INVESTIGATIONS ON THE ROLES OF CLASS 3 SEMAPHORINS IN THE ADULT HIPPOCAMPUS

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(M.Sc.)

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILSOPHY

DEPARTMENT OF PHYSIOLOGY

YONG LOO LIN SCHOOL OF MEDICINE

NATIONAL UNIVERSITY OF SINGAPORE

2014

Declaration

I hereby declare that the thesis is my original work and it has been written by me in its entirety.

I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Acknowledgements

I am indebted to my supervisor, Dr Eyleen Goh, for giving me the opportunity to work on this project, her generosity in allowing me to pursue a PhD on a part-time basis while working as a research associate at Duke-NUS Graduate Medical School, her time and effort invested in my graduate education and for providing invaluable insight and guidance for this project.

I am also grateful to Dr Ryu Jae Ryun, whom I collaborate closely, for performing part of the *in vitro* studies and for engaging discussions on the project. In addition, the members of the EG laboratory at Duke-NUS, especially Mr Terence Tan and Miss Rachel Choi, have rendered excellent technical assistance. I would like to acknowledge Mr Benjamin Chew, Dr Mattu Chetana Shivaraj and Dr Wong Peiyan of Duke-NUS and Dr Zoe Bichler of National Neuroscience Institute, Singapore for suggesting, performing and analyzing some of the behavioral experiments, Mr Guillaume Thierry Marcy for demonstrating adult neural precursor cell culture, and Drs Colin Su, Rye Jae Ryun and Lee Yie Hou for reading and commenting on this thesis.

I thank Drs David Ginty and Alex Kolodkin for sharing their NRP1 conditional knockout mice, and Sema3A-AP, Sema3F-AP and AP constructs.

I would also like to thank the members of my Thesis Advisory Committee, Drs Lim Kah Leong and Antonius VanDongen, for their time and commitment for the period of my PhD candidature.

Lastly, I am grateful for the love, encouragement and emotional support from my family and close friends throughout my life.

Table of Contents

Chapter 6

NRP2 specifically regulates cell-positioning of adult-born neurons via GSK3β

Chapter 7

NRP2 affect adult hippocampal functions

Chapter 8

Overall discussion and conclusion

Summary

Class 3 Semaphorins are important guidance cues for the development of the embryonic nervous system. However, the roles of Semaphorins in the adult nervous system have not been characterized. The aim of this study is to address the roles of Class 3 Semaphorins signaling in the dentate gyrus of the adult hippocampus. The dentate gyrus is a complex and heterogeneous structure made of diverse cell types, including embryonic-born and adult-born neurons. Using retroviral and lentiviral-mediated approaches, I investigated the roles and functions of Class 3 Semaphorins and elucidated their downstream signaling pathways in the adult dentate gyrus.

To investigate if Class 3 Semaphorins play any role in the development of adult-born neurons, I utilized retroviruses to specifically knock-down Class 3 Semaphorins receptors, Neuropilin (NRP) 1 or 2, in neural precursor cells in the adult mouse dentate gyrus. Silencing of NRP1 or NRP2 in adult neural precursor cells resulted in neurons with shorter dendritic length and simpler branching *in vivo*. Mechanistically, Sema3A and 3F increased dendritic length and branching complexity of cultured hippocampal neurons *in vitro* and serine phosphorylation of Focal Adhesion Kinase (FAK) through activation of Cyclin-dependent kinase 5 (Cdk5) was essential for such effects. Silencing of either Cdk5 or FAK led to very similar phenotypes in dendritic development as silencing of NRP1 or NRP2. Furthermore, *in vivo* overexpression of Cdk5 or FAK rescued the dendritic phenotypes seen in NRP1 and NRP2 deficient neurons.

Silencing of NRP2, but not NRP1, in neural precursor cells perturbed correct cell positioning of adult-born neurons within the dentate granule layer. Interestingly, silencing Glycogen Synthase Kinase 3β (GSK3β) in adult-born neurons phenocopied the cell positioning phenotype of shNRP2 neurons and overexpression of GSK3β restored correct cell positioning

of NRP2 deficient neurons *in vivo*. Hence, NRP2 specifically mediates cell positioning of adult-born neurons via a GSK3β signaling pathway.

Next, I investigated the roles of Sema3F/NRP2 signaling in the dentate gyrus, particularly in learning and memory. Using a lentiviral-mediated approach, I knocked down NRP2 in the dentate gyrus of adult mice and tested the animals in spatial and contextual fear learning tasks. Interestingly, shNRP2 mice were normal in contextual fear acquisition but impaired in contextual fear memory, compared to shCTR mice. On the other hand, both shNRP2 and shCTR mice performed similarly in spatial learning tasks, specifically in spatial learning tasks, such as the object-location learning tasks and in the Morris Water Maze test. These data suggested that Sema3F/NRP2 signaling in the adult animal is required for contextual fear learning and not for spatial learning.

In summary, this study shows that Class 3 Semaphorins play novel roles in promoting dendritic growth via a Cdk5-FAK pathway and in regulating cell positioning of newborn neurons in the adult hippocampus via the GSK3β pathway. In addition, Sema3F/NRP2 signaling in the adult dentate gyrus is critical for contextual fear learning. This study sets the stage for future investigations on how regulation of Class 3 Semaphorins contributes to brain functions in different physiological and pathological states and could imply potential clinical applications.

Abbreviations

ANOVA	analysis of variance
AP	Alkaline phosphatase
BSA	Bovine serum albumin
CNS	Central Nervous System
Cdk5	Cyclin-dependent kinase 5
DAPI	4’6’-diamidino-2-phenylindole
DCX	doublecortin
DG	dentate gyrus
EGF	Epidermal growth factor
FAK	Focal Adhesion Kinase
FGF	Fibroblast growth factor
GCs	granular cells (of dentate gyrus)
GSK3β	Glycogen Synthase Kinase 3β
MAP2	Microtubule associated protein-2
NPCs	Neural precursor cells
NRP1	Neuropilin-1
NRP1	Neuropilin-2
PBS	Phosphate buffered saline

Prox1	Prospero-related homeobox-1
SEM	Standard error of mean
Sema3A	Semaphorin 3A
Sema3F	Semaphorin 3F
SGZ	Subgranular zone
SVZ	Subventricular zone
TBS	Tris buffered saline
Tris	tris(hydroxymethyl)-aminomethane

List of Tables

List of Figures

Chapter 1

Introduction

1.1. Adult Hippocampus and Neurogenesis

1.1.1 Structure and Functions of the adult hippocampus

The adult hippocampus is crucial for episodic, contextual and spatial memory. The profound effects of medial temporal lobe resection in a famous patient, known by his initials H.M., provide strong evidence for the role of medial temporal lobe in episodic memory (Scoville and Milner, 1957; Squire et al., 2004). Similar conclusions were reached using a non-human primate model (Mishkin, 1982). Episodic memory is highly dependent on context (Tulving, 1977). Several lines of evidence suggest that the hippocampus is required for contextual memory (Maren et al., 2013; McHugh et al., 2007). In addition, it was hypothesized that the hippocampus forms the cognitive map for spatial navigation (Best et al., 2001; Jung and McNaughton, 1993; O'Keefe and Conway, 1978; O'Keefe and Dostrovsky, 1971).

The mammalian hippocampus is comprised of four distinct areas: the entorhinal cortex (EC), the subicular complex (Sub), the hippocampus proper or the cornu ammonis (subdivided into CA1, CA2 and CA3) and the dentate gyrus (DG) (Figure 1-1). Sensory information converges on the entorhinal cortex, transverses the entire hippocampal circuit and returns back to the entorhinal cortex. The transformations that take place through this traversal are presumably important for encoding, storage and retrieval of memory (O'Keefe and Dostrovsky, 1971; Rolls, 2010; Thierry et al., 2000; Treves and Rolls, 1992).

The entorhinal cortex (EC) integrates sensory information (auditory, visual, somatosensory, visuo-spatial) from other cortical regions (Brown and Aggleton, 2001; Eichenbaum, 2000) and sends episodic snapshots of the external environment to the CA3 and dentate gyrus (DG) through the perforant pathways (PP). However, the information from the EC is inherently noisy and prone to interference. Computational modeling suggested that the DG performs the function of pattern separation (the ability of the network to reduce overlap between similar inputs to prevent interference). The DG sparsely codes incoming cortical information to prevent interference and faithfully transfer the representation to the CA3 via mossy fibers (MF). On the other hand, the CA3 is important for pattern completion (the ability of the network to retrieve complete representations when presented with partial or degraded input patterns). It was hypothesized that the recurrent collaterals (RC) between CA3 neurons form the substrate for memory storage and retrieval (Bakker et al., 2008; Kesner, 2013; Kesner et al., 2004; Leutgeb et al., 2007; McHugh et al., 2007; Neunuebel and Knierim, 2014; Treves and Rolls, 1992, 1994). The CA3 projects to CA1 via the Schaffer collateral pathway (SC). The CA1 pyramidal cells project to the subicular complex, and outputs from the subicular complex project back to the layer V of the entorhinal complex, closing the circuit (Amaral and Witter, 1989; Andersen, 2007; Scoville and Milner, 1957; Shepherd, 2004).

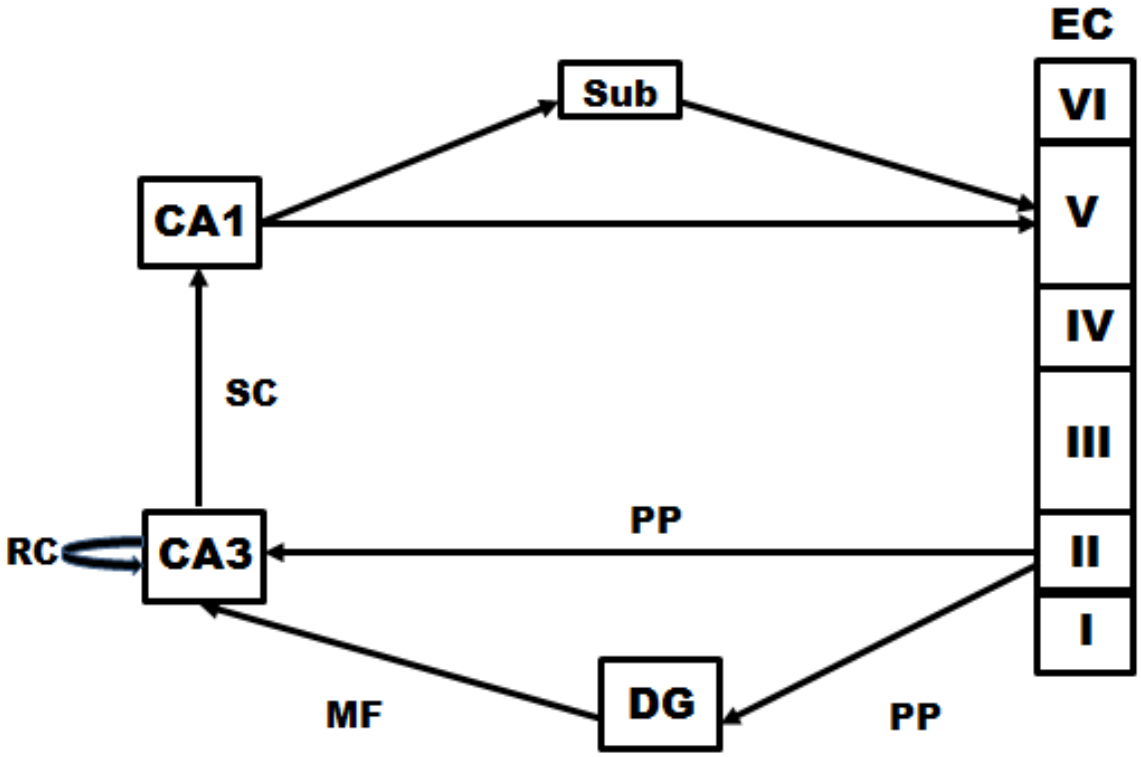


Figure 1-1. Neural circuitry of the adult hippocampus. The entorhinal cortex integrates sensory information and Layer II of the EC sends projections to the dentate gyrus (DG) or CA3 via the perforant pathways (PP). The dentate gyrus communicates with CA3 via mossy fibers (MF). CA3 neurons form recurrent collaterals (RC) among themselves and communicate with the CA1 via the Schaffer collateral (SC) pathway. CA1 sends projections directly to layer V of the EC or via the Subicular complex (Sub). EC: Entorhinal cortex (roman numbers indicate cell layers), DG: dentate gyrus, CA1: cornus ammonis 1, CA3: cornu ammonis 3, Sub: Subicular complex, PP: perforant pathway, RC: Recurrent collaterals, MF: mossy fibers, SC: Schaffer collateral pathway.

1.1.2 The Dentate Gyrus: Cell types and connections

The trisynaptic pathway originating from Layer II of the entorhinal cortex to the dentate gyrus and to the CA3 in the hippocampus is one of the most studied circuits in neuroscience (Figure 1-1). The dentate gyrus (DG) is the first relay station in this pathway and comprises of the molecular layer, the granular cell layer, subgranular zone and the hilus (Amaral and Witter, 1989; Andersen, 2007). The principal cell type in the DG is the embryonic-born, mature neuron in the granular layer (Figure 1-2). Granule cells (>1 million cells in the rat)

receive highly divergent inputs from the entorhinal cortex (~200,000 cells) and send sparse but powerful mossy fiber projections to CA3 cells (~12 per granule cell) (Henze et al., 2002). Cells in layer II of the lateral entorhinal cortex (LEC) send topographically organized projections to distal dendrites of granule cells in the outer one-third of the molecular layer while cells in the medial entorhinal cortex (MEC) send glutamatergic projections to dendrites in the middle one-third of the molecular layer (Amaral and Witter, 1989; Steward, 1976; Steward and Scoville, 1976). Mossy cells in the hilus send ipsilateral associational and commissural projections that terminate in the inner one-third of the molecular layer (Laurberg, 1979; Swanson et al., 1978). These terminals are asymmetric and excitatory, presumably provide the substrates for feedback loop (Kishi et al., 1980; Laatsch and Cowan, 1966).

Apart from excitatory inputs provided by cells in the entorhinal cortex and mossy cells, granule cells also receive inhibitory inputs from interneurons located in the granular layer and the hilus (Amaral, 1978; Freund and Buzsaki, 1996; Halasy and Somogyi, 1993), including Basket cells (Ribak and Seress, 1983), Chandelier cells (Somogyi et al., 1982; Soriano et al., 1990) and Somatostatin cells (Freund and Buzsaki, 1996).

Axons of granule cells (mossy fibers) arise from the hilar pole, project into the hilus and give off to small collaterals, and form glutamatergic terminals in the stratum lucidum of CA3 as thorny excrescence (Andersen, 2007). Because of the large size of mossy fiber terminals and proximal position to the soma of pyramidal cells, granule cells can greatly influence the activity of hippocampal pyramidal cells. When bursting, a single mossy fibre is capable of firing a downstream CA3 neuron (Henze et al., 2002). Another major target of granule cell axons are the mossy cells in the hilus (Frotscher et al., 1991), which in turn, forms synaptic connections with other granule cells, hence potentially providing for feedback loops within the dentate gyrus.

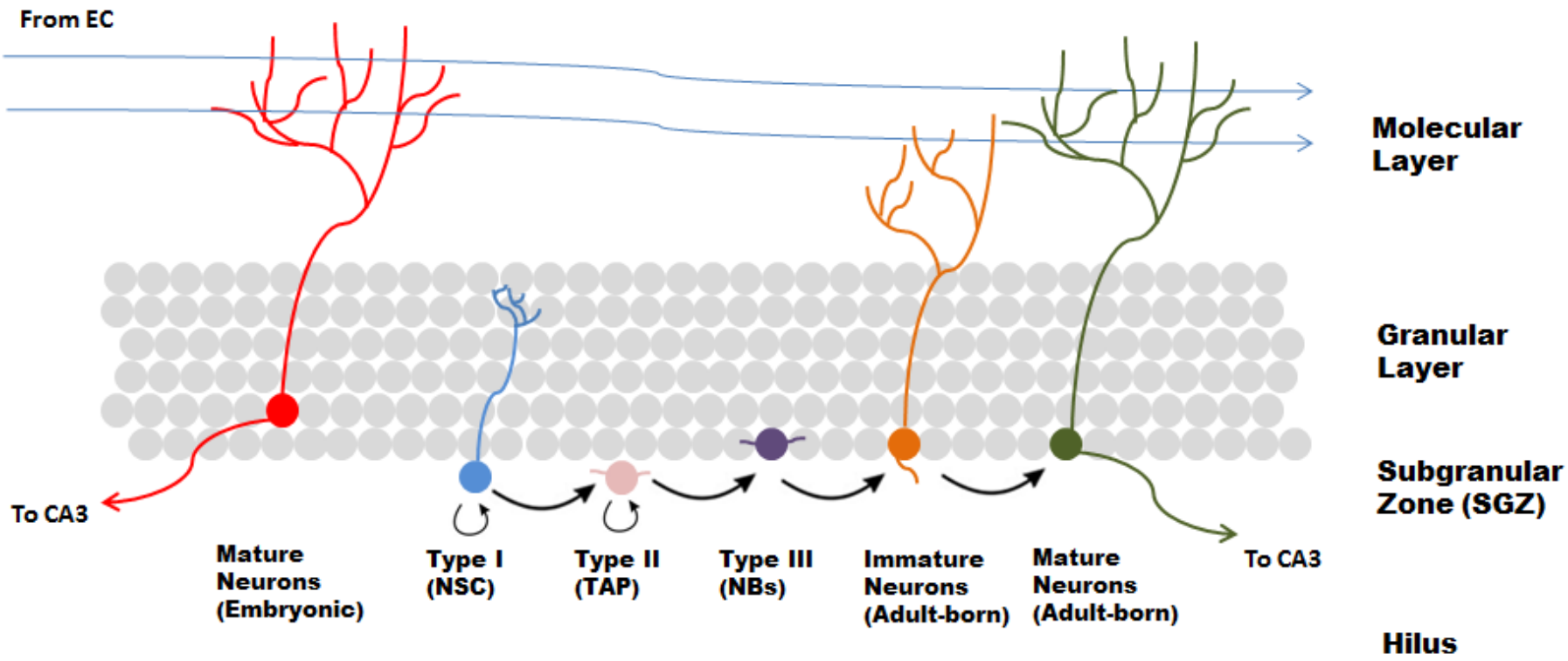


Figure 1-2. The dentate gyrus is a heterogeneous structure made of different cell types. Mature, embryonic-born neurons (red) are the predominant cell types in the granular layer. They receive information the entorhinal cortex (EC) and mossy cells (not shown) and send axonal projections to the CA3. A smaller population consists of adult neural precursors and adult-born neurons. Adult neurogenesis progresses from Type I, Type II, Type III, immature and mature neurons (see text). Type I cells (blue) are quiescent, radial-glial like neural stem cells (NSC). Type II cells (pink) are highly proliferative, also known as transit amplifying cells (TAP). Type III cells (purple) are committed neuroblasts (NBs). Immature, adult-born neurons (orange) are highly excitable while mature, adult-born neurons shared similar electrophysiological properties as mature, embryonic-born neurons. Mossy cells, Basket, Chandelier, Somatostatin and other cell types also reside in the dentate gyrus (not shown).

1.1.3 Neurogenesis persists in subgranular zone of the adult hippocampus

"*In the adult centers the nerve path are something fixed, ended, immutable. Everything may die, nothing may be regenerated*." – Santiago Ramon Y Cajal, "Degeneration and Regeneration in the Nervous System," 1928.

This long-held tenet became established as a dogma for much of the history of modern neuroscience and contested relatively late in the twentieth century. In 1965, Joseph Altman provided the remarkable insight that proliferating cells exist in the adult rat hippocampus and are able to incorporate tritiated thymidine (Altman, 1962a, b; Altman and Das, 1965a, b). It was shown that 20% of the normal number of dentate granule cells in the adult hippocampus are present in the rat at birth and the remaining are generated during postnatal stages and throughout adulthood (Bayer et al., 1982). Apart from the hippocampus, adult neurogenesis takes place in the olfactory system (Kaplan and Hinds, 1977). In 1998, Peter Ericksson provided evidence for adult neurogenesis in the human hippocampus (Eriksson et al., 1998). Using carbon dating technique, it is estimated that, 700 new neurons are added to each hippocampus per day in an adult human, corresponding to an annual turnover of 1.75% of neurons (Spalding et al., 2013; Spalding et al., 2005).

It is accepted now that there are two adult neurogenic regions in the brain, namely the subgranular zone in the hippocampus (SGZ) and subventricular zone in lateral ventricles (SVZ) under physiological conditions (Gage, 2000). While SGZ and SVZ neurogenesis share some similarities, there are fundamental differences between them. Neurogenesis in the SVZ produces several types of interneurons while neurogenesis in the SGZ produces only one type of excitatory neuron – the dentate granule cell. The interneurons modify outputs while the granule cells provide input nets. Neurogenesis in the SVZ is highly dependent on the turnover of sensory neurons in the olfactory epithelium and functions primarily as a means of

stabilizing neural networks. The turnover of neurons in the SGZ is low and addition of new neurons is believed to be adaptive and enhances performance (Appleby et al., 2011).

1.1.4 Development of adult-born neurons

Adult neurogenesis is enabled by the presence of neural precursor cells in the adult SGZ and SVZ. It encompasses the entire development of a stem cell into several fully functional adult-born neurons and this involved many cellular events, including proliferation, differentiation, migration, dendritic and axonal growth, survival, synaptogenesis, transmitter metabolism, network integration and functional maturation (Abrous et al., 2005; Ehninger and Kempermann, 2008; Song et al., 2005; Zhao et al., 2008).

Several types of cells have been characterized in the SGZ, including Type I, Type II, Type III, immature neurons and mature neurons. While it is generally accepted that adult neurogenesis progresses from Type I, Type II, Type III, immature neurons to mature neurons although the exact numbers and exact relationships between each cell types are still unclear (Figure 1-2) (Bonaguidi et al., 2012; Kempermann, 2011; Morrens et al., 2012; Muotri and Gage, 2006; Song et al., 2012; Yeo et al., 2008).

Type I cells

Type I cells are the putative stem cells, exhibiting the capacity for self-renewal and differentiation into multiple lineages. They are quiescent, have a radial-glial like appearance, express nestin, Sox2, glial fibrillary acidic protein (GFAP) but not calcium binding protein (S100β). It was proposed that Type I cells renew themselves by dividing symmetrically and can divide asymmetrically to generate a neuronal lineage-restricted progenitor cell (Type II)

and a glial restricted lineage or a transient stage between Type 1 and Type 2 cells (Seri et al., 2001; Steiner et al., 2004). Cells remain in the cell cycle 3 days after their initial division (Steiner et al., 2004).

Type II cells

Type II cells are highly proliferative neural stem cells and found in the SGZ. They are also known as transit amplifying cells and may be further divided into Type IIa or Type IIb, depending the expression of cell markers (Steiner et al., 2006). Excitatory inputs to these cells are GABAergic in nature (Tozuka et al., 2005; Wang et al., 2005).

Type III cells

Type III cells are less proliferative than Type II cells and are neuroblasts committed to becoming neurons. They migrate from the SGZ to the granular cell layer of the DG and commence early postmitotic differentiation to become immature neurons (Brandt et al., 2003).

Immature Neurons (adult-born)

As early as 1 day after birth, adult-born neurons are able to fire action potentials. These neurons lack distinguishing morphological features except for a few processes, start to express DCX, PSA-NCAM and Calretinin markers and potentially receive tonic GABA inputs from somatic receptors (Esposito et al., 2005).

A week following birth, at least 50% of adult-born neurons die through apoptosis (Kuhn et al., 2005). The surviving neurons show some neuronal features, extending non-oriented dendritic

processes. They receive somatic GABAergic inputs from local interneurons which are excitatory in nature, recapitulating development of embryonic born neurons. It was suggested that network activity can dynamically regulate proliferation and/or differentiation (Deisseroth et al., 2004; Song et al., 2012; Tozuka et al., 2005) and maturation of adult-born neurons (Ge et al., 2006) through these depolarizing GABAergic inputs.

Between 1 to 3 weeks after birth, adult-born neurons grow dendrites and axons rapidly. At 1.5 week after birth, a primary apical dendrite can be observed and dendritic arborization becomes more complex over several weeks. Mossy fibers extend to the hilar region at about 1 week after birth and reach the CA3 region at about 16 days after birth, organized in a laminar fashion (Sun et al., 2013; Toni et al., 2008; Zhao et al., 2006).

At 2 weeks after birth, spine formation occurs together with the onset of weak glutamatergic inputs (Esposito et al., 2005; Ge et al., 2006). Between 2 to 3 weeks after birth, the survival of adult-born neurons is dependent on N-methyl-D-aspartate (NMDA) signaling (Tashiro et al., 2006), In these immature stages, new-born neurons are highly excitable, having high input resistance and paired pulse facilitation, indicative of increased probability of vesicle release. They receive excitatory GABA inputs and exhibit lower activation threshold and increased long-term potentiation (LTP) amplitudes compared to mature neurons and (Ge et al., 2007; Schmidt-Hieber et al., 2004; Snyder et al., 2001; Wang et al., 2000). It was believed that this increased plasticity enables adult-born neurons to be particularly malleable and allows for the processing of new information during a critical developmental period (Aimone et al., 2011; Deng et al., 2010; Sahay et al., 2011b).

Mature neurons (adult-born)

When fully developed (~2 month), the mature (adult-born) neurons express Neuronal Nuclear Antigen (NeuN) and Calbindin markers, develop mature dendritic spines and project mossy fibers that form functional synapses with CA3 pyramidal cells. Mature neurons receive inhibitory GABAergic and excitatory glutamatergic inputs and share similar electrophysiological properties with neurons that were born in the embryonic brain, except for faster membrane time constant and smaller membrane capacitance (Ge et al., 2007; van Praag et al., 2002). In addition, mature neurons exhibit higher spine motility (Zhao et al., 2006) and they preferentially synapse with pre-existing axonal boutons (Toni et al., 2007).

1.1.5 Molecular regulation of adult neurogenesis

Factors regulating adult neurogenesis could be classified into intracellular factors, extracellular cues and cell interactions.

Intracellular Factors

In recent years, many important intracellular factors have been identified including, cell cycle regulators, transcription factors and epigenetics factors (Zhao et al., 2008). For example, Sox2 is required to maintain the pool of quiescent neuroprogenitors in the SGZ (Ehm et al., 2010; Favaro et al., 2009). Other transcription factors required for the survival and maturation of neurons include Prox1 (Lavado et al., 2010), NeuroD (Gao et al., 2009; Kuwabara et al., 2009), REST/NRSF (Gao et al., 2011), and Krüppel-like factor 9 (Scobie et al., 2009). Epigenetic factors also play important roles in coordinating gene expression during adult neurogenesis. For instance, Histone Deacetylase 2 (HDAC2) is required in maturation and

survival of neurons in adult brain but not required in embryonic neurogenesis (Jawerka et al., 2010). miR124, 137 and 184 were shown to fine tune the amount and timing of adult neurogenesis (Sun et al., 2011).

Extracellular cues

Many extracellular cues regulate adult neurogenesis. These include growth factors, morphogens, neurotransmitters, hormones. For instance, Insulin-like Growth Factor-1 (IGF-1) induces cell proliferation and net neurogenesis when infused peripherally or intracerebroventricularly (Aberg et al., 2003; Lichtenwalner et al., 2001). Glutamate regulates the survival of new born neurons in the adult SGZ through an NMDAR-dependent mechanism (Tashiro et al., 2006). GABA induces depolarization in new born neurons due to their high intracellular chloride content. Conversion of GABA-induced depolarization to hyperpolarization is required for proliferation, differentiation, synapse formation and dendritic development (Deisseroth et al., 2004; Ge et al., 2006; Song et al., 2012; Tozuka et al., 2005). Other external cues regulating the development of neural progenitors within the SGZ include, BDNF (Benraiss et al., 2001; Lee et al., 2002; Pencea et al., 2001), BMPs (Mira et al., 2010), Shh (Lai et al., 2003), Wnt (Karalay et al., 2011; Lie et al., 2005; Singh et al., 2011), EGF/FGF-2 (Doetsch et al., 2002; Kuhn et al., 1997; Palmer et al., 1999), VEGF (Calvo et al., 2011; Cao et al., 2004; Jin et al., 2002) and Notch (Shimojo et al., 2011).

Cell interactions

Radial glial cells were shown to form a scaffold to guide the growth of rudimentary apical dendrites of newborn neurons (Shapiro et al., 2007). Astrocytes promote neuronal

commitment of neural stem cells (Song et al., 2002) and are an important component of the neural stem cell niche (Horner and Palmer, 2003). Activated microglia induce transforming growth factors and promote neurogenesis (Battista et al., 2006). Primary cilia are critical for regulation of adult hippocampal neurogenesis (Breunig et al., 2008; Han et al., 2008). Endothelial cells regulate neural cell renewal and expansion in neurogenesis (Shen et al., 2004).

1.1.6 Activity-dependent regulation of adult neurogenesis

The process of adult neurogenesis is highly regulated by physiological stimuli, including enriched environment, learning, stress and ageing.

Enriched environment

Mice living in an enriched environment with voluntary exercise showed increased adult neurogenesis (Kempermann et al., 1998a; Kempermann et al., 1997, 1998b; van Praag et al., 1999a; van Praag et al., 1999b, 2000). Interestingly, mice living in social isolation showed an increase in neural stem cells, but not neurons (Dranovsky et al., 2011; Song et al., 2012). An enriched environment promotes survival of immature neurons (Kempermann et al., 1997, 1998b; van Praag et al., 1999b) during a critical period of a week after birth (Tashiro et al., 2007) and accelerates neuronal maturation of these immature neurons (Liu et al., 2012). Voluntary exercise increases both proliferation of neural progenitors and survival of adult-born neurons in young adult and aged mice (van Praag et al., 1999a; van Praag et al., 1999b; van Praag et al., 2005), improves spatial learning (van Praag et al., 1999a; van Praag et al.,

2005) and enhances LTP at medial perforant path synapses at the DG (van Praag et al., 1999a).

Stress

A stressful experience significantly reduces the rate of neural precursor proliferation in monkeys (Gould et al., 1998). Stress is known to strongly inhibit adult neurogenesis, via both glucocorticoids-dependent and independent pathways (Mirescu and Gould, 2006). Inhibiting adult neurogenesis prevented recovery of glucocorticoid levels induced in moderate stress and neurogenesis-deficient mice showed decreased sucrose preference and behavioral despair in forced swim test, suggesting that adult-born neurons play important roles in regulating the hypothalamic-pituitary-adrenal (HPA) axis (Snyder et al., 2011).

Learning

Learning increases survival of newborn neurons (Dupret et al., 2008; Gould et al., 1999a; Tashiro et al., 2006) and facilitates development of dendritic arbors (Tronel et al., 2010). Place learning in a Morris water maze and trace eyeblink conditioning increase the number of survival of neurons (Ambrogini et al., 2000; Gould et al., 1999a; Hairston et al., 2005). Late phase of spatial learning (Döbrössy et al., 2003) and associative LTP (Bruel-Jungerman et al., 2006) induce proliferation of NPCs in the dentate gyrus.

Ageing

Adult neurogenesis declines with increasing age in rodents (Kempermann et al., 2002; Kuhn et al., 1996; Seki and Arai, 1995) and non-human primates (Gould et al., 1999b). It was shown that adult neurogenesis was inhibited in young mice which were previously exposed to plasma from old mice. Chemokine CCL11 was identified to be a key molecule involved. These mice also showed impaired hippocampal functions, such as contextual fear conditioning and spatial memory (Villeda et al., 2011).

Activity	Effects on neurogenesis	References
Enriched Environment	Increases survival and maturation of immature neurons	Kempermann et al., 1998a; Kempermann et al., 1997, 1998b; Liu et al., 2002; Tashiro et al., 2007; van Praag et al., 1999b;
Exercise	Increases NPCs proliferation and survival of immature neurons	van Praag et al., 1999a; van Praag et al., 1999b; van Praag et al., 2005
Stress	Decreases NPC Proliferation	Gould et al., 1998; Mirescu and Gould, 2006; Snyder et al., 2011
Trace eyeblink	Increases survival of immature neurons	Ambrogini et al., 2000; Gould et al., 1999a; Hairston et al., 2005
Water maze (late phase)	Increases NPCs proliferation	Döbrössy et al., 2003
Ageing	Decreases NPCs proliferation	Gould et al., 1996b; Kempermann et al., 2002; Kuhn et al., 1996; Seki and Arai, 1995; Villeda et al., 2011

Table 1-1. Activity-dependent regulation of adult neurogenesis

1.1.7 The dentate gyrus is required for learning and memory

Based on anatomical evidence (Patton and McNaughton, 1995), electrophysiological studies (Jung and McNaughton, 1993), computational modeling (Marr, 1971; Rolls and Kesner, 2006), it was hypothesized that the dentate gyrus is critical for learning and memory, primarily as a pattern separator (by reducing overlaps between similar inputs to prevent interference).

The dentate gyrus is critical for spatial learning and memory. Reference memory is required to hold information on the relevant features of the tasks that are held constant between trials while working memory is required for to hold information on the relevant features of the current trial (Nadel and Hardt, 2011). In the reference memory protocol of the Morris water maze test, the location of the hidden platform was in a fixed location (Steele and Morris, 1999) while in the working memory protocol, the location of the hidden platform was moved to a new location each training day (Nakazawa et al., 2003). DG-lesions in mice impaired acquisition of both the reference memory (Conrad and Roy, 1993; McNaughton et al., 1989; Sutherland et al., 1983; Xavier et al., 1999) and working memory protocols of the Morris water maze (Jeltsch et al., 2001; Xavier et al., 1999). DG-lesioned mice were also impaired in both reference and working memories of the radial arm maze test (Jeltsch et al., 2001; McNaughton et al., 1989), in the temporal task of a delayed non-matching-to-place behavioral test (Costa et al., 2005) and in tasks requiring the detection of small spatial distances between objects (Gilbert et al., 2001; Goodrich-Hunsaker et al., 2005, 2008; Hunsaker and Kesner, 2008; Kesner et al., 2004). Inactivation of mossy fiber synapses and DG-lesion selectively impaired spatial learning tasks (Lassalle et al., 2000; Lee and Kesner, 2004a, b) while lesions to perforant inputs to CA3 region affected retrieval but spared learning (Lee and Kesner, 2004b). Elegant studies using transgenic animals strongly

supported the hypothesis that the DG is required for contextual learning and memory (McHugh et al., 2007; Nakashiba et al., 2012).

Apart from spatial learning, the dentate gyrus is required for contextual fear learning. DG lesioned mice were impaired in contextual fear acquisition and retrieval (Anagnostaras et al., 1999; Lee and Kesner, 2004a; Phillips and LeDoux, 1992, 1994), and contextual fear discrimination (Frankland et al., 1998). Transgenic mice with targeted deletion of NMDA receptors in DG cells showed impaired ability to distinguish between shocked and non-shocked context over time (McHugh et al., 2007).

It has been suggested that the dorsal and ventral hippocampi are functionally distinct structures. The dorsal hippocampus is required for cognitive functions while the ventral hippocampus is required for stress responses, emotions and affective behaviours (Fanselow and Dong, 2010; Moser and Moser, 1998). Several lines of evidences support this hypothesis. Firstly, the input and output connections to the dorsal and ventral hippocampi are anatomically distinct (Swanson and Cowan, 1977). A small lesion to the dorsal hippocampus is sufficient to affect spatial learning (Moser et al., 1995). Lesions to the ventral, but not the dorsal hippocampus, affect stress and emotional responses (Henke, 1990). More recently, it was shown that adult neurogenesis in the dorsal and ventral hippocampi are functionally dissociated. Optogenetic silencing of adult-born neurons in the dorsal, but not the ventral, hippocampus affected contextual fear learning and performance on the active place avoidance task. Optogenetic stimulation of adult-born neurons in the ventral hippocampus decreases innate anxiety but does not impair contextual learning (Kheirbek et al., 2013). This exciting finding has clinical implications in the treatment of anxiety disorders (Kheirbek and Hen, 2011; Kheirbek et al., 2012) and is further discussed in Section 1.1.9.

1.1.8 Adult-born neurons contribute to pattern separation and/or pattern integration

The phenomenon of adult neurogenesis poses the "stability-plasticity dilemma": new neurons potentially disrupt complex networks by interference yet networks that are too stable could not learn anything (Chambers et al., 2004; Deisseroth et al., 2004; Rakic, 1985). Computational modeling studies provide possible solutions to the dilemma. The Becker model suggests that the memory capacity of the hippocampus is increased by adult neurogenesis and turnover of neurons improves recall by minimizing interference (Becker, 2005). The Wiskott model suggests that interference is reduced by permitting new memories to be encoded by adult-born neurons, consistent with electrophysiological data showing that adult-born neurons display lower threshold for LTP (Wiskott et al., 2006). Mechanistically, adult-born neurons improve memory capacity and resolution by mediating pattern separation (Sahay et al., 2011b), consistent with the role of the dentate gyrus or by playing novel roles in pattern integration (Aimone et al., 2010; Aimone et al., 2006).

Adult-born neurons mediate pattern separation

Several lines of evidence suggest that dentate gyrus sparsely codes incoming entorhinal inputs to prevent interference through pattern separation (Jung and McNaughton, 1993; Marr, 1971; Patton and McNaughton, 1995; Treves and Rolls, 1992, 1994). It was proposed that adult-born immature neurons activate inhibitory neurons which increased sparseness of activity in the dentate gyrus and enabled pattern separation (Sahay et al., 2011b). Experimentally, it was shown that inhibiting adult neurogenesis in mice caused defects in pattern separation tasks (Clelland et al., 2009; Scobie et al., 2009; Tronel et al., 2012). A transgenic mutant mouse line, where mature granule cells were specifically inhibited leaving immature neurons intact, showed enhanced or normal pattern separation. Ablation of

immature neurons by X-ray irradiation abolishes pattern separation (Nakashiba et al., 2012). Conversely, increasing adult hippocampal adult neurogenesis enhances pattern separation tasks (Creer et al., 2010; Sahay et al., 2011a).

Inhibiting adult neurogenesis affects other forms of hippocampal-related learning, including trace eyeblink conditioning (Shors et al., 2001), trace fear conditioning (Shors et al., 2002), contextual fear conditioning (Dupret et al., 2008; Gu et al., 2012b; Ko et al., 2009; Saxe et al., 2006; Shors et al., 2002), spatial learning on the object location task (Goodman et al., 2010) and Morris water maze test (Dupret et al., 2008; Goodman et al., 2010; Gu et al., 2012b; Kee et al., 2007; Shors et al., 2002; Snyder et al., 2005; Zhang et al., 2008).

Adult-born neurons may play a role in pattern integration

Adult-born neurons could have different roles apart from pattern separation. It was proposed that adult-born neurons could play the novel role of pattern integration in the dentate gyrus (Aimone et al., 2010, 2011; Aimone et al., 2006, 2009). In other words, adult-born neurons could encode for relationships between patterns and thus improving memory resolution (Aimone et al., 2011). For instance, since adult neurogenesis occurs throughout the lifetime of the animal, sets of neurons born at different times could potentially correlate patterns and encode for events separated temporally (Aimone et al., 2006). While solid experimental evidence for this hypothesis is lacking, it was shown in a study that DG-lesions disrupt temporal associations between spatial events close in time (Morris et al., 2013a), suggesting that the adult-born neurons could play a novel role in temporal integration.

1.1.9 Clinical relevance of adult hippocampus neurogenesis

Neurological disorders are polygenic and multifactorial. While it is unclear if impaired adult neurogenesis is a robust etiological risk, aberrant neurogenesis and development of adult-born neurons with hippocampal dysfunction are common findings in diverse neurological diseases, including strokes, Alzheimer's disease, clinical depression, anxiety disorders, schizophrenia, and Rett Syndrome (Jagasia et al., 2006; Jessberger and Gage, 2009; Li et al., 2009; Lie et al., 2004; Mu and Gage, 2011; Smrt et al., 2007; Sun et al., 2009; Winner et al., 2011; Winner et al., 2012).

Strokes

The adult hippocampus is particularly susceptible to ischemic strokes (Nikonenko et al., 2009). Proliferation of adult-born neurons in the SGZ is upregulated following ischemic damage, presumably as a compensatory and neuroprotective mechanism (Kronenberg et al., 2003; Kronenberg et al., 2005; Kunze et al., 2006; Liu et al., 1998; Sun et al., 2003). Enhancing adult neurogenesis showed some promise in improving disease outcomes in animal models (Jin et al., 2010; Zhang et al., 2006).

Seizures

Seizures accelerate the proliferation and functional integration of adult-born granule cells in the hippocampus. Adult born neurons induced by status epilepticus exhibit connectivity that reduces brain excitability, suggesting that adult neurogenesis could function to mitigate pathological conditions (Jakubs et al., 2006; Kempermann, 2006; Overstreet-Wadiche et al., 2006; Scott et al., 2000). On the other hand, epilepsy-induced neurons showed aberrant

neuronal polarity and migration defects and it was proposed that integration of these neurons into stable networks could exacerbate, rather than improve, long term disease outcomes (Jessberger et al., 2007).

Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease with neuronal and synaptic loss in brain regions, such as the cortex, hippocampus and amygdala, and characterized by amyloid-β plaques (proteolysis products of Amyloid Precursor Protein (APP) by β and γ secretases) and neurofibrillary tangles (due to hyperphosphorylated tau) (Hardy and Selkoe, 2002). Amyloid Precursor Protein (APP), Presenilin-1 (PS1), tau and apolipoprotein E4 mutations are genetic risk factors in AD. Interestingly, impaired hippocampal neurogenesis were seen in AD rodent models, such as PS1M146V knock-in mice (Feng et al., 2001), triple transgenic AD mice (mutations in APP, PS1, and tau) (Fedele et al., 2011; Lim et al., 2011; Rodríguez et al., 2008; Wang et al., 2004b), and apolipoprotein E4 mice (Li et al., 2009). It has been suggested that impaired hippocampal neurogenesis may contribute to memory impairments seen in AD patients (Mu and Gage, 2011).

Clinical depression

New neurons are required for the formation of the hypothalamic-pituitary-adrenal (HPA) axis and suppressing neurogenesis led to anhedonia and behavioral despair which are associated with an impaired HPA axis (Snyder et al., 2011). While decreasing neurogenesis does not necessarily lead to depression, adult hippocampal neurogenesis is required to mediate the

beneficial effects of anti-depressants (Li et al., 2008; Santarelli et al., 2003; Surget et al., 2011).

Anxiety and panic disorders

Adult hippocampal neurogenesis has been implicated in some anxiety and panic disorders (Becker and Wojtowicz, 2007; Patricio et al., 2013; Pechnick and Chesnokova, 2009; Samuels and Hen, 2011). It was hypothesized that reduced adult hippocampal neurogenesis leads to deficits in pattern separation function and contributes to anxiety and panic disorders (Kheirbek et al., 2012). For instance, a war veteran suffering from post-traumatic stress disorder (PTSD) can fail to discriminate a novel and safe experience (campfire) from a previously stored experience of a traumatic event (fire in a war zone). The authors suggested that excessive generalization due to deficits in the pattern separation function of the DG can contribute to excessive fear responses of the war veteran upon seeing a neutral stimulus (campfire). Indeed, PTSD patients showed reduced DG and CA3 subfields (Wang et al., 2010), suggesting the hippocampal dysfunction could explain the etiology of post-traumatic stress disorder. It was shown that optogenetic stimulation of adult-born neurons in the ventral, and not the dorsasl, DG reduces anxiety (Kheirbek et al., 2013), suggesting that the ventral hippocampus regulates anxiety. Interestingly, selective serotonin reuptake inhibitors and tricyclic antidepressants selectively increase NPCs in the anterior (ventral) DG in human (Boldrini et al., 2009) and chronic treatment with agomelatine and 5-hydroxytryptamine receptor 2C selectively increases adult neurogenesis in the ventral DG of rodents (Boldrini et al., 2009), suggesting that these molecules could be viable candidates for treating anxiety disorders.

Schizophrenia

Post-mortem brain analysis showed reduced neurogenesis in schizophrenia patients compared to controls (Reif et al., 2006). Familial analysis has identified several schizophrenia susceptibility genes, including disrupted in schizophrenia 1 (DISC1) (Hennah et al., 2006), neuronal PAS domain-containing protein 2 (NPAS3) (Kamnasaran et al., 2003), and Neuregulin 1 (NRG1) (Harrison and Law, 2006). These genes have been subsequently shown to regulate the neuronal development of adult-born neurons. For example, DISC1 is critical for the dendritic growth and cell positioning of adult-born neurons (Duan et al., 2007; Kang et al., 2011; Kim et al., 2009; Kim et al., 2012a; Kvajo et al., 2011; Namba et al., 2011; Zhou et al., 2013), NPAS3 regulates survival of new neurons (Pickard, 2011; Pieper et al., 2010), and NRG1 has pro-neurogenic effects in the SGZ (Mahar et al., 2011).

Rett syndrome

Rett syndrome (RTT) is an autism spectrum disorder that primarily affects young girls, with early onset between 6 – 18 months of age. Patients showed progressive dementia, ataxia, loss of speech, loss of purposeful hand use and intellectual disability (Weaving et al., 2005). RTT is caused by mutations in the MeCP2 gene located in the X-chromosome (Amir et al., 1999). While RTT is considered to be a neurodevelopmental disorders, a landmark study in 2007 demonstrated that it is possible to reverse neurological defects by activating MeCP2 in a RTT adult mouse model (Guy et al., 2007). Furthermore, deletion of adult MeCP2 recapitulates RTT in mice (McGraw et al., 2011). These studies suggest that MeCP2 plays critical roles in the adult context. Indeed, it was shown that disrupting MeCP2 altered gene expression and delayed maturation of adult hippocampal neurons (Smrt et al., 2007), and perturbed morphological development of immature neurons (unpublished data from our lab). It will be

interesting to investigate if MeCP2 in adult-born neurons contributes to disease progression in RTT patients.

1.2. Class 3 Semaphorins

1.2.1 Structures of Class 3 Semaphorins

Semaphorins comprise of a large family of conserved proteins that function as guidance cues in diverse organisms (Tran et al., 2007). They are subdivided into eight classes: Class 1 and 2 Semaphorins have only been described in invertebrates, Classes 3 to 7 Semaphorins are found in vertebrates while Class V encodes for viral Semaphorins (Figure 1-3).

Class 3 Semaphorins is the most well-characterized in the nervous system (de Wit and Verhaagen, 2003; Mann et al., 2007; Pasterkamp, 2012; Pasterkamp and Giger, 2009a; Pasterkamp and Kolodkin, 2003; Pasterkamp and Verhaagen, 2001; Yoshida, 2012). They encode for 90 kDa secreted glycoproteins, with each molecule containing a conserved Sema domain for biological activity, a positively charged C terminus for potentiating activity and a Ig domain required for dimerization (Koppel et al., 1997; Koppel and Raper, 1998).

All Class 3 Semaphorins, except for Sema3E, bind to holoreceptors complexes comprising of Neuropilins and Plexins (Sharma et al., 2012). Neuropilins are the obligate ligand-binding receptors for Class 3 Semaphorins while Plexins are the signal transduction receptors. There are two members of Neuropilin identified in vertebrates, namely Neuropilin-1 (NRP1) and Neuropilin-2 (NRP2). In mammals, nine Plexin members have been identified and divided into subclasses: Plexin A1-A4, Plexin B1-B3, Plexin C1 and Plexin D1 (Kruger et al., 2005; Tran et al., 2007; Zhou et al., 2008). Besides Neuropilin and Plexins, Semaphorins require

IgCAMs L1 or NrCAM for specific repulsive or attractive responses (Bechara et al., 2008; Falk et al., 2005).

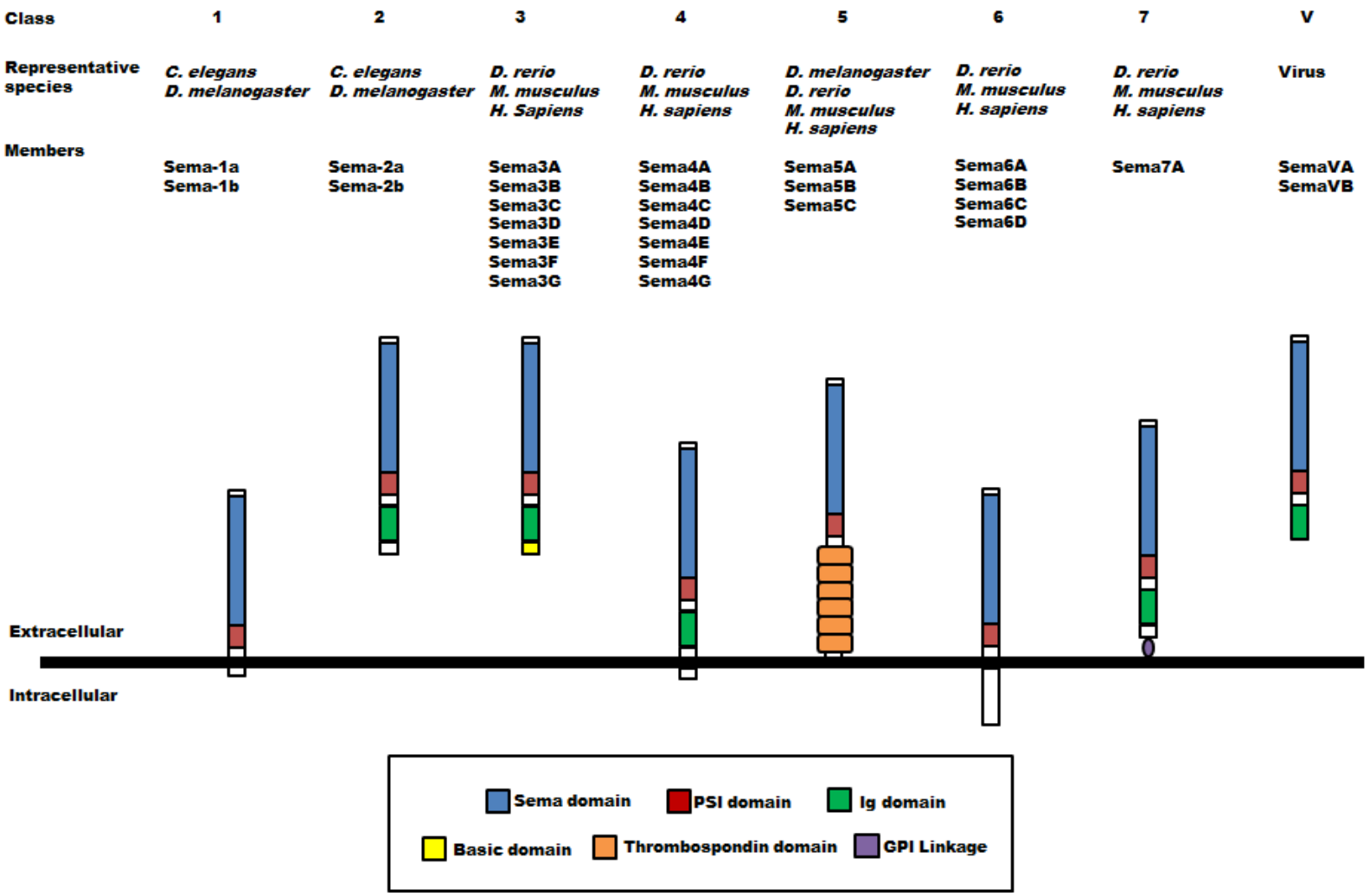


Figure 1-3. Semaphorins are conserved proteins expressed in diverse organisms. Class 1 and 2 Semaphorins have are found in invertebrates, Classes 3 to 7 Semaphorins are found in vertebrates while Class V encodes viral Semaphorins. Semaphorins are secreted or transmembrane proteins containing the conserved Sema domain. Other possible domains include the Plexin-Semaphorin-Integrin (PSI), Ig, Basic, thrombospondin domains and the GPI linkage.

1.2.2 Structures of Neuropilins

Two independent screens with cDNA libraries identified Neuropilin-1 (NRP1) as the binding receptor for Sema3A (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). NRP1 encodes for a 110 kDa transmembrane receptor. The protein consists of a signal peptide, two CUB (with homology to complement complement components C1r and C1s) domains (a1 and a2), two FV/VII (coagulation-factor-homology) domains (b1 and b2), one MAM (mephrin/A5/μ-phosphatase) domain, one transmembrane and a short cytoplasmic domain. Sema3A binds to NRP1 with high affinity (Kd = 0.325 to 1.5 nM). An antibody against NRP1 blocked ability of Sema3A to repel and induce growth cone collapse of DRG neurons (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997).

Neuropilin-2 (NRP2) another Class 3 Semaphorin receptor, closely related with NRP1 and sharing 44% identity (Chen et al., 1997). Like NRP1, the NRP2 protein consists of a signal peptide, two CUB domains (a1 and a2), two FV/VII domains (b1 and b2), one MAM domain, one transmembrane and a short cytoplasmic domain. Multiple isoforms of NRP2 arise by alternative splicing. While Sema3A shows detectable binding only to NRP1 (Kd = 0.325 to 1.5 nM), Sema3F shows higher affinity to binding to NRP2 (Kd = 0.09 nM) and lower affinity to NRP1 (Kd = 1.15 nM). Thus, Sema3A signaling is likely to regulate through NRP1 and Sema3F signaling through NRP2 (Chen et al., 1997). It is generally accepted now that Sema3A signals through NRP1-PlexinA4 complex and Sema3F signals through NRP2-PlexinA3 complex (Suto et al., 2005; Yaron et al., 2005).

1.2.3 Functions of Class 3 Semaphorins in the embryonic and adult CNS

The formation and functioning of neural circuit is a primary concern in neuroscience. Increasing evidence suggests that Class 3 Semaphorins regulate key cellular events in circuit formation, including axonal fasciculation, guidance, dendritic growth, migration, synaptogenesis, in the embryonic nervous system (Mann et al., 2007; Pasterkamp, 2012; Tran et al., 2007; Yoshida, 2012).

Axonal Guidance

Semaphorin-1a (Sema-1a) was first identified as an important axonal guidance cue and fasciculation factor for Ti neurons in the grasshopper (Kolodkin et al., 1992). In the Drosophila, Sema-1a and Sema-2a are expressed in subsets of CNS neurons and muscles (Kolodkin et al., 1993). In mutant Drosophila embryos lacking Sema-1a, motor axons stall and fail to fasciculate at specific choice points (Yu et al., 1998). Ectopic expression of Sema-2a in muscles that normally do not express it resulted in abnormal targeting of the RP3 motor neuron, suggesting that Sema-2a can function as a guidance cue as well as a selective inhibitor of specific synaptic arborisation for subsets of neurons (Matthes et al., 1995).

Class 3 Semaphorins were subsequently identified in the chick (Luo et al., 1993; Luo et al., 1995) and mouse (Kolodkin et al., 1993; Messersmith et al., 1995; Puschel et al., 1995). Similar to invertebrates, Semaphorins function as early axonal guidance cues in the developing nervous system of vertebrates. In the rodent spinal cord, Sema3A selectively repel afferents that normally terminate in the dorsal spinal cord, creating nonpermissive domains for neurite extension in the developing embryo (Messersmith et al., 1995; Puschel et al., 1995).

Sema3A $^{-/-}$ mice show abnormalities in many peripheral and cranial nerve projections (Behar et al., 1996; Catalano et al., 1998; Tanelian et al., 1997; Taniguchi et al., 1997). On the other hand, Sema3A is not required in the formation of some CNS tracts, including the climbing fibers, mossy fibers, thalamocortical and basal forebrain and cranial projections (Catalano et al., 1998). In Sema3F $^{-/-}$ mice, specific nerves are disorganized and defasiculated, including oculomotor, trochlear nerves, anterior commissure and stria terminalis of amydalar efferents (Sahay et al., 2003). In the hippocampus, the infrapyramidal tracts were projected abnormally in the Sema3F null mice (Giger et al., 2000; Sahay et al., 2003).

Compared to mice deficient in Class 3 Semaphorins, NRP1 $^{-/-}$ and NRP2 $^{-/-}$ display more severe axon guidance phenotypes, suggesting that the different members of Semaphorins can play redundant roles. NRP1 $^{-/-}$ mice show severe abnormalities could be seen in trajectory of cranial and spinal efferent fibers that express NRP1. For instance, the ophthalmic nerve defasiculated at 9.5 days post coitum (dpc) and overshoot their normal targets at 12.5 dpc. Spinal nerves are defasiculated and cross the dorsal midline (Gu et al., 2003; Kitsukawa et al., 1997). NRP2 $^{-/-}$ mice showed marked defects in development of cranial and spinal nerves. In the hippocampus, mossy fiber axons are also mis-targeted (Chen et al., 2000; Giger et al., 2000). Overexpression of NRP1 promotes neurite outgrowth (Hirata et al., 1993; Kawakami et al., 1996; Takagi et al., 1991; Takagi et al., 1995) and regeneration (Fujisawa et al., 1995).

Dendritic guidance

While Class 3 Semaphorins are generally chemorepellents for axons, they can function as chemoattractants for dendrites. Slice overlay experiments show that cortical axons are repelled by a gradient of Sema3A at the marginal zone while apical dendrites are attracted by it. It was suggested that asymmetric localization of soluble guanylate cyclase mediates such

effects (Polleux et al., 1998; Polleux et al., 2000). In the cortex of Sema3A $^{-/-}$ mice, many pyramidal cells have mis-oriented dendrites (Behar et al., 1996). It is also known that Class 3 Semphorins promotes dendritic growth of pyramidal neurons in the developing neocortex (Fenstermaker et al., 2004) and in hippocampal neurons (Schlomann et al., 2009).

Neuronal polarity

Sema3A promotes dendritic identity of immature neurites by inhibiting axon specification. Exposure of young neurons to Sema3A leads to an increase in cyclic GMP and a decrease in cyclic AMP. It is well-established that increasing cyclic GMP promotes dendritic specification and suppresses axon identity while raising cyclic AMP has the opposite effect (Shelly et al., 2011). In Xenopus spinal commissural interneurons, Sema3A application triggers cyclic GMP production and induces expression and targeting of $Ca_v2.3$ channels to immature neurites, promoting dendritic fate while suppressing axonal identity (Nishiyama et al., 2011).

Migration of neurons and neural crest cells

Sema3A and 3F prevent Neuropilin-expressing interneurons from invading the striatum of the developing brain (Marin et al., 2001), Interestingly, Robo1 interacts with NRP1 to regulate the migration (Hernandez-Miranda et al., 2011). Similarly, Sema3F expressed in the posterior sclerotome of developing somites to repel NRP2-expressing neural crest cells, restricting them to the anterior sclerotome (Gammill et al., 2006). In zebrafish, Semaphorins also played an evolutionary conserved role in guiding cranial neural crest cell migration in the developing brain (Yu and Moens, 2005).

Topographical map formation

In the olfactory epithelium, each olfactory sensory neuron (OSN) express a single type of receptor and axons from OSNs expressing the same receptor converge to a single glomerulus in the olfactory bulb. Intriguingly, axons are pre-sorted into sub-bundles before projecting into the olfactory bulb depending on the expression levels of Sema3A and NRP1. The anterior-posterior topographical olfactory connections are perturbed when Sema3A and NRP1 are genetically altered (Imai et al., 2009).

On the other hand, Sema3F regulates the dorsal-ventral patterning of olfactory connections. Sema3F-positive OSNs mature earlier and send out their axons earlier to dorsal regions of the olfactory bulb. NRP2-positive neurons mature later and their axons are repelled by Sema3F in the dorsal regions and move to the more ventral regions. Thus, the spatial-temporal expressions of Sema3F and NRP2 establish the dorsal-ventral topography of olfactory networks (Takeuchi et al., 2010).

Synaptic plasticity

Synaptic formation, maintenance and elimination are critical for formation of appropriate neural circuits in the embryo. During adulthood, synaptic plasticity allows for adaptation to physiological and environmental changes. In the *Drosophila* giant fiber system, the transmembrane Semaphorin 1a (Sema-1a) is required both pre- and postsynaptically in formation and assembly of a central synapse (Godenschwege et al., 2002).

The role of Sema3A in regulating synaptogenesis is still controversial. Sema3A treatment increased dendrite spines, clustering and co-localization of pre-synaptic marker synapsin-1 and post-synaptic marker PSD-95 in primary mouse cortical neurons and dendritic spines

density in layer V of cortex is reduced in Sema3A $^{-/-}$ adult mouse brains in one study and (Morita et al., 2006) but another study showed no evidence for the reduction in spines density in the cortical neurons in Sema3A null mice (Cao et al., 2004). In the hippocampus, it was shown that Sema3A treatment reduces accumulation of synaptophysin and PSD-95 in primary mouse hippocampal neurons (Bouzioukh et al., 2006b) while a separate study showed no differences in dendritic spines density in Sema3A null mice (Tran et al., 2009). In the cerebellum, Sema3A release by Purkinje cells inhibits synaptic pruning and innervation from climbing fibers (Uesaka et al., 2014). In the ventral spinal cord, astrocytic release of Sema3A is required for promoting both vGlut1 excitatory and VGAT inhibitory presynaptic puncta in α-motor neurons (Molofsky et al., 2014). Possibly, Sema3A may have divergent effects on neuronal subtypes or it could function in both synaptic formation and elimination.

Normally, few spines are present in the primary apical dendrites of cortical and hippocampal neurons. Mice defective in SEMA3F, NRP2 or PLXNA3 show increased spines in primary apical dendrites of dentate gyrus granule cell and cortical layer V pyramidal neuron of postnatal and adult CNS (Tran et al., 2009). In addition, Sema3F reduced the number of spines and PSD-95 positive puncta in primary cultures of dentate gyrus neurons. Interestingly, Sema3F treatment decreased the co-localization of excitatory synaptic markers PSD-95 and vGlut1 but not the markers for inhibitory synapses, vGAT and gephrin (Tran et al., 2009). Thus, Sema3F establishes synaptic specificity by synaptic elimination and negatively regulating synaptic structure (spine number and size) in excitatory synapses.

In adult mouse hippocampal slices, Sema3A decreases the number of field excitatory postsynaptic currents (EPSCs) in the CA1 (Bouzioukh et al., 2006b) while Sema3F increases the frequency of mini-EPSCs (Sahay et al., 2005). Dentate gyrus and cortical layer V from NRP2 $^{-/-}$ mice showed increased frequency of mini EPSCs and Sema3F $^{-/-}$ mice are prone to

seizures (Sahay et al., 2005; Tran et al., 2009). Furthermore, long-term potentiation in primary hippocampal neurons induces miR-188 which downregulates NRP2 protein expression and transfection of miR-188 can rescue NRP2-mediated decrease in dendritic spine density and synaptic transfection (Lee et al., 2012).

Importantly, paired-pulse amplitude ratios was not increased in NRP2 $^{-/-}$ mice compared to wild-type mice, suggesting that the increase in EPSCs is due to increased formation of spines and not increased pre-synaptic release probability (Tran et al., 2009). Future work is needed to demonstrate if Sema3F can modulate synaptic strength independently of its roles in synaptic formation in other neurons.

The expression and localization of Neuropilin receptors are critical in determining the synaptic plasticity of neurons. Sema3A/NRP1 signaling only controls basal dendritic arborization in layer V cortical neurons but not spine development while Sema3F/NRP2 negatively regulates spine formation in apical dendrites (Tran et al., 2009). These observations are attributed to the differential expression and localization of Sema3A and 3F and their respective receptors in the CNS. Specifically, NRP2 is expressed in apical dendrites of cortical pyramidal neurons, while NRP1 is more uniformly expressed in all processes (Tran et al., 2009).

1.2.5 Clinical relevance of Class 3 Semaphorins signaling

Class 3 Semaphorins have been implicated in some diseases, including spinal cord injury, seizures, neurodegenerative and psychiatric disorders.

Spinal Cord Injury

Following spinal cord injury, meningeal fibroblasts invade and populate the center of the scar tissue. Injured axons are unable to cross this zone and form swollen endings proximal to the scar tissue. It was shown the meningeal fibroblasts express Semaphorins and other inhibitory molecules, creating a non-permissive zone for axonal regeneration (Pasterkamp and Verhaagen, 2006). Sema3A inhibits extension in a human neuroma model (Tannemaat et al., 2007), adenoviral overexpression of Sema3A inhibits axonal regeneration in the spinal cord (Tang et al., 2007), and a specific Sema3A inhibitor facilitates axonal regeneration in a rat spinal cord injury model (Kaneko et al., 2006).

Seizures

Sema3F $^{-/-}$ and NRP2 $^{-/-}$ mice are prone to seizures (Sahay et al., 2005; Sahay et al., 2003; Tran et al., 2009). Sema3A and Sema3F were shown to be downregulated in injury-induced seizures (Barnes et al., 2003; Holtmaat et al., 2003; Yang et al., 2005). Aberrant Semaphorin signaling could explain the genetic predisposition seen in injury-induced seizures. For example, FVB/NJ mice, but not C57Bl/6J, developed chronic epilepsy and synaptic reorganization following kainic acid (KA) treatment. Interestingly, Sema3F was shown to be down-regulated in KA-treated FVB/NJ mice, but not C57Bl/6J (Barnes et al., 2003; Holtmaat et al., 2003; Yang et al., 2005).

Alzheimer's disease

A multi-protein complex isolated from degenerating brain tissue of Alzheimer's disease (AD) patients contains phosphorylated microtubule-associated protein (MAP) 1B, Sema3A, Collapsin response mediator protein 2 (CRMP-2), plexinA1 and A2. It was shown that the aberrant release of Sema3A in the subiculum, and its internalization by CA1 neurons contribute to the neurodegeneration of the CA1 field in the hippocampus (Good et al., 2004). The Ser522 and Thr509 phosphorylations of CRMP-2 by Cyclin-dependent Kinase 5 (Cdk5 and Glycogen Synthase Kinase 3β (GSK3β) respectively, reduce its affinity with tubulin, and are required for Sema3A-induced axonal collapse (Brown et al., 2004; Uchida et al., 2005; Yoshimura et al., 2005). Sema3A stimulation enhanced phosphorylation of CRMP-2 at both residues and it was speculated that hyper-phosphorylated CRMP-2 contribute to aggregation of microtubule associated proteins in neurofibrillary tangles seen in AD brain tissues (Uchida et al., 2005). Supressing CRMP-2 hyperphosphorylation rescued hippocampal damage and cognitive decline in AD models (Wang et al., 2013).

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is characterized by the progressive loss of motor neurons in the brain and spinal cord, resulting in loss of limb function and motor paralysis (Hardiman et al., 2011). It is known that Sema3A is required for axonal guidance of spinal motor neuron during development (Huber et al., 2005) and could be a genetic risk factor for ALS (Lesnick et al., 2008). In the $SOD1^{G93A}$ transgenic model for ALS, the selective increase of Sema3A expression in terminal Schwann cells (TSCs) of fast-fatigable type IIb and IIx muscle fibres is thought to contribute to denervation and degeneration of motor neurons at the neuromuscular junction (De Winter et al., 2006). It will be of interest to test if the application

of a Sema3A inhibitor (Kaneko et al., 2006) could mitigate the early-onset neurodegeneration of motor neurons in the ALS mouse models and motor dysfunction in human patients.

Schizophrenia

Several genetic studies associate Sema3A, Sema3D and plexinA2 with schizophrenia (Eastwood et al., 2003; Fujii et al., 2007; Fujii et al., 2011; Mah et al., 2006). Comparative proteomics studies implicate CRMP-2, a well-known downstream mediator of Class 3 Semaphorin signaling, in schizophrenia (Edgar et al., 2000; Zhou et al., 2012). These correlative studies suggest that Semaphorins contribute to synaptic pathology of schizophrenia (Eastwood, 2004; Eastwood et al., 2003; Zhou et al., 2012). The causative link between Semaphorins and schizophrenia needs to be more firmly established in future studies.

1.3. Aims of this study

Class 3 Semaphorins are critical for the development of the embryonic nervous system. Comparatively, their roles in the adult nervous system are less well-characterized. The expression patterns of Semaphorins ligands and Neuropilins receptors in the adult dentate gyrus suggested that they could play important roles in the development and functions of adult-born and embryonic-born neurons in the hippocampus. I carried out investigations to test the following hypotheses on the role of Class 3 Semaphorins signaling in the adult neurogenesis and adult hippocampal functions, particularly in learning and memory.

Hypothesis 1: Class 3 Semaphorins regulate key cellular events in the development of newborn neurons in the adult dentate gyrus

Although it was known that Class 3 Semaphorins are expressed in the adult dentate gyrus, there were no published studies on the roles of Class 3 Semaphorins in regulating the neuronal development of adult-born neurons. Adult neurogenesis is a process consisting of many cellular events, such as proliferation, differentiation, neurite formation, migration, synaptic formation and network integration. I characterized expression of Neuropilin receptors in adult neural precursor cells in the adult SGZ and used retroviral-mediated approaches to knockdown Neuropilins receptors in these cells (Chapter 3). The co-expression of GFP marker enables us to trace the morphological development of adult-born neurons *in vivo* and address if Class 3 Semaphorins regulate cellular events during adult neurogenesis (Chapter 4, 5, 6).

Hypothesis 2: Intracellular kinases act downstream of Class 3 Semaphorins in the cellular events during adult neurogenesis.

Class 3 Semaphorins signaling is known to be mediated by a wide range of kinases and different kinases regulate different cellular functions in neuronal development. I tested several candidate kinases as downstream mediators in cellular events induced by Class 3 Semaphorins in the adult brain (Chapter 5 and 6). Using retroviral-mediated approach, I can address if knocking down these kinases phenocopied Neuropilin-defective neurons and whether overexpression of the kinases can rescue the Neuropilin-defective phenotypes.

Hypothesis 3: Neuropilin-2 signaling in the dentate gyrus is required for learning and memory in the adult animal.

Sema3F regulates synaptic plasticity by negatively regulating dendritic spines formation during embryonic development. Synaptic plasticity plays an important role in learning and memory in the hippocampus. It is unknown if intact Sema3F/NRP2 signaling is required in the adult context after the embryonic neural circuit has formed. Using a lentiviral-mediated approach, I specifically target Neuropilin-2 in the dentate gyrus of adult mice and subject the animals to learning and memory tasks. This approach allowed us to address the contribution of Neuropilin-2 in the adult DG for hippocampal functions (Chapter 7).

In summary, I aim to address if Class 3 Semaphorins play any role in neuronal development of adult-born neurons and investigate downstream signaling pathways regulating these events. I am also interested in the functions of Neuropilins in adult hippocampal functions, especially in learning and memory.

Chapter 2

Materials and Methods

2.1. Reagents

2.1.1 Retroviral constructs

The pUEG vectors are engineered to express self-inactivating murine retroviruses which infect proliferating cells and their progeny (Ge et al., 2006). GFP expression was under the control of Elongation Factor 1α (EF1α) promoter and shRNA was co-expressed under the control of human U6 promoter in the same vector. shRNAs against different regions of mouse NRP1-782, NRP1-2231, NRP2-238, NRP2-1076, SEMA3A-714, SEMA3F-348, Cdk5-250, FAK-1412:, GSK3β-282 (Table 2-1) were cloned into *BglII* and *XhoI* sites downstream of the U6 promoter of pUEG constructs using standard molecular biology techniques.

The pCAG retroviral vector was used to overexpress transgenes driven by a CMV early chicken enhancer/chicken beta actin (CAG) promoter (Matsuda and Cepko, 2004). Transgenes include cDNAs of FAK, Cdk5, GSK3β and shRNA-resistant forms of NRP1 or NRP2 fused to mCherry via a T2A linker (Szymczak et al., 2004). Transgenes were amplified using primers (Table 2-2) and cloned into *AgeI* and *RsrII* sites downstream of CAG promoter of pCAG constructs using standard molecular biology techniques.

2.1.2 Lentiviral constructs

The pLL-synapsin lentiviral vector was modified from original pLL3.7 vector by replacing the Cytomegalovirus (CMV) promoter with a synapsin promoter (Rubinson et al., 2003). GFP expression was under the control of synapsin promoter and shRNA was co-expressed under the control of mouse U6 promoter in the same vector. shRNAs against different regions of mouse NRP1-782, NRP1-2231, NRP2-238, NRP2-1076, SEMA3A-714, SEMA3F-348, Cdk5-250, FAK-1412:, GSK3β-282 (Table 2-1) were cloned into *HpaI* and *XhoI* sites downstream of synapsin promoter of pLL-synapsin constructs using standard molecular biology techniques.

The FUGW lentiviral vector was used to drive transgenes under the human polyubiquitin promoter (Lois et al., 2002). cDNAs encoding FAK, Cdk5, GSK3β, shRNA-resistant forms of NRP1 or NRP2 were fused to mCherry via a T2A linker (Szymczak et al., 2004). Transgenes were amplified using primers (Table 2-2) and were cloned into *AgeI* and *EcoRV* sites downstream of CAG promoter of pCAG constructs using standard molecular biology techniques.

2.1.3 Mammalian expression constructs

For expression in HEK293 cells, the mammalian expression constructs pCDNA3.1(-)Myc-His (Invitrogen) and pEGFP-C1 (Clontech) were used to express transgenes fused to the myc epitope and EGFP respectively. Both constructs utilize the strong CMV promoter for overexpression. Wildtype and shRNA-resistant forms of NRP1 and -2 were cloned into pCDNA3.1(-)Myc-His vector while wildtype and dominant negative FAK were cloned into pEGFP-C1 vector using standard molecular biology techniques.

pAPtag constructs (GenHunter) were used to produce secreted Sema3A and Sema3F ligands fused to alkaline phosphatase and were kindly provided by Drs Alex Kolodkin and David Ginty of Department of Neuroscience, John Hopkins University, Baltimore, USA.

2.1.4 shRNA target sequences

shRNA sequences were designed against mouse NRP1, NRP2, Sema3A, Sema3F, FAK and GSK3β. Target sequences were checked for off-targets in the mouse genome by using the Basic Local Alignment Tool software available on the National Institute of Health website (Table 2-1).

2.1.5 Primers for cloning cDNAs

Primers were designed to amplify cDNAs using Primer3 software and ordered from IDT Singapore. Restriction enzyme sites were adding to 5' ends and 3' ends of the forward and reverse primers respectively to enable cloning into various vectors (Table 2-2).

2.1.6 Primers for site directed mutagenesis

Primers were designed for site-directed mutagenesis and ordered from IDT Singapore. Silent mutations were introduced to generate shRNA-resistant forms of NRP1 and NRP2. Single amino acid changes were introduced to generate dominant negative mutants for FAK (Table 2-3).

2.1.7 List of antibodies

Antibodies were ordered from commercial sources and tested for specificities to antigens using Western Blotting and/or immunohistochemistry (Table 2-4).

2.1.8 Animals

Adult C57/BL6 Jackson (6 weeks old) mice and time-mated (E18) female pregnant Sprague-Dawley (SD) rats were ordered from Centre for Animal Resources (CARE), Singapore. Upon delivery, time-mated pregnant rats were immediately sacrificed for hippocampal culture and mice were housed in the Duke-NUS Singhealth vivarium, under a 12 hour light: 12 hour dark cycle, with *ad libitum* access to food and water, housed in groups of 5 and allowed to habituate for one week before initiation of experiments.

Ethics Statement: All animal procedures were in accordance and approved with Institutional Animal Care and Use Committee (IACUC), National University of Singapore, with accordance with "Guide for the Care and Use of Laboratory Animals" and "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research", National Research Council.

shRNA	Target Sequences
shCTR	GTTCTCCGAACGTGTCACG
shNRP1-782	CGAATGTTCTCAGAACTAT
shNRP1-2231	CACAGAGAAGCCAACCATT
shNRP2-238	ACACGACTGCAAGTATGAC
shNRP2-1076	TCGTACAAGCTGGAAGTCA
shSema3A-714	CTATGCAAACGGAAAGAAC
shSema3F-348	CTTTCTGCTCAACACTACA
shFAK-1412	GCCTTAACAATGCGTCAGT
shcdk5-250	GATCAGGACCTGAAGAAAT
shGSK3-282	GAACCGAGAGCTCCAGATC

Table 2-1. shRNA target sequences

cDNA	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
T2A-mCherry	ACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAG	CTCGAGTGGGCCAGGATTCTCCTCGACGTCACCGCATGTTAGCAG ACTTCCTCTGCCCTCTCCACTGCCCTTGTACAGCTCGTCCATGCC
NRP1	CTCGAGATGGAGAGGGGGCTGCCGTTG	GAATTCTCACGCCTCTGAGTAATTACTCTG
NRP2	CTCGAGATGGATATGTTTCCTCTTACC	GCGGCCGCTCATGCCTCCGAGCAGCACTTCTG
Cdk5	CTCGAGATGCAGAAATACGAGAAA	GAATTCCTATGGGGGACAGAAGTC
FAK	CGCTCGAGCCGGGGGCCATGGAGCGAGTC	CGCGGACCGTTAGTGGGGCCTGGACTGGCT
GSK3β	GCCTCGAGATGTCGGGGCGACCGAGA	GCCGGACCGTCAGGTGGAGTTGGAAGC

Table 2-2. Primers used for cloning cDNAs

Mutant	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
NRP1-782	TCAAGAGAGGGCCCGAATGCTCACAAAACTATACAGCAC	GTGCTGTATAGTTTTGTGAGCATTCGGGCCCTCTCTTGA
NRP1-2231	ACCACTGTCCTGGCCACCGAAAAGCCGACCATTATAGAC	GTCTATAATGGTCGGCTTTTCGGTGGCCAGGACAGTGGT
NRP2-238	ATCGAGAAACATGATTGTAAGTACGACTTCATTGAGATT	AATCTCAATGAAGTCGTACTTACAATCATGTTTCTCGAT
NRP2-1076	TACTACGTCAAATCATATAAGCTAGAGGTCAGCACAAAT	ATTTGTGCTGACCTCTAGCTTATATGATTTGACGTAGTA
FAK-K38A	GCCATGGAGCGAGTCCTAGCGGTTTTTCACTACTTTGAA	TTCAAAGTAGTGAAAAACCGCTAGGACTCGCTCCATGGC
FAK-397F	ATCAGAAACAGATGACTTTGCAGAGATAATAGA	TCTATTATCTCTGCAAAGTCATCTGTTTCTGAT
FAK-732A	AGTGAAGGGTTTTATCCGGCTCCTCAGCATATGGTACAG	CTGTACCATATGCTGAGGAGCCGGATAAAACCCTTCACT
FAK-732D	AGTGAAGGGTTTTATCCGGACCCTCAGCATATGGTACAG	CTGTACCATATGCTGAGGGTCCGGATAAAACCCTTCACT
FAK-S732E	AGTGAAGGGTTTTATCCGGAACCTCAGCATATGGTACAG	CTGTACCATATGCTGAGGTTCCGGATAAAACCCTTCACT

Table 2-3. Primers used for site-directed mutagenesis

Antibody	Isotype	Company	Cat. No.	Western Blotting	Immunohistochemistry
anti-c-Myc tag	Mouse IgG	Sigma-Aldrich	M 4439	1:1000	Not tested
anti-c-Myc tag	Rabbit IgG	Abcam	ab9106	1:1000	Not tested
anti-GFP	Mouse IgG	Roche	11 814 460 001	1:1000	Not tested
anti-GFP	Goat IgG	Rockland	600-101-215	1:1000	1:1000
anti-α-tubulin	Mouse IgG	Sigma-Aldrich	T5168	1:1000	Not tested
anti-Cdk5	Mouse IgG	Abcam	ab28438	1:1000	Not tested
anti-Cdk5	Rabbit IgG	Abcam	ab40773	1:1000	Not tested
anti-FAK	Rabbit IgG	Millipore	06-543	1:1000	Not tested
anti-FAK-phospho-Y397	Rabbit IgG	Cell Signaling	3283	1:1000	Not tested
anti-FAK-phospho-S732	Rabbit IgG	Abcam	ab4792	1:1000	Not tested
anti-GSKβ	Rabbit IgG	Abcam	ab93926	1:1000	Not tested
anti-GSKβ-phospho-S9	Rabbit IgG	Abcam	ab75814	1:1000	Not tested
anti-NRP1	Goat IgG	R&D Systems	AF566	1:1000	1:1000 (with antigen retrieval)
anti-NRP1	Rabbit IgG	Cell Signaling	3725	1:1000	1:1000 (with antigen retrieval)
anti-NRP2	Rabbit IgG	Cell Signaling	3366	1:1000	1:1000 (with antigen retrieval)
anti-Sema3A	Rabbit IgG	Abcam	ab23393	1:1000	Not tested
anti-Sema3F	Rabbit IgG	Abcam	ab39956	1:500	Not tested
anti-DCX	Goat IgG	Santa Cruz	SC-8066	Not tested	1:250
anti-DCX-phospho-S297	Rabbit IgG	Abcam	ab23542	1:1000	Not tested
anti-Prox1	Rabbit IgG	Abcam	ab37128	Not tested	1:200
anti-Sox2	Rabbit IgG	Santa Cruz	sc-20088	Not tested	1:250
anti-Nestin	Goat IgG	Santa Cruz	sc-21248	Not tested	1:100
anti-MAP2	Rabbit IgG	Chemicon	AB5622	Not tested	1:500
anti-Tau1	Mouse IgG	Chemicon	MAB3420	Not tested	1:500
anti-TuJ1	Mouse IgG	Covance	MMS-435P	Not tested	1:250

Table 2-4. List of antibodies

2.2. Experimental protocols

2.2.1 Molecular Cloning

Standard molecular biology techniques were used to general recombinant constructs using protocols from "Molecular cloning. A laboratory manual" by Tom Maniatis and colleagues. Restriction enzymes (New England Biolabs), Calf intestinal alkaline phosphatase (Roche, Cat # 10713023001), and T4 DNA rapid DNA ligation kit (Roche, Cat #11635379001) were used for cloning.

2.2.2 Site-directed mutagenesis

The QuikChange II Site Directed Mutagenesis Kit (Stratagene, Cat #200524) was used to generate shRNA-resistant forms of NRP1 and -2 and mutant clones and FAK dominant negative mutants. DNA sequencing was performed to verify mutagenesis.

2.2.3 Mammalian cell cultures

HEK-293GP cells (Clontech) and 293FT (Invitrogen) cells were used as packaging cells for retrovirus and lentivirus production respectively. HEK293T cells (ACCA) were used for overexpression studies. NIH3T3 cells (ACCA) were used for viral titer determination. All cell types were maintained in DMEM culture medium (Life Technologies, Cat #11995073) containing 10% Fetal Bovine Serum (Life Technologies, Cat #16000044), 1x Penicillin-Streptomycin (Life Technologies, Cat #15140-148), in 37 ^{o}C incubator with 5% CO_2. For overexpression studies, HEK 293T were transfected with expression constructs using calcium phosphate (Jordan et al., 1996).

2.2.4 Production of Semaphorin-AP ligands

To produce Alkaline Phosphatase-fused Sema3A and 3F ligands, HEK cells were transfected with pAPTag constructs encoding Sema3A-AP and Sema3F-AP (obtained from Drs. Alex Kolodkin and David Ginty). After 24 hr post transfection, media was replaced with Serum free HEK 239 Freestyle media (Life Technologies, Cat #12338018) and cells were growth for a further 48 hrs. The media was concentrated using Centricon Plus-70 (Millipore, Cat #UFC710008) and the amount of secreted ligands were quantified with an enzymatic assay for alkaline phosphatase activity (Flanagan et al., 2000) with diethanolamine (Sigma-Aldrich, Cat #D8885) and p-Nitrophenyl Phosphatase substrate (Sigma-Aldrich, Cat #P4744). Efficacies of ligands were checked and found to be comparable to commercially produced ligands: Recombinant Mouse Semaphorin 3A/Fc Chimera (R&D Systems, Cat #1250-S3) and Recombinant Human Semaphorin 3F/Fc Chimera (R&D Systems, Cat #3237-S3).

2.2.5 Primary embryonic rat hippocampal cell culture

Hippocampal neurons were isolated from the hippocampi of embryonic stage E18 rats as previously described (Goh et al., 2008). Dissociated neurons were cultured on poly-l-lysine (Sigma-Aldrich, Cat #P2658) coated plates or coverslips. For biochemical analysis, neurons were treated with AraC (Sigma-Aldrich, Cat #C6645) to eliminate dividing astrocytes and used at 4-5 days after plating and starved in MEM (Life Technologies, Cat #11095-114) for 2-3 hrs prior treatment with Sema3A/3F-AP. For expression studies, primary neurons were mixed with plasmids in Amaxa Rat Neuron Nucleofector solution (Lonza, Cat #VPG-1003) and electroporated with a Nucleofector 2b device (Lonza, Model AAB-1001) using program G-013 before plating.

2.2.6 Adult neural precursor cell culture

Adult neural precursor cells were isolated from adult mice as described (Shivaraj et al., 2012). Briefly, eight 6 weeks old C57/BL6 mice were sacrificed and their hippocampi, were gently separated from the corpus collosum. The tissue samples were diced with a scalpel blade in Hanks' Balanced Salt Solution (HBSS) (Life Technologies, Cat #14175103) and centrifuged at 2000 rpm for 5 min. After removing the supernatant, tissue samples were enzymatically digested with 2 mg/ml papain (Worthington, Cat #LS003118), 250 U/ml DNase I (Sigma-Aldrich, Cat #D4263) and 1 U/ml Dispase II (Life Technologies, Cat #17105014) at 37°C for 30 min. Tissues were then dissociated into single cells and centrifuged at 2000 rpm for 5 min to obtain cell pellet. The pellet was resuspended into Dulbecco's Modified Eagle Medium (DMEM)/nutrient mixture F-12 (DMEM/F12) (Life Technologies, Cat #11320033) supplemented by 1x N2 supplement (Life Technologies, Cat #17502048), 1x Penicillin-Streptomycin (Life Technologies, Cat #15140-148) and 1 mM L-glutamine (Life Technologies, Cat #25030081). Finally, cells were washed and resuspended in culture media supplemented with 10 ng/ml recombinant Human Basic Fibroblast Growth Factor (rh bFGF) (STEMCELL Technologies, Cat #02634) and 20 ng/ml recombinant Human Epidermal Growth Factor (rh EGF) (STEMCELL Technologies, Cat #02633) in 2 µg/ml heparin/PBS (STEMCELL Technologies, Cat #07980). Primary hippocampal cells were incubated for 7 days to allow neurospheres expansion. The neurospheres were then dissociated using accutase (STEMCELL Technology, Cat #07920) and maintained as described below.

Adult NPC cells were maintained as monolayer in NeuroCult® NSC Basal Medium (Mouse) (STEMCELL Technology, Cat #05700) supplemented with NeuroCult® Neural Stem Cell Proliferation Supplements (Mouse) (STEMCELL Technologies, Cat #0571) in 35 mm culture plates coated with Matrigel (BD Biosciences Cat #352477). Media were supplemented with 10 ng/ml FGF and 20 ng/ml rh EGF daily and changed on alternate days.

2.2.7 Cell Proliferation Assay of adult NPCs

For proliferation, 20,000 cells/cm^2 per well were seeded in each 12 mm coverslip coated with 15 μg/ml of laminin (Sigma Cat# L2020) and growth in NeuroCult® NSC Basal Medium (Mouse) (STEMCELL Technology, Cat# 05700) supplemented with NeuroCult® Neural Stem Cell Proliferation Supplements (Mouse) (STEMCELL Technologies, Cat# 0571). After seeding, cells were treated with Sema3A-AP, Sema3F-AP or AP ligands (50, 100, 250 or 500 ng/ml) and allowed to proliferate for 48 hours and quantified with a standard MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay as described (Mosmann, 1983) with a MTT kit (Sigma Aldrich, Cat #M-0283).

2.2.8 Cell Differentiation Assay of adult NPCs

For differentiation, 50,000 cells/cm^2 were seeded in each 12 mm coverslip coated with 15 μg/ul of laminin (Sigma Cat# L2020) cells and growth into Dulbecco's Modified Eagle Medium (DMEM)/nutrient mixture F-12 (DMEM/F12) (Life Technologies, Cat #11320033) supplemented by 1x N2 supplement (Life Technologies, Cat #17502048), 1x B27 Supplement (Life Technologies, Cat #17504044), 1x Penicillin-Streptomycin (Life Technologies, Cat #15140-148) and 1 mM L-glutamine (Life Technologies, Cat #25030081). After seeding, cells were treated with 500 ng/ml Sema3A-AP, Sema3F-AP or AP ligands every alternate day for 7 days. Cells were fixed with 4% paraformaldehyde and immunostained for DCX, a cell marker for immature neurons.

2.2.9 Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was extracted from primary E18 rat hippocampal culture or adult progenitor culture using RNeasy Mini Kit (Qiagen, Cat #74104). First strand cDNAs were purified from 1 μg total RNA using the Superscript III First-Strand Synthesis System (Life Technologies, Cat #18080-51). Double stranded cDNAs were amplified using Platinum Taq High Fidelity Polymerases (Life Technologies, Cat #11304-011) with specific primers (Table 2-2).

2.2.10 Western Blotting

Protein concentration was determined by DC protein assay (Biorad, Cat #500-0112). Protein lysate (20 μg per well) was run using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (Biorad, Cat #162-0177) at 100V for 1 hour in an electrotransfer tank (Bio-rad, Cat #165-8000). Membrane was blocked in blocking buffer (5% milk in TBS with 0.1% Tween 20) for 1 hour at room temperature and incubated with primary antibodies in blocking buffer for overnight at 4 ^{o}C. The next day, the membrane was washed three times for 10 min each with TBS with 0.1% Tween 20. The membrane was then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies diluted 1:5000 in blocking buffer (Goat anti-Mouse-HRP: Thermoscientific, Cat #32430; Goat anti-Rabbit-HRP: Thermoscientific, Cat #32460) for 1 hour at room temperature, and washed three times for 10 min each with TBS with 0.1% Tween 20. For developing, the membrane was incubated in chemiluminescent substrate Super Signal West Pico Chemiluminescence detection kit (Thermoscientific, Cat #34077) and signals captured by exposure to X-ray film. Densitometry analysis was carried out in ImageJ (NIH, Bethesda).

2.2.11 Immunohistochemistry and immunocytochemistry

Adult C57/BL6 Jackson (6 weeks old) mice received an overdose of pentobarbital (150 mg/ml) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were extracted and post-fixed overnight. The brains were then transferred to 30% sucrose in 0.1 M phosphate buffer (pH 7.4) for cryoprotection at 4 ^{o}C overnight. The hippocampi were section at a thickness of 40 µm using a sliding microtome (Leica, Model SM2000 R) and stored in 0.1 M phosphate buffer at 4 ^{o}C until use for immunohistochemical studies.

Neurons were grown on poly-L-lysine coverslips and fixed with 4% paraformaldehyde/4% sucrose in 0.1 M phosphate buffer (pH 7.4) for 20 min. Cells were then washed three times with PBS and stored in PBS at 4 ^{o}C until use for immuncytochemical studies.

For immunostaining, brain slices or cells were washed three times for 10 min each with Tris-buffered saline with 0.1%-Triton X-100. Blocking was done for 1 hour with Tris-buffered saline with 0.1%-Triton X-100 and 5% Donkey serum. Brain slices or cells were incubated with primary antibodies diluted with Tris-buffered saline with 0.1%-Triton X-100 and 5% Donkey serum for overnight at 4 ^{o}C. The next day, they were washed three times for 10 min each with Tris-buffered saline with 0.1%-Triton X-100 before incubating with the appropriate secondary antibody (Alexa Fluor 488 Goat anti-Rabbit IgG: Life Technologies, Cat # A11080; Alexa Fluor 555 Goat anti-Mouse IgG: Life Technologies, Cat #A31570; Alexa Fluor 555 Donkey anti-Goat IgG: Life Technologies, Cat #A21432) diluted 1:500 with Tris-buffered saline with 0.1%-Triton X-100 and 5% Donkey serum for 1 hour at room temperature. Washing was performed three times for 10 min each with Tris-buffered saline with 0.1%-Triton X-100. Brain slices or cells were mounted on microscope slides and imaged with a confocal microscope (Carl Zeiss Microimaging GmbH, LSM 710).

2.2.12 Virus production and titer determination

For retrovirus production, HEK293GP cells (Ge et al., 2006) were cultured in DMEM culture medium (Life Technologies, Cat #11995073) containing 10% Fetal Bovine Serum (Life Technologies, Cat #16000044), 1x Penicillin-Streptomycin (Life Technologies, Cat #15140-148), in 37 ^{o}C incubator with 5% CO_2 in a 15 cm culture plate and transfected with pUEG or pCAG constructs using calcium phosphate (Jordan et al., 1996) and pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G). Transfection medium was replaced after 16 hours with 13 ml fresh DMEM. Media were collected every 12 hours and replaced with 13 ml fresh media. Pooled media containing viruses from 3 collections (~40 ml) were collected and filtered through a 0.45 μm syringe filter (Sartorius Stedim, Cat #16555K) and ultra-centrifuged at 50,000 x g for 1.5 hr at 4 ºC. 15 μl of DPBS (Life Technologies, Cat #14190250) was added to the pelleted virus and allowed to stand for at least 4 hours at 4 ºC before resuspension. For lentivirus production, FUGW or pLL constructs with packaging plasmids encoding for VSV-G, Gag, Pol, were transfected into HEK293FT cells (Invitrogen) using calcium phosphate (Jordan et al., 1996).

For viral titer determination, NIH 3T3 cells were plated at a density of 2 x 10^4 cells per well in a 24-well plate. The viruses were serially diluted at a dilution factor from 10^4 to 10^9. 48 hours post-infection, cells were checked for GFP expression. The number of colonies expressing GFP was counted and multiplied by dilution factor to give viral titer (colony forming units/ml or cfu/ml). Only high titers of engineered retroviruses (1 x 10^9 unit/ml) were used for *in vivo* experiments.

2.2.13 Stereotaxic injection of adult mice

Adult (5-6 weeks old) female C57Bl/6 mice (Charles River) housed under standard conditions were anaesthetized (100 μg ketamine, 10 μg xylazine in 10 μl saline per gram of animal) and mounted on a stereotaxic frame (David Kopf Instruments). The skin was wiped with 70% ethanol and cut to expose the skull. A dental drill (0.6 mm drill bit) was used to make four shallow holes at the following coordinates: anterioposterior = -2 mm from bregma; lateral = ±1.6 mm; ventral = 2.5 mm; anterioposterior = -3 mm from bregma; lateral = ±2.6 mm; ventral = 3.2 mm. The drill should penetrate the skull completely but not any further to prevent injury to the brain. A 1 μl Hamilton, flat-tip syringe was used to inject 0.5 μl retrovirus at a rate of 0.25 μl/min into the dentate gyrus, at the specified ventral depth for each co-ordinate. To prevent fluid backflow, a pause of 2 min was allowed before slowly withdrawing the syringe. The wound was closed with sterile surgical suture and returned to standard housing conditions. Mice (4-6 animals per experiment) were sacrificed at desired time points for morphological analysis. 40 μm sections were made along the entire septotemporal axis of the brains and only data from animals showing GFP+ signals in 50% of all hippocampal slices (in either the left or the right hippocampus) were used for behavioural analysis.

2.2.14 Confocal imaging and morphological analysis

Images were acquired on a Zeiss LSM 710 confocal system (Carl Zeiss, Singapore) and analysed using Zeiss Zen software and NIH ImageJ with the NeuronJ plugin. For analysis of the dendritic structure of newborn neurons, three-dimensional (3-D) reconstructions of the dendritic processes of each GFP^+ neuron were made from Z-series stacks of confocal images. The projection images were semi-automatically traced with NIH ImageJ using the NeuronJ

plugin. Measurements do not include corrections for inclinations of dendritic process in 3-D and therefore represent projected lengths. The distributions of the total dendritic length and branch number of each individual neuron under different conditions were shown in accumulative distribution plots. A total of 25-30 neurons from 4-6 animals injected with shRNA were analysed per group. Sholl analysis for dendritic complexity was done by counting the number of dendrites crossing a series of concentric circles at 20 μm intervals from the cell soma. Statistical significance ($P < 0.05$) was assessed using the student's t-test.

2.2.15 Open-field test

The open-field test measures the spontaneous locomotor activity of the mouse in an open arena. Each mouse was placed in the centre of an arena consisting of a 40 cm × 40 cm × 16 cm acrylic box (Versamax Animal Activity Monitor, AccuScan Instruments) and allowed to explore the arena for 5 min. Time spent in the centre of the area, time spent in the periphery, total distance moved and speed, are measured from video-recordings and coded using TopScan Version 3.0 (Clever Sys Inc), by an observer blind to the treatment of the animal.

2.2.16 Object-location learning test

For habituation, each mouse was placed in the centre of an arena consisting of an 40 cm × 40 cm × 16 cm acrylic box (Versamax Animal Activity Monitor, AccuScan Instruments) and allowed to explore the arena for 5 min. Twenty four hours later, the mice were exposed to two identical objects placed in centres of top-left and top-right quadrants of the arena for 10 min. The two identical objects were LEGO® bricks (16 mm x 32 mm x 9.6 mm). Testing for short term memory and long-term memory was performed 20 mins and 24 hours respectively

after training by changing the location of one of the objects. Different batches of animals were used for short-term and long-term memory tasks. The locations of the object are counterbalanced between subjects to prevent bias. The arena and objects were cleaned with 70% ethanol after each subject was tested. Time spent with an object was defined as the duration and number of contacts was defined as the bouts when the mouse is oriented towards the object and within half a body length of that object, and could include sniffing, touching, or climbing on the object. These tests were video-recorded and coded using TopScan Version 3.0 (Clever Sys Inc), by an observer blind to the treatment of the animal. Discrimination ratios were calculated as (Time spent with novel location – Time spent with familiar location) / (Total time spent with objects in both locations). Positive scores indicated preferences for the novel location, negative scores showed preferences for the familiar location, and scores approaching zero denoted no preference for either location.

2.2.17 Contextual and cued fear conditioning

Three days before training, mice were allowed to be habituated in the behavioural phenotyping room and handled by the same experimenter performing the behavioural tests daily for 5 min. Mice were trained in four identical conditioning chambers (30 x 25 x 25 cm; Med-Associates) during the light cycle of the animal facility. The conditioning chambers were placed in a sound-attenuating cubicle with a 60 dB background noise provided by a fan. The floor of each chamber were made of 32 stainless steel rods (2 mm diameter) placed 6 mm apart and wired to a shock generator and scrambler (Med-Associates, Inc) to deliver the foot shock. Each chamber was cleaned with 70% ethanol between tests. Freezing responses were scored using Video Freeze Version 1.20.5.0 (Med Associates) using the following parameters, Motion Threshold: 40 and Minimum Freeze Duration: 1 frame. To assess roles of

NRP2 in contextual learning tasks with different cognitive demands, different batches of mice were trained with a single trial (one shock) or multiple trials (five shocks) conditioning protocols. Contextual fear memory was assessed 24 hours after training.

Day 1: Contextual and cued fear training

Single shock protocol: Mice were placed in context A (conditioning chamber with stainless grid floor, ambient house lighting, and a 70% ethanol scent) and allowed to habituated for 3 min, followed by a 30 sec 90 Hz tone coupled with a 0.7 mA foot shock in the last 2 sec of the tone and followed by an additional 2 min observation period after the tone.

Multiple shocks protocol: Mice were placed in context A (conditioning chamber with stainless grid floor, ambient house lighting, and a 70% ethanol scent) and allowed to habituated for 3 min. A total of 5 shock trials were delivered. Each shock trial consists of a 30 sec 90 Hz tone coupled with a 0.5 mA foot shock in the last 2 sec of the tone and an inter-trial of 1 min without foot-shock. At the end of the fifth trial, the mouse was observed for a further 3 min. A multiple trials protocol elicits greater emotional stress and arousal compared to a single trial protocol and hence a lower foot-shock amplitude is used in the multiple trials protocol (Cravens et al., 2006).

Day 2: Contextual Fear Memory (24 hours after training)

Mice were placed back to context A and recordings of freezing behaviour were taken over a 5 min period and returned to home cages for 2 hours before testing for cued fear retrieval.

Day 3: Cued Fear Memory (26 hours after training)

Mice were placed in different context D (conditioning chamber with plastic insert covering the stainless grid floor, ambient house lighting blocked by a red plastic insert, and a lemon detergent scent) and allowed to habituate for 2 min, followed by a 30 sec 90 Hz tone without

foot shock and followed by an 4.5 min observation after the tone before returning to home cages.

2.2.18 Morris Water Maze Test

The water maze consists of a 120 cm diameter grey circular pool filled with water (23–26◦C, 40 cm deep) made opaque by adding non-toxic white paint (Crayola®). The pool is surrounded by several distant cues from the environment of the experimental room. The animals learns to find a transparent platform (10 cm in diameter) hidden 1 cm below the water surface, and which location remains constant throughout the experiment. Each animal is tested four times a day for five consecutive days, with intertrial of 20 to 40 min. For each trial, mice are released facing the tank wall from one randomly selected starting points (North, South, East or West), and are allowed to swim until they reach the platform. Animals which fail to find the platform within 1 min are gently directed to it and put on it for 15 sec. After the trial, mice are removed from the pool, gently dried with a towel and put individually in cage filled with paper towel and warmed with water bottles to avoid hypothermia. The criterion of learning success consists to reach the platform in less than 20 sec. On the 6th day, a 60-second probe trial is conducted without platform and the time spent in each quadrant is recorded. Trials are recorded with a video camera placed above the centre of the pool and the analysis is automated through the ANY-maze® video tracking system (Stoelting, USA).

Chapter 3

Retroviral and Lentiviral approaches to study adult neurogenesis

3.1. Introduction

3.1.1 Limitations in the use of knockout mice in studying adult neurogenesis

Sema3A knockout mice were generated by two independent groups. One group reported that Sema3A $^{-/+}$ heterozygous mice are normal while Sema3A $^{-/-}$ homozygous mice appeared normal at birth but within 24 hours, seemed weaker and had less milk in stomach. 70% die within first 3 days with major cardiac and skeletal defects and 17% die at weaning. A small percentage (12%) survived to adulthood but had difficulty maintaining upright posture (Behar et al., 1996). Strikingly, another group reported that Sema3A $^{-/-}$ mice were viable after birth with no apparent cardiac defects, with half of the weight compared to wildtype littermates at 4 weeks old (Taniguchi et al., 1997). The differences in mortality and cardiac defects were attributed to different genetic backgrounds, 129 strain (Behar et al., 1996) and C57BL/6 (Taniguchi et al., 1997) used in the studies. On the other hand, Sema3F null mice were viable. However, they exhibited severe defects in the axonal fasciculation and targeting in major limbic nerves and prone to seizures (Sahay et al., 2003).

NRP1 $^{-/-}$ mice were embryonically lethal. Live homozygous mice could be observed as late as 12.5 days postcoitus (dpc) (Kitsukawa et al., 1997). These NRP1-deficient mice showed severe abnormalities in the trajectory of efferent fibers of the peripheral nervous system (Kitsukawa et al., 1997). Chimeric mice over-expressing NRP1 were also embryonically lethal, exhibiting excess capillaries and blood vessels, dilation of blood vessels, malformed

hearts, ectopic sprouting and defasciculation of nerve fibers, and extra digits (Kitsukawa et al., 1995). Likewise, many NRP2 $^{-/-}$ mice were weak and did not survive into adulthood (Giger et al., 2000).

The lack of viability and survival into adulthood presents considerable limitations in using them to investigate their roles in adult neurogenesis and hippocampal functions. Furthermore, the expression of Semaphorins and their receptors in many non-neuronal tissues are confounding factors to investigate exact roles of Semaphorins in the adult neurogenic regions.

3.1.2 Advantages of retroviral and lentiviral approaches

5-bromo-2-deoxyuridine (BrdU), a thymidine analogue integrated during the S-phase of the cell cycle, is commonly used to label adult-born neurons. It is reliable and could be used for quantification work. However, BrdU are diluted with subsequent cell divisions and the nuclear expression of BrdU is not suitable for morphological studies.

Retroviruses require the breakdown of nuclear membrane during cell division for genomic integration and hence specifically target dividing cells into the adult neurogenic regions. The genomic integration of transgenes in target cells and their progenitors provide stable expression of marker protein, such as GFP, for morphological studies. However, the delivery and uptake of retroviruses are not ubiquitous and less efficient than BrdU uptake, rendering retroviral-mediated approaches less useful for quantification studies. However, by engineering retroviral constructs to express shRNA targeting genes in dividing neural progenitors, it is possible to investigate the genetic mechanisms by which neural progenitors mature into adult-born neurons in the adult neurogenic regions. Notably, the retroviral mediated approach specifically targets neural progenitors while sparing other cell types such

as mature neurons and vascular cells in the neurogenic niche. In addition, the approach could be used to label single isolated cells to dissociate between cell autonomous and non-cell autonomous effects. Retroviral mediated shRNA knockdown is a robust method and had been used extensively in many studies to study roles of genes in adult neurogeneis (Ge et al., 2007; Gu et al., 2012b; Jessberger et al., 2007; Laplagne et al., 2007a; Mathews et al., 2010; Toni et al., 2008; van Praag et al., 2002).

Lentiviruses transduce both actively dividing and non-dividing cells. It can be used to introduce a transgene or block the expression of a specific gene using RNA interference technology. By stereotaxic injection of lentiviruses, it is possible to target subregions of the hippocampus (for example, DG vs, CA3, dorsal vs. ventral hippocampus etc.) to investigate their specific roles in hippocampal functions. In the adult neurogenic regions, lentiviruses efficiently transduce large populations of both dividing progenitors and mature neurons, allowing us to investigate the functions of genes in regulating animal behaviour. For example, it has been showed that lentiviral-mediated expression of dominant negative CREB in the dorsal hippocampus and dorsal-lateral striatum impairs place learning and contextual fear conditioning (Kathirvelu and Colombo, 2013; Kathirvelu et al., 2013). Since lentiviruses do not discriminate between dividing and mature neurons, it is less useful for investigating the precise roles of adult-born neurons and mature neurons in hippocampal functions.

While Class 3 Semaphorins play important role in the development of the embryonic nervous system, it is unknown if they play any roles in the development of adult-born neurons and adult hippocampal functions. All Class 3 Semaphorins bind directly to holoreceptor complexes comprising of Neuropilins and Plexins (Sharma et al., 2012).

To study the roles of Class 3 Semaphorins in adult neurogenesis, I adopted the retroviral-mediated approach to label and knockdown Neuropilin-1 and -2 in neural progenitors in the

adult mouse SGZ. The retroviral constructs were engineered to co-express enhanced green fluorescent protein (GFP) under the EF1α promoter and gene-specific shRNAs under the human U6 promoter. By analysing the development of these neural progenitors at different stages through the use of markers (Figure 3-1), I could address if Class 3 Semaphorins signaling plays specific roles in diverse processes, such as proliferation, differentiation, migration, neurites formation and network integration in the adult SGZ (Chapter 4, 5, and 6). To address the role of NRP2 in adult hippocampal functions, I used the lentiviral-mediated approach to knockdown NRP2 in the adult dentate gyrus and assessed the animals in contextual and spatial learning tasks (Chapter 7).

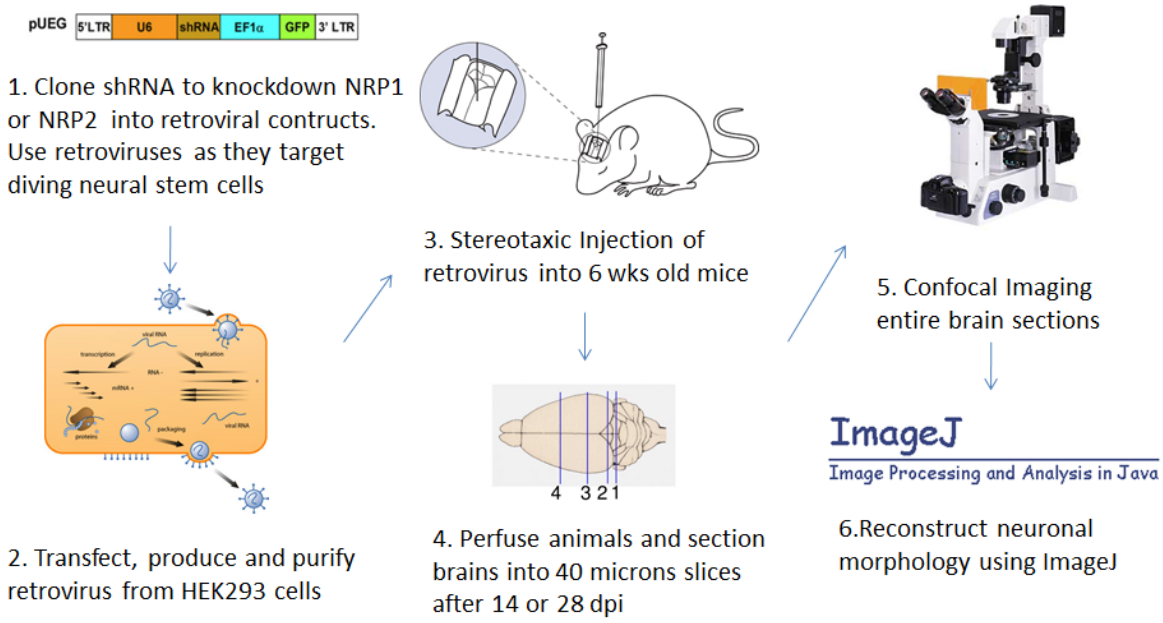


Figure 3-1. Retroviral mediated knockdown of adult-born neurons. Experimental procedures include generation of retroviral constructs, production and purification of retrovirus, transduction of adult-born neurons in vivo, confocal imaging and reconstruction of neuronal morphology.

3.2. Results

3.2.1 shRNAs effectively and specifically knockdown exogenous Neuropilins and Semaphorins

I generated shRNAs that effectively knockdown exogenously expressed NRP1 and NRP2 in HEK 293T cells as shown by Western Blotting (Figure 3-2). To check the specificity of the shRNAs against NRP1 and NRP2, RNAi refractory forms of NRP1 and NRP2 are generated. These RNAi refractory forms harbour silent mutations in target sequences of the shRNA such that their mRNAs encode for wildtype proteins but are resistant to shRNA knockdown. Western Blotting revealed that shRNAs effectively knocked down wildtype forms of NRP1 and NRP2 but not the RNAi refractory forms of NRP1 and -2 (~100 kDa), confirming their specificities (Figure 3-2).

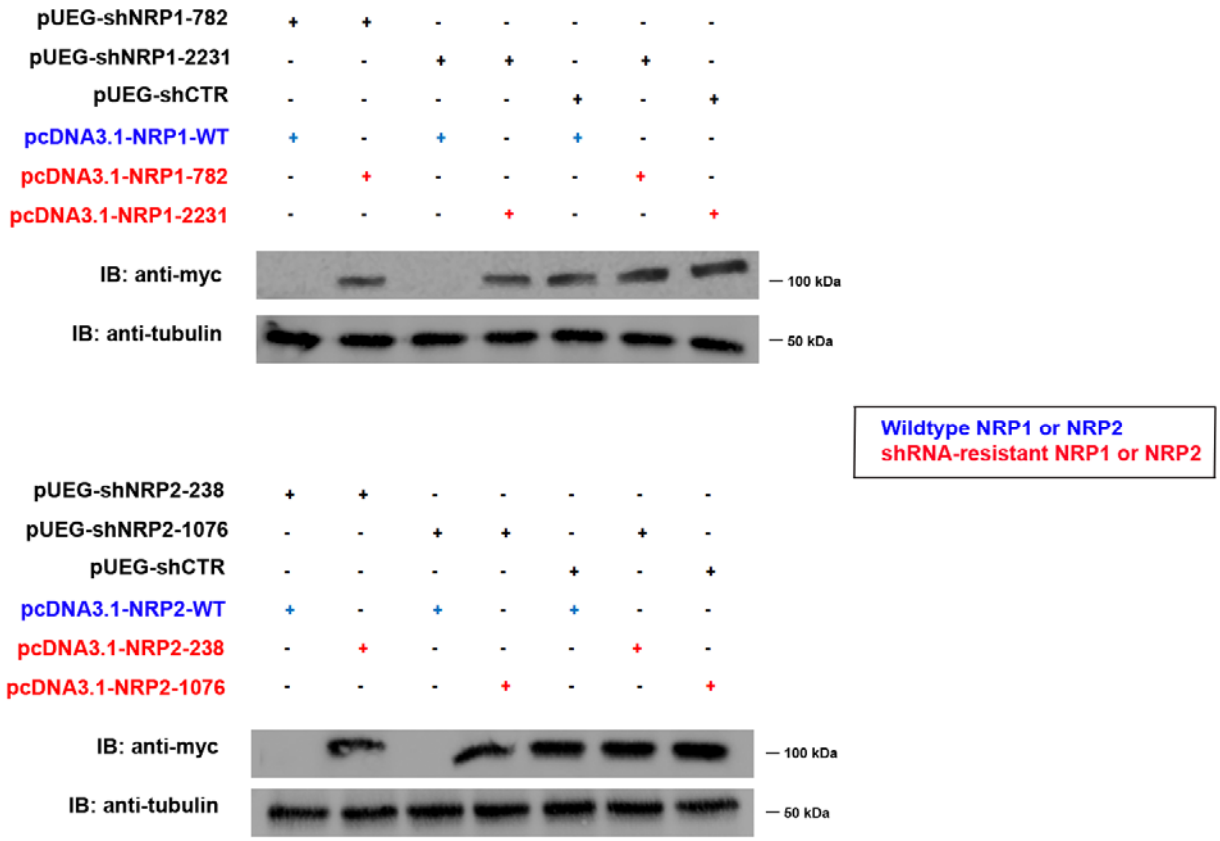


Figure 3-2. shRNAs effectively and specifically knockdown exogenous NRPs. Retroviral constructs expressing different shRNAs were co-transfected with the indicated myc-tagged overexpression constructs into 293T cells. Western Blot analysis showed effective knockdown of wild-type NRP1 and NRP2 by shRNAs for NRP1 and NRP2. pcDNA3.1-NRP1-782 and pcDNA3.1-NRP1-2231 express NRP1 that harbor silent mutations rendering them are resistant to knockdown by pUEG-NRP1-782 and pUEG-NRP1-2231, respectively. pcDNA3.1-NRP2-238 and pcDNA3.1-NRP2-1076 are resistant to knockdown by pUEG-NRP2-238 and pUEG-NRP2-1076, respectively.

In addition, I generated specific shRNAs against Sema3A and Sema3F. As Class 3 Semaphorins are secreted proteins, I co-transfected pAPtag constructs overexpressing Sema3A and 3F fused to an alkaline phosphatase (AP) tag in HEK 293T cells, collected both cell lysates and conditioned media after 48 hours post transfection. The conditioned cell media were filtered through a Centrico column to concentrate the secreted Semaphorins ligands. I performed Western blotting on cell lysates and concentrated media with specific antibodies against Sema3A and Sema3F. As expected, cell lysates do not contain any detectable Sema3A and Sema3F protein while concentrated media from cells contain specific ~150 kDa protein bands corresponding to Sema3A-AP and Sema3F-AP fusion proteins (Sema3A:89 kDa, Sema3F: 89 kDa, AP:67 kDa). Co-expression of shRNAs against Sema3A and Sema3F with pAPtag constructs showed effective knockdown of Sema3A and Sema3F in the concentrated cell media (Figure 3-3).

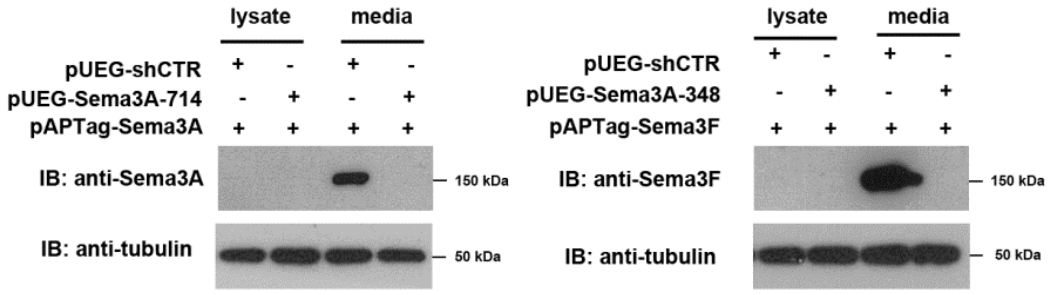


Figure 3-3. shRNAs effectively and specifically knockdown Semaphorins. Retroviral constructs expressing different shRNAs were co-transfected with the indicated overexpression constructs into 293T cells. Sema3A and 3F are secreted into the cell media and not detectable in the cell lysate fraction. Western Blotting showed shRNA effectively knockdown of exogenous Sema3A and 3F.

3.2.2 shRNAs effectively and specifically knockdown endogenous Neuropilins

Having shown that our shRNAs effectively and specifically knock down exogenous NRP1 and -2 in HEK 293T cells, I next checked if the shRNAs can knock down endogenous NRPs in primary neuronal cultures. Rat hippocampal neurons were electroporated with retroviral constructs and immunostained with antibodies against NRP1 and -2. At 6 days *in vitro* (6 DIV), NRP1 and -2 expression could be seen in both soma and dendrites in neurons electroporated with the shCTR construct. In neurons electroporated with shRNA against NRP1 and -2, the expression of NRP1 and -2 in both soma and dendrites are markedly reduced respectively (Figure 3-4). Thus, I validated that our shRNA could effectively and specifically knocked down endogenous NRP1 and -2 in primary neurons.

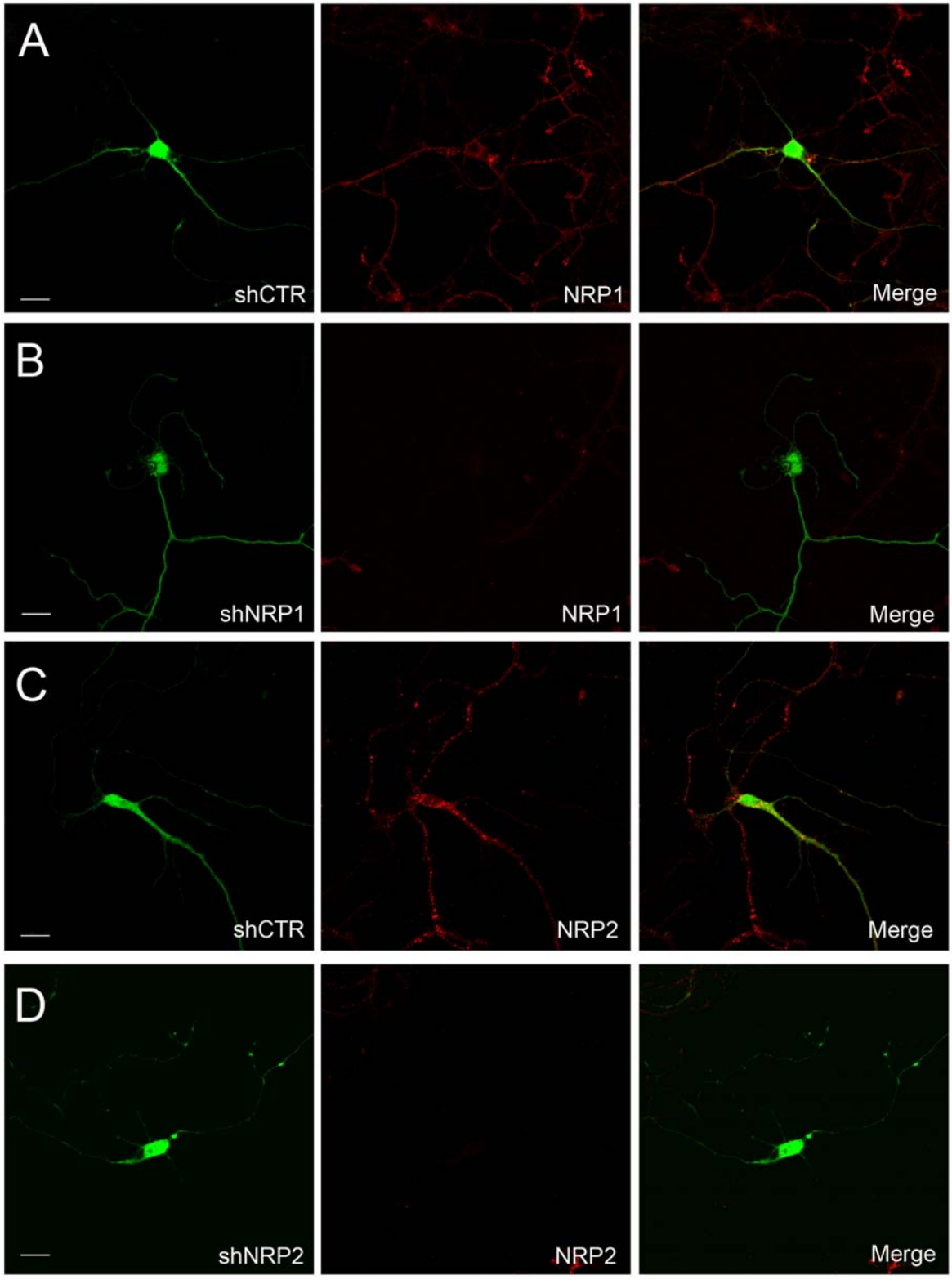


Figure 3-4. shRNAs effectively and specifically knockdown endogenous Neuropilins. E18 hippocampus neurons were electroporated with shCTR (A, C), shNRP1 (B) and shNRP2 (D) constructs. Neurons were fixed at 6 DIV and immunostained with anti-NRP1 and anti-NRP2. Representative images show effective knockdown of endogenous NRP1 (B) and NRP2 (D) in primary neurons. White arrows indicate GFP-shRNA positive neuronal cell bodies stained with anti-NRP1 or NRP2 antibodies as indicated. White arrowheads indicate dendrites. Scale bar = 20 µm.

3.3. Discussion

NRP1 and -2 knockdown transgenic animals were not viable and could not survive well to adulthood. Retroviral mediated knockdown approach allowed us to specifically target NRP1 and -2 in newborn neurons to investigate their roles in adult neurogenesis. On the other hand, lentiviruses effectively transduce both dividing and non-dividing cells, allowing us to address their roles in hippocampal function which may require large population of neurons.

In this chapter, I described the generation and validation of constructs expressing shRNA targeting Sema3A, Sema3F, NRP1 and NRP2. I checked that shRNAs are effective in knocking down exogenously expressed transgenes in HEK293T (Figure 3-2 and 3-3) and endogenous NRP1 and -2 in primary neurons (Figure 3-4). A primary criticism of RNAi technology is that shRNAs may not be specific and could have off-targets effects. I checked the shRNA sequences do not have off-targets by sequence alignments with the mouse genome (Blastn, NIH, Bethesda). Importantly, I also showed that shRNAs were unable to knockdown RNAi-refractory forms of NRP1 and -2, confirming their specificities (Figure 3-2).

Retroviruses enabled genomic integration of transgenes into target cells and their progenitors, resulting in stable expression of markers. Our retroviral constructs express a GFP marker under the strong EF1α promoter, allowing us to carry out morphological studies of target cells. In subsequent chapters (Chapters 4-6), I would use the retroviral constructs described here to interrogate the roles of NRP1 and -2 in the development of adult-born neurons.

Chapter 4

Neuropilins do not regulate proliferation and differentiation of adult-born neurons

4.1. Introduction

4.1.1 Class 3 Semaphorins and Neuropilins are expressed in the adult hippocampus

In the adult SVZ, Sema3A and its receptor NRP1 are expressed in the endothelial cells along the rostral migratory stream (Melendez-Herrera et al., 2008). Migratory neuroblasts undergo progressive differentiation along the rostral migratory stream before reaching their final targets in the olfactory bulb. Thus, endothelial cells along the rostral migratory could act as a source of Class 3 Semaphorins for migrating neuroblasts. Conversely, endothelial cells could act as targets of Semaphorins signaling as they expressed NRP1. Interestingly, migrating neuroblasts secret Slit1 which modify the surrounding Robo-expressing astrocytic cells, enabling their rapid migration along the rostral migratory stream (Kaneko et al., 2010). While not demonstrated, the expression of Sema3A and NRP1 in endothelial cells suggest they could regulate rostral migratory stream migration of neuroblasts in the adult SVZ (Melendez-Herrera et al., 2008).

In the adult hippocampus, *in situ* hybridization revealed that Sema3A mRNA expressions in the CNS of P15 and adult rats are virtually the same. Stellate cells in layer II and pyramidal cells in layer III of the entorhinal cortex provide the principal source for the perforant path to the hippocampal formation. Retrograde tracing combined with *in situ* hybridization demonstrated that axons of stellate cells provide the principal source of Sema3A, forming a

gradient in the outer two-thirds of the molecular layer that gradually declines towards the granule cell layers (Giger et al., 1998). Similarly, Sema3F ligand binding sites were found in the medial and lateral entorhinal cortex and in their axonal projection to the hippocampus (Tran et al., 2009). In the hippocampus, Sema3F ligand binding sites could be seen in dentate granular layer, inner molecular layer, mossy fibre projections, hilar cells and CA1-3 pyramidal cells (Tran et al., 2009).

Binding studies showed that NRP1 and -2 are localized in the inner molecular layer of the dentate gyrus of adult mice and the stratum lucidum of the CA3 (Sahay et al., 2005), suggesting robust expression of NRP1 and -2 in dendrites and axons, and absence of expression in the soma of granule cells (Kawakami et al., 1996; Tran et al., 2009). Dendritic fields of CA1-3 pyramidal cells also showed NRP1 and -2 labelings, with weaker expression in the adult stage compared to post-natal stages (Sahay et al., 2005; Tran et al., 2009).

4.1.2 Proliferation and differentiation of adult NPCs

While it is demonstrated clearly that Semaphorins and Neuropilins are expressed in the adult hippocampus (Giger et al., 1998; Sahay et al., 2005; Tran et al., 2009), it is unknown if they are expressed in the adult neural precursor cells (NPCs). Also, it is unclear if Semaphorins play any role in the proliferation and differentiation of adult NPCs.

To investigate these issues, I cultured adult NPCs from the hippocampus of 6 weeks old mice and characterized the expression of NRP1 and NRP2 in these cells by Western Blotting. To address if Semaphorins regulate the proliferation and differentiation, I treated adult NPCs culture with Sema3A and Sema3F ligands in proliferation and differentiation assays. Finally,

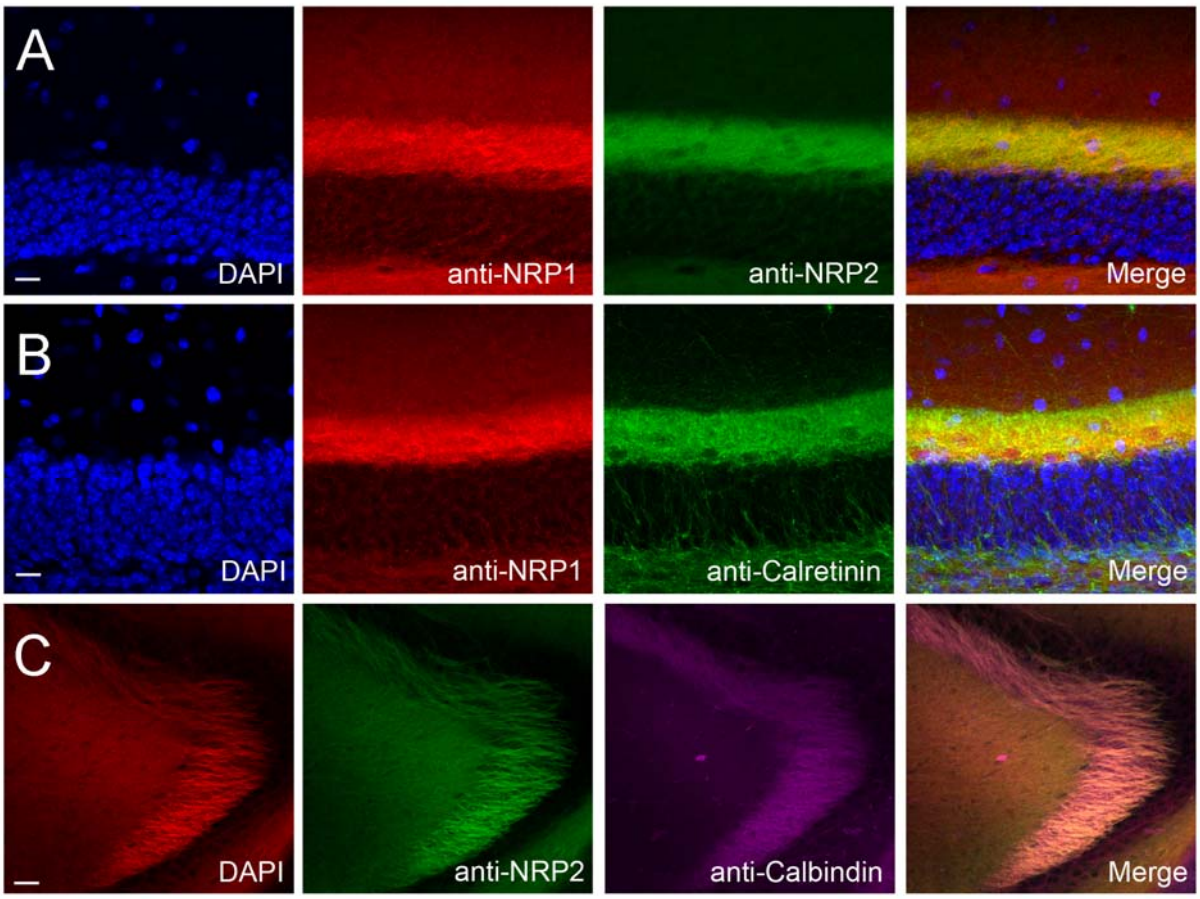
I carried out retroviral mediated knockdown of NRP1 and -2 in the adult hippocampus to investigate if knock down of NRP1 and -2 affect the cell fate of adult NPCs *in vivo*.

4.2. Results

4.2.1 Adult hippocampal tissues express Semaphorins and Neuropilins

I first confirmed the expression patterns NRP1 and -2 in adult hippocampal tissues and the specificity of antibodies used in this study. Immunostaining of 6 weeks old adult mouse hippocampal brain sections showed that NRP1 and NRP2 are strongly expressed in the inner molecular layer of the dentate gyrus (Figure 4-1A and B) but not in the soma of granule cells as described previously (Kawakami et al., 1996; Tran et al., 2009). Mossy fibers terminating in the CA3 regions also expressed NRP1 and NRP2 (Figure 4-1C). The staining patterns confirmed published data showing that NRP1 and NRP2 are expressed in dendrites and axons of granule cells in the adult dentate gyrus (Kawakami et al., 1996; Sahay et al., 2005; Tran et al., 2009) and provided evidence showing the our antibodies are specific for NRP1 and -2.

I attempted to perform immunohistochemistry using antibodies against Sema3A and 3F but I was unable to obtain specific staining on the brain sections (data not shown). While the antibodies can detect overexpressed Sema3A and 3F (Figure 3-3), the antibodies may not have sufficiently high titers or sensitivity to detect endogenously Sema3A and 3F in brain section. It is also possible that Sema3A and 3F are expressed at very low levels, beyond the detection limits of the antibodies. Another possible explanation is that the antibodies detect only the denatured forms of Sema3A and Sema3F in Western Blots but not their native conformations in brain tissues. Nonetheless, in-situ hybridization and binding studies have provided strong evidence showing that Sema3A and 3F are expressed in the adult dentate gyrus (Giger et al., 2000; Giger et al., 1998; Sahay et al., 2005; Tran et al., 2009). The principal source of Semaphorin ligands appeared to be secreted from axons of entorhinal cortex while other sources may be from hilar interneurons or granule cells.


A
DAPI
anti-NRP1
anti-NRP2
Merge
B
DAPI
anti-NRP1
anti-Calretinin
Merge
C
DAPI
anti-NRP2
anti-Calbindin
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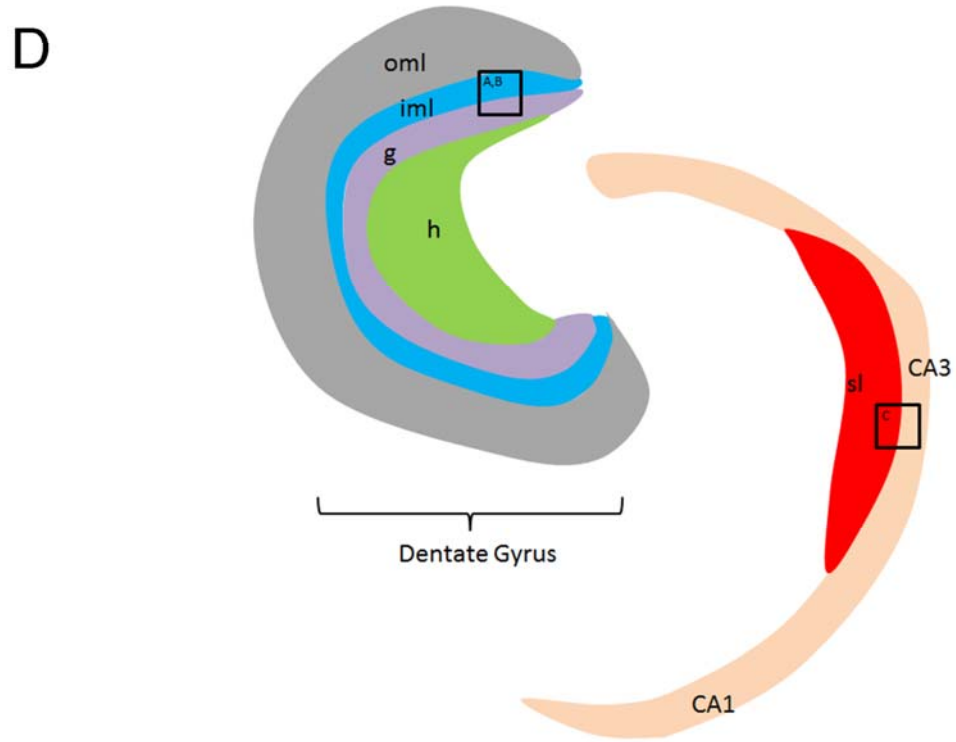
D
oml
iml
g
h
A,B
sl
CA3
C
CA1
Dentate Gyrus

Figure 4-1. Adult hippocampal tissues express Neuropilins. (A) NRP1 (Red) and NRP2 (Green) are expressed in the inner molecular layer of the adult dentate gyrus. (B) NRP1 (Red) co-localizes with calretinin (Green), a marker for the inner molecular layer of the dentate gyrus. (C) NRP1 (Red) and NRP2 (Green) are expressed in the mossy fibers of the dentate granule cells terminating at the CA3 region. Calbindin is used as a marker for mossy fibers. (D) Schematic diagram of the hippocampus. Strong expressions of Neuropilin-1 and -2 in (A) and (B) are seen in the inner molecular layer (boxed inserts labelled A, B) and expressions in (C) are seen in the stratum lucidum of CA3 (boxed insert C). h: hilus, g: granular layer, iml: inner molecular layer, oml: outer molecular layer, sl: stratum lucidum of CA3. Scale bar = 20 μm.

4.2.2 Adult NPCs express Semaphorins and Neuropilins

I cultured adult neural precursor cells from the hippocampus of 6 week old adult mice. The adult NPCs formed neurospheres, expressed stem cell markers, Nestin and Sox2 (Figure 4-2), demonstrated capacity for self-renewal through self-culturing and ability to differentiate into immature neurons, suggesting that our cultures comprised of bona fide adult neural precursor cells.

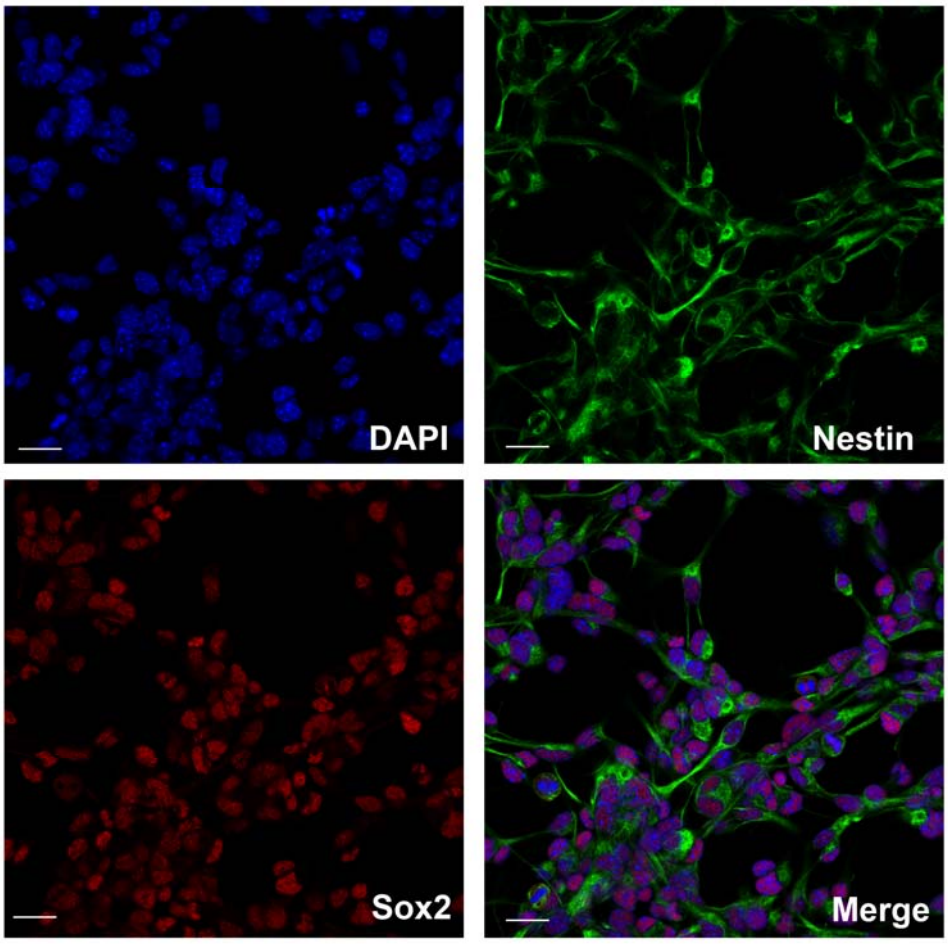

Figure 4-2. Adult neural precursor cells express stem cells markers. Adult NPCs co-express Nestin (Green) and Sox2 (Red). Panels show DAPI (Blue), Nestin (Green), Sox2 (Red) and merged channel. Scale bar = 20 μm.

I next checked if adult NPCs express NRP1 and -2 receptors. To do so, I extracted protein lysate from cortical, hippocampal primary neurons from adult animals, cultured adult NPCs, and HEK cells overexpressing NRP1 and -2. Western blotting revealed specific 100 kDa bands corresponding to NRP1 and -2 in cultured adult NPCs, cortical and hippocampal primary neurons and HEK overexpressing NRP1 and -2 (Figure 4-3). Hence, adult NPCs expressed Neuropilin receptors, suggesting that they could be important for neuronal development of adult-born neurons.

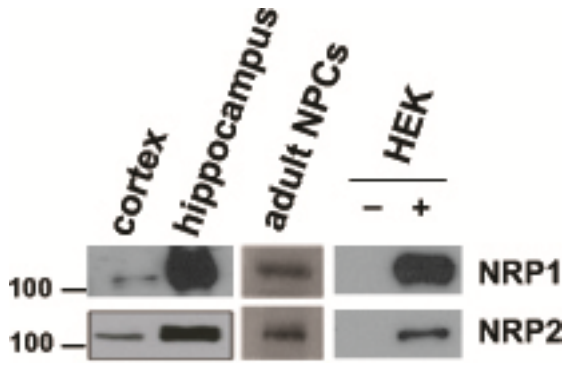

Figure 4-3. Adult neural precursor cells express Neuropilins. Representative western blots showing that NRP1 and NRP2 are expressed in cortical and hippocampal primary neuronal culture and adult hippocampal neural progenitor cells (NPCs). HEK 293 cells were overexpressed with NRP1 ('+', upper panel) and NRP2 ('+', lower panel) and probed with anti-NRP1 and anti-NRP2 respectively. Untransfected HEK 293 cell lysate ('-', upper and lower panels).

4.2.3 Semaphorins do not affect proliferation of adult NPCs

Having demonstrated that adult NPCs express NRP1 and -2, I first check if Semaphorins can regulate the proliferation and viability of adult NPCs culture. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a commonly used to measure proliferation and cell viability (Mosmann, 1983). It is based on the principle that NAD(P)H-dependent cellular oxidoreductase enzymes represent the number of viable cells under defined conditions, and reduce the water soluble MTT to an insoluble formazan. The formzan can then be solubilized in an organic solvent and the concentration determined by optical density at 570 nm, which give an estimate of the number of viable cells.

To check if Semaphorins regulate proliferation and cell viability in adult NPCs after seeding, I treated adult NPCs with different doses of AP, Sema3A-AP and Sema3F-AP (50, 100, 250, and 500 ng/ml) and allowed cells to proliferate for 48 hours prior to MTT assay. At seen in Figure 4-4, Sema3A and 3F treatments did not affect cell proliferation of adult NPCs *in vitro* at all doses tested, suggesting that Semaphorins do not affect cell proliferation and viability of adult NPCs *in vitro*.

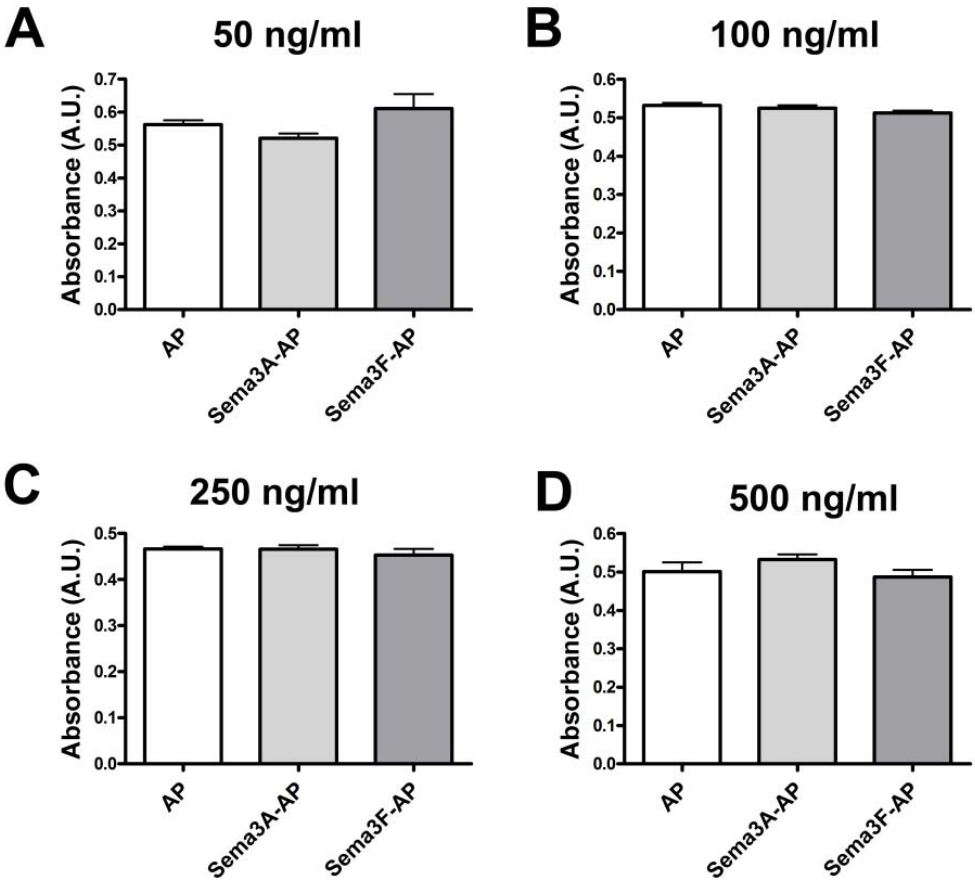

Figure 4-4. Semaphorins do not affect cell proliferation of adult NPCs. AP, Sema3A-AP, Sema3F-AP at different concentrations (A) 50 ng/ml (B) 100 ng/ml (C) 250 ng/ml and (D) and 500 ng/ml were added to adult NPCs for 48 hours. Cells were fixed for MTT assay and absorbances (OD_{570}) were measured. Sema3A-AP and Sema3F-AP do not affect proliferation at all doses tested. Values represent mean ± s.e.m. Statistical test was performed with 1-way ANOVA. No significant differences were found.

4.2.4 Semaphorins do not affect differentiation of adult NPCs

Next, I investigated if Semaphorins can regulate the differentiation of adult NPCs *in vitro*. Adult NPCs were induced to differentiate into immature neurons and treated with 500 ng/ml of AP, Sema3A-AP and Sema3F-AP respectively for 7 days. Cells were then fixed and stained with anti-DCX, a cell marker for immature neurons. The percentages of DCX+ cells in different treatment groups were quantified. Sema3A-AP and Sema3F-AP treatments

resulted in similar percentages of DCX+ immature neurons as AP control treatments. Hence, Semaphorins do not appear to regulate the differentiation of adult NPCs *in vitro* (Figure 4-5).

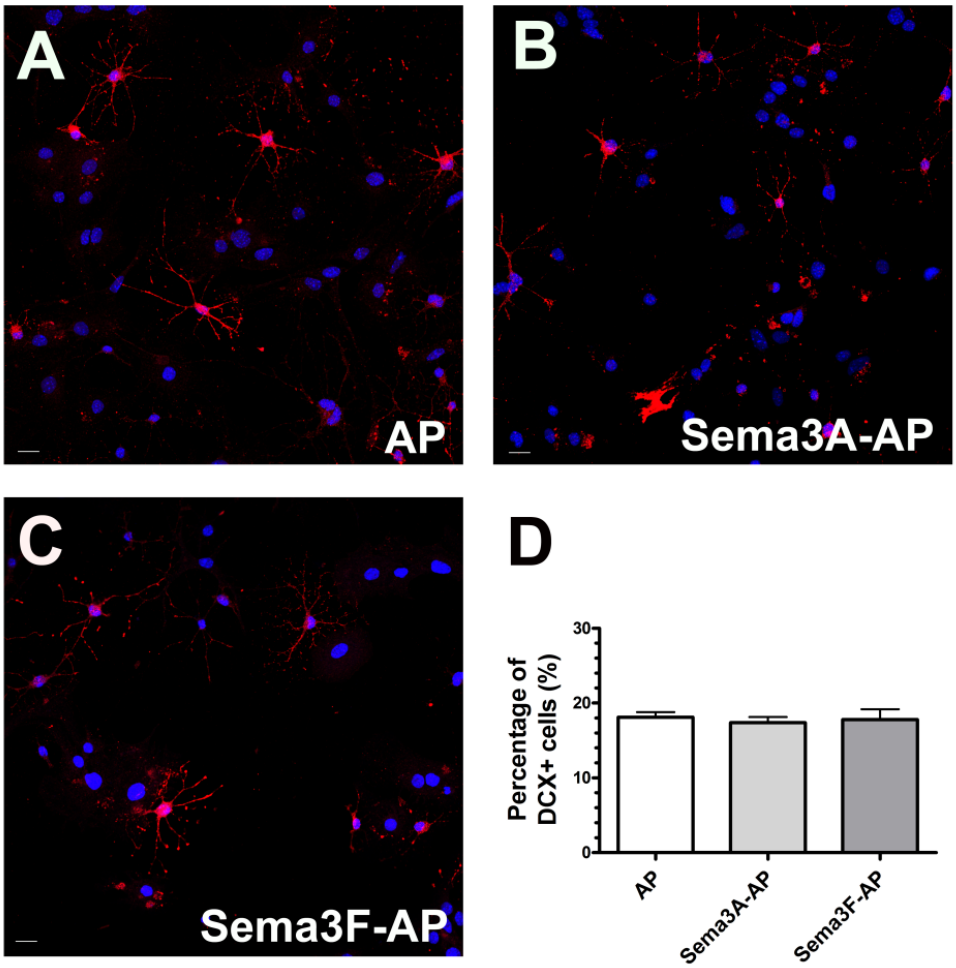

Figure 4-5. Semaphorins do not affect differentiation of adult NPCs. Representative images showing DCX+ cells (Red) in AP (A), Sema3A-AP (B) and Sema3F-AP (C) treatments. (D) Quantification of DCX+ cells in different treatment groups. Scale bar = 20 µm. Sema3A-AP and Sema3F-AP treatments have similar effects on adult NPCs differentiation as AP controls. Scale bar = 20 µm. Values represent mean ± s.e.m. Statistical test was performed with 1-way ANOVA. No significant difference was found.

4.2.5 Neuropilins do not affect neuronal differentiation *in vivo*

Finally, I checked if Neuropilin affects neuronal differentiation of adult-born neurons *in vivo*. To do so, I transduced adult-born neurons with stereotaxic injection of retroviruses into the DG of adult mice. These retroviruses express shRNA sequences that has no known target (shCTR) or sequences that knockdown Neuropilin-1 (shNRP1) or Neuropilin-2 (shNRP2). All retroviral-infected neurons (n=30) are positive for DCX and Prox1 expression, which are specific markers for immature neurons and dentate granule cells respectively after 14 days post infection (DPI) (Figure 4-6). Therefore, NRP1 or NRP2 knockdown *in vivo* do not appear to affect neuronal differentiation during adult hippocampal neurogenesis.

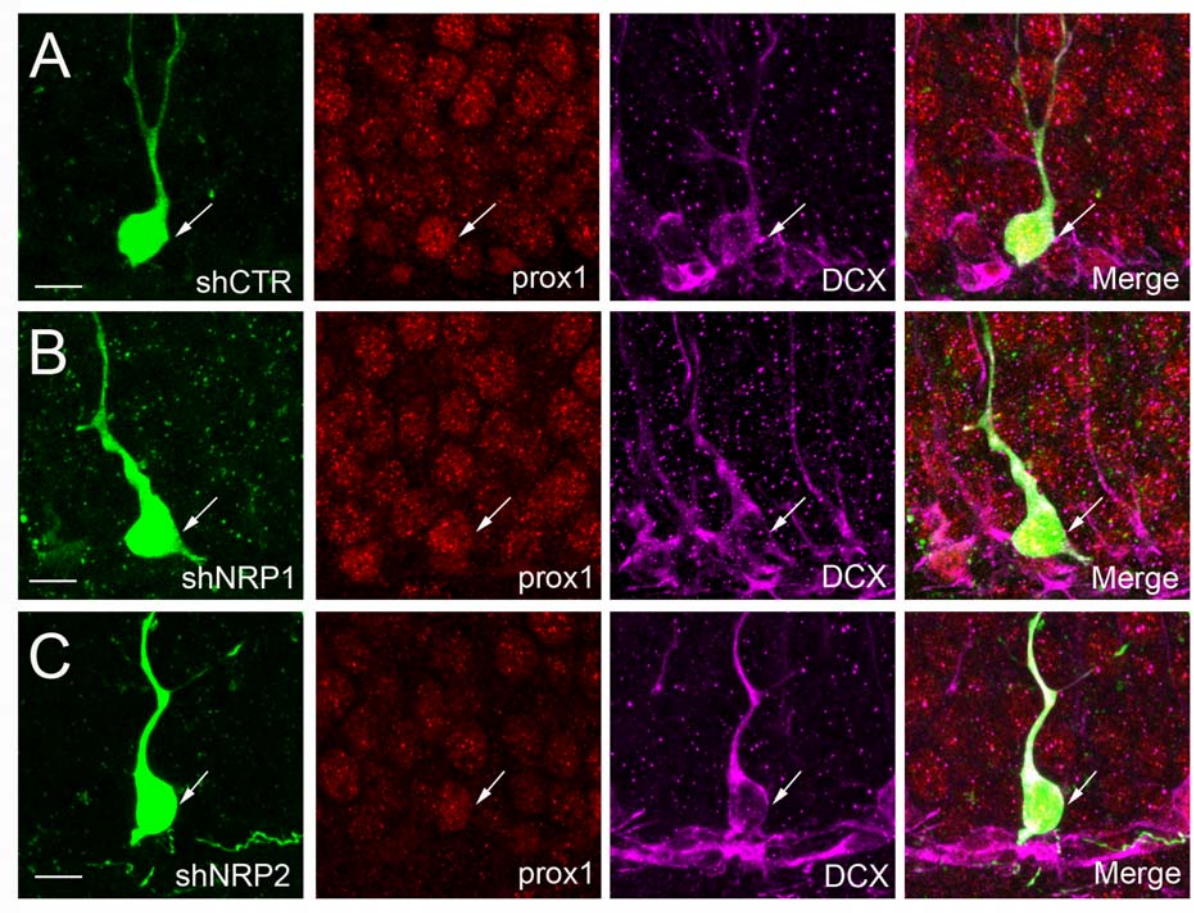

Figure 4-6. Neuropilins do not affect neuronal differentiation of adult-born neurons. Characterization of retrovirus labeled newborn cells in the adult DG. Retrovirus labeled GFP-positive cells express DG granular neuronal marker, Prox1 (red) and immature neuronal marker, DCX (pink). White arrows indicate colocalizations of different markers. Scale bar = 10 μm.

4.3. Discussion

In the adult hippocampus, Semaphorins RNA transcripts are found in axonal projections of entorhinal cortex (Giger et al., 1998), dentate granule cells (Barnes et al., 2003; Hirsch et al., 1999; Holtmaat et al., 2002), pyramidal cells of CA1 and CA3 (Barnes et al., 2003; Gong et al., 2003; Holtmaat et al., 2002) and interneurons (Alme et al., 2010; Barnes et al., 2003; Gong et al., 2003). NRP1 and -2 RNA transcripts are found in dentate granule cells, CA3 pyramidal cells and at lower levels in CA1 pyramidal cells (Giger et al., 2000; Holtmaat et al., 2003). High levels of NRP1 and -2 proteins are found in the axons and dendrites of dentate granule cells (Tran et al., 2009). I confirmed the expression of NRP1 and NRP2 proteins in the inner molecular layer and the stratum lucidum of the CA3 using specific antibodies against NRP1 and NRP2 respectively (Figure 4-1).

It was not clear in NRP1 and NRP2 are expressed in the adult neurogenic regions. To check this, I cultured adult neural precursor cells (NPCs) from hippocampal DG of 6 weeks old adult mice (Figure 4-2) and showed that NRP1 and -2 are expressed in these cells (Figure 4-3). The robust expression of Neuropilin receptors in the adult neural precursor cells led us to speculate that they could play important roles in adult neurogenesis.

Treating adult neural precursor cells with Sema3A and Sema3F ligands at different doses did not affect their proliferation and cell viability *in vitro*, as shown by the MTT assay (Figure 4-4). It was shown previously that Semaphorin 3A mediate apoptosis in neural progenitor cells (Bagnard et al., 2001). On closer inspection, the authors used a neuroectodermal progenitor

cell line that was derived from a medulloblastoma. It was also shown in other studies that Class 3 Semaphorins induce apoptosis in other tumor cell lines (Castro-Rivera et al., 2004; Wu et al., 2011a). These tumor cell lines required immortalization for passaging resulting in change the properties of the cells and would not serve as a good model for adult stem cells. Arguably, our NPCs derived from adult hippocampal tissue are a superior system to assess specifically the cell proliferation and viability of adult NPCs.

Semaphorins are required for differentiation of precursor cells into diverse cell types. For instance, the transmembrane proteins Sema6A regulate oligodendrocytes differentiation (Bernard et al., 2012) and Sema4C is required for myogenic differentiation (Ko et al., 2005). Class 3 Semaphorins have been implicated in osteoclast differentiation (Fukuda et al., 2013; Hayashi et al., 2012; Verlinden et al., 2013), pulmonary epithelial cell differentiation (Becker et al., 2011) and podocyte differentiation (Reidy et al., 2009). However, there were no published studies on whether Semaphorins affect the differentiation of adult neural precursor cells into neurons. In our study, Sema3A and Sema3F treatments (500 ng/ml) did not appear to affect the differentiation of adult progenitor cells into neuronal cells *in vitro* (Figure 4-5), suggesting that Class 3 Semaphorins are not required for differentiation of adult neural precursor cells. It would be interesting to test if other doses of Class 3 Semaphorins can promote or inhbibit diffentiation of adult neural precursor cells in future experiments. The adult neural precursor cells could be producing Class 3 Semaphorins and masking the effects of exogenous addition of Semaphorins ligands on differentiation. It would be interesting to check the expression of Semaphorins in adult NPCs culture and inhibit the endogenous expression by using anti-NRPs antibodies. Hence, our *in vitro* data supported the idea that Semaphorins do not affect the differentiation of adult-born neurons. All cells infected with retroviruses encoding shNRP1, shNRP1 and shCTR (n=30, per group), are co-labelled with Prox1 and DCX, which are markers for immature neurons (Figure 4-6).

In this chapter, I showed that Class 3 Semaphorins do not affect proliferation and differentiation of adult neural precursor cells *in vitro* and differentiation of adult neural precursors into immature neurons *in vivo*. Thus, Class 3 Semaphorins do not appear to regulate early proliferation and differentiation events during adult neurogenesis. In the next and subsequent chapters, I checked if Semaphorins can regulate other cellular events in adult neurogenesis, including the dendritic growth and cell positioning of adult-born neurons *in vivo*.

Chapter 5

Neuropilin-1 and -2 regulate dendritic growth of adult-born neurons via Cdk5/FAK

5.1. Introduction

5.1.1 Class 3 Semaphorins regulate dendritic growth of embryonic neurons

Sema3A is required for proper dendritic branching in CA1. In wildtype mice, approximately 7% of CA1 pyramidal cells display bifurcation at the proximal dendrite. This percentage was increased to 32% in Sema3A mutant mice (Nakamura et al., 2009). Only 30% of neurons cultured from Sema3A mutant mice showed pyramidal like morphology (Nakamura et al., 2009). CA1 pyramidal neurons from p35 mutant mice display similar proximal bifurcation defects, suggesting that p35/Cdk5 may act downstream of Sema3A in regulating dendritic branching (Nakamura et al., 2009).

A separate study confirmed the proximal bifurcation phenotype in CA1 pyramidal cells of Sema3A mutant mice (Schlomann et al., 2009). The authors also showed that dissociated hippocampal neurons from Sema3A mutant mice have shorter dendritic length compared to wildtype, and dendritic growth could be rescued by treatment with Sema3A ligands (Schlomann et al., 2009). Sema3A-induced dendritic growth in primary neurons are blocked in neurons defective in Focal Adhension Kinase (FAK), suggesting that FAK is an important downstream mediator of Semaphorin signaling in regulating dendritic growth (Schlomann et al., 2009).

Apart from the hippocampus, Sema3A also appears to regulate dendritic branching and length in the cortex (Fenstermaker et al., 2004). Scholl analysis revealed that cortical neurons in brain slices from Sema3A mutant mice are less complex than wild type animals (Fenstermaker et al., 2004). Exogenous addition of Sema3A could rescue the dendritic growth of cortical neurons in Sema3A mutant mice (Fenstermaker et al., 2004).

5.1.2 Do Class 3 Semaphorins regulate dendritic growth of adult-born neurons?

While it was shown that Semaphorins regulates dendritic growth of cortical pyramidal and hippocampal CA1 neurons in the embryonic nervous system (Fenstermaker et al., 2004; Nakamura et al., 2009; Schlomann et al., 2009), it is unclear if Semaphorins can regulate the dendritic growth of adult-born neurons. Using retroviral-mediated approach, I attempted to knockdown Neuropilin-1 and -2 in neural precursor cells in the adult SGZ and analysed the dendritic morphology of adult-born neurons.

In addition, it is apparent from many studies that Class 3 Semaphorins activate a plethora of intracellular kinases to regulate diverse cellular effects during embryonic development (Franco and Tamagnone, 2008; Zachary, 2011). Some of the important kinases include cdk5 (Sasaki et al., 2002), FAK (Bechara et al., 2008; Chacon et al., 2010) and GSK3β (Cole et al., 2006; Lerman et al., 2007; Shelly et al., 2011). It is plausible that the same intracellular kinases act downstream of Class 3 Semaphorin signaling in adult neurogenesis.

Using neural progenitors and primary neuronal culture, I can address if these kinases are important downstream mediators. Specifically, I tested Cdk5 and FAK as both molecules have been implicated in dendritic growth of hippocampal neurons (Nakamura et al., 2009; Schlomann et al., 2009). To confirm the effects *in vivo*, I adopted the same retroviral

mediated approach to target Cdk5 and FAK in the adult SGZ and ask if the effects phenocopy that of neuropilins knockdown. Lastly, to prove that these intracellular kinases are indeed downstream mediators of Class 3 Semaphorins, I can overexpress transgenes to rescue neuropilins knockdown phenotypes in the adult SGZ.

5.2. Results

5.2.1 NRP1 and -2 regulate the dendritic development of adult-born neurons

To determine the role of Sema3A/NRP1 and Sema3F/NRP2 signaling in adult neurogenesis, I employed a retrovirus-mediated method for birth-dating and genetic manipulation of individual newborn neurons in the adult mouse dentate gyrus. I stereotaxically injected high titers of engineered retroviruses into the hilar region of the adult C57BL/6 mouse hippocampus to infect proliferating neural progenitors *in vivo*. Mice were analyzed at 14 days post-infection (dpi). Representative images of the dendritic arborization of these labelled neurons are shown in Figure 5-1A. Interestingly, neurons expressing shNRP1 or shNRP2 exhibited significantly shorter total dendritic length (Figure 5-1B) and total branch number (Figure 5-1C) compared to neurons expressing shCTR at 14 dpi. Sholl analysis further demonstrated a decrease in the dendritic complexity of these neurons (Figure 5-1D).

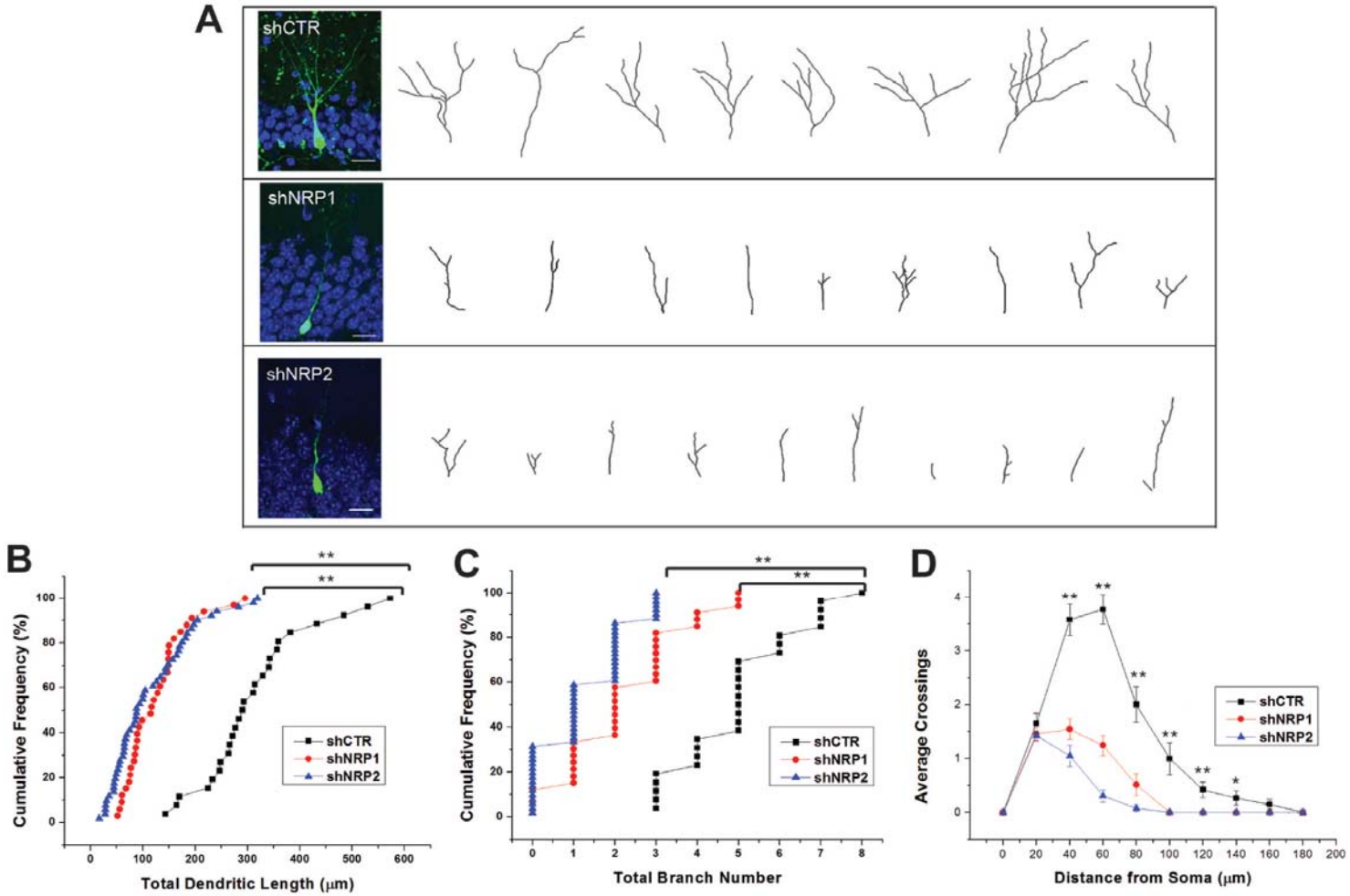

Figure 5-1. NRP1 and NRP2 regulate dendritic morphology of adult-born neurons (14 DPI). (A) Representative images and tracings of dendrites of control, NRP1 or NRP2-shRNA expressing DGCs at 14 dpi. Scale bar = 20 μm. (B, C) Quantification of total dendritic length and branch number of newborn DGCs. Each symbol represents a single DGC. (**P<0.01, 1-way ANOVA with Newman-Keuls' post hoc test). (D) Graph showing dendritic complexity of GFP^+ DGCs. Values represent mean ± s.e.m. (**P<0.01, *P<0.05, 1-way ANOVA with Newman-Keuls' post hoc test).

Adult-born neurons increase their dendritic complexity during their maturation. To check if Class 3 Semaphorins Signaling is required for continued dendritic growth during development, mice injected with shNRP1 and shNRP2 were fixed at 28 dpi. Adult-born neurons expressing shNRP1 exhibited shorter dendritic length (Figure 5-2A) and total branch number (Figure 5-2B) compared to neurons expressing shCTR at 28 dpi. Taken together, these data suggest that Class 3 Semaphorins regulate the initial dendritic elaboration and subsequent dendritic growth of adult-born neurons.

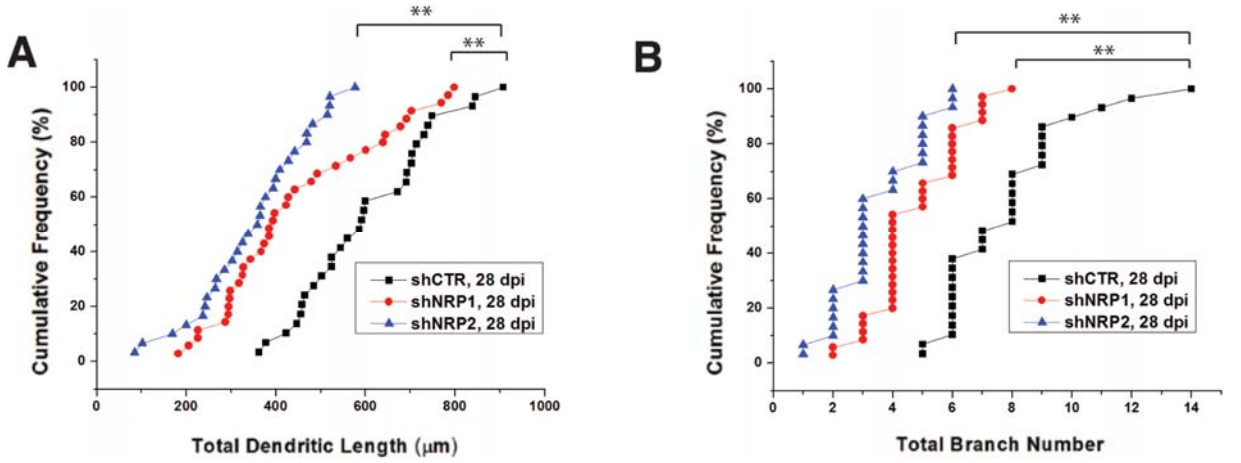

Figure 5-2. NRP1 and -2 regulate dendritic morphology of adult-born neurons (28 DPI). Quantification of total dendritic length and branch number of newborn DGCs at 28 DPI. Graph showing dendritic length (A) and total branch no (B) of GFP^+ DGCs. Each symbol represents a single DGC. (**P<0.01, 1-way ANOVA with Newman-Keuls' post hoc test). Values represent mean ± s.e.m. (**P<0.01, *P<0.05, 1-way ANOVA with Newman-Keuls' post hoc test).

5.2.2 RNAi refractory forms of NRP1 and -2 rescue dendritic phenotypes of shNRP1 and shNRP2 adult-born neurons respectively

To confirm the specificity of shRNA manipulations *in vivo*, I investigated if the dendritic phenotypes of NRP1 and -2 knockdown neurons could be reversed or rescued by cDNA encoding for RNAi refractory forms of NRP1 and -2 respectively. These mutated NRP1 and NRP2 are resistant to knockdown by the shRNAs used (Figure 3-2) and could completely

rescue the dendritic phenotypes of the neurons deficient in NRP1 or NRP2 (Figure 5-3) respectively. These results demonstrated the specificities of the shRNA used and confirmed the role of NRP1 and 2 in dendritic outgrowth of adult-born neurons *in vivo*.

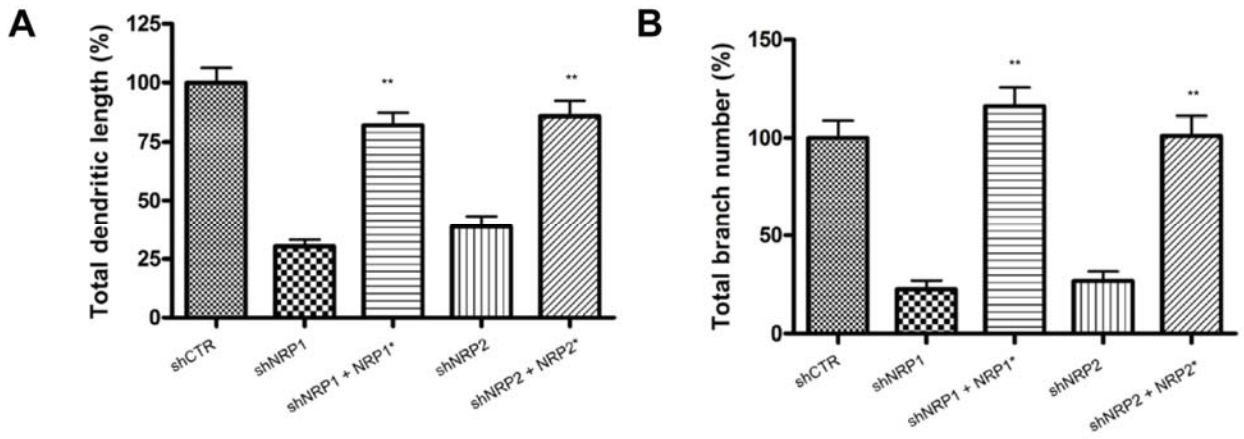

Figure 5-3. RNAi refractory forms of NRP1 and -2 rescue dendritic phenotypes of shNRP1 and shNRP2 adult-born neurons respectively. Overexpression of shRNA-resistant NRP1 and NRP2 rescues phenotype induced by NRP1 and NRP2 shRNA. Mixture of retrovirus expressing the indicated constructs was injected into dentate gyrus. Quantification of total dendritic length (A) and branch number (B) of newborn DGCs at 14 DPI. 30-50 neurons from each group were used for analysis. (**P<0.01, 1-way ANOVA with Newman-Keuls' post-hoc test). NRP1*: NRP1 shRNA-resistant NRP1, NRP2*: NRP2 shRNA-resistant NRP2.

5.2.3 NRP1 mediated dendritic development of adult-born neurons is independent of vascular endothelial growth factors (VEGF)

Since NRP1 can bind to VEGF (Gu et al., 2003), I next sought to prove that these phenotypes are not linked to the function of VEGF. I made use of a knockin line (KI) that expresses a NRP1 variant that lost the ability to bind to Sema3A but has intact VEGF binding site (Gu et al., 2003). Expressing Cre recombinase (Cre) in neural progenitor cells in NRP1 conditional knockout mice (CRE NRP1 Floxed/Floxed) and another transgenic line expressing a single copy of NRP1 variant (CRE NRP1 Floxed/KI) resulted in adult-born neurons with impaired dendritic formations compared to mice expressing a single copy of wild type NRP1 (CTR

NRP1 Floxed/KI) (Figure 5-4). These data confirming our results using shRNA against NRP1 and provided direct evidence that the dendritic phenotypes of NRP1 deficiency I observed was not linked to the function of VEGF.

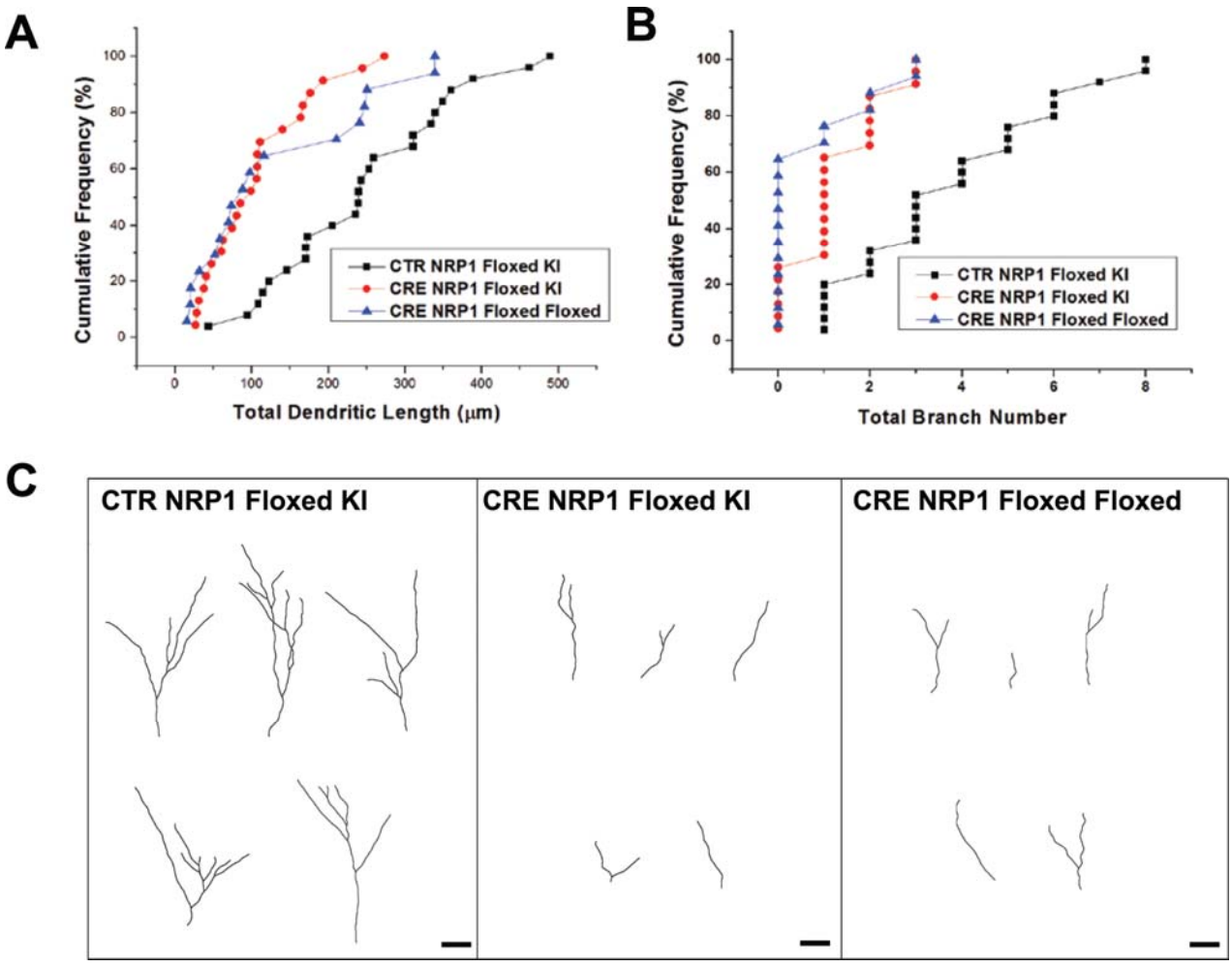

Figure 5-4. NRP1 mediated dendritic development VEGF. Single cell knockout of NRP1 in neural progenitors results in neurons with impaired dendritic formations is independent of vascular endothelial growth factor (VEGF)-binding of NRP1. (A) Graphs showing quantification of total dendritic length (A) and branch number (B) of newborn DGCs expressing GFP (CTR) or Cre-recombinase (CRE) at 14 DPI in NRP1 conditional knockout mice (NRP1 Floxed/Floxed) or mice expressing one single copy of Floxed-NRP1 and a knockin (KI) of an altered ligand binding site variant of NRP1 (NRP1 Floxed/KI). (C) Representative tracings of dendritic morphology of adult-born neurons from the above indicated groups. Scale bar = 20 μm.

5.2.4 Knockdown of Sema3A and 3F affects dendritic morphology of adult-born neurons

The principal source of Sema3A appears to be secreted by axons of layer II neurons, forming a gradient in the outer two-thirds of the molecular layer, which gradually declines towards the granule cell layers (Giger et al., 1998). Interestingly, the expression of Sema3A and Sema3F in various cell types of adult hippocampus, including the dentate granule cells (Sahay et al., 2005; Tran et al., 2009), suggests that there could be additional autocrine and/or paracrine sources of Class 3 Semaphorins. I was intrigued if adult progenitor cells secrete Class 3 Semaphorins which direct their own dendritic growth cell autonomously. To test this, I employed the same retroviral mediated approach to knock down Sema3A and Sema3F in neural precursor cells *in vivo*. Adult-born neurons expressing shSema3A or shSema3F exhibited significantly shorter total dendritic length (Figure 5-5A) and total branch number (Figure 5-5B) compared to neurons expressing shCTR at 14 dpi. These data suggests that Sema3A and Sema3F could contribute cell-autonomously to the dendritic development of adult-born neurons *in vivo*.

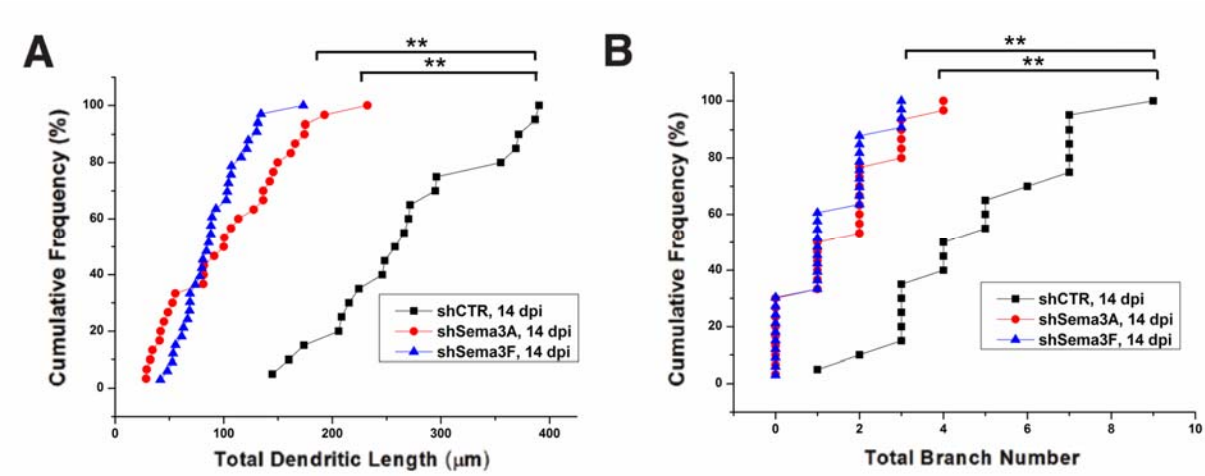

Figure 5-5. Knockdown of Sema3A and 3F affect dendritic morphology of adult-born neurons. Quantification of total dendritic length and branch number of shSema3A and shSema3F neurons at 14 DPI. Graph showing dendritic length (A) and total branch no (B) of GFP^+ DGCs. Each symbol

represents a single DGC. (**P<0.01, 1-way ANOVA with Newman-Keuls' post hoc test). Values represent mean ± s.e.m. (**P<0.01, *P<0.05, 1-way ANOVA with Newman-Keuls' post hoc test).

5.2.5 Sema3A and 3F stimulate dendritic growth in cultured neurons

Since primary neurons and adult-born neurons shared many signaling mechanisms, I explored the signaling mechanisms underlying Semaphorin regulation of dendritic growth using primary hippocampal neurons that allow isolation of bigger amount of cells as a model system. I would then investigate if the same signaling mechanism is conserved in adult-born neurons *in vivo*. Class 3 Semaphorin was initially identified to be axon repulsive cues (Tran et al., 2007), so I first checked the efficacy of our Semaphorins ligands. Sema3A-AP and Sema3F mediates axonal collapse but not dendritic growth cone collapse of hippocampal neurons after 30 min after addition of ligand to the media (Figure 5-6).

Next, I tested the effects on Sema3A and Sema3F in dendritic growth of primary embryonic hippocampal neurons. Alkaline phosphatase (AP) fused semaphorin 3A (AP-Sema3A) treatment at 2 DIV (days in vitro) for 16 hrs increased the total dendrite length by 60 % at 189.10 ± 13.59 μm, as compared to 118.30 ± 6.55 μm in AP-treated control neurons (Figure 5-7B). Similarly, AP-fused semaphorin 3F (Sema3F) treatment also increased the total dendrite length (184.30 ± 8.64 μm) (Figure 5-7B). The number of branches per neuron was also significantly increased upon Sema3A or Sema3F treatment (Figure 5-7C). Axonal length, on the other hand, was not significantly affected by treatment with either Sema3A or 3F (Figure 5-7D).

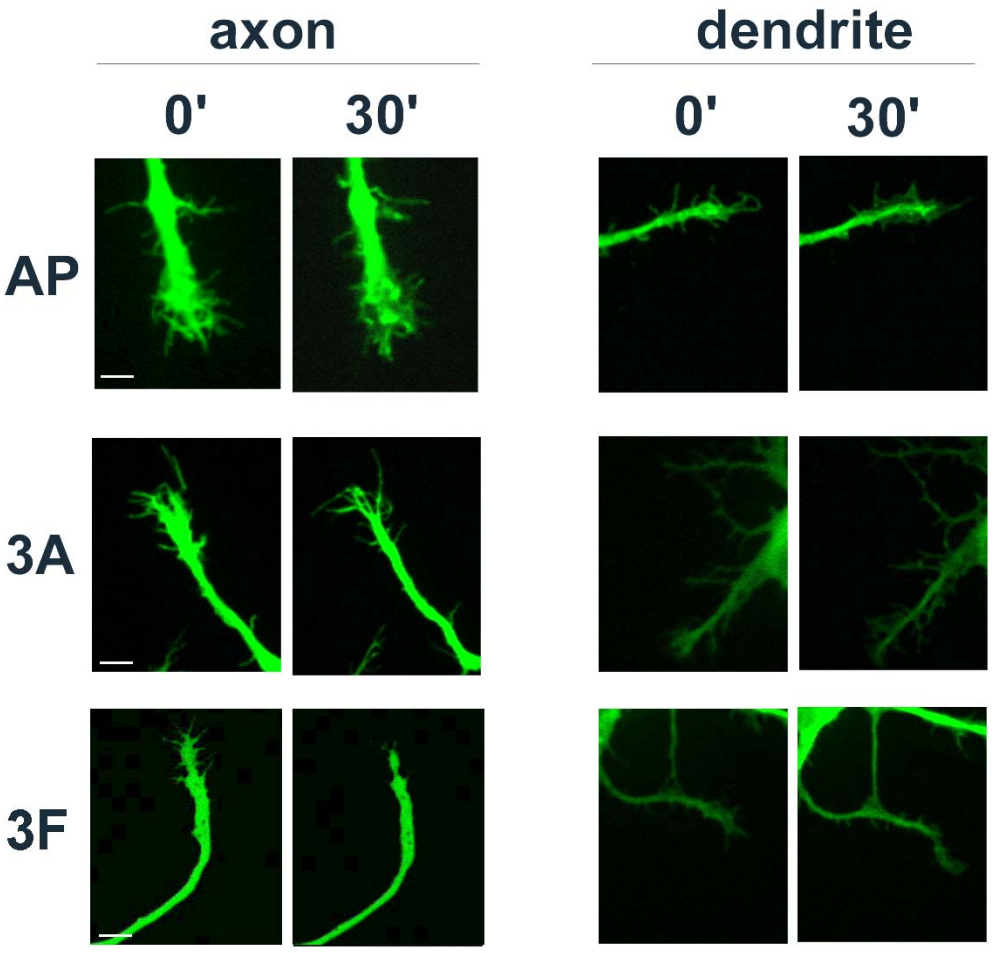

Figure 5-6. Sema3A-AP and Sema3F mediates axonal collapse but not dendritic growth cone collapse. Hippocampal neurons treated with AP, Sema3A-AP and Sema3F-AP ligands and imaged for 30 minutes. Semaphorins mediate axonal, but not dendritic growth cone collapse. Scale bar = 10 μm.

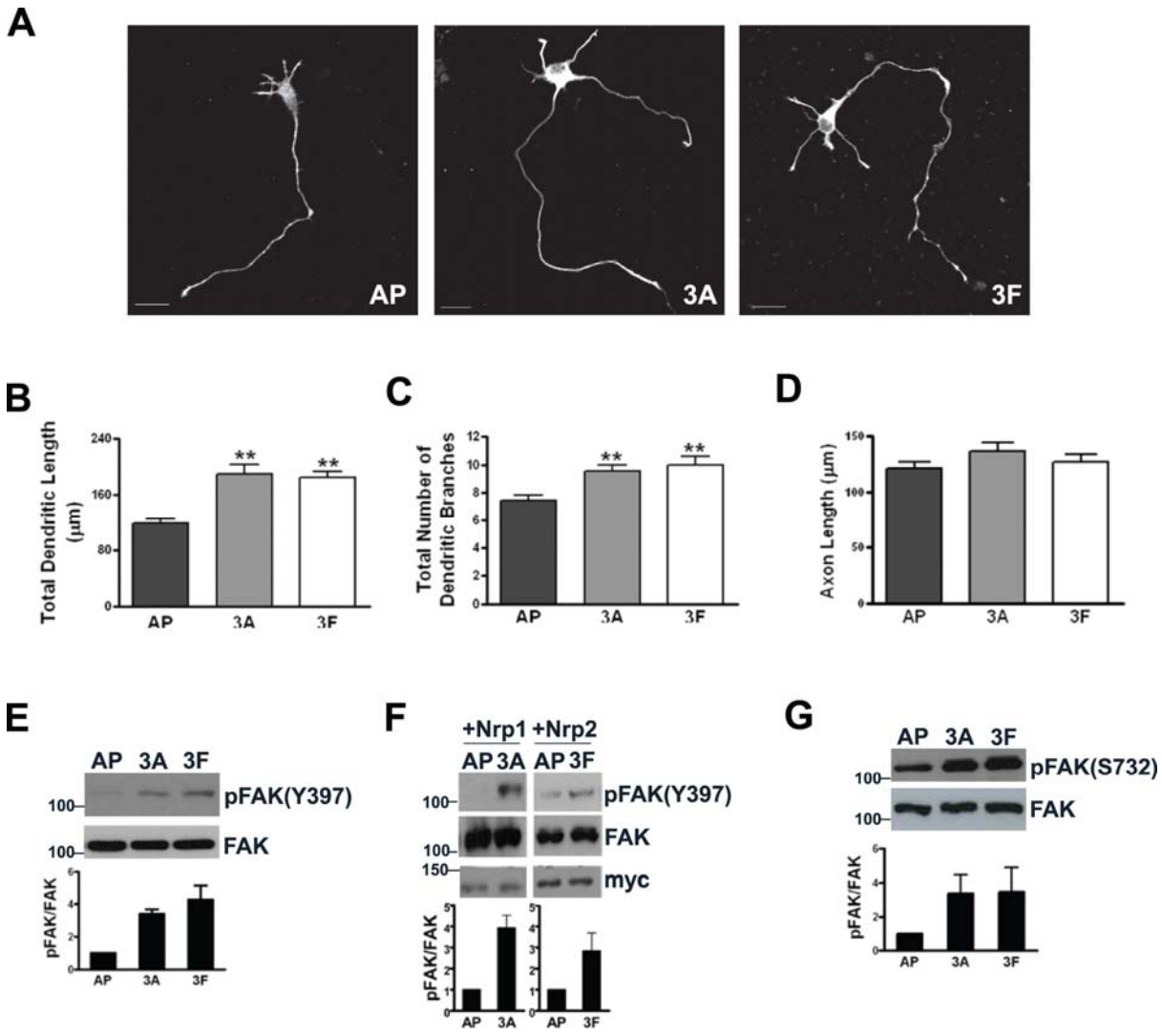

Figure 5-7. Semaphorin 3A/3F increased dendritic growth in primary neurons and induce tyrosine and serine phosphorylation of FAK. (A) Representative images showing neurons treated with AP-control (AP), Sema 3A-AP (3A) and 3F-AP (3F). Scale bar = 20 μm. (B, C and D) Graphs showing total dendritic length (B), total number of dendritic branches (C) and axon length (D) of primary hippocampal neurons treated with AP control, Sema3A or Sema 3F. (E, F and G) Representative blots showing phosphorylation of FAK in primary neurons (E and G) and 293T cells (F) treated with AP control, Sema3A or 3F. Graph displays change relative to total FAK and values are mean ± s.e.m, n=3 independent experiments. 40-50 neurons from each group were used for analysis. Values represent mean ± s.e.m. (**P<0.01, 1-way ANOVA with Newman-Keuls' post-hoc test).

5.2.6 Phosphorylation of FAK regulates dendritic outgrowth in cultured neurons

I next examined the potential involvement of FAK signaling in Semaphorin-mediated dendritic growth in cultured hippocampal neurons. Tyrosine residue (Tyr) 397 and serine residue (Ser) 732 were both phosphorylated in response to Sema3A or 3F in cultured hippocampal neurons (Figure 5-7E, G) and HEK293T cells (Figure 5-7F). Since 293T cells express endogenous Plexin As and L1, but not NRP1 and -2, I overexpressed NRP1 or NRP2 for Sema3A or Sema3F stimulation respectively. These results indicated that Sema3A and 3F signaling led to tyrosine and serine phosphorylation of FAK in primary hippocampal neurons and that phosphorylation of these two residues might be involved in dendritic growth regulated by Sema 3A and 3F.

5.2.7 Tyrosine and serine phosphorylation of FAK are are essential for Sema3A and 3F-induced dendritic growth

To dissociate the role of individual critical tyrosine and serine residues in dendrite growth, single tyrosine mutant FAK-Y397F, single serine mutant FAK-S732A and the double mutant, FAK-Y397F/S732A, were generated and overexpressed in HEK293T cells (Figure 5-8B). Mutation of S732 did not affect phosphorylation at Y397 and S732 phosphorylation was also not affected in FAK-Y397F expressing cells (Figure 5-8B), indicating that phosphorylation of serine and tyrosine residues of FAK were regulated independently. Expression of these phosphorylation deficient FAK mutants decreased basal dendritic growth of hippocampal neurons and also abolished dendritic growth induced by Sema3A or 3F (Figure 5-8C, D and E). Quantitatively, dendritic growth inhibition by the double mutant FAK-Y397F/S732A was similar to that seen with each single mutant. These results indicated that phosphorylation of both tyrosine and serine residues of FAK were required for dendritic growth induced by Sema3A or 3F (Figure 5-8C, D and E). In particular, the involvement of serine phosphorylation of FAK in dendritic growth by Seamphorin led us next to explore upstream signaling molecules of FAK in Semaphorin signaling pathway.

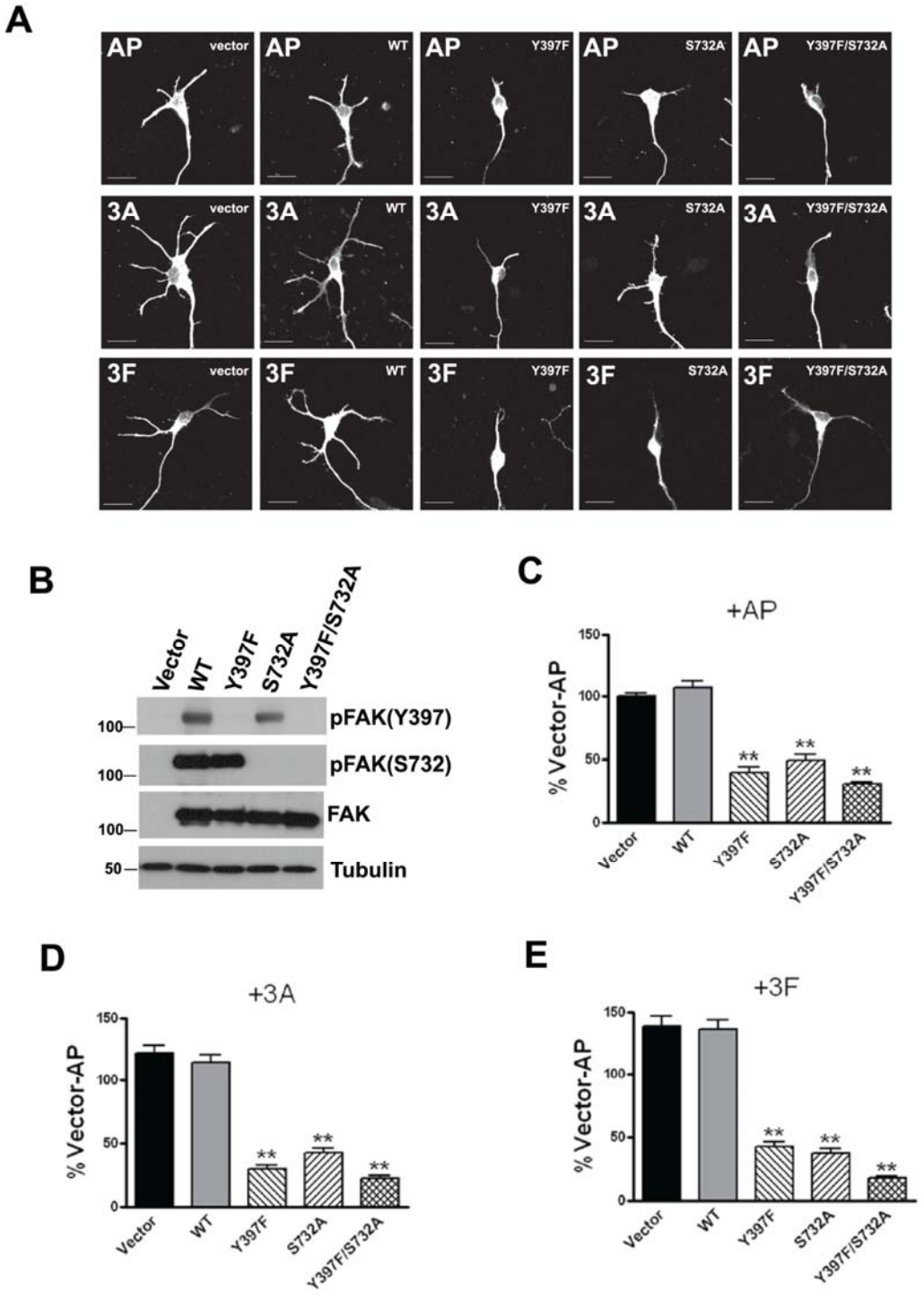

Figure 5-8. Expression of FAK phosphorylation-defective mutants in primary neurons impaired semaphorin-induced dendritic growth. (A) Representative images of primary hippocampal neurons transfected with vector, wild-type FAK (WT), Y397F FAK (Y397F), S732A FAK (S732A) and Y397F/S732A FAK (Y397F/S732A). Neurons were treated with AP control (AP), 3A-AP (3A) or 3F-AP (3F). Scale bar = 20 μm. (B) HEK293T cells transfected with the indicated constructs encoding FAK or its mutants validating the phosphorylation status of these constructs. (C, D and E) Graphs showing percentage dendritic growth of primary hippocampal neurons expressing FAK mutant constructs as indicated, treated with AP control (C), Sema3A (D) or 3F (E), with percentages normalized to Vector+AP. 30-100 neurons from each group were used for analysis. Values represent mean ± s.e.m. (**P<0.01, 1-way ANOVA with Newman-Keuls' post-hoc test).

5.2.8 Serine phosphorylation of FAK is dependent on cyclin dependent kinase 5 (Cdk5)

Cdk5 mediates regulation of dendrite orientation by Sema3A in the cerebral cortex (Lin et al., 2004). To determine if Cdk5 is involved in Sema3A or 3F-induced dendritic growth, I examined the activation of Cdk5 upon Sema3A or 3F treatment in HEK 293T cells transfected with NRP1 or 2. Addition of Sema3A/3F resulted in significant activation of Cdk5 as compared to control (Figure 5-9A). The phosphorylation of DCX, another known substrate of Cdk5, confirmed Cdk5 activation upon treatment with Sema3A or 3F in hippocampal neurons (Figure 5-9B). Serine phosphorylation of FAK and DCX induced by Sema3A or 3F were decreased upon treatment with roscovitine, which inhibits the activity of Cdk5 (Figure 5-9B). As expected, tyrosine phosphorylation of FAK and paxillin were not affected by roscovitine treatment (Figure 5-9B). These results suggested that FAK acted downstream of Cdk5 in Sema3A or 3F signaling. Next, I examined if the activation of Cdk5 was required for the increased in dendritic growth upon Sema3A or 3F treatment. Roscovitine treatment inhibited Sema3A or 3F-mediated increase in total dendritic length of the cultured hippocampal neurons (Figure 5-9C) indicating that Cdk5 played a role in dendritic growth regulated by Sema3A or 3F, likely through the activation of FAK.

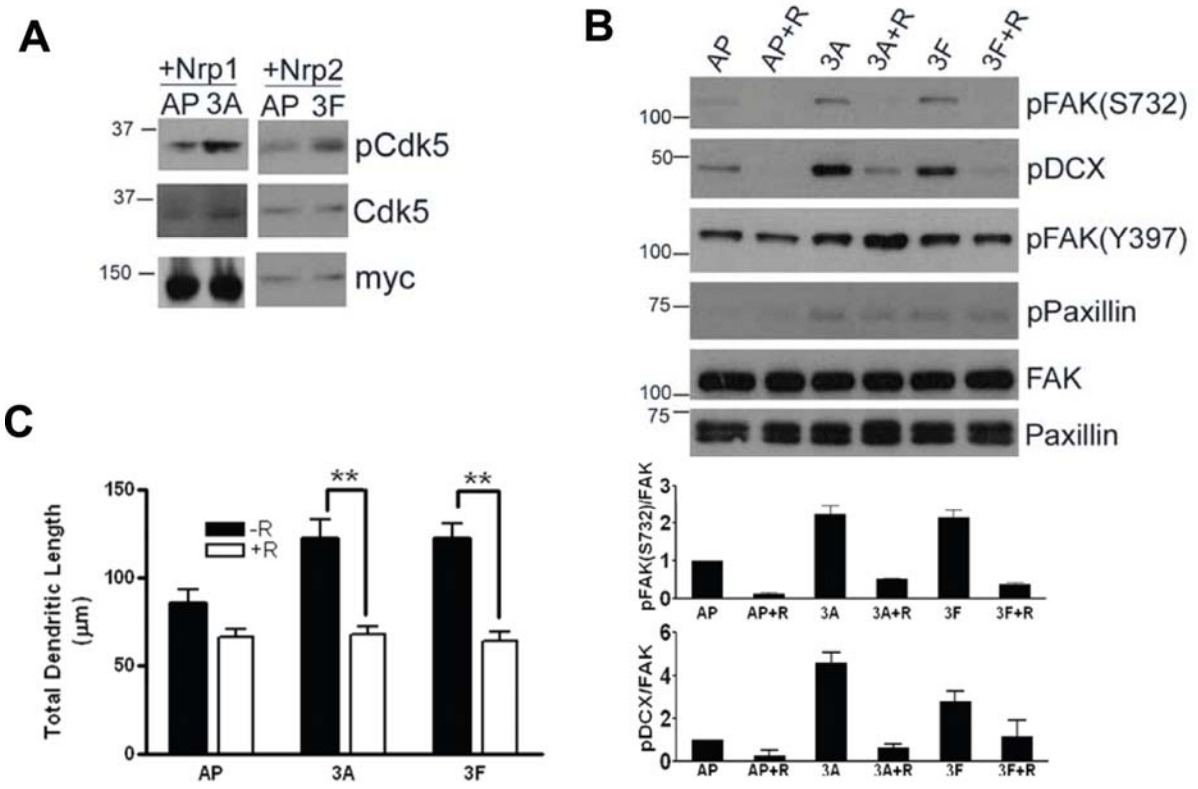

Figure 5-9. Cdk5 was required for regulating dendritic morphology of primary neurons. (A) Representative blots showing phosphorylation of Cdk5 in HEK293T cells transfected with NRP1 or 2 as indicated, treated with AP control, Sema3A or 3F. (B) Representative blots showing phosphorylation of FAK, paxillin and DCX in primary hippocampal neurons pretreated with vehicle (-R) or roscovitine (+R) prior to AP-control, Sema3A or 3F treatment. Graphs displays changes relative to FAK and values are mean ± s.e.m, n=3 independent experiments. (C) Graph showing percentage dendritic growth of primary hippocampal neurons treated with vehicle (-R) or roscovitine (+R) together with AP, Sema3A or 3F. 50 neurons from each group were used for analysis. Values represent mean ± s.e.m. (**P<0.01, 2-way ANOVA with Bonferroni's post-hoc test).

5.2.9 Overexpression of FAK rescues inhibition of dendritic outgrowth by roscovitine

To address if Cdk5 mediates dendritic growth of Sema3A or 3F through FAK, I examined if activation of FAK could rescue the inhibition of dendritic growth by roscovitine in Sema3A or 3F treated neurons. I found that overexpression of wild type FAK could overcome the inhibition of serine phosphorylation by roscovitine in HEK cells (Figure 5-10B). Next, I overexpressed wild-type FAK in primary neurons to determine if dendritic growth is restored in the presence of roscovitine. Overexpression of wild-type FAK alone did not affect the dendritic length in basal state comparable to GFP (vector) expression in neurons (Figure 5-10A and C). Treatment with Sema3A/3F increased dendrite length to a similar extent in GFP or wild-type FAK expressing neurons (Figure 5-10C, D and E). Addition of roscovitine significantly reduced the total dendrite length of GFP expressing neurons (Figure 5-10B, C and D). However, this reduction in dendritic length was rescued by overexpression of wild-type FAK (Fig 5-10B, C and D). There is residual S732 phosphorylation of FAK even after roscovitine inhibition in HEK cells (Figure 5-10B), suggesting that these amount of phosphorylation is sufficient to rescue the dendritic phenotype in primary neurons. These results demonstrated that Cdk5-mediated dendritic growth stimulated by Sema3A or 3F through the activation of FAK.

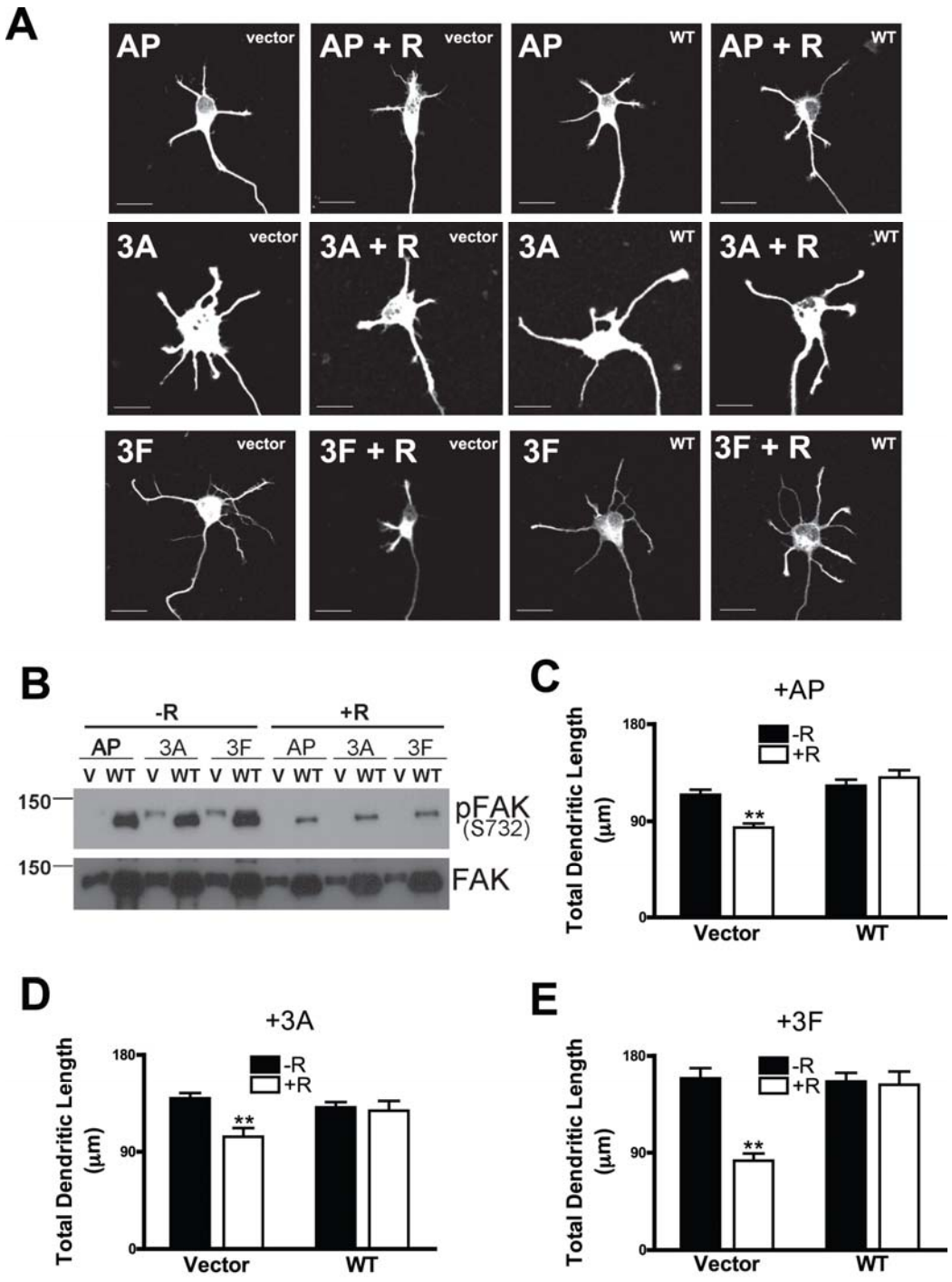

Figure 5-10. Roscovitine inhibition of Sema3A or 3F-induced dendritic growth was rescued by overexpression of wild-type FAK. (A) Representative images of primary hippocampal neurons transfected with vector or wild-type FAK (WT). Neurons were treated with AP control (AP), 3A-AP (3A), 3F-AP (3F), AP with roscovitine pre-treatment (AP + R), 3A-AP or 3F-AP with roscovitine pre-treatment (3A + R). Scale bar = 20 μm. (B) Representative blots showing phosphorylation of FAK in primary hippocampal neurons transfected with vector or WT-FAK treated with AP, Sema3A or Sema 3F together with vehicle (-R) or roscovitine (+R). (C, D and E) Graphs showing total dendritic length of primary hippocampal neurons expressing GFP (vector) or WT-FAK and treated with vehicle or roscovitine together with AP control (C), Sema3A (D) or 3F (E). 30-100 neurons from each group were used for analysis. Values represent mean ± s.e.m. (**P<0.01, 2-way ANOVA with Bonferroni's post-hoc test).

5.2.10 Cdk5 could not rescue dendritic morphology of neurons expressing FAK dominant negative constructs

To further confirm that Cdk5 is upstream of FAK, I reasoned that Cdk5 would not rescue dendritic morphology of neurons transfected expressing S732A FAK mutant construct. I co-expressed single serine mutant FAK-S732A in neurons with either wildtype Cdk5 or empty vector (mCherry) (Figure 5-11). FAK-S732A inhibited Semaphorin-induced dendritic growth and overexpression of WT Cdk5 was not able to rescue the effect of this dominant negative FAK-S732A (Figure 5-11). This result demonstrated that FAK is downstream of Cdk5 in Semaphorin signaling pathway.

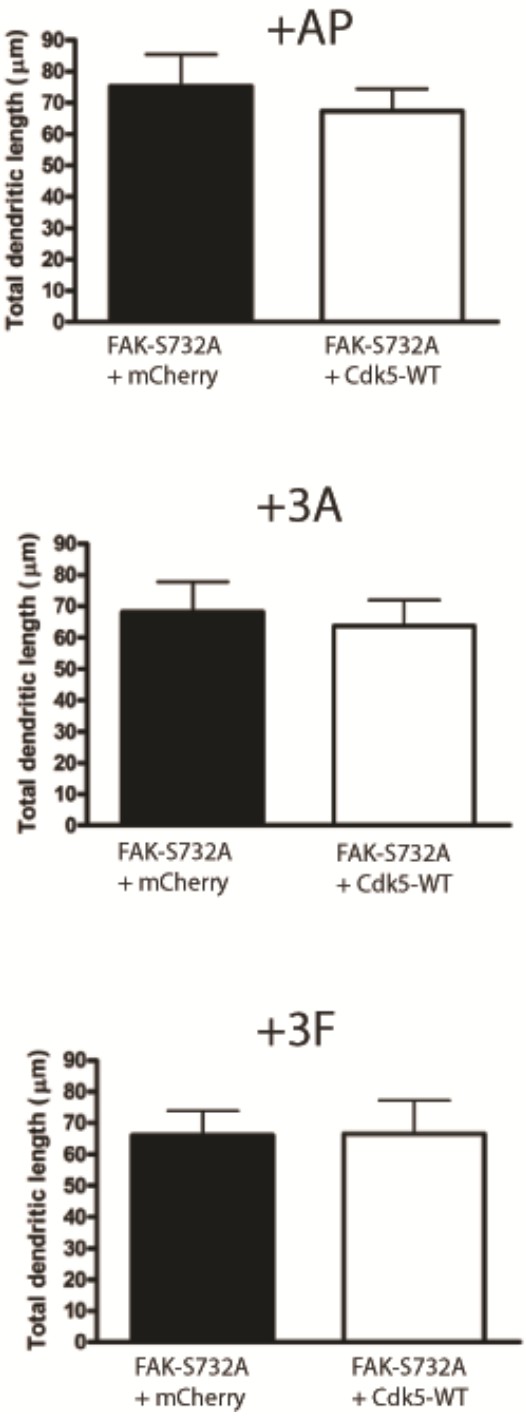

Figure 5-11. Serine phosphorylation of FAK by Cdk5 is important for Sema3A or 3F-induced dendritic growth. (A) Graphs showing total dendritic length of primary hippocampal neurons expressing single serine dominant negative mutant FAK-S732S together with control vector (mcherry) or with Cdk5-WT treated with AP control (top), Sema3A (middle) or 3F (bottom). 30 neurons from each group were used for analysis. Values represent mean ± s.e.m.

5.2.11 Constitutively active FAK rescues roscovitine inhibition on Semaphorin-induced dendritic growth

If FAK is downstream of Cdk5 in regulating Semaphorin-induced dendritic growth, constitutively active FAK should overcome roscovitine inhibition on Semaphorin-induced dendritic growth. To confirm this, I express a constitutively active form of FAK (S732D FAK) in primary hippocampal neurons pre-treated with roscovitine. Overexpression of S732D FAK overcame the effect of roscovitine on the inhibition of dendritic growth induced by Sema 3A and 3F (Figure 5-12A and B), suggesting that FAK acts downstream of Cdk5 in regulating dendritic growth in neurons.

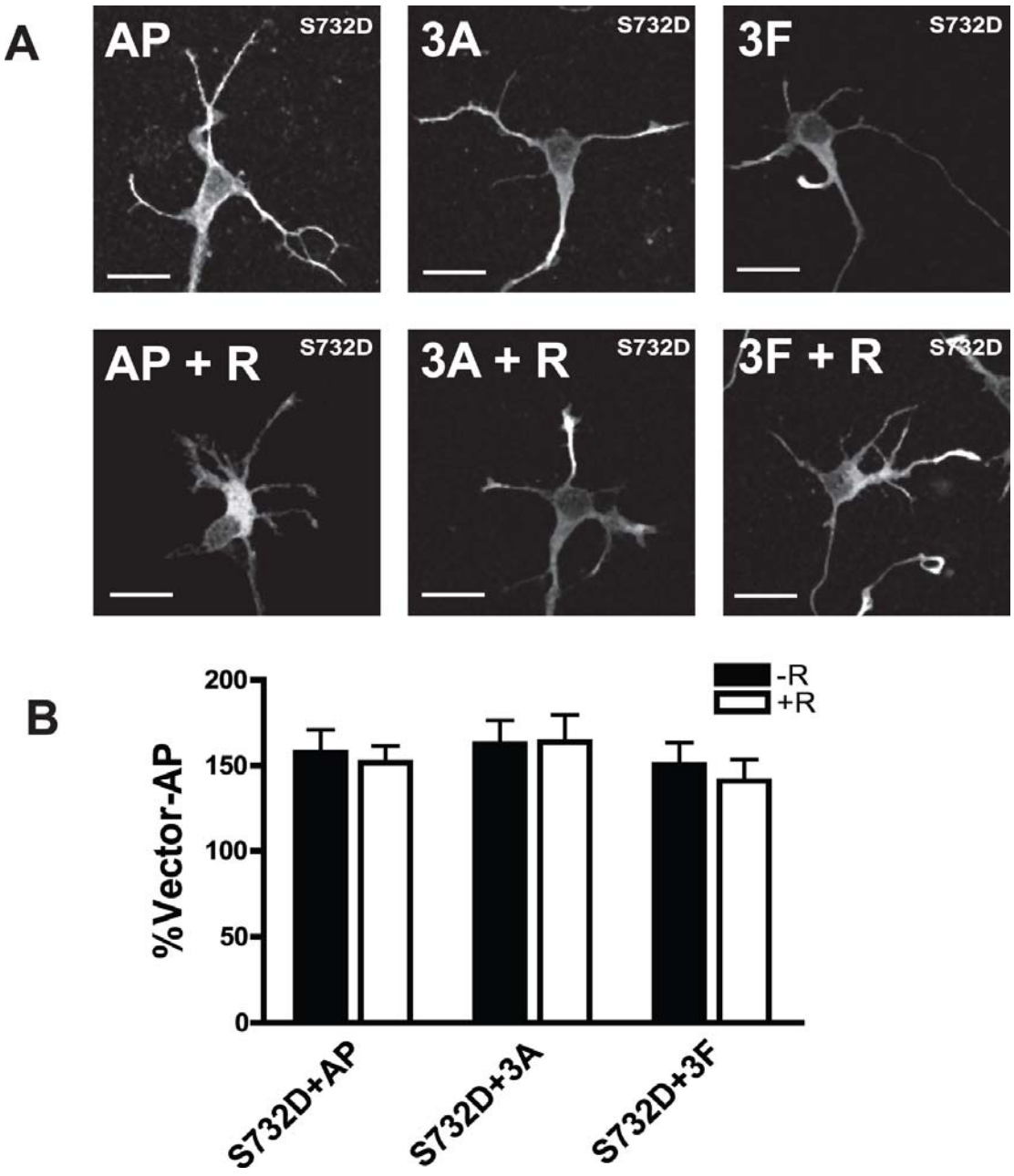

Figure 5-12. Overexpression of constitutively active FAK rescues the effects of roscovitine inhibition of semaphorin-induced dendritic growth. (A) Representative images of primary hippocampal neurons transfected with constitutively active FAK (S732D). Neurons were treated with AP control (AP), 3A-AP (3A), 3F-AP (3F), AP with roscovitine pre-treatment (AP + R), 3A-AP or 3F-AP with roscovitine pre-treatment (3A + R). Scale bar = 20 μm. (B) Graphs showing total dendritic length of primary hippocampal neurons expressing S732D FAK treated as indicated above. 40-50 neurons from each group were used for analysis. Values represent mean ± s.e.m. (No significant difference by 2-way ANOVA).

5.2.12 FAK and Cdk5 are downstream mediators of Semaphorin signaling in newborn neurons in the adult CNS *in vivo*

To determine if the effects of Cdk5 and FAK on dendritic development of cultured hippocampal neurons could also be observed in newborn neurons in adult CNS *in vivo*, I employed the same retroviral strategy for knocking down of Cdk5 and FAK in progenitor cells *in vivo*. Cdk5 or FAK deficiency resulted in impaired dendritic development of these newborn neurons (Figure 5-13A, B and C), with a severity that is comparable to the knockdown of NRP1 or NRP2 (Figure 5-1). This suggested that Cdk5 and FAK also played an important role in dendritic development of adult-born neurons *in vivo* and similar semaphorin signaling pathway might be conserved in embryonic neurons and newborn neurons in adult.

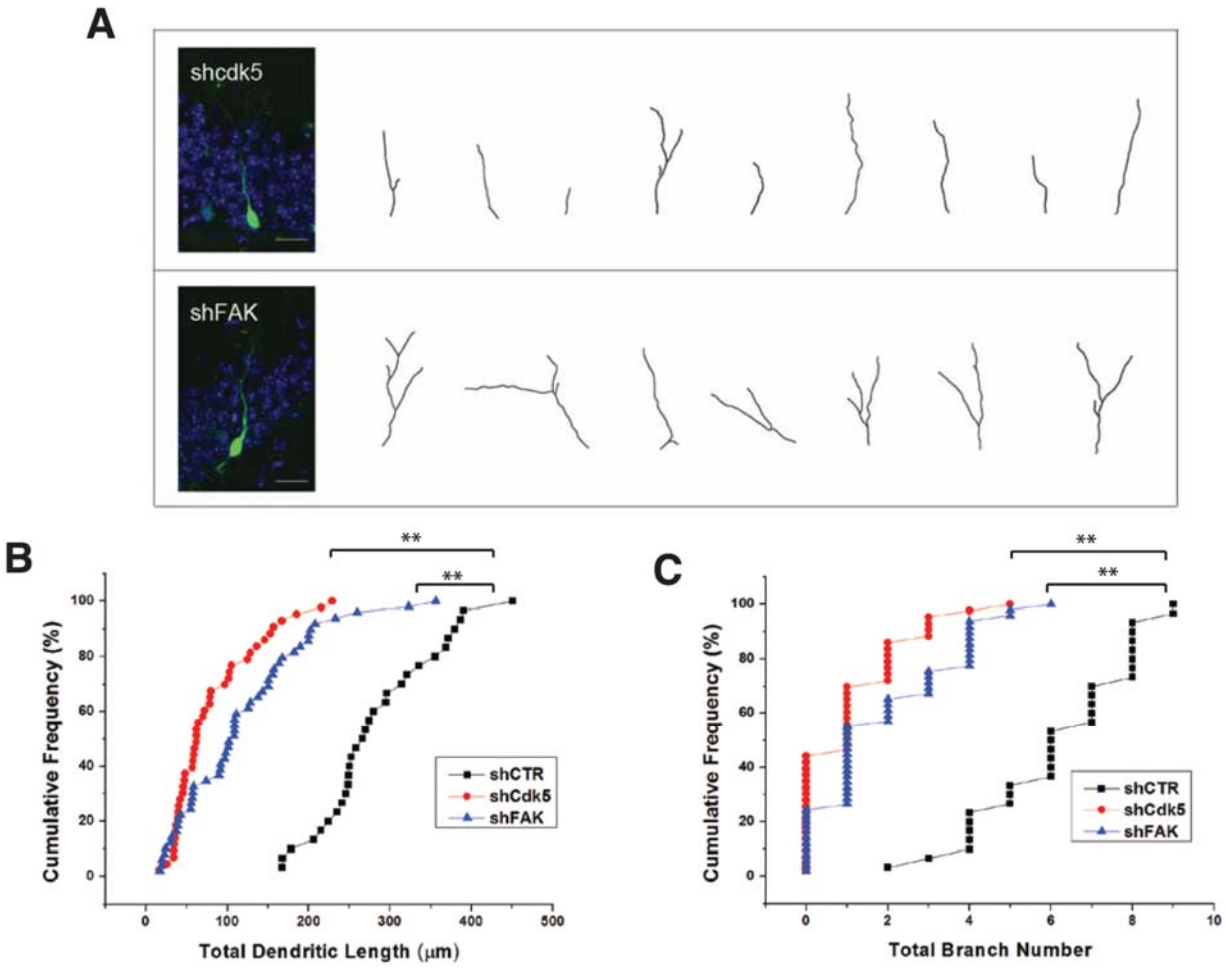

Figure 5-13. Knockdown of downstream mediators of semaphorin signaling, Cdk5 and FAK, induced defective dendritic morphologies of newborn DGCs in adult brain. (A) Representative images and tracings of dendrites of control, Cdk5 or FAK-shRNA expressing DGCs at 14 dpi. Scale bar = 20μm. Graphs showing quantification of total dendritic length (B) and branch number (C) of newborn DGCs. Each symbol represents a single DGC at 14 dpi. (**P<0.01, 1-way ANOVA with Newman-Keuls' post-hoc test).

To prove that semaphorin regulated dendritic development of adult-born neurons via activation of Cdk5 and FAK, I expressed Cdk5 or FAK in NRP1 or -2 deficient neurons. Overexpression of FAK or Cdk5 rescued the dendritic outgrowth phenotypes, by restoring (at least in part) the total dendritic length (Figure 5-14D, F) and branching (Figure 5-14E, G) of NRP1 or 2 silenced neurons. These evidences showed that Cdk5 and FAK were both downstream mediators of Sema3A/NRP1 and Sema3F/NRP2-induced dendritic development in newborn neurons in the adult CNS *in vivo*.

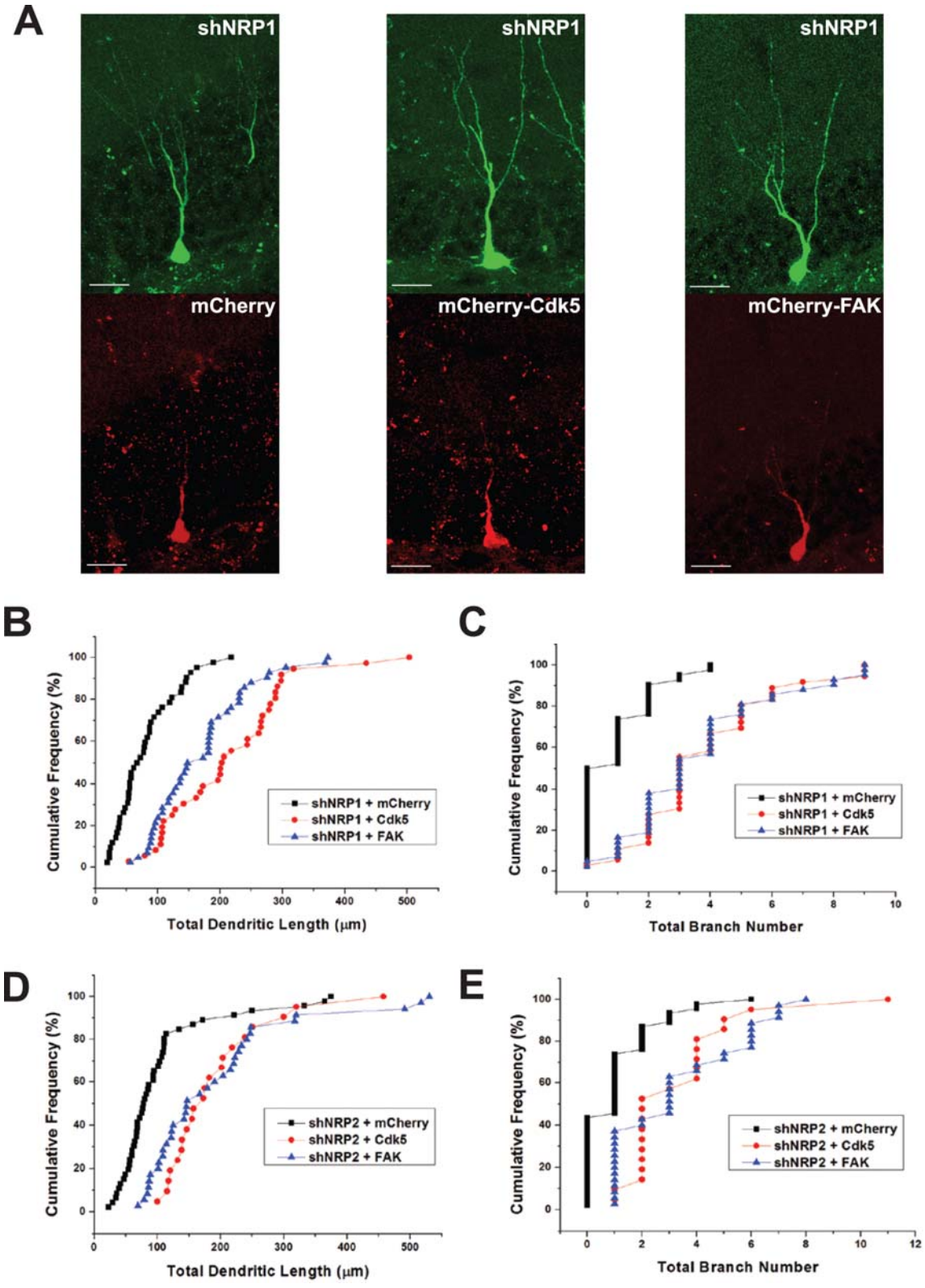
A
shNRP1
shNRP1
shNRP1
mCherry
mCherry-Cdk5
mCherry-FAK
B
Cumulative Frequency (%)
Total Dendritic Length (μm)
shNRP1 + mCherry
shNRP1 + Cdk5
shNRP1 + FAK
C
Cumulative Frequency (%)
Total Branch Number
shNRP1 + mCherry
shNRP1 + Cdk5
shNRP1 + FAK
D
Cumulative Frequency (%)
Total Dendritic Length (μm)
shNRP2 + mCherry
shNRP2 + Cdk5
shNRP2 + FAK
E
Cumulative Frequency (%)
Total Branch Number
shNRP2 + mCherry
shNRP2 + Cdk5
shNRP2 + FAK

Figure 5-14. Overexpression of Cdk5 or FAK rescued dendritic phenotypes of NRP1 and -2 deficient neurons. (A) Representative images of adult-born neurons co-infected with retrovirus expressing shNRP1 and mCherry shNRP1 and mCherry-T2A-Cdk5 and shNRP1 and mCherry-T2A-FAK. Scale bar = 20 μm. Quantification of total dendritic length (B, D) and branch number (C, E) of newborn DGCs at 14 dpi. Each symbol represents data from a single DGC at 14 dpi. (**P<0.01, 1-way ANOVA with Newman-Keuls' post hoc test).

5.3. Discussion

Semaphorins are essential for the developing nervous system. Class 3 Semaphorin was initially identified to be axon repulsive cues and, subsequently, was demonstrated to have both attractive and repulsive effects in various systems (Tran et al., 2007). Sema3A regulates dendritic growth of cortical pyramidal and hippocampal CA1 neurons during development (Fenstermaker et al., 2004; Nakamura et al., 2009; Schlomann et al., 2009). In addition, Sema3A and 3F play important roles in dendritic spine maturation (Morita et al., 2006; Tran et al., 2009) and synaptic transmission (Bouzioukh et al., 2006a; Sahay et al., 2005). More recently, it was reported that Sema3A plays a critical role in axonal-dendritic polarity in neurons (Nishiyama et al., 2011; Wang et al.). Despite their better-known functions during development, the mechanisms of class 3 Semaphorin signaling and their effects in the adult central nervous system remained largely unknown. However, the presence of Sema3A and 3F and their receptors (including NRP1 and 2) throughout adulthood suggests possible functional roles of Sema3A and 3F in the adult nervous system (Tran et al., 2007). Recently, it has been shown that Sema3F/NRP2 signaling decreases spine morphogenesis (spine number and size) or its distribution in dentate gyrus (DG) granule cell (GC) and cortical layer V pyramidal neuron of postnatal CNS (p21) and adult CNS. In this study, I have shown clearly that Sema3A/NRP1 and Sema3F/NRP2 play an important role in dendritic development in adult hippocampus. Knockdown of NRP1 and NRP2 affected both dendritic length and branching complexity of newborn neurons in adult *in vivo*. It has been shown that

defects in dendritic development of newborn granule neurons lead to defects in synapse formation and network integration of newborn neurons in adult, which are important in learning and memory (Ge et al., 2006; Ge et al., 2007; Gu et al., 2012a; Kumamoto et al., 2012). Defects in adult neurogenesis are increasingly reported in neurobehavioral disorders (Kempermann et al., 2008; Kim et al., 2012a). Semaphorins have been implicated in spinal cord injury and various neurological disorders such as Rett syndrome, autism, epilepsy and Alzheimer's disease, but the cellular basis and underlying mechanisms are still poorly understood (de Anda et al., 2012; Degano et al., 2009; Gant et al., 2009; Good et al., 2004; Holtmaat et al., 2003; Kaneko et al., 2006; Pasterkamp and Giger, 2009b). Our study sets the stage to investigate if Semaphorin signaling during adult neurogenesis plays a role the etiology of diseases.

Interestingly, I also showed that knockdown of Sema3A and 3F perturbs dendritic development of adult-born neurons (Figure 5-5), suggesting that Sema3A and 3F can act cell-autonomously. Recently, it was shown that the paracrine actions of Class 3 Semaphorins can have dramatic effects (Fukuda et al., 2013; Hayashi et al., 2012). While the principal source of Semaphorins appear to originate from the entorhinal cortex (Giger et al., 1998), it is shown here that dentate granule cells and newborn neurons could be potential sources of Class 3 Semaphorins. It would be interesting in the future to dissect the exact functions of Semaphorins that originate from the entorhinal cortex or local sources in the dentate gyrus by using transgenic animals or inhibitors.

FAK functions as an important downstream mediator of semaphorin signaling during axonal remodeling and dendritic growth (Bechara et al., 2008; Chacon et al., 2010; Schlomann et al., 2009). In these studies, semaphorin signaling induces tyrosine phosphorylation and subsequent activation of FAK. The interaction of the Ig superfamily cell adhesion molecule L1, but not Plexin-As, with NRP1 specifically mediates the activation of FAK–mitogen-

activated protein kinase (MAPK) signaling pathway controlling the repulsive behavior, and the disassembly of adhesion points of the growth cones in the developing cerebral cortex (Bechara et al., 2008). Activation of FAK is also required for chemorepellent or chemoattractant effects stimulated by multiple attractive and repulsive cues (Chacon and Fazzari, 2011). Sema3A and netrin, exert their opposing chemorepellent or chemoattractant effects possibly by differentially stimulating phosphorylation of FAK at different tyrosine sites. (Chacon et al., 2010; Li et al., 2004; Liu et al., 2004). Tyrosine phosphorylation of FAK has been extensively studied, especially Tyr 397, which is the FAK autophosphorylation site and is the first event in FAK activation. FAK initiates its activation by autophosphorylating its Y397 and triggering a series of tyrosine phosphorylation of FAK by other proteins including Src family kinases (SFKs) and integrin (Schaller et al., 1994). Functional outcome of FAK activation could be multifaceted, depending not only on the phosphorylation per se, but also largely dependent on the specific tyrosine residues that are being phosphorylated, the intensities and durations of the specific phosphorylation (Chacon et al., 2010). Our study showed that Sema3F as well as Sema3A stimulate dendritic outgrowth through tyrosine phosphorylation of FAK at Y397. Our data from using tyrosine-nonphosphorylated mutant of FAK confirmed this finding.

Notably I found that serine residue, S732 of FAK plays an important role in Semaphorin-induced dendritic growth. I also demonstrated that serine phosphorylation of FAK is mediated by activation of Cdk5. Serine phosphorylation of FAK is not well studied, except that serine residue of FAK is involved in microtubule organization and neuronal migration in endothelial cells and neurons (Park et al., 2009; Xie et al., 2003). It is very interesting to find how serine phosphorylation affects cellular function because kinase activity of FAK is not directly regulated by serine phosphorylation. Our mutant experiment indicated that Tyr397 and Ser732 are regulated independently. Therefore, it is unlikely that serine phosphorylation

regulates dendritic growth through tyrosine phosphorylation. However, I cannot completely exclude the possibility the functional interplay between tyrosine and serine phosphorylation of FAK because Ser732 phosporylation is known to affect Tyr407 phosphorylation of FAK (Le Boeuf et al., 2006).

In summary, I provided strong evidence showing that Class 3 Semaphorins regulate the dendritic development of primary hippocampal neurons and adult-born neurons via a Cdk5/FAK pathway (Figure 5-15).

It has been reported that many functional events during neuronal development are recapitulated in adult neurogenesis. For instance, occurrence of GABA mediated-input before glutamatergic influence in developing neurons is also observed in both embryonic and adult neurons (Ge et al., 2006). It is fascinating that a classical embryonic guidance cue, such as Semaphorins, can be co-opted to function in the adult context. Indeed, Cdk5 dependent FAK activation by Semaphorin is conserved both in dendritic development of embryonic neurons and newborn neurons of adult, supporting conserved signaling pathways between embryonic development and adult neurogenesis. The various environmental and physiological cues encountered by the animal during adulthood are likely to affect the nature of neurogenesis. The precise regulation of Semaphorin pathways during adulthood certainly deserves future lines of investigation.

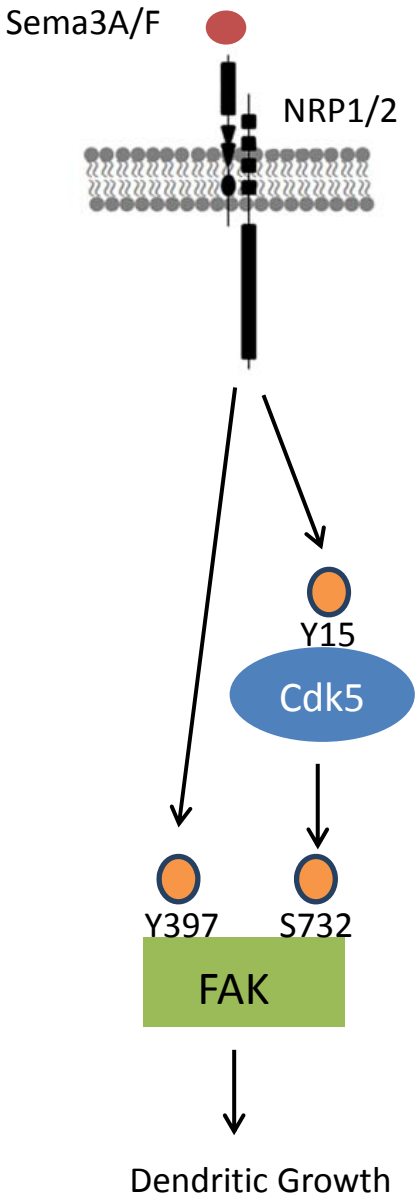

Figure 5-15. Class 3 Semaphorins regulate the dendritic development of primary neurons and adult-born neurons via Cdk5/FAK pathway.

Chapter 6

Neuropilin-2 regulates cell-positioning of adult-born neurons via GSK3β

6.1. Introduction

6.1.1 Cell positioning and organization of granule cells during embryonic development

Precise cell positioning of migrating neurons during development is essential for proper wiring and functioning of neural circuits (Ghashghaei et al., 2007). The dentate gyrus is a laminated structure in the hippocampus, forming a compact layer of 5-6 cells in the adult nervous system (Förster et al., 2006; Kempermann et al., 1998a). It is formed during the late embryonic to early postnatal stages in the mouse. Granule cells precursors undergo proliferation in the ventricular zone between E10 and E16 (Angevine and Sidman, 1961; Deguchi et al., 2011). They are guided by the chemokine, Stromal cell-derived factor 1 (SDF1), and tangentially migrated to the sub-pial space or the prospective hilus (Bagri et al., 2002). The cells are then guided by radial glial cells and radially migrated to form the suprapyramidal blade of the dentate gyrus around E18-E20 (Bagri et al., 2002; Barry et al., 2008; Nakahira and Yuasa, 2005). The radial migration takes place in an outside-in fashion, while the outermost granule cells are filled first, followed by the innermost cells (Nakahira and Yuasa, 2005). The second proliferative zone is formed within the future hilus, containing stem cells that originated from the ventricular zone. These cells migrated to form the infrapyramidal blade of the dentate gyrus at P5. The tertiary proliferative zone is also found

in the subgranular zones of both the suprapyramidal and infrapyramidal blades, providing for adult-born neurons during adulthood (Danglot et al., 2006).

Several signaling pathways have been implicated for the proper cell positioning and organization of the granule cells in the dentate gyrus. Cells with defects in Cxcr4, the Stomal derived factor-1 (SDF1) receptor, accumulate in the tangential migration pathway (Bagri et al., 2002). In nuclear factor 1b (Nfib) mouse mutants, granule cells were stalled in sub-pial tangential migration and could not form the granular layer (Barry et al., 2008). In a mouse model of lissencephaly, granule cells showed ectopic positions in the dentate gyrus (Fleck et al., 2000). Mice with mutations in the Reelin signaling pathway showed loose distribution of granule cells across the DG (Sheldon et al., 1997; Stanfield and Cowan, 1979; Trommsdorff et al., 1999; Zhao et al., 2004). Similar phenotypes were seen in Cdk5-deficient mice (Ohshima et al., 1996) and p35/p39 deficient mice (Ko et al., 2001). Interactions between Reelin and Cdk5 pathways are important for proper cell positioning of granule cells (Beffert et al., 2004).

6.1.2 Cell positioning and organization of newborn neurons in the adult animal

Adult neural precursors reside in the tertiary proliferative zone or the subgranular zone of the dentate gyrus in the hippocampus. During the first week of development, neuroblasts in the SGZ radially migrate a short distance towards the granular layer (Kempermann et al., 2004; Song et al., 2005). Typically, adult-born neurons are found mainly in the inner third of the granule cell layer and some are found in the middle third of the granule layer after 4 weeks (Duan et al., 2007; Esposito et al., 2005; Kempermann et al., 2003). There are no adult-born neurons found in the outer third granular layer and in the molecular layer of the dentate gyrus after 4 weeks (Duan et al., 2007). It is still unknown if adult-born neurons maintain their cell

positioning in the dentate granular layer or migrate towards the outermost layers during development. Radial migration of embryonic-born neurons take place in an outside-in fashion while adult-born neurons mainly reside in the inner third of the granular layer, suggesting that signaling mechanisms would be different. Interestingly, synaptically connected interneurons translocate in the opposite direction, form the molecular layer to granule layer, and they maintain old and form new synaptic contacts during migration, leading the authors to speculate that migration of interneurons and granule cells may be co-ordinated and could be important for neuroplasticity (Morozov et al., 2006).

Compared to embryonic development, there are limited studies on the signaling pathways regulating the cell-positioning of newborn neurons in the adult. GABA signaling appears to be important for proper cell positioning. Adult-born neurons lacking the α2-$GABA_A$ receptor migrated further into the granule cell layer compared to wildtype neurons (Duveau et al., 2011). Deletion of primary cilia affect proper positioning of adult-born neurons (Kumamoto et al., 2012). Down-regulation of Disrupted-In-Schizophrenia 1 (DISC1) and resulted in mis-positioning of adult-born neurons in the dentate gyrus (Duan et al., 2007). DISC1-NDEL1, but not DISC1-FEZ1 interaction, appeared to regulate cell positioning of adult-born neurons (Duan et al., 2007; Kang et al., 2011). Early rapamycin treatment rescued the cell positioning phenotype of DISC1 neurons (Kim et al., 2009; Zhou et al., 2013). NMDA receptor mediated signaling regulating DISC1 expression is necessary for proper cell positioning (Namba et al., 2011).

The functional implications of cell positioning in the adult-born neurons are unclear and difficult to work out. Firstly, there are no comparative electrophysiological studies performed on adult-born neurons found in the inner, middle, outer thirds of the granular layer and in the molecular layer to show functionality and integration to neural networks. Secondly, even if electrophysiological studies were performed on mis-positioned neurons in DISC1 and cilia-

defective animals, it is hard to prove causality on cell positioning as these neurons also showed defects in dendritic development (Duan et al., 2007; Kumamoto et al., 2012). Even though DISC1 knockdown in adult-born neurons resulted in cognitive and affective deficits, including decreased performance in object-place recognition test and hidden platform version of water maze test, depression-like phenotypes and increased anxiety (Zhou et al., 2013), it is unlikely that the cognitive and affective deficits found in DISC1 animals are solely dependent on cell positioning effects (Zhou et al., 2013). Hence, it would be interesting to screen for genes or drugs that specifically affect cell positioning.

6.2. Results

6.2.1 Knockdown of NRP2, but not NRP1, affects cell-positioning of adult-born neurons

The dentate gyrus consists of a compact granular cell layer made out of 5-6 cells, subdivided into the inner (layer 1), middle (layer 2) and the outer layers (layer 3) and the molecular layer (layer 4) (Figure 6-1A). Typically, adult-born neurons are found in the inner two-third of granular cell layer after 2 weeks of development (Duan et al., 2007; Esposito et al., 2005; Kempermann et al., 2003). To investigate if Neuropilins regulate cell positioning of adult-born neurons in the dentate gyrus, I stereotaxically injected retroviruses encoding for shCTR, shNRP1, and shNRP2 into the hippocampus of 6 weeks old adult mice and quantified the cell positions of adult-born neurons in the dentate gyrus after 28 days post infection (28 DPI).

Approximately 95% of control neurons mainly resided in inner granular layer (layer 1) with a small percentage (5%) of neurons positioned in the middle granular layer (layer 2), confirming published data that wild-type neurons are found in inner two-third of granular cell layer (Duan et al., 2007; Esposito et al., 2005; Kempermann et al., 2003). Knocking down Neuropilin-1 has no effect on cell positioning, approximately 95% and 5% of shNRP1 neurons resided in the inner and middle granular layers respectively. Surprisingly, I observed that knocking down Neuropilin-2 affected the cell positioning of adult-born neurons in the granular cell layer. At 4 WPI, Approximately 60% of shNRP2 neurons are found in the inner granular layer, 30% found in the middle granular layer and 10% found in the outer granule layer (Figure 6-1C). Hence, it seemed that Neuropilin-2, not Neuropilin-1, specifically regulate cell positioning of adult-born neurons in the dentate gyrus *in vivo*.

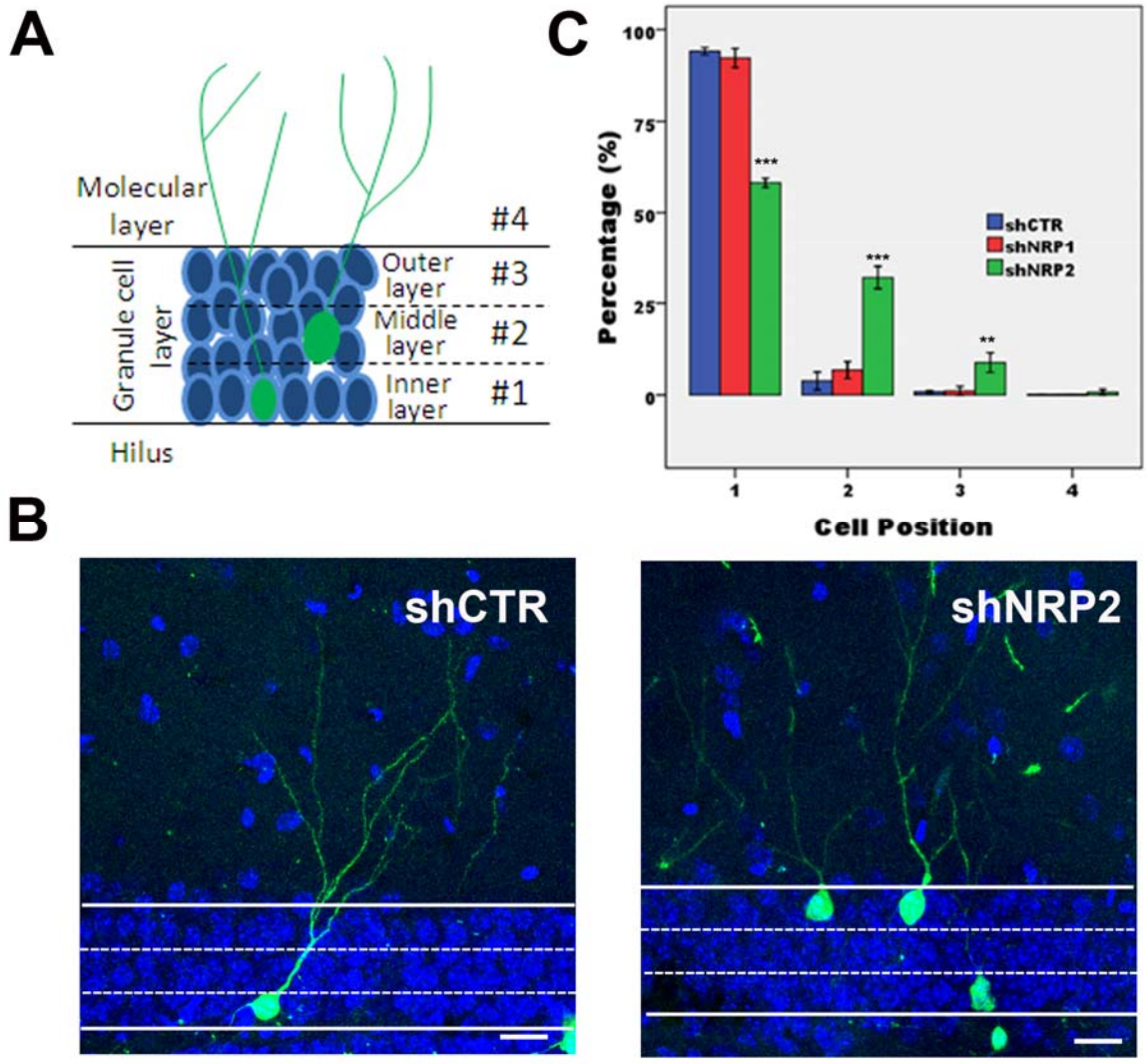

Figure 6-1. Neuropilin-2 regulates cell positioning of adult-born neurons in the DG. (A) The dentate gyrus consists of the granule cell layer (layer 1-3) and the molecular layer (layer 4). The granular cell layer is subdivided into the inner (layer 1), middle (layer 2), and the outer layer (layer 3). (B) Representative images showing shCTR and shNRP2 neurons residing in layer 1 and layer 3 respectively. (C) Quantification of cell positioning of adult-born neurons infected with retrovirus after 28 days post infection (28 DPI). Bars represent percentages of cells found in the inner (layer 1), middle (layer 2), outer granular layer (layer 3) and molecular layer (layer 4). Values represent mean ± s.e.m. Statistical test (**P<0.01, 1-way ANOVA with Newman-Keuls' post hoc test).

6.2.2 Time course analysis of cell positioning phenotype of shNRP2 neurons

To investigate the temporal changes in neuronal positioning during adult neurogenesis, I examined positions of newborn neurons expressing *in vivo* shNRP2 and shCTR *in vivo* at 5, 10 and 14 days post infection (DPI). Time-course analysis suggested that at 5 DPI, all shCTR and shNRP2 cells resided in the subgranular zone or inner granular layer (Figure 6-2A). At 10 DPI, shCTR neurons were mainly found in the inner granular layer (95%) with some found in the middle layer (5%). In contrast, 70% of shNRP2 are found in the inner granule layer and 30% of cells are found in the number of shNRP2 cells in the middle granular layer (Figure 6-2B). The cell positions of both shCTR and shNRP2 neurons did not appear to change significantly from 10 DPI to 14 DPI. shCTR neurons mainly occupied the inner granular layer while shNRP2 occupied the inner (70%) and middle (30%) granular layer (Figure 6-2C). Taken together, this time course analysis showed that the cell positioning phenotype of shNRP2 neurons occurred between 5 to 10 DPI.

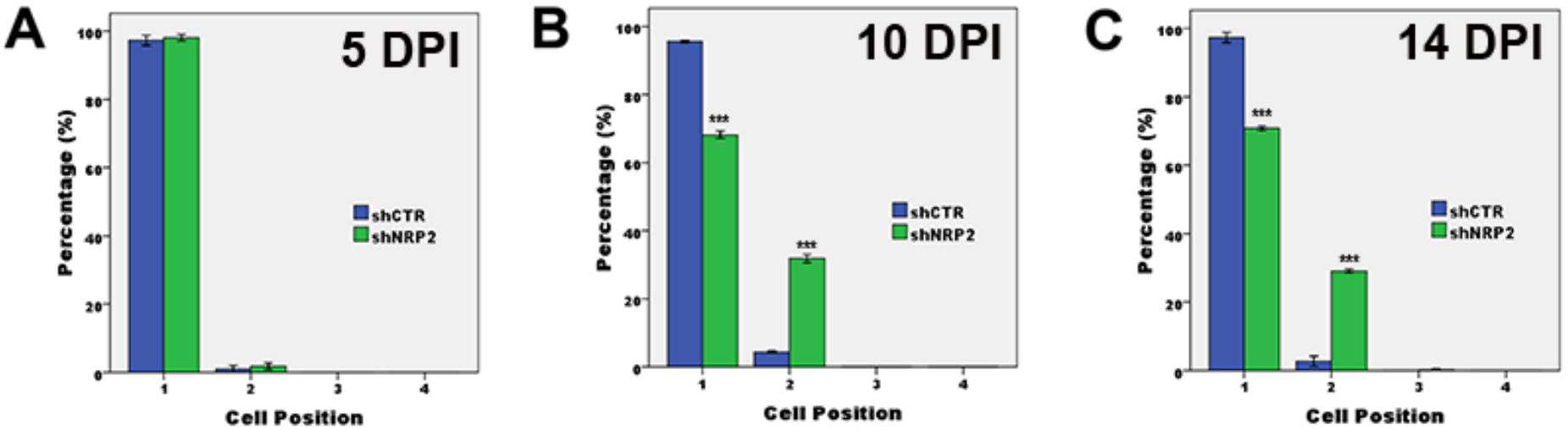

Figure 6-2. Time course analysis of cell positioning phenotypes of shNRP2 neurons. Quantification of cell positioning of adult-born neurons infected with retrovirus after 5 DPI (A), 10 DPI (B), 14 DPI (C). Bars represent percentages of cells found in the inner (layer 1), middle (layer 2), outer granular layer (layer 3) and molecular layer (layer 4). Values represent mean ± s.e.m. Statistical test (***P<0.001, Student's t-test).

6.2.3 Knockdown of GSK3β phenocopies shNRP2 cell positioning phenotype and does not affect dendritic growth

I showed previously that knockdown of Cdk5 and FAK affects dendritic development of adult-born neurons. Cdk5 and FAK could rescue the dendritic morphology of both shNRP1 and -2 neurons. These data suggest Class 3 Semaphorins regulate dendritic morphology of adult-born neurons via a novel Cdk5/FAK pathway (Chapter 5). I wondered if Cdk5 and FAK act downstream of Neuropilin-2 in regulating cell positioning of adult-born neurons in the dentate gyrus. I performed retroviral-mediated knockdown of FAK and Cdk5 and quantified the cell positioning of adult-born neurons in the dentate gyrus. I did not observe any apparent mis-positionings defects of shFAK and shCdk5 neurons at 28 DPI (Figure 6-3), suggesting that FAK and Cdk5 do not regulate cell positioning of adult-born neurons. Hence, I hypothesized that a different kinase acts downstream of Neuropilin-2 in regulating cell positioning of adult-born neurons.

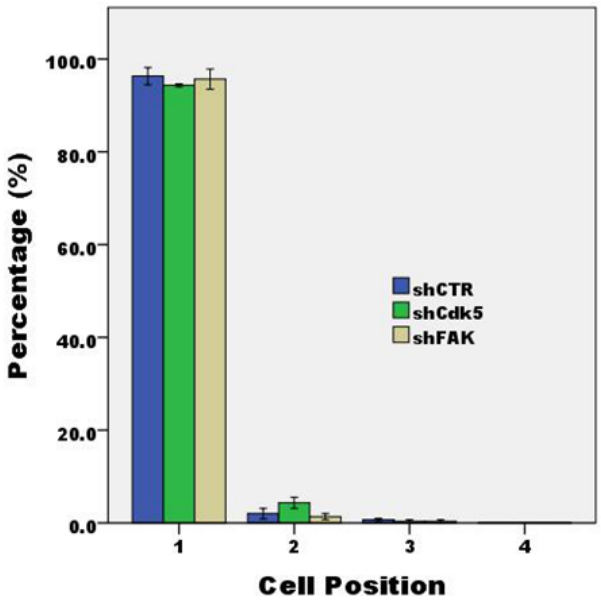

Figure 6-3. Knockdown of Cdk5 and FAK did not affect cell positioning of adult-born neurons. shCdk5, shFAK and shCTR neurons are mainly positioned in the inner third of the granular layer of the dentate gyrus at 28 DPI. Values represent mean ± s.e.m. ANOVA with Newman-Keuls' post hoc test, No significant differences found.

Glycogen synthase kinase 3β (GSK3β) is a key regulator of neuronal development (Hur and Zhou, 2010; Kim et al., 2011) and has been implicated in neuronal migration (Asada and Sanada, 2010; Ghashghaei et al., 2007). Several lines of evidences suggested that GSK3β is downstream of Semaphorin signaling. Sema3A activates GSK3β at leading edge of neuronal growth cones and GSK3β is required for Sema3A-induced growth cone collapse (Eickholt et al., 2002). It was also shown that Sema3A activates GSK3β by suppressing PI3K signaling and decreasing Akt phosphorylation (Chadborn et al., 2006). More recently, it was shown that Sema3A activate GSK3β by reducing cyclic AMP (cAMP) and protein-kinase A (PKA) activity, supressing PKA-dependent inhibition of GSK3β (Shelly et al., 2011). Since GSK3β regulate many key events in neuronal migration and was shown to be downstream of Class 3 Semaphorin signaling, I hypothesized that GSK3β is a good candidate for Sema3F-induced cell positioning of adult-born neurons in the dentate gyrus.

I performed retroviral mediated knockdown of GSK3β and quantified cell positioning of adult-born neurons in the dentate gyrus. At 28 DPI, shCTR neurons are mainly found in the inner (95%) and middle (5%) granule layer. In contrast, shGSK3β neurons are found in the inner (45%), middle (40%) and outer (10%) granular layer (Figure 6-4A). Therefore, shGSK3β phenocopied the cell positioning phenotype of shNRP2 neurons (Figure 6-1B and 6.2D). In addition, I measured the total dendritic length of shGSK3β and shCTR neurons at 28 DPI. Interestingly, knocking down GSK3β did not affect the dendritic morphology of adult-born neurons (Figure 6-4B). Therefore, GSK3β specifically regulates cell positioning of adult-born neurons but not dendritic growth. On the other hand, Cdk5 and FAK regulate dendritic morphology but not cell positioning (Chapter 5). These data suggest the neuronal development of adult-born neurons is regulated by distinct mechanisms.

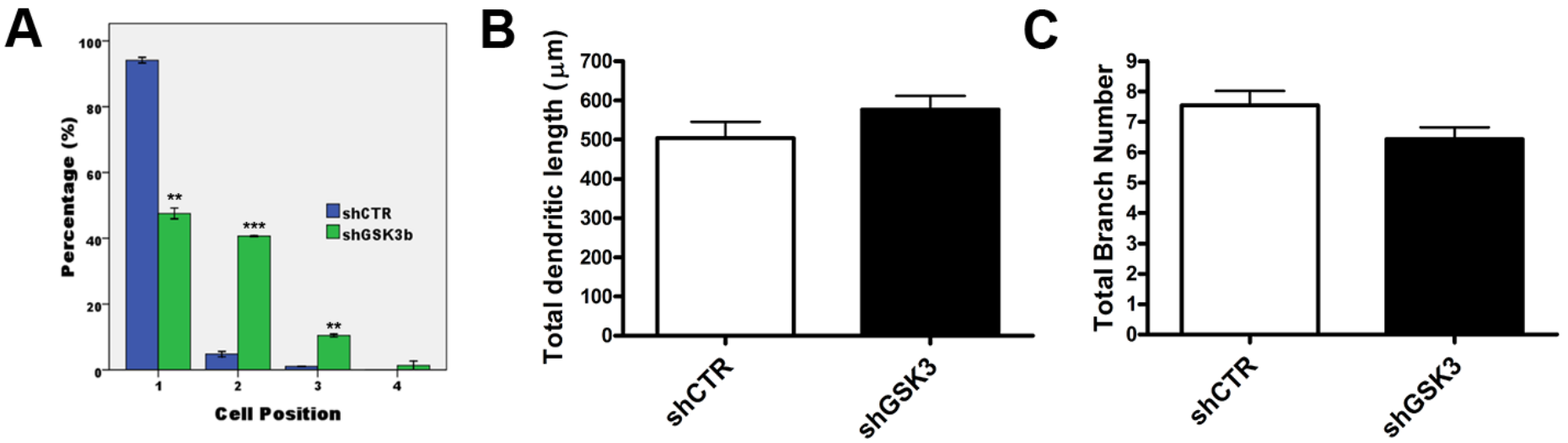

Figure 6-4. Knockdown of GSK3β affects cell positioning but not dendritic growth. (A) Quantification of cell positioning of adult-born neurons infected with retrovirus at 28 DPI. Values represent percentages of cells found in the inner (layer 1), middle (layer 2), outer granular layer (layer 3) and molecular layer (layer 4). (B) Total dendritic length and (C) Total branch number of shCTR and shGSK3β neurons at 28 DPI. Values represent mean ± s.e.m. Student's t-test, No significant differences found.

6.2.4. GSK3β overexpression rescues cell-positioning phenotypes but not dendritic growth of shNRP2 neurons

I have showed that Neuropilin-2 regulate both dendritic development of adult-born neurons (Chapter 5) and cell positioning in the dentate gyrus (Figure 6-1 and 6-2). Knockdown of GSK3β phenocopied cell positioning phenotype of shNRP2 neurons (Figure 6-4A) but did not affect dendritic development (Figure 6-4B). To prove that GSK3β specifically act downstream of Neuropilin-2 in regulating cell positioning, I attempted to rescue the cell positioning phenotype of shNRP2 neurons by overexpressing GSK3β *in vivo*. I co-injected retroviruses encoding shCTR or shNRP2 together with another retroviruses encoding mCherry or mCherry-T2A-GSK3β. shCTR/mCherry and shCTR/mCherry-T2A- GSK3β neurons showed correct cell positioning, suggesting that overexpressing GSK3β do not affect cell positioning of adult-born neurons (Figure 6-5A). shNRP2/mCherry neurons were located in the inner (60%), middle (30%) and outer (10%) granular layer (Figure 6-5A). Rescuing shNRP2 with GSK3β overexpression dramatically improved cell positioning defects as shNRP2/mCherry-T2A-GSK3β neurons are found in inner (90%) and middle (10%) granular layer (Figure 6-5A). In addition, I also attempted to rescue the dendritic phenotype of shNRP2 neurons by overexpressing GSK3β. Total dendritic length (Figure 6-4B) and total branch number (Figure 6-5C) of shNRP2/mCherry-T2A-GSK3β neurons were not significantly different from shNRP2/mCherry neurons, suggesting that GSK3β overexpression could not rescue dendritic phenotype of shNRP2 neurons. Therefore, GSK3β appeared to specifically act downstream of Neuropilin-2 in regulating cell positioning of adult-born neurons.

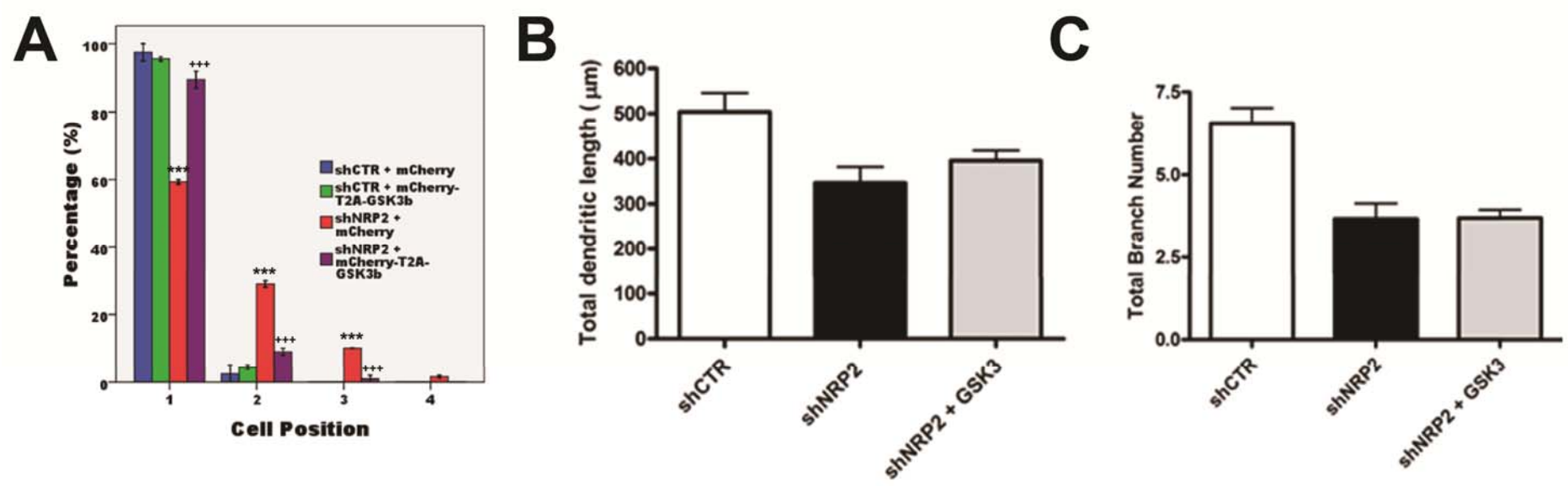

Figure 6-5. GSK3β rescues cell positioning phenotype but not defects in dendritic growth of shNRP2 neurons. (A) Quantification of cell positioning of adult-born neurons co-infected with retroviruses (28 DPI). Bars represent percentages of cells found in the inner (layer 1), middle (layer 2), outer granular layer (layer 3) and molecular layer (layer 4). (B) Total dendritic length of adult-born neurons co-infected with retroviruses at 28 DPI. Values represent mean ± s.e.m. Statistical test (**P<0.01, 1-way ANOVA with Newman-Keuls' post hoc test).

6.3. Discussion

In this study, I showed that Sema3F/NRP2 signaling specifically regulates the cell positioning of adult-born neuron via GSK3β. The involvement of NRP2 or Sema3F in neuronal migration leading to correct cell positioning has been reported in embryonic developmental studies. Neuropilin signaling is required for radial migration of cortical neurons during embryonic development (Chen et al., 2008; Shelly et al., 2011). Cells differentiated from whole neocortical area are mostly confined to the surface of the telencephalon through ventral migration in wildtype, but they spread over the deep medial region of the telencephalon in NRP2 and Sema3F mutant (Ito et al., 2008). Migration of ganglionic eminence cells is also regulated by Sema3F/NRP2 interactions (Tamamaki et al., 2003). Sorting of striatal and cortical interneurons depends on Semaphorins signaling. Migrating interneurons expressing NRPs are excluded from the striatum expressing Semaphorins which form an exclusion zone for interneurons while interneurons lacking NRPs are sorted to the striatum (Marin et al., 2001).

In young and adult animals, Semaphorin-3A and its receptor Neuropilin-1 are expressed in endothelial cells along the rostral migratory stream of young and adult animals and it was suggested that Semaphorin signaling is involved in the migration of neuroblasts from the adult SVZ (Melendez-Herrera et al., 2008). I was interested to address if Sema3F/NRP2 signaling is required for the migration and cell positioning of adult-born neurons in the dentate granule layer. Retrograde tracing combined with *in situ* hybridization demonstrated that entorhinal cortex express Sema3A which forms a gradient in the outer two-thirds of the molecular layer that gradually declines towards the granule cell layers (Giger et al., 1996). Similarly, Sema3F protein expression was found in the medial and lateral entorhinal cortex and in their axonal projection to the hippocampus (Tran et al., 2009). Sema3F protein expression could also be seen in granular cells in the adult DG (Tran et al., 2009).

Conceivably, Sema3F could forms an exclusion zone in the DG to regulate the migration and cell positioning of NRP2 expressing newborn neurons during adult neurogenesis. This model would be consistent with our data showing that shNRP2 neurons were found in the middle and outer thirds of the granular layer of the DG.

Dendritic growth and cell positioning of adult-born neurons could be regulated by distinct mechanisms. In the previous chapter, I showed that NRP1 and NRP2 are required for the dendritic development of adult-born neurons via a Cdk5-FAK signaling pathway. Cdk5 (Beffert et al., 2004; Ohshima et al., 1996) and its co-activators p35/p39 (Ko et al., 2001) are critical for the proper migration of granule cells during embryonic development and serine phosphorylation of FAK by Cdk5 is required for embryonic corticogenesis (Xie et al., 2003). In this study, knockdown of both FAK and Cdk5 did not affect cell positioning of adult-born neurons, suggesting that Cdk5-FAK signaling could be regulated differently during the development of the embryonic and adult nervous systems. Since Glycogen synthase kinase 3β (GSK3β) was shown to be downstream mediator of Semaphorin signaling (Chadborn et al., 2006; Eickholt et al., 2002; Shelly et al., 2011) and had been implicated in cortical migration (Asada and Sanada, 2010; Ghashghaei et al., 2007), I examined if GSK3β could regulate the dendritic development and neuronal positioning of adult-born neurons. Interestingly, knocking down of GSK3β specifically affects cell positioning while sparing dendritic growth of adult-born neurons. Importantly, overexpression of GSK3β rescued the cell positioning, but not dendritic deficits, of shNRP2 neurons. Therefore, dendritic growth mediated by NRP2 is apparently uncoupled from neuronal positioning in adult-born neurons, indicating the existence of independent regulation of these two distinct phenotypes. The idea that dendritic growth and cell positioning of adult-born neurons are regulated independently is also supported by other published studies. For instance, it was showed that while both $GABA_AR$ and DISC1-deficient neurons had mis-positioning defects, $GABA_AR$-deficient neurons

showed impaired dendritic growth (Duveau et al., 2011) and DISC1-deficient neurons have increased dendritic length (Duan et al., 2007).

Proper cell positioning of granule cells is required for neuronal function. In LIS1 mutant mice, ectopic positioning of granule cells in the molecular layer of the dentate gyrus resulted in neurons exhibiting aberrant Excitatory Post-Synaptic Currents (EPSCs) (Hunt et al., 2012). DISC1 silencing in adult-born neurons induced dendritic structural abnormalities together with mispositioning and resulted in severe cognitive and affective deficits (Zhou et al., 2013). Fitzsimons et al. also showed that abnormal dendritic complexity as well as ectopic positioning of newborn granular cells induced by glucocorticoid receptor knockdown impaired memory consolidation for contextual fear conditioning (Fitzsimons et al., 2013). Similarly, phospholipase C-β1 knockout mice exhibited abnormal migration of adult-born neurons and these mice showed a deficit in hippocampal-dependent location recognition task (Manning et al., 2012). Although these memory defects could also be attributed to other functional phenotypes, the mispositioning phenotype that was common among different systems suggests a possible link between memory defects and mispositioning of newborn neurons. I showed in the present study that GSK3β silencing specifically affects cell positioning but not dendritic development of adult-born neurons. Hence, manipulating GSK3β *in vivo* could be an excellent model system to examine if proper cell positioning in DG is essential for functioning of adult-born neurons without being complicated by other defective morphological phenotypes.

In conclusion, I showed that NRP1 and NRP2 regulate dendritic growth via Cdk5-FAK signaling and NRP2 regulates the cell positioning of adult-born neurons via GSK3β (Figure 6-6). I identified a new role for NRP2 in cell positioning of adult newborn neurons in DG, acting via GSK3β. Our findings showed that NRP2 mediates GSK3β-dependent neuronal

migration that is independent of its effect on dendritic development of newborn neurons in the adult neurogenic region. This finding would contribute towards understanding the etiology of neuronal migration related brain disorders.

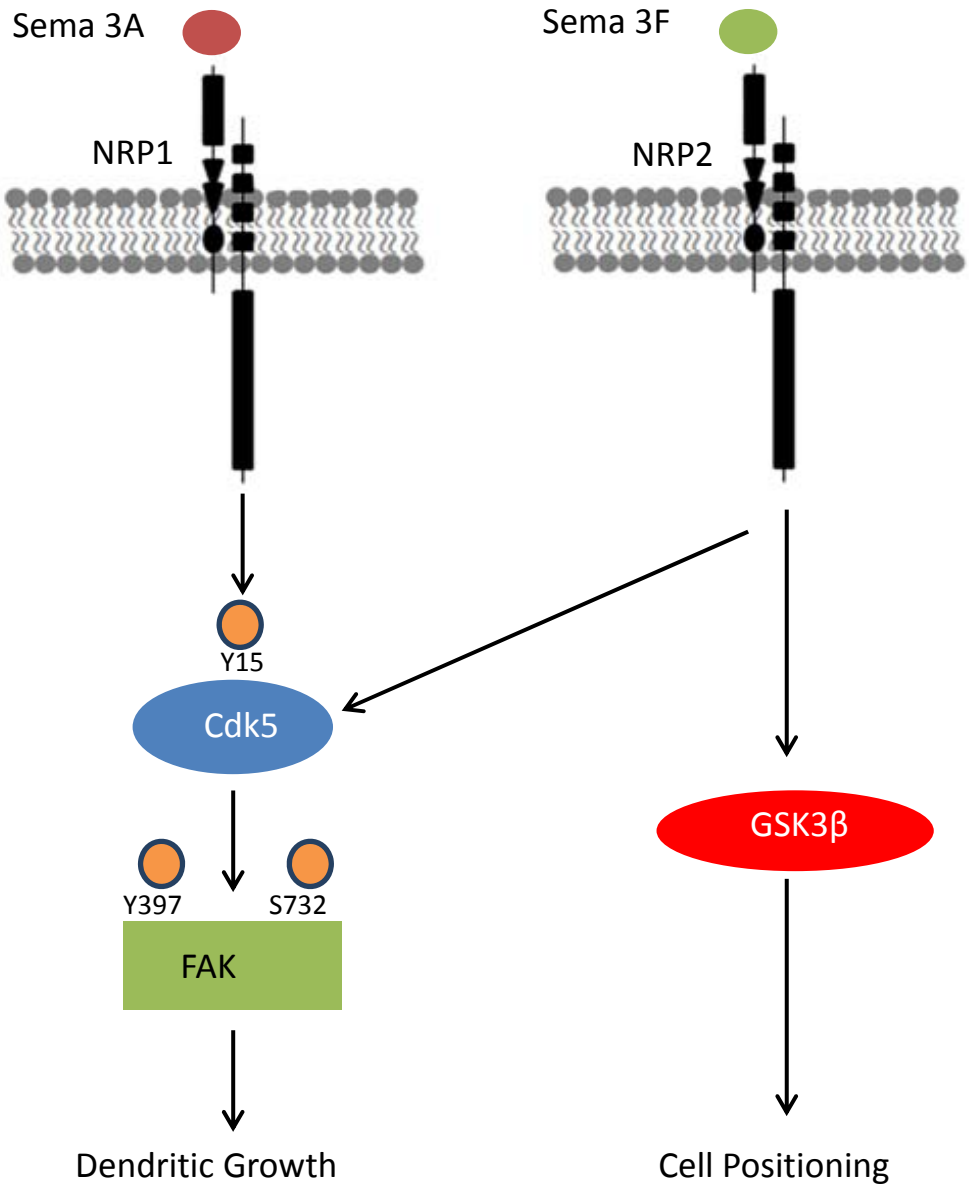

Figure 6-6. Sema3F specifically regulates the cell positioning of adult-born neurons in the dentate gyrus via a novel GSK3β signaling pathway.

Chapter 7

Neuropilins regulate adult hippocampal function

7.1. Introduction

7.1.1 Hippocampal sub-regions subserve distinct functions

It has been appreciated for more than half a century that the hippocampus is critical for episodic/declarative memory (Burgess et al., 2002; Scoville and Milner, 1957; Squire et al., 2004), contextual memory (Maren et al., 2013) and spatial memory (O'Keefe and Dostrovsky, 1971).

For memory, we need to build a mental pattern using environmental cues and compare the mental pattern to one that was previously encountered. This requires two opposing yet complementary processes, namely pattern separation and pattern completion (Colgin et al., 2008; Wilson, 2009). Pattern separation is defined as the processes by which overlapping or similar inputs as transformed into less similar outputs. Many episodes we experience shared many similarities, and pattern separation allows us to memorize distinct features of a particular episode. Pattern completion is defined as the reconstruction of complete stored representations from partial inputs. As specific episodes are rarely replicated in full, pattern completion enables us to reactivate previously acquired memories using partial cues.

David Marr put forth an influential and elegant mathematical framework for understanding the role of the hippocampal in memory (Marr, 1971). In order to effectively retrieve a memory from partial cues (pattern completion), he proposed that CA3 pyramidal cells need to form recurrent collaterals between each other. Other theorists also favoured the notion that

the autoassociative and recurrent CA3 network form the engram for pattern completion (Hasselmo et al., 1995; Marr, 1971; O'Reilly and McClelland, 1994; Treves and Rolls, 1994). Experimentally, transgenic mice with N-methyl-D-asparate (NMDA) receptors ablated specifically in CA3 pyramidal cells, showed impaired retrieval of spatial reference memory when presented with partial cues (Nakazawa et al., 2002), lending experimental support that CA3 is required for pattern completion.

Marr's treatment of the hippocampus ignored the role of the dentate gyrus. Layer II of the entorhinal cortex can send direct perforant pathway projections to the CA3, bypassing the dentate gyrus. The same entorhinal cortical neurons can also form indirect perforant pathway connections with granule cells in the dentate gyrus, which in turn, send mossy fibers to the CA3 (Witter, 2007). It is thus fascinating to ask what is the function of the dentate gyrus, which effectively duplicates the afferent inputs to the CA3?

McNaughton and Morris observed that mossy fibers projections synapse proximately to the soma of pyramidal cells and are potentially powerful. When bursting, a single mossy fiber is capable of firing a downstream CA3 neuron. They proposed that these 'detonator' synapses regulate associative plasticity and encoding of information (McNaughton and Morris, 1987). Such strong mossy fibers inputs could be unsupervised and potentially result in (a) to *catastrophic interference* of old memories stored in the CA3 network by new ones (French, 1999) and (b) interference when representations are highly similar and overlapping (Clelland et al., 2009; Hunsaker and Kesner, 2008; Hunsaker et al., 2008). It was proposed that both types of interferences are solved because the dentate gyrus experience a high level of tonic inhibition and granule cells make connections to only a dozen CA3 cells and fire only sparsely (Jung and McNaughton, 1993; Leutgeb et al., 2007; O'Reilly and McClelland, 1994). In other words, the dentate granule cell properties and duplication of the afferent inputs to the

CA3 essentially allow the dentate gyrus to solve interferences by orthogonalizing inputs to perform the classical function of pattern separation.

7.1.2 The roles of the DG in spatial memory and pattern separation are supported by animal behavioural studies

The medial perforant pathway mediates spatial information via activation of NMDA receptor while the lateral perforant pathway mediates visual information via the activation of opioid receptors (Hargreaves et al., 2005; Witter et al., 1989). Infusion of a NMDA antagonist to the dentate gyrus disrupts detection of a novel object location but not the detection of a novel object while the infusion of μ-opiod antagonist disrupt both spatial and visual information (Hunsaker et al., 2007). Animal with DG-lesions are impaired in olfactory cue-context associations compared to controls (Morris et al., 2013b). Hence, the dentate gyrus appears to be important for conjunctive encoding of spatial information paired with a visual or olfactory cue.

DG-lesion animals showed impaired performance in behavioural tasks, including radial arm maze (Emerich and Walsh, 1989; McLamb et al., 1988; Tilson et al., 1987; Walsh et al., 1986), Morris water maze (Jeltsch et al., 2001; Nanry et al., 1989; Sutherland et al., 1983; Xavier et al., 1999), contextual fear conditioning (Lee and Kesner, 2004a) and object-context recognition tasks (Dellu et al., 1997; Mumby, 2001; O'Brien et al., 2006; Piterkin et al., 2008). Transgenic mice with targeted deletion of NMDA receptors in DG cells showed impaired ability to distinguish between shocked and non-shocked context over time (McHugh et al., 2007). Functional MRI showed that presentations of highly similar but non-identical objects elicit increased blood flow in DG/CA3 regions in humans (Bakker et al., 2008).

Interestingly, rats were trained to discriminate between a sample and a foil object placed at five different spatial distances apart. DG-lesioned rats are impaired in this task compared to control animals and showed linear improvement as a function of increasing distances between objects (Gilbert et al., 2001). Thus, the dentate gyrus is required for metric distance changes for spatial pattern separation. On the other hand, the dentate gyrus is not required for topographical space as DG-lesioned rats do not show deficits in detecting the changes in topographical locations of four distinct objects placed in a cheeseboard maze (Goodrich-Hunsaker et al., 2008)

7.1.3 Class 3 Semaphorins signaling may be required for adult hippocampal functions.

Class 3 Semaphorins play critical roles in the assembly of neural circuits in the embryonic and adult nervous system. Particularly in the embryonic hippocampus, Sema3A regulates dendritic growth (Fenstermaker et al., 2004; Nakamura et al., 2009; Schlomann et al., 2009), dendritic spines formation (Bouzioukh et al., 2006b; Morita et al., 2006; Yamashita et al., 2007) and mediates synaptic transmission (Bouzioukh et al., 2006b). Sema3F regulates dendritic spines formation (Tran et al., 2009), mediates synaptic transmission (Sahay et al., 2005; Tran et al., 2009) and long term potentiation (Lee et al., 2012). Furthermore, Sema3F is required for the pruning of the infra-pyramidal tract of mossy fibers during embryonic development (Bagri et al., 2003; Sahay et al., 2003).

Although it was known that Sema3F/NRP2 signaling is critical for the development of the embryonic nervous system and regulating synaptic plasticity in the adult DG, it is unclear if Sema3F/NRP2 signaling in the adult hippocampus is required for learning and memory. One possible reason is that Sema3F null animals are prone to seizures (Sahay et al., 2003) and NRP2 null animals are weak and do not survive well to adulthood (Giger et al., 2000),

precluding the use of knockout animals in behavioural studies. Furthermore, in Sema3F$^{-/-}$ and NRP2$^{-/-}$ mice, ectopic dendritic spines were formed in dentate granule cells (Tran et al., 2009) and axonal pruning of mossy fibres failed to occur (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003). The embryonic loss of Sema3F and NRP2 disrupted hippocampal circuits and knockout animals would not be useful for addressing the specific roles of Sema3F/NRP2 signaling in the adult context for learning and memory. To circumvent these issues, I specifically target Neuropilin-2 in the dentate granule cells of adult mice using lentivruses after the embryonic hippocampal circuits have formed and are intact. To address if Sema3F/NRP2 signaling is required for learning and memory, I subjected shNRP2 and shCTR animals to different behavioral tasks, including contextual fear conditioning, Morris water maze test and the object-location learning task. Our data suggests that Sema3F/NRP2 signaling is required for contextual fear learning, specifically in the consolidation and/or retrieval of contextual fear memory.

7.2. Results

7.2.1 Sema3F/NRP2 signaling in the adult DG is required for contextual fear memory

I checked if Sema3F/NRP2 signaling is required in the dentate gyrus for contextual fear memory and auditory cue fear memory. Lentiviruses encoding for shCTR and shNRP2 were stereotaxically injected into the dentate gyri of adult animals and the mice were allowed to recover for 1 month in home cages for knockdown effects to occur before behavioral testing. Mice were sacrificed after behavioral testing to check for transduction efficiencies. Lentiviral transductions of dentate granule cells are highly efficient (Figure 7-1). 40 μm sections were made along the entire septotemporal axis of the brains and only data from animals showing GFP+ signals in 50% of all hippocampal slices (in either the left or the right hippocampus) were used for behavioural analysis.

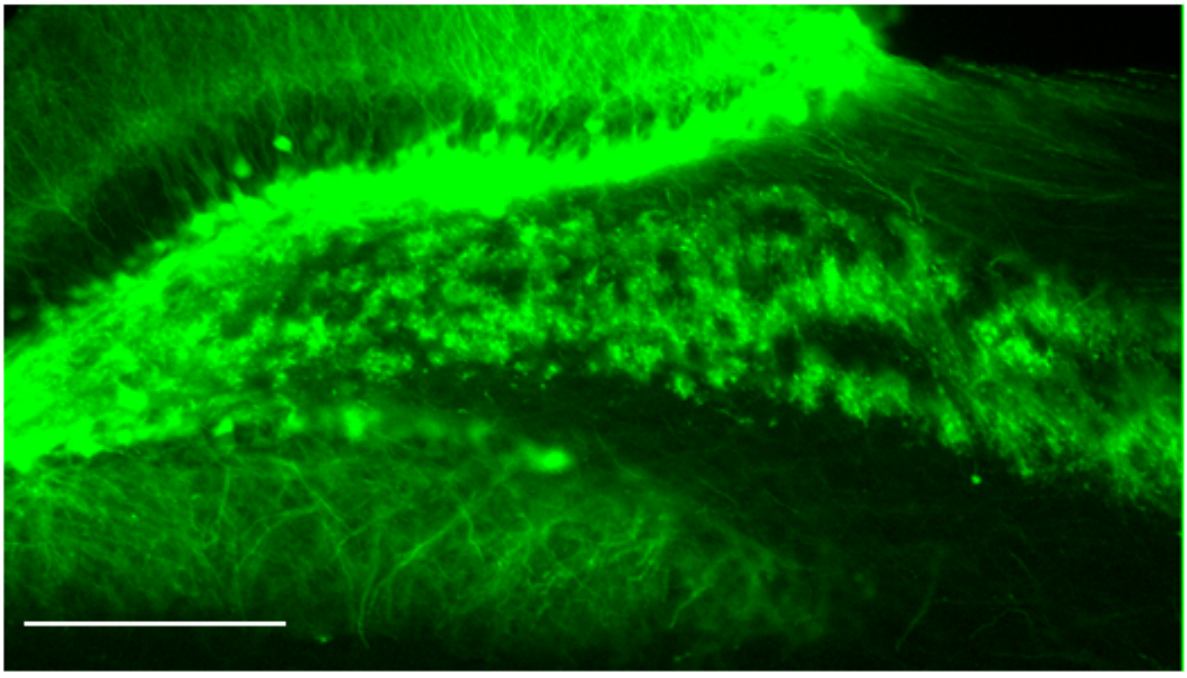

Figure 7-1. Efficient lentiviral transudction of granule cells in the dentate gyrus. A representative hippocampal slice showing large numbers of granule cells infected after stereotaxic injection of lentiviruses encoding shCTR or shNRP2. Scale bar = 200 μm.

For contextual and cued fear training, mice were allowed to habituate for 3 min in Context A in a conditioning chamber before a trial of 30 sec auditory tone with a 2 sec footshock at during the last 2 sec of the tone was administered. Mice were observed for a further 2 min before they were returned to home cages (Figure 7-2A). Freezing behaviors (defined as the absence of movement except for respiration) were measured during 3 min habituation and 2 min after tone/foot-shock pair. Brains were fixed after behavioral tests to check for the efficiency of lentiviral transduction. Both shCTR and shNRP2 mice exhibited absence of freezing during 3 min pretone and robust increase in freezing 2 min after the administration of the tone/foot-shock pair (Figure 7-2B).

24 hours after training, I tested for contextual fear memory. The mice were put back to Context A in the fear conditioning chamber without foot shock and observed for 5 min. Total freezing responses of the mice in 5 min were quantified from video recordings. shNRP2 animals showed significant lower freezing compared to shCTR animals in context A (Figure 7-2C), suggesting that Sema3F/NRP2 signaling in the adult dentate gyrus is required for contextual fear memory.

To check the cued fear memory, I returned the mice back to home cages for 2 hours after contextual fear testing. Mice were then placed in a novel context and allowed to habituate for 2 min in the new context. A 30 sec tone was administered without the accompany footshock and observed for 4 min after tone. Total freezing responses of the mice during the 30 sec tong and 4 min after the tone were quantitated from video recordings (Figure 7-2C). Both shNRP2 and shCTR showed similar responses during the 4.5 min cue test, suggesting that Sema3F/NRP2 signaling in the adult DG is not required for cue fear memory.

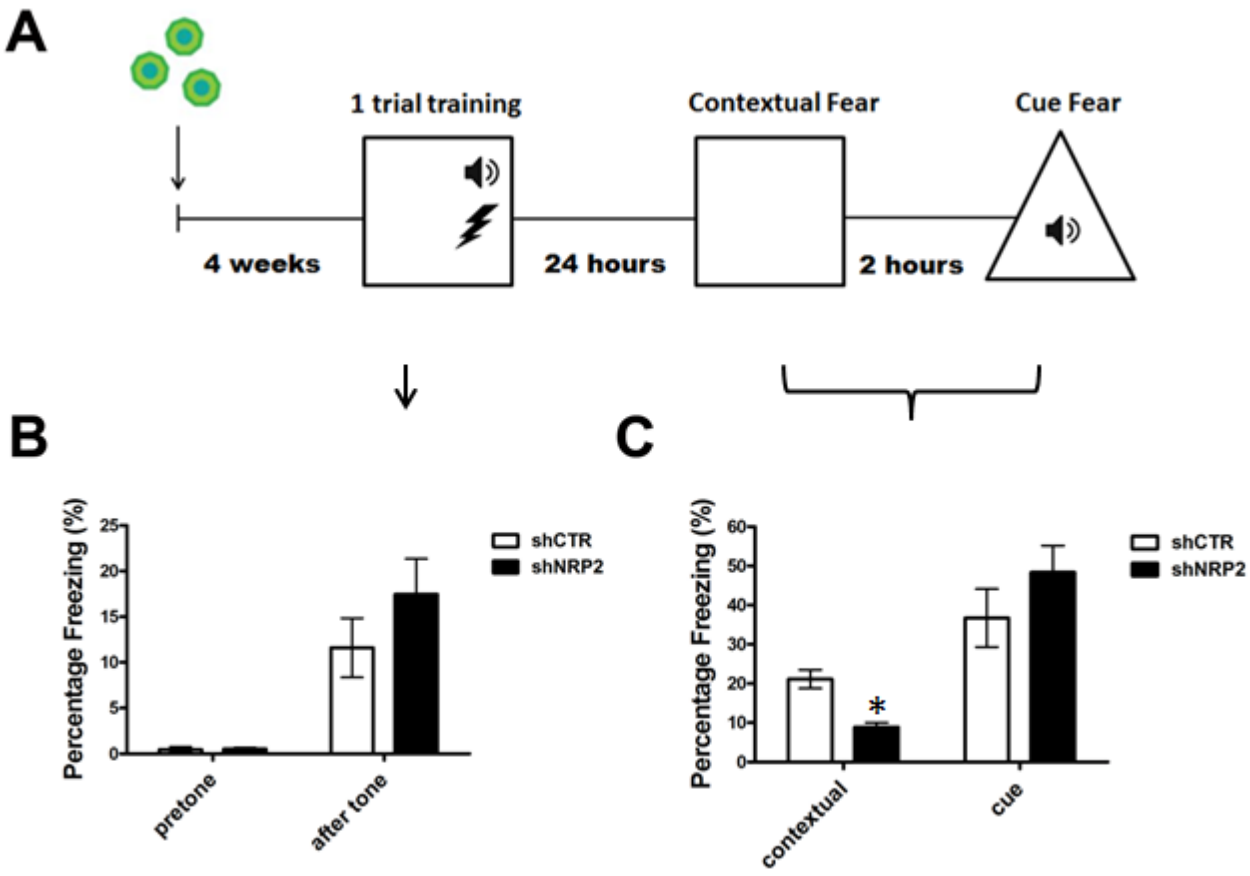

Figure 7-2. Sema3F/NRP2 signaling in the adult DG is required for contextual fear memory and not essential for cue fear memory. (A) Lentiviruses encoding for shCTR and shNRP2 (n = 10 per group) were injected into the DG of 6 weeks old male mice and returned to home cages for 4 weeks for knockdown effects to occur. For one trial fear training, shCTR and shNRP2 were allowed to habituate for 3 min in Context A in a fear conditioning chamber. A single trial consisting 30 sec auditory tone and co-terminated with a single 0.7 mA foot-shock during the last 2 sec of the tone was administered after habituation. Mice were then observed for 2 min after tone/footshock pair. 24 hours after training, freezing responses of mice were measured for 5 mins in Context A without footshock for contextual fear memory. Mice returned to home cages for 2 hours. To test for cue fear memory, mice were placed in a novel context B and played a 30 sec tone without footshock. Freezing responses were measured during the 30 sec tone and 4 mins after tone (B) Both shCTR and shNRP2 mice showed near absence of freezing during 3 min pretone habituation and increase in freezing after 2 min after administration of tone/foot-shock pair. (C) shNRP2 mice showed significantly lower freezing responses than shCTR mice during 5 min contextual test while both sNRP2 and shCTR showed similar freezing responses during 4.5 min cue fear test. Values represent mean ± s.e.m. (Student's t-test, *$p<0.05$).

7.2.2 Sema3F/NRP2 signaling in the adult DG is not required for fear acquisition

To check if Sema3F/NRP2 signaling in the adult DG is required for fear acquisition, I used a multiple trials protocol to measure fear learning during training. Lentiviruses encoding for shCTR and shNRP2 were stereotaxically injected into the dentate gyri of adult animals and the mice were allowed to recover for 1 month in home cages for knockdown effects to occur. Mice were then allowed to habituated for 3 min in Context A in a fear conditioning chamber 5 trials of tone/footshock pairings separated by an inter-trial interval of 1 min were administered. Each trial consists of a 30 sec auditory tone with a 2 sec footshock co-terminated the last 2 sec of the tone. After the 5^{th} trial, mice were observed for 2 min before they were returned to home cages. 24 hours after training, mice were tested for contextual fear memory by measuring the freezing responses of the mice for 5 min in Context A without footshock (Figure 7-3B). Both training, shCTR and shNRP2 mice showed absence of freezing during the 3 min habituation. With subsequent application of tone/footshock pairs, both shCTR and shNRP2 mice learned to associate tone with shock as shown by the increase in freezing responses following subsequent inter-trial intervals. Two minutes after the end of the 5^{th} trial, both shCTR and shNRP2 mice showed similar freezing responses (Mixed model ANOVA with repeated measures, genotype x time: $F(5, 110) = 0.45$, $p=0.8147$, genotype: $F(1, 110) = 0.3918$, $p = 0.3918$) . Hence, it appeared that Sema3F/NRP2 signaling at the adult DG is not required for fear acquisition (Figure 7-3B). 24 hours after training, I tested for contextual fear memory. Although both shNRP2 and shCTR animals showed similar fear learning, shNRP2 animals showed significant impairment in 24 hour contextual fear memory compared to shCTR animals (Figure 7-3C).

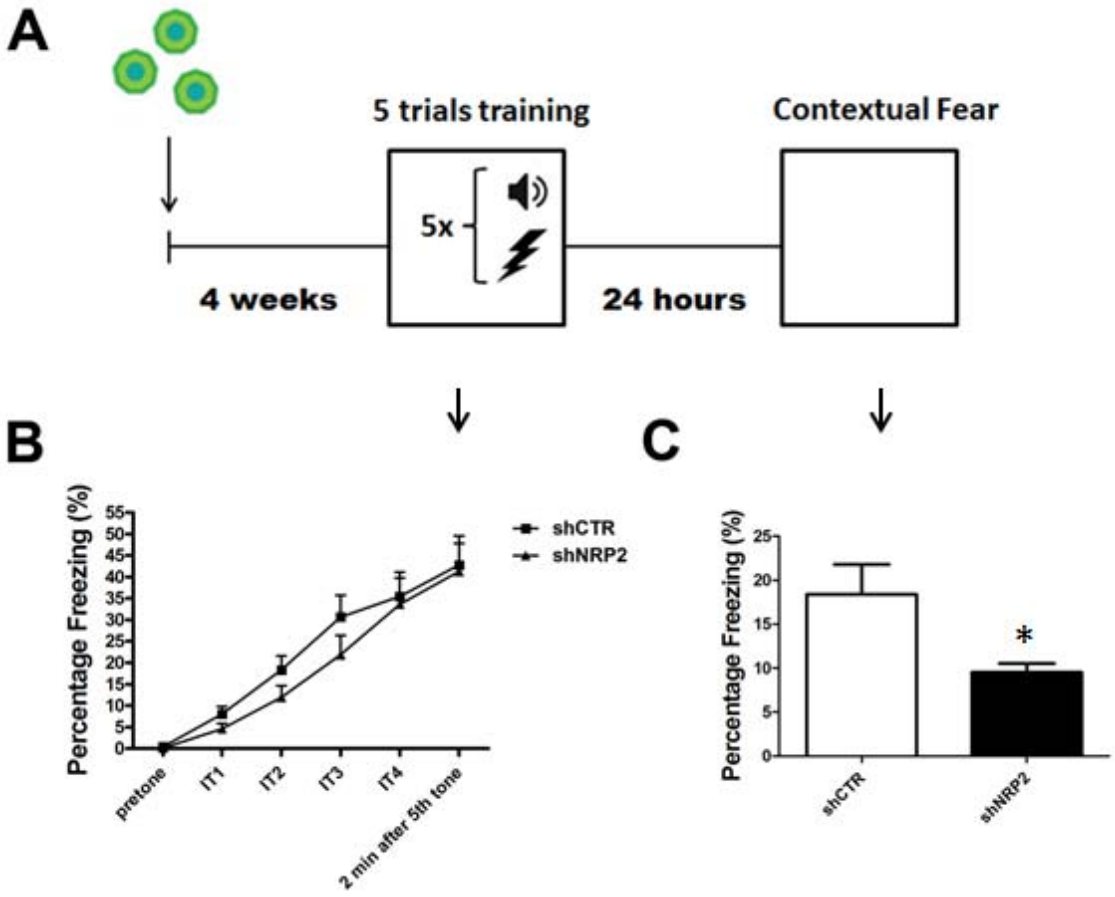

Figure 7-3. Sema3F/NRP2 signaling in the adult DG is not required for fear accquisition. (A) During training, mice were allowed to habituate for 3 min in a conditioning chamber followed by 5 trials of shock with 1 min inter-trial interval. Each shock trial consists of a 30 sec auditory tone was played and co-terminated with a 2 sec footshock. 24 hours after training, mice were placed for 5 mins in Context A without footshock and freezing responses of the mice were measured (B) Both shCTR and shNRP2 mice showed similar fear acquisition during 5 trials training Graphs show freezing responses during habituation, inter-trials intervals (IT1-4) and 2 min after 5th tone. (Mixed model ANOVA with repeated measures, genotype x time: $F(5, 110) = 0.45$, $p=0.8147$, genotype: $F(1, 110) = 0.3918$, $p = 0.3918$). (C) shNRP2 mice showed significant impairment in contextual fear memory. Values represent mean ± s.e.m. (Student's t test, *$p<0.05$).

7.2.3 Sema3F/NRP2 signaling in the adult DG does not affect anxiety levels or locomotor activity

To check if that the contextual freezing responses were not due to differences in anxiety or locomotor activity, I tested shCTR and shNRP2 animals in the open field test. Typically, mice would spend more time in the periphery compared to the center of the arena. Mice displaying lower anxiety levels would spend more time in the center of the arena compared to control animals. Both shCTR and shNRP2 animals spent the similar amounts of time in the periphery (Figure 7-4A) and center of the arena (Figure 7-4B), suggesting that Neuropilin-2 knockdown in the adult DG did not affect anxiety in adult mice. Furthermore, shNRP2 animals did not have locomotive deficits. Both shNRP2 and shCTR animals covered the similar total horizontal distance (Figure 7-4C) and with similar velocities (Figure 7-4D). Therefore, the impairments in contextual fear seen in shNRP2 animals were not due to differences in anxiety and locomotor activities between shNRP2 and shCTR animals.

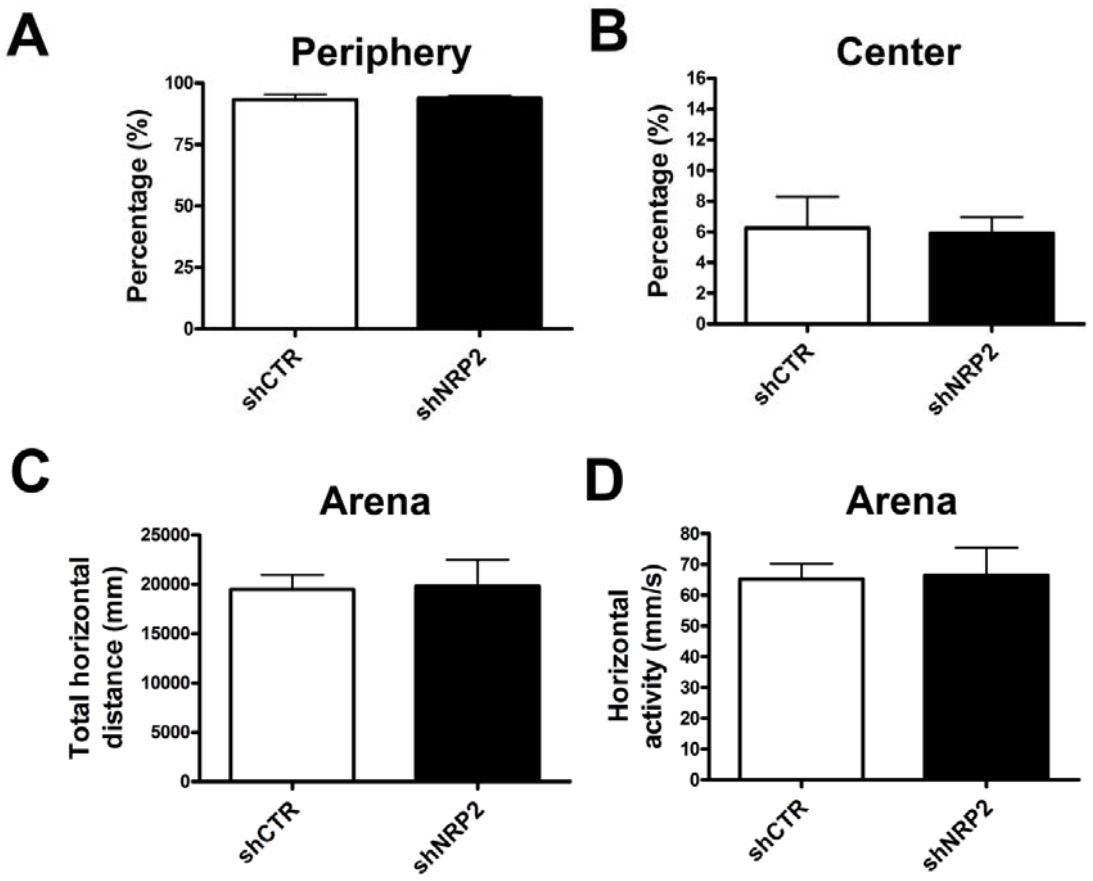

Figure 7-4. Sema3F/NRP2 signaling in the adult DG does not affect anxiety levels or locomotor activities. shNRP2 and shCTR animals spent similar amounts of time in the periphery (A) and in the center (B) of the arena when mice were allowed to explore for 5 min in an open field arena. Horizontal distances travelled (C) and velocities (D) were also similar in shCTR and shNRP2 mice. Values represent mean ± s.e.m. Student's t-test, no significant differences found.

7.2.4 Sema3F/NRP2 signaling in the adult DG is not required for spatial learning in the object-location learning task

I was interested to address if Sema3F/NRP2 signaling in the adult dentate gyrus is required for spatial learning in the object-location learning task. In this behavioral paradigm, I trained the mice to recognize two objects placed in two locations in an arena. After 20 min (short term memory) or 24 hour (long term memory), I changed the location of one of the objects to a new location (novel) while keeping in the other object in the old location (familiar) and

observed the amount of time the mice spent in exploring the object in the familiar and novel location. Typically, mice would spend more time exploring the object placed in the novel location compared to the object located in the familiar location. The preference for the object was measured by the discrimination ratio (Time spent in exploring object in novel location – Time spent in exploring object in familiar location / Total time spent in exploring both objects). Positive scores indicated preferences for the object in the novel location, negative scores showed preferences for the object in the familiar location, and scores approaching zero denoted no preference for the object in either location.

Both shCTR and shNRP2 animals have positive scores, showing preferences for the object in the novel location. Importantly, both shCTR and shNRP2 showed similar discrimination ratios after 20 min (Figure 7-5A) and 24 hour (Figure 7-5B), suggesting that Sema3F/NRP2 signaling is not required for spatial learning in the object-location task.

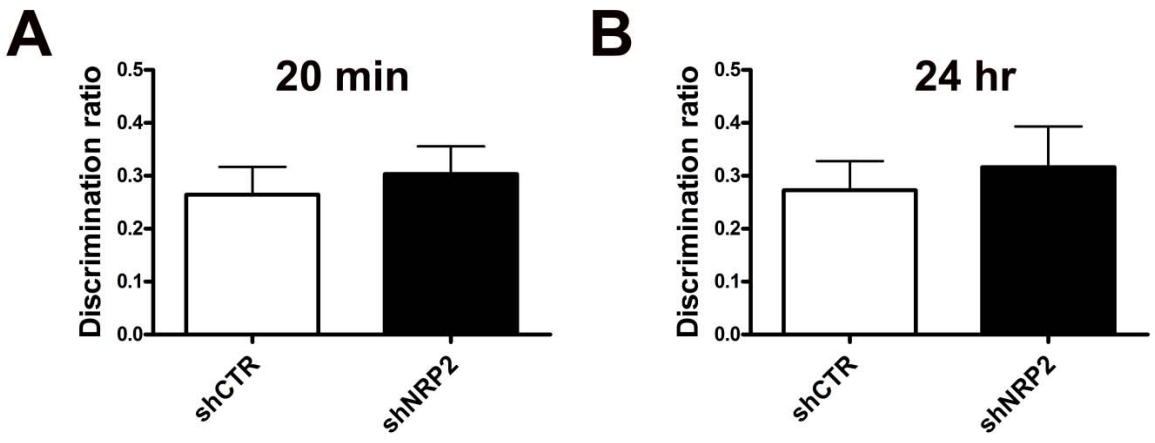

Figure 7-5. Sema3F/NRP2 signaling in the adult DG is not required for spatial learning in the object-location learning task. Both shCTR and shNRP2 mice showed preferences for the object in the novel location and showed similar discrimination ratios after (A) 20 min and (B) 24 hr. Values represent mean ± s.e.m. Student's t-test, no significant difference found.

7.2.5 Sema3F/NRP2 signaling in the adult DG is not required in the learning phase of the Morris water maze test

Next, I checked in Sema3F/NRP2 signaling in the adult DG is required for spatial learning in the Morris water maze test. shCTR and shNRP2 mice were trained in a pool to rely on extra-maze cues in the room to navigate to a hidden platform hidden 1 cm below the water surface.. Each animal is tested four times a day for five consecutive days, with inter-trial of 20 to 40 min. For each trial, mice are released facing the tank wall from one randomly selected starting points (North, South, East or West), and are allowed to swim until they reach the platform. In our protocol, the location of the platform remains constant throughout the experiment and the criterion of learning success consists to reach the platform in less than 20 sec. A well-trained mouse would showed decreasing escape latencies over successive training days and trials. Both shCTR and shNRP2 (n = 11 per group) mice showed decreasing and similar escape latencies over successive training (Figure 7-6A) days and (Figure 7-6B) trials. Importantly, both shCTR and shNRP2 animals reached the successful training criterion of 20 sec by the third day of training (Figure 7-6B). Therefore, Sema3F/NRP2 signaling in the adult DG is not required in the learning phase of the Morris water maze test

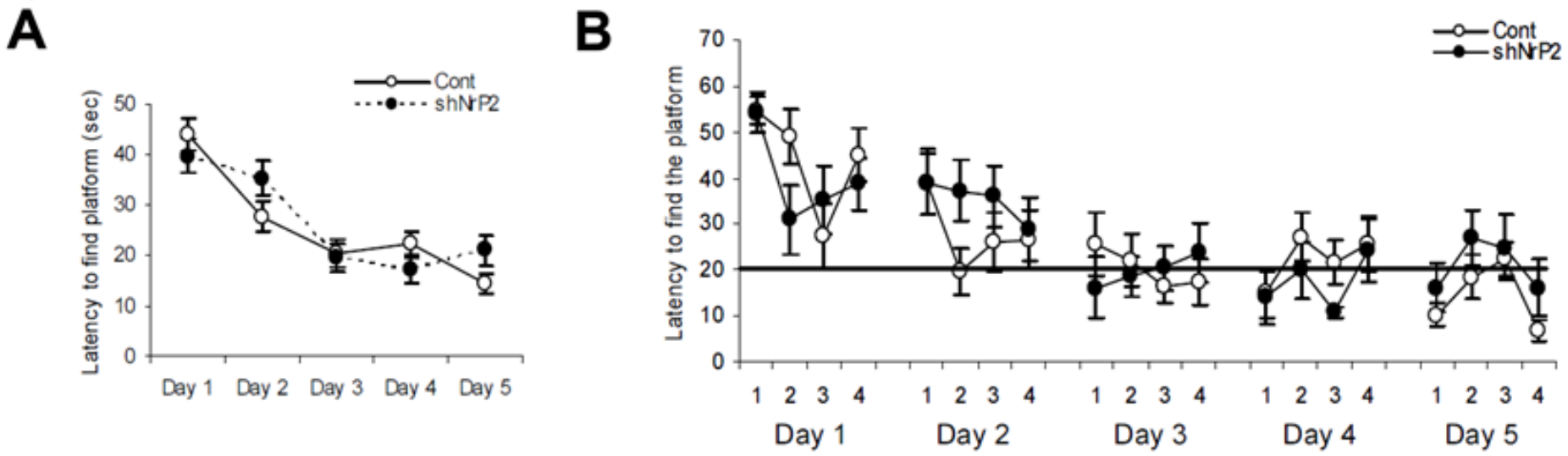

Figure 7-6. Sema3F/NRP2 signaling in the adult DG is not required in the learning phase of the Morris water maze test. The learning phase consists of four trials (with a maximum of 60 sec) per day with 30-45 min inter-trial intervals. Location of the hidden platform was fixed throughout the training. Both shCTR and shNRP2 (n = 11 per group) mice showed decreasing and similar escape latencies over training (A) days and (B) trials and reached successful training criterion of 20 sec at third day of training. Values represent mean ± s.e.m. Student's t-test, no significant differences found.

7.2.6 Sema3F/NRP2 signaling in the adult DG is not required for spatial reference memory of the Morris water maze test

Lastly, I checked if Sema3F/NRP2 signaling in the adult DG is required for spatial reference memory. I conducted the probe test where I removed the hidden platform on Day 6, after 5 days of learning phase of the Morris water maze test, and allowed the mice to search for the platform in 60 sec. A well-trained mouse would spend make more entries into the target quadrant (the quadrant where the platform was previously located) compared to other quadrants. The spatial bias indicates the spatial reference memory of the animal. In the probe test, both shCTR and shNRP2 animals made significantly more entries into the target quadrant (Figure 7-7A) compared to opposite and adjacent quadrants in 60 sec. In addition, shCTR and shNRP2 mice made similar entries into the area previously occupied by the platform in the 60 sec probe test (Figure 7-7B). Hence, knocking down NRP2 in the adult DG did not affect performance in the probe test. Taken together (Figure 7-5 and 7-6), Sema3F/NRP2 signaling in the adult DG is not required for spatial learning and memory in the Morris water maze test.

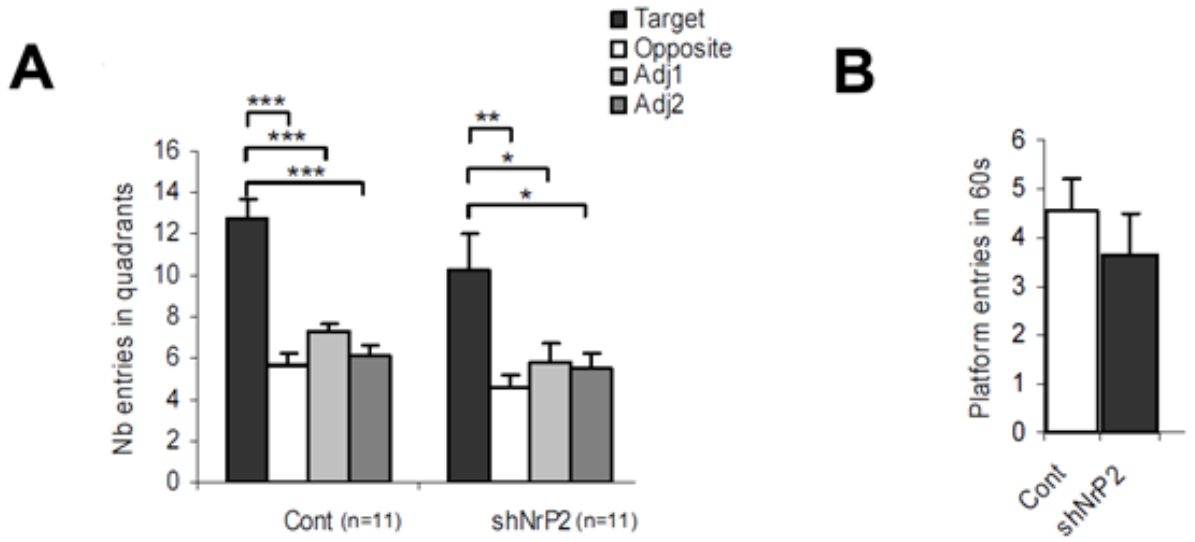

Figure 7-7. Sema3F/NRP2 signaling in the adult DG is not required for probe phase of the Morris water maze test. The probe test was carried out on Day 6, 24 hour after the last trial on training day 5 of the Morris water maze test. In the probe test, the hidden platform was removed and the mice were given 60 sec to search for the platform. (A) Both shCTR and shNRP2 (n = 11 per group) mice made significant more entries into the target quadrant compared to opposite and adjacent quadrants. ANOVA followed by post-hoc comparisons with quadrants (*p<0.05, **p<0.01, ***p<0.001) (B) Both shCTR and shNRP2 mice made similar entries in the area previously occupied by the platform. Student's t test, no significance found. Values represent mean ± s.e.m.

7.3. Discussion

The dentate gyrus is critical for learning and memory (Marr, 1971; Patton and McNaughton, 1995; Rolls and Kesner, 2006). In the adult DG, Semaphorins RNA transcripts are found in axonal projections of entorhinal cortex (Giger et al., 1998), dentate granule cells (Barnes et al., 2003; Hirsch et al., 1999; Holtmaat et al., 2002) and interneurons (Alme et al., 2010; Barnes et al., 2003; Gong et al., 2003). NRP1 and -2 RNA transcripts are found in dentate granule cells with high levels of NRP1 and -2 proteins are found in the axons and dendrites of dentate granule cells (Tran et al., 2009). The embryonic loss of Sema3F and NRP2 led to aberrant synaptic transmission and ectopic dendritic spines in dentate granule cells, leading us to hypothesize that Sema3F/NRP2 signaling in adult is required for learning and memory (Sahay et al., 2005; Tran et al., 2009). In this study, I used lentiviruses to knockdown NRP2 receptors in adult dentate granule cells and tested the animals in behavioural paradigms. The lentiviral approach confers clear advantages over the use of knockout animals, allowing us to directly address the specific roles of cells in the adult dentate gyrus in learning and memory (Kathirvelu and Colombo, 2013; Kathirvelu et al., 2013) and to circumvent developmental defects associated with Sema3F and NRP2 knockout animals (Sahay et al., 2005; Tran et al., 2009).

My data showed the lentiviral mediated knockdown of Neuropilin-2 in the adult dentate gyrus led to impaired contextual fear memory but spared cue fear training following one-trial and multiple trials training. It is known that contextual fear conditioning is highly dependent on the hippocampus and the amygdala while cue fear training is dependent on the amygdala (Phillips and LeDoux, 1992). After one trial training, shNRP2 animals showed impaired contextual fear training, suggesting that Sema3F/NRP2 signaling in the adult DG is critical for contextual fear memory. Both shCTR and shNRP2 mice show similar freezing curves during multiple-trials training, suggesting that impairments in contextual fear memory is not

due to deficits in fear acquisition.

On the other hand, shNRP2 and shCTR animals performed similarly in the object-location tasks and in the Morris water maze test, suggesting that Sema3F/NRP2 signaling in the adult DG is not required for spatial learning. These data seemingly contradict that idea that the dentate gyrus is required for both contextual and spatial memory as shown by lesion studies. However, it should be noted that contextual and spatial learning are regulated by different neural circuits. Contextual fear learning is highly dependent on neural connections between hippocampus and amygdala (Maren, 2011; Maren et al., 2013). In addition, lesions of the perirrhinal or postrhinal cortex which send projections to the hippocampus, specifically affect contextual fear training but spare spatial learning (Bucci et al., 2000; Bucci et al., 2002; Burwell et al., 2004). On the other hand, spatial learning is dependent on neural networks between grid cells in the hippocampus, entorhinal cortex and head cells in other brain region (Ainge and Langston, 2012). Moreover, contextual and spatial learning tasks could be independently regulated, depending on the strains (d'Isa et al., 2011) or gender (Dachtler et al., 2011) of the mice used. Lastly, Morris water maze protocols test for different forms of memory (Vorhees and Williams, 2006). In our protocol, I fixed the location of the platform throughout the training days and showed that knocking down NRP2 in the adult DG had no effect on spatial reference memory. By varying the Morris water maze protocols, I can check if Sema3F/NRP2 signaling in the dentate gyrus is required for other forms of spatial memory, such as spatial working memory (Steele and Morris, 1999) and reversal learning (Lipp and Wolfer, 1998).

Hippocampal subregions play distinct roles in learning and memory: the dentate gyrus is critical for pattern separation while the CA3 is required for pattern completion (Bakker et al., 2008; Treves and Rolls, 1992, 1994), it would be of great importance to address if Sema3F/NRP2 signaling is required in pattern separation tasks, such as delayed non-

matching-to-place task (Costa et al., 2005) and metric pattern separation (Gilbert et al., 2001; Goodrich-Hunsaker et al., 2005, 2008; Hunsaker and Kesner, 2008; Kesner et al., 2004; Lee and Solivan, 2010) in future studies. While this study focused on the roles of the adult DG, NRP2 are also expressed in CA3 pyramidal cells (Barnes et al., 2003; Gong et al., 2003; Holtmaat et al., 2002). It will also be of considerable interest to target NRP2 in CA3 cells to address if retrieval of memory from partial cues is affected (Nakazawa et al., 2002).

In conclusion, Sema3F/NRP2 signaling in the adult DG is required for contextual fear memory but spared spatial learning. This finding contributes to the molecular mechanisms underlying learning and memory in the adult hippocampus.

Chapter 8

Overall discussion and conclusion

8.1 Summary of findings

Although the roles of Class 3 Semaphorins have been well-characterized in developing embryonic CNS, there are no studies about their roles in adult neurogenesis. While Class 3 Semaphorins have been implicated in regulating the dendritic spines development and synaptic plasticity of DG cells during embryonic and post-natal development, it is not known if Class 3 Semaphorin signaling in the adult animal is required for hippocampal-dependent learning and memory. This study was set to investigate the roles of Class 3 Semaphorins in the adult DG by addressing the following questions:

1. What are the roles of Class 3 Semaphorins, if any, in regulating the neuronal development of adult-born neurons in the DG?
2. What are the downstream mediators of Class 3 Semaphorins signaling regulating these cellular events?
3. Is Class 3 Semaphorins signaling in the DG involved in hippocampal-dependent functions, particularly in learning and memory?

In this study, I explored the above questions and provided experimental evidence supporting the hypothesis that Class 3 Semaphorins are involved in the neuronal development of adult-born neurons and hippocampal-dependent learning and memory. This is the first study showing that Neuropilin-1 and -2 are expressed in adult SGZ neural precursor cell culture, promoting me to address if Class 3 Semaphorins regulate the neuronal development of adult-born neurons in the SGZ. Addition of Class 3 Semaphorins did not affect the proliferation

and differentiation of adult neural precursor cells *in vitro*. Moreover, both control and NRPs deficient adult-born neurons express Prox1 and DCX (markers of immature neurons) at 14 DPI, suggesting that Neuropilins do not regulate the *in vivo* differentiation of adult-born neurons in the SGZ. However, knocking down NRP1 and -2 in neural precursor cells resulted in adult-born neurons with several dendritic defects. On the other hand, knocking down NRP2 specifically affected the cell positioning of adult-born neurons in the dentate granule layers. I uncovered downstream mediators regulating these cellular events: Cdk5/FAK signaling pathway is activated by NRP1 and -2 and required for dendritic growth of adult-born neurons and GSK3β signaling is regulated by NRP2 for required for proper cell positioning in the adult hippocampus. Importantly, shCdk5 and shFAK neurons show defects in dendritic development and normal cell positioning while shGSK3β neurons how normal dendritic growth but cell positioning defects, suggesting that dendritic growth and cell positioning of adult-born neurons are regulated by independent pathways. Importantly, I was able to rescue the dendritic and cell migration defects by over-expressing Cdk5/FAK and GSK3β respectively. Lastly, I showed that lentiviral-mediated disruption of Sema3F/NRP2 signaling in the adult dentate gyrus impaired contextual fear learning. shNRP2 mice showed similar anxiety and locomotor activity levels as shCTR animals. In addition, Sema3F/NRP2 signaling does not seem to affect spatial learning and memory, as measured by similar performances of shNRP2 and shCTR animals in the Morris water maze test and object-location learning tasks. On the other hand, shNRP2 mice showed impaired contextual memory for the single and multiple trials contextual fear conditioning tasks, suggesting that Sema3F/NRP2 signaling is required for contextual learning and memory. Taken together, this study reveals the novel roles of Class 3 Semaphorins in the neuronal development of adult-born neurons and in regulating learning and memory in the adult hippocampus.

8.2 Class 3 Semaphorins regulate neuronal development of adult-born neurons

The hippocampus was first described in 1587 by the Venetian anatomist Julius Caesar Aranzi, who likened it to a seahorse-shaped ridge running along the temporal horn of the lateral ventricle. The hippocampus is formed during embryogenesis but most of the neurons are generated during post-natal stages (Bayer, 1980; Bayer et al., 1982). The discovery of adult neurogenesis in the SGZ significantly enhances the cellular and functional complexity of the hippocampus. It is proposed that the adult neural stem cell in the SGZ has an embryonic origin (Li et al., 2013). A plethora of signaling pathways regulating the development and functioning of neurons in the hippocampus are still being uncovered, some of these pathways are conserved between embryonic and adult neurogenesis (Ming and Song, 2011; Rolando and Taylor, 2014; Sun et al., 2011; Zhao et al., 2008). Many of these studies are focused on the early events of neurogenesis, such as proliferation, differentiation, and fate specification, comparatively much less is known about the signaling pathways regulating later stages of neurogenesis, such as dendritic and axonal growth, migration, and integration into neural networks.

In the embryonic and post-natal hippocampus, there is no published evidence suggesting that Class 3 Semaphorins are involved in the proliferation or differentiation of neural stem cells although there is a study showing that Sema3A is required for early specification of neuronal polarity by promoting dendritic identity and supressing axonal identity (Shelly et al., 2011). In addition, Class 3 Semaphorins are involved in dendritic branching and growth of CA1 pyramidal cells and in primary neuronal cultures (Nakamura et al., 2009; Pozas et al., 2001; Rehberg et al., 2014; Schlomann et al., 2009), axonal pruning of DG mossy fibers (Bagri et al., 2003; Chen et al., 2000; Giger et al., 2000), guidance of septal and hippocampal axons (Catalano et al., 1998; Chedotal et al., 1998; Pascual et al., 2005; Pozas et al., 2001), regulating synapse formation (Bouzioukh et al., 2006b; Lee et al., 2012; Tran et al., 2009)

and synaptic transmission (Bouzioukh et al., 2006b; Lee et al., 2012; Sahay et al., 2005; Tran et al., 2009).

This study demonstrated the novel role of Class 3 Semaphorins regulating the dendritic development of adult-born neurons. Retroviral-mediated knock down of NRP1 or NRP2 in adult neural precursor cells severely impaired dendritic development of adult-born neurons. Using primary neuronal culture, this study confirmed that tyrosine phosphorylation of FAK at Y397 is required for Semaphorin-induced dendritic growth (Schlomann et al., 2009) and uncovered data suggesting the novel role of Cdk5/FAK signaling in dendritic growth, apart from its known functions in microtubule organization and neuronal migration (Park et al., 2009; Xie et al., 2003). Knocking down Cdk5 or FAK phenocopied the dendritic phenotypes of Neuropilins-deficient neurons and overexpression of Cdk5 and FAK can rescue these dendritic defects. Taken together, these data suggest that Class 3 Semaphorin signaling is required for the dendritic development and is largely conserved between embryonic and adult neurogenesis.

While it was shown that Sema3F/NRP2 signaling is required for long range radial migration (Chen et al., 2008; Shelly et al., 2011) and tangential migration of cortical neurons during embryonic development (Ito et al., 2008; Marin et al., 2001; Tamamaki et al., 2003), migration of adult-born neurons from the subgranular zone to their proper positions in the granular layer cannot be readily extrapolated from embryonic studies of cortical migration due to region-specific, spatial and temporal differences. Intriguingly, knocking down NRP2 and GSK3β resulted in adult-born neurons occupying ectopic positions in the middle and outer thirds of the granule cells and overexpression of GSK3β restored the proper cell positioning of shNRP2 neurons. The data suggests that Sema3F/NRP2 signaling plays a novel role in regulating the cell positioning of adult-born neurons. This cell positioning phenotype has not been previously described in Semaphorins literature and advances

knowledge of the limited signaling pathways known to affect this process (Duan et al., 2007; Fitzsimons et al., 2013; He et al., 2014; Hunt et al., 2012; Manning et al., 2012; Zhou et al., 2013).

Cdk5 (Beffert et al., 2004; Ohshima et al., 1996) and its co-activators p35/p39 (Ko et al., 2001) are critical for the proper migration of cortical neurons during embryonic development and serine phosphorylation of FAK by Cdk5 is required for embryonic corticogenesis (Xie et al., 2003). In this study, knockdown of both FAK and Cdk5 did not affect cell positioning of adult-born neurons, suggesting that Cdk5-FAK signaling could be regulated differently during the development of the embryonic and adult nervous systems. Interestingly, knocking down of GSK3β specifically affects cell positioning while sparing dendritic growth of adult-born neurons. Importantly, overexpression of GSK-3β rescued the cell positioning, but not dendritic deficits, of shNRP2 neurons. Hence, I showed that dendritic growth and cell positioning of adult-born neurons could be regulated by distinct mechanisms and this idea is also supported by other published studies (Duveau et al., 2011).

8.3 Class 3 Semaphorins signaling is likely to be required for neuronal functions of adult-born neurons in the SGZ

Proper development of dendrites is critical for proper neuronal functions. Dendrites must fulfil three important criteria for proper neuronal functions: (1) dendrites must cover its receptive field encompassing the sensory and/or synaptic inputs of the neuron (MacNeil and Masland, 1998; Wässle and Boycott, 1991), (2) the dendritic branching density and pattern must be suitable for sampling and processing the converging signals on the dendritic field (Hu et al., 2008; Spruston, 2008), and (3) dendrites must exhibit structural plasticity to adjust to changing experience and activity (Espinosa et al., 2009; Jaworski et al., 2009). The hippocampus is highly organized in a laminated fashion (Foster et al., 2000). Dendrites of

granule cells process information from diverse sources: in the inner third of the molecular layer, they receive projections of mossy cells (Laurberg, 1979; Swanson et al., 1978), in the middle third of the molecular layer, they receive projections from the medial entorhinal cortex, and in the outer third of the molecular layer, they receive projections from the lateral entorhinal cortex (Amaral and Witter, 1989; Steward, 1976; Steward and Scoville, 1976).

Both adult-born neurons with deficient Neuropilins showed severely impaired dendritic development, often with only one main apical dendrite with very few branches. These neurons clearly showed defects in dendritic targeting in the molecular layer, as measured by measurements of total dendritic length, branch numbers and dendritic complexity. Without forming a proper dendritic field, these neurons presumably will not be able to sample and process the rich cortical signals converging onto the dentate gyrus and receive feedback information from hilar mossy cells within the DG. Given that adult-born neurons in the SGZ are critical for pattern separation by sparsely coding cortical inputs to prevent interference (Clelland et al., 2009; Creer et al., 2010; Nakashiba et al., 2012; Sahay et al., 2011a; Scobie et al., 2009; Tronel et al., 2012), it is highly likely that knocking down Neuropilins in adult-born neurons will adversely affect pattern separation due to the perturbed dendritic targeting and the consequential defects in sampling of cortical signals by these neurons.

Cell positioning of adult-born neurons is required for proper neuronal functions. For example, LIS1 mutant mice, ectopic positioning of granule cells in the molecular layer of the dentate gyrus resulted in neurons exhibiting aberrant Excitatory Post-Synaptic Currents (EPSCs) (Hunt et al., 2012). Sema3F/NRP2 signaling regulate cell positioning of adult-born neurons, presumably by acting as a STOP signal to prevent the ectopic migration of adult-born neurons beyond the inner third of the DG. However, it is presently difficult to assess the contribution of cell positioning to the functions of adult-born neurons as shNRP2 have both dendritic and cell positioning defects. On the other hand, shGSK3β neurons appear to have

only cell positioning defects. Therefore, GSK3β signaling is potentially a good system to assess the functional significance of cell positioning of adult-born neurons. One could start by measuring and comparing the electrophysiological properties of shGSK3β "mis-positioned" neurons in the middle third and outer granular layer and shGSK3β "normally positioned" neurons in the inner third of the granular layer. It is also possible to check the integration of shNRP2 and shGSK3β adult-born neurons in the neural network by injecting neural tracers.

8.4 Sema3F/NRP2 signaling contributes to the molecular mechanisms underlying learning and memory

The synapse occupies a central stage in learning and memory by permitting neural transmission and enabling structural changes during synaptic consolidation. It has been shown that Sema3F/NRP2 signaling in required for dendritic spines and synaptic transmission during embryonic and post-natal stages (Sahay et al., 2005; Sahay et al., 2003; Tran et al., 2009). However, it has not been demonstrated that Sema3F/NRP2 signaling is required for learning and memory, possibly because the Sema3F null animals are prone to seizures and NRP2 null animals are weak and do not survive well to adulthood, precluding them from behavioural analysis.

In this study, I set out to address if Sema3F/NRP2 signaling is required in the adult DG for learning and memory. I showed that lentiviral mediated knockdown of NRP-2 in the adult dentate gyrus led to impaired contextual fear memory following one-trial training. Interestingly, multiple-trials training showed that shCTR and shNRP2 mice had similar fear learning curves, suggesting that the impairment in fear memory is not due to defects in fear acquisition. On the other hand, shNRP2 and shCTR animals performed similarly in the object-location tasks and in the Morris water maze test, suggesting that Sema3F/NRP2

signaling in the adult DG is not required for spatial learning. These novel findings contribute to the molecular mechanisms underlying learning and memory in the adult hippocampus.

In the adult dentate gyrus (DG) and other CNS regions, neuronal connectivity is not thought to be hardwired and mature neurons retain a limited degree of structural plasticity, which is tightly regulated by signaling pathways throughout adulthood (Yuste and Bonhoeffer, 2004). It was shown that Sema3F/NRP2 signaling in required for dendritic spines and synaptic transmission during the embryonic and post-natal stages (Sahay et al., 2005; Sahay et al., 2003; Tran et al., 2009), I am currently checking the morphological development of shNRP2 neurons in the adult DG.

8.5 Is Sema3F/NRP2 signaling required in other hippocampal functions, apart from learning and memory?

It is known that the dorsal hippocampus is required for cognitive functions while the ventral hippocampus is required stress responses, emotions and affective behaviours (Fanselow and Dong, 2010; Moser and Moser, 1998). Likewise, adult-born neurons in the dorsal and ventral hippocampi are also functionally dissociated. It could be hypothesized that Class 3 Semaphorins signaling specifically regulates cognitive learning and affective behaviour in the dorsal and ventral hippocampus respectively. In this study, I injected lentiviruses expressing shCTR and shNRP2 along the spatiotemporal axis of the hippocampus and did not discriminate between dorsal and ventral hippocampus. While shNRP2 mice showed impaired performance in contextual fear learning compared to shCTR mice, shNRP2 mice showed similar anxiety levels and exploratory activities as shCTR mice in the open field test. It is premature to conclude that Sema3F/NRP2 signaling in the ventral DG is not required for anxiety from these experiments and more work is required to test this hypothesis more rigorously. For example, shNRP2 lentiviruses can be specifically targeted to the ventral or

dorsal DG and the animals tested for affective behavioural paradigms, such as open field, zero maze and forced swim tests. In addition, it was also showed that adult neurogenesis in the dorsal hippocampus is required for contextual fear learning while the ventral hippocampus regulates innate anxiety levels (Kheirbek et al., 2013). It will be interesting to investigate if Sema3F/NRP2 signaling in adult-born neurons is specifically required for cognitive and affective behaviours by targeting high titer retroviruses expressing shNRP2 in the dorsal and the ventral DG and carrying out the behavioural tests on the animals. the dentate gyrus is critical for pattern separation while the CA3 is required for pattern completion (Bakker et al., 2008; Treves and Rolls, 1992, 1994), it would be of great interest to address if Sema3F/NRP2 signaling is required in pattern separation tasks, such as delayed non-matching-to-place task (Costa et al., 2005) and metric pattern separation (Gilbert et al., 2001; Goodrich-Hunsaker et al., 2005, 2008; Hunsaker and Kesner, 2008; Kesner et al., 2004; Lee and Solivan, 2010).

8.6 Clinical Implications of this study

Many neurological disorders are attributed to disruptions in signaling pathways regulating synaptic plasticity in mature neurons (van Spronsen and Hoogenraad, 2010). It is also appreciated that dysfunctional signaling can result in aberrant development of adult-born neurons and impaired neurogenesis is a common finding in many neurological disease (Ruan et al., 2014).

Synaptic failure is believed to be a major pathological mechanism underlying many neurological diseases (Selkoe, 2002; van Spronsen and Hoogenraad, 2010). For example, in a human amyloid precursor protein (hAPP) transgenic model of Alzheimer's disease (AD), adult born neurons showed aberrant development of dendritic arbors and synaptic spines (Li et al., 2009; Sun et al., 2009). Previous studies have shown that Sema3F/NRP2 signaling is

required for proper spines formation and synaptic transmission (Sahay et al., 2005; Sahay et al., 2003; Tran et al., 2009) and I show here that inhibiting Sema3F/NRP2 signaling in the adult mice affected contextual learning, a common behavioral deficit found in AD mouse models and human patients (Hamann et al., 2002). It could be fruitful to explore the tangible links between Semaphorin signaling and neurodegenerative diseases, such as Alzheimer's disease.

Defects in neuronal maturation and dendritic arborization in the hippocampus and cortex have been implicated in neurodevelopmental diseases, such as Rett Syndrome (Smrt et al., 2007; Zhao et al., 2003) and morphological development of immature neurons (unpublished data from our lab). Interestingly, it was shown that Semaphorin 3F is a transcriptional target of MeCP2 in mouse models of Rett Syndrome (Degano et al., 2009). It will be of considerable interest to check if Semaphorins are transcriptional targets using samples from Rett Syndrome human patients. Pharmacological inhibitors of Semaphorins could have potential therapeutic applications in treating these diseases.

8.7 Future Work

The fundamental difference between embryonic and adult neurogenesis is that development of adult-born neurons takes place in a mature environment and the neurons have to integrate into existing neural circuits. Emerging evidence suggests that although embryonic and adult neurogenesis shared conserved signaling mechanisms, the spatial-temporal regulations are distinct due to differences in the environments (Ming and Song, 2011). For instance, it was known that GABA regulates both the neuronal maturation of embryonic neurons (Rivera et al., 1999) and adult-born neurons (Ge et al., 2007), evoking similar electrophysiological properties in both cell types (Laplagne et al., 2007b). However, in early development, GABA release is non-synaptic, genetically specified and occurs in defined embryonic time windows

(Haydar et al., 2000; LoTurco et al., 1995; Manent et al., 2005; Owens and Kriegstein, 2002; Represa and Ben-Ari, 2005) while GABA release in the adult CNS is synaptically released by interneurons within the dentate gyrus and non-synaptically by adult precursor cells, activity-dependent and occurs throughout the adult life span (Doetsch et al., 1997; Ge et al., 2006; Wang et al., 2003).

It is imperative to characterize the detailed spatial and temporal expression patterns of Semaphorins and other guidance cues in order to fully understand their roles in neuronal development and hippocampal functions. For instance, region-restricted astrocytic release of Sema3A is required for axonal initial segment orientation, synaptic formation and survival of α motor neuron in the ventral spinal cord (Molofsky et al., 2014). A combinatorial Semaphorins code instructs different stages of neural circuit assembly the Drosophila CNS (Wu et al., 2011b) and is hypothesized to regulate the development of subpopulations of motor neurons in the spinal code (Cohen et al., 2005). It was shown that Neuropilins interact with other receptors, such as Robo1 receptor (Hernandez-Miranda et al., 2011) and VEGF receptors (Favier et al., 2006), suggesting that it is important to characterize the expression patterns of other guidance cues as these could potentially modulate Neuropilin signaling. Lastly, the expression of Semaphorins and Neuropilin are temporally regulated and activity-dependent, induced by electrical activity, physical exercise, learning and environmental enrichment (Cao et al., 2004; Coma et al., 2011; Elfving et al., 2013; Lee et al., 2012). In this study, I showed that knocking down Sema3A and 3F in adult-born neurons affect their dendritic development, suggesting that Class 3 Semaphorins regulate the dendritic development of adult-born neurons in a cell-autonomous manner. Future studies are required to assess the contributions of Semaphorins from other cell types in the hippocampus, such as endothelial and vascular cells in the neural stem cell niche, astrocytes, hilar interneurons and mature granule cells along the septotemporal axis of the dentate gyrus. It is equally important

to investigate the temporal patterns and activity-dependent regulation of Semaphorins and Neuropilins, in both physiological and pathological conditions.

Apart from dendritic growth and cell positioning of adult-born neurons, it is still unclear if Class 3 Semaphorins regulate neuronal development of adult-born neurons, including dendritic spines and mossy fibers development. The defects in dendritic growth and cell positioning of shNRP1 and -2 neurons implied that these adult-born neurons could not integrate properly into the existing neural circuits. To prove that it is the case, single cell electrophysiology of neurons deficient in NRP1 or -2 should be performed. Alternatively, one could use retrograde and anterograde labelling techniques to show if the adult-born neurons could make proper synaptic connections with the entorhinal cortex and CA3 regions. The Synaptotrophic Hypothesis proposes that inputs from a presynaptic to postsynaptic cell eventually change the course of synapse formation. The metabolic cost of dendritic elaboration and the need to cover the receptive field determines the size and shape of dendritic arbors (Cline and Haas, 2008). Entorhinal axons are the principal source of Class 3 Semaphorins in the molecular layer of the DG (Giger et al., 1998; Tran et al., 2009). It will be very interesting to investigate if synaptic activities in the entorhinal cortex are critical for dendritic elaboration, and possibly cell positioning, of adult-born neurons through the regulated release of Semaphorins in the entorhinal cortex.

The dentate gyrus is a heterogeneous structure comprising of embryonic-born, mature neurons and adult-born, immature neurons. Immature, adult-born neurons make up a small population of the DG and showed increased intrinsic excitability and plasticity compared the majority of mature, embryonic-born neurons (Esposito et al., 2005; Ge et al., 2007). Adult-born neurons mature after a few weeks and become indistinguishable from mature neurons (Laplagne et al., 2006). Adding to this complexity, neurons are born different rates throughout adulthood in response to various inputs so that heterogeneity of the DG changes

over time (Deng et al., 2013; Deng et al., 2009; Kim et al., 2012b). Using lentiviruses, I showed here that Sema3F/NRP2 signaling in the adult-born and mature neurons is critical for learning and memory. Embryonic loss of Sema3F and NRP2 resulted granule cells with ectopic spines formation and exhibited increased EPSCs frequency during embryonic and post-natal stages (Tran et al., 2009). It has not been formally demonstrated here that the impairment of contextual fear memory is due to disruption to dendritic spines formation and synaptic plasticity of adult-born and mature neurons as there could be differences between embryonic and adult neurogenesis. It is important to check the cellular morphology and electrophysiological properties of both adult-born and mature neurons after lentiviral transduction. It is still presently Class 3 Semaphorins signaling in adult-born and mature neurons has distinct roles in learning and memory. A critical experiment is to inject high titer retroviruses encoding for shNRP2 into the DG and subject the animals in learning and memory tasks. By comparing the performances of these animals to the lentiviral transduced animals in this study, one can assess the relative contributions of Semaphorin signaling in adult-born and mature neurons to learning and memory. In addition, the systemic and activity-dependent regulation of Semaphorins and Neuropilins (Cao et al., 2004; Elfving et al., 2013; Lee et al., 2012) presumably has consequences on the morphology and functioning of embryonic and adult-born neurons. It is conceivable to investigate the relationships between systemic regulation, Semaphorin-induced structural changes and its consequential effects on learning and memory.

Lastly, while retroviral and lentiviral-mediated approaches are powerful and established techniques used in assessing the morphological and behavioral analysis, they suffer from several disadvantages. It is highly unlikely to infect all the granule cells along the septotemporal axis of the DG (1 million cells in the rodent DG), even with highly successful stereotaxic injection and the infection efficiency depends on the delivery and uptake of viral

particles, rendering them less useful for quantification studies (for example, in quantifying the proliferation of adult-born neurons). Recently, specific CreER promoter lines for adult-born neurons and mature neurons have been generated (DeCarolis et al., 2013; McHugh et al., 2007; Nakashiba et al., 2012; Sun et al., 2014). These lines could be used to generate inducible and conditional knockout lines to confirm the data in this study and investigate the aspects of spatial and temporal regulation that are not readily addressable by viral-mediated techniques.

8.8 Conclusion

The mechanisms and functional implications of neuronal development of adult neurogenesis and hippocampal functions are still far from clear. Dysregulation of adult neurogenesis and hippocampal functions could explain neurobehavioral disorders. Our study provided strong and novel evidence implicating Class 3 Semaphorins in neuronal development of adult-born neurons and in adult hippocampal functions, particularly in learning and memory. It is hoped that this study sets the cornerstone for future investigations in the Semaphorins-dependent mechanisms of adult neurogenesis and hippocampal functions in normal physiology and neurological and psychiatric diseases. Studies on the dysregulation of these signaling pathways could explain etiologies of brain diseases and potentially have clinical and therapeutic applications.

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