaboration with Dr. Renping Zhou's lab at Rutgers University)

2.8 Molecular Biology

DNA

EphA5(K-)-GFP and EphA3(K-)-GFP constructs are EphA5 or EphA3 kinase domain deficient DNA linked with GFP, which are generous gifts from Dr. Renping

Zhou (Yue et al., 2002). Enhanced GFP (EGFP) construct was used as the control.

Neuronal Transfection

The neurons were typically transfected at DIV 5 and recorded 9 days later. The Plasmid-Ca-Pi coprecipitation was carried out on dissociated hippocampal neuronal cultures. Briefly, for each 35 mm dish, 3 ug of DNA (at 0.5 ug/ul) was mixed with 2 M $CaCl_2$ and 20 ul sterile water, and then added with 30 μl 2XHBSS by drop while vortexing. 2XHBSS contained 50 mM HEPES, 1.5 mM 280 mM NaCl, 10 mM KCl, and 15 mM glucose (all from Sigma). After keeping the DNA mixture for 20 minutes precipitation in the dark, cells were washed with DMEM (Sigma) three times, 80% of the medium exchanged each time. 1 ml DMEM was left in the dishes after the final round of washing. 60 μl of the DNA-Ca-Pi mixture was added to each dish. Cells were incubated with the DNA in for 40 minutes in the incubator. The medium was then discarded, the dish washed with SFM three times, and incubated for 1 hour at 37° C in 5% CO2 for the thorough clearing of the remaining DNA-Ca-Pi reagents. Finally, the medium was replaced with the original conditioned medium taken from the original dishes and the cells were returned into the incubator.

CHAPTER 3

AN IN-VITRO MODEL FOR STUDYING SPECIFICITY OF SYNAPTIC CONNECTIVITY

As stated in "Aims of the Thesis", it is essential to establish that elements of in vivo synaptic connectivity can be reproduced in vitro. This is a critical first step needed to perform our research on the interaction of ephrin-A/EphA and BDNF in the development of Schaffer collateral connection in the hippocampus. What is necessary is a good model where the selectivity still exists but where conditions can be easily manipulated and reproducible results obtained. Dissociated primary hippocampal neuronal culture is a good candidate since it is amenable to electrophysiological recording, immunocytochemistry is practical, and more functional synaptic connections can be studied between cell pairs.

The question then comes: does the dissociated neuronal culture have the same unidirectional CA3 neuron projection pattern onto CA1 neurons as in the intact hippocampus? If so, are there any obvious candidate molecules that could account for this specificity? Experiments using immunocytochemistry combined with electrophysiology were done to answer these questions.

3.1 Most Functional Synaptic Connections in Primary Hippocampal Neuronal Cultures are Unidirectional

Although cells from different hippocamal subregions has been dissociated and mixed in the culture, they might still retain the determinant factors (as yet unknown) that establish the unidirectional connection pattern of hippocampal circuitry. Our hypothesis regarding this research project was that, in the hippocampal neuronal culture, cells from CA3 regions still project to CA1 neurons while cells from CA1 do not synapse onto CA3 neurons. In the other words, CA1 pyramidal neurons can only serve as postsynaptic cell (in CA3 → CA1 synapses) while CA3 cells can be either presynaptic (in CA3 → CA1 synapses) or postsynaptic cells (in CA3 → CA3 synapses). If this hypothesis is true, utilizing the CA1 pyramidal neuron marker SCIP and paired-cell recording, presynaptic cells should generally be SCIP negative (CA1 pyramidal cell/Dentat granule cell) whereas postsynaptic cells could either be SCIP negative or SCIP positive. Conversely, if we see equal proportions of presynaptic SCIP positive and SCIP negative cells, we would have to conclude that synapse formation in the cultured cells is no longer representative of cell type specificity and instead depends on other factors such spatial proximity or simply random interactions.

The most straightforward way to gain some idea about cell type selectivity in functional synapse formation is to characterize electrophysiologically the synaptically connected pyramidal cells by paired-cell recordings. In this set of recordings on cells

studied after DIV 14, one pyramidal cell (cell 1) was depolarized by current injection to fire an action potential while the other nearby pyramidal cell (cell 2) was voltage clamped to see whether an excitatory postsynaptic current (EPSC) was elicited right after the action potential. Then, the two cells were reversed regarding the pre- post-relationship, which means that cell 2 would be depolarized to fire an action potential while cell 1 would be in the voltage clamp mode to test the related EPSC.

A stable EPSC evoked reliably a short latency by a single presynaptic action potential indicates that a successful synaptic connection has been formed between a pair of neurons (Figure 3.1a). Inhibitory postsynaptic synaptic current (IPSCs) were also seen, which indicated that the presynaptic cell was an inhibitory neuron. If no postsynaptic current was elicited by the presynaptic action potential, then a functional synapse was not formed between this particular set of presynaptic cell and postsynaptic cells (Figure 3.1b). All the recorded cell pairs were categorized based on these criteria for synapse formation (Figure 3.1c). As shown in Fiture 3.1d, out of the 289 paired-cell recordings, very often (52% of the recordings), we saw no synpase in either direction (0---0) which means that the two cells didn't form synapse onto each other at all or they did not simply select cells in close proximity as their targets. When an EPSC was observed (23% of the recordings), it was typically unidirectional (E---0) which suggested a selective synaptic connection (maybe CA3 → CA1). Only very rarely (3% of the recordings) were bi-directional excitatory (E---E) connections observed. In addition, 16% of the cell pairs showed inhibitory synapse in one direction with no synapse in the other direction (I---0), 6% showed inhibitory synapse in one way and excitatory synapse in other (E---I, an

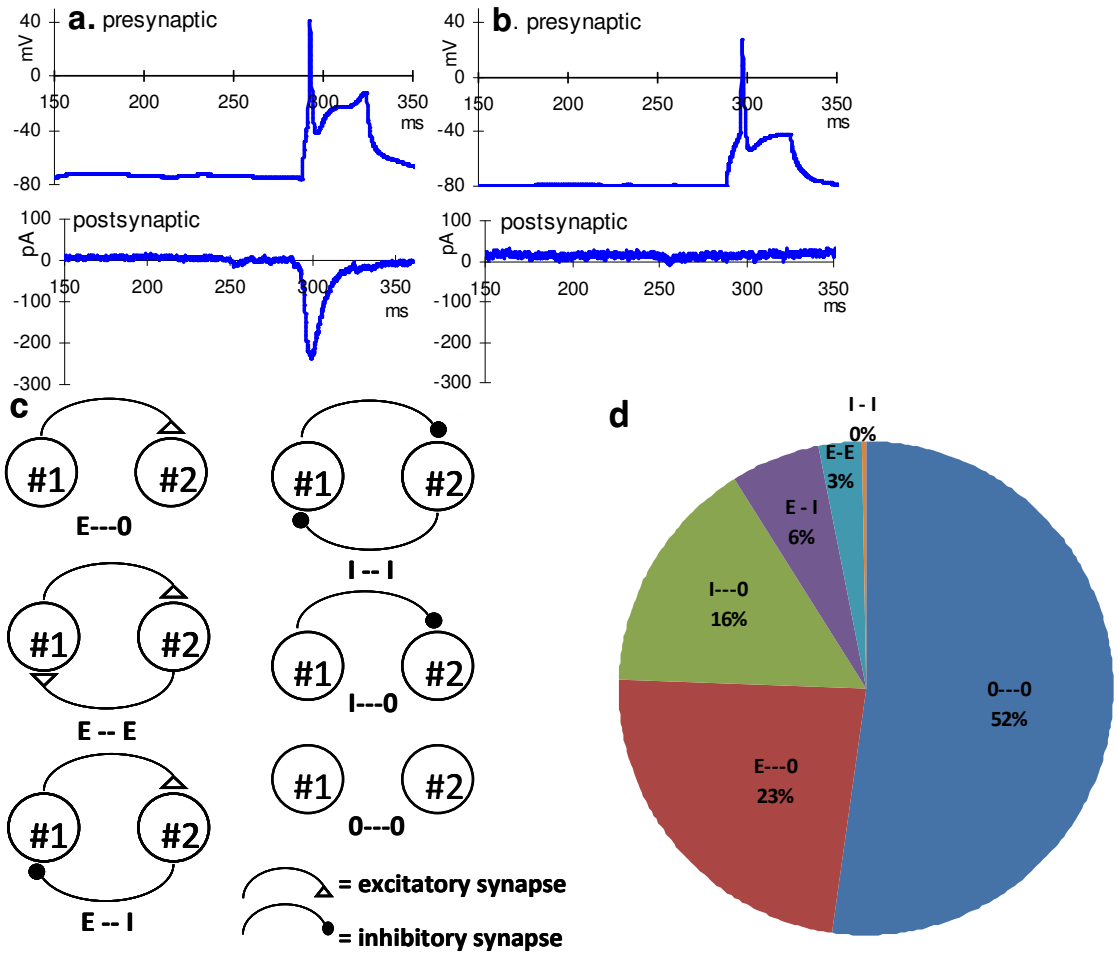

Figure 3.1 The rates for unidirectional and bi-directonal connections in embryonic hippocampal neuron cultures

a. An example of an excitatory connection. The upper trace is an action potential fired by a presyaptic cell, and the lower trace shows an EPSC in the postsynaptic cell. **b.** An example of a paired recording without synapse identified. The upper trace is an action potential fired by a presyaptic cell, while the lower trace does not show any corresponding current in the postsynaptic cell. **c**. The diagram shows how to categorize the synapses into 6 categories based on the paired cell recordings: unidirectional excitatory connection (E---0), bi-directional excitatory connection (E--E), excitatory—inhibitory connection (E--I), bi-directiaon inhibitory connection (I—I), unidirectional inhibitory connection (I---0), and no synapse (0---0). **d**. There are 289 recordings included in this figure. 52% of all the recordings showed 0---0 connection, 23% of all the recorded cell pairs formed E---0 connection, and 3% of all the cell pairs formed E---E. 16% cell pairs showed I---0, 6% were E---I and only 1 recording revealed I---I.

inhibitory cell and an excitatory cell form synapses onto each other), and 0.34% (1 recording out of 289 recordings) revealed bi-directional inhibitory synapses between two GABAergic neurons (Figure 3.1d). In no case in these cultures were any autapses observed.

Of the synaptically connected cell pairs, the E---0 and I---0 connections predominated. There were significantly fewer E---E connections and only 1 I---I connection. This result strongly suggested that the dissociated hippocampal neurons did not form synapses simply as a result of proximity. I hypothesized that there must be some intrinsic factors determining where the axon of a neuron should target, and these factors remain functional in the culture as replicate processes that occur in vivo. Thus, our assumption that selectivity of synaptic connectivity was preserved in the embryonic hippocampal neuronal cultures was strongly supported. But, more direct evidence was required to reach the conclusion.

3.2 Immunocytochemical identification of CA1 Pyramidal Cells

The previous section provides support for the generally unidirectional nature of synaptic connectivity in tissue culture, but does not demonstrate that this occurs according to cell type. In order to test this prediction, a cell type-specific marker is essential to distinguish the cells in the culture that came from CA1 region and those came from CA3 region. It has been shown that SCIP (a suppressed cyclic AMP-

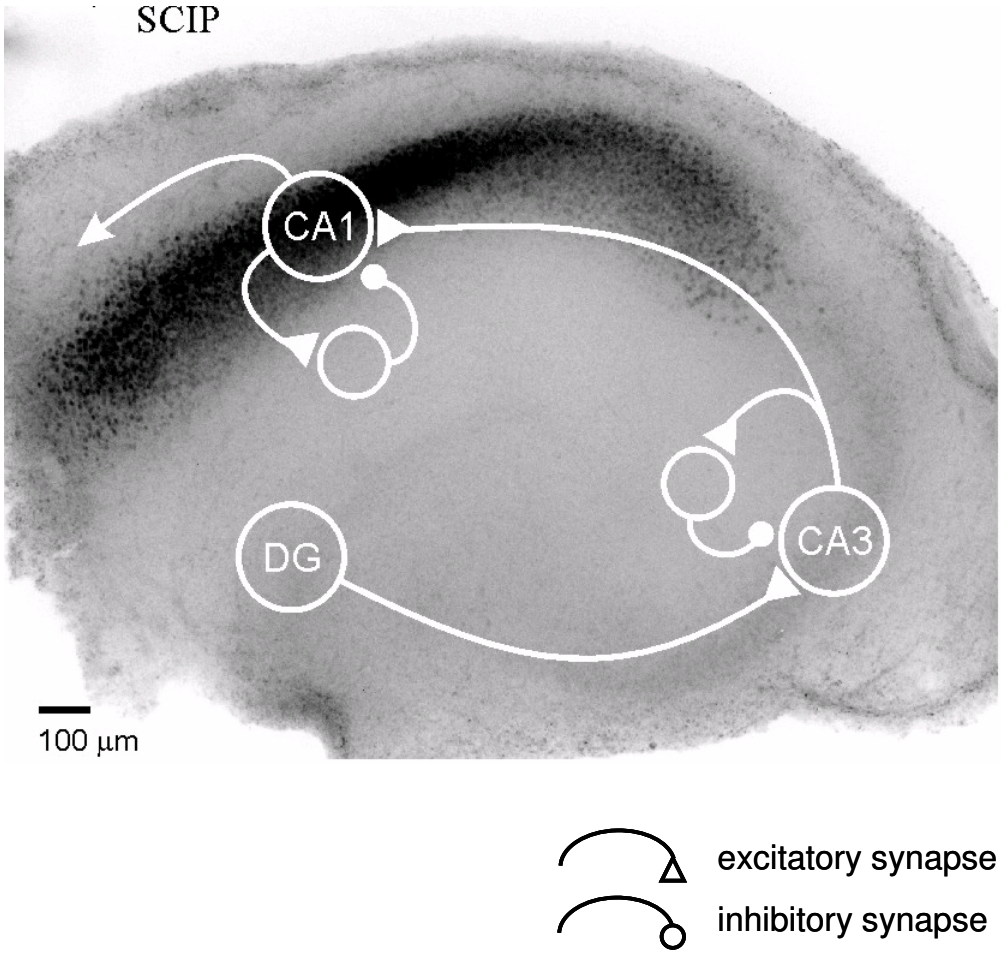

Figure 3.2 SCIP is selectively expressed in hippocampal CA1 region

A P4 rat hippocampal slice was used for the SCIP immunohistochemistry. The primary antibody was rabbit anti-SCIP, and the secondary antibody was goat anti-rabbit alexa 546. The black granules in the picture are SCIP stained cells. Notice that the SCIP positive cells are all in the CA1 region, which told us that this SCIP antibody can be a marker for CA1 pyramidal cells.

inducible POU domain protein) is an early gene expressed in CA1 pyramidal cells specifically after the stage of field-specific differentiation (~E14.5) during hippocampal development (Tole et al. 1997; Ilia et al., 2002; Grove and Tole, 1999; Tole & Grove, 2001). So, SCIP has been used as a CA1 marker, but mostly in tissue slices (Zorick et al., 1996; Ilia et al., 2002; McEvilly et al., 2002; Tole, et al. 1997; Tole & Grove, 2001).

To test the specificity of the anti-SCIP antibody for the hippocampal CA1 region before using it for our study, immunohistochemistry was done on acutely cut hippocampal slices from postnatal rats and mice. The slice was fixed in PFA and Methanol, blocked in NGS/BSA/PBS, stained with the primary antibody rabbit anti-SCIP (a generous gift from Dr. Dies Meijer), and then detected by the secondary antibody goat anti-rabbit alexa 594. The SCIP expression was found to be exclusively in CA1 region of the hippocampal slice (Figure 3.2), thus this antibody was considered as an ideal marker for discriminating CA1 neurons from other pyramidal cells.

3.3 The CA1 Pyramidal Cells did not Project Back to CA3 Cells

With a CA1 marker in hand, the next step was to provide direct evidence that the unidirectional excitatory synaptic connections matched those expected from the in vivo situation. To accomplish this, I combined electrophysiology with immunocytochemistry (for methods, see Figure 2.1) to identify the type of the pre- and postsynaptic cells. If our prediction is true, then cells labeled with anti-SCIP should only receive excitatory

connections and not make them since CA1 cells do not project onto CA3 or DG cells in vivo.

Hippocampal neurons at DIV 14 or older were used for this purpose. Two relatively close pyramidal-like cells were selected for paired-cell recording. This allowed us to tell whether and how those two cells formed synapses. Our modified internal solution was supplemented with the fluorescent dye pyranine, allowing enabling me to know which cells I had recorded from. Immediately after recording, and immunocytochemistry was in the usual way for anti-SCIP staining. Finally, images were taken and analyzed.

I found that in the unidirectional connection, when one of the cells was SCIP-positive, the presynaptic cell was almost always SCIP negative while the postsynaptic cell was SCIP positive (Figure 3.3, upper panel), reminiscent of a CA3 pyramidal neuron projecting to a CA1 pyramidal neuron. There were two exceptions where the presynaptic cell was identified as SCIP positive. In once case, the postsynaptic neuron was an inhibitory interneuron, entirely consistent with in vivo hippocampal connectivity. In the other case, the presynaptic CA1 connection may have been onto an excitatory neuron, perhaps reflecting an immature connection that would be eliminated with time (discussed more in Chapter 5). For the bi-directional connections that were observed, neither neuron was labeled by anti-SCIP (Figure 3.3, lower panel), indicating the possible CA3 → CA3 commissural connections. Taken together, these results gave the strongest evidence that neurons in dissociated tissue culture retained the ability to form synapses according to cell type-specificity.

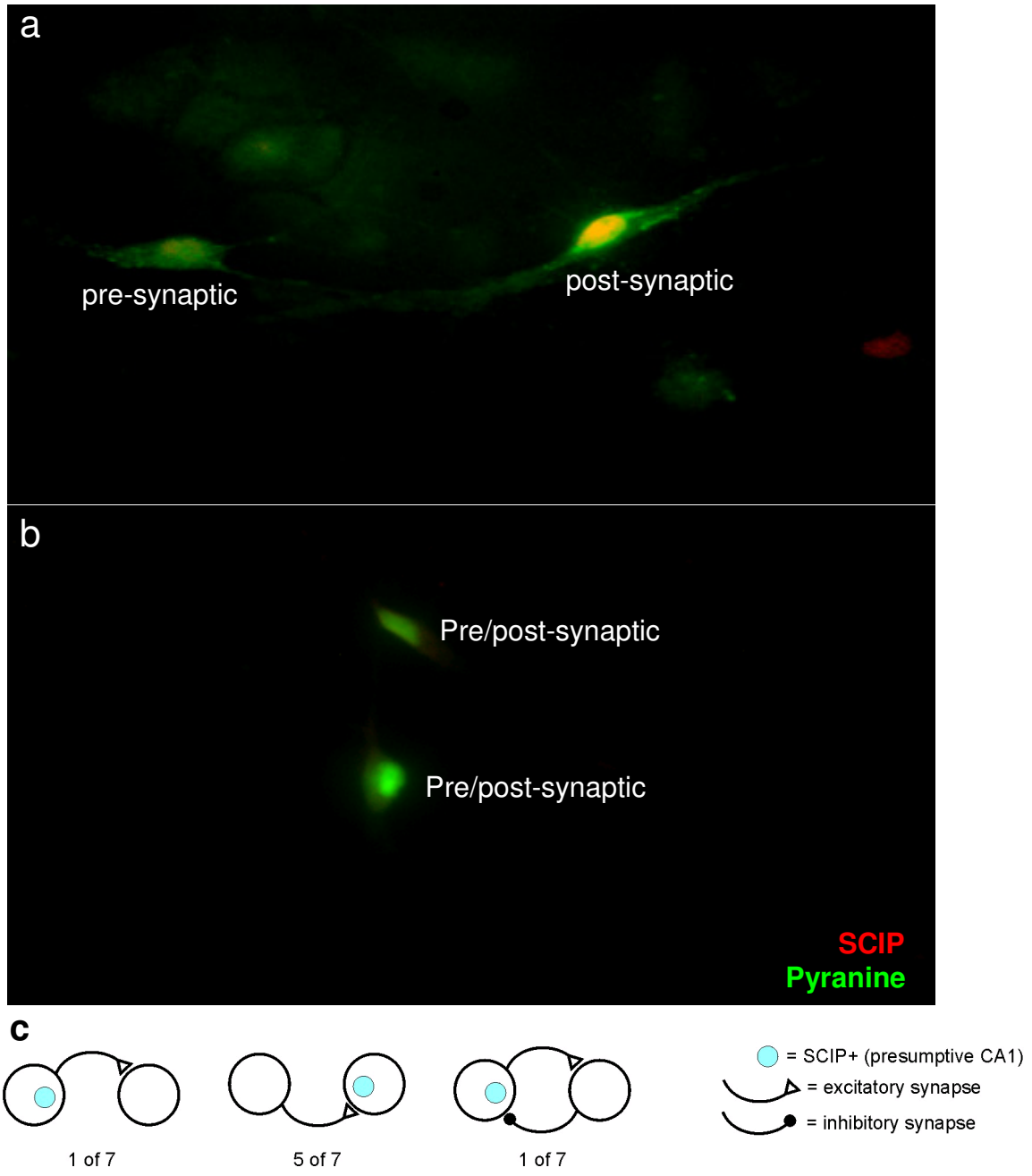

Figure 3.3 Identities for neurons after paired recording

After paired recordings, the recorded cells were stained for SCIP. The green channel is the Pyranine dye in the recording pipette, and the red is the SCIP staining. **a**. a pair of neurons with postsynaptic neuron as SCIP+ and presynaptic cell as SCIP- (CA3/DG → CA1), which is what was seen most of the time. **b**. The rare bi-directional connections with both neurons as SCIP- cells (very likely CA3 → CA3). **c**. Diagram for recordings from pairs of cells in which one is SCIP positive.

In order to see this result in a clearer way, statistics were done on the total of 16 pairs of cells that formed unidirectional excitatory synapses. Table 3.1a showed the frequency of different types of synaptic connectivity for a variety of cell type combinations, such as a presynaptic SCIP- cell synapsing onto a postsynaptic SCIP+ cell, a presynaptic SCIP- synapsing onto a postsynaptic SCIP- cell, and so on. Notice that there was only one pair of neurons with a SCIP+ presynaptic cell, and a SCIP- postsynaptic cell (Table 3.1a). Typically, SCIP- cells were presynaptic and formed synapses onto either SCIP- or SCIP+ cells. For the bi-directional synapses (Table 3.1b), I only labeled two cell pairs successfully, and out of those only one survived the immunocytochemical staining procedure. In that case both cells were SCIP-. For the other pair, only one cell survived after staining and was found to be SCIP-, which is still consistent with our hypothesis. The Fisher exact probability test was employed to confirm that the conclusion of SCIP+ cells (CA1 pyramidal neurons) not projecting back to CA3 pyramidal cells is of statistical significance (P=0.5, accept the hypothesis H0: the observed proportion (1/6) of presynaptic cells that are SCIP- are not significantly different from the expected proportion (0/6), which support the predition that SCIP positive cells mostly do not serve as presynaptic cells.).

3.4 The Predominant Presynaptic Ephrin-A and Postsynaptic EphA Expression Pattern in E18 Hippocampal Neuronal Cultures

a. Unidirectional synapses (N = 16)

Postsynaptic neuron / Presynaptic neuron	SCIP +	SCIP -	Not identified
SCIP +	**0**	**1**	**0**
SCIP -	**4**	**6**	**2**
Not identified	**2**	**2**	**0**

b. Bi-directional synapses (N = 2)

Pre/post-synaptic neuron / Post/presynaptic neuron	SCIP +	SCIP -
SCIP +	**0**	**0**
SCIP -	**0**	**1**
Not identified	**0**	**1**

Table 3.1 CA1 pyramidal cells did not serve as presynaptic cells

This figure shows the frequency for each kind of cell type combination between the presynaptic and postsynaptic cells for unidirectional (a) and bi-directional excitatory connections (b). The values indicate the number of recordings that showed the corresponding row label (identity of postsynaptic cell) and column label (identity of presynaptic cell) combination. "Not identified" means the cell was lost after staining. Notice that SCIP+ cells were rarely found to be presynaptic . On the other hand, SCIP- cells were presynaptic to either SCIP- or SCIP+ cells. A total 16 pairs of cells that formed unidirectional excitatory synapses and 2 pairs of cells that formed bi-directional excitatory synapses were included in this analysis.

Since ephrin/Eph expression pattern has been shown to be critically involved in the topographic mapping of neuronal projections in a variety of systems (McLaughlin and O'Leary, 2005; Knoll and Drescher, 2002), I was interested in how they are expressed in our hippocampal neuronal cultures.

Immunocytochemistry was used to identify the synaptic localization pattern of EphA and ephrin-A. Firstly, ehrin-A5-Fc was used to label endogenous EphA receptors and EphA5-Fc to label endogenous ephrin-A5 ligand in 12-14 days old cultures. Cells were then double stained with anti-synaptophysin (presynaptic marker) and anti-MAP2 (neuronal dendritic marker) to determine whether the ephrin-A5 or EphA5 labeling was pre- or postsynaptic. It was shown that most ephrin-A5-Fc labeled puncta were apposed to but not overlapping with synaptophysin (neighboring red and green puncta in Figure 3.4a, bottom panels), suggesting the predominantly postsynaptic localization of EphA5 receptors. Ephrin-A5 distribution was in an opposite pattern as revealed by Eph-A5-Fc labeling. Ephrin-A5 was also found on dendritic spines but its distribution was different from EphA, was primarily co-localized with synaptophysin (yellow puncta in Figure 3.4b, bottom panels), suggesting a predominant presynaptic expression of ephrin-A ligands.

Notice that, despite of the predominant (not exclusive) distribution pattern that may have occurred during the development of connectivity, the pictures also showed some presence of EphA and ephrin-A at both pre- and postsynaptic sites.

This presynaptic ephrin-A and postsynaptic EphA localization pattern suggested that ephrin-A/EphA singaling might be involved in synapse formation and potentially

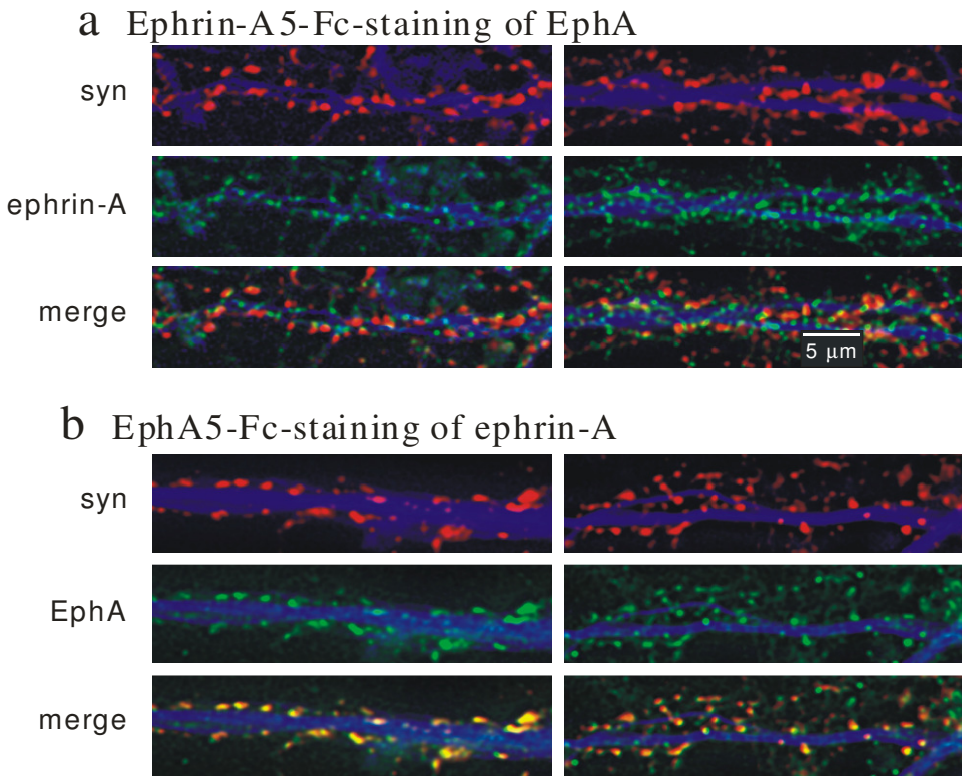

Figure 3.4 Predominant presynaptic ephrin-A ligand and postsynaptic EphA receptor expression in 12-14 DIV embryonic hippocampal neuron cultures

a. Synaptic distribution of endogenous EphA receptor labeled by ephrin-A5-Fc (FITC). Top panels: Anti-synaptophysin (Alexa 546) labeling of presynaptic terminals and anti-MAP2 labeling of dendrites. Middle panels: Labeling for EphA receptor and MAP2. Bottom panels: Merged images of the top and middle panels. Left and right sets of images were from different platings. The ephrin-A5-Fc labeling revealed that EphA can be both presynaptic (yellow puncta of EphA overlapping wtih MAP2) and postsynaptic (green puncta apposed to red puncta), with the postsynaptic sites as the predominant location. b. Synaptic distribution of ephrin-A labeled by EphA5-Fc (FITC). Top panel: Double labeling for synaptophysin (Alexa 546) and MAP2. Middle panel: Labeling for ephrin-A and MAP2. Bottom panels: Merged images of the top and middle panels. Both pre- and post-synaptic localization was also observed for ephrin-A, but the mainly presynaptic distribution was suggested by the substantial abundance of yellow puncta.

projection specificity. I therefore decided to determine whether EphA5 was distributed in a cell type-specific manner.

3.5 The EphA5 Expression was Correlated with SCIP Localization in E18 Hippocampal Culture

So far, it has been suggested that CA1 pyramidal cells are postsynaptic in the hippocampal culture in vitro as well as in the hippocampus in vivo. Based on the previous results about the postsynaptic EphA expression (Figure 3.4), I speculated that EphA might be correlated with CA1 pyramidal cells in the dissociated hippocampal culture. To test this prediction, immunocytochemical experiments were pursued on cultured hippocampal neurons culture to look for colocalization of EphA3 or EphA5 with SCIP.

Hippocampal neurons at DIV 14 or older were fixed using 4% paraformaldehyde and 100% Methanol, blocked with 10% DS, and incubated with antibodies against EphA3 or EphA5, followed by the secondary antibody detection. A second round of staining was then done to label SCIP. Cells were blocked again with 5% NGS, incubated with antibodies against SCIP and MAP2, and detected by other secondary antibodies (for detail, see the methods section in Chapter 2). Images were taken and analyzed with IPLab.

I found that almost all of the SCIP positive cells were also labeled by anti-EphA5

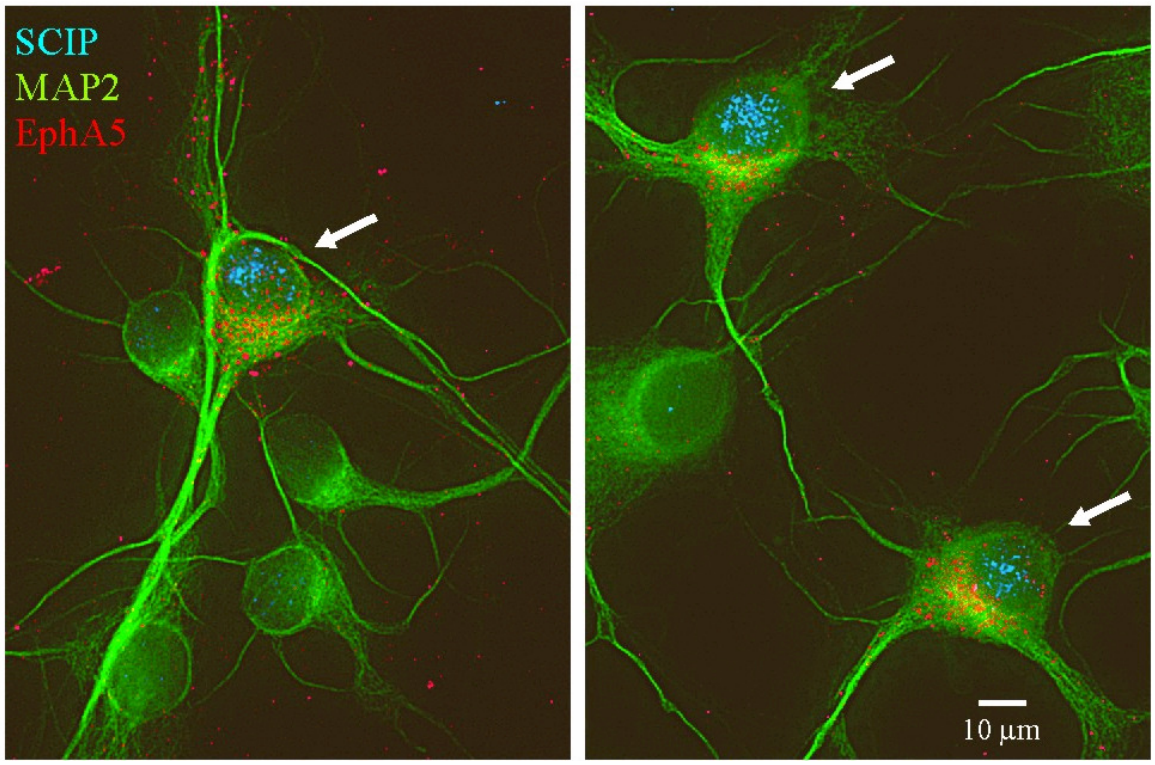

Figure 3.5 SCIP positive cells also express EphA5

Cultured hippocampal neurons used for the EphA5 – SCIP – MAP2 staining. The blue is ant-SCIP, the green is anti-MAP2, and the red is anti-EphA5. I found that almost all the SCIP labeling positive cells (arrows in the pictures) were also labeled by anti-EphA5 while most SCIP negative cells were also EphA5 negative.

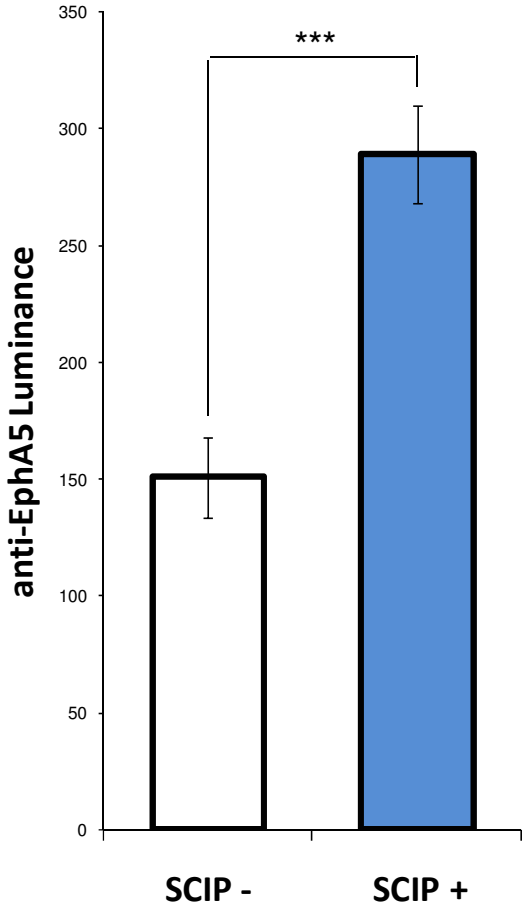

Figure 3.6 Quantification anti-SCIP and anti-EphA5 labeling luminance in hippocampal neuron cultures

Morphologically identified pyramidal neurons from different platings were selected up for luminance analysis for both SCIP and EphA5. Cells with SCIP luminance below 150 (background level) were considered to be SCIP negative cells wherease cells with SCIP luminance higher than 150 were considered to be SCIP positive. All the SCIP positive cells were grouped together to get the average EphA5 intensity (y axis in the figure) as were the SCIP negative group. This figure shows that SCIP positive cells have dramatically higher EphA5 expression than SCIP negative cells. N = 53. ***P < 0.001 by Student's t-test.

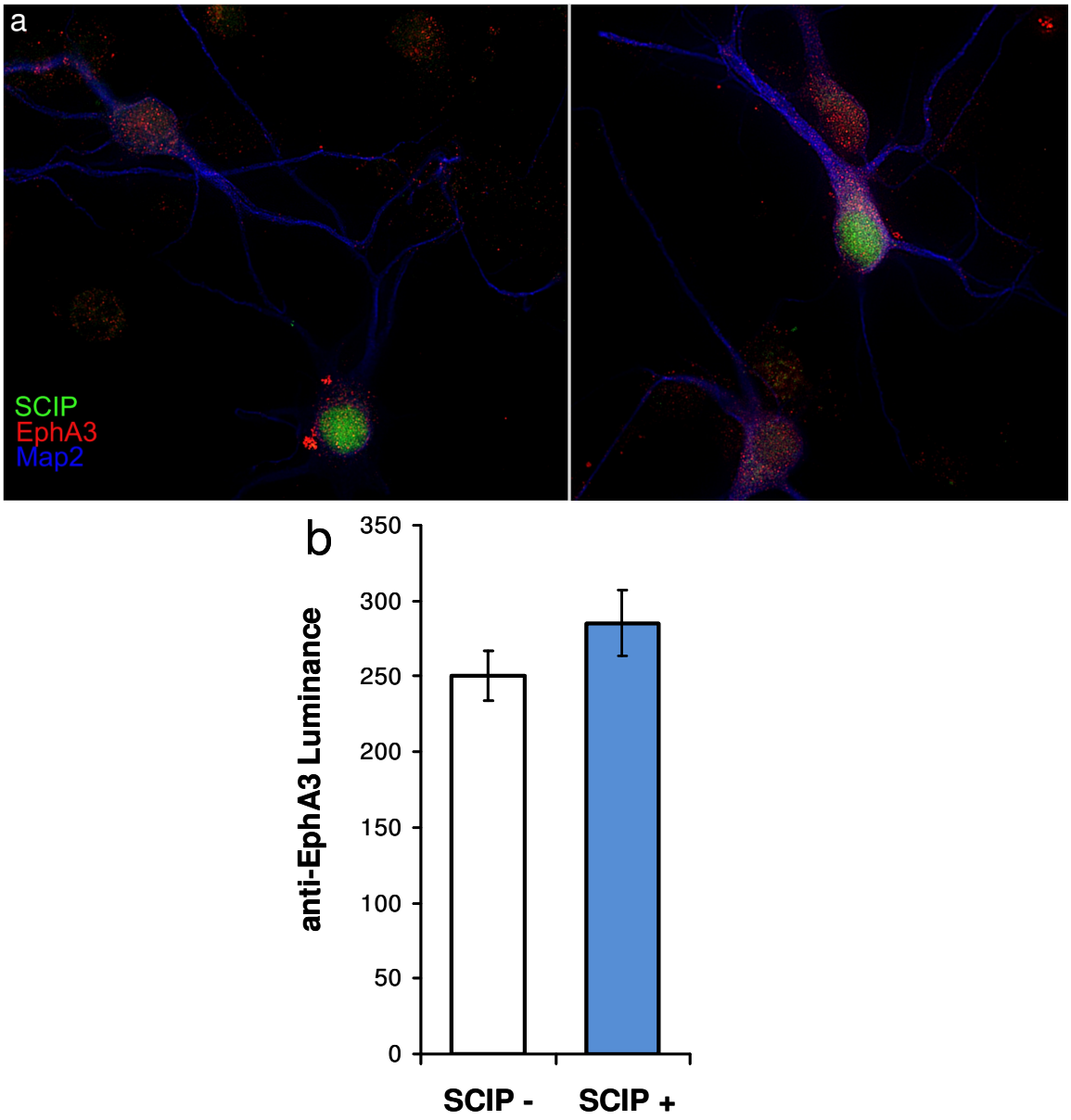

Figure 3.7 EphA3 expression was not correlated with anti-SCIP labeling

a. Cultured hippocampal neurons used for the EphA3 – SCIP – MAP2 staining. Green is anti-SCIP, red is anti-EphA3, and blue is anti-Map2. Note that almost all the neurons were labeled by anti-EphA3 regardless of whether they were SCIP positive or SCIP negative. Left and right panels are from different fields in the dish. Pictures were taken at 40X. **b**. Quantification for the luminance analysis for SCIP and EphA3 labeling in the same way as the analysis in Figure 3.6. N = 23, P > 0.15 (no significant difference) by Student's t-test.

whereas most of the SCIP negative cells were EphA5 negative (Figure 3.5). The results were quantified correlating anti-SCIP staining intensity and EphA5 intensity in individual cells (Figure 3.6). The results suggested that EphA5 is predominantly expressed on CA1 pyramidal neurons. Interestingly, however, EphA3 localization did not show this relationship with anti-SCIP labeling, with almost all the neurons expressing this EphA3 receptor (Figure 3.7).

This finding strongly suggested that the specific EphA5 expression in CA1 pyramidal cells might be a critical factor determining the specific hippocampal projection formation. In addition, this feature is special for EphA5 since another member in the Eph family EphA3 did not show the same result.

In summary, the data shown in this chapter revealed strong electrophysiological and immunocytochemical evidence that embryonic neurons in dissociated embryonic hippocampal culture preserve features of the in vivo synaptic circuitry. In addition, the positive correlation between EphA5 expression and the CA1 pyramidal cell marker expression was consistent with the result of postsynaptic labeling of EphA receptors in mature hippocampal cultures (Figure 3.4), suggesting that EphA/ephrin-A signaling might be contributing to this selectivity of connection (Figure 3.8). Thus embryonic hippocampal neuron cultures can serve as a useful model for the next two chapter's investigation into the interaction between ephrin/Eph – BDNF in regulating the selectivity of synapse formation selectivity in the hippocampus.

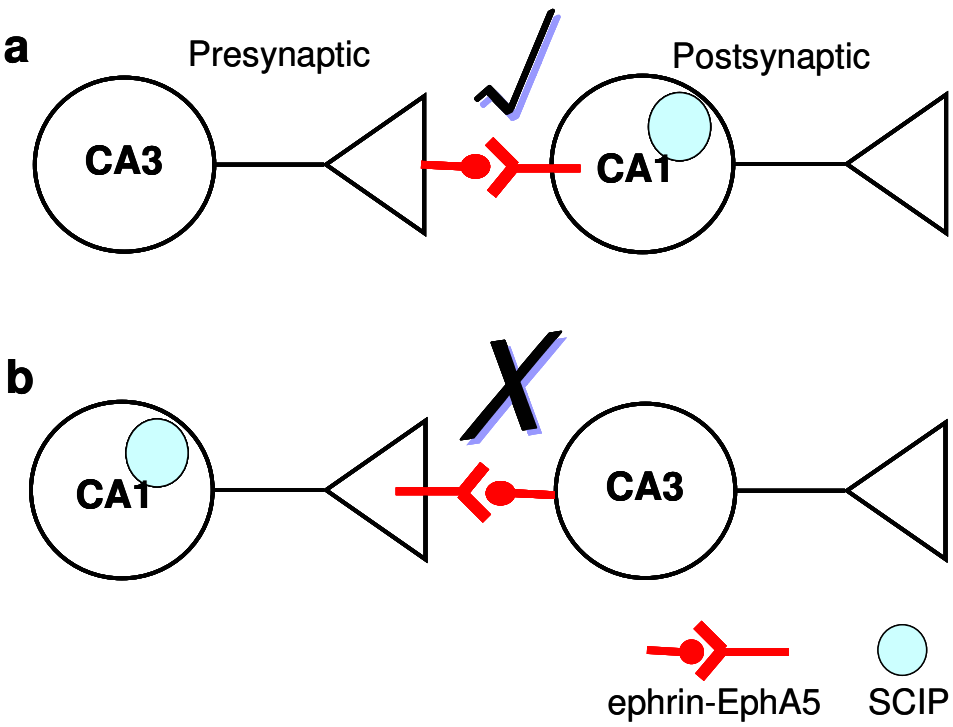

Figure 3.8 Summary of a possible mechanism for producing selective CA3 → CA1 synapse formation

Two possible orientations of EphA5 expression in hippocampal neurons. **a**. Most CA1 pyramidal cells (SCIP+) express EphA5 while the presynaptic CA3 neurons express predominantly a corresponding ligand. Potential synapses of this configuration will survive until maturation. This pattern is also consistent with the result of postsynaptic labeling of EphA receptors in mature hippocampal culture (Figure 3.4). **b**. The opposite configuration will be EphA5 expression in presynaptic CA1 cells and the ephrin-A ligand in postsynaptic CA3 neurons. Potential synapses of this configuration will not survive so it was not observed as predominant pattern in mature hippocampal culture (Figure 3.4).

CHAPTER 4

EPHRIN/EPH – BDNF INTERACTION IN SYNAPTIC TRANSMISSION

To pursue our hypothesis proposed in the previous chapter (Figure 3.8) that presynaptic EphA activation by postsynaptic ephrin-A ligands binding prevents hippocampal synapse formation, we turned to electrophysiology for studying ephrin-A/EphA involvement in synaptic activity, which has been extensively proved to be critical for synapse formation and maintenance. In particular, BDNF modulation has been shown to be an important player during synapse development, so we will next focus on the interaction between ephrin-A/EphA and BDNF signaling in synaptic activity in hippocampal neurons.

The electrophysiology studies in this chapter can be divided into three parts. First, I identified the direct effects of the endogenous EphA or ephrin-A signaling activation by ephrin-A5-Fc or EphA5-Fc on synaptic transmission. Second, the effect of ephrin-A5-Fc or EphA5-Fc application on the BDNF modulation of synaptic transmission was examined. Third, the possible mechanism for the interaction was investigated.

4.1 Ephrin-A5-Fc and EphA5-Fc Increase Synaptic Activity in Embryonic Hippocampal Neurons

To gain some idea about whether ephrin-A5/EphA5 affects neuronal electrophysiology, we examined the effects of ephrin-A5-Fc or EphA5-Fc application alone on ongoing synaptic activity in cultured embryonic hippocampal neurons at DIV 14 or greater. Pyramidal-like neurons were recorded under voltage clamp mode and held at -50mV to record ongoing synaptic activity. After baseline activity during NRS perfusion had been stabilized for several minutes, the perfusion solution was switched to soluble ephrin-A5-Fc or EphA5-Fc (2 μg/ml) for 20 minutes (Figure. 4.1a). Both of these molecules increased synaptic activity rapidly and transiently in most neurons recorded. Synaptic charge significantly increased within a minute of ephrin-A5-Fc application, and returned to baseline within 10 minutes (Figure 4.1b, d). Similarly, application of EphA5-Fc produced a rapid increase in synaptic activity that also decayed to baseline within 10 minutes (Figure 4.1c, e). Note the decline of increased activity despite of the maintained presence of ephrin-A5 or EphA5.

4.2 The Potential Postsynaptic Mechanism for the Excitatory Effects of Ephrin-A5-Fc and EphA5-Fc

4.2.1 NMDA involvement in the excitatory effect of Ephrin-A5-Fc and EphA5-Fc on synaptic activity

Since ephrin-B ligands have has been reported to be associated with NMDA

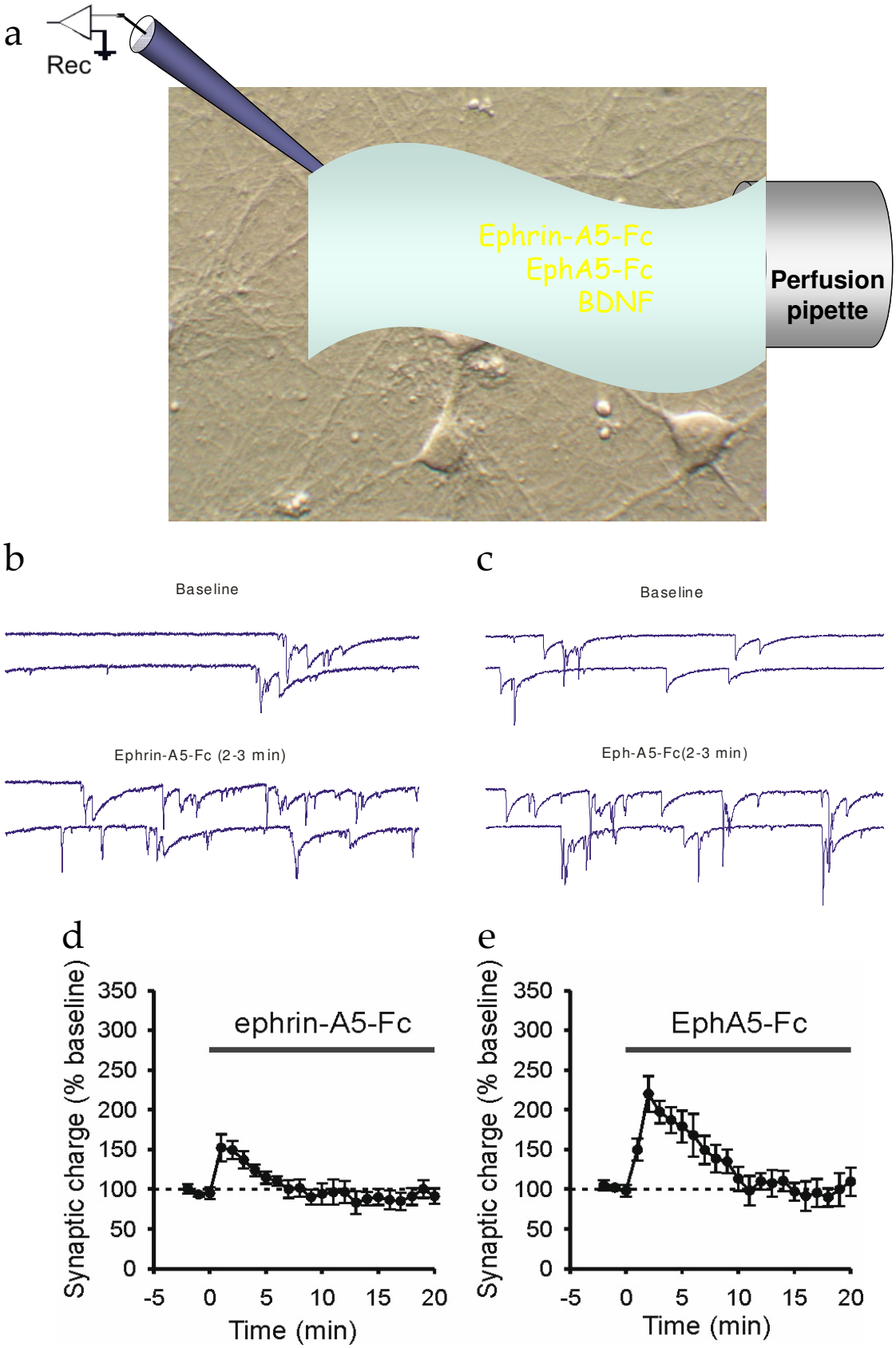
a
Rec
Ephrin-A5-Fc
EphA5-Fc
BDNF
Perfusion pipette
b
Baseline
Ephrin-A5-Fc (2-3 min)
c
Baseline
Eph-A5-Fc(2-3 min)
d
ephrin-A5-Fc
Synaptic charge (% baseline)
Time (min)
e
EphA5-Fc
Synaptic charge (% baseline)
Time (min)

Figure 4.1 Ephrin-A5-Fc/EphA5-Fc application elicited an acute and transient increase in synaptic activity in cultured embryonic hippocampal neurons

a. Diagram of the experimental setup. A pyramidal-like neuron in the culture dish was selected. Once the whole cell recording has been stabilized, experimental reagents such as NRS, ephrin-A5-Fc, EphA5-Fc or BDNF etc. were applied through the perfusion pipette. **b**. Example traces characterizing the baseline synaptic activity (upper panel) of neurons cultured in vitro for 12 – 15 days, and showing the increase in ongoing neuronal activity 2-3 minutes after the onset of 2g/ml ephrin-A5-Fc application (lower panel). **c**. Upper traces showing the baseline activity and lower traces revealing the increased synaptic activity by 2 ug/ml EphA5-Fc applications. **d-e**. Time course showing the minute-binned response of hippocampal neurons to 20 min application of 2g/ml ephrin-A5-Fc (c, N = 8) or EphA5-Fc (d, N = 6). In both cases, the increase in activity occurred rapidly and transiently, and the activity returned to baseline within 10 minutes. In this and subsequent figures, activity was quantified as synaptic charge and error bars indicate s.e.m.).

receptor function (Takasu et al., 2002; Henderson et al., 2001; Dalva et al., 2000; Armstrong et al., 2006), and EphB and ephrin-A family-cross binding has also been shown, it is possible that the excitatory effects of ephin-A5 and EphA5 are related with NMDA receptor function.

We began by testing possible NMDA receptor involvement in the ephrin-A5-Fc effect. The same experimental protocol as above was used but with the NMDA antagonist AP5 present during the recording. First, we confirmed that the vehicle for ephrin-A5/EphA5-Fc didn't produce any effect on synaptic activity in the presence of AP5 (Figure 4.2). Then, when ephrin-A5-Fc or EphA5-Fc were applied in the presence of AP5, virtually no response was seen (Figure 4.2), indicating that inhibiting NMDA receptors blocked or dramatically reduced the increase in synaptic activity elicited by ephrin-A5-Fc/EphA5-Fc. It is thus suggested that ephrin-A5/EphA5 might be exerting their direct excitatory effects mainly through synaptic NMDA receptors.

4.2.2 Ephrin-A5-Fc and EphA5-Fc did not affect presynaptic transmitter release

To examine whether Ephrin-A5-Fc and EphA5-Fc also exerted their excitatory effects through presynaptic mechanism, we used miniature excitatory postsynaptic current (mEPSC) frequency as an index of presynaptic release probability. The amplitude of mEPSCs was also analyzed since it could be an indicator for both presynaptic and postsynaptic mechanisms. TTX was applied during the whole recording to isolate the miniature events. Once the baseline has been stabilized for 5 minutes, ephrin-A5-Fc or EphA5-Fc would be applied for 20 minutes. It was found that

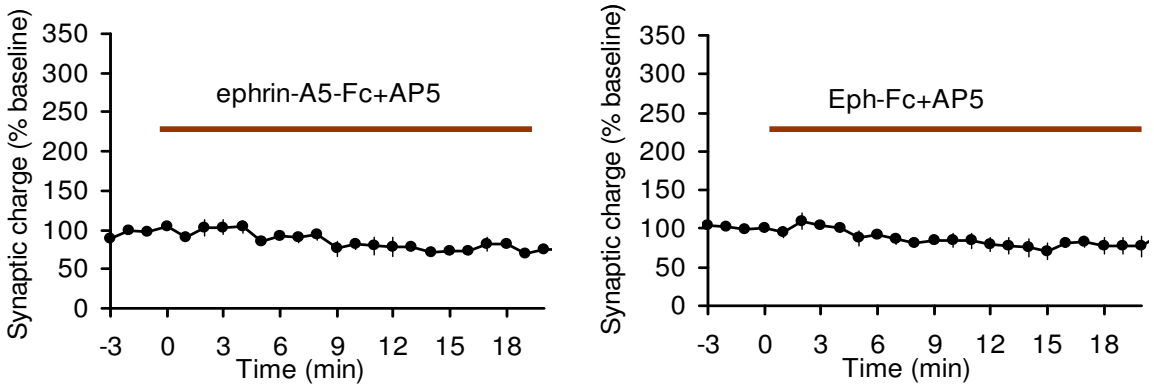

Figure 4.2 NMDA antagonists blocked the excitatory effect of Ephrin-A5-Fc and EphA5-Fc on synaptic activity

Recordings of synaptic activity from DIV14 hippocampal neuron cultures. AP5 was added to NRS, ephrin-A5-Fc and EphA5-Fc perfusion solution to block NMDA receptor activity. Time 0 minutue is the onset of ephrin-A5-Fc and EphA5-Fc application. The plot represents the minute-binned synaptic charge that was normalized to the baseline (100%). Note that in the presence of AP5, neither ephrin-A5-Fc (N = 6) nor EphA5-Fc (N = 5) showed any increase effect on synaptic activity, compared with the control effect shown in Figure 4.1.

neither ephrin-A5-Fc nor EphA5-Fc had any effect on the miniature frequency or amplitude (Figure 4.3).

4.3 Ephrin-A5 Inhibited the Synaptic Actions of BDNF

We employed the combination of ephrin/Eph perfusion and BDNF perfusion in the same recording to examine ephrin/Eph - BDNF interactions. After the baseline was stabilized for at least 3 minutes, one test substance was applied for 5 minutes followed by a second test substance for 15 minutes. We started with the possible effect of ephrin-A5-Fc on BDNF modulation of synaptic transmission. When applied alone, BDNF (20 ng/ml) caused a long-lasting and dramatic increase of synaptic activity (averaged at 8-9 min: 241±18%, N = 4) when preceded with vehicle application which had no effect on synaptic activity (Figure 4.4a). However, when ephrin-A5-Fc was pre-applied, subsequent application of BDNF didn't produce an obvious response (Figure 4.4b)，and synaptic activity did not increase compared to where it would have been (solid line in Figure 4.4b) if the normal BDNF response (doubling of activity) was added to the response to ephrin-A5 (Figure 4.4c; 100 ±11%, N = 18).

Interestingly, this inhibition was not reciprocal. When I reversed the order of BDNF and ephrin-A5 application, the inhibition of pre-applied BDNF on the response to ephrin-A5 was not observed. When ephrin-A5 application was preceded by vehicle, the transient increase in activity was observed as ephrin-A5 alone (Figure 4.4d; 190±12%, N = 6). When ephrin-A5 was preceded by BDNF, it still produced a normal increase in

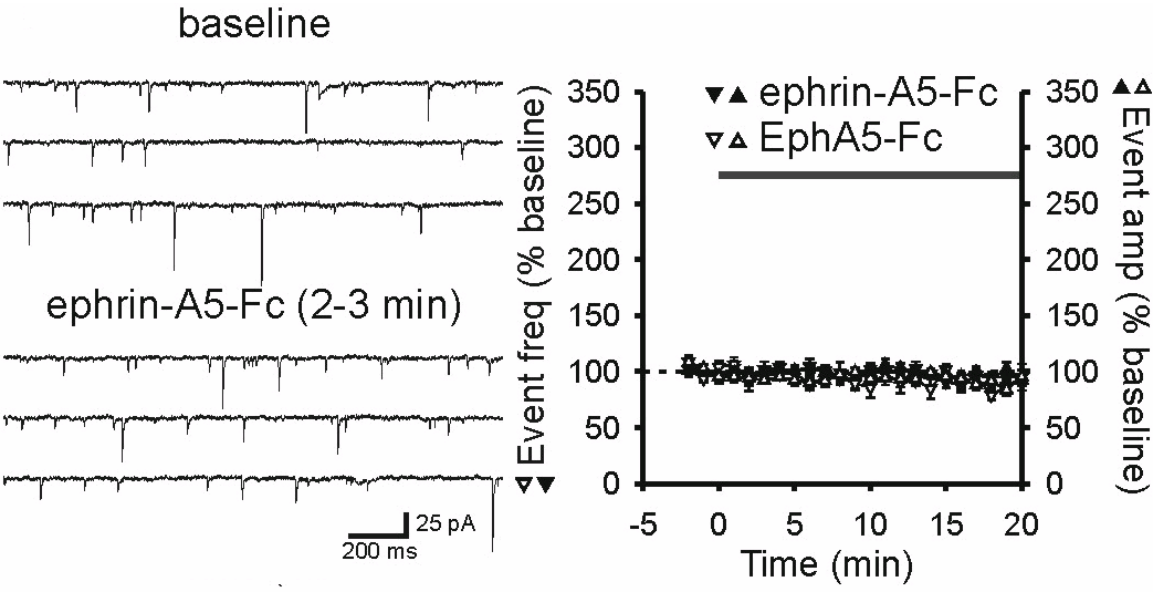

Figure 4.3 Ephrin-A5-Fc and EphA5-Fc did not affect spontaneous presynaptic transmitter release

Recordings of miniature synaptic currents were made from DIV14 hippocampal neuron cultures. Average mEPSC frequency and amplitude were analyzed and binned to plot the time course. Time 0 is the onset of ephrin-A5-Fc and EphA5-Fc application. Compared to the baseline (100%), neither ephrin-A5-Fc nor EphA5-Fc showed any effect on mEPSC frequency or amplitude. For ephrin-A5-Fc, N = 6; EphA5-Fc, N = 5.

synaptic activity (data not shown; 163±40%, N = 10).

To exclude the possibility that the absence of an effect of BDNF on ephrin-A5 –Fc might result from washout of BDNF during ephrin application, we used co-application ephrin-A5-Fc and BDNF instead of sequential application following BDNF pre-application, which showed similar results with normal ephrin-A5 response after BDNF (Figure 4.4d, e; 172±38%, N = 5).

4.4 EphA5 Reduced, but did not Eliminate, the Synaptic Actions of BDNF

Since ephrin/Eph signaling has been shown to be bi-directional, with both the ligand and receptor capable of activating intracellular signaling pathways, we examined whether binding to ephrin-A could also modulate the synaptic responses to BDNF by conducting similar experiments with EphA5-Fc (Figure 4.5). We found that pre-application of EphA5-Fc also reduced the synaptic response to subsequently applied BDNF. But unlike ephrin-A5, EphA5-Fc application especially inhibited the late phase of BDNF application while the early phase of BDNF modulation was not affected so much as by ephrin-A5-Fc (Figure 4.5a; 140±17%, N = 5 at 8-9 min, compare to response following vehicle in Figure 4.4a), suggesting that EphA5 might inhibit postsynaptic component of BDNF response. We also did co-application of BDNF and EphA5 after 5 min exposure to EphA5 to control for the possibility of washout of EphA5 during the

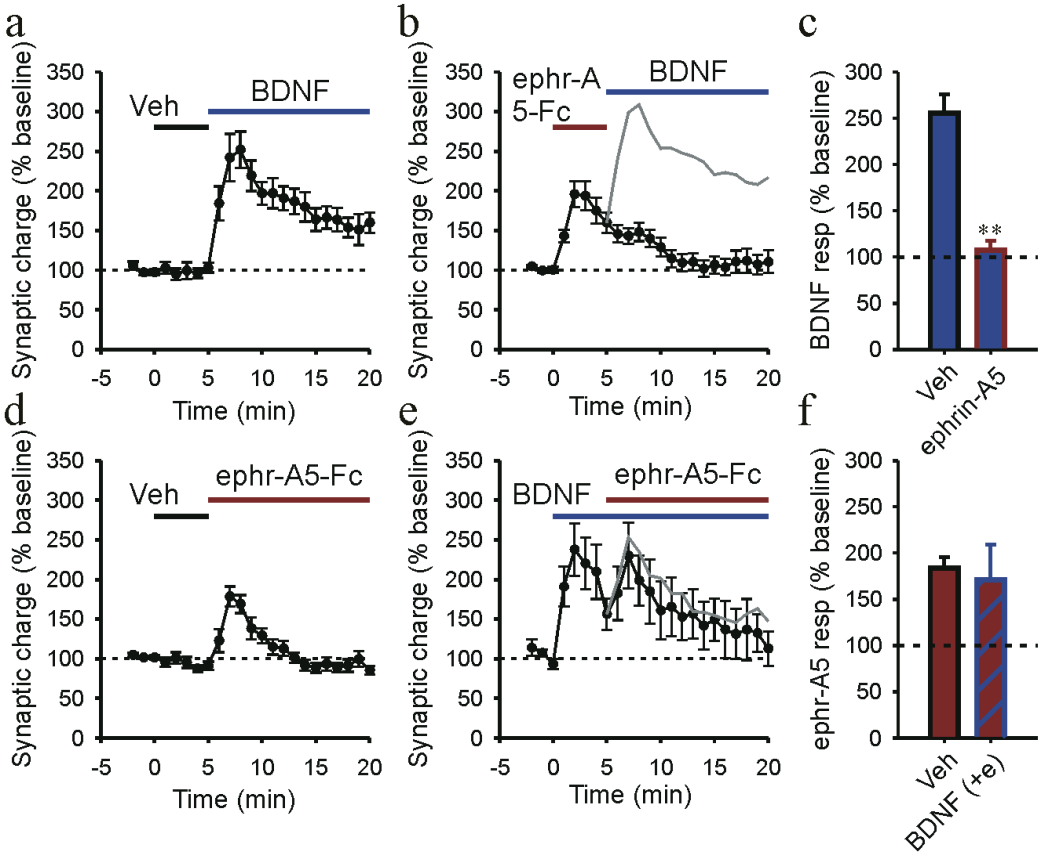

Figure 4.4 Ephrin-A5-Fc pre-exposure prevented the BDNF-induced increase in synaptic activity

a. Time course of synaptic activity during sequential application of vehicle for 5 min and 20 ng/ml BDNF for 15 min. Vehicle didn't have a significant effect and BDNF produced a large and comparatively long-lasting increase in synaptic activity (N = 4). **b.** Time course of response to 15 min of BDNF application preceded by exposure to ephrin-A5-Fc instead of vehicle (N = 18). Note the failure of BDNF to induce a response after ephrin-A5-Fc application (compared to the brown line, which is the superimposed response if BDNF showed a response normally as shown in a). **c.** Quantification of BDNF peak response (at 7-8 min) in the cases of Figure 4.4a and Figure 4.4b (** = $p < 0.01$). **d.** Time course for sequential 5 min application of BDNF vehicle and 15 min application of ephrin-A5-Fc (N = 6). Note the transient response to ephrin-A5 as normal (compared to Fig. 1c). **e.** Time course of response to 15 min ephrin-A5 co-applied with BDNF when preceded by BDNF application instead of vehicle (N = 5). Note the similar increase in activity by ephrin-A5 as in d (brown line). **f.** Quantification of ephrin-A5 peak response (at around 7-8 min) in the cases of Figure 4.4d and Figure 4.4e ($p > 0.65$).

BDNF perfusion being responsible for the lack of complete inhibition (Figure 4.5b). No significant difference in the response was observed between co-application and sequential application (170±17%, N = 7), and the amplitude of the BDNF peak response was significantly lower when preceded by EphA5 exposure (Figure 4.5c).

We next tested for potential BDNF - EphA5 interactions. As with ephrin-A5-Fc, the EphA5-induced response was not altered significantly by pre-exposure to BDNF in both sequentially applied (191±26%, N = 5 vs. 164±18%, N = 5; not shown) and co-applied conditions (Figure 4.5d-f; 191±26%, N = 5 vs. 163±29%, N = 5).

4.5 The Inhibitory Effect of Ephrin-A5 is Presynaptic

To examine how and where ephrin-A5 was exerting its effects, we firstly took miniature excitatory postsynaptic current (mEPSC) frequency as an index of presynaptic mechanisms. The same experimental strategy was used as above. As the control, BDNF application increased mEPSC frequency significantly when preceded with vehicle that by itself produced no response (Figure 4.6a; N = 8). However when preceded by ephrin-A5 application, subsequent applied BDNF did not increase mEPSC frequency (Figure 4.6b, c; N = 5), and ephrin-A5 didn't change mEPSC frequency either.

These data suggest that ephrin-A5, although it has no effect of its own on mEPSC frequency, inhibits BDNF action presynaptically. EphA5 was also tested for potential presynaptic effects. EphA5 had no effect on mEPSC frequency and also did not affect

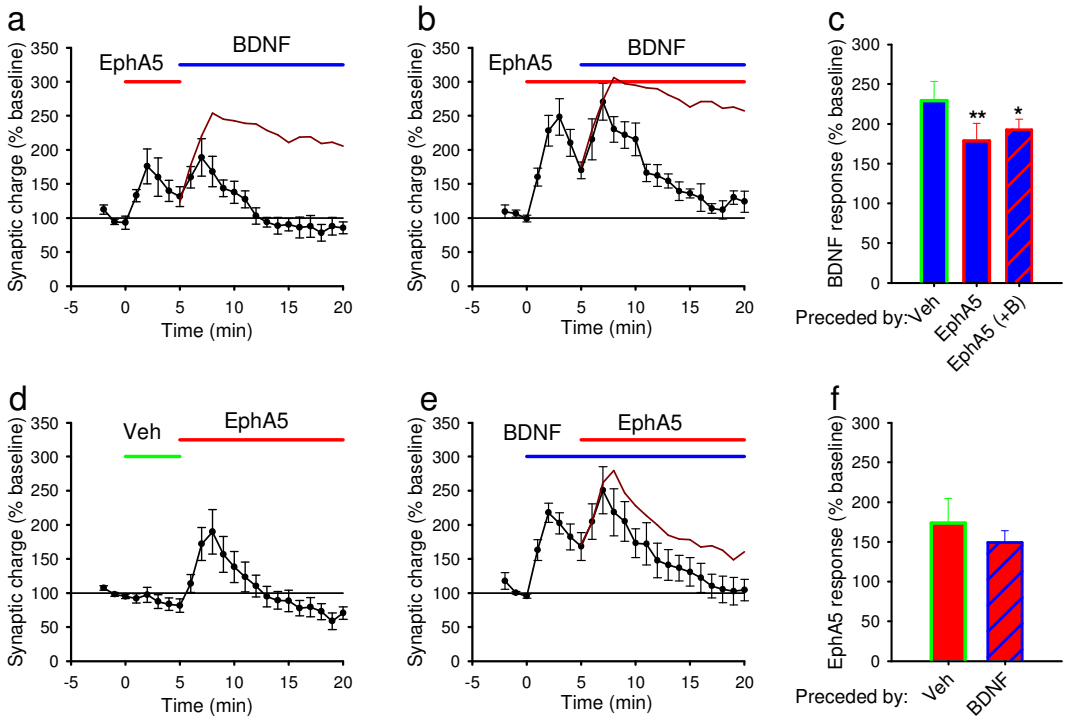

Figure 4.5 EphA5-Fc application partially inhibited the BDNF-induced increase in synaptic activity

a. Time course of synaptic activity change during sequential 5 min application of EphA5-Fc and 15 min application of BDNF (N = 5). Note the smaller and shorter-lasting BDNF response after EphA5-Fc (compared to the brown line representing the superimposed response if BDNF had the same response as in figure 2a). **b.** Time course of response to BDNF co-applied with and proceeded by EphA5-Fc application (N = 7). The response was similar to that observed in sequential application (a). **c.** Quantification of BDNF peak response (at around 8-9 min) in the cases of Fig. 4.4a, Fig. 4.5a and 4.5b (** = $p < 0.01$, * = $p < 0.05$). **d.** Time course of sequential 5 min application of BDNF vehicle and 15 min application of EphA5-Fc. Note the absence of a response to vehicle and the transient response to EphA5 produced a synaptic charge (N = 5). **e.** Time course of response to 15 min EphA5 co-applied BDNF when preceded by BDNF (N = 5). Note the similar increase in activity as in d (brown line). **f.** Quantification of EphA5 peak response (at around 7-8 min) in the conditions of Fig. 4.5d and 4.5e ($p > 0.4$).

the BDNF response. There was no statistical difference in the magnitude of BDNF response in either sequential application (Figure 4.6b, c; N = 10). This suggested the possibility that EphA5-Fc application might inhibit BDNF modulation on synaptic transmission postsynatically, which is actually consistent with the EphA5-Fc inhibition on the late phase (the postsynaptic component of BDNF effect; Alder et al., 2005) BDNF effect on synaptic activity.

4.6 EphA Receptors Mediate the Excitatory Effects of Ephrin-A5-Fc on Synaptic Activity and its Inhibition on BDNF Effect

It has been known that ephrin family ligands can bind approximately to various Eph receptor members, so the ephrin-A5-Fc application might have activated more than one kind of EphA receptors. It would be very meaningful to identify which receptors are involved in this modulation process. It has been demonstrated that ephrin-A5 can bind to the general EphA family receptors, but EphA3 and EphA5 are the highest affinity receptors for ephirn-A5-Fc (Himnen et al., 2004). The best candidate we have is EphA5 because of its selective distribution in SCIP positive cells. EphA3 isn't as good of a candidate due to its lack of region specificity in expression. So we started by testing the candidacy of EphA5 as a partner of ephrin-A5-Fc application for increasing synaptic activity.

The EphA5 kinase domain negative (EphA5(K-)-GFP) and EphA3(K-)-GFP

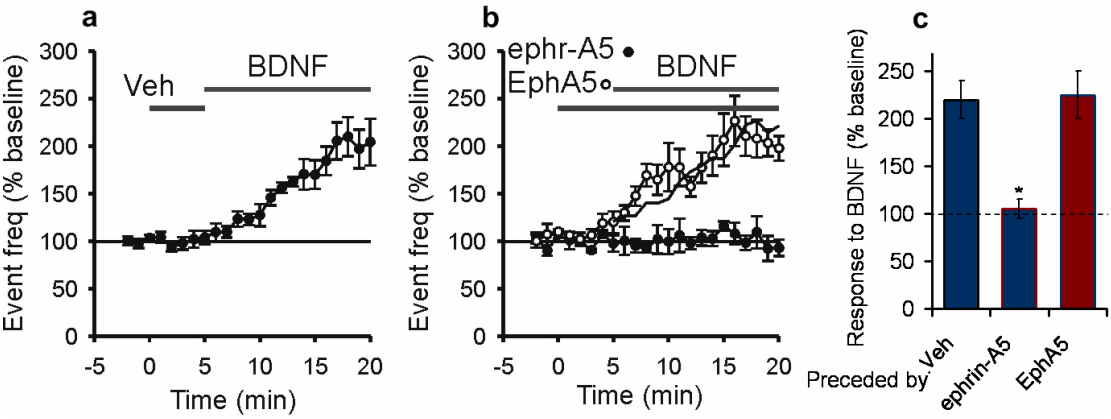

Figure 4.6 Ephrin-A5, but not EphA5 abolished the presynaptic modulation of BDNF on mEPSC frequency

a. Time course of mEPSC event frequency during sequential application of 5 min vehicle and 15 min BDNF. Vehicle had no effect whereas BDNF increased the mEPSC frequency (N = 8). **b.** Time course of response to 15 min application of BDNF preceded by 5 min ephrin-A5-Fc affliction (closed circle, N = 5) or EphA5-Fc application (open circle, N = 10). Note the lack of increased response by BDNF after ephrin-A5 application and the normal increased response by BDNF after EphA5 application (compared to the curve line without data point symbols which is the normal BDNF response as in a). **c.** Quantification of the averaged BDNF response (10-15 min) in the conditions of a and b (* = $p < 0.05$).

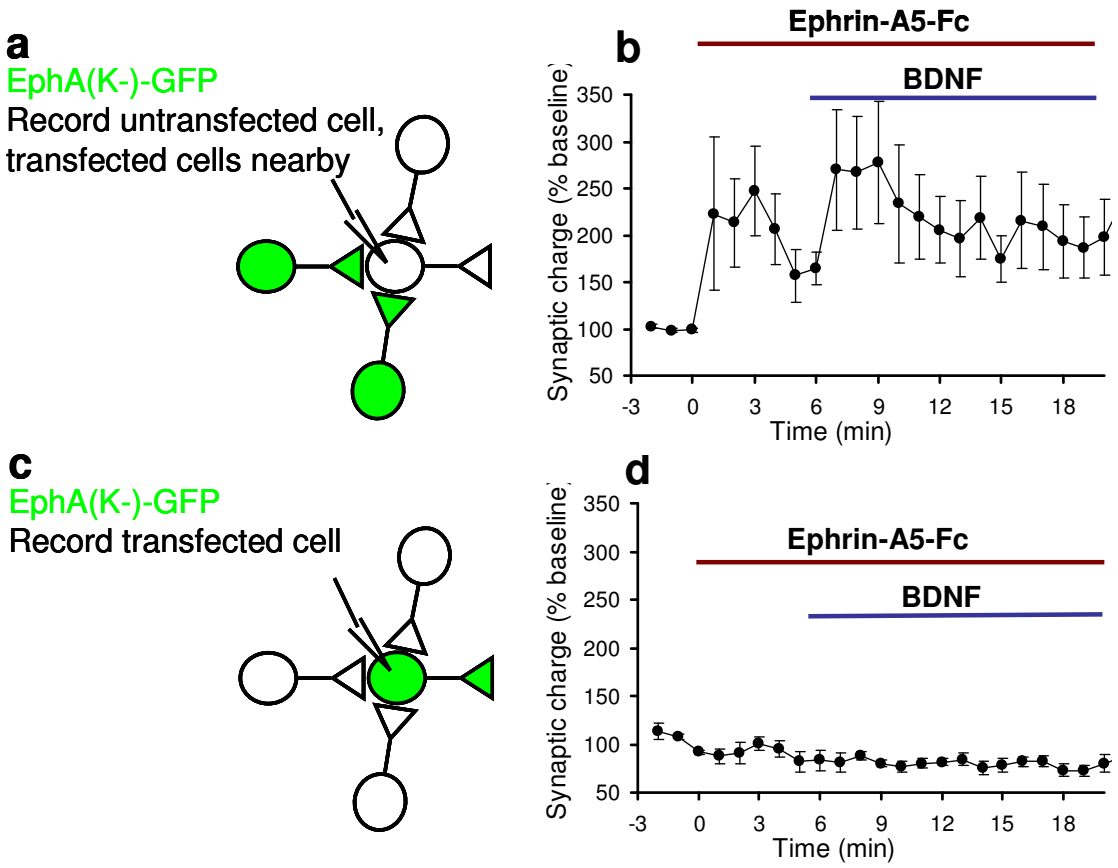

Figure 4.7 EphA signaling interruption blocked the excitatory effect of ephrin-A5-Fc on synaptic activity

Hippocampal neurons were transfected with EphA5(K-)-GFP or EphA3(K-)-GFP construct on DIV 5, and recorded around DIV 14. The data from EphA5(K-)-GFP or EphA3(K-)-GFP groups were averaged together as EphA(K-)-GFP to plot the graph b and d. **a**. Demo of the configuration of recordings (in b) from an untransfected cell with EphA(K-)-GFP transfected cells nearby. **b.** The untransfected cells still showed an increase in activity by ephrin-A5-Fc, but did not prevent the subsequent BDNF action as usual (N = 5). **c**. Demo of the configuration of recordings (in d) from the EphA(K-)-GFP transfected cell. **d.** The transfected cells did not show an increase in activity by ephrin-A5-Fc, but the subsequent BDNF action was prevented as usual (N = 7). This suggests that the presynaptic EphA receptors are responsible for the inhibitory effect of ephrin-A5-Fc on BDNF modulation, while the postsynaptic EphA receptors are mediating direct excitatory effect of ephrin-A5-Fc on synaptic activity.

constructs linked with GFP (generous gifts from Dr. Renping Zhou) were used to transfect dissociated hippocampal neurons on DIV 5. It has been shown that the EphA5(K-) over-expression inhibited both the endogenous EphA5 and EphA3 activation (Yue et al., 2002), and EphA3(K-) over-expression will have the similar inhibition on both EphA5 and EphA3 signaling due to the promiscuous binding between ephrin-A and EphA. So, transfection with either of these two constructs of EphA5(K-) and EphA3(K-) might interfere with the activation of multiple members of the endogenous EphA family. A construct containing only EGFP was used to transfect neurons as the control group. Synaptic charge recordings were done on the transfected pyramidal neurons at DIV 14 to match the previous recordings. The recording and drug application strategy was the same as in the previous experiment (Section 4.1).

To address the consequence of the kinase dominant negative I recorded from both transfected cells (Figure 4.7c) and untransfected cells found in the vicinity of transfected cells (Figure 4.7a). The untransfected cell close to transfected cells still showed increased in activity in response to ephrin-A5-Fc application (Figure 4.7b), which was expected since it still had the normal EphA receptors that are responsible for the postsynaptic action of ephrin-A5-Fc application. However, in this configuration of recording, ephrin-A5-Fc pre-application did not inhibit the effects of subsequent BDNF application, as shown in figure 4.7b. This is in keeping with our hypothesis in that the recorded cell very likely received synapse from the nearby EphA(K-) transfected cells, which means that its presynaptic EphA signaling was reduced by the EphA(K-)

overexpression. This is entirely consistent with the idea that the inhibitory effect of ephrin-A5-Fc on BDNF action is mediated by presynaptic EphA.

For the opposite configuration of experiments as shown in Figure 4.7c where the EphA(K-) transfected cell with no EphA(K-) nearby was recorded, neither ephrin-A5-Fc nor the subsequent BDNF produced an increase in activity (Figure 4.7d). In other words, the direct excitatory effect of ephrin-A5-Fc was inhibited, but its inhibition on the BDNF modulation was not prevented in the EphA signaling deficient cell itself since its presynaptic cells still have the normal EphA receptors that can mediate the presynaptic inhibition of ephrin-A5-Fc on the BDNF effect.

These results strongly suggested that EphA receptors are involved in the electrophysiological effect of ephrin-A5-Fc on synaptic activity. Specifically, the presynaptic EphA receptors are responsible for the inhibitory effect of ephrin-A5-Fc on BDNF modulation, while the postsynaptic EphA receptors are mediating direct excitatory effect of ephrin-A5-Fc on synaptic activity. Due to the reported promiscuous binding between EphA and ephrin-A, the question of any specific Eph receptor mediating any particular effect is left open for now, and will be investigated in the future.

4.7 A Proposed Model to Link the Electrophysiology Effect of ephrin/EphA with Synapse Formation

At first glance, the results shown in the previous section about the presynaptic inhibition by ephrin-A5-Fc and the potential postsynaptic inhibition by EphA5-Fc on BDNF modulation on synaptic transmission would predict the presence of presynaptic EphA receptors (to mediate the presynaptic inhibitory effect of ephrin-A5-Fc on the BDNF-induced increase in mEPSC frequency) and the postsynaptic presence of existence of ephrin-A (to mediate the potential postsynaptic inhibition of the response to BDNF by EphA5-Fc), as shown by the model in the left panel of Figure 4.8. As I showed earlier, however, in figure 3.4 in Chapter 3, this is not the predominant pattern. While there is clearly the presence of presynaptic EphAs ligands labeled by ephrin-A5-Fc (colocalized with presynaptic marker synaptophysin) and postsynaptic ephrin-As receptors labeled by EphA5-Fc (opposite to synaptophysin, and colocalized with MAP2), this is not the primary distribution, at least in more mature cultures.

So why is the opposite distribution pattern (presynaptic ephrin-As and postsynaptic EphAs) predominant in the neuronal culture after two weeks in vitro? Regarding synapse formation, the pattern that supports ephrin-A/EphA inhibition of BDNF actions in synaptic transmission, however, might not be preferred in vivo since this will maximize inhibition of the synapse promoting actions of BDNF and may prevent formation of synaptic connections. Instead, the opposite distribution pattern might be expected to optimize the influences on synapse formation (Figure 4.8, right). As a consequence, the "incorrect" potential synapses that possess the presynaptic EphA5 and postsynaptic ephrin-A5 pattern, such as synapses formed by CA1 projecting to CA3, might be eliminated during development. On the other hand, the synapse candidates

with presynaptic ephrin-A5 and postsynaptic EphA5 pattern will be supported by BDNF modulation, allowing this pattern of distribution to be selected as the predominant global localization as hippocampal development proceeds.

To summarize, this chapter presents evidence that both ephrin-A5-Fc activated EphA signaling and EphA5-Fc activated ephrin-A signaling pathway have the excitatory effects on synaptic activity, which might work through NMDA receptors postsynatically. More interestingly, ephrin-A5-Fc prevents the BDNF modulation of presynaptic transmitter release, and EphA5-Fc might inhibit the postsynaptic action of BDNF on synaptic transmission, while BDNF does not inhibit the excitatory effect of either ephrin-A5-Fc or EphA5-Fc. This pattern of ephrin-A/EphA – BDNF interaction suggests that ephrin-A/EphA signaling has a higher priority than BDNF signaling. The positive effect of BDNF and the inhibitory effect of ephrin-A/EphA signaling on BDNF modulation might be critical for synaptic network refinement by promoting formation of the correct synapses and pruning incorrect synapses during hippocampal development. In order to explore this issue, the next chapter will provide evidence that ephrin-Eph signaling and its interaction with BDNF has a mechanistic role in functional synapse formation in the hippocampus.

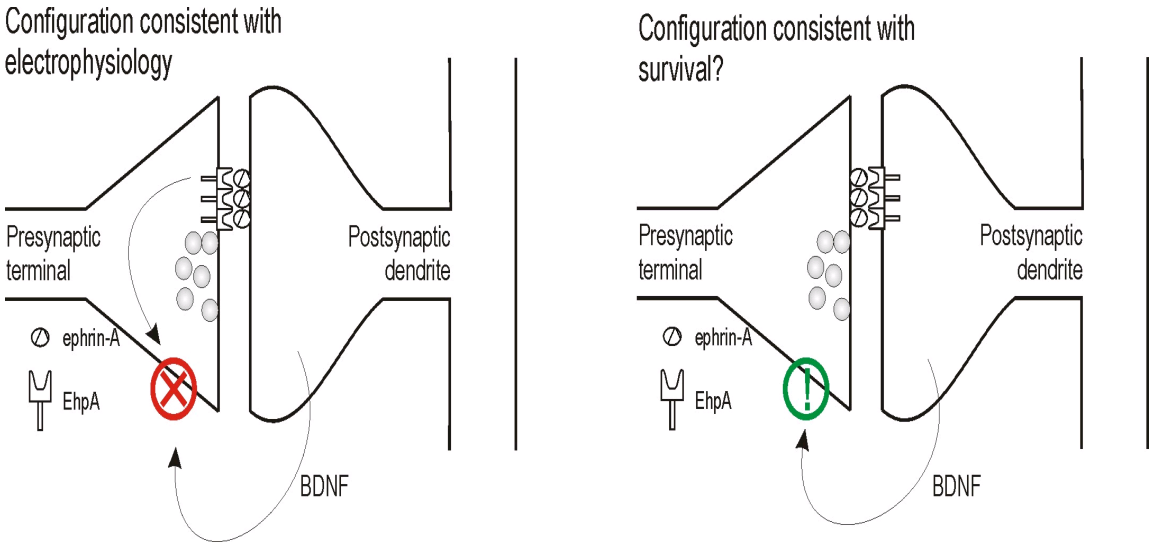

Figure 4.8 A model of how the presynaptic/postsynaptic orientation of ephrin-A ligands and EphA receptors could have opposite effects on synapse formation

Models for two patterns of synaptic distribution of ephrin-A ligands and EphA receptors are illustrated in this figure. **Left panel**: The configuration of the presynaptic presence of EphA receptors (to mediate the presynaptic ephrin-A5-Fc inhibition of the action of BDNF on mEPSC frequency) and the postsynaptic presence of existence of ephrin-A (to mediate the potential postsynaptic inhibition of the response to BDNF by EphA5-Fc) are essential to support the electrophysiological effects that I observed, which would be predicted to be detrimental to synapse survival. **Right panel**: The opposite configuration of presynaptic ephrin-A ligands and postsynaptic EphA receptors would leave the synaptic actions of BDNF intact and therefore could promote the actions of BDNF and thus optimize the influences on synapse formation.

CHAPTER 5

EPHRIN/EPH – BDNF INTERACTIONS AND SELECTIVE HIPPOCAMPAL SYNAPSE FORMATION

This chapter focuses on the involvement of ephrin-A/EphA – BDNF interaction in hippocampal synapse formation, since it has been strongly suggested by the results from the previous chapters. The ephrin/Eph – BDNF signaling balance was manipulated by EphA(K-)-GFP construct overexpression and a function blocking anti-BDNF antibody, and the potential effects on the selectivity of synapse formation in the hippocampus were studied.

5.1 EphA5 and EphA3 Signaling Contributes to the Cell Type Selectivity of Hippocampal Synapse Formation

Since all the evidence so far strongly supports the hypothesis that ephrin-A/EphA signaling is critical for the development of the selectivity of CA3 → CA1 synaptic connection in the hippocampus, a "loss of function" experiment would be very important to test this a more direct way – functional synapse analysis by paired recordings.

In order to interfere with the endogenous EphA function, the truncated form (kinase domain negative) of EphA5 or EphA3 constructs were transfected into hippocampal neuron cultures at DIV5 and recorded around DIV14. EGFP was used as a control. Paired recordings were done on cells pairs with at least one neuron transfected. All the cell pairs recorded were categorized and counted. It was found that in either EphA5(k-) (Figure 5.1b) or EphA3(k-) (Figure 5.1c) transfected cultures, the chance to get a bi-directional excitatory (E---E) connection between two pyramidal neurons was dramitically higher than that in the control culture (Figure 5.1a), while both the E---0 connections and the pairs without synapses (0---0) formed were much less than in the EGFP control culture (Figure 5.1). In general, through the comparison between Figure 5.1a with Figure 5.1d and the quantification data in Fiture 5.1f, we see that interrupting the endogenous EphA signaling decreased the specificity in hippocampal synapse formation, resulting in the much increased E---E cell pairs and less E---0 connections.

This result supports the idea that EphA5 signaling contributes to the selectivity of synapse formation in hippocampal circuitry by removing "incorrect" synapses. But, is this synapse refinement function of EphA signaling related to the ephrin/Eph– BDNF interaction? The following section will try to answer this question.

5.2 The EphA Modulation of the Selective Hippocampal Synapse Formation Depends upon BDNF signaling

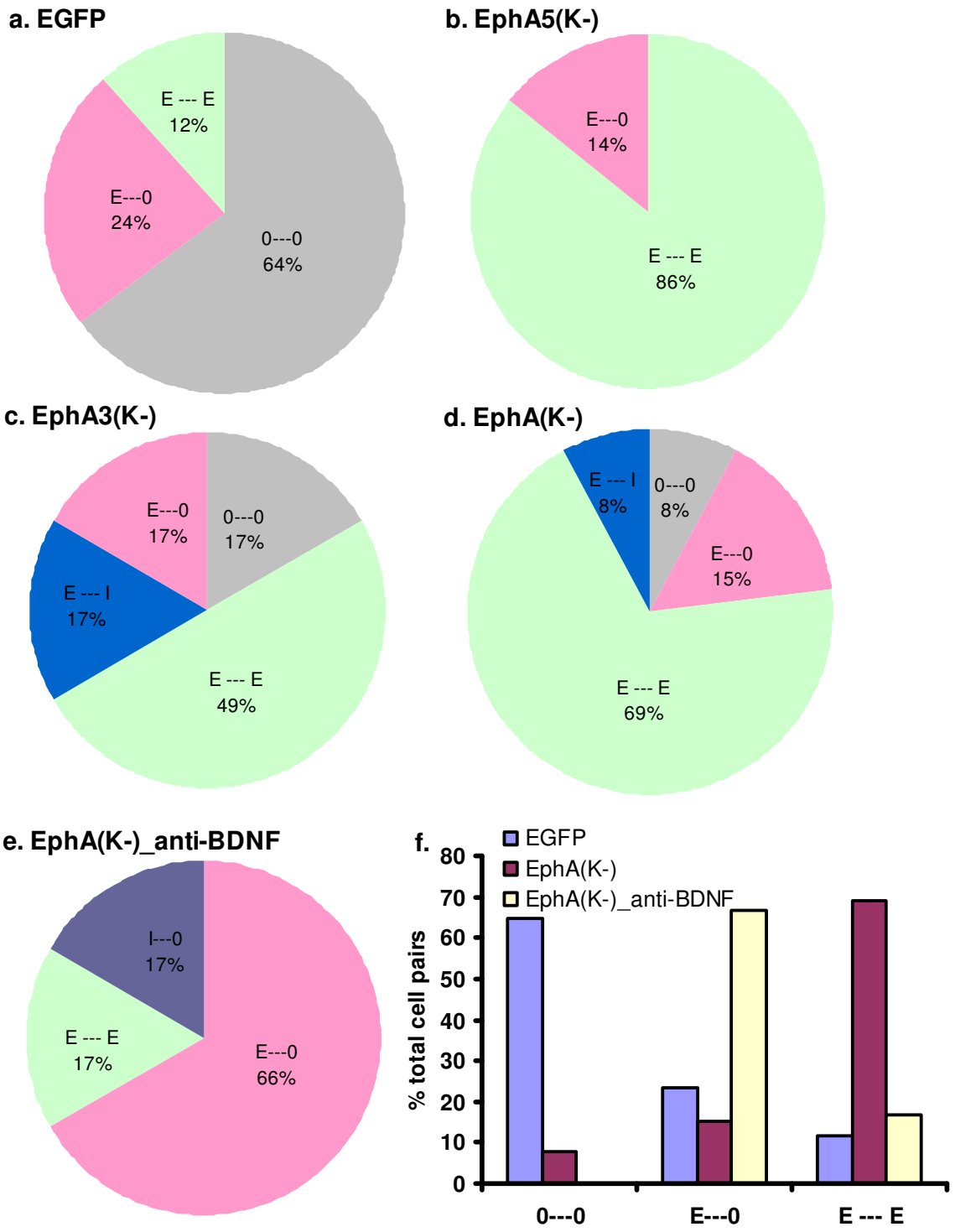
a. EGFP
E --- E
12%
E---0
24%
0---0
64%
b. EphA5(K-)
E---0
14%
E --- E
86%
c. EphA3(K-)
E---0
17%
0---0
17%
E --- I
17%
E --- E
49%
d. EphA(K-)
E --- I
8%
0---0
8%
E---0
15%
E --- E
69%
e. EphA(K-)_anti-BDNF
I---0
17%
E --- E
17%
E---0
66%
f.
EGFP
EphA(K-)
EphA(K-)_anti-BDNF
% total cell pairs
80
70
60
50
40
30
20
10
0
0---0
E---0
E --- E

Figure 5.1 Kinase domain negative EphA5 and EphA3 overexpression affect the synaptic connection selectivity in a TrkB signaling related manner

The EphA5(K-)-GFP, EphA3(K-)-GFP, or EGFP were transfected into hippocampal neuron cultures at DIV5 and recorded at DIV14. The percentages of every type of synaptic connection of the total recorded cell pairs were plotted as shown in the pie graphs. **a**. Control cultures with EGFP transfection (N = 17). **b**. Cultures with EphA5(k-) overexpresstion (N = 7). **c**. Cultures with EphA3(k-) transfection (N = 6). Note that in either EphA5(k-) (b) or EphA3(k-) (c) transfected cultures, the chance to get a bi-directional excitatory (E---E) connection between two pyramidal neurons was dramatically higher than in the control cultures (a), while both unidirectional excitatory synaptic (e---0) connections and pairs without synapses formed (0---0) less frequently than in the EGFP control cultures, with even a complete absence of no synapse formed pairs in EphA5(k-) transfected cells (b). **d**. Cultures with EphA signaling interfered by either EphA5(k-) (c) or EphA3(k-) (d) (N = 13). This is the sum of b and c. **e**. The functional blocking antibody to BDNF was applied to the culture right after transfection of EphA5(k-) or EphA3(k-) and at 4 day intervals thereafter (N = 6). Note the decreased frequency of E---E connections and the increased frequency of E---0 connections. **f**. Quantification of the rate of 0---0, E---0 and E---E types of cell pairs for the groups of EGFP control (blue bar), EphA(K-) (purple bar) and EphA(K-)_anti-BDNF (yellow bar). Note that anti-BDNF treatment reversed the effects of EphA(K-) on the ratio of E---0 and E---E synapses.

To examine the involvement of BDNF signaling in the synaptic pruning process, the function blocking anti-BDNF antibody (Promega) was added to the hippocampal neuron cultures at a concentration of 4 μg/ml right after transfection of EphA5(K-)-GFP and EphA3(K-)-GFP to block the BDNF function by scavenging the endogenous BDNF (Magby et al., 2006, Flores-otero et al., 2007). To avoid being depleted during this time, anti-BDNF was replenished at 4 day interval.

Paired-cell recording and data analysis were done in the same way as in the previous section. The major finding of this experiment was that the number of bi-directional excitatory connections was much lower than in the EphA(K-) transfected groups, and close to the EGFP control (Figure 5.1e, f). On the other hand, the percentage of unidirectionally connected cell pairs was greatly increased compared to the EphA(K-) transfection group. Note that the N is small for this experiment, only 6 pairs of cells were recorded, so this result might need to be consolidated in the future. It is thus suggested that reduction of EphA signaling with the EphA(K-)-GFP construct resulted in an increased number of bi-directional synaptic connections because of unregulated promotion of synaptic connectivity by BDNF.

In summary, this chapter shows that interruption of endogenous EphA signaling resulted in increased functional synapse formation and apparently decreased connection specificity in embryonic hippocampal neurons, which could be partially reversed by blocking BDNF-TrkB signaling. This supports our hypothesis that the inhibitory effect of ephrin-A/EphA on BDNF signaling plays a critical role in refining hippocampal synapse formation possibly by eliminating the potential “incorrect” synapses. So, the

dis-inhibition of BDNF signaling by kinase negative EphA5 or EphA3 overexpression caused a loss of the synaptic projection selectivity in the hippocampus.

CHAPTER 6

DISCUSSION

The major finding of this thesis is that there is a cross-talk between BDNF and ephrin-A/EphA signaling pathways, and that this interaction playa an important role in both hippocampal synaptic transmission and synapse formation. Interruption of EphA signaling greatly decreased the selectivity of synapse formation onto CA1 pyramidal neurons in the hippocampus, which is rescued by the functional blocking of BDNF signaling by the anti-BDNF antibody. All of these suggest that ephrin-A/EphA signaling contributes to the selective Schaffer collateral synapse formation by its inhibitory effect on the positive modulation of BDNF in synapse development and maturation. At present I cannot discriminate between possible of DG → CA1 synapses and CA3 → CA1 synapses since I have not yet found a good CA3 marker to identify the presynaptic cell as CA3 pyramidal cell. Although this ambiguity must be addressed in future work, it does not compromise the conclusions of this study because one of the key findings in this thesis is that CA1 pyramidal cells do not project back to their presynaptic neurons. Nonetheless, my data is consistent with the well known repellent role of ephrin/Eph guidance in topographic mapping in a variety of systems such as the visual system, the olfactory system and motor neuron innervation (Cutforth et al., 2003; Dufour et al., 2003; Eberhart et al., 2004; Dickson, 2002; Rasband et al., 2003; Nakagawa et al., 2000).

6.1 The Unidirectional CA3 → CA1 Projection in the Hippocampus

The hippocampus is comprised of a predominantly unidirectional tri-synaptic circuit, a pathway that relays information from the perforant path to the dentate gyrus, dentate to area CA3 via mossy fibers, and CA3 to area CA1 via the Schaffer axon collaterals. It has been proposed that hippocampal information processing occurs along the tri-synaptic circuit, making this specific projection orientation crucial for various types of information processing (Goodrich-Hunsaker et al., 2008; Kesner, 2007; Bernard and Wheal, 1994; Andersen et al., 1971). Our study has focused on the unidirectional projection from CA3 pyramidal cells to CA1 pyramidal cells, an intensely investigated synaptic connection most well know for NMDA receptor dependent LTP (Huang et al., 2005; Malenka and Bear, 2004). The data presented in this thesis will contribute to the understanding of how the hippocampus develops into such a unique and interesting brain region.

6.2 Preservation of the Unidirectional Synaptic Connections in Dissociated Cultures of Embryonic Hippocampal Neurons

To better understand the fundamental question addressed in this thesis, my study focused on functional synaptic connections at the level of single cell-pairs. I first

established an in vitro model for studying selectivity of connectivity onto CA1 neurons. By combining paired-cell recording with immunocytochemical identification of CA1 pyramidal cell with anti-SCIP, I found that proximity is definitely not the most important determinant of whether a synapse will be formed because cells that did not receive synaptic input from nearby cells still receive remote input, and the unidirectional projection from CA3 (or potentially DG) neurons to CA1 pyramidal cells still exists in the dissociated hippocampal cultures. This finding suggested that there must be some endogenous mechanisms that determine the destination of the neuronal axon terminal and the fate of the potential synapse, and these endogenous factors are preserved even after being dissociated and cultured in vitro.

Using this model is what enabled my study to be successful. Although the hippocampal slice preparation might preserve the endogenous synaptic circuit better and represent a more physiological condition, it greatly limits the ways in which hypotheses can be tested. Cultured neurons that reproduce the unidirectional CA3 → CA1 projection are significantly easier to manipulate with pharmacological, electrophysiological and molecular biological tools, and also provide reproducibility and comparability due to the large number of plates that can be obtained from the same animal dissection.

The hippocampal culture preparation is not unique for preserving selective synapse formation in vitro. For example, it has been reported that the cultured dissociated *Aplysia californica* neurons form electrical synapses in a selective way: pairs of neurons derived from the same ganglion, or homoganglionic pairs, form electrical

connections with high coupling coefficients in culture; by contrast, pairs of neurons from different ganglia, or heteroganglionic pairs, form electrical connections with lower coupling coefficients (Carrow et al., 1989; Hawver et al., 1993). Further more, this specific manner can be manipulated by some molecules such as a lectin, Con A (Lin et al., 1987). Hawver's work on promoting (faciculation) and inhibiting factors (avoidance) influencing *Aplysia* neurons' target selecting decisions showed that *Aplysia* L10 neurons selectively formed chemical connections with RB neurons rather than RUQ neuron targets whereas R2 neurons preferred RUQ neuron as the reliable synaptic targets. In contrast, L10 usually failed to form detectable chemical connections with RUQ targets in co-cultures (Hawver et al., 1993). Selective fasciculation and avoidance was then suggested to aid in target selection by regulating the amount of contact between presynaptic processes and potential target cells (Hawver et al., 1993). In my study, BDNF and ephrin/Eph were suggested to be the promoting and inhibiting factors respectively affecting the synaptic connection decisions by the hippocampal neurons.

6.3 Facilitative Factors in Synapse Formation

During the development of the nervous system, many factors have been identified that promote synapse formation. There are many trans-synaptic adhesion molecules including cadherins, integrins, NCAM, nectins, neuoligins, SynCAMs, and SALMs which are attractive candidates for regulation of synapse formation by initiating bi-directional signaling simultaneously, and which are also involved in the maintenance of

synapses and dendritic spines, or even synaptic plasticity (Kimberley Mcallister, 2007; Akins and Biederer 2006; Scheiffele, 2003; Waites et al., 2005, Li and Sheng, 2003). Besides the trans-synaptic molecules, there are also many secreted molecules that promote synapse formation, such as glutamate, neurotrophins, Wnt-7a, Wnt-3, FGF, and TGF (Kimberley Mcallister, 2007; Hall et al., 2000; Krylova et al., 2002; Umemori et al., 2004; Marques et al., 2002).

Among these, the neurotrophin BDNF probably has been the mostly thoroughly described with regard to its role in central nervous system (CNS) synapse formation. BDNF treatment can increase the number of functional synapses, including both excitatory and inhibitory synapses, whereas NT-3 induced formation of only excitatory synapse (Vicario-Abejón, et al., 1998; Vicario-Abejón, et al., 2003). Using conditional knockout mice with a deleted TrkB gene in pre- and postsynaptic cells at different developmental stages, it has been demonstrated that TrkB-BDNF signaling is required for Schaffer collateral synapse development at both presynaptic and postsynaptic sites (Luikart et al., 2005). Presynaptically, BDNF-TrkB signaling can facilitate synaptic vesicle docking at the active zone and thus promote synapse formation (Pozzo-Miller et al., 1999), and increase presynaptic transmitter release (Magby et al., 2006). Postsynaptically, BDNF-TrkB can regulate the surface expression of AMPA and GABA receptors probably through post-transcriptional and post-translational mechanisms (Narisawa-Saito et al., 1999; Narisawa-Saito et al., 2002; Vicario-abejon et al., 2003), induce rapid surface translocation of the GluR2 subunit, and promote NMDA receptor function and GABA receptor maturation (Yamada et al., 2002; Elmariah et al., 2004).

6.4 Potential Inhibitory Factors in Synapse Formation

To form an efficiently functional neuronal network, it is not enough to just have factors that promote synapse formation. Factors that limit synapse formation are also essential for the development of proper, refined and specific neuronal circuitry. There are a large number of molecules that can decrease synapse number such as Myocyte enhancer factor 2 (MEF2) families (Flavell et al., 2006), major histocompatibility complex I molecules (MCH I) (Wampler and McAllister, 2005), ephrin-A and ephrin-B families (Brownlee et al., 2000; Murai et al., 2003; Hoogenraad et al., 2005).

What's interesting about ephrin families is that they are not just simple repulsive guidance molecules as they are generally regarded. Both ephrin-A and ephrin-B members can have branch-promoting activity in addition to the process collapsing effects during neuronal development (Mann et al., 2002; Zhou et al., 2001; Gao et al., 1999). Regarding synapse formation and maturation, the EphA and EphB family signaling have also both been implicated as a positive signal in spine formation, synapse development and neural connectivity of hippocampus (Palmer and Klein, 2003; Martinez et al., 2005; Otal et al., 2006) in addition to their down regulation of synaptogenesis.

The integration of these various promoting factors and inhibitory factors for synapse development will help to form the final functional neuronal network. This

thesis particularly focused on the ephrin – BDNF interaction in the CA3 → CA1 synapse selection process during development.

6.5 The Direct Excitatory Effect of Ephrin-A5-Fc and EphA5-Fc on Synaptic Activity

In this project, the experiment to check whether ephrin-A/EphA signaling is involved in hippocampal synaptic transmission examined the electrophysiological effect of acutely applied ephrin-A5-Fc or EphA5-Fc on synaptic activity in hippocampal neuron culture. The exogenous ephrin-A5-Fc or EphA5-Fc has been indicated to activate the endogenous EphA or ephrin-A signalings since they are of the dimerized form (Davy et al., 1999; Gerlai et al., 1999; Bluckner et al., 1997; Zamora et al., 2005). It was shown that both ephrin-A5-Fc and EphA5-Fc application induced a rapid (1 – 2 minutes right after application) and transient (lasting 4 – 5 minutes) increase in synaptic activity. We do not know the exact meaning of the activity, but it represents the ongoing neuronal activity due to the intrinsic neuronal transmitter transmission release between synapses, which has been indicated to be important for neuronal synaptogenic activity.

It has been known that synapse formation, selection, maturation and maintenance involve numerous activity-dependent processes (Nelson, et al., 1990; Tarsa and Goda, 2002; Constantine-Paton and Cline, 1998; Harms et al., 2005; Herrera and Zeng, 2003; Barber and Lichtman, 1999; Buffelli et al., 2002), such as postsynaptic neurotransmitter

receptor clustering and relocation. Nascent synapses can be eliminated if there is insufficient activity to consolidate the synapse. So, the excitatory action of ephrin-A5 and EphA5 singling activation are strongly implicated in some stage of the modulation in synaptic development.

6.6 Interaction between Ephrin-A/EphA and BDNF Signaling in Synaptic Transmission in Dissociated Hippocampal Neuron Culture

Since we are interested in studying the interaction between ephrin-A/EphA signaling and BDNF signaling in synapse formation, and it was shown in this thesis that both BDNF and ephrin-A/EphA signaling are involved in modulation of synaptic transmission, it became very promising to study the electrophysiological consequences of ephrin-A/EphA – BDNF interaction.

What I discovered in this study is that pre-exposure of hippocampal neurons to ephrin-A5-Fc prevented the excitatory effect of subsequent BDNF application on synaptic activity, and the pre-exposure to EphA5-Fc inhibited the late phase of BDNF modulation without interfering with the early phase of BDNF modulation. Conversely, BDNF has little if any effect on subsequently applied ephrin-A5-Fc or EphA5-Fc. This result indicated that different factors that function during synapse development could be categorized hierarchically, with the contact-mediated signaling (ephrin-A/EphA in

this case) taking precedence over the diffusion-mediated effect (BDNF release induced modulation).

BDNF has been demonstrated to have antagonistic relationships with other factors in other studies. In the auditory system, BDNF and NT-3 elicit opposite effects on neuronal phenotype (Adamson et al., 2002) and it has been shown that chronic treatment with NT-3 can prevent the presynaptic effects of BDNF (Paul et al., 2001). One important special point about our results, however, is that the inhibitory effects of ephrin-A5-Fc and EphA5-Fc application on BDNF signaling modulation we observed are non-reciprocal. It is predicted that, in this case at least, contact-mediated interactions can overrule diffusion-mediated ones. Although the activity-dependent release of BDNF provides some spatial specificity, it can not reach the precision of cell-to-cell contact-mediated interaction. Such an attraction followed by pruning model has already been suggested (Gao et al., 1999); this could provide an important mechanism which limits the action of released BDNF to appropriate targets.

6.7 Mechanisms of the Ephrin-A/EphA - BDNF Interaction in Hippocampal Synaptic Transmisson

The next question that I addressed was how ephrin-A/EphA signaling interacts with BDNF/TrkB function. Previous work from our laboratory and others has shown that BDNF acts both presynaptically and postsynaptically (Lessmann, 1998; Lu and

Gottschalk, 2000; Magby et al., 2006) with the postsynaptic actions involving a variety of ion channels and neurotransmitter receptors (Rose et al., 2004) including NMDA receptors (Jarvis et al., 1997; Song et al., 1998; Lin et al., 1998; Levine et al., 1998; Lin et al., 1999; Crozier et al., 1999; Levine & Kolb, 2000; Arvanian & Mendell, 2001; Slack et al., 2004; Kolb et al., 2005). Even more, the pre- and postsynaptic actions of BDNF can occur independently and can be studied in isolation (Alder et al., 2005).

Since ephrin-A5-Fc and EphA5-Fc have different representations of the inhibitory effect on the BDNF modulation, we predict that they interfere with the BDNF signaling at different synaptic loci. In order to better understand the inhibitory mechanisms, we dissected out the presynaptic mechanisms out by isolating spontaneous mEPSCs in hippocampal neurons and examining the frequency of mEPSCs as an indicator of presynaptic release probability (Bouron, 2001). The results showed that application of ephrin-A5-Fc itself did not change mEPSC frequency, but prevented the effect of subsequently applied BDNF on presynaptic release probability, indicating that ephrin-A5-Fc is working presynaptically to prevent the BDNF-induced upregulation of synaptic activity. On the other hand, application of EphA5-Fc by itself did not have effect on mEPSC frequency either, but the neuronal response to the subsequently applied BDNF was normal, suggesting that EphA5-Fc is acting postsynaptically to interfere with BDNF modulation. It has been reported that application of EphA5-IgG to hippocampal slices could impair LTP induction without affecting baseline transmission (Gao et al., 1998), which is consistent with our finding that EphA5-Fc inhibits the postsynaptic effects of BDNF. It was also suggested that perfusion with ephrin-A5–IgG induced a sustained

increase in normal synaptic transmission via presynaptic mechanisms. It is interesting that the presynaptic/postsynaptic relationship for ephrin-A5 and EphA5 effect in electrophysiology in our study is the same as in Gao's study (Gao et al., 1998).

As to the molecular mechanisms, the inhibitory effect of ephrin-A5 on BDNF-induced neural activity is predicted to be mediated by the down-regulation of MAP kinase activity. This prediction is supported by the biochemical data showing that BDNF-induced phophorylation of MAPK was reduced by ephrin-A5 (unpublished data by Yue and Zhou). In the presence of ephrin-A5, BDNF failed to induce the rise of Erk/MAP kinase activity. Ephrins have been shown to inhibit Erk activation by a number of extracellular ligands including platelet-derived growth factor, epidermal growth factor, and glutamate (Elowe et al., 2001; Miao et al., 2001; Grunwald et al., 2001). There is evidence that the inhibition is mediated by RasGAP (Elowe et al., 2001). RasGAP is physically associated with Eph receptors, and a dominant-negative mutant of RasGAP prevented MAP kinase down regulation by ephrin-B2 (Elowe et al., 2001). How signals from Trk and Eph receptors are integrated is not known. One candidate, the ankyrin repeat-rich membrane spanning protein (ARMS), could be a convergence point for BDNF and ephrin/Eph pathways. It has been shown that ARMS can bind to both TrkB and ephrin/Eph signaling (Kong et al., 2001; Luo et al., 2005), and may be a key to signal integration between different receptors. Altogether, these observations suggest that Eph receptors converge onto the Erk/MAP kianse pathway to modulate Trk receptor activity.

6.8 Involvement of EphA Receptors in the Electrophysiological Effect of Ephrin-A5-Fc

So far, we showed that ephrin-A/EphA activation had dual actions of both excitation and inhibition. The promiscuous binding of ephrins ligands and their Eph receptors makes it uncertain; however, which ligand/receptor combination responsible for the inhibition of the BDNF effect is also responsible for the enhancement of synaptic activity. We predict that the exogenous ephrin-A5-Fc induced effects may be mediated by different binding partners and synaptic effectors and through different downstream signaling mechanisms.

We began with EphA5 and EphA3 examination since they have been reported to be the high affinity receptors for ephrin-A5 (Himanen et al., 2004). Based on our experiments using kinase negative constructs for EphA3 and EphA5 to interfere with the endogenous EphA3 or EphA5 signaling, it is suggested that both EphA3 and EphA5 are involved in the ephrin-A5-Fc induced excitatory effect on synaptic activity. Note that we can not exclude the possibility of other Eph receptors to be involved in this modulation since other Eph members might also be affected by these kinase negative EphA constructs with low effectivity due to the promiscuous binding between ephrin-A and EphA. The specific test of individual EphA type will be the future direction for this research.

6.9 Involvement of EphA Receptors in the Unidirectional Synapse Connections

It is widely accepted that the regulation of synapse formation is activity-dependent and that the stimulating factors in synaptic plasticity can also induce changes in synaptic morphology (Marty et al., 2000; Frost, 2001; Poo, 2001; Zhou et al., 2004). Based on the already characterized neuronal function of ephrin/Eph, it is quite possible that ephrin is also involved in the activity-dependent formation of neuronal networks. So, the kinase negative constructs for EphA3 and EphA5 were also used to test the potential involvement of EphA signaling in the specificity of hippocampal CA3 → CA1 synapse formation.

Our results showed that both EphA5(K-)-GFP and EphA3(K-)-GFP over-expression in hippocampal neurons produced a much lower rate of unidirectional synaptic connectivity and a much higher rate of bi-directional synaptic connections compared to the GFP over-expression control, which is suggestive of a dramatic loss of the unique CA3 → CA1 synaptic projection identified by the paired recordings and immunocytochemistry. This is quite consistent with the hypothesis above about EphA involvement in the selective formation of the intra-hippocampal neuronal connections.

6.10 Possible Mechanisms for EphA Modulation in Selective Synapse Formation: Ephrin-A/EphA – BDNF Interaction

Although EphA involvement in shaping the intra-hippocampal synaptic projections has been suggested above, a more important issue will be how this is accomplished. Since ephrin-A/EphA signaling have excitatory effects on synaptic activity and inhibitory effects on the synaptic modulation of BDNF, we predict that the endogenous EphA signaling might contribute to the CA3 → CA1 synapse specificity by eliminating the "incorrect" potential synapses such as CA1 → CA3 connections due to the interference with synaptic effects of BDNF.

We used the function blocking antibody anti-BDNF to interfere with the endogenous BDNF signaling in cells that over-expressing the kinase negative EphA5 or EphA3. Interestingly, we found that the unidirectional synaptic connection rate between hippocampal pyramidal neurons was higher and the bi-directional rate was lower in the EphA(K-)-GFP transfected and anti-BDNF treated cells than that without anti-BDNF treatment. Although the sample number was small (N = 6), the result was very consistent and obvious. It implicates the need for BDNF promotion of synaptic connectivity in order to see the mis-targeting effects of EphA inhibition. It is thus suggested that the EphA modulation in synapse selectivity might be due to their interference interaction with BDNF signaling at certain cross points on their downstream signaling pathways.

6.11 Distribution of Ephrin-A/EphA in Hippocampal Neurons

All the modulatory effects of ephrin-A5-Fc and EphA5-Fc application should be supported by the corresponding presence of appropriate Eph receptors or ephrin ligands. So, the immunocytochemical experiments were done to localize the receptors or ligands for ephrin-A5-Fc and EphA5-Fc. Although the presynaptic Eph receptors or ephrin-A5-Fc and postsynaptic ephrin ligands for Eph-A5-Fc were shown to be present to mediate the presynaptic inhibitory effect of ephrin-A5-Fc and postsynaptic effect of ephrin-A5-Fc, the predominant localization pattern was presynaptic ephrin ligands for Eph-A5-Fc and postsynaptic Eph receptors for ephrin-A5-Fc.

Combining the immunocytochemical results with the electrophysiological effects of ephrin-A/EphA signaling and BDNF signaling, a model we propose is that BDNF provides a general beneficial and activity-dependent influence on neurite outgrowth and synapse development. Presynaptic EphA or postsynaptic ephrin-A initiated signaling can disrupt the action of BDNF while postsynaptic EphA or presynaptic ephrin-A mediated signaling doesn't inhibit BDNF modulation significantly. Therefore, when cell pairs with EphA expressed presynaptically and ephrin-A expressed postsynaptically make contact, they may become non-responsive to the synapse promoting effects of BDNF and thus experience a repellent effect instead. Conversely, synaptic connections would be able to be established between cells with presynaptic ephrin-A and postsynaptic EphA since this pattern of ephrin-A/EphA distribution doesn't override the BDNF response. This could account for the distribution pattern of predominant presynaptic ephrin-A and postsynaptic EphA protein that we observed in mature neuronal cultures with immunohistochemistry. So, at the very early stage of

hippocampal synaptic development, there should be more hippocampal neurons possessing presynaptic EphA and postsynaptic ephrin-A proteins than the mature hippocampus since the synapse selection has not been accomplished.

A more specific result about EphA5 localization is from the double labeling with the antibodies for SCIP and EphA5. It showed an excellent correlation between EphA5 expression and SCIP labeling, which implied that EphA5 is mostly expressed in CA1 pyramidal cells. This EphA5 expression gradient along the intra-hippocampal circuitry might be one of the important contributors to the connection selectivity between CA3 and CA1 hippocampal neurons.

Thus, the unidirectional CA3 to CA1 projection might be shaped by specific ephrin-A/EphA localization characteristics in hippocampus in vivo and the ephrin-A/EphA – BDNF interaction.

Interestingly, BDNF-induced modulation of synaptic transmission by EphA and ephrin-A may also be present in the adult nervous system, since EphA and ephrin-A are present postnatally as well as in the developing hippocampus (Zhou, 1998). The expression of EphA and ephrin-A in the adult brain suggests that the ephrin/Eph - BDNF interaction may persist into adulthood and have an impact on synaptic plasticity and cognitive function.

6.12 Conclusion and Future Directions

It is well known that hippocampal neuronal circuits are assembled during development between thousands of differentiating neurons. Proper synapse formation during childhood provides the substrate for cognition, whereas improper formation or function of these synapses leads to neurodevelopmental disorders, including mental retardation and autism. To understand the causes of brain disorders, it is very important to understand the mechanisms underlying synaptogenesis and cell type-specific target selection. Recent work has begun to identify some of the early cellular events in synapse formation as well as the molecular signals that initiate this process.

Based on the already characterized neuronal function of BDNF and ephrin/Eph, this study shed light on the issue about how neurotrophin and ephrin/Eph families would be integrated in synapse function and development. This thesis proposed that EphA family signaling was involved in the development of synaptic selectivity in the hippocampus, through its interaction with BDNF modulation on synapse formation.

However, it is still not clear which specific EphA member was responsible for the specific process. This will be a big question that our future work will try to answer. Since we have indicated the correlation between EphA5 (but not EphA3) and the CA1 pyramidal cells (Figure 3.6, 3.7), EphA5 is more likely to be the contributor than EphA3. One thing that might be helpful is to study extensively the distribution of ephrin-A5 and other ephrin-A ligands or EphA receptors within the hippocampus. There also might be a compensatory expression of another ligand, such as ephrin-A5, in a different region like CA3. Once we filter out some candidates, we can do the further study to specify the target. ShRNA to each specific candidacy (for example, EphA5) will be used to

specifically knock down the endogenous signaling, and the consequences for connection selectivity in the hippocampus will be analyzed.

In addition, a good CA3 marker will also be employed in order to critically conclude about the involvement of the interaction between ephrin/Eph signaling and BDNF-TrkB signaling in the CA3 → CA1 projection specificity. With both the CA1 and CA3 pyramidal cell markers in hand, it will be very helpful to identify wheether the excess bi-directional connections in the EphA(K-) overexpressed culture are "mistakes" in that CA1 neurons are frequently the presynaptic neuron whereas the CA3 pyramidal cells are the postsynaptic neurons.

The development of hippocampal circuitry is an extremely complex process, involving numerous factors. The results presented in this thesis, complemented with the future study, will contribute by providing a valuable mechanism for hippocampal synapse development and function through balancing opposing influences of various modulating factors at specific developmental phases.

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