UNIVERSITY OF CALIFORNIA, SAN DIEGO

Regulation of Hippocampal Synapse Formation and Specificity

A Dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Neurosciences

by

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2008

The Dissertation of Elizabeth Kathryn Davis is approved, and it is acceptable in quality and form for publication on microfilm:

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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ABSTRACT OF THE DISSERTATION

Regulation Hippocampal Synapse Formation and Specificity

by

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Doctor of Philosophy in Neurosciences

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Professor Anirvan Ghosh, Chair
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Synapse formation is a complex process that requires coordination of multiple steps. The factors that control this process are only beginning to be understood. Several molecules have already been implicated, including cell adhesion molecules like cadherins, synCAM, and neuroligin and its binding partner β-neurexin as well as secreted factors like FGF22, thrombospondin, and Wnts. Wnts are an intriguing class of molecules that have been reported to be involved in everything from regulating cell fate in stem cells to cancer. However, Wnts have only recently been investigated in synapse formation. Studies exploring Wnts in synapse formation have yielded conflicting results and it is unclear whether this disagreement is a result of the variety of systems or the variety of Wnt proteins used in these studies.

I show here that Wnts are expressed in all regions of the forebrain during the first two weeks of postnatal rodent development, a time at which synapses rapidly form. Using hippocampal cultures, I show that several Wnts expressed in the hippocampus can mediate various signaling pathways. We show that Wnts 3a, 7a, and 7b, which are able to upregulate β-catenin, have a positive effect on synapses, while Wnt5a, which is unable to stabilize β-catenin, has a negative effect on synapses. This bidirectional control of synapses is likely due to the differential effects of these Wnts on stabilization of β-catenin.

In addition to the coordinated assembly of synapses, it is also important for these synapses to be targeted to the correct postsynaptic cell. This directed synapse formation creates functional circuits of the brain. In collaboration with Megan Williams, we explore the specificity of the DG synapse *in vitro* using a combined functional and anatomical approach. We find that DG neurons in the absence of axon guidance cues make more synapses and evoke larger postsynaptic currents in their correct postsynaptic targets, CA3 neurons, than in any other cell type. These results lay the groundwork in which to explore potential mediators of synaptic specificity, including Wnts.

CHAPTER 1: Introduction and Dissertation

1.1. Introduction

The brain is comprised of millions of neurons which communicate with each other by both electrical and chemical signals through distinct structures named synapses. Chemical synapses make the majority of synapses in the central nervous system and are composed of several specialized compartments, including a presynaptic terminal with an active zone, a synaptic cleft, and a postsynaptic density. The presynaptic terminal is filled with hundreds of small vesicles that hold neurotransmitters. When a depolarizing pulse travels along an axon, this depolarization opens calcium channels in the terminal leading to influx of calcium. The calcium binds to proteins at the active zone of the presynaptic terminal, causing fusion of vesicles and release of neurotransmitter into the synaptic cleft. The fusion of vesicles is localized to the active zone which is directly juxtaposed to the postsynaptic density. The fusion of vesicles translates an electrical pulse in the presynaptic cell into a chemical signal. The main neurotransmitters of the brain are GABA, an inhibitory neurotransmitter, and glutamate, an excitatory neurotransmitter.

The postsynaptic density at excitatory synapses is composed of scaffolding proteins which serve to cluster receptors. There are two ionotropic receptors at excitatory synapses, AMPA and NMDA receptors. The receptors open upon binding to glutamate to flux ions, translating the chemical signal back into an electrical one. It is this type of communication through synapses that is the underlying basis of our thoughts. Understanding the process by which synapses are formed is fundamental in

our understanding of the development of the most human of traits, personality and emotions. While the characteristics of mature synapses have been studied in depth, how synapses arrive at that mature state and the factors that influence the development of synapses are still unclear.

1.2. Synapse Formation

The general process of synaptogenesis on the scale of the whole brain or an entire circuit may occur over weeks or even months, depending on the species. In the cortex of primates, for example, synaptogenesis occurs at a high rate beginning approximately two months before birth and continues for two months after birth (Bourgeois and Rakic, 1993 and Bourgeois et al, 1994). In the visual cortex of rodent, this process takes place largely in the first three postnatal weeks (Fiala et al, 1998 and Blue and Parnavelas, 1983) whereas at the rat NMJ, acetylcholine receptors (AChRs) begin to cluster under nerve terminals at E16 and synapse maturation and elimination is complete by P14 (Lomo, 2003). However, an individual synapse in culture can form within one to two hours (Ahmari et al, 2000; Friedman et al, 2000; Okabe et al, 2001; and Washbourne et al, 2002). Synapse formation begins with contact between pre- and postsynaptic cells. At the NMJ, this contact occurs between the muscle and the growth cone of an axon on a motor neuron, while in the CNS, contact may be between an axon and dendritic shaft or an axon and filapodia (Ahmari et al, 2000; Fiala et al, 1998; Gerrow et al, 2006; Jontes et al, 2000; Niell et al, 2004; and Ziv and Smith et al, 1996). The steps that come after this initial contact can vary greatly among systems.

The accessibility and simplicity of the vertebrate NMJ has made the NMJ a good starting point in which to study and derive a model for the development of synapses. After an axon of a motor neuron exits the spinal cord, it travels to a single muscle and makes contact with multiple muscle fibers. Upon first contact, these axon terminals are capable of neurotransmitter release (Kidokoro and Yeh, 1982; Chow and Poo, 1985; and Xie and Poo, 1986), but have a low efficacy (Nakajima et al, 1980). These terminals have few vesicles and no active zones. Over the span of a week, the synapse becomes fully functional by accumulating synaptic vesicles, cytoskeletal elements, and active zone proteins (Takahashi et al, 1987; Lupa and Hall, 1989; and Ko 1985). The organization of the presynaptic terminal is likely due to target derived factors such as FGF2 and laminin. FGF2 has been found to help localize synaptic vesicles (Dai and Peng, 1995) while laminin have been found to stop the growth of axons and cause accumulation of vesicles (Porter et al, 1995).

Postsynaptic differentiation occurs concurrently with presynaptic organization as AChRs on muscles aggregate just as the motor neuron makes contact. Agrin is well known to be a target derived factor in the organization of the postsynaptic density at NMJs. Agrin is a heparin sulfate proteoglycan (Denzer et al, 1995 and Tsen et al, 1995) that is synthesized by motor neurons and released from motor axon terminals (Neuhuber and Daniels, 2003 and Ma et al, 2000). Overexpression of agrin in the absence of nerve terminals leads to clustering of AChRs (Godfrey et al, 2000; Jones et al, 1997; Meier et al, 1997; and Cohen et al, 1997) while agrin mutant mice have impaired postsynaptic differentiation (Gingras et al, 2007; Gingras et al, 2002; and

Gautam et al, 1996). MuSK appears to be the best candidate receptor for agrin signaling. MuSK mutants show a similar phenotype to agrin mutants and fail to form AChR clusters even in the presence of agrin (Zhou et al, 1999) despite normal levels of AChRs (DeChiara et al, 1996). Reintroduction of MuSK rescues this phenotype (Zhou et al, 1999). Downstream of MuSK is a protein named rapsyn that exists in the postsynaptic density at a 1:1 ratio with AChRs. Rapsyn is thought to bind directly to AChRs (Sealock et al, 1984) and rapsyn knockout mice display no AChR clusters (Gautam et al, 1995). In addition to the clustering of AChRs, part of the process of forming a functional NMJ is the repression of AChR clustering in extrasynaptic sites on the muscle. Shortly, after the motor neuron makes contact, the site directly under the motor neuron increases the density of AChRs while sites outside decrease in AChR density. This repression is due to electrical stimulation of the muscle (Sanes and Lichtman, 1999).

In the CNS, presynaptic proteins are delivered to the synapse byway of two types of vesicles: piccolo transport vesicles (PTVs) and synaptic vesicle protein transport vesicles (STVs). PTVs were discovered in hippocampal neurons due to the expression of the scaffolding protein piccolo. Purification of membrane fractions that associated with piccolo showed that these vesicles carry a number of active zone proteins along with scaffolding proteins such as piccolo and bassoon (Zhai et al, 2001). PTVs move rapidly in young neurons (Shapira et al, 2003) and arrive at the synapse within minutes (Friedman et al, 2000). STVs carry synaptic vesicle proteins and proteins required for exo- and endocytosis to the synapse (Ahmari et al, 2000).

Like PTVs, STVs are highly mobile in developing neurons (Nakata et al, 1998 and Dai and Peng, 1996). STVs have also been shown to be associated with the microtubule motor proteins kinesin-1, KIF1a, and KIF1Bβ2 (Nakamura et al, 2002; Okada et al, 1995; and Sato-Yoshitake et al, 1992).

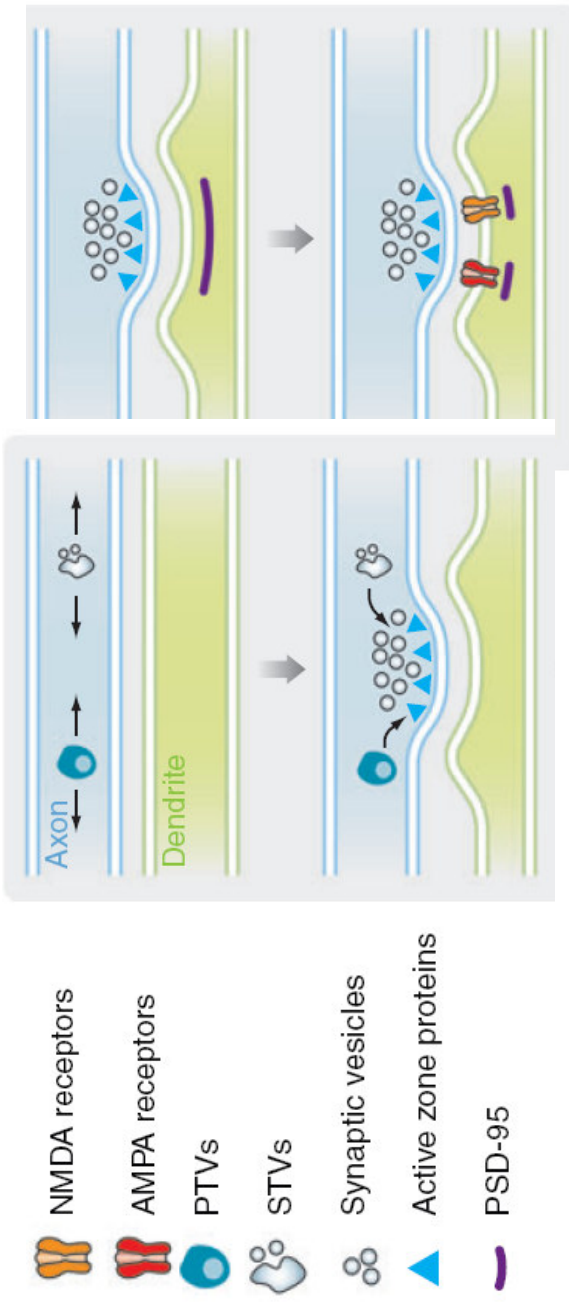
On the postsynaptic side, scaffolding proteins are thought to arrive first and then NMDA and AMPA receptors (Bresler et al, 2004; Washbourne et al, 2002; and Friedman et al, 2000). It is currently unclear which side of the synapse is organized first (Gerrow et al, 2006, Friedman et al, 2000). Upon maturation of the synapse, the number of AMPA receptors increases, indicated by an increase in the AMPA to NMDA receptor ratio (Hsia et al, 1998; Crair and Malenka, 1995; Lu et al, 2001). This change in receptor composition also leads to an expansion of the spine head (Passafaro et al, 2003 and Matsuzaki et al, 2001). Eventually, the synapse develops the appropriate electrophysiological and structural characteristics to become fully mature. This process is illustrated in Figure 1-1 (McAllister, 2007).

1.3. Molecular Mechanisms of CNS Synapse Formation

The process of CNS synapse formation is controlled by many different factors, including cell adhesion molecules, glial derived molecules, secreted molecules (Waites et al, 2005), and activity (Knott et al, 2002 and Maletic-Savatic et al, 1999). While activity is a major determinant in synapse maturation and stabilization (Zhang

Figure 1-1. Synapse formation in the CNS.

In a young axon, PTVs and STVs are highly mobile. When contact occurs between a pre- and a postsynaptic neuron, PTVs deliver scaffolding molecules and active zone proteins to the presynaptic terminal while STVs deliver synaptic vesicle related proteins and proteins necessary for exo- and endocytosis. On the postsynaptic side, PSD-95 accumulates and then AMPA and NMDA receptors are recruited to the membrane (adapted from McAllister, 2007).


Axon
Dendrite
NMDA receptors
AMPA receptors
PTVs
STVs
Synaptic vesicles
Active zone proteins
PSD-95

and Poo, 2001) my dissertation will focus primarily on the molecular basis of synapse formation. Currently, there are several known molecules that participate in synapse formation in the CNS, including cell adhesion factors such as synCAM, neuroligin and its binding partner, β-neurexin, and cadherins (Waites et al, 2005 and Shapiro et al, 2007). Both synCAMs and neuroligins are families of four different transmembrane proteins. SynCAMs demonstrate heterophilic binding with different members of the same family (Fogel et al, 2007), while neuroligins at postsynaptic densities (Song et al, 1999) bind to β-neurexins at presynaptic terminals (Dean et al, 2003). SynCAM and neuroligin have become well-known due to their ability to initiate functional presynaptic terminal formation onto nonneuronal cells when either of the two molecules is transfected with a glutamate receptor into HEK cells (Bierderer et al, 2002; Scheiffele et al, 2000; and Sara et al, 2005). Likewise, β-neurexins have shown a similar ability to organize postsynaptic densities through neuroligin binding in both excitatory and inhibitory neurons (Graf et al, 2004; Chih et al, 2005; and Nam and Chen, 2005).

Cadherins are another intriguing family of molecules that may be involved in synapse formation. Cadherins are cell adhesion molecules that are localized to the synapse (Kohmura et al, 1998; Benson and Tanaka, 1998; and Jontes et al, 2004). Expression of a dominant negative construct in the horizontal cells in the chick retina or in hippocampal cultures leads to spine elongation, or persistent filapodia, which normally disappear at later ages, suggesting cadherins play a role in spine formation (Togashi et al, 2002 and Tanabe et al, 2006). Cadherins are also thought to be

responsible for presynaptic terminal organization through binding of β-catenin. B-catenin is thought to localize presynaptic vesicles to the membrane by binding Veli and CASK through a PDZ domain (Bamji et al, 2003). While the precise role of cadherins at the synapse is unknown, synapses formed onto hippocampal neurons with altered cadherin expression have decreased synaptic vesicle recycling and decreased spontaneous EPSCs while EM shows immature looking synapses in flies (Bozdagi et al, 2004 and Iwai et al, 2002).

Other types of synapse formation regulators are secreted molecules. These include FGF22, Wnts, and glial derived factors (Waites et al, 2005). FGF22 was discovered by purification of brain extracts which were found to promote synapse formation in cultured neurons. When FGF22 is applied to cultured neurons, it leads to longer neurites and more presynaptic puncta. Additionally, when it is knocked down, presynaptic differentiation of axons both *in vivo* and *in vitro* is inhibited (Umemori et al, 2004). Glia have also been shown to play a role in synapse formation through the secretion of molecules (Ullian et al, 2001 and Pfeiger and Barres, 1997). Thrombospondins and cholesterol have both been purified from glia conditioned media and have been shown to induce synapse formation when applied to cultures (Mauch et al, 2001 and Christopherson et al, 2005). Finally, as the subject of my dissertation, Wnts were first identified as inducers of synapse formation in the cerebellum. Wnts are a large class of signaling molecules that have been implicated in a variety of processes throughout development such as body axis specification, neural tube development, and synaptogenesis (Ciani and Salinas, 2005).

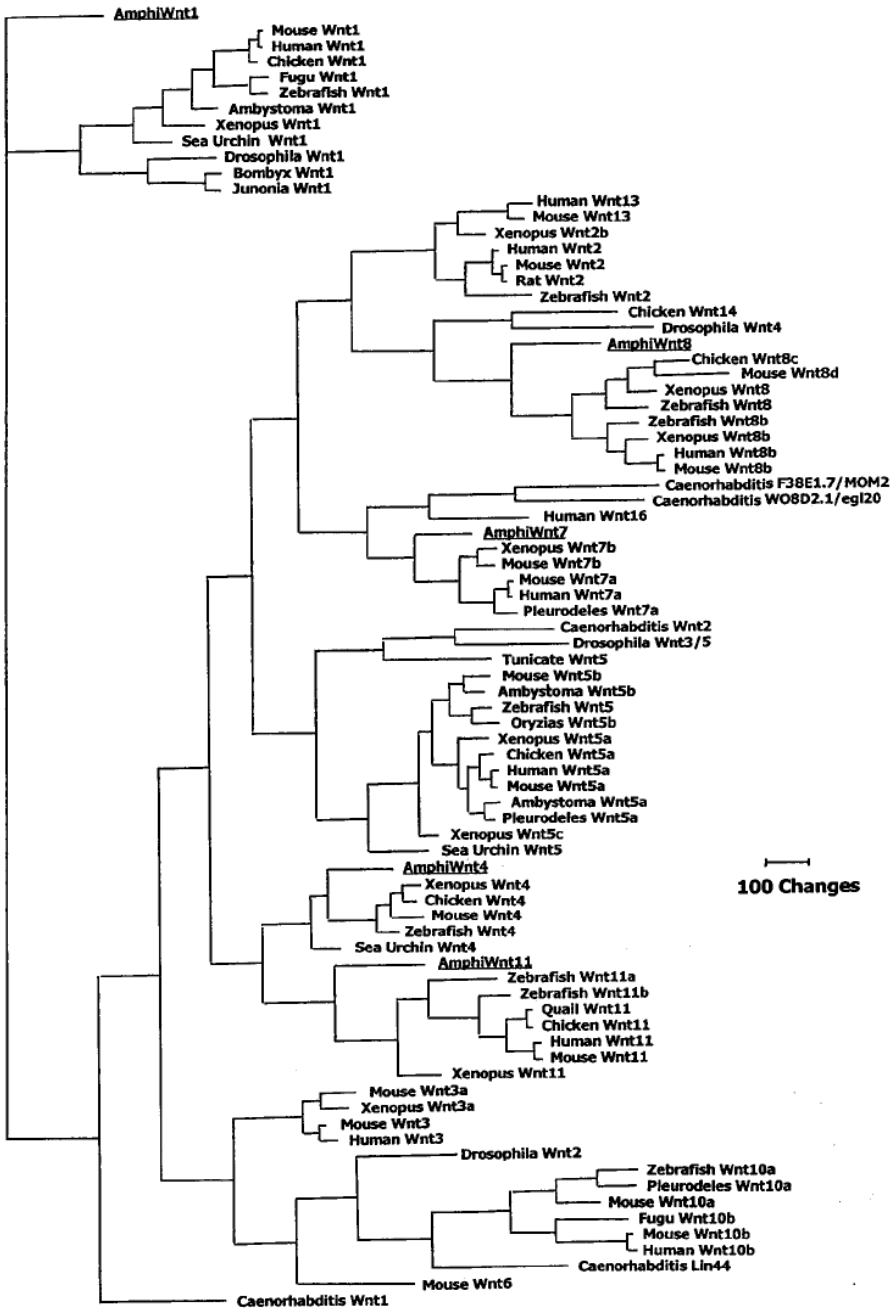
1.4. Wnt Proteins: A Diverse Signaling Network

Wnts are a conserved group of proteins across numerous species from sea urchins and *C. elegans* to humans, although the largest family exists in mammals. The relationships between Wnt proteins between species based on sequence homology can be seen in Figure 1-2 (Schubert et al, 2000). Wnts were first discovered in drosophila and named Wingless. In mammals, Wnts were initially named Ints. Eventually, the two names were combined and the family was named Wnts. Today, Wingless is known to be one of seven Wnts in drosophila and is closely related to a canonical pathway activator, mammalian Wnt1 (The Wnt Homepage). *C. Elegans* have only five Wnts, cwn-1, cwn-2, lin-44, mom-2, and egl-20 (Eisenmann, 2005) and rodents have 19 members (The Wnt Homepage). Wnts in all species contain a conserved cysteine enriched region which is palmitoylated (Logan and Nusse, 2004).

In vertebrates, Wnts can bind to multiple receptors, Frizzleds (Fzd), Ryk, and ROR2 (Kikuchi et al, 2007), although Fzds are the most common receptors for Wnts. Fzds are seven pass transmembrane proteins that are thought to be coupled to G proteins. Currently, there is no direct evidence for Fzds binding directly to G proteins, however, inhibition of $G_{i/o}$ and G_q type proteins disrupts multiple pathways mediated by Fzd (Ma and Wang, 2006; Slusarski et al, 1997; and Liu et al, 2001). There are ten members in the Fzd family in rodents (The Wnt Homepage). Wnts have several different pathways through which they can act, but the best studied is the canonical pathway. In this pathway Wnt binds to a LRP5/6 and Frizzled co-receptor, which

Figure 1-2. Phylogenetic Tree of the Wnt Gene Family.

Wnts are a conserved family across species. There are 19 members in rodents, 7 in drosophila, and 5 in nematodes. Each of these proteins is produced from a distinct gene. Wnt genes have sequence homology between 35% and 83%. This figure shows the relationship between all Wnts with full-length sequences in different species based on sequence homology (Schubert et al, 2000).


AmphiWnt1
Mouse Wnt1
Human Wnt1
Chicken Wnt1
Fugu Wnt1
Zebrafish Wnt1
Ambystoma Wnt1
Xenopus Wnt1
Sea Urchin Wnt1
Drosophila Wnt1
Bombyx Wnt1
Junonia Wnt1
Human Wnt13
Mouse Wnt13
Xenopus Wnt2b
Human Wnt2
Mouse Wnt2
Rat Wnt2
Zebrafish Wnt2
Chicken Wnt14
Drosophila Wnt4
AmphiWnt8
Chicken Wnt8c
Mouse Wnt8d
Xenopus Wnt8
Zebrafish Wnt8
Zebrafish Wnt8b
Xenopus Wnt8b
Human Wnt8b
Mouse Wnt8b
Caenorhabditis F38E1.7/MOM2
Caenorhabditis WO8D2.1/egl20
Human Wnt16
AmphiWnt7
Xenopus Wnt7b
Mouse Wnt7b
Mouse Wnt7a
Human Wnt7a
Pleurodeles Wnt7a
Caenorhabditis Wnt2
Drosophila Wnt3/5
Tunicate Wnt5
Mouse Wnt5b
Ambystoma Wnt5b
Zebrafish Wnt5
Oryzias Wnt5b
Xenopus Wnt5a
Chicken Wnt5a
Human Wnt5a
Mouse Wnt5a
Ambystoma Wnt5a
Pleurodeles Wnt5a
Xenopus Wnt5c
Sea Urchin Wnt5
AmphiWnt4
Xenopus Wnt4
Chicken Wnt4
Mouse Wnt4
Zebrafish Wnt4
Sea Urchin Wnt4
AmphiWnt11
Zebrafish Wnt11a
Zebrafish Wnt11b
Quail Wnt11
Chicken Wnt11
Human Wnt11
Mouse Wnt11
Xenopus Wnt11
Mouse Wnt3a
Xenopus Wnt3a
Mouse Wnt3
Human Wnt3
Drosophila Wnt2
Zebrafish Wnt10a
Pleurodeles Wnt10a
Mouse Wnt10a
Fugu Wnt10b
Mouse Wnt10b
Human Wnt10b
Caenorhabditis Lin44
Mouse Wnt6
Caenorhabditis Wnt1
100 Changes

signals downstream to Disheveled (Dvl). Dvl then inhibits a trio of proteins, GSK3β, Axin, and APC. Inhibition of these proteins leads to dephosphorylation of β-catenin, thus activating it and allowing translocation to the nucleus to activate TCF/LEF transcription factors (Polaskis, 2000 and Logan and Nusse, 2004; Figure 1-3). While many genes have been identified as transcriptional targets of the canonical Wnt pathway, a couple of genes are of interest, including EphB1 and ephrinB1 (Tice et al, 2002 and Batlle et al, 2000), which have been shown to cluster NMDA receptors (Dalva et al, 2000).

Several other pathways, including BMP, FGF, and cadherin signaling pathways, intersect at junctions within the Wnt canonical pathway. FGF and Wnt both converge when they cause upregulation of β-catenin signaling through inhibition of GSK3β (Katoh and Katoh, 2007). Cadherins are also well known to bind β-catenin, which then binds to α-catenin. A-catenin then anchors the complex to actin. Due to its ability to bind β-catenin, cadherins may even play a regulatory role in Wnt signaling by sequestering β-catenin (Wheelock and Johnson, 2003). GSK3β also regulates cell-cell communication by cadherin through presinilin1 (Uemura et al, 2007). The BMP-MAPK pathway also regulates the canonical Wnt pathway by activating Nlk which inhibits β-catenin dependent transcription (von Bubnoff and Cho, 2001).

In addition to the canonical pathway, Wnts can also activate a variety of noncanonical pathways. Most of these pathways also involve the activation of Dvl, but Fzds can also act directly through adenylyl cyclase (Chen et al, 2005) or casein kinase

Figure 1-3. Canonical Wnt Signaling Pathway.

The canonical signaling pathway can be considered as a two state model: one state in which Wnt is not bound and one state in which Wnt is bound to its receptor. When Wnt is absent, a complex of proteins consisting of APC, axin, and GSK3β phosphorylate β-catenin. This phosphorylation leads to the degradation of β-catenin. When Wnt binds to Fzd LRP5/6 co-receptors, Dvl is phosphorylated and binds to axin so that it is recruited to the surface of the cell to bind to LRP5/6. The complex is then broken apart and β-catenin levels within the cell rise. B-catenin can then translocate to the nucleus to regulate transcription.

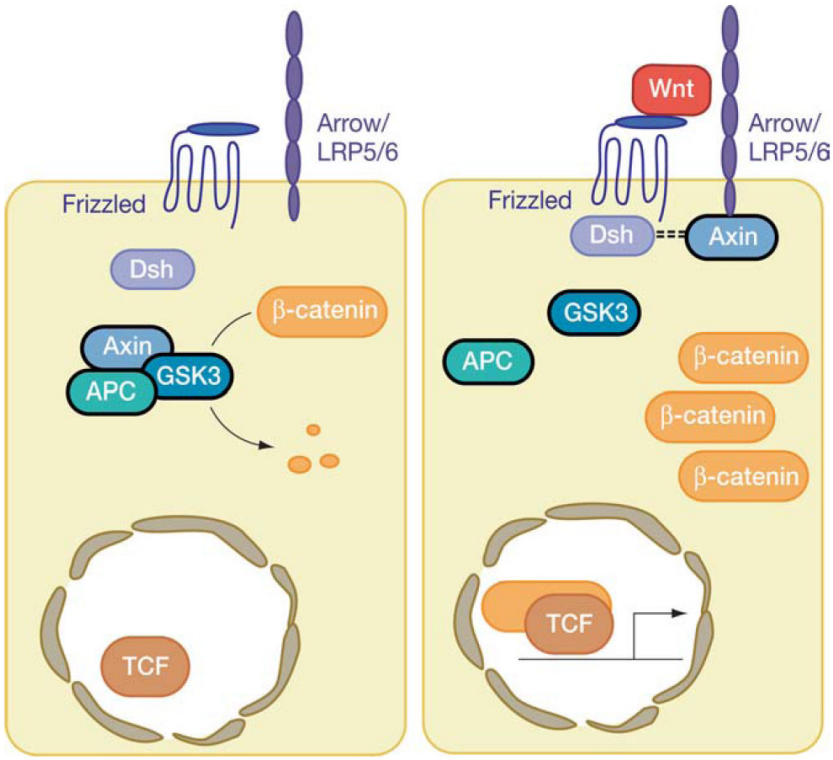
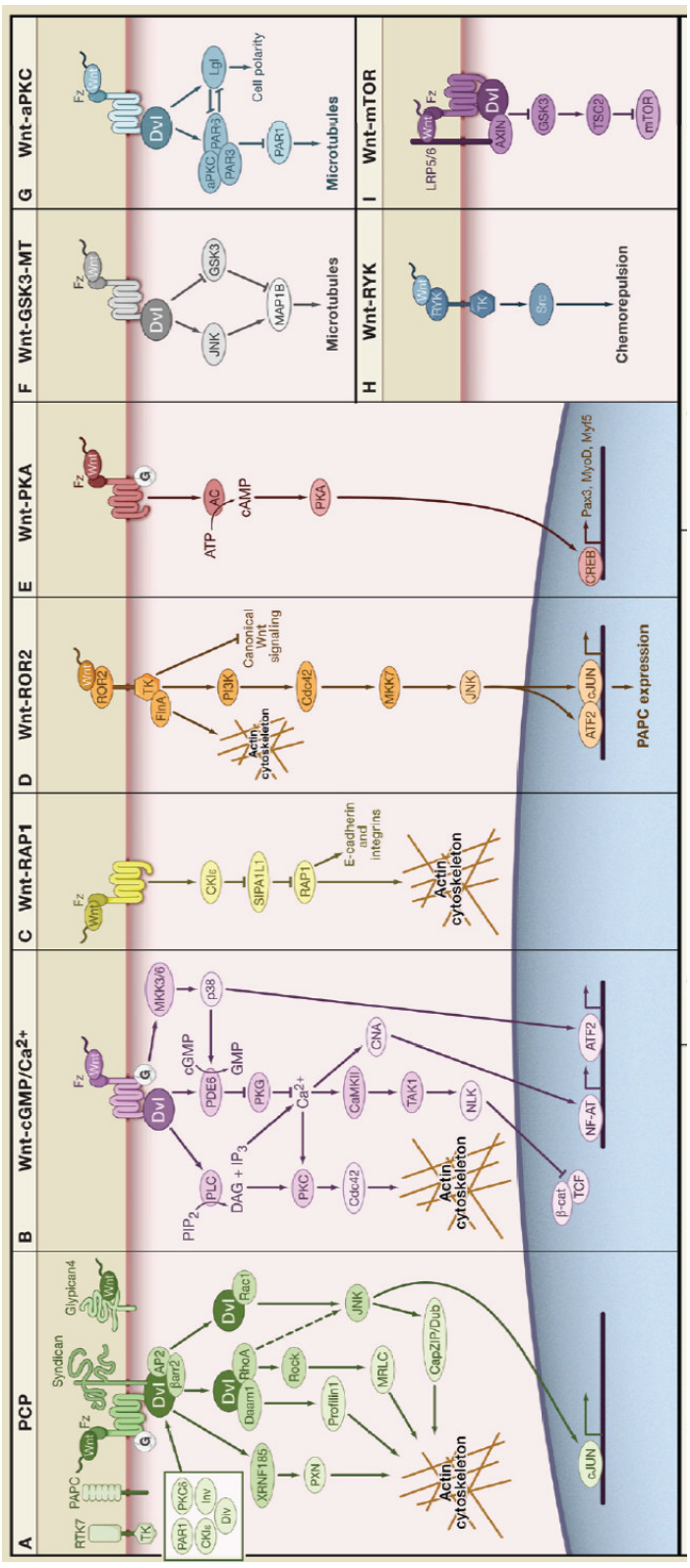

Arrow/
LRP5/6
Frizzled
Dsh
β-catenin
Axin
GSK3
APC
TCF
Wnt
Arrow/
LRP5/6
Frizzled
Dsh
Axin
GSK3
APC
β-catenin
β-catenin
β-catenin
TCF

Figure 1-4. Noncanonical Signaling Pathways.

Wnts can signal through several pathways other than the canonical pathway shown in Figure 1-3. These pathways are all considered noncanonical Wnt signaling pathways. Most noncanonical signaling pathways are mediated by the receptor Fzd, but Wnts can also signal through ROR2 and Ryk. Many of these pathways ultimately lead to transcription, like the canonical pathway. Additionally, some noncanonical pathways actually inhibit the canonical pathway (Semenov et al, 2007).


A PCP
B Wnt-cGMP/Ca2+
C Wnt-RAP1
D Wnt-ROR2
E Wnt-PKA
F Wnt-GSK3-MT
G Wnt-aPKC
H Wnt-RYK
I Wnt-mTOR
RTK7
PAPC
Syndecan
Glypican4
Wnt
Fz
G
TK
PAR1
PKCδ
CKIε
Inv
Dlv
Dvl
AP2
βarr2
Dsam1
RhoA
Rac1
Rock
Profilin1
MRLC
JNK
CapZIP/Dub
XRNF185
PXN
Actin cytoskeleton
cJUN
PIP2
PLC
DAG + IP3
PKC
Cdc42
cGMP
PDE6
GMP
PKG
Ca2+
CaMKII
CNA
TAK1
NLK
MKK3/6
p38
β-cat
TCF
NF-AT
ATF2
CKIε
SIPA1L1
RAP1
E-cadherin and integrins
ROR2
Ptk7
PI3K
Canonical Wnt signaling
Cdc42
MKK7
JNK
ATF2
cJUN
PAPC expression
ATP
AC
cAMP
PKA
CREB
Pax3, MyoD, Myf5
GSK3
JNK
MAP1B
Microtubules
aPKC
PAR3
PAR6
Lgl
PAR1
Cell polarity
RYK
TK
Src
Chemorepulsion
LRP5/6
AXIN
GSK3
TSC2
mTOR

I (Price et al, 2006) to initiate a cellular response. Alternately, Wnts can bind to and activate other receptors such as ROR2 and Ryk. Both of these receptors are tyrosine kinases which activate PI3K and Src respectively. Many of these noncanonical pathways ultimately lead to transcription (Semenov et al, 2007; Figure 1-4). Two of the best studied noncanonical pathways in vertebrates are the planar cell polarity pathway where Dvl activates JNK and Rho GTPases (Veeman et al, 2003 and Montcouquiol 2006) and the Wnt/calcium pathway where CAMKII and PKC are activated (Kuhl 2000). The fact that the components of Wnt signaling pathways are involved in so many other pathways, and that Wnts can activate such a variety of pathways makes Wnt signaling much more of a component of a signaling network than a specific signaling pathway.

1.5. Wnt Secretion and Activity Dependence

Many of the responses that are elicited in the target cell have been intensely studied; however, how Wnts are secreted is still unclear. There are two proteins that are involved in Wnt secretion, although their precise roles or whether they have additional roles in Wnt secretion is not known. The first of these proteins is Porcupine. Porcupine has been shown to be localized to the endoplasmic reticulum (Kadowaki et al, 1996) and porcupine mutants prevent secretion of Wingless (van den Huevel et al, 1993). The lipid modification of Wnts (Willert et al, 2003 and Takada et al, 2006) is thought to be regulated by Porcupine which shares some resemblance to a family of o-acyl transferases (Hofmann, 2000 and Kadowaki et al, 1996). Porcupine is clearly

important in Wnt secretion, although there is no direct evidence of Porcupine as the lipid modifier of Wnts.

The second protein crucial for Wnt secretion is Wntless (Wls), also called Evenness Interrupted (EVI) or Sprinter (SRT). Wls was also identified in Drosophila and is a transmembrane protein (Banziger et al, 2006, Bartscherer et al, 2006, and Goodman et al, 2006). Wls is thought to be located either in the Golgi apparatus (Banziger et al 2006 and Port et al, 2008) or the cell surface (Bartscherer et al, 2006) of Wingless secreting cells. Wls has been shown to be required in Wingless signaling (Bartscherer et al, 2006 and Banziger et al, 2006) and it is currently thought that Wls accompanies Wingless from transport through the Golgi apparatus to the cell surface (Franch-Marro et al, 2008) due to Wingless accumulation in the Golgi apparatus in Wls mutants (Port et al, 2008).

Wnt secretion in neurons specifically has been linked to both pre (Ahmad-Annuar 2006 and Hall et al 2000) and postsynaptic cells (Packard et al, 2002). In the cerebellum, Wnts are expressed in granule cells (Lucas and Salinas, 1997) and have been proposed to be secreted by granule cells to act on presynaptic terminals of the mossy fibers (Hall et al, 2000). In drosophila, Wingless is secreted from both sides of the NMJ and acts in different ways to affect the pre- and postsynaptic terminals (Packard et al, 2002). In neuronal cultures, hippocampal slices, and at the NMJ, Wnts secretion has been found to be activity dependent. Chen and others (2006) found that Wnt3a immunoreactivity was lowered in sections of the DG in hippocampal slices when tetanus stimulation was given to fibers from the entorhinal cortex. This decrease

in immunoreactivity was interpreted as an increase in Wnt3a release. Yu and Malenka (2003) also found that Wnt stimulation increases dendritic branching and outgrowth. The effects of Wnts were enhanced when cultures were stimulated with high potassium. In addition, Ataman and others (2007) found that activity promotes morphological changes in presynaptic terminals at the fly NMJ. With the same stimulation protocol used to induce these changes, they found that Wingless secretion is enhanced using immunostaining at pre- and postsynaptic junctions.

1.6. Wnts: Secreted Factors in Synapse Formation

Previous studies in invertebrates and vertebrates have implicated Wnts as both positive and negative regulators of synaptogenesis. Studies in cultures of mouse cerebellum show that Wnt7a conditioned media increases the number and size mossy fiber terminals (Hall et al, 2000). This signal is thought to act through Dvl, which colocalizes with presynaptic makers. Cultures of Dvl knockout mice also show a decrease in presynaptic puncta, but postsynaptic components were not examined (Ahmad-Annuar et al, 2006). Studies *in vivo* show a decrease in the size of presynaptic synapsin positive rosettes at P10, but this decrease disappeared completely by P15 (Hall et al, 2000), perhaps due to the presence of other Wnts in the cerebellum (Salinas et al, 1994). This decrease was exacerbated slightly in double knockouts of Wnt7a and Dvl (Ahmad-Annuar et al, 2006). While this evidence is convincing, the authors had limited functional data on synapses. Electrophysiology did not show significant differences, but a trend toward a lower mini-EPSC frequency in granule cells,

meaning Wnts either caused a decrease in probability of release or a decrease in the number of synapses. They also showed that the presynaptic puncta induced by Wnt7a were functional with FM studies (Ahmad-Annuar et al, 2006). Evidence from mice lead to a model where Wnt is secreted by the postsynaptic cell, granule neurons, which signals to the presynaptic axon, mossy fibers, and acts through Dvl to increase the size of the growth cone.

At the fly neuromuscular junction, Wnts are thought to be secreted by the presynaptic terminal and act on both active zone assembly and postsynaptic organization. Wingless mutants show a decrease in the number of presynaptic boutons and a change in the cytoskeleton of these terminals. EM also shows that a subset of wingless mutants had only an assembly of synaptic vesicles, but no active zone complex. Mutants also show irregular glutamate receptor expression. Consistent with glutamate receptor redistribution, EM studies show that adjacent to a subset of active zones in wingless mutants, there were enlarged postsynaptic pockets devoid of mitochondria and subsynaptic reticulum (Packard et al, 2002).

However, Wnts have also been shown to be antisynaptogenic in drosophila embryos and *C. elegans*. In 2007, Klassen and Shen used the DA9 neuron in *C. elegans*, which has an asynaptic region at the tail end of the animal and a synaptic region more anterior. Klassen and Shen (2007) show that Wnts act as negative regulators of synaptogenesis using a synaptobrevin-1 (snb-1) mutant *C. elegans* under a mig-13 promoter, to label only the DA9 neuron. They first looked at Wnts as a candidate for this process because lin-44 (Wnt) is secreted by four hypodermal cells in

the tail, which creates a Wnt gradient. Given the developmental processes in which Wnt is involved, this seemed an ideal choice. Mutations in lin-44 led to an increase in the number of snb-1 positive puncta in the previously asynaptic portion of the axon, suggesting that Wnt signaling may negatively regulate synapse formation in the tail region. This phenotype was exacerbated by mutation of another Wnt, egl-20, which is expressed on the anterior and ventral side of the tail. The phenotype was mimicked when lin-17 (Fzd) was mutated. Lin-17 was also found to be localized to the asynaptic region of the DA9 axon in lin-17::YFP mutants, strengthening the prospect of Wnt as a negative regulator of synaptogenesis.

In addition to Klassen and Shen (2007), Inaki et al (2007) found that Wnt4 in drosophila embryos provides a negative synapse regulation signal. This group looked at the NMJ of the drosophila embryo. In drosophila, the RP5 neuron extends its axon exclusively to muscle 12 (M12) and not the adjacent M13. Using a subtractive drosophila gene chip screen between M12 and M13 they found 26 molecules that could be responsible for the specificity of the RP5 neuron. One of these molecules was Wnt4 which was expressed in M13, but not in M12. In Wnt4 knockout embryos, innervation of M13 is expanded while innervation of M12 is reduced. When Wnt4 expression was induced in M12, axons stopped short of innervation of their target. These results suggest that Wnt4 is a negative regulator of synapses at the embryonic drosophila NMJ. A summary of the results just discussed are presented in Table 1-1.

The vast array of Wnt and Fzd genes might account for differences in findings between mice, drosophila, and *C. elegans*. It is still unclear how exactly a cell chooses

Table 1-1. Summary of Wnts Role in Synapse Formation.

System	Wnt Ligand	Mammalian Homologue (if applicable)	Influence on synapse formation	Reference(s)
Rodent Cerebellum	Wnt7a and Wnt7b		Positive	Hall et al, 2000
Rodent Hippocampus	Wnt7a		Positive	Ahmad-Annuar et al, 2006 and Cerpa et al, 2008
C. Elegans NMJ	Lin-44	Wnt10a and Wnt10b	Negative	Klassen and Shen, 2007
Embryonic Drosophila NMJ	DWnt4	Wnt9a and Wnt9b	Negative	Inaki et al, 2007
Drosophila NMJ	Wingless	Wnt1	Positive	Packard et al, 2001 and Ataman et al, 2008

to respond to Wnt signals. It is hypothesized that there are noncanonical and canonical classes of Wnts, however, the receptor type on the responding cell is also thought to be able to determine which pathway Wnt acts through (Mikels et al, 2007). An intriguing possibility for Wnts is if one neuron reacts one way to the Wnt signal and another reacts the opposite due to differential expression of receptor and/or ligand type. Because Wnts are such a large family of proteins and because there are several pathways through which Wnts may act, Wnts are candidates for this sort of mediation of synapse formation.

1.7. Determinants of Synaptic Specificity

While understanding how synapses form is certainly important, it is the directed formation of synapses that allow for the proper formation of complex circuits in the brain. When circuits are not formed properly, developmental diseases result, including autism and epilepsy (Belmonte et al, 2004 and Dudek and Sutula, 2007). The formation of these circuits can ultimately be explained by the way that cells make targeted synapses onto a subset of cells rather than every cell it comes in contact with. We call the process of this directed formation of synapses synaptic specificity. It is likely that synaptic specificity is regulated by both positive and negative influences on synapse formation, making Wnts candidate molecules. Sperry (1963) was one of the first to suggest that the correct targeting of synapses in the brain is mediated by individual chemical tags on neurons. He coined this type of synaptogenesis regulation the chemoaffinity hypothesis. Indeed, there are several such examples where axons

bypass entire regions to synapse with specific cells. For example, thalamocortical axons from the lateral geniculate nucleus must travel through the internal capsule and layers V and VI before making synapses onto neurons in layers IV (Benson et al, 2001). Nowadays, we know that the ability for an axon to make the correct choice of which cell to synapse onto is dependent on both activity and molecular identification tags on neurons.

Recently, progress has been made in understanding how neurons use molecular signals to make appropriate synapses on target cells. There are currently four families of molecules that have been shown to provide specificity signals to axons looking for postsynaptic partners: Syg, Dscam, sidekicks, and Wnts. Syg-1 and -2 were discovered in *C. elegans*. These two molecules serve as a guide for the HSNL motor neuron. Without syg-1, located in the surrounding epithelial cells where the HSNL neuron should synapse, or syg-2, the receptor for syg-1, located on the HSNL axon, ectopic synapses are made in surrounding areas on incorrect target cells (Shen and Bargmann, 2003 and Shen et al, 2004).

Another family of molecules that has been shown to effect synaptic specificity is Dscam. Dscams are a family of proteins that are able to undergo alternative splicing to form over 38,000 different mRNAs (Schmucker et al, 2000) and many of these exhibit isoform specific binding (Wojtowicz et al, 2007). These properties make Dscams an ideal family of molecules to uniquely label the many types of neurons within the brain and guide synapse specificity. Dscams were first implicated in synaptic specificity in the olfactory receptor neurons projecting to the antennal lobe in

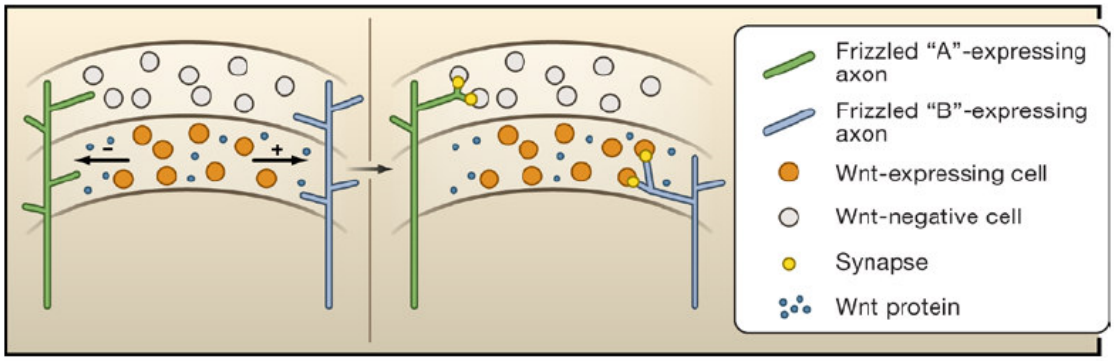
drosophila. Upon mutation of Dscam, olfactory neurons terminated at ectopic sites in the antennal lobe (Hummel et al, 2003). Dscam also promotes synaptic specificity of retinal ganglion cells in the mammalian retina (Yamagata and Sanes, 2008).

Sidekicks are a group of molecules that have been shown to regulate the laminar specificity of retinal ganglion cells in chicks. Retinal ganglion cells project both axons and dendrites in a laminar specific manor. Sdk-1 and -2 are expressed in different layers of the retina corresponding to different populations of retinal ganglion cells. Ectopic expression of sdks leads to a diversion of processes to sdk positive layers (Yamagata et al, 2002). This effect is also seen in the mammalian retina (Yamagata and Sanes, 2008).

Finally, Wnts have been shown to regulate synaptic specificity in *C. elegans*. Wnts create a gradient in the tail of the animals where the highest expression is more caudal and the lowest is more dorsal. In the regions where the highest Wnt expression is found, there are no synapses in the DA9 axon. By misexpression of Wnts to other regions, a different pattern of synapses are made by the DA9 axon, suggesting that differential patterns of Wnts and Fzds can control where synapses are formed and where they are not (Klassen and Shen, 2007). An example of how different Fzds or Wnts may mediate synapse formation axons entering a laminar structure such as the cortex is shown in Figure 1-5.

Figure 1-5. Model of how Wnts might regulate synaptic specificity.

The axons expressing different Wnt receptors (Frizzled "A" and Frizzled "B") are initially intermixed in Wnt-positive and Wnt-negative zones. Frizzled "A" mediates an antisynaptogenic Wnt response, whereas Frizzled "B" mediates a prosynaptogenic response. This leads to segregation of the synapses from two axon populations to the Wnt-negative and Wnt-positive zones.


Frizzled "A"-expressing axon
Frizzled "B"-expressing axon
Wnt-expressing cell
Wnt-negative cell
Synapse
Wnt protein

1.8. Dissertation: *In vitro* Hippocampal Synapse Formation

The studies discussed here have shown Wnts to have a variety of effects at the synapse. In my dissertation, I will report results on the role of Wnts in synapse formation. However, before I could investigate the role of Wnts in synaptic development, I needed to know which Wnts are expressed in the developing hippocampus. The most complete information on Wnt expression exists in the adult mouse. Given that synapses are formed during the first two postnatal weeks in rodents, the adult expression patterns of Wnts cannot definitively reveal which Wnts might be acting as synapse formation mediators. In Chapter 2 of my dissertation I show results from an in situ hybridization screen on P7 and P14 mice to see which Wnts are expressed during the crucial time of synapse formation in rodents. Along with the expression of several Wnts in the hippocampus, I find that many Wnts are expressed in other regions of the brain.

In Chapter 3 of my dissertation, I explore the function of different Wnt signaling pathways on synapse formation. Wnts have been shown to have a variety of effects on synapse formation, but it is still unclear whether the differences in Wnt activity on synapse formation reflect the differences between species or between different types of Wnt proteins. I show here that the ability to activate different signaling pathways may determine whether a Wnt is a positive or negative regulator of synapses.

Finally, I explore the problem of synapse specificity in Chapter 4. In a collaboration with Megan Williams that combines a functional and anatomical

approach to exploring synaptic specificity *in vitro*, we find that the DG synapse retains specificity to CA3 neurons even in the absence of secreted axon guidance cues. Megan Williams did experiments pertaining to anatomy and counted the number of synapses DG cells made onto DG, CA3, and CA1 neurons. I used a dual patch clamping approach to explore the time course of functional synapse formation and the relative sizes of evoked currents from presynaptic DG cells in postsynaptic DG, CA1 and CA3 neurons. Our results provide the framework to explore potential mediators of synaptic specificity, including Wnts.

1.9. Acknowledgments

Chapter 1, in part, has been published in Cell, 2007. Davis, E.K. and Ghosh, A., 2008. The dissertation author was the primary author.

CHAPTER 2: Expression of Wnts in the developing mouse brain

2.1. Introduction

Wnt proteins have been demonstrated to be involved in many embryonic developmental processes, such as cell fate specification, body axis specification, and axon guidance (Ciani and Salinas, 2005). Wnts have recently been demonstrated to be involved in postnatal processes such as synapse formation. Most synapse formation in rodents occurs during the first two weeks of an animal's life (Fiala et al, 1998 and Blue and Parnavelas, 1983). However, the best expression data available in mammals is in the adult mouse. In the adult mouse, several Wnts are expressed in the hippocampus (compiled from Allen Brain Atlas; Figure 2-1A) along with many of the downstream canonical signaling components (compiled from Allen Brain Atlas; Figure 2-1B). Most of the Wnts expressed in the hippocampus are consistently expressed in all regions of the hippocampus (Wnt1, 2, 5a, 7b, 8b, and 10b) while only one, Wnt8a, is expressed in a distinct subregion, the DG.

In addition to expression data on different Wnts, there has also been work on different Frizzled (Fzds) expression in the adult brain. The Allen Brain Atlas has shown Fzds to be expressed either in all regions (Fzd3 and 8) or in subregions. Fzd1 expression is restricted to the DG and CA1 regions while Fzd7 expression is restricted to the CA3 region (Figure 2-1A). While expression data in adult mouse is useful, it cannot inform us which Wnts might be involved in synapse formation.

Figure 2-1. Wnt, Fzd, and canonical Wnt signaling component expression in the adult hippocampus.

Compiled information of expression patterns in the adult hippocampus on Wnts and Fzds (A) and on components of the canonical Wnt signaling pathway (B). Most Wnts and Fzds expressed in the adult mouse have consistent expression throughout all regions of the hippocampus, with only a few expressed in distinct subregions (A). Members of the canonical signaling pathway are expressed in all subregions of the hippocampus in the adult mouse. Only inhibitors (sFRPs) of Wnts show spatial restriction to certain subregions of the hippocampus (B). Compiled from the Allen Brain Atlas.

Figure 2-1A

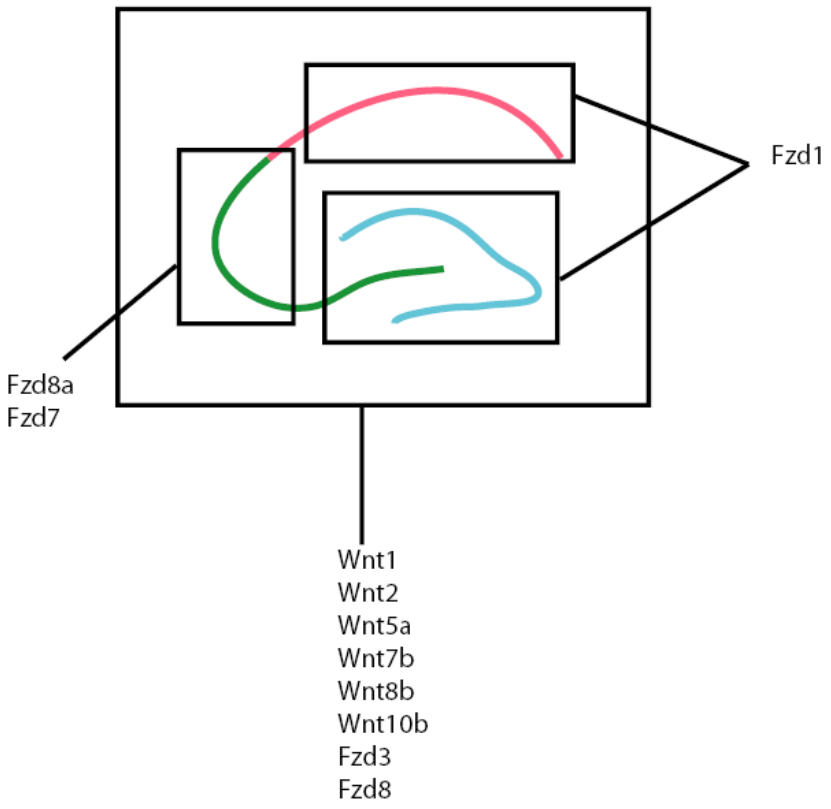

Fzd1
Fzd8a
Fzd7
Wnt1
Wnt2
Wnt5a
Wnt7b
Wnt8b
Wnt10b
Fzd3
Fzd8

Figure 2-1B

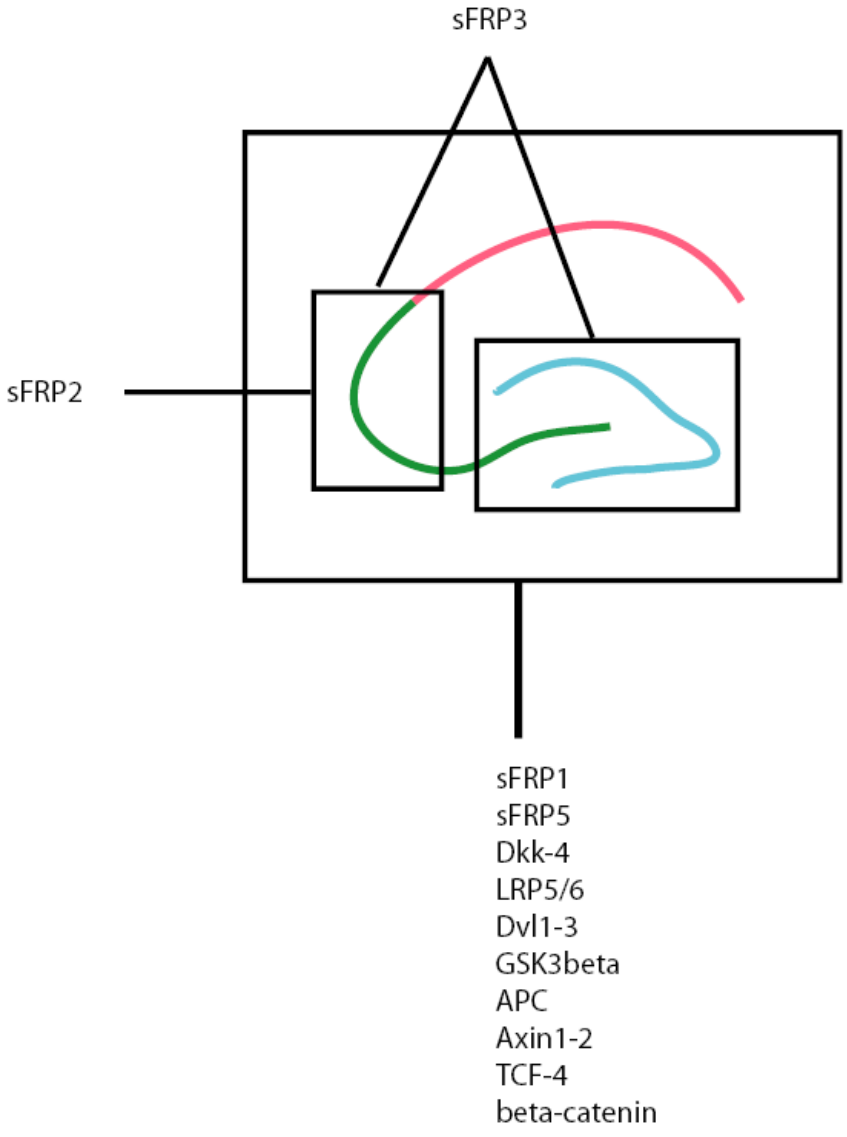


Figure 2-1 Continued

2.2. Results

2.2.1. Hippocampal Wnt and Fzd expression

To determine which Wnts might be involved in synapse formation, we performed an *in situ* hybridization screen to identify Wnts that are expressed in the hippocampus during synaptogenesis. In rodents, most synapses in the hippocampus are formed within the first two weeks after birth (Fiala et al, 1998 and Blue and Parnavelas, 1983). We therefore chose to observe the expression of Wnts in horizontal mouse brain slices at postnatal day 7 (P7) and P14. Using DIG-labeled probes to all 19 Wnt genes, we found that only four, Wnt5a, Wnt7a, and Wnt7b, and low levels of Wnt3, were expressed (Table 2-1, Figure 2-2). Two of these Wnts, Wnt5a and Wnt7b, were expressed in all subregions of the hippocampus at both P7 and P14 (Figure 2-2B, D). Wnt7b had a particularly interesting expression pattern at P14. While still expressing in all subregions, cells in the CA2 and dentate gyrus (DG) regions had particularly high expression of Wnt7b. Wnt3 was only expressed in a small number of cells in the DG hilus (Figure 2-2I-J). The expression of Wnt7a was dynamically regulated between P7 and P14. At P7, Wnt7a was only expressed in the DG and by P14 expression of Wnt7a expanded to include both the DG and CA1 subregions (Figure 2-2E-F).

To determine if hippocampal neurons express Wnt receptors, we examined the expression of the most common Wnt receptor, Fzd, by *in situ* hybridization. Using DIG-labeled probes to six of the Fzd family members, Fzd1, Fzd2, Fzd3, Fzd5, Fzd8, and Fzd9, we found three Fzds were expressed in the hippocampus during

Figure 2-2. Wnt expression in the developing hippocampus.

A and B. Negative control *in situs* at P7 (A) and P14 (B). C-H. Three Wnts are broadly expressed during synaptogenesis in the hippocampus. Wnt5a is expressed in all subregions at both P7 (C) and P14 (D). Wnt7a is expressed in the DG at P7 (E) and in the DG and CA1 regions at P14 (F; arrowheads). Wnt7b is expressed in all subregions at P7 (G) and P14 (H). Wnt3 was only expressed at P14 in the DG (I, J). Expression was determined using 20x magnification to differentiate signal from background.

Figure 2-2

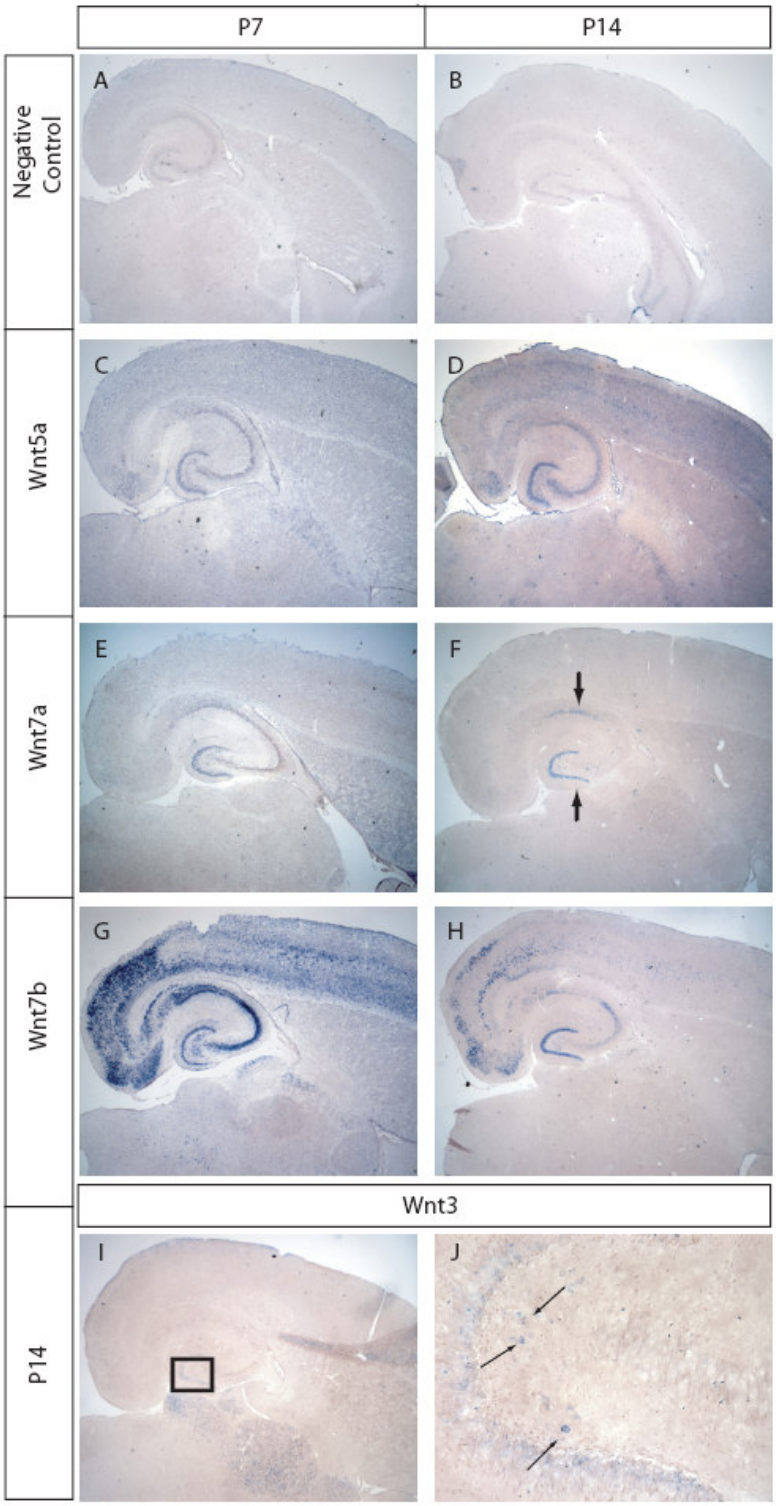
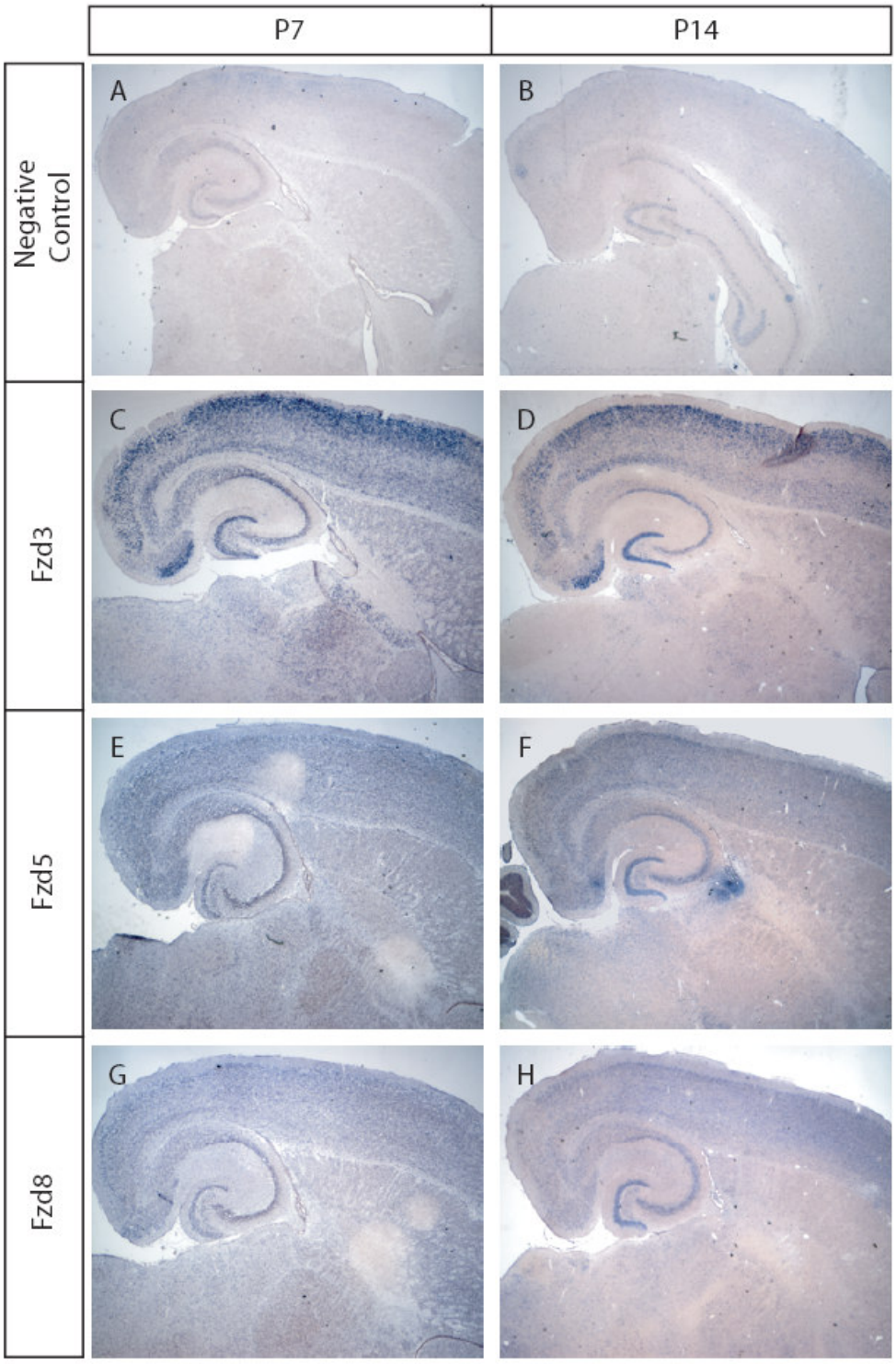

P7
P14
Negative Control
A
B
Wnt5a
C
D
Wnt7a
E
F
Wnt7b
G
H
Wnt3
P14
I
J

Figure 2-3. Fzd expression in the developing hippocampus.

A and B. Negative control *in situs* at P7 (A) and P14 (B). C-H. Three of six Fzds tested are expressed in the hippocampus during development of synapses. All three Fzds, Fzd3 (C, D), Fzd5 (E, F), and Fzd8 (G, H) are expressed in all three subregions at both P7 and P14. Expression was determined using 20x magnification to differentiate signal from background labeling.

Figure 2-3



synaptogenesis (Figure 2-3). The three Fzds identified, Fzd3, Fzd5, and Fzd8, had consistent spatial expression patterns between P7 and P14 and were expressed in all regions (Figure 2-3). These results indicate that Wnts and their receptors are expressed during synapse formation.

2.2.2. Expression of Wnts in Other Regions of the Forebrain

In addition to Wnt expression in the hippocampus, several Wnts were also expressed in the cortex, thalamus, or olfactory bulb (OB). These expression patterns in other regions of the brain are shown at P14. Those that were expressed in the cortex included Wnt1, 4, 5a, 7b, and 9a. These Wnts were expressed in different layers as well as specialized regions of the cortex. Wnt1, 4, and 5a were expressed in L2/3 while Wnt9a was expressed in L4. Wnt1, 5a and 7b were all expressed in L5/6 (Figure 2-4A). Wnt5a, and 7b were also highly expressed in the entorhinal cortex (Figure 2-2C-D, G-H) while Wnt1, 4, and 7b were expressed in the piriform cortex (Figure 2-4B).

A few Wnts also were also expressed in the olfactory bulb. Wnt7a was expressed in the inner granular cell layer and the glomerular cell layer. Wnt7b was expressed in scattered cells in the mitral cell layer while Wnt5a was expressed in all regions (Figure 2-4C). Wnt6 was expressed in the dura outside of the glomerular cell layer in the olfactory bulb and in a thin layer around the rest of the brain, but had no expression in the brain itself (data not shown). Also, Wnts 2b, 3, 4, 5a, 9b, and 16 were all expressed in various nuclei in the thalamus and/or midbrain (Figure 2-4D).

There were also several Wnts that were not expressed in any region of the developing brain, which included Wnt2, 3a, 5b, 8a, 8b, 10a, 10b, and 11.

Unlike expression of certain Wnt ligands, Fzd expression within the developing brain seemed ubiquitous. Fzd3 for example, was expressed in almost every region we examined, the olfactory bulb (Figure 2-4C), piriform cortex (Figure 2-4B), hippocampus and cortex (Figure 2-3), and thalamus (Figure 2-4D). In addition, a secreted inhibitor of Wnts, sFRP2 was also expressed in multiple places, the cortex (Figure 2-4A), piriform cortex (2-4B) and thalamus (Figure 2-4D).

2.3. Discussion

2.3.1. Expression of Wnts and Fzds in the developing hippocampus

Our results indicate that several Wnts are expressed in the hippocampus during the peak of synapse formation in development. Using *in situ* hybridization, we found that of the 19 Wnt ligands, only a few are expressed in the hippocampus, including Wnts 3, 5a, 7a, and 7b. These Wnts, based on sequence are all loosely related, as seen in Figure 1-3. Previous studies investigating the developmental regulation of Wnt expression have shown similar results. Shimogori (2004) examined the developmental expression profile of several Wnts (1, 2b, 5a, 7a, 7b, 8b) in multiple regions of the brain. They found that Wnt5a, Wnt7a, and Wnt7b are all expressed in the hippocampus from P0 through P20, consistent with our results, although the areas showing expression are slightly different. This could be due to the difference in

Figure 2-4. Wnt, sFPRP2, and Fzd3 expression in the forebrain at P14.

Expression of Wnts in different areas of the forebrain including the cortex (A), the piriform cortex (B), the olfactory bulb (C), and the thalamus (D). Due to the expression of Wnts in almost every region of the developing forebrain, Wnts are likely to play a role in many postnatal developmental processes. All in situ hybridizations were done with negative controls using a sense probe to Wnt5a under identical conditions. Expression was determined using 20x magnification to differentiate signal from background labeling.

Figure 2-4A

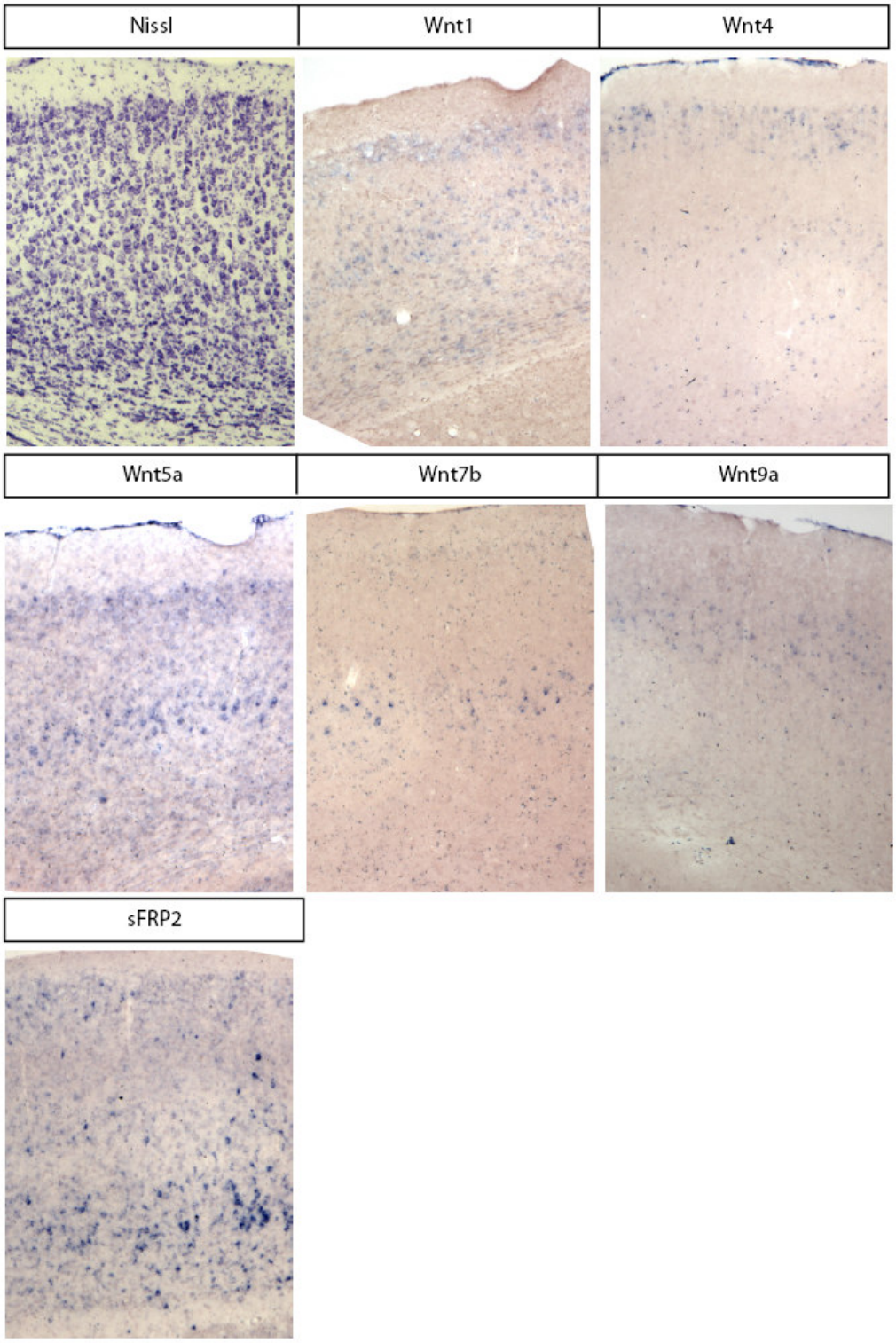


Figure 2-4B

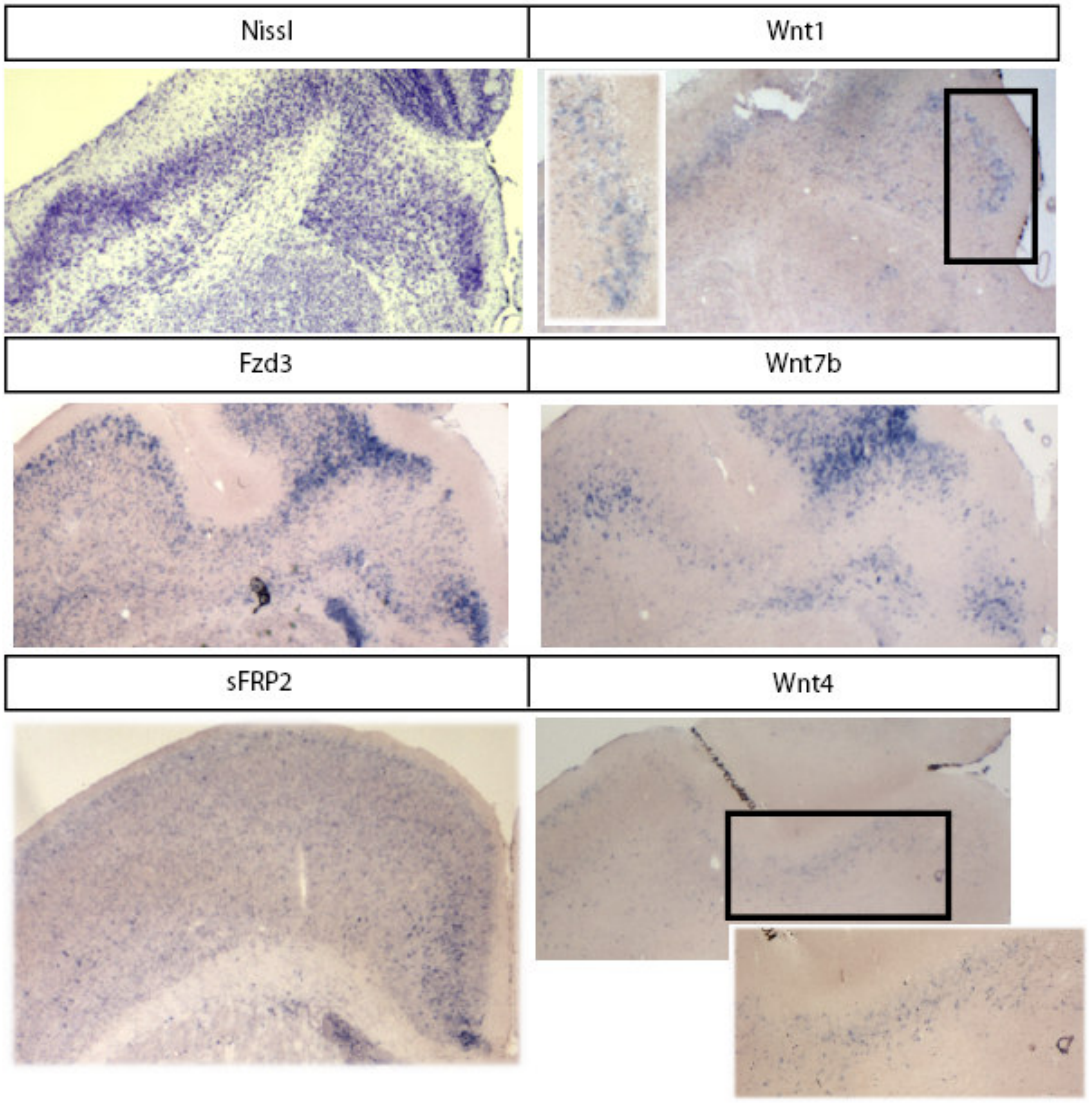


Figure 2-4 Continued.

Figure 2-4C

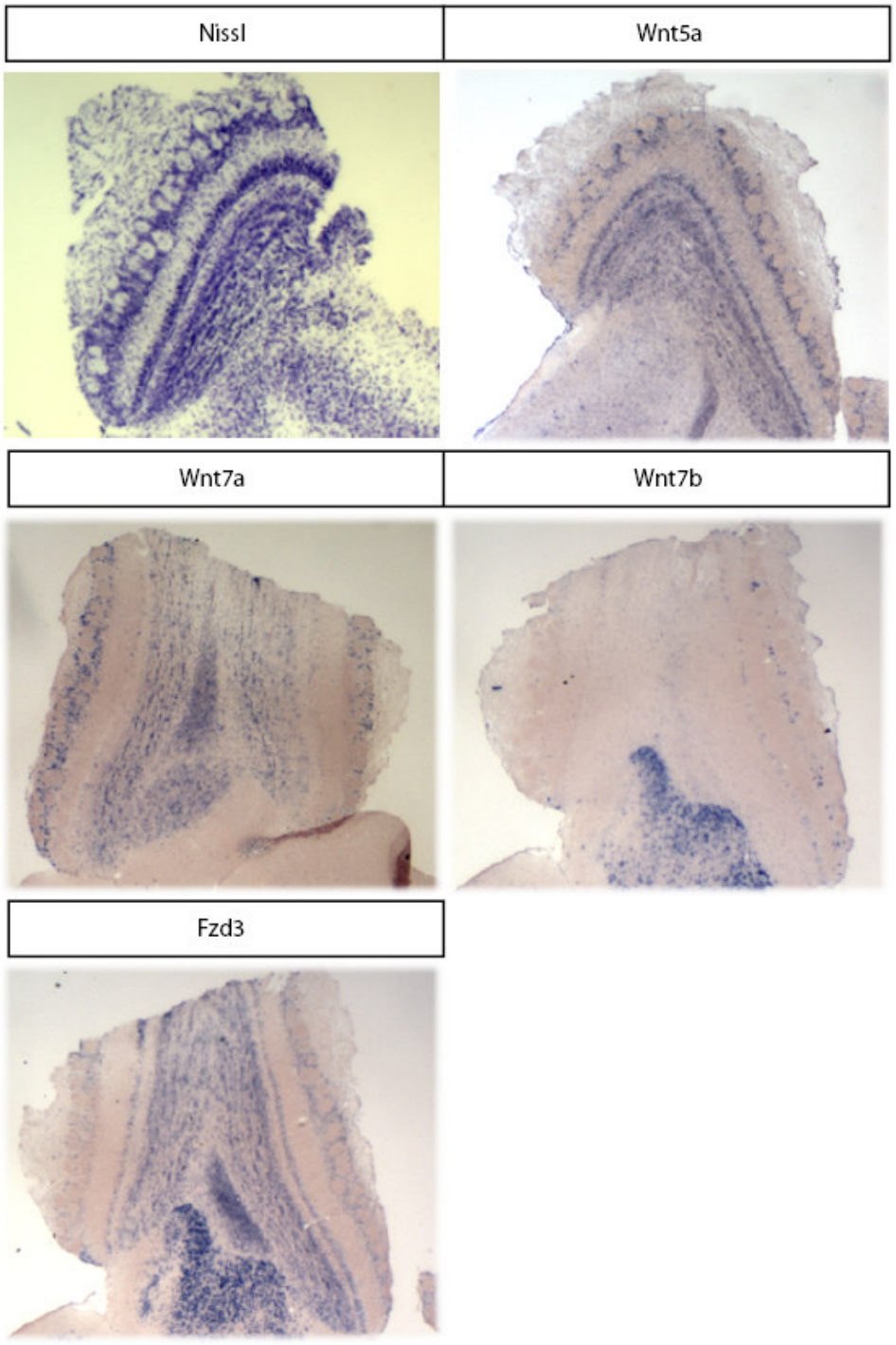


Figure 2-4 Continued

Figure 2-4D

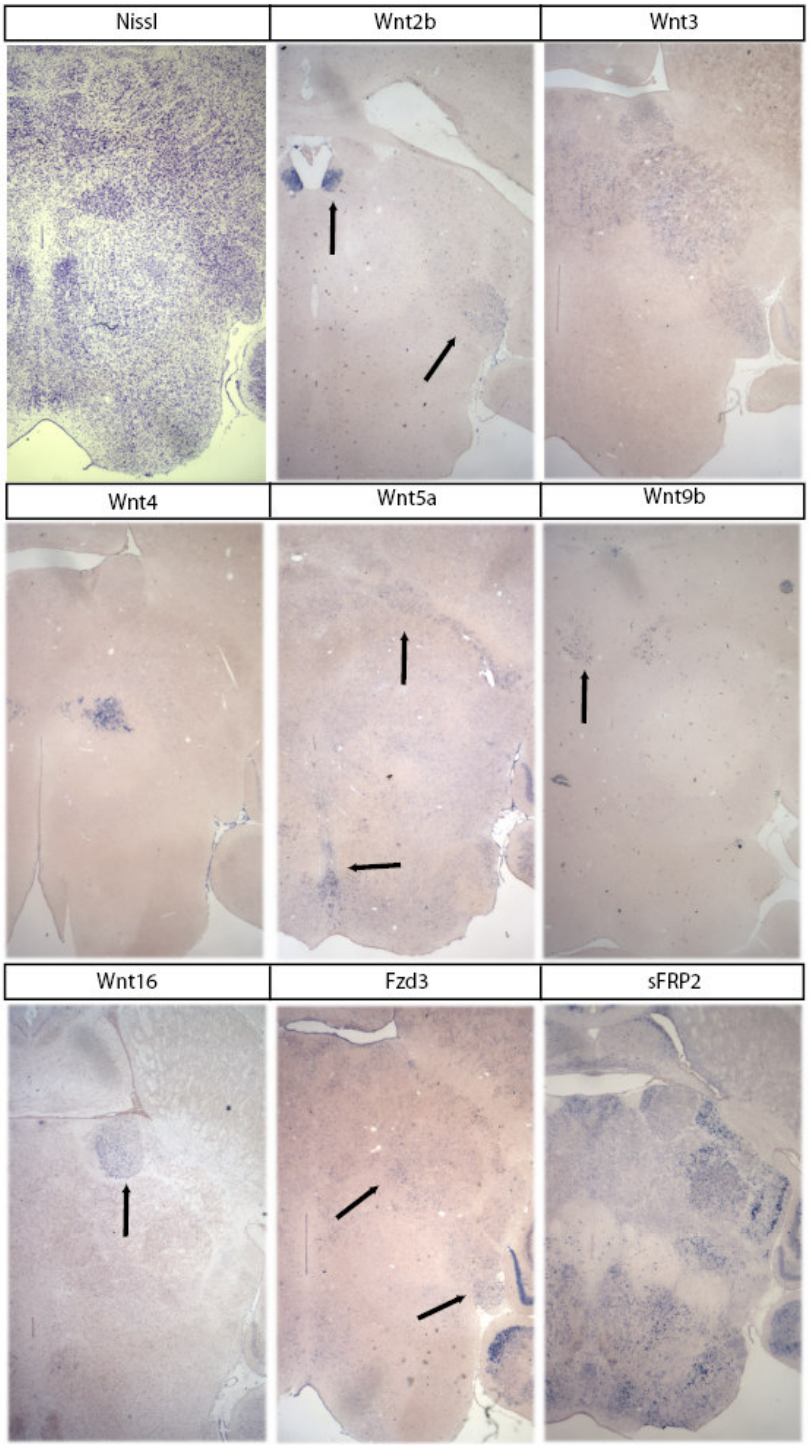


Figure 2-4 Continued

preparation of the probes. Shimogori (2004) used full length cDNAs to prepare the probes, whereas the probes used in this study were taken partially from the 3' untranslated region and partially from the coding region at the carboxy terminus of each gene. Shimogori (2004) also saw expression of Wnt8b in the DG hilus at P20. We did not see Wnt8b expression, but Wnt8b may start expressing shortly after our P14 time point. Cerpa (2007) found through real time PCR, in addition to Wnt5a and Wnt7a, that Wnt11 and Wnt4 are expressed in hippocampal cultures at 10DIV. We did not find any Wnt4 expression in the hippocampus, though *in situ* hybridization is not sensitive enough to detect low signal levels from background. We did, however, find expression of Wnt4 in the cortex. We did not detect any Wnt11 expression in the brain at P7 or P14. Compared to the adult mouse, the expression patterns of Wnts changed only slightly. In addition to Wnt5a and Wnt7b, the Allen Brain Atlas found that Wnts 1, 2, 8a, 8b, and 10b were also expressed in the hippocampus. We did not find expression of Wnts 8a, 8b, and 10b anywhere in the brain at P7 or P14. Wnt8a and 8b could be expressed shortly after our P14 time point due to expression at P20.

We also found that three of the six Fzd receptors we tested are expressed during hippocampal development. These Fzds were Fzd3, Fzd5, and Fzd8. At present, we do not know which Fzd interacts with each Wnt, although it is likely that the Wnts present in the hippocampus bind to multiple Fzd receptors (He et al, 1997). Indeed, Wnt5a has been shown to interact with nearly all of the Fzd receptors (Weeraratna et al, 2002; Kawasaki et al, 2007; Sheldahl et al, 1999; Umbhauer et al, 2000). Additionally, we do not know which pathway each Fzd is activating. It is currently

unknown how Wnts create such a large variation in responses of the targeted cell, although this could be attributed to the diversity of Fzds and Wnts. Previous work has shown all three of the Fzds we found expressed to activate both canonical and noncanonical pathways (Kawasaki et al, 2007; Sheldahl et al, 1999; Umbhauer et al, 2000; Kemp et al, 2007; Takada et al, 2005; and Cavodeassi et al, 2005). It is also possible that other receptors for Wnts are present within the developing hippocampus (Oishi et al, 2003) which may mediate some of the effects of Wnt proteins in synapse formation.

2.3.2. Expression of Wnts in Other Regions of the Forebrain

Shimogori (2004) found that Wnt2b, 5a, and 7a were expressed in different layers of the olfactory bulb at P20. We also found Wnt5a and Wnt7a, but did not find Wnt2b expression in any region of the brain except the thalamus. Since Wnt2b expression was found in the thalamus, we believe Wnt2b must therefore be expressed later than our P14 time point. Shimogori (2004) also found Wnt2b, 5a, and 7b expression in the cortex. We found expression of Wnt5a and 7b among other Wnts, but no expression of Wnt2b. Wnt2b could, again, start expressing shortly after our latest time point.

A summary of our findings of Wnts expressed in the hippocampus and in the entire forebrain can be found in Figure 2-8A and Figure 2-8B, respectively. We show that several Wnts are expressed in all distinct areas of the forebrain, implicating a role for Wnt signaling in postnatal development. The complexity of the Wnt signaling

network makes Wnts ideal candidates to regulate numerous processes in the postnatal developing brain.

2.4. Acknowledgments

Chapter 2, in part, and Chapter 3, in full, has been submitted for publication in Neural Development, 2008. Davis, E.K., Zou, Y., and Ghosh, A., 2008. The dissertation author is the primary investigator and author of this paper.

Figure 2-5. Summary of Wnt and Fzd expression patterns in the developing forebrain.

A. A summary of Wnt expression in the developing hippocampus at P14. We find that 50% of Wnts expressed in the hippocampus at this age are expressed in all subregions. Wnt3 was only expressed in the DG subregion while Wnt7a was expressed in the DG and CA1 subregions. Fzds in the hippocampus were expressed in all subregions.

B. A summary of Wnts in the developing forebrain at P14. Wnts were expressed in every major region we examined – piriform cortex, olfactory bulb, cortex, hippocampus, and thalamus.

Figure 2-5A

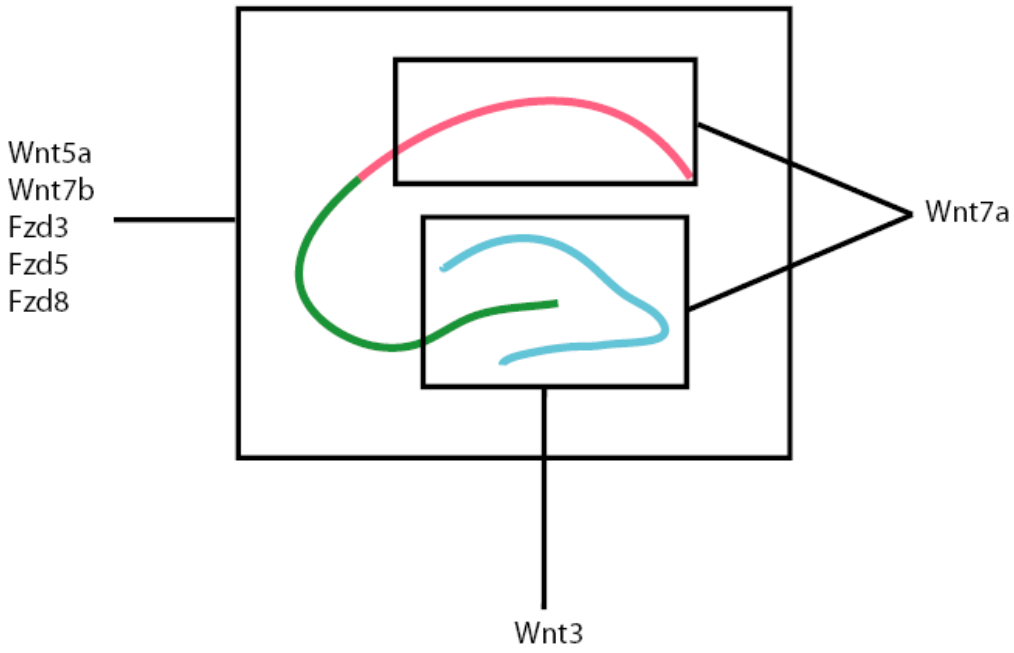


Figure 2-5B

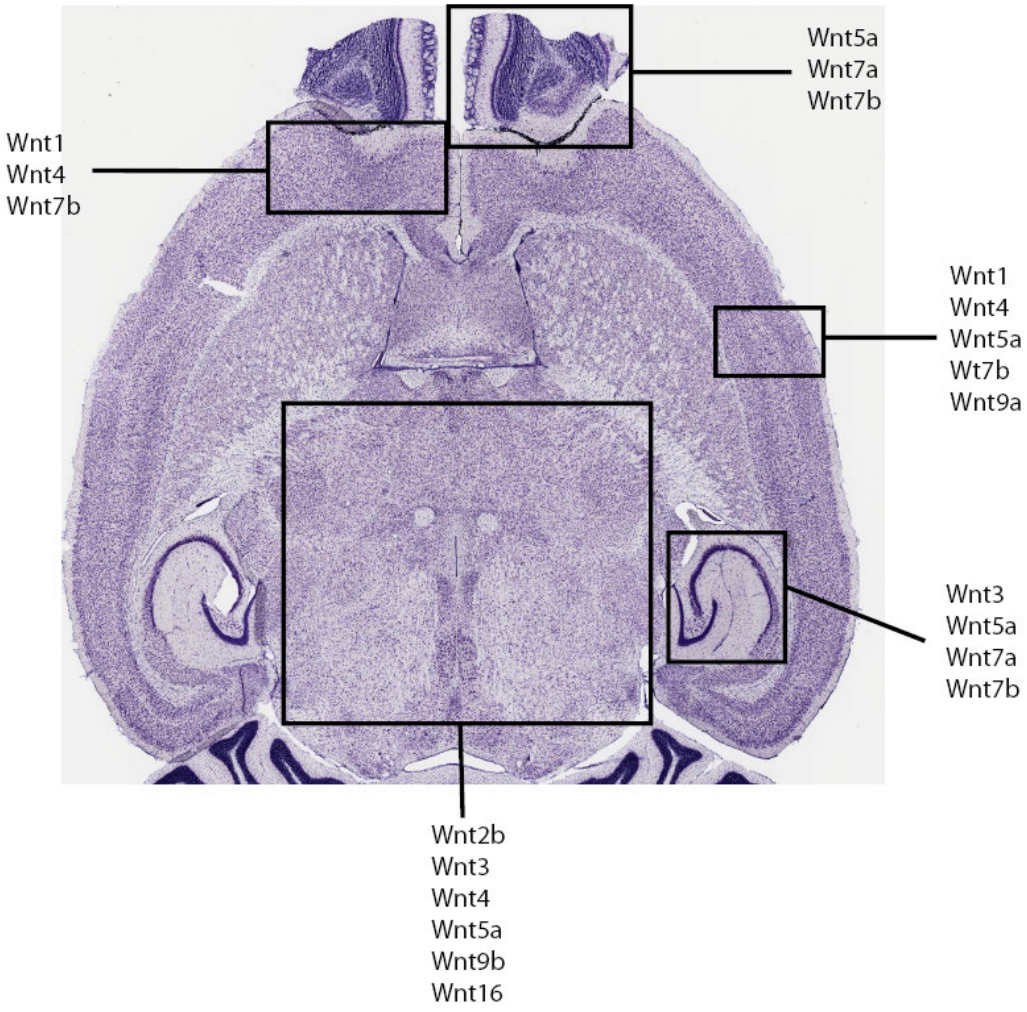


Figure 2-5 Continued

Table 2-1. Expression of Wnts in the developing forebrain.

Gene	Hippocampus	Piriform Cortex	Entorhinal Cortex	Olfactory Bulb	Cortex	Thalamus
Wnt1	-	+	-	-	+	-
Wnt2	-	-	-	-	-	-
Wnt2b	-	-	-	-	-	+
Wnt3	+	-	-	-	-	+
Wnt3a	-	-	-	-	-	-
Wnt4	-	-	-		+	+
Wnt5a	+	+	+	+	+	+
Wnt5b	-	-	-	-	-	-
Wnt6	-	-	-	-	-	-
Wnt7a	+	-	-	+	-	-
Wnt7b	+	+	+	+	+	+
Wnt8a	-	-	-	-	-	-
Wnt8b	-	-	-	-	-	-
Wnt9a	-	-	-	-	-	+
Wnt9b	-	-	-	-	+	-
Wnt10a	-	-	-	-	-	-
Wnt10b	-	-	-	-	-	-
Wnt11	-	-	-	-	-	-
Wnt16	-	-	-	-	-	+

CHAPTER 3: The role of Wnt signaling in synapse formation

3.1. Introduction

Wnts are a large family of 19 different secreted ligands that has been described in numerous developmental processes. Wnts can signal through several different types of receptors, but the most widely recognized Wnt receptors are Frizzled proteins (Fzd). Fzds are a family of 10 different G-coupled protein receptors (Kikuchi et al, 2007). Wnts are able to elicit a variety of responses in the target cell through Fzd activation, but the best studied is the canonical signaling pathway. The canonical pathway begins with activation Fzd and a LRP5/6 coreceptor resulting in the phosphorylation of Disheveled (Dvl). Dvl then disrupts a complex of proteins that includes Axin, APC, and GSK3β which normally degrades β-catenin through phosphorylation. This results in stabilization of β-catenin levels. B-catenin is then actively transported to the nucleus to regulate transcription (Logan and Nusse, 2004). Several other noncanonical Wnt pathways that signal through Fzd have been identified including the planar cell polarity pathway and the Calcium/Wnt pathway (Montcouquiol et al, 2006). However, new pathways for Wnt signaling through Fzd and various other receptors are emerging (Semenov et al, 2007).

Several observations suggest that Wnt proteins are involved in synapse formation. First, many Wnt signaling components are present at the synapse (Matsumine et al, 1996 and Hirabayashi et al, 2004). Second, studies have implicated Wnts in synapse formation, both as pro- (Hall et al, 2000; Ahmad-Annuar et al, 2006;

Cerpa et al, 2008; and Packard et al, 2002) and antisynaptogenic (Klassen and Shen, 2007 and Inaki et al, 2007 molecules. In vertebrates, Wnts promote growth cone expansion and increase presynaptic puncta formation (Hall et al, 2000 and Ahmad-Annuar et al, 2006). In Drosophila, Wnts act both pre- and postsynaptically to positively regulate presynaptic bouton number, presynaptic active zone assembly, and postsynaptic glutamate receptor distribution (Packard et al, 2002). Conversely, in *C. elegans*, Wnts are antisynaptogenic molecules and prevent synapse formation near the tail of the animal (Klassen and Shen, 2007). It is not known whether these different effects of Wnt signaling reflect different roles of individual Wnt proteins or whether they reflect the activity of different Wnts in different organisms.

In this study we explore the function of Wnt proteins in synapse formation by examining the effects of several developmentally expressed Wnts (see Chapter 2) on hippocampal cultures. We show here that Wnts have differential synaptogenic activity according to the activation of canonical or noncanonical pathways.

3.2. Results

3.2.1. Development of hippocampal synapses *in vitro*

To explore the effects of Wnts on synapse formation, we first established the time window of synaptogenesis in P0 hippocampal cultures. We immunostained cultures at 8DIV and 14DIV with an excitatory presynaptic marker, Vesicular Glutamate Transporter 1 (VGLUT1), and a dendritic marker, Microtubule Associated Protein 2 (MAP2; Figure 3-1A). We found that during the second week *in vitro*, the

number of presynaptic inputs increased dramatically (Figure 3-1A). We also found that 85±2% of VGLUT1 puncta colocalized with postsynaptic Membrane Associated Guanylate Kinases (MAGUKs; Figure 3B; left), determined by staining with an anti-pan-MAGUK antibody. Additionally, 87±2% of VGLUT1 colocalized with postsynaptic Glutamate Receptor 2/3 (GluR2/3; Figure 3-1B; right). These high rates of colocalization with postsynaptic markers indicate that the number of VGLUT1 puncta associated with dendrites is representative of the number of synapses and suggest that the increase in the number of VGLUT1 puncta between 8DIV and 14DIV reflects an increase in the number of synapses during this period. Consistent with this interpretation, there is a marked increase in synaptic currents in hippocampal cultures between 8DIV and 14DIV (see Chapter 4).

3.2.2. Subcellular Localization of Fzd3

One major question in exploring the role of Wnts in synapse formation is which side of the synapse Wnts signal to. In Chapter 2, I show that Fzd3 is widely expressed in the developing brain during synapse formation. Using an antibody to Fzd3, I examined the subcellular localization of Fzd3 in hippocampal cultures, which might give clues as to which side of the synapse Wnts act. GFP transfected neurons were immunostained for GFP, GFAP, a protein expressed in glial cells, and Fzd3 at 14DIV. We find that Fzd3 is expressed heavily in glia cells and in neurons. Fzd was localized primarily to cell bodies, dendrites and axons, but was not present in spines (Figure 3-2A). To explore Fzd3 puncta associated with presynaptic puncta, we also

Figure 3-1. Development of synapses in P0 hippocampal cultures.

A. Representative images of immunofluorescence staining of MAP2 (blue) and VGLUT1 (green) at 8DIV (left) and 14DIV (right). Scale bar = 2μm. Bottom, quantification of presynaptic inputs per area of dendrite at 8DIV and 14DIV. Error bars represent SEM. To ensure equivalent staining, cells were fixed at respective days and then stained with a single reaction. The number of inputs at 14DIV is normalized to the number of inputs at 8DIV. n = 47 fields of view, *$p<0.001$, students t-test.

B. Colocalization of VGLUT1 puncta (red) with MAP2 (blue) and either MAGUKs (green; left) or GluR2/3 (green; right).

Figure 3-1A

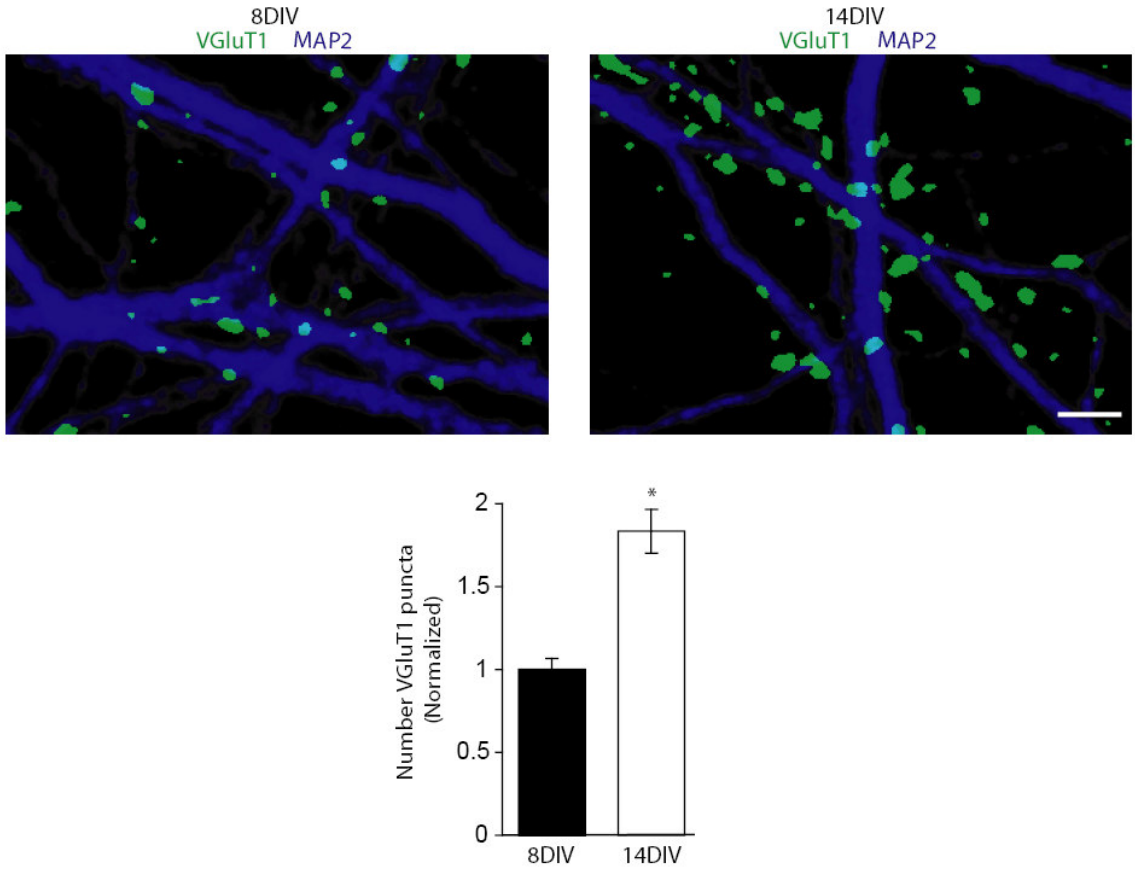

8DIV
VGluT1 MAP2
14DIV
VGluT1 MAP2
Number VGluT1 puncta (Normalized)
2
1.5
1
0.5
0
*
8DIV
14DIV

Figure 3-1B

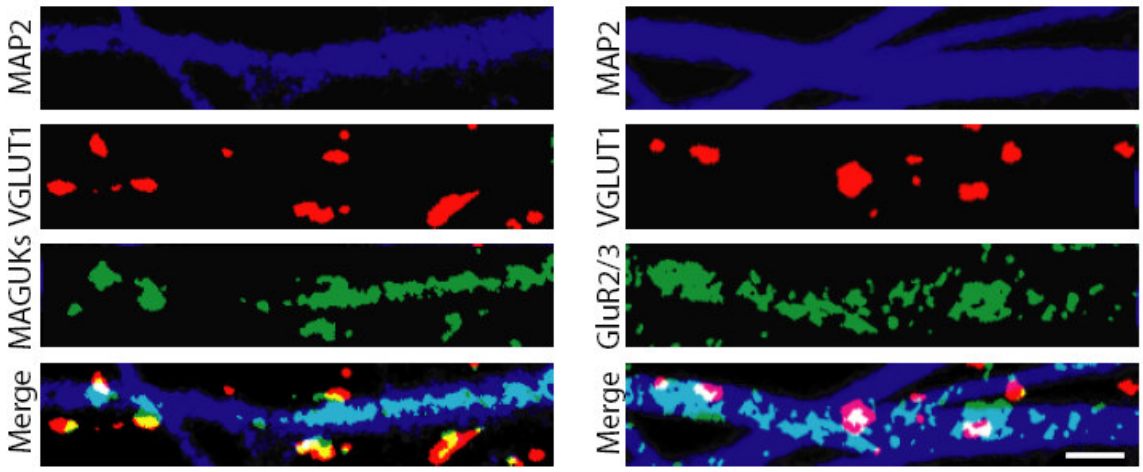


Figure 3-1 Continued

Figure 3-2. Subcellular localization of Fzd3.

A. Images of GFP transfected hippocampal neurons stained with antibodies to GFP (top, green; bottom, blue), Fzd3 (top, red; bottom, green), and GFAP (blue). Fzd staining seemed to be localized to axons (top) and dendrites (bottom), but not spines (*, bottom). Bar = 2μm.

B. Images of 12DIV hippocampal cultures stained with anti-Fzd3 (green), anti-VGLUT1 (red), and anti-MAP2 (blue). Some VGLUT colocalized with Fzd3 puncta (arrows), but some did not (*). Bar = 2μm.

Figure 3-2A

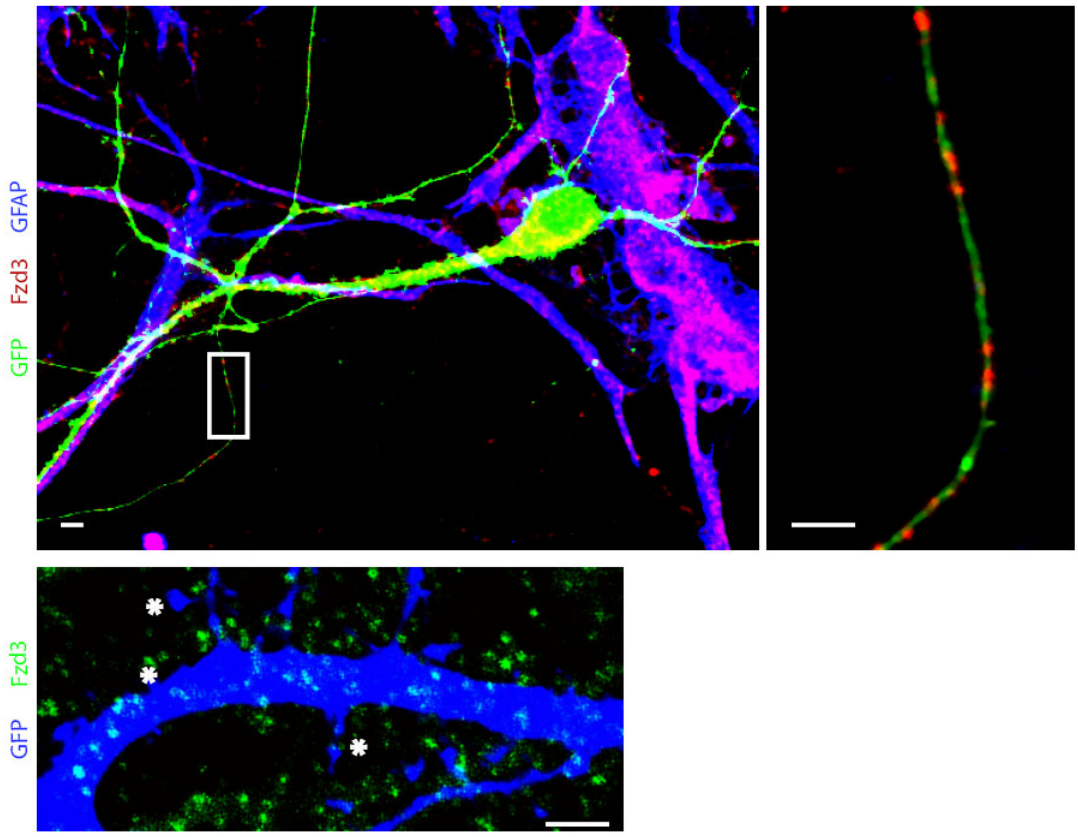


Figure 3-2B

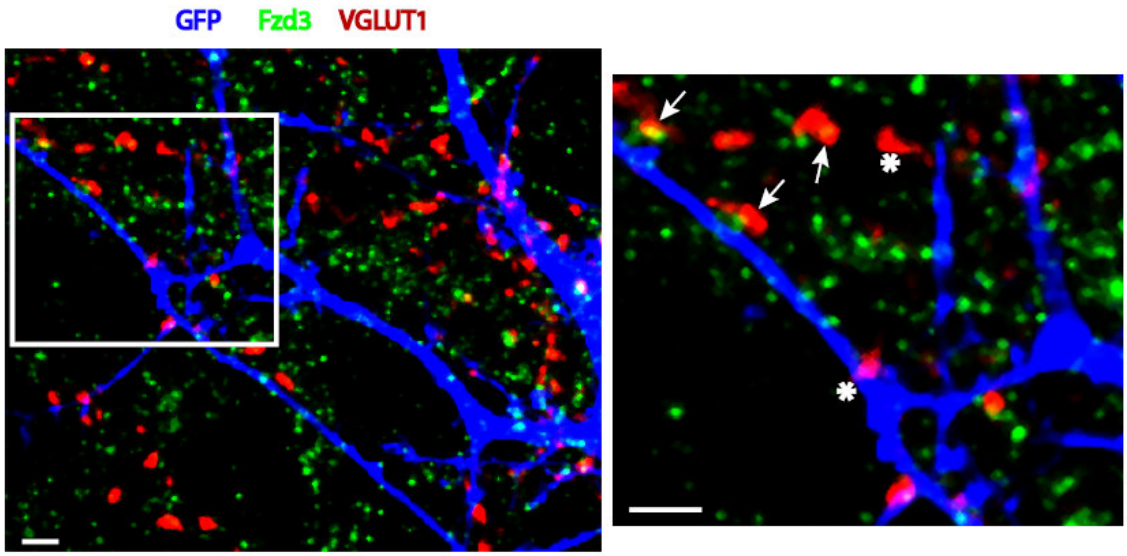


Figure 3-2 Continued

stained for VGLUT1 with GFP and Fzd3. We found that Fzd3 colocalized with some VGLUT1 puncta (Figure 3-2B; arrows), but there was a lot that did not colocalize (Figure 3-2B; asterisks). These results suggest that Wnts probably act at presynaptic terminals and may act indirectly on postsynaptic cells through extrasynaptic receptors on dendrites and cell bodies.

3.2.3. Activation of the canonical Wnt pathway promotes synapse formation

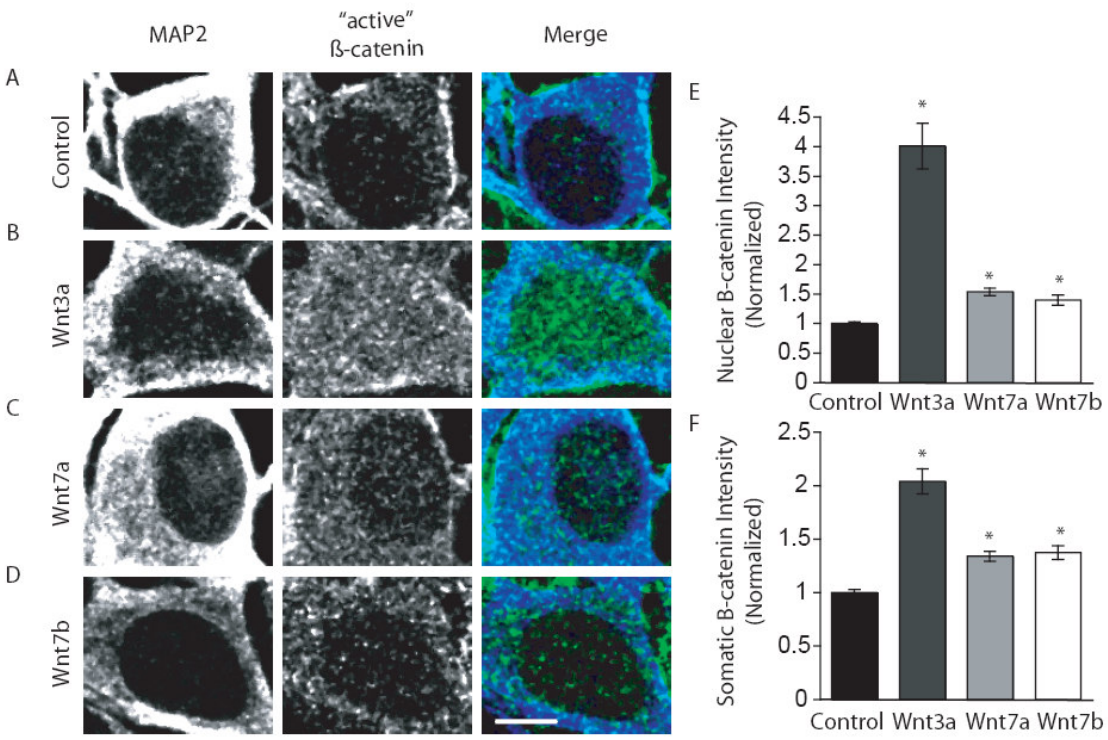
The Wnts expressed in the hippocampus at the peak of synapse formation have been implicated in the activation of both canonical and noncanonical pathways. Wnt7a and Wnt7b specifically have been identified as canonical Wnt pathway activators. To verify their role as canonical pathway inducers, we examined the consequences of exposing P0 hippocampal cultures to either recombinant Wnt protein or Wnt conditioned media for 48 hours. The cellular response to different Wnts was determined using an anti-"active" β-catenin antibody which identifies non-phosphorylated β-catenin by binding to the GSK3β phosphorylation site on β-catenin (van Noort et al, 2002). In our cultures, application of LiCl, an inhibitor of GSK3β, produced a strong increase in the intensity of "active" β-catenin in both the nucleus and soma, verifying the effectiveness of the antibody (Figure 3-3).

We first examined the intensity of "active" β-catenin at 10 days *in vitro* (DIV) after the application of Wnt3a. Wnt3a is strongly associated with activation of the canonical pathway (Galceran et al, 1999 and Willert et al, 2003) and consequently served as a positive control. As expected, application of Wnt3a resulted in greater than

Figure 3-3. Wnt3a, Wnt7a, and Wnt7b activate the canonical pathway in hippocampal cultures.

A-D. Right, representative images of "active" β-catenin stabilization by endogenous Wnts (A), addition of recombinant Wnt3a (B; 150ng/mL), and addition of recombinant Wnt7a (C; 200ng/mL), or Wnt7b conditioned media (D). Bar = 6μm. E. Quantification of "active" β-catenin immunofluorescence intensity by Wnts in the nucleus (top) and soma (bottom). Each condition was normalized to the control condition. Error bars represent SEM. n = 428 cells; *$p<0.001$. Statistics performed using t-test for each control and experimental group pairing.

Figure 3-3


MAP2
"active" ß-catenin
Merge
A
Control
B
Wnt3a
C
Wnt7a
D
Wnt7b
E
Nuclear B-catenin Intensity (Normalized)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control Wnt3a Wnt7a Wnt7b
F
Somatic B-catenin Intensity (Normalized)
2.5
2
1.5
1
0.5
0
Control Wnt3a Wnt7a Wnt7b

a two-fold increase in "active" β-catenin intensity in both the soma and the nucleus (Figure 3-3). This increase in β-catenin indicates that cultured hippocampal neurons have the ability to relay a canonical Wnt signal. Application of recombinant Wnt7a or Wnt7b conditioned media also led to an increase in intensity of "active" β-catenin immunofluorescence (Figure 3-3), indicating that these Wnts activate canonical Wnt signaling in hippocampal neurons.

To determine if activation of canonical Wnt signaling influences synapse formation we examined the effects of exposing hippocampal cultures to recombinant Wnt proteins or Wnt conditioned media. We treated cultures at 8DIV and examined the number of VGLUT1 puncta per area of dendrite after 48 hours. Application of Wnt3a, Wnt7a, or Wnt7b, increased the number of excitatory presynaptic puncta (Figure 3-4A-C), suggesting that canonical Wnt signaling positively influences synapse formation.

To further explore the role of canonical Wnt signaling in synapse formation, we examined the effects of manipulating different parts of the canonical signaling pathway. We first inhibited canonical Wnt signaling at the level of the receptor by incubating cultures with Dickkopf-1 (Dkk-1; Glinka et al, 1998). Dkk-1 promotes internalization of the LRP5/6 coreceptor, which is required to relay a canonical signal, but not a noncanonical signal. Addition of recombinant Dkk-1 from 12-14DIV resulted in both decreased β-catenin (Figure 3-5A) and decreased excitatory presynaptic puncta number (Figure 3-5B) indicating that activation of the endogenous canonical pathway contributes to synapse formation.

Figure 3-4. Wnt proteins that activate the canonical pathway increase presynaptic inputs.

A-C. Left, representative images of control conditions, Wnt3a (A; right) Wnt7a (B; right), or Wnt7b (C; right) treated cultures immunostained with VGLUT1 (green) and MAP2 (blue). Cultures were treated for 48 hours and stained at 10DIV. The same concentrations of proteins were used as in Figure 3-3. Bar = 2μm. A-C. Right, quantification of the number of VGLUT1 puncta per area of dendrite for Wnt3a (A; n = 101 fields of view; *$p<0.001$, students t-test), Wnt7a (B, n = 96 fields of view; *$p=0.001$, students t-test), or Wnt7b (C; n = 59 fields of view, *$p<0.05$, student's t-test) treated cultures. Error bars represent SEM. All conditions were normalized to control.

Figure 3-4A

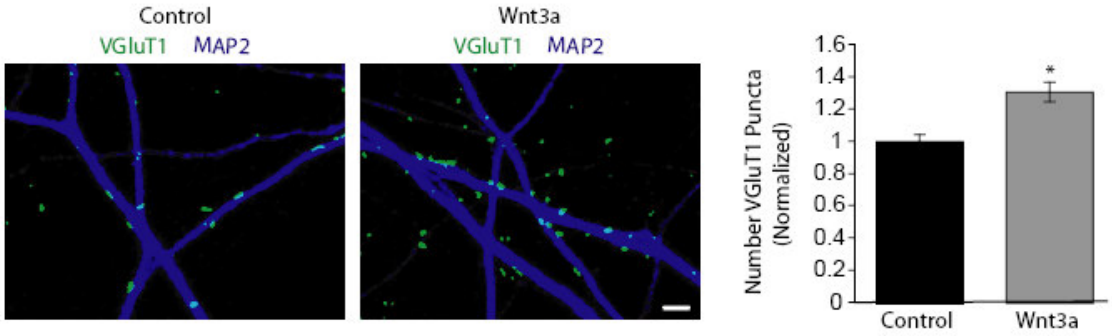

Control
VGluT1 MAP2
Wnt3a
VGluT1 MAP2
Number VGluT1 Puncta (Normalized)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Wnt3a

Figure 3-4B

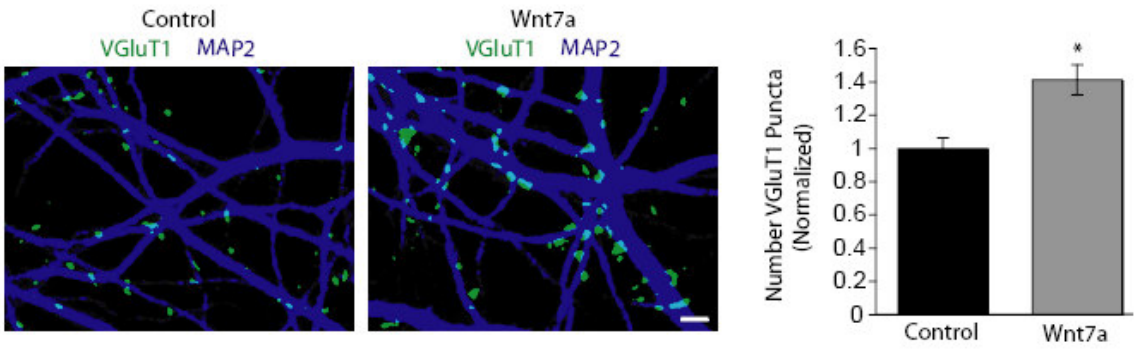


Figure 3-4 Continued

Figure 3-4C

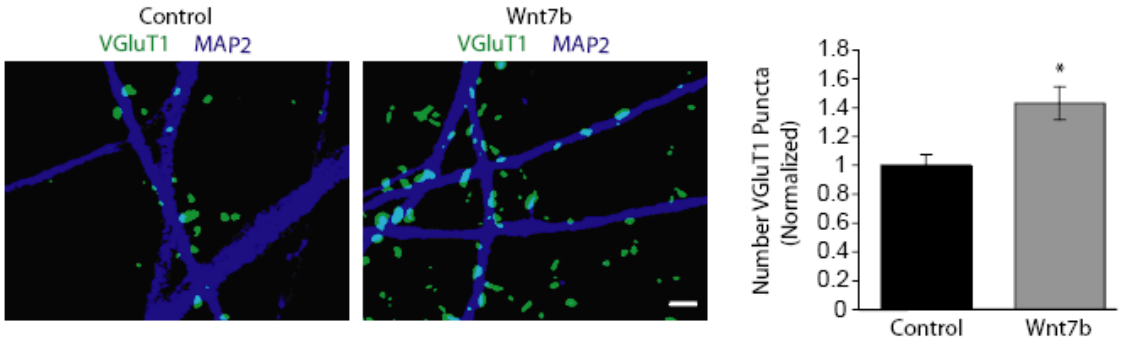


Figure 3-4 Continued

Figure 3-5. Dkk-1 decreases presynaptic inputs in hippocampal cultures.

A. Right, representative images of neurons stained with anti-"active" β-catenin in control (top) or Dkk-1 (bottom; 1μg/mL) conditions. Bar = 6μm. Left, quantification of "active" β-catenin immunofluorescence in the nucleus (top) and some (bottom). Error bars represent SEM. n = 211cells, *$p<0.0001$, student's t-test. Dkk-1 treatment condition was normalized to control.

B. Left, representative images of control (left) and Dkk-1 (right; 1ug/mL) treated cells stained with VGLUT1 (green) and MAP2 (blue) at 14DIV after 48hours of treatment. Bar = 2μm. Right, quantification of the number of VGLUT1 puncta per area of dendrite. Error bars represent SEM. Dkk-1 treatment was normalized to control treatment. n = 80 fields of view, *$p<0.05$, students t-test.

Figure 3-5A

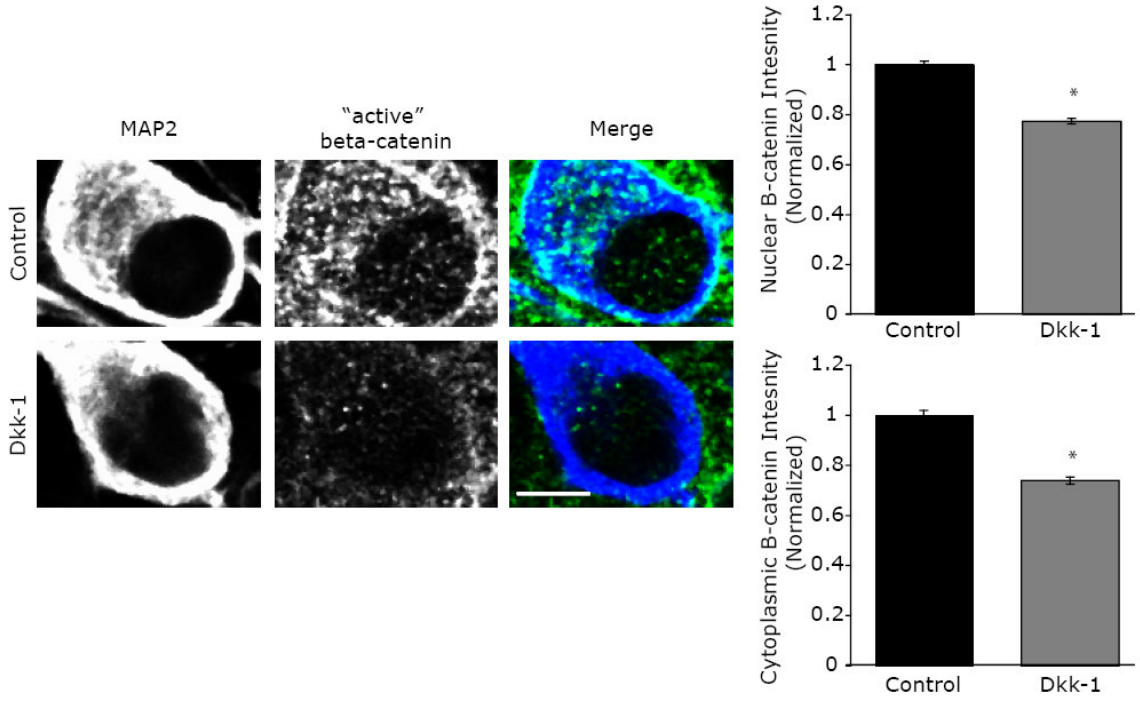

MAP2
"active" beta-catenin
Merge
Control
Dkk-1
Nuclear B-catenin Intesnity (Normalized)
Cytoplasmic B-catenin Intesnity (Normalized)
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Dkk-1

Figure 3-5B

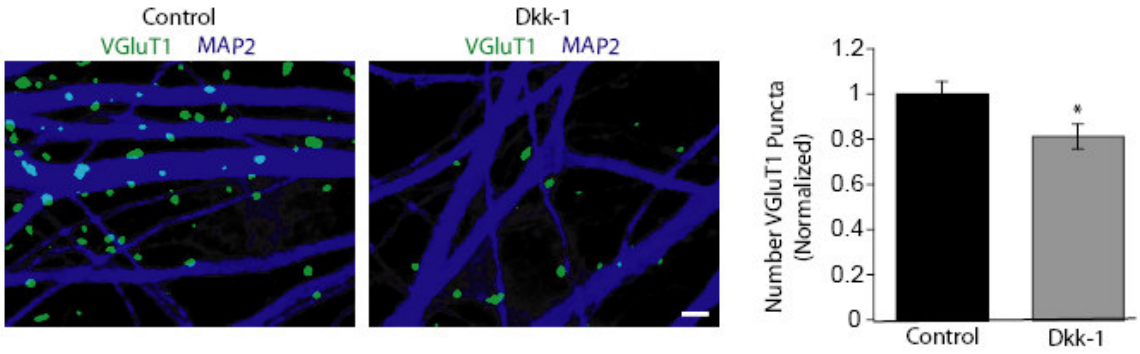


Figure 3-5 Continued

Next, we examined the consequences of manipulating downstream canonical signaling components. To mimic a canonical Wnt signal, we applied lithium chloride (LiCl) at 12DIV for 48 hours which leads to stabilization of β-catenin by inhibiting GSK3β (Klein and Melton, 1996; Figure 3-6A). Consistent with a role of canonical signaling in promoting synapse formation, we observed an increase in the number of presynaptic inputs per area of dendrite in response to LiCl treatment (Figure 3-6B).

Additionally, neurons transfected with a constitutively active form of β-catenin at 7DIV showed an increase in the number of presynaptic inputs per area of dendrite at 14DIV (Figure 3-6C; middle). Conversely, neurons transfected with full length axin, which inhibits canonical signaling, decreased the number of presynaptic puncta (Figure 3-6C, right). These results show that mimicking the canonical Wnt signal increases the number of presynaptic inputs and inhibiting the canonical Wnt signal decreases the number of presynaptic inputs. Taken together, these results suggest that activation of canonical Wnt signaling promotes synapse formation.

3.2.4. Noncanonical Wnt signaling inhibits presynaptic inputs

Of the Wnts expressed in the developing hippocampus, only one, Wnt5a, has been strongly implicated as a noncanonical Wnt pathway activator. However, Wnt5a has also recently been shown to signal through the canonical pathway (Mikels and Nusse, 2006). Therefore, we wanted to determine whether Wnt5a behaves as a canonical or noncanonical Wnt in hippocampal cultures. We exposed cultures to recombinant Wnt5a at 8DIV for 48 hours. We found that Wnt5a decreases “active” β-

Figure 3-6. The canonical Wnt signaling pathway increases presynaptic inputs.

A. Left, lithium chloride increases "active" β-catenin. Images of control (top) and LiCl (bottom) treated neurons stained for MAP2 and "active" β-catenin. Bar = 6μm. Right, bar histograms showing normalized intensity of "active" β-catenin immunofluorescence increases in the nucleus and soma with lithium chloride. Error bars represent SEM. n = 67 cells, *$p<0.001$, students t-test.

B. Left, representative images of MAP2 (blue) and VGLUT1 (green) staining at 14DIV after 48hours of treatment with NaCl (control; left; 4mM) or LiCl (right; 4mM). Bar = 2μm. Right, quantification of the number of VGLUT1 puncta per area of dendrite. Error bars represent SEM. LiCl treatment was normalized to the control condition. n = 73 field of views; *$p<0.001$, students t-test.

C. Top, representative images of dendrites immunostained with GFP (blue) and VGLUT1 (green) at 14DIV after transfection of GFP (left), constitutively active β-catenin (middle) or axin (right) at 7DIV. Bar = 2μm. Bottom, bar histogram showing the quantification of the number of VGLUT1 puncta per area of dendrite. Error bars represent SEM. All conditions were normalized to the control condition. n = 134 cells; *$p<0.05$. Statistics performed using three- way ANOVA, then Tukey test.

Figure 3-6A

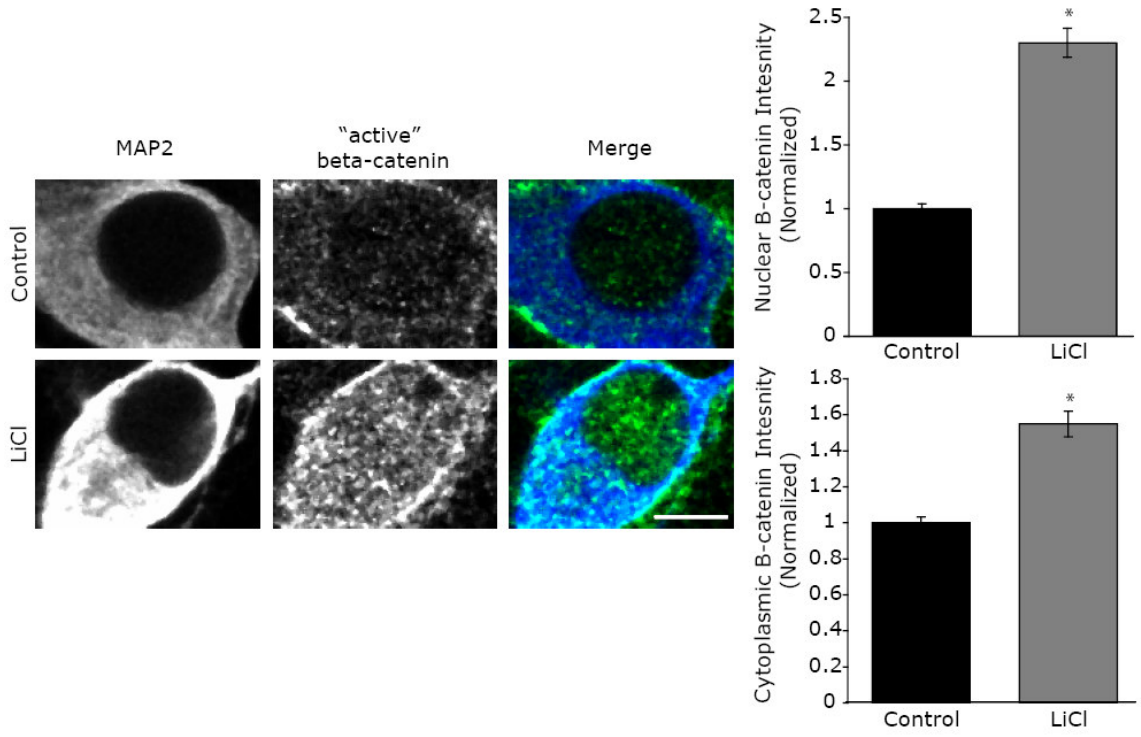

MAP2
"active" beta-catenin
Merge
Control
LiCl
Nuclear B-catenin Intesnity (Normalized)
2.5
2
1.5
1
0.5
0
Control
LiCl
*
Cytoplasmic B-catenin Intesnity (Normalized)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
LiCl
*

Figure 3-6B

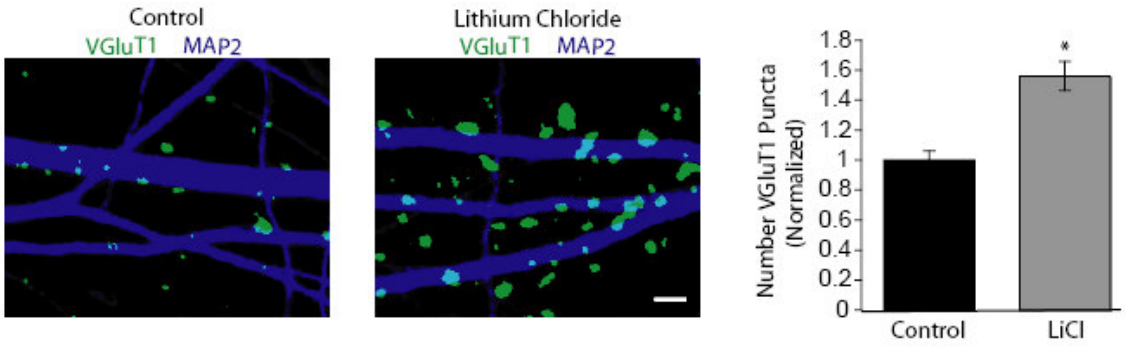


Figure 3-6 Continued

Figure 3-6C

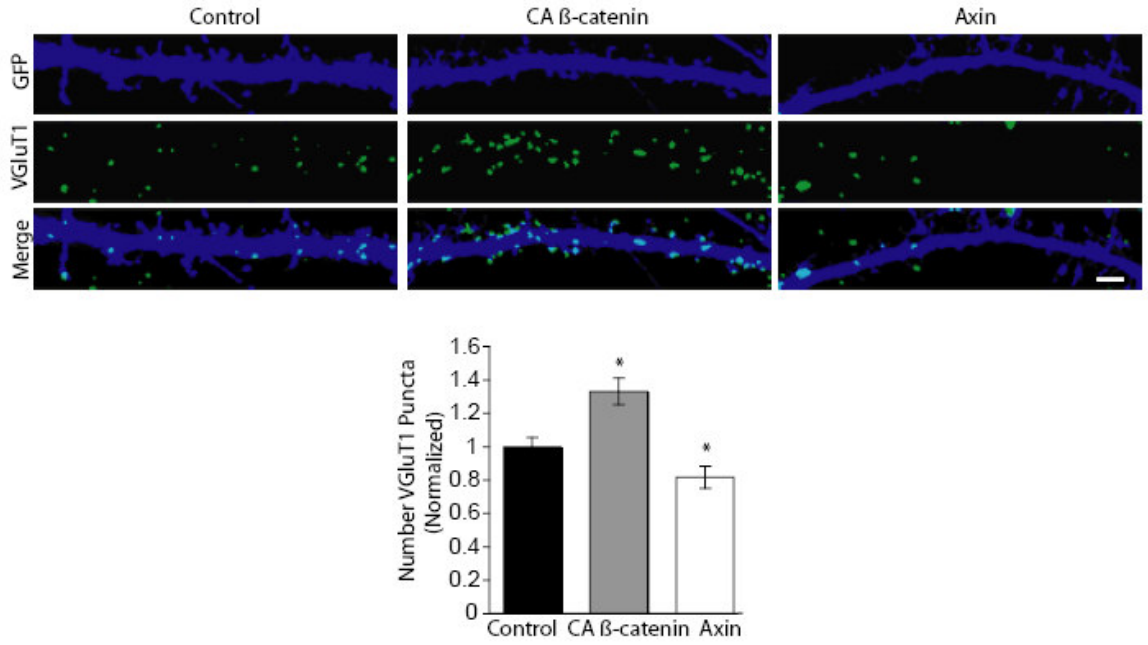


Figure 3-6 Continued

catenin immunofluorescence intensity, indicating that Wnt5a does not activate the canonical pathway and may act as an antagonist to canonical signaling (Figure 3-7A).

To determine if Wnt5a affects hippocampal synapse formation, we exposed cultures to Wnt5a at for 48 hours and observed the effect on the number of VGLUT1 puncta. In contrast to canonical Wnts, exposure to Wnt5a led to a significant decrease in the number of presynaptic terminals (Figure 3-7B), implying that the activation of a noncanonical pathway negatively regulates synapse formation.

Finally, we wanted to examine the net effect of Wnt signaling on synapse formation due to the differential effect of Wnts on VGLUT1 puncta number. To broadly inhibit Wnt signaling, we applied secreted frizzled related protein 2 (sFRP2) to hippocampal cultures. SFRP2 is an endogenous secreted protein that has a binding domain similar to Fzd (Wawrzak et al, 2007). We found that a two day incubation with sFRP2, at 12-14DIV, led to an increase in excitatory presynaptic puncta number (Figure 3-7C). These results suggest that, overall, endogenous Wnts act as negative regulators of synapse formation. However, the effect of Wnts on a given cell is likely determined by the relative activation of canonical and noncanonical pathways.

3.3. Discussion

3.3.1. Canonical Wnt signaling promotes synapse formation

We find that the Wnts expressed in the developing hippocampus have differential effects on stabilization of β-catenin. Based on our evidence of increased β-catenin levels when Wnt7a and Wnt7b is applied, and that of previous work (Cerpa et

Figure 3-7. Wnt5a acts through a non-canonical pathway and decreases the number of presynaptic inputs.

A. Left, representative images of "active" β-catenin immunostaining in control (top) and Wnt5a (bottom; 200ng/mL) treated cells. Bar = 6μm. Right, quantification of "active" β-catenin immunofluorescence intensity in the nucleus (top) and soma (bottom). Error bars represent SEM. Wnt5a treated cultures were normalized to the control condition. Statistics performed using student's t-test. n = 220 cells, *$p<0.001$.

B. Left, representative images of control (left) and Wnt5a (right; 200ng/mL) treated cells immunostained with VGLUT1 (green) and MAP2 (blue) at 10DIV. Bar = 2μm. Right, quantification of VGLUT1 puncta per area of dendrite. Error bars represent SEM. Statistics performed using student's t-test. n = 119 fields of view, *$p<0.0015$.

C. Left, cultures in which endogenous Wnts were inhibited with sFRP2. Control (left) and sFRP2 (right; 200ng/mL) treated cells were stained at 14DIV with VGLUT1 (green) and MAP2 (blue). Bar = 2μm. Right, quantification of sFRP2 effects on the number of VGLUT1 puncta per area of dendrite. Error bars represent SEM. n = 120 fields of view; *$p<0.001$. Statistics performed using student's t-test.

Figure 3-7A

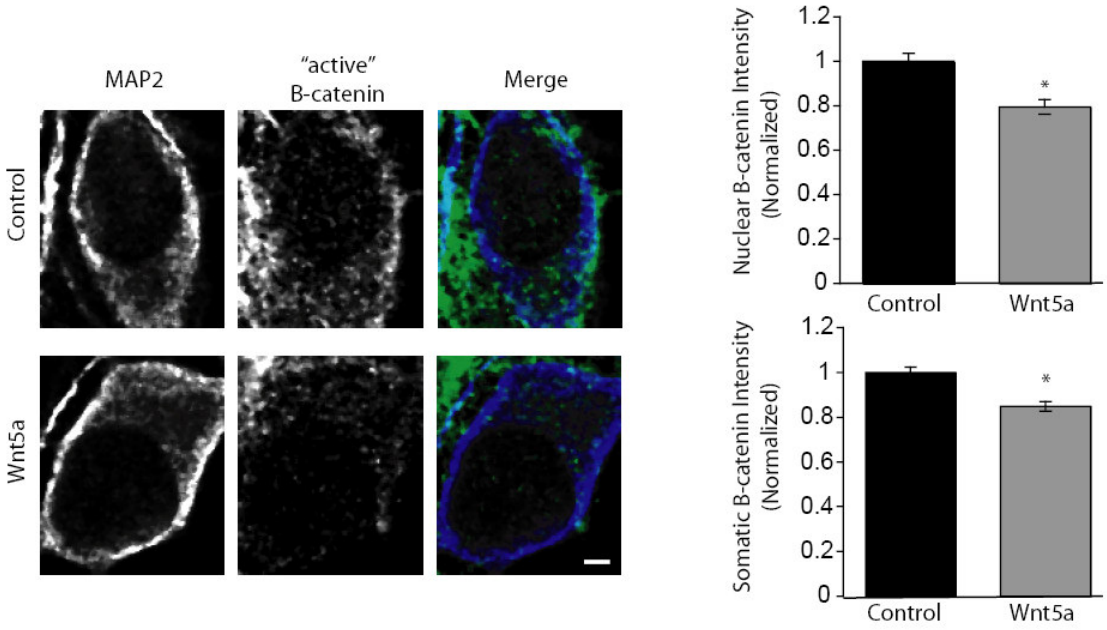

MAP2
"active"
B-catenin
Merge
Control
Wnt5a
Nuclear B-catenin Intensity
(Normalized)
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Wnt5a
Somatic B-catenin Intensity
(Normalized)
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
Wnt5a

Figure 3-7B

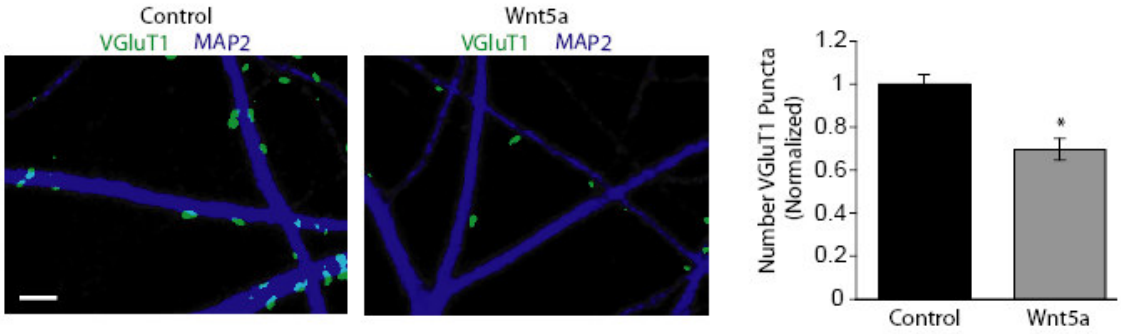


Figure 3-7 Continued

Figure 3-7C

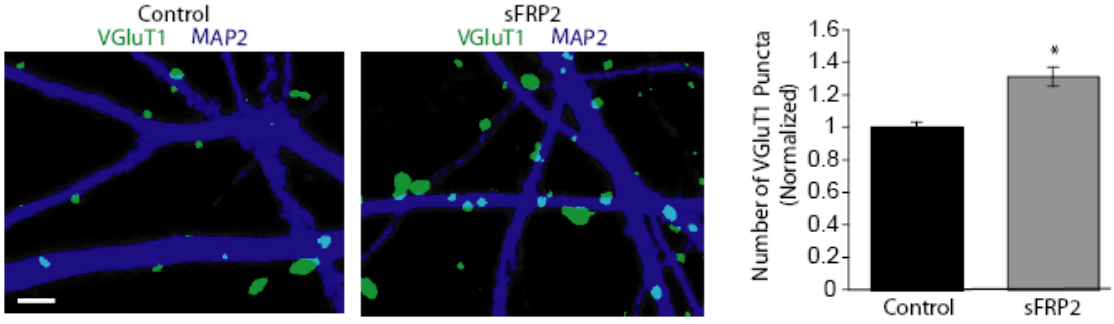


Figure 3-7 Continued

al, 2008), it is likely that Wnt7a and Wnt7b act through the canonical pathway. We also find that Wnt5a behaves in hippocampal cultures as previously described (Torres et al, 1996; Liu et al, 2005; Westfall et al, 2003; and Topol et al, 2003). Because Wnt5a antagonizes the canonical pathway in our cultures, it is possible that it acts through the Wnt/Ca2+ pathway (Ishitani et al, 2003) or even through an alternate receptor, ROR2.

We found that activation of the canonical Wnt pathway promotes synapse formation based on the evidence that Wnts that activate canonical signaling increase the number of presynaptic terminals per area of dendrite. The canonical pathway regulates presynaptic inputs through a series of events that begins with Wnts binding to the Fzd-LRP5/6 coreceptor, as indicated by the reduction of presynaptic inputs when this complex is inhibited by Dkk-1. The activation of the coreceptors leads to a stabilization of β-catenin, indicated by the increase in presynaptic inputs when β-catenin levels are increased either by inhibition of GSK3β by LiCl or transfection of a constitutively active form of β-catenin. Additionally, when Wnt signals are blocked by transfection of axin, which is part of the complex that degrades β-catenin, we see a reduction of inputs.

There are several mechanisms by which increased β-catenin levels might promote synapse formation. First, canonical Wnts might act solely to stabilize β-catenin, which can in turn stabilize N-cadherin, and therefore act as part of a cell adhesion mechanism (Togashi et al, 2002 and Yu and Malenka, 2003). Second, canonical Wnts could activate the classical canonical pathway to initiate transcription

of synaptic components such as EphB/EphrinB (Batlle et al, 2002 and Tice et al, 2002), which have been shown to cluster NMDA receptors (Dalva et al, 2003). Third, activation of the canonical pathway might act to localize presynaptic vesicles at the synapse through β-catenin stabilization (Bamji et al, 2003). And important goal of future experiments will be to identify the mechanisms by which canonical Wnt signaling promotes synapse formation.

3.3.2. Noncanonical Wnt signaling inhibits synapse formation

In addition to effects of the canonical Wnt pathway, we also wanted to explore the effect of a noncanonical Wnt pathway on the development of synapses. We found that in hippocampal cultures, Wnt5a decreased β-catenin levels and reduced number of presynaptic puncta. We do not yet know the pathway by which Wnt5a exerts its effect, but it is likely to be a noncanonical pathway that antagonizes the canonical pathway (Semenov et al, 2007). By antagonizing the canonical pathway, Wnt5a may inhibit any of the previously proposed mechanisms for prosynaptogenic effects of canonical pathway activation.

3.3.3. Opposing roles of Wnts during synapse formation

Our results show that inhibition of endogenous Wnts by sFRP2 leads to an increase in presynaptic inputs, suggesting that Wnt5a is the dominant Wnt during synapse formation in hippocampal cultures. This could be due to higher Wnt5a expression than Wnt7a and Wnt7b combined or a higher affinity of Wnt5a for Fzd

receptors, either of which would lead to more noncanonical pathway activation than canonical pathway activation. While the mechanism of competition between canonical and noncanonical pathway activators is not yet known, others have shown similar results when both types of pathways are activated at once (Nemeth et al, 2007).

Previous studies have reported divergent effects of Wnts in synapse formation. Wnts have been shown to be antisynaptogenic in *C. elegans* by a mechanism that does not involve the canonical pathway (Klassen and Shen, 2007). Wnt4 in drosophila embryos also has an antisynaptogenic activity (Inaki et al, 2007). Although the authors do not suggest a pathway through which this is mediated, Wnt4, which is most closely related to mammalian Wnt9a and Wnt9b, has previously been described as a noncanonical Wnt (Montcouquiol et al, 2006). Conversely, in mammals, Wnts have been shown to be pro-synaptogenic. Wnt7a, which can activate the canonical Wnt signaling, was found to alter growth cone complexity and promote synapse formation in the cerebellum (Hall et al, 2000). Based on these observations, together with the findings presented here, we suggest that the pro- and anti-synaptogenic effects of Wnts on synapse formation are generally mediated by the level of activation of canonical and noncanonical signaling pathways.

3.4. Acknowledgments

Chapter 2, in part, and Chapter 3, in full, has been submitted for publication in Neural Development, 2008. Davis, E.K., Zou, Y., and Ghosh, A., 2008. The dissertation author is the primary investigator and author of this paper.

CHAPTER 4: Synapse Specificity *in vitro*

4.1. Introduction

The hippocampus is an ideal system to study synaptic specificity due to the simplicity of its excitatory circuitry. The excitatory circuitry of the hippocampus consists of three major synapses between three cells types. First, dentate gyrus (DG) granule neurons project axons (mossy fibers, MF) onto pyramidal cells of the CA3 region. Next, CA3 cells can synapse onto other CA3 cells through a recurrent pathway or onto CA1 pyramidal cells through Schaffer collaterals (SC). CA1 neurons project onto cells in the subiculum and cells in the subiculum project out into the cortex. The connections between cells types in the hippocampus are strict; DG neurons will never synapse onto CA1 pyramidal cells and likewise, CA3 cells never project axons into the DG region.

The structure and electrophysiological properties of excitatory synapses in the hippocampus can vary greatly. The main differences are between the MF-CA3 synapse and synapses between pyramidal cells. MF synapses consist of a giant presynaptic terminal that surrounds spines on the proximal dendrite of CA3 cells, named thorny excrescences (Chicurel and Harris, 1992). Each MF presynaptic bouton has many active zones juxtaposed to different postsynaptic densities from the same pyramidal cell (Acsady et al, 1998, Chicurel and Harris, 1992). Each DG cell will only make synapses with 11-15 CA3 cells (Amaral and Dent, 1981). The CA3-CA1 and CA3-CA3 synapses, in contrast, consist of a single bouton and a single postsynaptic

density and are similar in structure to synapses within the cortex or other regions of the brain. Unlike granules cells, each CA3 cell will contact many target neurons (Li et al, 1994).

The electrophysiological properties of synapses in the hippocampus also vary greatly. The MF-CA3 synapse has very unique electrophysiological properties compared to the SC-CA1 or CA3-CA3 synapses. For example, short term plasticity, a direct correlate of probability of release, can be measured by paired pulse facilitation or depression. The MF-CA3 synapse is very facilitating, indicating a very low probability of release. Through development, this facilitation increases from P10 to adult (Hussain and Carpenter, 2001). The mature SC-CA1 synapse has less facilitation than the MF-CA3 synapse (Hsia et al, 1998). This facilitation starts off very low at P5 and increases until P12 (Wasling et al, 2004) and then decreases slightly as the animal matures to adulthood (Hussain and Carpenter, 2001).

How these synapses come to their complete and mature state and why DG neurons form specialized synapses with a select type of neuron, CA3 cells, is of great interest. The process of specificity occurs through several complex steps that have not been completely characterized. In order for a presynaptic neuron to target its correct postsynaptic cell, an axon must extend, sometimes through entire incorrect target areas to arrive at the correct target area. Once at the correct area, a selection process occurs where the cell either makes synapses directly onto its target neurons or makes synapses with all cells in the general area and then eliminates those that are incorrect and matures those that are correct (Benson et al, 2001). Little is known about this

process or what factors are involved to help neurons identify a correct or incorrect target.

In this study, I collaborated with Megan Williams to examine the DG synapse in culture. We first developed an *in vitro* system to study how DG cells decide which cell type to synapse onto. This environment would ensure that the ability of the DG cell to recognize its target is not an effect of a diffusible factor gradient or a consequence of axon guidance cues. I first characterized the development of synapses on these microislands using evoked EPSCs. I found that synapses start to form around 7DIV and are mature over the next week *in vitro*. Additionally, I found that evoked currents in CA3 neurons from DG neurons are larger than in CA1 or DG cells. M.W. found that DG neurons transfected with synaptophysin-GFP make more synapses onto its correct target, CA3 neurons. These results suggest that DG cells are able to identify its correct target by a molecular tag. This tag could be either a positive cue on CA3 cells, which would promote synapse formation or a negative cue on DG and CA1 neurons, which would inhibit synapse formation.

4.2. Results

4.2.1. Preparation of Hippocampal microislands and identification of cell types (M.W. and E.K.D.)

In order to examine the mechanism by which DG cells select correct synaptic targets, we developed an *in vitro* system in which DG neurons would have a choice between CA3, CA1, and other DG neurons. Using an *in vitro* environment would

ensure that the DG neuron would be able to recognize its correct target in the absence of axon guidance or experience-dependent cues. Briefly, hippocampal neurons from neonatal rats were dissociated and plated on glial microislands on so that a small number of neurons were each island contained a small number of isolated neurons (Segal and Furshpan, 1990; Figure 4-1). These islands provided a small network of cells in which monosynaptic connections were common. To determine whether DG neurons recognize correct targets in microcultures, we first needed a way to identify cell types within the cultures. Using a combination of antibodies to Prox1, a transcription factor specifically expressed by DG neurons (Bagri et al, 2002), PY, a cytoskeletal protein expressed by CA3 pyramidal neurons and a few scattered interneurons (Woodhams et al, 1989), and CTIP2, a transcription factor expressed by CA1 pyramidal neurons and most DG neurons but not CA3 pyramidal neurons (Arlotta et al, 2005), each major excitatory cell type in the hippocampus could be identified.

4.2.2. Functional development of synapses *in vitro* (E.K.D.)

After developing the microisland cultures, we next wanted to examine the development of functional synapses in cultures in order to find the time window for which synaptic specificity could be occurring. For these experiments, two neurons on individual islands were patched simultaneously and tested for synaptic connectivity (Figure 4-2; top). One neuron was held in current clamp and stimulated to produce

Figure 4-1. Preparation of microislands and immunostaining of microislands. Top, a schematic of microisland preparation. Coverslips were first coated in agarose and then sprayed with a poly-d-lysine and collagen mixture. Glia was plated onto islands and then dissociated neurons onto glia. Bottom, left, a DIC picture of live 14DIV hippocampal neurons on microislands during a recoding session, performed as described in Figure 4-2. Middle, the same neurons as in left panel, after fixation and staining with an antibody to the neuronal marker MAP2 (purple). Right, the same field of view as in right panel, but immunostained with antibodies against Prox1 (green), PY (red), and CTIP2 (blue) to identify DG, CA3, and CA1 neurons respectively.

Figure 4-1

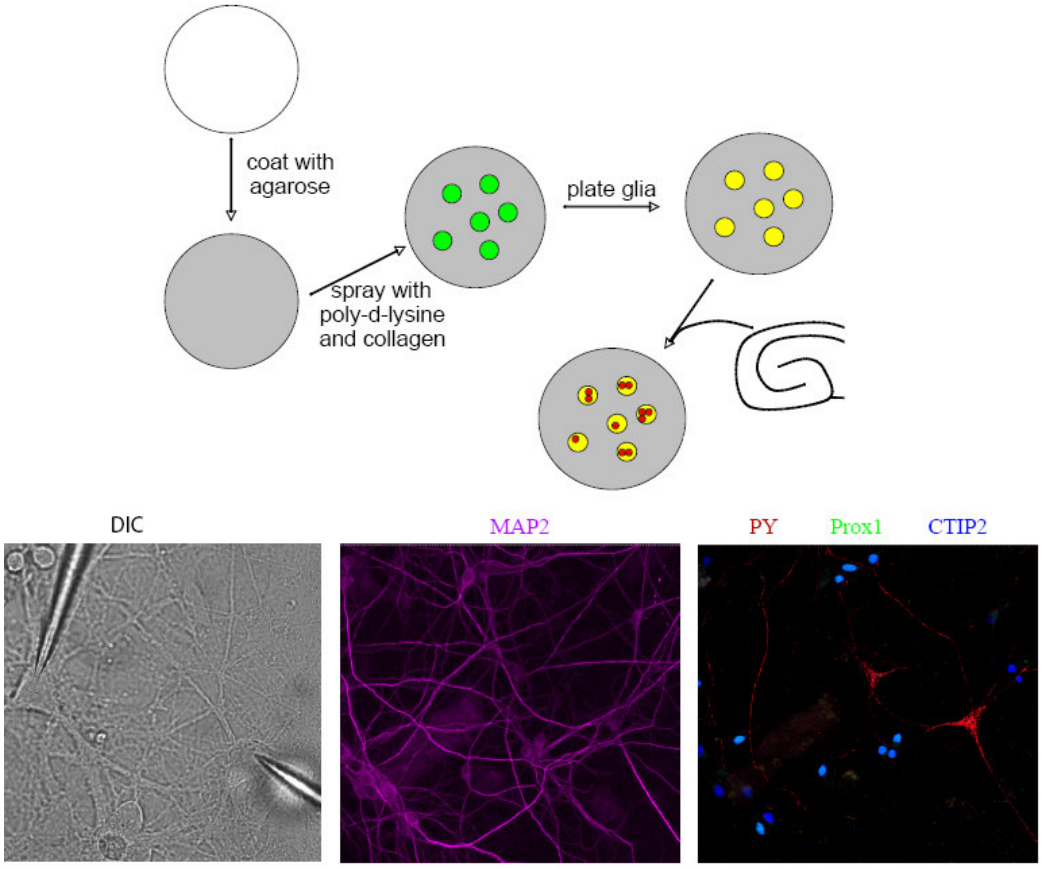
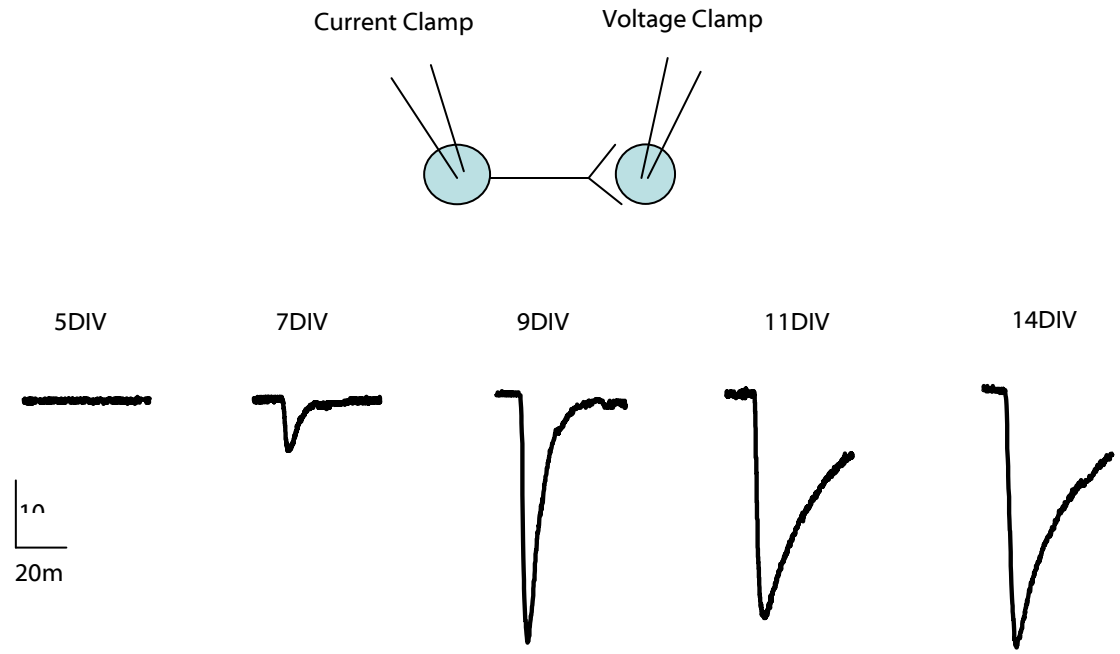

coat with
agarose
spray with
poly-d-lysine
and collagen
plate glia
DIC
MAP2
PY
Prox1
CTIP2

Figure 4-2. Development of synaptic currents in P0 hippocampal microisland cultures.

Top, a schematic of a dual patch clamp technique using hippocampal microislands where the presynaptic cell was held in current clamp and the postsynaptic cell in voltage clamp. A single action potential was evoked in the presynaptic cell and the corresponding current recorded in the postsynaptic cell. Bottom, representative traces of evoked currents at various time points. At 5DIV (n=8), currents were never found, however, shortly after, at 7DIV, currents were found, but were often small. These currents became larger between 7DIV and 9DIV and persisted through the second week *in vitro*.

Figure 4-2



single action potentials and, at the same time, EPSCs were recorded from the second neuron held in voltage clamp. We found that evoked EPSCs were completely absent at 5DIV (n=8), but that currents were found shortly after, at 7DIV. During the second week *in vitro* these currents increased in amplitude through 14DIV (Figure 4-2; bottom). These results indicate that functional synapses in microislands are largely formed between 7DIV and 14DIV. We therefore studied the specificity of the DG synapse between 11-14DIV.

4.2.3. Synapse specificity in hippocampal neurons is preserved *in vitro* (M.W.)

To determine if DG neurons preferentially innervate CA3 neurons given a choice of potential targets, cultures were transfected with synaptophysin-GFP at the time of plating to visualize the synaptic terminals of transfected cells (4-2). For analysis, only islands where a single DG neuron was transfected were selected so that every synaptophysin-GFP puncta could be uniquely associated with the transfected neuron (Figure 4-2A). By 12DIV the neurons have extended elaborate axons and dendrites, and the distinct synaptophysin puncta can be clearly identified. An example of such a culture is shown in Figure 4-2D-E. This island has one transfected DG neuron (indicated by arrow) and about 20 surrounding neurons, stained with anti-MAP2 (Figure 4-2D). At higher magnification the presence of puncta on dendrites of some of the neurons identifies putative synaptic sites (Figure 4-2E). Co-staining with anti-Prox and anti-Py antibodies indicates that in this field, the synapses are concentrated on CA3, as opposed to other DG neurons (Figure 4-2F).

To analyze the distribution of synaptic terminals on the different classes of target neurons the total number of presynaptic terminals on each of the cells on the islands was counted. As shown in Fig. 1G, this ranged from 1-50 synaptophysin-GFP puncta per neuron. To represent the number of synapses formed on individual neurons for each cell type, we graphed the raw data as a bar-code where each vertical stripe represents data from a single target neuron (Figure 4-2G). The bars are color-coded such that dark blue represents zero synaptophysin-GFP puncta per neuron while red indicates a maximum of 50 synaptophysin-GFP puncta per neuron. From this representation it is evident that the highest number of synaptophysin-GFP puncta, depicted by green, yellow, and red bars, fall on correct CA3 target cells. In contrast, the incorrect DG and CA1 target neurons are innervated at very low levels as indicated by the color blue (Figure 4-2G). Quantification of these results indicated that DG neurons showed a strong preference for CA3 neurons over CA1 neurons and other DG neurons. The total number of DG synaptic inputs sorted by target cell types showed that CA3 neurons receive 4 times as many synaptophysin-GFP puncta per neuron than DG or CA1 neurons (Figure 4-2H). These observations indicate that DG neurons have the ability to distinguish between correct and incorrect targets, even under these minimal conditions that lack any positional cues or other information that might be present *in vivo*.

Figure 4-3. DG neurons make more synaptophysin-GFP puncta on CA3 neurons than on other types of neurons of the hippocampus.

A. A 12DIV microisland with a single synaptophysin-GFP transfected neuron was immunostained with antibodies against MAP2 (purple) and GFP (green) and the merged areas appear white. Note that the entire island did not fit in one field of view and three views are merged in one image.

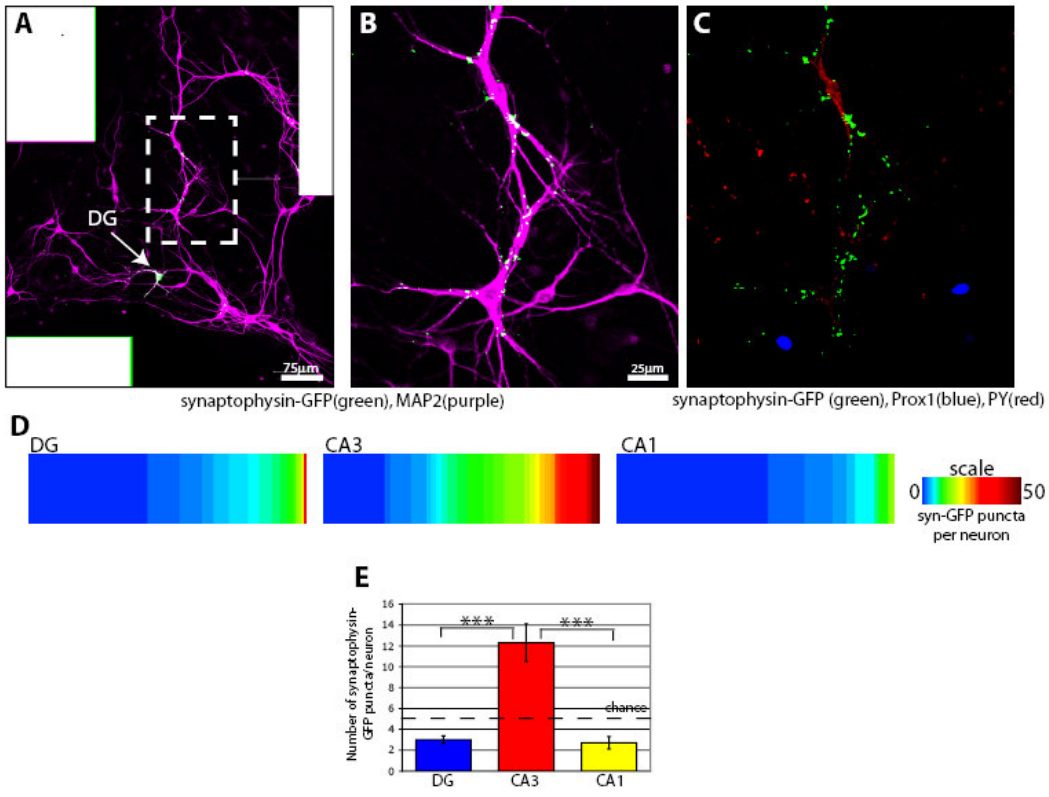
B. Magnified image of the region outlined in A.

C. The same magnified image shown in B was also immunostained with antibodies against Prox1 (blue) and PY (red) to identify cell types and the GFP channel (green) is shown again to identify the location of the synaptophysin-GFP puncta.

D. The number of synaptophysin-GFP puncta located on each target neuron is shown. Each data point is represented as a band of color based on the scale shown at right. (n>150 cells, 21 islands).

E. Graph of the average data collected for each target cell type as indicated. Error bars represent SEM. Dotted line represents the average expected by chance without regard to cell type. Statistics were performed using three-way ANOVA then post test. n>150 cells, 21 islands; ***$p<0.001$ and **$p<0.01$.

Figure 4-3


A
B
C
DG
75µm
25µm
synaptophysin-GFP(green), MAP2(purple)
synaptophysin-GFP (green), Prox1(blue), PY(red)
D
DG
CA3
CA1
scale
0
50
syn-GFP puncta per neuron
E
Number of synaptophysin-GFP puncta/neuron
***
***
chance
DG
CA3
CA1

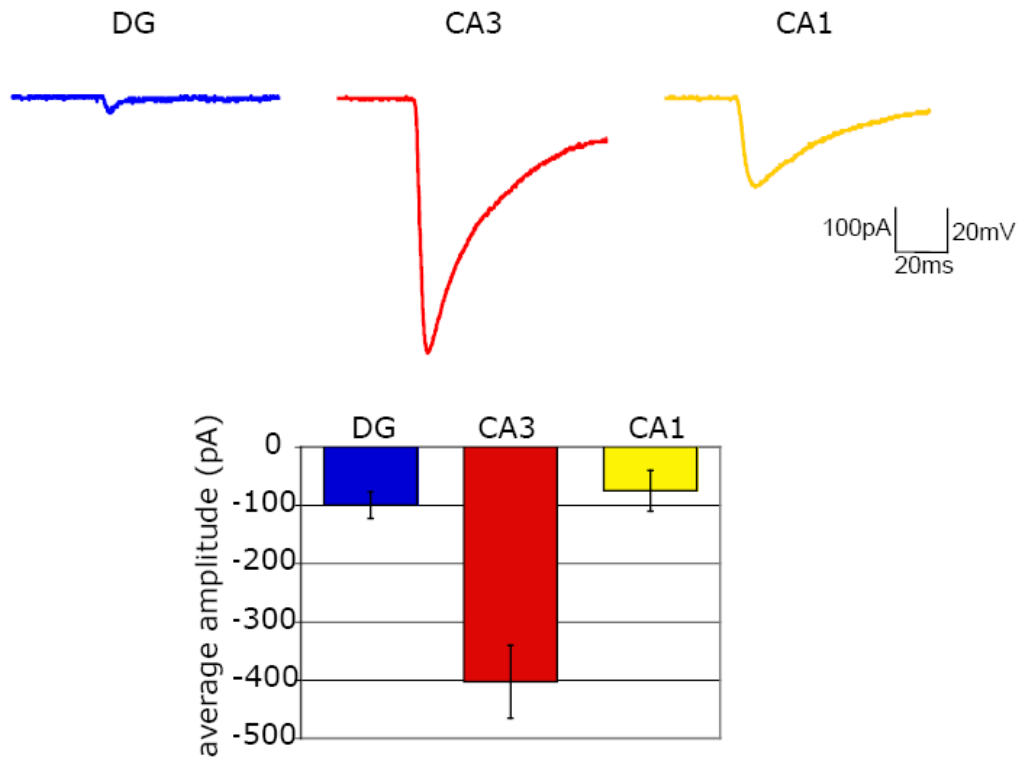
4.2.4. DG neurons produce larger EPSCs in CA3 neurons than in incorrect target cells (E.K.D.)

Analyzing the number of synaptophysin-GFP puncta is a good measure of the anatomical synapses made from DG neurons onto different cell types. However, we cannot conclude that the number of synaptophysin-GFP puncta reflect the number of functional puncta from DG neurons onto various cells types. We chose to investigate the functional preference of DG neurons for CA3 neurons through paired whole cell recordings. As briefly described before, the presynaptic cell was held in whole cell current clamp to elicit a single action potential while the postsynaptic cell in whole cell voltage clamp simultaneously recorded the response. This recording protocol was followed by immediate fixation and immunostaining for cell type specific markers, as described in Figure 4-1. Experiments in which a DG neuron was the presynaptic cell showed variability in the postsynaptic response depending on the identity of the postsynaptic cell. DG-DG and DG-CA1 pairs of neurons showed relatively weak postsynaptic responses, whereas DG-CA3 pairs showed much stronger responses. Representative traces of each cell type pairing can be seen in Figure 4-4 (top). When averages are taken from each cell pairing, it is clear that the average EPSC amplitude is much higher with DG-CA3 pairs than with DG-DG or DG-CA1 pairs (Figure 4-4; bottom). These results confirm our previous anatomical findings and strongly suggest that DG neurons are able to identify their correct physiological target in the absence of any axon guidance or experience-dependent cues.

Figure 4-4. Biased functional connections develop in microcultures.

Top, representative traces of paired recordings from pre- and post-synaptic cells of known cell types. In all cases the presynaptic cell is a DG neuron. Bottom, graph of the average EPSC amplitudes from postsynaptic cells of different cell types. Error bars represent SEM. Statistics were performed using three way ANOVA followed by post-tests where ** indicates $p<0.01$. n = at least 5 pairs per cell type.

Figure 4-4


DG
CA3
CA1
100pA
20mV
20ms
DG
CA3
CA1
average amplitude (pA)
0
-100
-200
-300
-400
-500

4.3. Discussion

The results shown here demonstrates that hippocampal cultures develop synapses over the first week *in vitro* and that DG neurons are able to correctly identify their *in vivo* target, CA3 neurons, in both the number of synapses and the strength of the evoked response. It would be interesting to examine the properties of the synapses DG neurons make onto CA3 neurons *in vitro* and compare their properties to that of DG-CA3 synapses in slices or *in vivo*. It would also be interesting to investigate the properties of incorrect synapses made from DG neurons onto either other DG cells or CA1 cells. Are these synapses merely immature mossy fiber synapses or do DG neurons make an entirely differently type of synapse onto incorrect targets? One study suggests that it might be the target neuron that determines the type of synapse made. In Campell and Frost (1987), they rerouted retinal ganglion cell (RGC) projections from their original target, the dorsal lateral geniculate (LGd) nucleus, to synapse onto the ventrobasal (VB) thalamic nucleus. The VB nucleus is normally where somatosensory neurons synapse. They then used electron microscopy to look at the differences between RGC synapses onto incorrect and correct targets as well as correct somatosensory synapses. They found that the RGC-VB synapses looked much more like somatosensory-VB synapses than RGC-LGd synapses suggesting that the target cell determines the synaptic properties of the pre and postsynaptic terminals.

Our results showing that DG neurons maintain their ability to correctly synapse on their target neurons in culture suggests that DG neurons identify this target by a molecular tag due to the absence of axon guidance cues in an *in vitro* environment.

This molecular tag could be either a positive or negative signal to DG neurons. A positive tag would be expressed only on CA3 neurons while a negative cue would be expressed on DG and CA1 neurons. Therefore, any surface molecule that is differentially expressed in the hippocampus could be a potential mediator of this specificity. Candidate molecules with differential expression patterns include LRRTM4, netrin-G1, and netrin-G2 (Allen Brain Atlas). However, other candidate molecules exist due to previous reports of involvement in synapse specificity. Previous studies have implicated Dscam (Hummel et al, 2003) and Syg-1 and -2 (Shen and Bargmann, 2003 and Shen et al, 2004) as positive cues and Wnts as negative cues (Klassen and Shen, 2007).

4.3. Acknowledgments

Chapter 4 was a collaborative effort between Dr. Megan Williams (M.W.) and the dissertation author (E.K.D.).

CHAPTER 5: Conclusions and Future Directions

5.1. Conclusions

Since the discovery of Wnts, there has been an explosion of research on Wnt signaling and the functions of Wnts in development. The variety of functions Wnts have been shown to be involved in – from cell fate to cancer – underlines its importance in development and beyond. This vast signaling network has been shown to even be involved in synapse formation, although this function of Wnt signaling has not been fully explored. There have only been a handful of studies investigating the role of Wnts in synapse formation and these studies have yielded conflicting results. Studies in mice and at the fly NMJ suggest that Wnts are positive synaptogenesis regulators, while studies in drosophila embryos and C. *elegans* suggest that Wnts negatively regulate synapse formation. The purpose of my dissertation was to resolve this issue by exploring the role of Wnts in synapse formation in hippocampal cultures.

Chapter 2 of my dissertation examines the expression of Wnts in the developing mouse brain. We find that out of 19 Wnt probes, 11 Wnt proteins are expressed throughout various regions of the brain. We found that the hippocampus expressed four Wnts, Wnt3, 5a, 7a, and 7b. The expression patterns were unique for each Wnt. We also found that Wnts 1, 4, 5a, 7b, and 9a were expressed in the cortex and that Wnts 5a, 7a, and 7b were expressed in different regions of the olfactory bulb. Many of the Wnts were also expressed in various nuclei of the thalamus. We also examined Fzd and sFRP2 expression and found expression of these proteins was fairly ubiquitous.

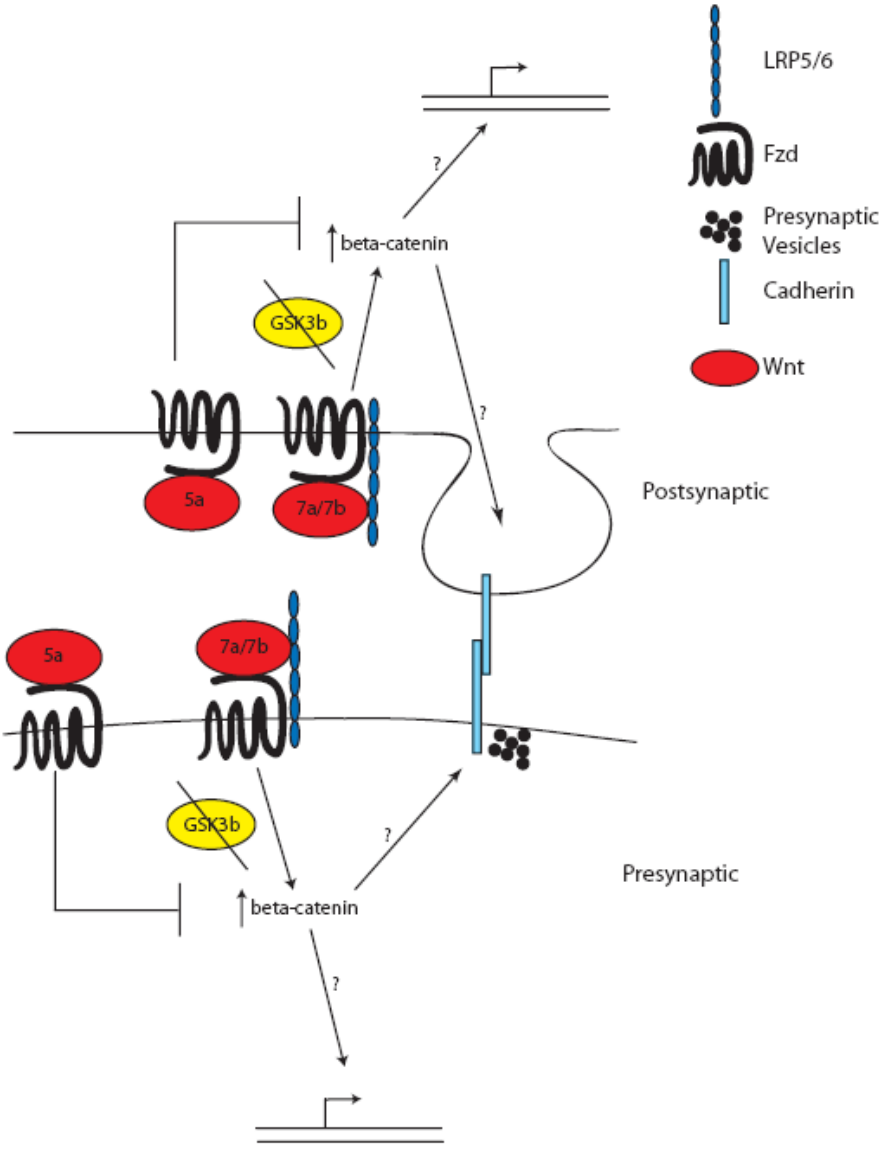
Chapter 3 of my dissertation explores the role of Wnts in synapse formation. We used recombinant Wnt protein or Wnt conditioned media to explore the effects of Wnts on synapses in hippocampal cultures. We found that the Wnts expressed in the developing hippocampus are able to activate either canonical (Wnt7a or Wnt7b) or noncanonical (Wnt5a) pathways. The activation of the canonical pathway, either through pathway manipulations or through Wnt stimulation, increases presynaptic inputs. In contrast, exposure to Wnt5a, which activates a noncanonical signaling pathway, decreases the number of presynaptic terminals. Our observations suggest that the pro- and antisynaptogenic effects of Wnt proteins are associated with the activation of the canonical and noncanonical Wnt signaling pathways. These results suggest that the effect of Wnts on a particular cell is dependent on the relative activation of canonical and noncanonical pathways.

In summary, a model of how Wnts might work at the synapse is shown in Figure 5-1. When Wnt7a or Wnt7b bind to Fzd-LRP5/6 co-receptor the trio of proteins that normally degrade β-catenin is disrupted. This then leads to a rise in the level of β-catenin. B-catenin then leads to localization of presynaptic vesicles (Bamji et al, 2003), transcription of synaptic proteins such as EphB/EphrinB (Batlle et al, 2002 and Tice et al), or simply cell adhesion through a cadherin dependent mechanism. Since we cannot show that transcription is necessary for the effect of Wnts at the synapse, we can only conclude that Wnt-β-catenin signaling is necessary for the

Figure 5-1. Model of Wnts working at the synapse.

It is currently unknown whether Wnts act pre- or postsynaptically, although data examining Fzd3 subcellular localization suggests that they are able to act on both. On the presynaptic side, when Wnt7a or Wnt7b binds to the Fzd-LRP5/6 coreceptor, it inhibits GSK3β and causes an increase in β-catenin levels. This could lead to transcription of synaptic components, increased adhesion through cadherins or even localization of presynaptic vesicles in presynaptic terminals. On the postsynaptic side, an increase in β-catenin levels could activate cadherin dependent cell adhesion or transcription of synaptic components to positively regulate synapse formation. Wnt5a would act in a similar manner on both the pre- and postsynaptic side of the synapse and could inhibit any of the proposed mechanisms.

Figure 5-1


LRP5/6
Fzd
Presynaptic Vesicles
Cadherin
Wnt
?
beta-catenin
GSK3b
5a
7a/7b
Postsynaptic
5a
7a/7b
GSK3b
?
Presynaptic
beta-catenin
?

role of Wnts to positively regulate synapse formation. When Wnt5a binds to Fzd, the canonical pathway is inhibited and there is a decrease in β-catenin (Topol et al, 2003). This leads to inhibition of any one of the proposed mechanisms. In summary, my research both advances our knowledge of the way Wnts work at the synapse and lays the groundwork for additional research on Wnts in synaptic specificity.

While the work that I have presented in this dissertation addresses some questions about how Wnts act during synapse formation, further questions arise. First, it would be useful to know whether Wnts are acting pre- or postsynaptically. Previous data would suggest that Wnts can signal to presynaptic terminals due to colocalization of canonical Wnt signaling components with presynaptic puncta (Matsumine et al, 1996 and Hirabayashi et al, 2004) and evidence that Wnts act on lone axons growing from explants to increase synaptic puncta number (Ahmad-Annuar et al, 2006). However, other studies exploring the role of Wnt in dendrites (Russo et al, 2005 and Yu and Malenka, 2003) suggest that Wnts may also act postsynaptically. In addition, I show that Fzd3 expression is localized to both axons and dendrites. While I do not know which Wnt is binding to Fzd3, it would suggest that Wnts can act anywhere in the cell, both in axons and dendrites.

Knowing whether Wnts act on the pre- or postsynaptic side of the synapse would give clues to exactly what Wnt does in synapse formation, whether this is recruitment of receptors, transcription of synaptic proteins, or promoting cell adhesion through cadherin interactions. This role of Wnts has already been somewhat explored. Yu and Malenka (2003) suggest that the effects of Wnts on dendrites are mediated by

cadherin signaling as a dominant negative TCF did not yield an effect on dendritogenesis. However, we do not know the precise role of Wnts directly at synaptic junctions.

Chapter 4 of my dissertation investigates whether synaptic specificity is maintained in culture. In collaboration with Megan Williams, we used a combined anatomical and functional approach in exploring the specificity of the DG synapse *in vitro*. Using microislands with hippocampal neurons, we first looked at the development of functional synapses in microislands and found that functional synapses start to form about 7DIV. Next we found that DG neurons make more synapses and evoke a larger current in its correct targets, CA3 neurons, than in other cell types, DG or CA1 neurons.

One final future direction of my research is whether Wnts are involved in synaptic specificity. Wnts have been implicated in synaptic specificity at both the embryonic drosophila NMJ and the C. *elegans* NMJ. Generally, Wnt expression gradients are thought to be the mechanism by which Wnts guide synapse specificity. In nematodes, Wnts create a gradient at the tail end of the animal. This gradient leads to the prevention of synapse formation in axons that express Fzds (Klassen and Shen, 2007). In the embryonic drosophila, Wnts are differentially expressed in muscles. This differential expression inhibits axons with Fzds from extending into the wrong target area (Inaki et al, 2007). There are several ways in which a gradient could guide synapse specificity. One such mechanism is shown in Figure 1-4. In this cartoon, two axons express different Fzds which react differently to Wnt proteins. This leads to

layer specific segregation of axons in a laminated structure such as the cortex. An alternative possibility would be if different Wnts that have different effects on synapse formation, such as Wnt7a and Wnt5a, were expressed in different layers. This would cause one axon that expresses Fzd receptors to only synapse in the Wnt7a positive zone and another which does not express Fzd receptors to synapse in both zones.

In chapter 4 we explore whether DG synaptic specificity is retained *in vitro*. We are particularly interested in how specificity is maintained and what molecular mechanism is used to guide the specificity of this synapse. Due to the differential effects of Wnts expressed in the hippocampus on synapse formation, a combination of differential expression patterns of multiple proteins could predict where mossy fibers make synapses. Currently, we do not know the full spectrum of receptors expressed in the developing hippocampus nor the relative levels of each of the Fzd receptors, or even which pathway each Fzd signals through. This signal transduction could even be different in different types of neurons. In addition, we do not know the expression patterns of all the Wnt inhibitors. All of these factors could play a role in the correct synapse formation of mossy fibers in the hippocampus. Due to the difficulties of mammalian genetics in misexpressing Wnts, one way in which we can further explore these kinds of possibilities is to use microisland cultures as discussed in chapter 4. Postsynaptic targets could then overexpress one of the Wnt proteins or an inhibitor of Wnts. With the presynaptic DG cell expressing synaptophysin-GFP, the number of puncta can be counted on each cell type to see whether the expression of Wnts in the postsynaptic cell changes the specificity of the DG neuron.

The possibilities for Wnts as bidirectional regulators of synapse formation are intriguing. In recent years numerous molecules have been identified as positive synapse formation signals, but very few have been implicated as negative regulators of synapse formation. It is likely that specificity is dependent on both positive and negative cues to form synapses on the correct target cell, however, there have only been a few such examples of negative cues. One family of molecules is Wnts, as previously described, but other likely candidates in the negative regulation of synapse formation are Tolls and Semaphorins. Toll is a cell surface molecule that is expressed in the drosophila embryo in muscles 15 and 16 around the time that synaptogenesis occurs, approximately 14 hours. Toll helps to create the correct synaptic zone for an ISNb neuron, RP3, which innervates muscles 6 and 7. When Toll is knocked out, RP3 axons stop just short of their correct target area, muscles 15 and 16. When Toll is misexpressed, RP3 reaches muscles 6 and 7, but never form stereotypical presynaptic terminals (Rose et al, 1997 and Rose and Chiba, 2000). However, Toll cannot be an anti-synaptogenic for all neurons within the drosophila embryo because some members of the ISNd class of neurons synapse in exactly the same place as where Toll is normally expressed, muscles 15 and 16 (Kraut et al, 2001).

Semaphorins are a large class of both secreted and transmembrane proteins that have become well known as negative axon guidance cues. However, a small number of studies have also identified semaphorins as negative synaptogenic signals in drosophila. The giant fiber system in drosophila consists of a small number of interneurons that extend axons out of the brain and synapse in the thorax where they

drive motor neurons. When Sema-1a is overexpressed in the interneurons of the giant fiber system, the axons stop short of their target in the thorax, suggesting that Sema-1a acts as a repulsive cue to the axon terminal (Godenschwege et al, 2002). An additional study shows that Sema II can be used in combination with other synaptogenic cues to predict where a synapse is to form. Like studies observing the role of Toll on synapse formation, Winberg and others (1998) used the targeting of the RP3 axon to muscles 6 and 7 to investigate the role of various synaptogenic cues. They observed that a Sema II mutation leads to abnormal synapses onto neighboring muscles and that overexpressing Sema II decreases appropriate innervation, providing additional evidence for the role of semaphorins as negative synaptogenic cues. It will be exciting in the future to see how Wnts, semaphorins, toll, and other antisynaptogenic molecules coordinate with prosynaptogenic signals to produce the amazing organization of circuits in the brain.

APPENDIX: Methods and Experimental Procedures

Hippocampal Cultures

Hippocampi were dissected from P0 Long Evans rats, dissociated, and plated onto 8-well LabTek chamber slides (Nunc; Rochester, NY) coated with poly-d-lysine (Millipore; Billerica, MA) and laminin (Sigma; St. Louis, MO) at a density of 80,000cells/well. Cultures were grown for 8 or 12 days at 37ºC with 5% carbon dioxide atmosphere. Cells were then treated with for 48 hours with lithium (4mM), sFRP2 (200ng/mL), recombinant Dkk-1 (1μg/mL), recombinant wnt3a (150ng/mL), recombinant Wnt5a (200ng/mL), recombinant Wnt7a (200ng/mL) or Wnt7b conditioned media. All proteins, apart from sFRP2, were obtained from R&D Systems (Minneapolis, MN). Cells treated with Wnt proteins were changed into a 5% FBS media upon addition of the protein.

Wnt7b conditioned media was prepared by plating HEK293T cells in 60mm dishes at a density of 2million cells/dish in 10% FBS media. HEK293T cells were then transfected 24 hours after plating with either Wnt7b or GFP using Fugene (Roche; Mannheim, Germany). The Fugene transfectant was washed off 24 hours later and cells were changed into a 2% FBS media. HEK293T cells were incubated for 72 hours. Wnt7b or control conditioned media was then supplemented with B27 and Glutamax (Invitrogen; Carlsbad, CA), and glucose (to 120mM) and then added directly to hippocampal cultures.

Preparation of sFRP2

SFRP2 protein was expressed by viral infection in an SF9 insect cell line and expanded until 500mL of cultures were obtained (Lyuksyutova et al, 2003). The cells were then spun down and the resulting media was incubated with charged nickel beads for two hours at 4ºC. A column bed was made by allowing the media to flow through an empty column. SFRP2 was then purified using the QIAexpressionist protocol. Elutions showing protein, determined by a protein gel and Coomassie blue staining, were concentrated using a 32kDa MW cutoff filter.

Calcium Phosphate Transfection and Constructs

The constitutively active β-catenin and full length axin constructs were generously donated by Dr. Tannishtha Reya. PCR amplification was used to purify the inserts with the following primer pairs: axin – forward: CGC CCG CGC CCA TGC AGA GTC CCA AAA TGA ATG TCC, reverse: CGC CCC GGG TCA GTC CAC CTT TTC CAC CTT GCC; constitutively active β-catenin – forward: CGC GGC CGC CCA TGG CTA CTC AAG CTC ACC TGA TG, reverse: CGC CCC GGG TTA CAG GTC AGT ATC AAA CCA GGC CAG. Inserts were then ligated into an IRES-GFP construct.

Constructs were transfected into hippocampal cultures at 7DIV by calcium phosphate transfection. Briefly, low glucose DMEM (Dulbecco's Modified Eagle's Medium; Invitrogen; Carlsbad, CA) was added to cells just before preparation of transfectant. DNA was mixed with 250mM calcium chloride and then added to an

equal volume of 2x HEBS. Twenty five microliters of transfectant solution was added to each well. Cells were incubated at 37ºC for approximately 20min and then washed with low glucose DMEM. DMEM was replaced with fresh media after the last wash. Cells were incubated for an additional seven days before fixation.

Immunohistochemistry

Hippocampal cultures were briefly washed with 1xPBS and then fixed in 4% paraformaldehyde/4% sucrose solution for 20min. Cells were permeated with a blocking solution of 3% Bovine serum albumin and 0.1% Triton-X for 30min in 1xPBS. Cells were immunolabeled with anti-"active" β-catenin (Millipore; Billerica, MA; 1:1000), anti-VGLUT1 (Millipore, 1:5000), anti-pan MAGUK (NeuroMAb; Davis, CA; 1:500), anti-GluR2/3 (Millipore; 1:300), and/or anti-MAP2 (Sigma; St. Louis, MO; 1:5000) for 2 hours at room temperature or overnight at 4ºC. Cultures were washed four times with blocking solution and then incubated with secondary antibodies (Jackson Immunoresearch, West Grove, PA, or Molecular Probes, Eugene, OR; 1:1000) for 1 hour at room temperature. Cells were coverslipped using Fluormount-G (Southern Biotech; Birmingham, AL).

Imaging and Data Analysis

Z-stacks of random views of VGLUT1, MAP2, and MAGUKs or GluR2/3 immunofluorescence were captured with a Leica confocal microscope with conditions blinded to the experimenter. At least 10 images were taken per experiment and all

experiments were repeated three or more times. Flattened Z stack images were first median filtered with radius of one pixel to reduce background noise and then thresholded to obtain discrete VGLUT1 puncta. The same threshold values were used throughout an entire set of images. The dendrite area was measured and the number of puncta that colocalized with the dendrite was quantified.

For β-catenin experiments, random images were taken of cell bodies stained with MAP2 and "active" β-catenin. At least six fields of view were taken of each experiment and experiments were repeated at least three times. A region of interest was identified within the nucleus and soma for each cell and the average intensity was measured for each region of interest.

All data analysis was performed using ImageJ. Student's t-tests were used to determine significance between control and the experimental group. For results with more than two conditions, a single factor ANOVA was first performed and following statistical significance, a Tukey test was performed. Images prepared for figures have been enhanced in Photoshop 6.0 using thresholding for VGLUT1 puncta and any postsynaptic markers and a median filter to decrease background noise.

Glial Cultures and Microisland Preparation

The cortex and hippocampus was dissected out of P1-P3 rats, the meninges removed and the brain chopped into smaller pieces. The tissue was incubated at 37degreesC for 30 minutes in 12mL 1xHBSS, 1.5mL 1% DNase I, and 1.5 mL 2.5% trypsin (Invitrogen; Carlsbad, CA). Cells were then triturated multiple times to break

apart the tissue. The cells were plated onto 250mm dishes at a density of one brain per dish in glia media consisting of 10% FBS (Invitrogen), 100 units Penicillin/Streptomycin (Invitrogen), and 20mM glucose. Glia was grown to confluency, approximately two weeks.

Before plating onto microislands, glia were frozen for up to four months. Each 250mm dish of glia cultures was rinsed once with 1xPBS and then removed from the culture dish with a 5 minute incubation with 0.25% trypsin (Invitrogen). Glia media was used to stop the reaction. The cells were then spun down for 5minutes at 1000rpm and the resultant supernatant suctioned off. The glia was resuspended in 1.5mL glia media per dish and stored in cryotubes with 50uL DMSO. Approximately one week before plating, glia was unfrozen and plated into a 250mm culture dish.

To make microislands, treated coverslips (Carolina Biological; Burlington, NC) were covered with 40uL 0.2% agarose type II and allowed to dry. Coverslips were then sprayed by a picospritzer with poly-d-lysine and then sanitized under ultraviolet light for 10-30 minutes. Glia was then plated onto coverslips and the glia was allowed to grow to confluency on the microislands for 3-5 days. P0 hippocampal cultures were then prepared, as previously described, and plated on to the microislands at a density of 40,000 cells per coverslip.

Electrophysiology

Microisland cultures were grown on 18mm coverslips for 10-14 days and were then transferred to a recording chamber and perfused with artificial cerebrospinal fluid

(ACSF – in mM: NaCl 124, KCl 5, NaHCO3 26, NaH2PO4 1.25, Glucose 10, $MgCl_2$ 1, CaCl2 2) saturated with 95% O2 and 5% CO2. Recordings were done at room temperature using Multiclamp700B (Axon Instruments). The presynaptic cell was held in current clamp at -55mV while the postsynaptic cell in voltage clamp at -65mV. The presynaptic cell was stimulated for 20ms at the minimum threshold to produce an action potential. Presynaptic cells were stimulated 10 times at a rate of 0.66Hz. Cells were recorded at a sampling rate of 100kHz. Neurons with a leak current below -500pA or a resting membrane potential above -30mV, and an access resistance above 25MΩ were excluded from analysis. Electrode resistance was 3-6MΩ. Electrode solutions were either Cs based (in mM: CsCl 10, CsMeSO3 105, NaCl 8, ATP 0.5, GTP 0.3, HEPES 10, TEA 5, $MgCl_2$ 2, EGTA 1, QX314 2) or K gluconate based (in mM: K gluconate 125, NaCl 8, D-glucose 5, HEPES 5, ATP 0.5, GTP 0.3, $MgCl_2$ 2, EGTA 1).

After recording, coverslips were transferred from the recording chamber to a 12-well plate and washed with 1xPBS. Neurons were immediately fixed for 25minutes in 4%PFA/4%sucrose. Cells were then stained for chick anti-MAP2 (Sigma; St. Louis, MO; 1:5000), rabbit anti-Prox1 (Covance; Princeton, NJ; 1:1000), IgM anti-PY (1:20), and rat anti-CTIP2 (Abcam; Cambridge, MA; 1:1000). Cell types were identified by taking pictures using a Leica confocal microscope, as previously described. Cell types were identified according to the immunofluorescence.

In situ hybridization

P7 and P14 mice were perfused with 4%PFA in 0.1M phosphate buffer (PB). The brains were removed from the skull and post-fixed in 4% PFA in 0.1M PB for 24 hours at 4ºC. The tissue was then washed with 250mM EDTA in 0.1M PB for 24 hours and then incubated in 25% sucrose for up to 72 hours at 4ºC. Brains were embedded in TissueTek (Sakura; Torrance, CA), frozen, and then stored at -80ºC. Thirty micron horizontal sections were then cut using a cryostat.

In situ hybridization was performed on sections with a probe concentration of 600ng/mL in hybridization solution at 58°C for 36 hours. Hybridization was followed by sequential washes with 5x (5 min), 2x (1min), 50% formamide in 0.2x (30 min), and 0.2x (5min) SSC at 58ºC. Sections were then blocked for 1 hour in 1% Blocking reagent (Roche; Mannheim, Germany) followed by a three hour incubation at room temperature in anti-DIG-AP (Roche; Mannheim, Germany; 1:3000). Signals were detected using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) for at least 2 hours and no more than 12 hours at room temperature. The hybridization signal was compared with sections using a sense probe under identical conditions. All probes were designed and made previously by the Yimin Zou lab (Liu et al, 2005).

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