Determining the role of Wnt signaling during BDNF-induced cortical neuron growth and dendritic spine formation

By

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ABSTRACT

Brian Gibson Hiester (Ph.D., Molecular, Cellular, and Developmental Biology)
Determining the role of Wnt signaling during BDNF-induced cortical neuron growth and dendritic spine formation.
Thesis directed by Associate Professor Kevin R. Jones

Dendritic spines are major sites of excitatory synaptic transmission and changes in their densities and morphologies have been linked to neurodevelopmental disorders and neurodegenerative diseases. The Jones lab has previously shown using a forebrain-specific BDNF knockout mouse (fsBDNF-KO) that loss of BDNF leads to a significant reduction in dendritic spine density and a loss of dendrites in cortical neurons. However, the mechanisms by which BDNF regulates dendrites and dendritic spine formation remain unclear. I propose that one mechanism by which BDNF regulates these processes is by controlling the expression of other secreted signaling proteins, thereby establishing bidirectional communication between neurons. Global transcriptional analysis of fsBDNF-KO mice revealed that several Wnt genes are dysregulated along with other components of Wnt signaling pathways. The Wnts are a large family of secreted signaling proteins known to be regulated by neural activity and to regulate synapse function. However, a role for the Wnts in BDNF-mediated synapse formation in the CNS has not yet been described. Here, I demonstrate that blocking Wnt signaling in cortical neurons *in vitro* using four different Wnt signaling inhibitors impairs cortical dendrite growth and dendritic spine formation. Additionally, I show that Wnt signaling is required for both BDNF-induced dendritic spine formation and BDNF-induced changes in dendritic spine morphology associated with dendritic spine maturation. Further, I show

that BDNF increases the expression of an individual Wnt gene, Wnt2, and may decrease the expression of another Wnt gene, Wnt4. Lastly, I demonstrate that Wnt2 is sufficient to increase dendrite growth, increase dendritic spine density and promote dendritic spine maturation, while Wnt4 is sufficient to increase dendritic spine density. Together, these data suggest that BDNF and Wnt signaling may cooperatively regulate dendritic spine formation and that BDNF may regulate dendritic spine formation in part by regulating expression of different Wnt genes.

DEDICATION

This thesis is dedicated to the memory of Dr. Art Champlin, who was my first scientific mentor. I thank him dearly for giving me my first opportunity to perform laboratory research and for laying the seeds of my career path. Most importantly, in times of struggle and frustration, I can always hear him reminding me that, “these things happen, Brian. These things happen.” Thanks, Art.

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CHAPTER 1: Introduction

The mammalian brain is an astoundingly complex organ. Although the brain contains a remarkable diversity of different cell types, the fundamental unit that mediates long-distance connectivity and information processing in the brain is the neuron. Neurons are morphologically unique cells that possess an ability to form connections with one another at specialized structures called synapses. Each neuron in the brain is capable of forming thousands of synaptic connections. The human brain contains trillions of neurons, thus it contains approximately 10^{14} synapses. Despite the enormous connective potential of the brain, these synapses are organized into precise anatomical circuits. This remarkably organized connectivity and its inherent plasticity is ultimately what underlies brain function. Importantly, the ability of neurons to modulate the formation and strength of synaptic connections within circuits in response to experience is what endows the brain with the functional flexibility needed to regulate biological phenomena as complex as human behavior.

1.1 Neurons communicate through electrochemical synapses

Neurons are unique cell types that process and transmit electrical and chemical signals, and communicate with other neurons through asymmetric structures known as electrochemical synapses. A typical synapse consists of two major components, an axonal presynaptic specialization and a dendritic postsynaptic specialization (Fig 1.1).

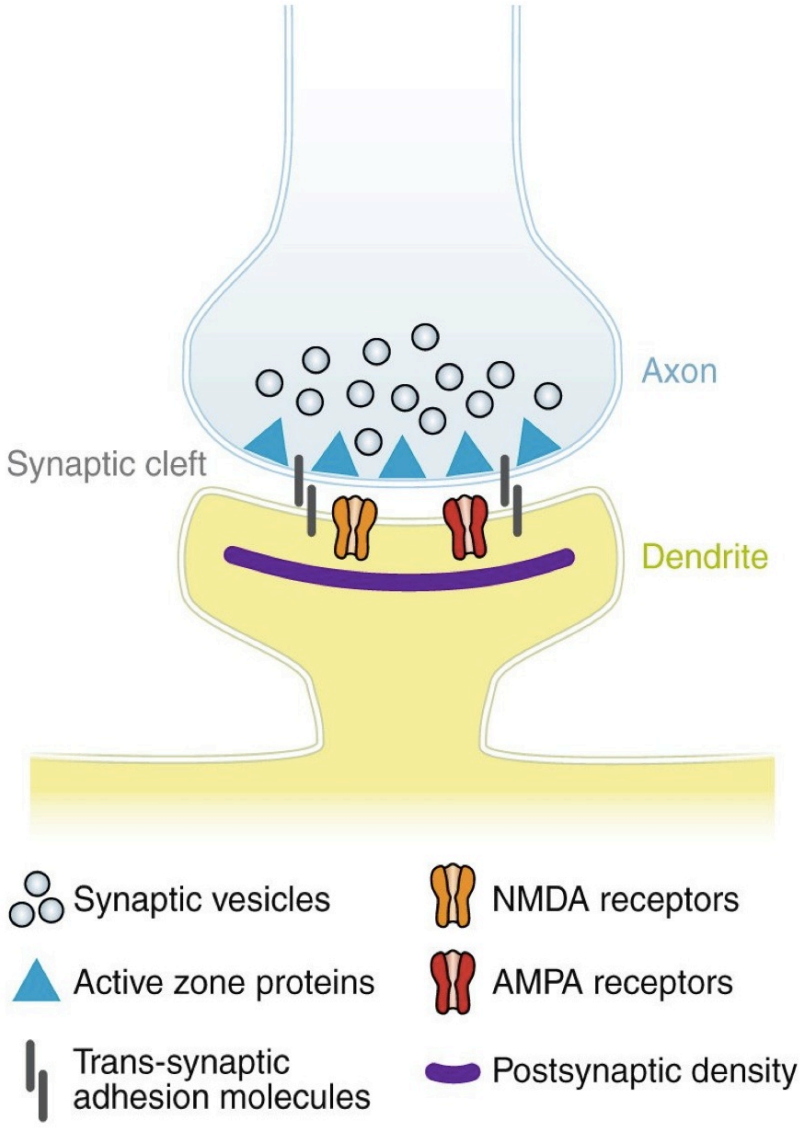

Figure 1.1. Diagram illustrating some of the basic components of a typical CNS excitatory synapse. Figure taken from McAllister (2007).

The purpose of the synapse is to convert an electrical signal, in the form of action potentials transmitting down axons into the presynaptic bouton, into a chemical signal that crosses the synaptic cleft, in the form of neurotransmitters released from the presynaptic cell (Burns and Augustine, 1995). The neurotransmitters transduce a signal to the postsynaptic dendrite by binding to neurotransmitter receptors located on the surface of the postsynaptic dendrite (Kennedy, 2000). The neurotransmitter receptors act to convert the chemical signal from the neurotransmitters back into an electrical signal within the postsynaptic cell. This series of events is known as synaptic transmission and forms the basis for the majority of information transfer within the central nervous system (CNS).

There are two major classes of synapses within the CNS, excitatory and inhibitory. Excitatory synapses transmit information using the amino acid neurotransmitter glutamate, which signals through glutamate receptors to increase the activity of the postsynaptic neuron (McAllister, 2007). In contrast, inhibitory synapses use the neurotransmitter gamma-Aminobutyric acid (GABA), which binds GABA receptors and decreases activity in the postsynaptic neuron (Markram et al., 2004). The balance of excitatory and inhibitory input into a given neuron regulates its excitability and, ultimately, its functional role within a given neural circuit (Levinson and El-Husseini, 2005). As the purpose of this thesis is to examine the formation of a specialized type of excitatory synapse, I will focus further discussion on this topic to the composition and regulation of excitatory synapses in particular.

As previously mentioned, the excitatory synapse is an asymmetric structure, consisting of a presynaptic terminal and a postsynaptic density (PSD) (Fig. 1.1). The presynaptic active zone is characterized by the presence of an electron-dense meshwork of proteins and synaptic vesicles that are embedded within this matrix, some of which are docked to the synaptic membrane (Burns and Augustine, 1995; Hirokawa et al., 1989; Landis, 1988; Phillips et al., 2001; Waites et al., 2005). Directly apposed to the presynaptic active zone is the postsynaptic density (PSD), which is another electron-dense meshwork of proteins. The PSD serves to cluster neurotransmitter receptors, voltage-gated ion channels, and various kinases and second-messenger signaling molecules that act to transduce the synaptic signal into the postsynaptic cell (Sheng, 2001; Waites et al., 2005). Embedded within both the active zone and the PSD are various classes of trans-synaptic adhesion molecules that physically connect the pre- and postsynaptic compartments and also to specify proper neuronal connections during synapse formation (Scheiffele, 2003; Waites et al., 2005). Synapse formation can generally be described as the coordinated regulation of the assembly of pre- and postsynaptic components (Craig et al., 2006; Garner et al., 2006; Lin and Koleske, 2010; McAllister, 2007; Waites et al., 2005).

1.2 Dendritic spines are specialized excitatory synapses

The majority (>90%) of excitatory synapses in the CNS form on dendritic spines, which are specialized actin-rich postsynaptic structures that emanate from the dendritic shaft (Harris and Kater, 1994; Nimchinsky et al., 2002).

Dendritic spines are, in essence, a unique compartmentalized synapse in which the postsynaptic compartment is isolated from the dendritic shaft. The physical separation of the synapse from the dendritic shaft allows dendritic spine synapses to function with varying degrees of independence from the dendritic shaft.

Dendritic spines were first discovered over a century ago by the renowned neuroscientist Ramón y Cajal while studying the dendrites of Purkinje cells in hens stained using the Golgi method (Cajal, 1891). Cajal expanded his study of dendritic spines to several different types of cells and species and was the first to propose a functional role for dendritic spines in connecting neurons and regulating the flow of information between them. An example of some of his drawings is shown in Figure 1.2. Dendritic spines occur on the majority of principal neuron classes in the brain, including the pyramidal neurons of the cortex, the medium spiny neurons of the striatum, and the Purkinje cells of the cerebellum. Despite their seeming ubiquity, dendritic spine distribution can vary widely depending on the cell type throughout the brain, and the mechanisms underlying spine formation and maintenance in different brain regions can vary quite considerably (Garcia-Lopez et al., 2010; Yuste and Bonhoeffer, 2004).

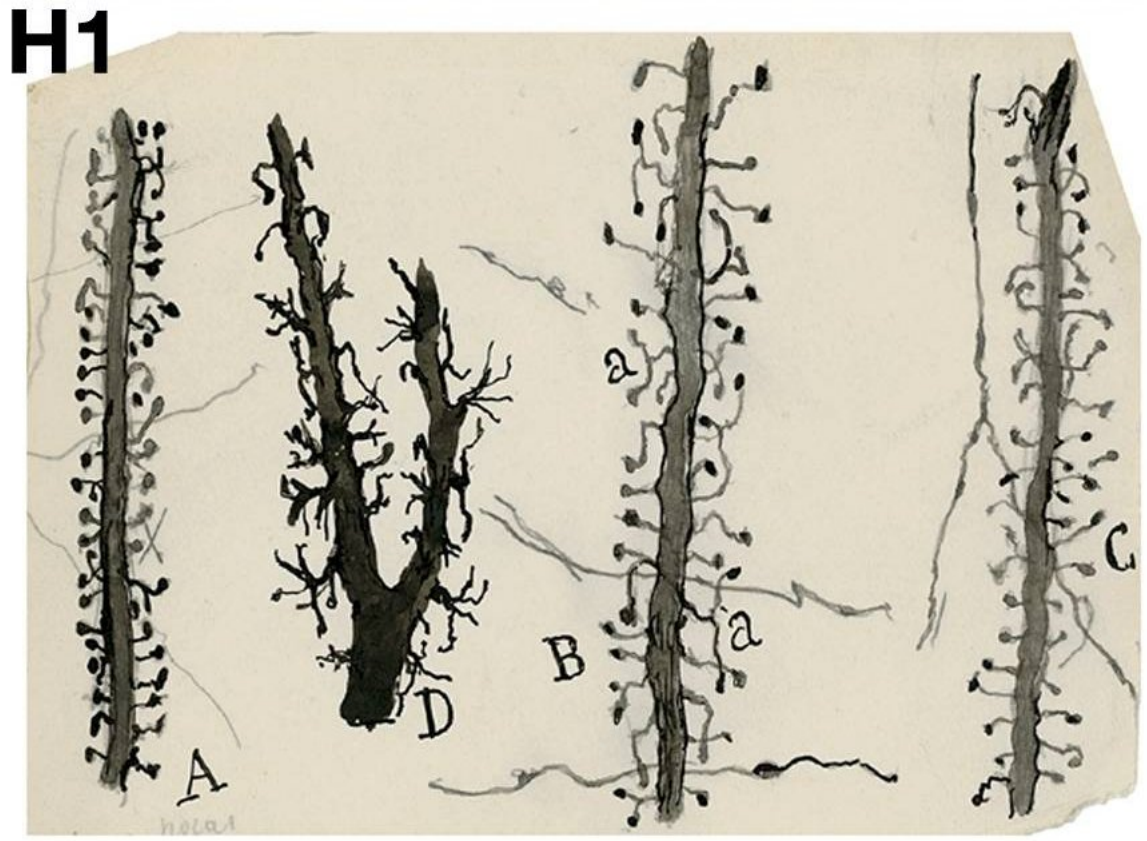

Figure 1.2. Examples of drawings made by Cajal from his pioneering studies in the discovery of dendritic spines. Image taken without permission from Garcia-Lopez et al. (2010).

1.3 Dendritic spine formation correlates with spine morphology

Due to their distinct morphology, the formation and function of dendritic spines have most frequently been examined in a morphological manner. Typically, dendritic spines consist of a bulbous spine head that is connected to the dendritic shaft through a thin spine neck that varies in length (Nimchinsky et al., 2002). However, spine shape is very heterogeneous. Extensive studies using both light and electron microscopy revealed that there is a huge spectrum of different spine types. The first rigorous classification of spine shape categorized spines into three main categories: mushroom, stubby or thin (Peters

and Kaiserman-Abramof, 1970). These spine types are determined by the ratio of the width of the spine head to the length of the spine neck. Subsequent studies identified a fourth spine shape, termed filopodial spines, which lack a discernible spine head (Fiala et al., 1998; Papa and Segal, 1996; Ziv and Smith, 1996). Figure 1.3 shows a diagram of these four main spine morphologies.

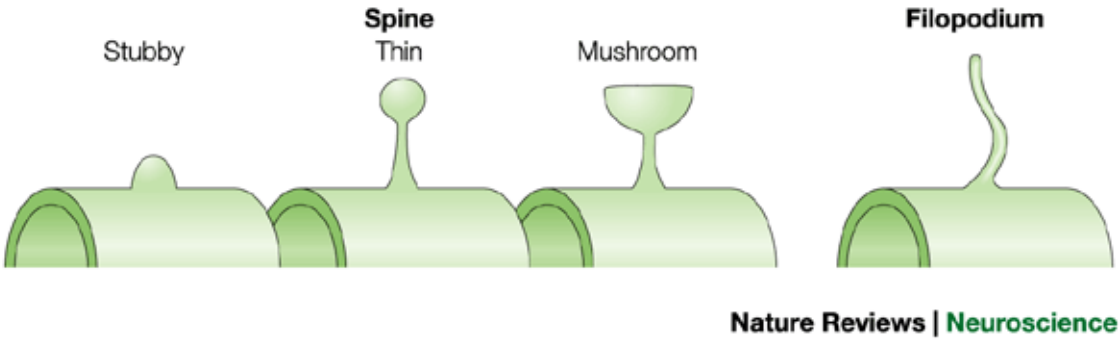

Figure 1.3. General classification of dendritic spine shapes in the CNS. Image taken without permission from Yuste and Bonhoeffer (2004).

The more recent use of live cell fluorescence microscopy has helped to illustrate the dynamic nature of dendritic spines and has revealed substantial information regarding the stability of dendritic spine contacts throughout development. Several *in vivo* studies examining the dynamics of dendritic spine formation in the mouse cortex indicate that while rates of spine formation are higher in younger animals, a greater percentage of those spines are less stable. Additionally, as animals age, spine formation rates decrease, but the portion of dendritic spines that remain stable increases (Grutzendler et al., 2002; Holtmaat et al., 2005; Majewska et al., 2006; Trachtenberg et al., 2002; Xu et al., 2007; Zuo et al., 2005). Although there are differences in spine formation rates and

stabilities depending on the specific type of neuron imaged and the imaging technique used, dendritic spine structural plasticity declines with age.

Until very recently, the nature of the relationship between dendritic spine formation and synaptogenesis remained unclear. Although stable dendritic spines contain all of the components of an excitatory synapse and the vast majority of dendritic spines receive excitatory input (Arellano et al., 2007; Harris, 1999) it was unknown how soon nascent dendritic spines formed functional synaptic connections. Experiments utilizing live cell fluorescence microscopy in combination with techniques used to assay levels of neural activity have further clarified the relationship between dendritic spine formation and synaptogenesis. Notably, Lohmann and Bonhoeffer (2008) demonstrated that nascent filopodial spines from hippocampal neurons generate glutamate signaling-independent local dendritic calcium currents within seconds of establishing contact with a presynaptic axon, and the frequency of these calcium transients correlates with the stability of the contact (Lohmann and Bonhoeffer, 2008). Additionally, Zito et al. (2009) showed that newly formed (<2.5 hours) hippocampal spines form functional glutamatergic synapses and that the amplitude of the glutamate current correlates with the volume of the dendritic spine (Zito et al., 2009). The combined results of these recent studies together with the earlier anatomical studies of dendritic spines helped to formulate and support the filopodial model of dendritic spine formation. The filopodia model postulates that dendritic spines are first formed as motile filopodia that extend from the dendritic shaft in search of a potential presynaptic partner. Once contact is made, the nascent filopodium

begins to form a spine head containing synaptic components, and, as this synaptic contact matures, the spine head continues to grow and the spine neck grows smaller as the spine head retracts toward the dendritic shaft (Fiala et al., 1998; Holtmaat et al., 2005; Ziv and Smith, 1996; Zuo et al., 2005). It should be noted, however, that the percentage of filopodial spines that are stabilized by presynaptic contact is very low, and that most filopodia retract without having made a stable contact (Holtmaat et al., 2006; Lohmann and Bonhoeffer, 2008; Zuo et al., 2005). Presynaptic neural input plays a key role during the stabilization of nascent filopodial contacts, which will be discussed further below.

While the filopodia model may be the most widely accepted model of dendritic spine formation, there are exceptions. Notably, some cerebellar Purkinje cell dendritic spines are able to form in the absence of presynaptic axonal input. Several lines of inquiry support this statement. First, analysis of Weaver mutant mice in which granule cells, the presynaptic partners of most Purkinje cells, are absent, demonstrated that Purkinje cell dendrites form dendritic spines even in the absence of presynaptic axon terminals (Hirano and Dembitzer, 1973; Landis and Reese, 1977; Rakic and Sidman, 1973; Sotelo, 1975). In addition, analysis of Reeler mutant mice in which the migration of neuronal precursors in the cerebellum is grossly perturbed demonstrated that ectopic Purkinje cells that develop in the absence of granule cells are able to form morphologically normal dendritic spines (Mariani et al., 1977). Further, selective ablation of cerebellar granule cells by X-irradiation of neonatal rats does not prevent Purkinje cells from developing dendritic spines (Sotelo, 1977).

Together, these studies formed the basis of the Sotelo model of spine formation, which postulates that spine formation is an intrinsic and cell-autonomous property of the neuron and can occur independently of presynaptic contact. In stark contrast to Purkinje cells, striatal medium spiny neurons (MSNs) grown *in vitro* do not form dendritic spines unless they are co-cultured with excitatory cortical neurons, indicating that spine formation in these cells absolutely requires excitatory presynaptic input (Segal et al., 2003). The difference between these two cell types supports the idea that there is considerable variability in the way that dendritic spines are formed throughout the brain.

1.4 Dendritic spine formation and maintenance is altered in neuropathological states

An emerging theme from work examining the causes of both neurodegenerative and neurodevelopmental disorders is that the underlying pathophysiological mechanisms of these diseases is rooted in improper regulation of synapse formation or maintenance. In the case of neurodevelopmental disorders, the pathology arises from improper establishment of neural connectivity. Interestingly, dendritic spine formation is impaired in a number of neuropathologies, suggesting that the regulation of dendritic spine formation or maintenance may contribute to the aberrant neural connectivity in these diseases.

Early studies examining post-mortem brain tissue of humans that suffered from various forms of mental retardation revealed reduced dendritic spine density and abnormal morphology of dendritic spines in the cortex (Huttenlocher, 1970,

1974; Purpura, 1974, 1975). In contrast, a recent study examining brain tissue from humans with autism spectrum disorder (ASD) indicated that dendritic spine density increased in cortical pyramidal neurons (Hutsler and Zhang, 2010). Interestingly, autism is thought to arise from a pathological increase in local connectivity within the cortex, which would be consistent with increases in dendritic spine density. Two diseases that are often comorbid with autism, Angelman's syndrome and Fragile-X syndrome, also display dendritic spine abnormalities. Angleman syndrome is a genetic disorder caused by maternal deletion or mutation of *UBE3A*, a gene that encodes for an E3 ubiquitin ligase (Mabb et al., 2011). Hippocampal and cortical pyramidal neuronal dendritic spine densities are decreased in mouse models for Angleman syndrome (Dindot et al., 2008; Yashiro et al., 2009). Fragile-X syndrome is caused by reduced expression the RNA-binding protein Fragile-X mental retardation protein (FRMP) due to transcriptional silencing of the *Fmr1* gene (He and Portera-Cailliau, 2012). Analysis of both human postmortem tissue and brains of mice lacking FMRP1 indicates that dendritic spine densities are increased in Fragile-X, but that there is an overabundance of long, immature dendritic spines (Antar et al., 2006; Irwin et al., 2001; Nimchinsky et al., 2001).

Premature loss of dendritic spines may be a contributing factor during neurodegenerative diseases. Schizophrenia is a heterogeneous cognitive disorder that emerges in late adolescence and young adulthood, and is characterized by pathological loss of gray matter in the brain. Studies examining postmortem tissue from Schizophrenic individuals revealed that reduced dendritic

arbors and loss of spines is a major component of the reduction in gray matter. Notably, these studies revealed that spine density is specifically reduced in the cortex and hippocampus (Glantz and Lewis, 2000; Kolluri et al., 2005; Kolomeets et al., 2005; Sweet et al., 2009).

Loss of grey matter and reductions in overall brain mass are also associated with Alzheimer's disease (AD). Research dedicated toward understanding the mechanistic causes of AD suggests that impairments in synaptic connectivity precede the massive degeneration of tissue throughout the brain. Analysis of human postmortem tissue from AD patients indicates that dendritic spine density is reduced in both the hippocampus and cortex (Baloyannis et al., 1992; Einstein et al., 1994; Ferrer et al., 1990; Probst et al., 1983). Further, analysis of several different mouse models of AD that display age related increases in amyloid plaques similar to human AD indicates that dendritic spine density is reduced in the hippocampus and cortex (Alpar et al., 2006; Grutzendler et al., 2002; Spires et al., 2005). An additional study that analyzed spine formation in a temporal manner revealed that spines form normally in an AD mouse model, but that there is an increased amount of ectopic spine elimination, suggesting that dendritic spine contacts are destabilized during AD (Spires-Jones et al., 2007). Interestingly, dysregulation of Wnt signaling is suggested to mediate synapse stability during the progression of AD (Purro et al., 2012), which I will discuss further in subsequent chapters.

1.5 Increased neural activity promotes dendritic spine stability and maturation

The previously mentioned study demonstrating that dendritic spines on striatal MSNs do not form in the absence of excitatory input (Segal et al., 2003) highlights the important role of neural activity during spine formation. Several studies demonstrated that synaptic activation and induction of long-term potentiation (LTP), a long-lasting form of synaptic plasticity that increases synaptic strength, triggers the growth of dendritic filopodia and the formation of new spines (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999; Yuste and Bonhoeffer, 2001). More recent studies have begun to elucidate the mechanisms underlying neural activity-mediated induction of dendritic spine formation. Notably, the intracellular kinase CaMKII is required for LTP-induced dendritic spine formation (Jourdain et al., 2003). Additionally, the actin binding protein neurabin-1 promotes dendritic filopodial formation through its ability to bundle f-actin, a major component of dendritic spines (Terry-Lorenzo et al., 2005).

There is accumulating evidence that neural activity is also critical for the stabilization and subsequent maturation of dendritic spine synapses in a process that is independent of the initiation of filopodial growth. Several studies utilizing EM to identify morphologically mature synapses within newly formed dendritic spines indicate that spine growth precedes synapse formation (Arellano et al., 2007; De Roo et al., 2008; Knott et al., 2006). Additionally, as previously discussed, detailed time-course analysis of dendritic spine formation combined with electrophysiological recording of individually stimulated spines revealed that

nascent spines acquire the ability to transmit glutamate-dependent signals soon after being formed (Zito et al., 2009). Together, these studies indicate that a dendritic spine is not able to respond to excitatory neural input until just after it is formed, and suggest that neural activity may promote the subsequent stabilization and maturation of a dendritic spine. Indeed, the enlargement of the stabilized and maturing spine head shows similarities with the spine growth associated with the induction of LTP at pre-existing dendritic spines (De Roo et al., 2008; Ehrlich et al., 2007; Harvey and Svoboda, 2007; Kopec et al., 2007; Matsuzaki et al., 2004).

Precisely how neural activity promotes stabilization of dendritic spine contacts and subsequent growth of the dendritic spine head is unclear, but recent studies indicate that activity-mediated regulation of actin dynamics may play a key role. In cultured hippocampal neurons, glutamate signaling through N-methyl-D-aspartate receptors (NMDARs), a specific subtype of ionotropic glutamate receptor, targets the actin binding protein profilin to dendritic spines where it promotes spine growth (Ackermann and Matus, 2003). Additionally, induction of LTP in hippocampal neurons leads to increased actin polymerization within dendritic spines and an increase in the size of spine heads (Okamoto et al., 2004). This increase in spine head growth is followed by a further increase in glutamate receptor currents, suggesting that larger spines transmit stronger synaptic signals (Matsuzaki et al., 2004). Increases in glutamate receptor currents are likely due to addition of glutamate receptors to the postsynaptic dendritic spine surface (Malinow and Malenka, 2002). Further, several studies

demonstrated that inhibition of actin polymerization impairs the induction of LTP (Fukazawa et al., 2003; Kim and Lisman, 1999; Krucker et al., 2000; Ramachandran and Frey, 2009). Together, these studies highlight the crucial role of neural activity in promoting the maturation of dendritic spines.

Despite the large body of evidence indicating that neural activity promotes dendritic spine formation, some studies examining dendritic spine formation in hippocampal and cortical pyramidal neurons indicate that neural activity is not strictly required for synapse and dendritic spine formation. Deletion of *munc-13* or *munc-18*, two genes that are required for synaptic vesicle exocytosis and neurotransmitter release, completely abolishes electrical activity in the developing brain, yet seemingly morphologically complete synapses are able to form in these mice (Varoqueaux et al., 2002; Verhage et al., 2000). Although *munc-13* knockout mice die at birth, hippocampal neurons from these mice grown for 21 days in vitro (DIV) appear to be able to form dendritic spines (Varoqueaux et al., 2002), suggesting that neural activity is not required for the initial formation of dendritic spines. However, the authors in this study did not perform a detailed morphological analysis of the dendritic spines on these neurons, so it is possible that theses spines may have initially formed but did not fully mature.

1.6 Decreased neural activity leads to dendritic spine shrinkage and loss

Dendritic spines are additionally capable of undergoing specific morphological changes associated with decreased synaptic strength. Long-term

depression (LTD) is a form of long-lasting synaptic plasticity characterized by a reduction in synaptic strength (Collingridge et al., 2010). LTD is important for a variety of physiological functions including hippocampal dependent learning and memory (Brigman et al., 2010; Nicholls et al., 2008; Zeng et al., 2001) and development of the visual cortex (Bear et al., 1987; Crozier et al., 2007; Heynen et al., 2003).

Dendritic spine structure is influenced by the induction of LTD. LTD induction of hippocampal slices using low frequency stimulation results in a reduction of dendritic spine volume in a Ca^{2+}-dependent manner (Zhou et al., 2004). Additionally, hippocampal LTD increases the rate of dendritic spine retraction (Nagerl et al., 2004). Further, hippocampal spine retraction induced by LTD is associated with a reduction in pre- and postsynaptic contacts, indicating that regulation of dendritic spines by LTD can affect overall neural connectivity (Bastrikova et al., 2008). Combined, these studies further support the importance of neural activity in regulating dendritic spine formation. Additionally, the studies examining the effects of LTP and LTD suggest that the neural activity-induced alterations in dendritic spine structure may represent a structural mechanism by which changes in neural input affect the activity of the postsynaptic neuron.

1.7 BDNF is a widely expressed member of the neurotrophin family

The neurotrophins, consisting of nerve growth factor (NGF), Brain-derived neurotrophic factor (BDNF), neurotrophic factor-3 (NT-3) and neurotrophic factor-

4/5 (NT-4/5), are a family of secreted signaling proteins that convey critical signals during neural development and during the maintenance of the adult nervous system (Bibel and Barde, 2000; Huang and Reichardt, 2001). Individual neurotrophins signal by acting on specific members of the Trk family of transmembrane tyrosine kinase receptors: NGF activates TrkA, BDNF and NT-4/5 activate TrkB, NT-3 activates TrkC and also TrkA and TrkB in some cellular contexts (Patapoutian et al., 1999). Additionally, the neurotrophins can also signal via $p75^{NTR}$, a member of the tumor necrosis factor receptor (TNFR) family (Kaplan and Miller, 2000; Reichardt, 2006).

BDNF is the most abundant neurotrophin in the adult neocortex and hippocampus (Maisonpierre et al., 1990) and was first identified as a factor that allowed survival of neurons that were unresponsive to NGF (Barde et al., 1982). Subsequent work implicates BDNF in a plethora of brain functions, including, but not limited to, cell migration, neurite growth, synaptic transmission and potentiation, synapse formation and dendritic spine formation. BDNF is expressed throughout the brain during development and into adulthood. BDNF mRNA is found in the cortex, hippocampus, thalamus, cerebellum, ventral midbrain and other areas (Conner et al., 1997; Nawa et al., 1995). Of these, the cortex and hippocampus express the highest levels of BDNF within the CNS. To a large extent, expression of the *bdnf* gene is responsive to neural activity-mediated regulation of Ca^{2+}-dependent signaling cascades (Shieh and Ghosh, 1999; Tabuchi et al., 2002; Tao et al., 2002). Indeed, reduction of neural activity in the visual cortex by continuous visual deprivation causes reduced BDNF

mRNA levels (Capsoni et al., 1999). Shorter periods of visual deprivation cause an increase in the amount BDNF mRNA, but a decrease in the total amount and dendritic localization of BDNF protein (Tropea et al., 2001). Further, short exposure to light following visual deprivation is sufficient to restore BDNF protein levels (Tropea et al., 2001).

1.8 BDNF production and secretion

BDNF, like all neurotrophins, is synthesized as a pre-proBDNF precursor that undergoes post-translational modifications within the ER and Golgi prior to secretion. ProBDNF is cleaved and converted into mature BDNF by endoprotease-mediated cleavage in the trans-Golgi or by proprotein convertases within immature secretory granules (Mowla et al., 1999). More recent studies demonstrated that uncleaved proBDNF is secreted from neurons and is converted to mature BDNF by extracellular proteases including plasmin and membrane matrix metalloproteinases (Lessmann and Brigadski, 2009). Indeed, some studies suggest that the primary form of BDNF secreted from cultured neurons is actually proBDNF (Chen et al., 2003; Mowla et al., 1999). Importantly, extracellular cleavage of proBDNF by plasmin plays a key role during hippocampal LTP (Pang et al., 2004).

BDNF protein is secreted through a variety of different pathways in both a constitutive and activity-dependent manner (Mowla et al., 1999). The majority of BDNF release is activity-dependent. Interestingly, the valine66-methionine single nucleotide polymorphism located within the pro domain of proBDNF increases

the proportion of BDNF release that is constitutive (Egan et al., 2003). In addition, BDNF is released from multiple neuronal compartments. BDNF produced in the cell body is delivered to presynaptic axon terminals in secretory vesicles by anterograde transport (Adachi et al., 2005; Fawcett et al., 1998; Kohara et al., 2001). A recent study suggests that, within the hippocampus, most, if not all, BDNF is secreted in an anterograde manner from axons (Dieni et al., 2012). However, overexpression of epitope-tagged BDNF suggests that BDNF is also transported in a retrograde manner into dendrites for postsynaptic release (Adachi et al., 2005; Brigadski et al., 2005; Goodman et al., 1996; Hartmann et al., 2001; Haubensak et al., 1998; Kohara et al., 2001). Further support for retrograde BDNF release comes from studies demonstrating that BDNF mRNA is also trafficked to portions of the dendritic arbor, thus allowing for localized translation of BDNF protein near sites of synaptic activation within dendrites (An et al., 2008; Bramham and Wells, 2007; Tongiorgi et al., 1997). Recent evidence indicates that the dendritic transport of BDNF mRNA may be dependent on a dendritic targeting signal located in the long 3'UTR of BDNF produced by alternative splicing (An et al., 2008).

1.9 BDNF signals through the receptor TrkB

BDNF is a secreted signaling protein and signals through the receptor TrkB, a receptor tyrosine kinase. Although BDNF, and specifically its unprocessed precursor proBDNF, also signals through the $p75^{NTR}$ receptor, I will focus on signaling mediated by TrkB. BDNF binds the TrkB receptor as a dimer

and induces subsequent dimerization of TrkB and autophosphorylation of conserved tyrosine residues in the TrkB intracellular domain (McCarty and Feinstein, 1998). Following activation of TrkB, several different signaling cascades can be activated, depending on the cellular context. BDNF signaling through TrkB activates three main signaling cascades: Ras-stimulated activation of mitogen-activated protein (MAP) kinase cascades, phosphatidyl inositol-3 (PI3) kinase-mediated activation of Akt, and phospholipase C (PLC)-γ-1-dependent regulation of calcium signaling cascades through generation of inositol triphosphate (IP3) and diacylglycerol (DAG) (Cunha et al., 2010; Huang and Reichardt, 2001). A general schematic of BDNF signaling (through both TrkB and $p75^{NTR}$ is shown in Figure 1.4.

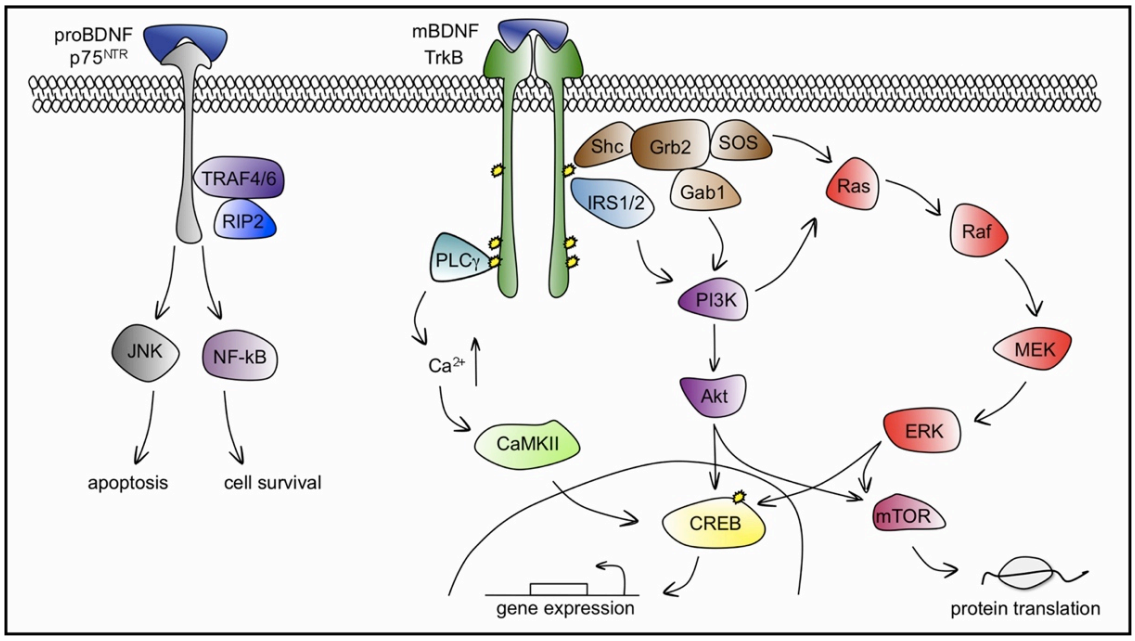

Figure 1.4. Signaling cascades initiated by BDNF signaling through either TrkB or $p75^{NTR}$. Image taken without permission from Cunha et al. (2010).

Similar to BDNF, TrkB is widely expressed throughout the brain. TrkB mRNA and protein can be found in the cortex, hippocampus, striatum, cerebellum and brain stem (Carvalho et al., 2008). TrkB receptors are localized to both pre- and postsynaptic compartments and are present in approximately one-third of glutamatergic synapses (Pereira et al., 2006). Interestingly, most surface localization of TrkB protein in excitatory neurons is restricted to dendritic spines (Drake et al., 1999; Pereira et al., 2006), suggesting that the majority of BDNF/TrkB signaling occurs at sites of synaptic contact.

1.10 BDNF promotes synaptic potentiation

An increasing portion of research examining the function of BDNF in the CNS is focused on the ability of BDNF to potentiate excitatory synapse function. BDNF modulates the formation and function of inhibitory synapses as well (Baldelli et al., 2005; Frerking et al., 1998; Hong et al., 2008; Matsumoto et al., 2006; Ohba et al., 2005). However, I will focus on the role of BDNF at excitatory synapses in this thesis. It should be noted that cortical neurons form inhibitory synapses, and this process may be regulated by BDNF. Early studies examining a role for BDNF at excitatory synapses demonstrated that BDNF treatment of hippocampal neurons was sufficient to increase excitatory transmission in a TrkB-dependent manner (Kang and Schuman, 1995; Messaoudi et al., 1998). Subsequent studies determined that BDNF regulates excitatory transmission in multiple fashions. BDNF stimulates Ca^{2+}-dependent release of glutamate from hippocampal and cortical nerve terminals (Canas et al., 2004; Jovanovic et al.,

2000; Pereira et al., 2006; Sala et al., 1998). Additionally, BDNF increases the expression and surface localization of both types of ionotropic glutamate receptors, AMPA receptors (Caldeira et al., 2007a; Nakata and Nakamura, 2007) and NMDA receptors (Caldeira et al., 2007b). BDNF also increases the expression of glutamate receptor-interacting proteins, which ultimately may lead to increased surface localization of these receptors (Jourdi et al., 2003).

1.11 BDNF regulates the induction of LTP

As mentioned previously, LTP is a unique form of synaptic potentiation that is long lasting and is thought to be the cellular mechanism underlying learning and memory. The role of BDNF during induction of LTP is well documented. Hippocampal LTP is impaired in reduced BDNF or TrkB-knockout mice (Chen et al., 1999; Korte et al., 1995; Minichiello et al., 1999; Patterson et al., 1996; Xu et al., 2000a). Importantly, the impairment of LTP in BDNF-knockout mice is rescued by acute application of BDNF (Figurov et al., 1996; Patterson et al., 1996; Pozzo-Miller et al., 1999). BDNF mediates LTP induction both pre and postsynaptically. Presynaptically, BDNF enhances early stages of LTP by enabling sustained glutamate release during bursts of increased neural activity (Gottschalk et al., 1998; Jovanovic et al., 2000; Pozzo-Miller et al., 1999; Tyler and Pozzo-Miller, 2001; Xu et al., 2000a). BDNF also contributes to LTP induction in a postsynaptic manner, but the mechanisms are unclear. The previously mentioned roles for BDNF in regulating the trafficking of glutamate

receptors to the synaptic surface may mediate the postsynaptic effect of BDNF during LTP, but this has yet to be directly demonstrated.

Interestingly, BDNF may promote LTP, and synaptic potentiation generally, by regulating local translation of proteins in postsynaptic dendritic compartments. BDNF-induced potentiation in the hippocampus requires cell body-independent protein translation (Kang and Schuman, 1995). Additionally, BDNF stimulates protein synthesis of a dendritically targeted translational reporter in mechanically isolated dendrites (Aakalu et al., 2001). BDNF-dependent stimulation of local protein translation may be mediated in part by increased expression and activation of eukaryotic initiation factor-4E (eIF4E), a key translation factor (Kanhema et al., 2006). It is also likely that BDNF stimulates dendritic translation by activating the mTOR pathway, as rapamycin treatment blocks BDNF-induced translation of activity-regulated cytoskeleton-associated (Arc) protein and CaMKII in synaptoneurosomes (Takei et al., 2004).

1.12 Alterations in BDNF expression affect dendritic spine formation

A growing body of literature indicates that BDNF signaling through TrkB specifically regulates the formation and maintenance of dendritic spines in several regions of the mammalian brain. TrkB receptors are localized to postsynaptic densities in the cortex and hippocampus (Wu et al., 1996). Further, TrkB receptors are trafficked in both axons and dendrites in cultured cortical neurons and are enriched on the surface of glutamatergic synapses (Gomes et al., 2006). Chronic (2-week) treatment of cultured cerebellar Purkinje cells with

recombinant BDNF increases dendritic spine density (Shimada et al., 1998). Additionally, treatment of hippocampal slices with recombinant BDNF increases dendritic spine density and promotes the formation of mature spine types (Alonso et al., 2004; Tyler and Pozzo-Miller, 2003). Together, these studies suggest that BDNF/TrkB signaling is sufficient to increase dendritic spine formation.

Several strategies have been used to determine a requirement for BDNF/TrkB signaling during dendritic spine formation. Elimination of BDNF expression using Cre-mediated recombination in the entire CNS results in a 95% reduction of BDNF protein levels in the brain and causes a reduction of dendritic spine density in the striatum and defects in spine maturation in hippocampal CA1 neurons (Rauskolb et al., 2010). Experiments using a more focused strategy of conditionally eliminating BDNF expression in the mouse forebrain demonstrated that BDNF is required both for the formation (Zeiler et al., unpublished) and maintenance (Vigers et al., 2012) of cortical dendritic spines. Additionally, striatal dendritic spine formation is also reduced in these mice (Baquet et al., 2004), which is particularly interesting because the striatum does not express BDNF on its own and is, instead, dependent on BDNF secreted from cortical neuronal axons innervating the striatum, suggesting that anterograde BDNF signaling regulates spine formation. Cell-autonomous inhibition of TrkB signaling by expression of a truncated form of TrkB in visual cortical neurons reduced dendritic spine density and also resulted in an increased proportion of immature spine types (Chakravarthy et al., 2006). Interestingly, dendritic spines on hippocampal CA1 neurons were unaffected in this mouse (Chakravarthy et al.,

2006). In contrast, TrkB deletion in pre and postsynaptic neurons in the hippocampus reduced dendritic spine density (Luikart et al., 2005). Additionally, aged mice that are heterozygous for TrkB display reductions in dendritic spine density and increases in dendritic spine length in CA1 (von Bohlen und Halbach et al., 2008). Together, these studies indicate that BDNF/TrkB signaling is required for dendritic spine formation in several brain regions, but additionally that these regions may have different directional requirements for BDNF/TrkB signaling.

1.13 Mechanisms of BDNF-induced dendritic spine formation

While it is clear that BDNF is a potent modulator of synapse and dendritic spine function, a precise means by which BDNF regulates this process is unclear. Only recently has work elucidated some of the mechanisms governing BDNF-induced dendritic spine formation. Recombinant BDNF treatment of hippocampal slices increases dendritic spine density by activating the ERK-signaling pathway (Alonso et al., 2004). BDNF treatment of hippocampal slices also induces a slowly activating and long lasting inward cationic current mediated by transient receptor potential canonical-3 (TRPC3) voltage-gated calcium channels, which is required for dendritic spine formation (Amaral and Pozzo-Miller, 2007). Further, BDNF promotes the surface localization of TRPC3 channels through activation of PI3K (Amaral and Pozzo-Miller, 2007). BDNF also increases the expression and synaptic localization of PSD95 (Yoshii and Constantine-Paton, 2007). Importantly, increases in PSD95 expression are

sufficient to induce dendritic spine formation in hippocampal neurons (El-Husseini et al., 2000) while PSD95 knockdown causes defects in spine maturation (Ehrlich et al., 2007). Lastly, BDNF increases the expression of Ryanodine receptors (RyRs) in hippocampal neurons, and BDNF-induced dendritic spine formation in these neurons requires expression of RyRs (Adasme et al., 2011). Together, these studies highlight the wide variety of means by which BDNF is known to regulate dendritic spines. However, the relative lack of mechanistic understanding of how BDNF regulates dendritic spines emphasizes a need for further research in this area. Additionally, studies examining the molecular mechanisms of BDNF-induced dendritic spine formation have focused primarily on the hippocampus. While cortical and hippocampal excitatory neurons are similar in many ways, it is possible that dendritic spine formation in these two brain regions is differentially regulated by BDNF, thus necessitating the study of cortex-specific spine formation.

1.15 Specific Aims

The goal of this thesis is to summarize the work that I performed in order to characterize the requirement for Wnt signaling in the growth and development of cortical neuron connectivity. The main hypothesis of my project is that Wnt signaling is required during BDNF-induced dendritic spine formation. The experimental results presented in this thesis pertained to three specific objectives. First, using several different strategies to inhibit Wnt signaling, I

determined the role of Wnt signaling during cortical neuron dendrite growth and dendritic spine formation. Second, by combining these Wnt inhibition strategies with a BDNF-overexpression paradigm, I determined whether Wnt signaling is required for BDNF-induced cortical neuron dendrite growth and dendritic spine formation. Third, I examined the function of two individual Wnt proteins in order to determine if they may mediate the effects of BDNF during cortical dendritic spine formation.

CHAPTER 2: Examining the requirement for Wnt signaling during cortical dendrite growth and BDNF-induced dendritic spine formation

2.1. INTRODUCTION

2.1.1 Wnts were discovered as key regulators of development

Wnt proteins are one of the major families of biologically important signaling molecules and regulate several fundamental processes in both invertebrate and vertebrate development. The founding members of the Wnt gene family were identified independently in mice and *Drosophila*. The first mouse Wnt gene, initially termed *int-1*, was discovered as a proto-oncogene activated by integration of mouse mammary tumor virus in mammary tumors (Nusse and Varmus, 1982; van Ooyen and Nusse, 1984). In *Drosophila,* wingless *(wg)* was identified as a segment polarity gene required for wing development (Baker, 1987; Sharma and Chopra, 1976). Work demonstrating that *wg* and int-1 encoded homologous secreted glycoproteins led to the derivation of the name "Wnt" as a combination of "wingless" and "*int-1*" (Cabrera et al., 1987; Rijsewijk et al., 1987).

Early phenotypic analysis identified Wnt genes as important developmental regulators. Work in *Drosophila* identified a variety of requirements for *wg* during embryonic development (Klingensmith and Nusse, 1994). In mice, *int-1/Wnt1* gene disruption led to pronounced neural developmental defects (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Additionally, ectopic expression of *Wnt1* in Xenopus led to duplication of the embryonic axis and promoted the formation of two-headed tadpoles (McMahon and Moon, 1989). Further, disruption of several Wnt genes in mice

resulted in an array of developmental phenotypes (Cadigan and Nusse, 1997). Overall, the past two decades of work examining Wnts in several organisms indicates that Wnt signaling is critical in a wide variety of developmental processes such as axis patterning, cell fate specification and cell proliferation (Cadigan and Nusse, 1997; Logan and Nusse, 2004; Wodarz and Nusse, 1998).

The initial indication of the importance of Wnt genes in embryonic development led to a subsequent search for Wnt homologs throughout eukaryotic organisms. To date, more than 100 Wnt genes have been identified in several organisms, including *C. elegans*, *Drosophila*, Zebrafish, Chicken, *Xenopus*, Mouse and Humans. Notably, Wnt genes are present in Cnidarians, an ancient metazoan phylum, indicating that Wnts are conserved throughout metazoan evolution (Croce and McClay, 2008). There are 19 different mammalian Wnt proteins, reflecting the diverse array of functions regulated by Wnt signaling.

2.1.2 Wnts signal through multiple signaling mechanisms

Early experiments in *Drosophila* utilizing forward genetic screens, genetic epistasis experiments and other techniques were instrumental in identifying some of the core Wnt signaling transduction components (van Amerongen and Nusse, 2009). Chief among these components is the seven-pass transmembrane protein Frizzled (Fzd). Binding of Wnts to the extracellular cysteine-rich domain of Fzd receptors is the initial step in Wnt signal transduction (Bhanot et al., 1996). There are 10 different mammalian Fzd proteins. Subsequent work demonstrated

that members of the lipoprotein receptor-related protein (LRP) family of single-pass transmembrane proteins are also required for Wnt signal transduction, indicating that Fzd and LRP function as co-receptors for Wnt proteins (Tamai et al., 2000; Wehrli et al., 2000).

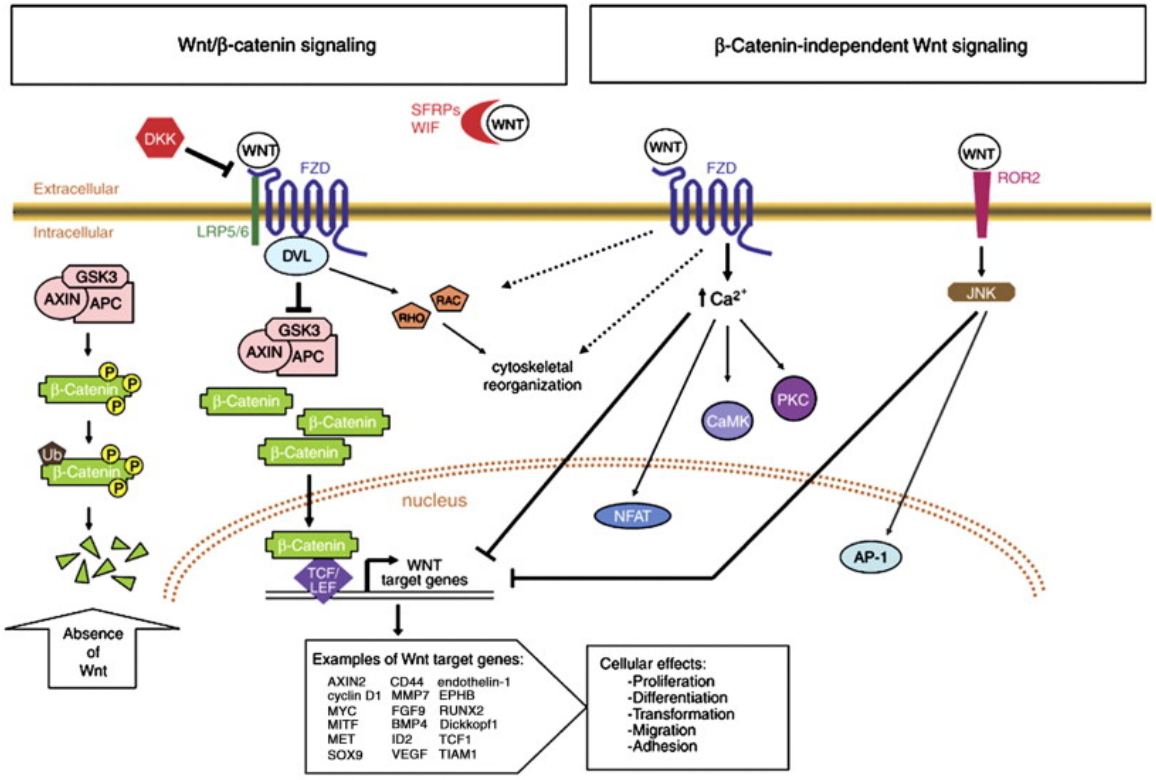

Figure 2.1. Schematic illustration of the various Wnt signaling cascades including both ß-catenin-dependent and ß-catenin-independent pathways. Image taken without permission from Freese et al. (2010).

Binding of Wnts to their receptors activates a number of intracellular signaling cascades (Fig. 2.1). The first and most characterized signaling cascade is the “Canonical” Wnt pathway, in which binding of Wnts to the Fzd-LRP receptor complex activates Dishevelled (Dvl), which in turn promotes the stabilization of ß-catenin by inhibiting the activity of a “destruction complex” consisting of Axin, Adenomatous Polyposis Coli (APC), Glycogen Synthase

Kinase-3ß (GSK-3ß) and Casein Kinase I (CKI) (Freese et al., 2010; Huang and He, 2008). In the absence of a Wnt signal, the destruction complex phosphorylates ß-catenin, which is then targeted for proteasome-dependent degradation. Wnt-mediated inhibition of the destruction complex results in elevation of cytoplasmic ß-catenin levels, leading to its translocation to the nucleus where it regulates transcription of Wnt target genes via association with the TCF/LEF family of transcription factors.

In addition to the canonical Wnt pathway, two other Wnt signaling pathways have been elucidated that utilize many of the same signaling components. The "non-canonical" Wnt signaling pathways signal independently of ß-catenin and are known as the planar cell polarity (PCP) pathway and the Wnt-calcium (Wnt-Ca^{2+}) pathway. The Wnt-PCP pathway involves small GTPase-mediated activation of c-Jun-terminal kinase (JNK) by Dvl, and results in changes in cytoskeleton dynamics (Wang and Nathans, 2007). The Wnt-Ca^{2+} pathway involves Dvl-mediated activation of phospholipase C (PLC), which then results in an increase in intracellular Ca^{2+} levels and subsequent activation of protein kinase C (PKC), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and calmodulin (Kohn and Moon, 2005). The Wnt-Ca^{2+} pathway regulates both the cytoskeleton and transcriptional events (Kohn and Moon, 2005). Although the majority of work examining Wnt signaling has focused on events downstream of the Fzd receptor, Wnts can also initiate signaling cascades independently of the Fzd transmembrane receptors. Recent work indicates that Wnts can also bind the tyrosine kinase receptors Derailed (Drl)/Ryk and Ror and activate

distinct signaling cascades downstream of those receptors (van Amerongen and Nusse, 2009).

Several secreted protein families antagonize and therefore modulate Wnt signaling. These Wnt inhibitors can be separated into two major classes. The first class of inhibitors acts by binding Wnt ligands, thus preventing them from interacting with their receptors and initiating signal transduction. This class includes the secreted Frizzled-related proteins (SFRPs) and Wnt inhibitory proteins (WIFs). The SFRPs are the largest family of Wnt inhibitors and bind to Wnt ligands through a cysteine-rich domain that is similar to those found in Fzd receptors (Bovolenta et al., 2008). However, further work indicates that the SFRPs have additional functions independent of their ability to antagonize Wnt signaling. SFRPs can directly bind and activate Fzd receptors and can interact with proteins unrelated to Wnt signaling (Bovolenta et al., 2008). The WIF proteins antagonize Wnt signaling in a manner similar to the SFRPs by binding Wnt ligands, but do this through an n-terminal WIF domain (Malinauskas et al., 2011). Although the SFRPs and WIF proteins are typically characterized as antagonists of Wnt signaling, it is possible that the ligand binding capability of these types of proteins aid in establishing Wnt gradients (Tabata and Takei, 2004). A second distinct class of Wnt antagonists that includes the Dickkopf (Dkk) family of secreted proteins acts by disrupting the interaction between the Fzd receptor and the LRP co-receptor (MacDonald et al., 2009). Dysregulation of both major classes of Wnt inhibitors is associated with a variety of human diseases (Bovolenta et al., 2008; MacDonald et al., 2009; Rubin et al., 2006).

2.1.3 Wnt signaling regulates the development of the mammalian nervous system

Research utilizing loss-of-function and gain-of-function assays demonstrated that Wnt signaling controls anterior-posterior axis formation and neural patterning during early vertebrate development (Takahashi and Liu, 2006). Specifically, Wnt signaling is critical for the development of the mammalian nervous system. As previously mentioned, the initial studies examining the role of Wnt signaling in mice demonstrated that *int-1/Wnt1* gene disruption led to pronounced but specific neural developmental defects. Inactivation of the *Wnt1* gene by homologous recombination resulted in severe abnormalities in the development of the mesencephalon and the metencephalon, which includes the mid-brain and the cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). More recent work demonstrated a specific requirement for Wnt signaling during the development of the mammalian forebrain, which includes the hippocampus and cortex. In Wnt3a mutant mice, the hippocampus fails to form, presumably due to reduced proliferation of a specific population of neural precursor cells (Lee et al., 2000). Additionally, overexpression of a stabilized version of ß-catenin results in over proliferation of cortical neural precursor cells and an enlargement of the cortex (Chenn and Walsh, 2002), while elimination of ß-catenin expression in cortical neural precursor cells results in reduced cell proliferation and premature neural differentiation (Woodhead et al., 2006). The requirement for Wnt signaling during neural precursor proliferation is maintained in the adult as disruption of Wnt signaling reduces adult neurogenesis in both the

subgranular zone of the hippocampus and the subventricular zone (Adachi et al., 2007; Lie et al., 2005).

2.1.4 Wnt signaling regulates the development of neural circuitry

Research using both invertebrate and vertebrate organisms implicates Wnt signaling as a crucial regulator of the development of neural circuitry. Extensive work indicates that Wnts regulate the growth, guidance and remodeling of neuronal axons and dendrites. The first demonstration that Wnt signaling regulated axon guidance was accomplished in *Drosophila* by ubiquitously overexpressing DWnt5 (then DWnt3), which resulted in severe disruption of CNS commissural axon tracts (Fradkin et al., 1995). Further work demonstrated that DWnt5 signals through the atypical Wnt receptor Derailed/Ryk to regulate this process (Yoshikawa et al., 2003).

Several studies in mice have identified clear roles for Wnt signaling in regulating axon growth and guidance in many regions of the mammalian nervous system. Wnt3 expression in spinal cord motor neurons is proposed to regulate axon branching and axon growth cone size of innervating sensory neurons (Krylova et al., 2002). In the developing spinal cord, Wnt4 signaling through Fzd3 functions as an attractive guidance cue for commissural axons after midline crossing (Lyuksyutova et al., 2003). Additionally, targeted deletion of Fzd3 results in a complete loss of several major axon tracts within the forebrain including the thamalmocortical, corticothalamic and nigrostriatal tracts, and of the anterior commissure and corpus callosum (Wang et al., 2002). In the

cerebellum, Wnt7a promotes axon growth and branching by increasing microtubule dynamics, presumably by inhibiting GSK3ß-mediated phosphorylation of MAP-1B, a key microtubule stabilizing protein (Lucas et al., 1998).

The role of Wnt5a during axon growth and guidance has been studied extensively. Wnt5a expression in developing sympathetic neurons mediates Nerve Growth Factor-mediated axon branching (Bodmer et al., 2009). Wnt5a promotes cortical axon growth by signaling through Ryk receptors and induces repulsive axon turning by signaling through both Fzd and Ryk receptors (Li et al., 2009). Both the axon growth and axon repulsive functions of Wnt5a are mediated by alterations in intracellular Ca^{2+} (Hutchins et al., 2011). In addition, Wnt5a expression in the developing midbrain promotes dopaminergic axon growth and regulates the proper targeting of these axons through a non-canonical Fzd-mediated signal (Blakely et al., 2011).

In addition to regulating axon growth, Wnt signaling regulates the development of neural circuitry in the mammalian brain by influencing the growth of dendrites. Wnt7b is expressed in the mouse hippocampus and promotes dendrite branching and growth through a non-canonical signaling mechanism involving Dvl1-mediated activation of JNK signaling (Rosso et al., 2005). Also in the hippocampus, Wnt2 mediates neural activity-induced dendrite branching and growth, though the mechanism underlying this process is unknown (Wayman et al., 2006).

2.1.5 Wnt signaling regulates neural connectivity by regulating synapse and dendritic spine formation

Research utilizing both invertebrate and vertebrate systems over the last decade indicates that one of the key roles for Wnt signaling in the developing and adult nervous system is to regulate the formation and function of neural synapses. Although the majority of research demonstrates that Wnt signaling promotes synapse formation, accumulating evidence indicates that, in some cases, Wnt signaling can also inhibit synapse formation. Together, this suggests that the actions of Wnts in synapse formation and maintenance are complex.

Studies of the *Drosophila* neuromuscular junction (NMJ), an invertebrate model of an excitatory glutamatergic synapse, have provided many insights into the roles of Wnt signaling during synapse formation. *Wg* is expressed and secreted postsynaptically by motor neurons at the *Drosophila* NMJ. Loss of *Wg* leads to impairments in pre- and postsynaptic assembly (Packard et al., 2003). Further research into the role of Wg at the Drosophila NMJ revealed that Wg acts to promote synapse formation through a unique mechanism involving the cleavage and nuclear translocation of the cytoplasmic tail of DFzd2 (Ataman et al., 2008; Mathew et al., 2005). The *Drosophila* LRP6 co-receptor homolog Arrow is localized both pre- and postsynaptically at the NMJ, and *arrow* mutants display synaptic impairments similar to *wg* mutants, including fewer synaptic boutons that are smaller and have abnormal morphology (Miech et al., 2008). Expression of Arrow in either the presynaptic motor neuron or the postsynaptic muscle cell partially rescues these impairments, suggesting that this receptor functions at both sides of the NMJ (Miech et al., 2008). Interestingly, the activity

of Arrow seems to be independent of ß-catenin, suggesting that Arrow participates in a divergent pathway related to canonical Wnt signaling in order to regulate synapse formation (Miech et al., 2008).

Wnt signaling also inhibits synapse formation at the *Drosophila* NMJ. DWnt4 expression in the M13 muscle cell prevents motor neurons from innervating M13 and forming synapses (Inaki et al., 2007). Similarly, at the *C. elegans* NMJ, Wnt/Lin-44 signals through Frizzled/LIN-17 to inhibit DA9 motor neuron synapse formation onto dorsal muscle cells (Klassen and Shen, 2007).

A role for Wnt signaling in regulating NMJ synapse formation is conserved in vertebrates. In zebrafish, Wnt11r binds and signals through MuSK, a receptor tyrosine kinase that also binds Agrin, in order to regulate the clustering of postsynaptic acetylcholine receptors (AChRs) (Jing et al., 2009). Similarly, in the chick wing Wnt3 is expressed in the presynaptic motor neuron and promotes AChR clustering, while inhibition of Wnt signaling using Sfrp1 decreases AChR clustering (Henriquez et al., 2008). Wnt3-induced AChR clustering requires Dvl, MuSK and Rac1 (Henriquez et al., 2008; Luo et al., 2002). In contrast to the role of Wnt3, Wnt3a expression in cultured myotubes induces dispersal of AChR clusters, possibly by signaling in a ß-catenin dependent manner (Wang and Luo, 2008).

In addition to regulating vertebrate peripheral synapses, Wnts are crucial regulators of synapse formation in the CNS. The first demonstration that Wnt signaling regulates central synapse formation was done using cultured mouse cerebellar neurons and showed that Wnt7a induces presynaptic clustering of

Synapsin-1 (Lucas and Salinas, 1997). Subsequent work *in vivo* demonstrated that Wnt7a signals in a retrograde manner to promote synapse formation between mossy fiber axons and granule cell dendrites at cerebellar glomerular rosettes (Hall et al., 2000), and that Wnt7a signaling activity requires Dvl1 (Ahmad-Annuar et al., 2006).

Wnt signaling is also a key regulator of hippocampal synapse formation. Research thus far indicates that several Wnts promote hippocampal synapse formation through a variety of mechanisms. Wnt3a signaling through Fzd1 in hippocampal neurons regulates presynaptic protein clustering and synaptic vesicle recycling (Varela-Nallar et al., 2009). Wnt7a regulates presynaptic function in hippocampal neurons by inducing the clustering of nicotinic AChRs (nAChRs) through a mechanism involving APC, a component of the ß-catenin destruction complex (Farias et al., 2007). Additionally, Wnt7a enhances excitatory neurotransmission by increasing the number of presynaptic vesicle release sites in a ß-catenin independent manner (Cerpa et al., 2008). The activity of Wnt7a may be mediated by the receptor Fz5 (Sahores et al., 2010). Wnt7b also promotes synapse formation in hippocampal neurons by inducing assembly of the presynaptic protein VGlut1 (Davis et al., 2008).

Wnt5a also regulates hippocampal synapse formation, but its role is somewhat controversial. In one study, exposure of hippocampal neurons to Wnt5a decreased the number of presynaptic puncta, suggesting that Wnt5a inhibits synapse formation (Davis et al., 2008). However, in another study, Wnt5a increased synapse density in hippocampal neurons by signaling through

the atypical Wnt receptors Ror1 and Ror2 to induce clustering of the presynaptic protein Synaptophysin (Paganoni et al., 2010). Further, multiple studies demonstrated that Wnt5a promotes synapse formation by signaling at the postsynaptic specialization. Wnt5a enhances excitatory synaptic transmission by signaling through JNK to induce the clustering of the postsynaptic protein PSD-95 in hippocampal neurons (Farias et al., 2009; Varela-Nallar et al., 2010). Additionally, Wnt5a promotes the formation of inhibitory synapses by increasing the recycling of $GABA_A$ receptors, key mediators of fast synaptic inhibition (Cuitino et al., 2010). Together, the work examining the function of Wnt5a illustrates the burgeoning complexity of synaptic Wnt signaling.

The ability of Wnt signaling to promote synapse formation may also play an important role during neurodegenerative diseases such as Alzheimer's disease (AD). Exposure of hippocampal neurons to a mixture of Amyloid-ß oligomers induces a rapid transcriptional upregulation of the Wnt inhibitor Dkk-1 (Purro et al., 2012). Further, treatment of hippocampal neurons with Dkk-1 induces rapid but reversible synaptic disassembly, suggesting that dysregulation of Wnt signaling may play a causal role in the synaptic pathophysiology associated with AD (Purro et al., 2012).

Despite the growing appreciation for the role of Wnt signaling in regulating synapse formation, research examining the role of Wnt signaling during dendritic spine formation is only just beginning to emerge. Notably, two individual Wnts, Wnt5a and Wnt7a, increase dendritic spine density in hippocampal neurons. Treatment of hippocampal neurons Wnt5a rapidly increases dendritic spine

formation, presumably due to the ability of Wnt5a to induce clustering of PSD-95 (Farias et al., 2009). Wnt7a increases dendritic spine density and maturation in hippocampal neurons through a mechanism that requires Dvl1 and CaMKII (Ciani et al., 2011). Lastly, application of Fzd2CRD, a soluble version of the Wnt receptor Fzd2 that blocks Wnt signaling, decreases dendritic spine density in neurons (Varela-Nallar et al., 2010). To date, no work has examined a role for Wnt signaling during dendritic spine formation in cortical neurons.

2.1.6 Wnts are required for activity dependent neural growth and synapse formation

As previously mentioned, neural activity is a key regulator of both dendrite growth and synapse formation (See Chapter 1). Interestingly, recent work indicates that Wnt signaling is a critical component of activity-induced neural growth and can mediate the effects of neural activity during dendrite growth and synapse formation. ß-catenin, a downstream component of Wnt signaling, is not only required for activity-induced dendrite growth of hippocampal neurons (Yu and Malenka, 2003), but it is also re-localized into dendritic spines after neuronal depolarization where it then promotes the growth of hippocampal dendritic spines (Murase et al., 2002). Wnt2 expression is increased by neural activity in hippocampal neurons and Wnt signaling is required for activity-induced dendrite growth in hippocampal neurons (Wayman et al., 2006). Treatment of hippocampal slices with the Wnt inhibitor Fzd8CRD, a soluble version of the receptor Fzd8, blocks the induction of LTP (Chen et al., 2006). Increased neural activity induced by exposing mice to an enriched environment (EE) increases

synapse size and strength in the hippocampus and stimulates the expression of Wnt7b (Gogolla et al., 2009). Further, treatment with Sfrp1 blocks the EE-induced increase in synapse size (Gogolla et al., 2009). Finally, high frequency stimulation (HFS) of hippocampal slices induces surface localization of Fzd5, which in turn mediates Wnt7a induced increases in synapse formation (Sahores et al., 2010).

2.2 EXPERIMENTAL METHODS

2.2.1 Microarray Analysis of Emx-BDNF^{KO} Mice

Conditional forebrain-specific BDNF^{KO} mice were generated as previously described (Gorski et al., 2003). Cortical (anterior and posterior), striatal and cerebellar tissue was harvested from 5 week-old (P35) mice. Tissue from six mice of each BDNF genotype (*Emx-BDNF^{KO}*, *BDNF$^{+/-}$,* wild type) was used for analysis. Total RNA was harvested using Qiazol reagent (Qiagen, Valencia, CA) according to manufacturer's instruction. Residual salts and proteins were removed from the total RNA using a Qiagen RNeasy kit (Qiagen) according to manufacturer's instructions. 2µg of total RNA was used to synthesize biotinylated-cRNA using the Affymetrix single-cycle probe synthesis kit (Affymetrix, Santa Clara, CA) according to manufacturer's instructions. Synthesized cRNA was then hybridized to Affymetrix MOE430A 2.0 arrays (Affymetrix) containing 22,690 probesets. Affymetrix microarrays were analyzed using RMA, R version 2.3, and the Bioconductor packages *affy* and *limma*

(http://www.bioconductor.org). Differential gene expression was assessed relative to wild type using paired tests.

2.2.2 Cortical Neuron Cultures

Cortices were dissected from postnatal day 0-1 CD1 mouse pups and incubated in papain (Worthington, Lakewood, NJ) for 45 minutes at room temperature. The tissue was triturated to obtain a single-cell suspension, and the cells were plated into 12-well cell-culture dishes coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO) at a concentration of $2.5x10^6$ cells/cm^2. Cells were grown initially in DMEM (Life Technologies Corporation, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals, Lawrenceville, GA) and Penicillin-Streptomycin (Life Technologies Corporation). After 24 hours, the culture medium was changed to Neurobasal-A medium (Life Technologies Corporation) supplemented with B27 (Life Technologies Corporation), Glutamax (Life Technologies Corporation) and Penicillin-Streptomycin (Life Technologies Corporation). The anti-mitotic drug 5-Fluorodeoxyuridine (Sigma-Aldrich) was added to the Neurobasal-A medium to prevent glial cell proliferation. Cultures were maintained at 5% CO_2 for 10 days *in vitro* (DIV) before beginning experimental procedures, with half of the medium changed on DIV9. For all experiments, neuron cultures were fixed with HEPES-buffered (ThermoFisher Scientific, Waltham, MA) 4% paraformaldehyde (Sigma-Aldrich), 4% Sucrose (ThermoFisher Scientific) solution in PBS (pH 7.4). After fixation, neuron cultures

were mounted in Fluoromount-G (Southern Biotech, Birmingham, AL) for subsequent imaging analysis.

2.2.3 Neuron Transfection

Neuron cultures were transfected using Lipofectamine 2000 (Life Technologies Corporation) according to manufacturer's instruction. Briefly, plasmid DNA was prepared for transfection at a DNA (μg):Lipofectamine (μl) ratio of 1:3 in serum-free Neurobasal-A (Life Technologies Corporation). 1.5μg of total plasmid DNA was used per well of a 12-well cell-culture dish. In all experiments, 500ng of each individual plasmid was used for transfection. Appropriate empty vector controls were added to ensure that the total amount of transfected DNA was 1.5μg. Neurons were transfected for 2 hours, at which point the transfection medium was replaced with fresh culture medium. Transfection efficiency was approximately 5-10% using this method.

2.2.4 Expression Plasmids

pCMV-BDNF was constructed by inserting the ORF for murine BDNF into the pEGFP-N1 backbone (Clontech, Mountain View, CA). pCS2+-Sfrp1 (Addgene plasmid 16693, Cambridge, MA) expresses murine Sfrp1 pRK5-mFzd8CRD-IgG. (Addgene plasmid 16689) expresses a fusion protein consisting of the extracellular domain of the murine Frizzled-8 protein fused with the human immunoglobulin heavy chain (Semenov et al., 2001). pCS2+-mDvl1ΔPDZ-HA expresses a C-terminally HA tagged murine Dishevelled-1 with a deletion of

amino acids 276-336 (Rosso et al., 2005). pEGFP-N1 was used to express cytoplasmic GFP. pTRE-tight-BDNF was constructed by inserting the murine BDNF ORF from pCMV-BDNF into the pTRE-tight backbone (Clontech). pTRE-tight-EGFP was constructed by inserting the coding sequence for EGFP from pEGFP-N1 into the pTRE-tight backbone. pTRE-tight-BDNF and pTRE-tight-EGFP were co-transfected with pCMV-rtTA, which was constructed by inserting the coding sequence for the Reverse-Tet-Transactivator protein into the pEGFP-N1 backbone. BDNF expression was induced from pTRE-tight-BDNF by adding 1 µg/ml doxycycline (BD Biosciences, San Diego, CA) to the culture medium. pTRE-tight-EGFP was used to express cytoplasmic GFP in order to visualize the architecture of transfected neurons and to control for tetracycline-mediated activation of transcription.

2.2.5 Image Acquisition and Analysis

Dendritic protrusion and primary dendrite images were collected using Metamorph software (Molecular Devices, Downington, PA) and a Nikon Eclipse TE2000-U microscope (Nikon, Melville, NY) fitted with a spinning disk confocal system (Solamere Technology Group, Salt Lake City, UT) and a Cascade II 16-bit EMCCD camera (Photometrics, Tucson, AZ). Dendritic arbor images were collected using Zen software (Carl Zeiss, Germany) and a Zeiss LSM 510 Meta confocal system (Carl Zeiss, Germany).

To quantify dendritic protrusion density and length, 8-10 neurons per coverslip from a total of 3-4 coverslips per treatment were imaged using a

1.40NA 100X objective (Nikon). Neurons that had a pyramidal-shaped cell body and a clear apical dendrite were chosen for analysis. 5 dendritic segments (3 apical segments and 2 basal segments) per neuron were imaged in 0.2μm Z-steps. Z-stacks were then loaded into ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij) for analysis. Dendritic spine density was measured using uncompressed Z-stacks. All dendritic protrusions less than 5 μm in length were counted as dendritic spines and then quantified as the number of dendritic spines per μm of dendrite length. Average apical and basal spine densities for each neuron were calculated from the apical and basal segments, respectively. The total spine density per neuron was calculated as the average of the apical and basal spine density.

In order to measure dendritic spine length and dendritic spine head width, Z-stacks were flattened using the stack focuser plug-in for ImageJ (n x n Kernel = 11). Dendritic spines emanating in a perpendicular direction from the dendrite shaft in the X/Y plane were quantified. Spine length was defined as the distance between the dendrite shaft and the tip of the dendritic spine and was measured using the freehand line drawing tool in ImageJ. Spines emerging either above or below the dendrite were not measured in order to minimize the effects of the Z-projection on spine length. Dendritic spine head width was defined as the widest part of the dendritic spine head and was measured using the segmented line drawing tool in ImageJ. Spine head width measurement was restricted to dendritic protrusions that had formed some type of a bulbous spine head, thus

excluding filopodial spines that lacked a spine head. Using these parameters, approximately 50% of total spines were measured for length and width.

To measure the number of primary dendrites, the cell body of each neuron that was used to measure dendritic protrusions was imaged in 0.5μm Z-steps using a 1.40NA 100X objective. Uncompressed Z-stacks were analyzed in ImageJ. Primary dendrites were defined as all neurites that emerged directly from the cell body of the neuron.

To quantify dendritic arbors, 10-20 neurons per coverslip from a total of 3-4 coverslips per treatment were imaged using a 0.8NA 20X objective (Carl Zeiss) in 1.0μm Z-steps. Using ImageJ, Z-stacks were flattened using a Max-point Z-projection. Dendritic arbors were traced using the NeuronJ plugin for ImageJ (Eric Meijering, Biomedical Imaging Group, Erasmus MC, University Medical Center, Rotterdam, Netherlands). NeuronJ settings were as follows: Neurite appearance: Dark; Hessian smoothing scale: 2.0; Cost-weight factor: 0.7; Snap window size: 5x5; Path search window: 2500x2500; Tracing smoothing range: 5; Tracing subsampling factor: 5; Line width: 1. Dendritic arbor traces were skeletonized to a pixel width of 1.0 and then converted into a binary image. Total dendrite length was calculated by measuring the total pixel count for each tracing and then converting the pixel count into a length measurement (1.0μm dendrite length per 2.27 pixels) for each neuron that was traced. Sholl analysis was performed on the skeletonized dendritic arbor traces using the automated Sholl analysis plugin for ImageJ (Ghosh Lab, University of California, San Diego). Sholl analysis settings were as follows: Starting Radius: 20μm; Ending Radius:

200μm; Radius Step Size: 20μm; Radius Span: 0.00; Span Type: median. Dendrite endpoints were also measured in ImageJ and were defined as any time a dendrite branch terminates. All imaging and analysis was performed in a blinded fashion with respect to neuron treatment.

2.2.6 Measuring c-Fos induction by BDNF

To measure the induction of c-Fos expression by BDNF, cortical neurons were co-transfected on DIV10 with pTRE-tight-BDNF (500ng), pTRE-tight-EGFP (400ng) and pCMV-rtTA (100ng). For samples testing the effects of Wnt inhibition on BDNF-induced c-Fos expression, neurons were additionally co-transfected with plasmids expressing one of the four Wnt inhibitors (500ng) or an empty vector control (500ng). Similar to other experiments, a total of 1.5μg of plasmid DNA was transfected per well. Neurons were allowed to express the inhibitors for 2DIV at which point BDNF expression was induced by adding 1μg/mL doxycycline to the culture medium. 12 hours after induction of BDNF, neurons were fixed and prepared for c-Fos immunostaining. Fixed neurons were rinsed with PBS twice, then once with PBS+0.5% Tween. Neuron were incubated in blocking solution (PBS+0.5% Tween+10% Normal Goat Serum) for 1 hour at room temperature, then stained overnight at 4°C with the primary antibodies directed against c-Fos (1:500, Rabbit-anti-c-Fos SC-52, Santa Cruz Biotechnology, Santa Cruz, CA) and GFP (1:1,000, Mouse-anti-GFP, Roche, Madison, WI) diluted in blocking solution. Following primary staining, neurons were rinsed three times in PBS+0.5% Tween. Neurons were then stained for 2

hours at room temperature in secondary antibody (Alexa Fluor Goat-anti-Rabbit 555, Alexa Fluor Goat-anti-Mouse 488, Life Technologies, Grand Island, NY) diluted 1:1,000 in blocking solution. Finally, neurons were rinsed twice with PBS+0.5% Tween, once with PBS, and then mounted with Fluoromount. Neurons were stained with DAPI (1:10,000) during the final PBS rinse.

To quantify c-Fos induction,10 neurons per treatment were imaged in a manner similar to the way that primary dendrite images were acquired. Briefly, neural cell bodies were imaged in 0.5μm Z-steps using a 1.40NA 100X objective. Cell bodies were imaged using three channels. Nuclear DNA was imaged using the DAPI channel (500 ms exposure), the outline of the cell body as indicated by GFP expression was imaged using the FITC channel (100 ms exposure), and c-Fos was imaged using the TR channel (250 ms exposure). Laser settings were kept consistent throughout image acquisition. In order to eliminate sample bias and to ensure that neurons were not selected for imaging based on observed c-Fos immunoreactivity during image acquisition, neurons were selected for imaging using only their morphology as indicated by GFP fluorescence. The first 10 neurons meeting the proper morphological criteria (Pyramidal shaped cell body, one clear apical dendrite) were imaged in this manner.

c-Fos expression was quantified by loading uncompressed Z-stacks into ImageJ. Using the images from the DAPI channel, I identified the specific image plane at which the nucleus was at maximum size, then outlined a ROI around the nucleus using the segmented line drawing tool. I then measured the integrated density of the corresponding image in the TR channel within the ROI and divided

this by the area of the ROI in order to quantify the normalized nuclear c-Fos immunoreactivity for each neuron. The relative c-Fos intensity for each treatment was calculated as the average of 10 neurons.

2.2.7 Statistical Analysis

Statistical significance for experiments comparing two populations was determined using a two-tailed unpaired Student's t-test. For experiments comparing three or more populations, a One-way ANOVA with Tukey's post-hoc test was used. For spine length frequency distributions and Sholl analysis distributions, statistical significance was determined using a Two-way ANOVA with a Bonferroni post-test to compare means at individual data points. All statistical analyses were performed using Graphpad Prism (Graphpad Software, Inc., La Jolla, CA). All data are presented as the mean ± SEM with n = number of neurons. Data presented here are representative of results from at least two separate experiments.

2.3 RESULTS

2.3.1 Components of Wnt signaling are dysregulated in Emx1-BDNF^{KO}Mice

Examination of a microarray-based transcriptional analysis of Emx1-BDNF^{KO} mice revealed that expression levels of several components of the Wnt signaling pathway are dysregulated in comparison with wild type mice (Strand et al., 2007). Wnt signaling components whose expression levels differed

significantly from wild type in either the anterior cortex, posterior cortex or striatum are listed in Table 1.1 along with their respective fold change of expression and p-value. Dysregulated components included Wnt ligands, Wnt receptors, intracellular signaling components, and secreted Wnt inhibitors. Interestingly, the Wnt ligands themselves constituted the largest fraction of dysregulated Wnt signaling components. The expression level of some Wnt ligands decreased (Wnt2, Wnt4, Wnt10a, Wnt11), while others increased (Wnt5a, Wnt7a, Wnt7b).

Gene Symbol		Anterior Cortex	p-value	Posterior Cortex	p-value	Striatum	p-value
Wnt2		0.69	***	0.79	**	0.62	***
Wnt4		0.70	***	0.75	**	0.84	**
Wnt10a		0.83	**	0.78	**		
Wnt11		0.85	*				
Wnt7a						1.19	**
Wnt5b		1.17	*				
Wnt7b		1.13	*	1.22	*	1.17	*
Wnt5a		1.20	***	1.15	*		
Fzd2						0.86	*
Fzd3						0.89	*
Fzd7		1.20	**				
Fzd6		1.12	*				
Fzd6		1.17	**				
Lrp5		0.91	*				
Axin2		0.88	**				
Catnb	(ß-Catenin)	1.17	***	1.26	*	1.14	*
Ror1		0.82	*			0.83	*
Sfrp1		1.28	***				
Sfrp2		2.01	***	1.80	***		
Wif1				0.90	*		
Dkk3		0.72	***	0.75	***		
Dkk2						0.84	***

Table 1.1. Fold changes in gene expression levels of Wnt signaling components in Emx1-BDNF^{KO} mice compared to WT mice. Increases in gene expression are highlighted in red. Decreases in gene expression are highlighted in green. *p<0.05, **p<0.01, ***p<0.001.

2.3.2 Wnt inhibition impairs cortical dendrite elaboration

To investigate the role of Wnt signaling in cortical neuron growth I expressed four different Wnt inhibitors, Wif1, Sfrp1, mFzd8CRD-IgG and mDvl1ΔPDZ in cultured cortical neurons. Wnts signal through both canonical (Logan and Nusse, 2004) and non-canonical signaling cascades, and these inhibitors can interfere with both types of Wnt signaling cascades. Wnt-inhibitor Factor-1 (Wif1) and Secreted Frizzled-Related Protein-1 (Sfrp1) are endogenous secreted proteins that can bind to Wnt ligands in the extracellular space and prevent them from binding their receptor (Malinauskas et al., 2011; Rattner et al., 1997). mFzd8CRD-IgG is a secreted fusion protein consisting of the extracellular domain of the murine Wnt receptor Frizzled-8 fused to the human immunoglobulin light chain. mFzd8CRD-IgG also binds to Wnt ligands in the extracellular space and prevents them from binding their receptor (Hsieh et al., 1999). mDvl1ΔPDZ is a deletion mutant of the murine Dishevelled-1 protein, an essential intracellular component of both canonical and non-canonical Wnt signaling cascades (Gao and Chen, 2010). mDvl1ΔPDZ lacks the PDZ domain that is required for Dvl1 to promote hippocampal dendrite growth (Rosso et al., 2005).

As previously mentioned, elaboration of hippocampal neuronal dendrites requires Wnt signaling. Hippocampal neurons either treated with Sfrp1 or expressing Dvl1ΔPDZ display reduced dendrite growth and branching (Rosso et al., 2005). I first determined whether dendrite growth in cortical neurons is similarly affected by the inhibition of Wnt signaling. Cultured cortical neurons

were co-transfected on DIV10 with 500ng of a plasmid expressing one of the four different Wnt inhibitors, 500ng of a plasmid expressing cytoplasmic GFP in order to visualize neuron morphology, and 500ng of an empty vector plasmid (1.5μg total DNA/well). Neurons were then fixed and imaged on DIV14 after expressing Wnt inhibitors for 4 days. I quantified dendritic arbors by measuring three aspects of dendrite elaboration: dendrite length, number of dendrite endpoints and total dendritic complexity as determined using a Sholl analysis (Sholl, 1954). Representative images of cortical neurons expressing each of the four Wnt inhibitors are shown in Fig 2.1A. Quantification of total dendrite length reveals that the four Wnt inhibitors variably affected dendrite growth. While both Sfrp1 and Dvl1ΔPDZ caused a significant decrease in total dendrite length, neither Wif1 nor Fzd8CRD caused a significant decrease (Fig. 2.1B). Three of the four Wnt inhibitors decreased the number of dendrite endpoints per neuron, suggesting that inhibiting Wnt signaling causes a reduction in total dendritic branching (Fig. 2.1C). Indeed, Sholl analysis revealed that the same three inhibitors that decreased the number of dendrite endpoints (Sfrp1, Fzd8CRD, Dvl1ΔPDZ) also decreased overall dendritic complexity (Fig. 2.1D-G), further suggesting that Wnt inhibition causes decreased dendritic branching. In contrast, none of the Wnt inhibitors caused a decrease in the number of primary dendrites (Fig. 2.5B). Together, these data indicate that inhibiting Wnt signaling leads to a decrease in dendritic arborization in cortical neurons.

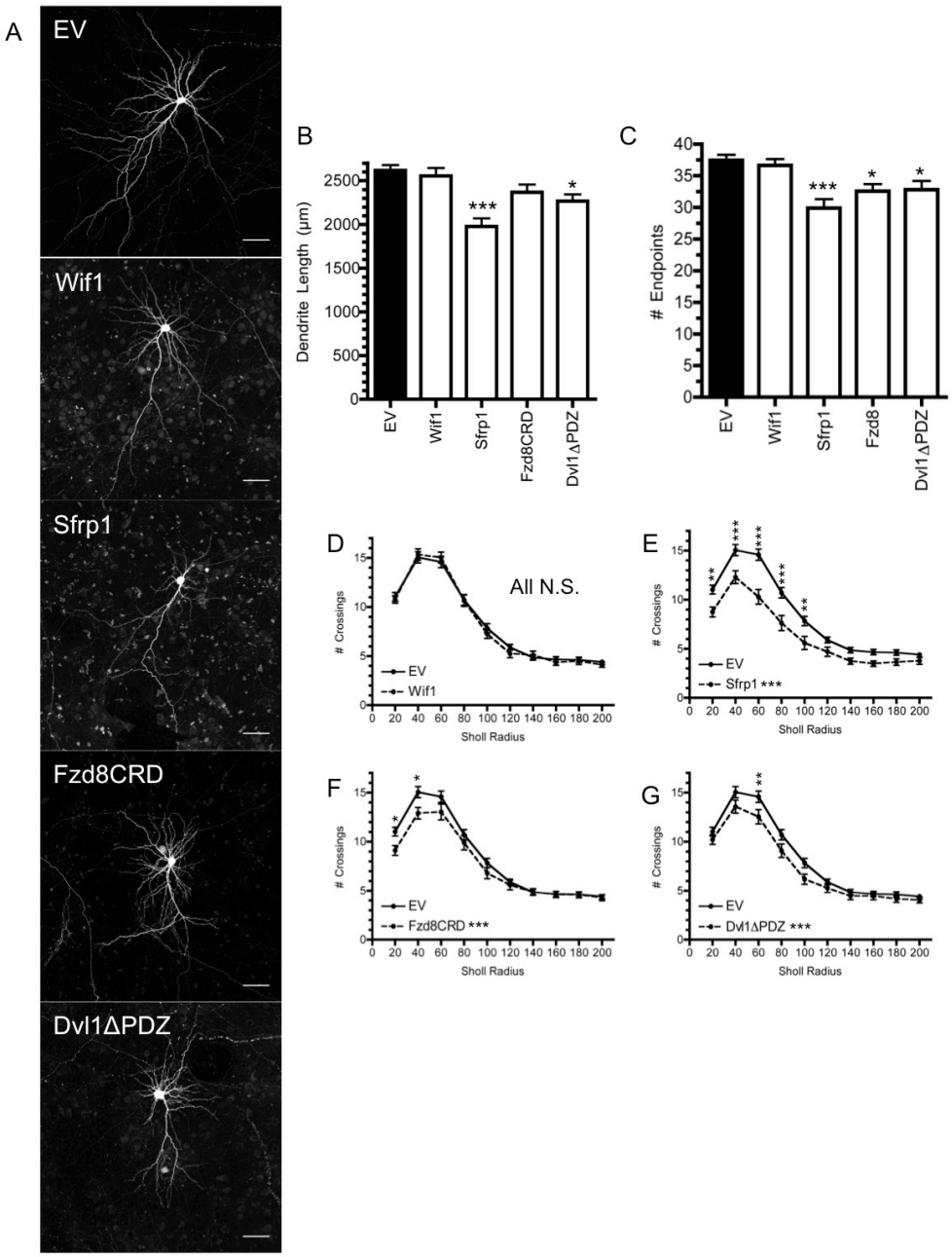

Figure 2.1. Wnt inhibition results in decreased dendrite elaboration. (A) Representative cortical neurons expressing Empty Vector (EV), Wif1, Sfrp1, Fzd8CRD or Dvl1ΔPDZ. (B) Quantification of the total dendrite length per neuron for each treatment. (C) Quantification of the number of dendritic endpoints per neuron for each treatment. (D-G) Sholl analysis of dendritic complexity comparing neurons treated with each Wnt inhibitor to control neurons. ***p<0.001, **p<0.01, *p<0.05. n=number of neurons: EV n=56, Wif1 n=49, Sfrp1 n=39, Fzd8CRD n=39, Dvl1ΔPDZ n=39 Scale Bar: 50μm.

2.3.3 Wnt inhibition modestly impairs cortical dendritic spine formation

I next determined whether Wnt signaling is required for dendritic spine formation in cortical neurons. Representative images of dendritic segments from neurons from empty vector (EV) control or Wnt-inhibited neurons are shown in Fig 2.2A. Quantification revealed that although all four Wnt inhibitors appeared to decrease dendritic spine density only Sfrp1 caused a significant decrease (Fig. 2.2B). Similarly, quantification of dendritic spine length revealed that only Wif1 and Dvl1ΔPDZ significantly increased dendritic spine length (Fig. 2.2C). However, relative frequency distribution plots of spine lengths indicate that all four Wnt inhibitors caused a significant decrease in the fraction of short spines and a significant increase in the fraction of long spines when compared to control (Fig. 2.2E-H). Lastly, quantification of dendritic spine head width revealed that three of the four Wnt inhibitors (Wif1, Sfrp1 and Fzd8CRD) caused significant decreases (Fig 2.2D). Dendritic spines are highly plastic structures that undergo dramatic changes in morphology after they emerge and as they mature. New dendritic spines emerge as long filopodia, which, throughout the course of spine maturation, decrease in length and retract toward the dendritic shaft as the nascent synapse develops (Fiala et al., 1998; Knott et al., 2006; Lohmann and Bonhoeffer, 2008; Maletic-Savatic et al., 1999; Zito et al., 2009). Together, these data suggest that inhibiting Wnt signaling in cortical neurons modestly impairs dendritic spine formation by inhibiting their maturation.

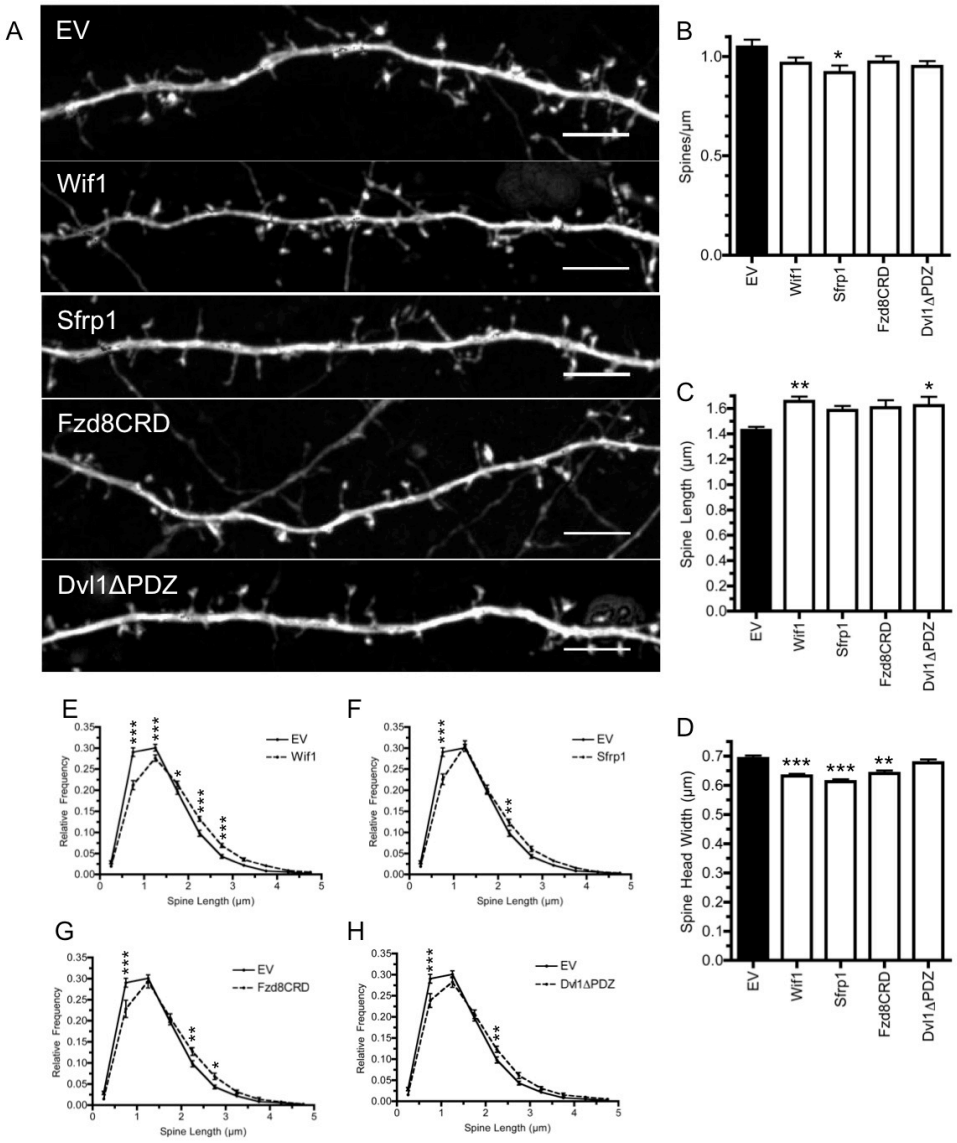

Figure 2.2. Wnt inhibition results in altered dendritic spine development. (A) Representative dendritic segments of cortical neurons expressing EV, Wif1, Sfrp1, Fzd8CRD or Dvl1ΔPDZ. Quantification of dendritic spine density (B), average spine length (C) and average spine head width (D) for each treatment. (E-H) Relative frequency distributions comparing the distribution of spine length for each Wnt inhibitor compared to control. ***p<0.001, **p<0.01, *p<0.05. n=number of neurons: EV n=31, Wif1 n=34, Sfrp1 n=23, Fzd8CRD n=25, Dvl1ΔPDZ n=25. Scale bar: 5µm.

2.3.4 The effect of Wnt inhibition on BDNF-mediated cortical dendrite elaboration

Inhibition of Wnt signaling caused some defects in dendritic spine and dendritic arbor development in cortical neurons cultured in standard conditions. However, the primary goal for these experiments was to determine the requirement for Wnt signaling during BDNF-induced cortical neuron growth. To begin to address this role, I analyzed the effects of BDNF on the growth and elaboration of cortical dendrites, and further examined how these effects were modulated by Wnt inhibition. Using the same 4DIV expression paradigm, I co-expressed each of the four Wnt inhibitors with 500ng of a plasmid encoding BDNF (in place of the 500ng of empty vector plasmid from Figs. 2.1, 2.2). Representative images of neurons co-expressing BDNF with each Wnt inhibitor are show in Fig. 2.3A. Although previous work from other labs demonstrated that BDNF promotes the growth of cortical dendrites (Horch et al., 1999; McAllister et al., 1996, 1997; Wirth et al., 2003), I demonstrate here that BDNF expression did not significantly affect total dendrite length of cultured cortical neurons (Fig. 2.4B), and that total dendrite length may actually have slightly decreased. In fact, separate analysis of the effects of BDNF on either apical (Fig. 2.4C) or basal (Fig. 2.4D) dendrite length reveals that BDNF significantly decreased apical dendrite length. Additionally, BDNF did not significantly increase the number of dendrite endpoints (Fig. 2.4E), suggesting that BDNF expression did not increase dendrite branching. Sholl analysis of dendrite complexity indicates that BDNF increased the complexity of proximal dendritic arbors while simultaneously decreasing the complexity of distal dendritic arbors (Fig. 2.4F). The increase in

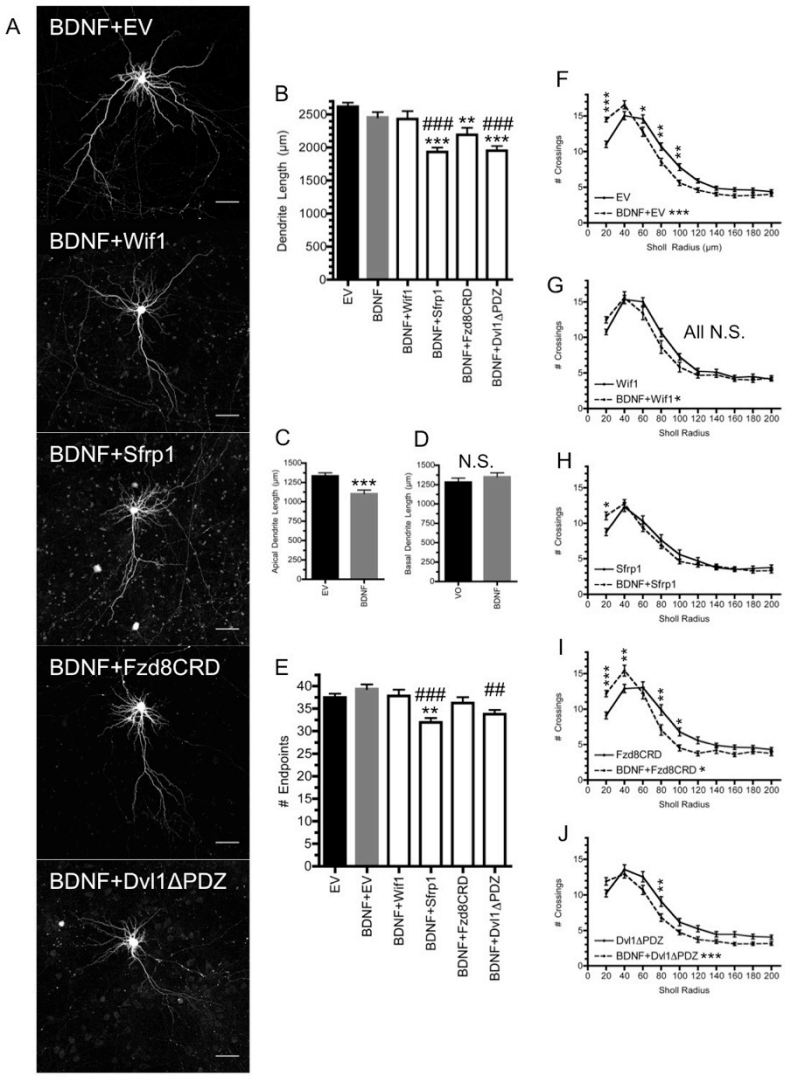

Figure 2.3. BDNF expression increases dendritic complexity without increasing total dendrite length. (A) Representative cortical neurons co-expressing BDNF with EV, Wif1, Sfrp1, Fzd8CRD and Dvl1ΔPDZ. (B) Quantification of total dendrite length for each treatment. Quantification of the effect of BDNF expression on (C) apical and (D) basal dendrite length. (E) Quantification of the number of dendrite endpoints for each treatment. (F) Sholl analysis of dendritic complexity comparing neurons expressing BDNF to EV. (G-J) Sholl analysis of dendritic complexity comparing neurons co-expressing BDNF with each Wnt inhibitor to neurons expressing BDNF alone. Asterisks indicate comparisons to EV. Hash marks indicate comparisons to BDNF+EV. ***p<0.001, **p<0.01, *p<0.05. n=number of neurons: EV n=56, BDNF+EV n=52, BDNF+Wif1 n=38, BDNF+Sfrp1 n=39, BDNF+Fzd8CRD n=39, BDNF+Dvl1ΔPDZ n=50. Scale Bar: 50μm.

proximal arbor complexity may be a result of the ability of BDNF to induce primary dendrite formation, which I explore further in the next section.

The effects of BDNF on dendrite arbors that I observed complicated the interpretation of the effects of co-expression of the Wnt inhibitors with BDNF. Co-expression of three of the Wnt inhibitors (Sfrp1, Fzd8CRD and Dvl1ΔPDZ) with BDNF caused a significant decrease in total dendrite length when compared with EV control (Fig 2.4B), while only one Wnt inhibitor (Sfrp1) caused a significant decrease in the number of dendrite endpoints (Fig. 2.4E). Sholl analysis of dendritic complexity revealed that the same general pattern of BDNF expression increasing proximal dendritic complexity while decreasing distal complexity remained (Fig. 2.4G-J), but the absence of significant differences at individual data points for some of the Wnt inhibitors makes this conclusion difficult to support. In general, the only statement that I feel comfortable making is that BDNF is unable to rescue any defects in dendrite growth or elaboration caused by Wnt inhibition. However, because BDNF expression on its own was unable to clearly increase any of the parameters associated with dendrite growth and elaboration, it is unclear if BDNF should function in this manner. Reasons for the observed effects of BDNF on dendrite growth will be further addressed in Chapter 4.

2.3.5 Wnt signaling is required for BDNF-induced cortical dendritic spine formation.

The main hypothesis of my work is that Wnt signaling is required during BDNF-induced dendritic spine formation. To test this I measured dendritic spine density of neurons expressing BDNF in combination with each of the four Wnt inhibitors. Representative images of dendritic segments are shown in Fig 2.4A. Quantification of dendritic spine density revealed that BDNF expression led to a significant 27±3%, increase in dendritic spine density (Fig. 2.4B). Importantly, this increase was blocked by co-expression of all four of the Wnt inhibitors (Fig. 2.4B), suggesting that Wnt signaling is required for BDNF-induced formation. Wnt inhibition alone appeared to impair dendritic spine maturation. I additionally determined whether Wnt inhibition prevents BDNF-induced dendritic formation by impairing spine maturation. Previous work demonstrated that BDNF promotes dendritic spine formation in part by regulating the maturation of dendritic spine synapses (Tyler and Pozzo-Miller, 2003). In order to determine if Wnt inhibition blocked the ability of BDNF to increase dendritic spine maturation, I measured dendritic spine length and dendritic spine head width of dendritic spines from neurons co-expressing BDNF and the four Wnt inhibitors. Quantification of dendritic spine length revealed that BDNF expression alone had no effect on average dendritic spine length (Fig. 2.4C). Additionally, effects of BDNF co-expression with the Wnt inhibitors varied. While Wif1 and Dvl1ΔPDZ no longer induced a significant increase in dendritic spine length in comparison to EV control, both Sfrp1 and Fzd8CRD did (Fig. 2.4C). Similar to the results obtained during Wnt inhibition alone, all four Wnt inhibitors decreased the fraction of short

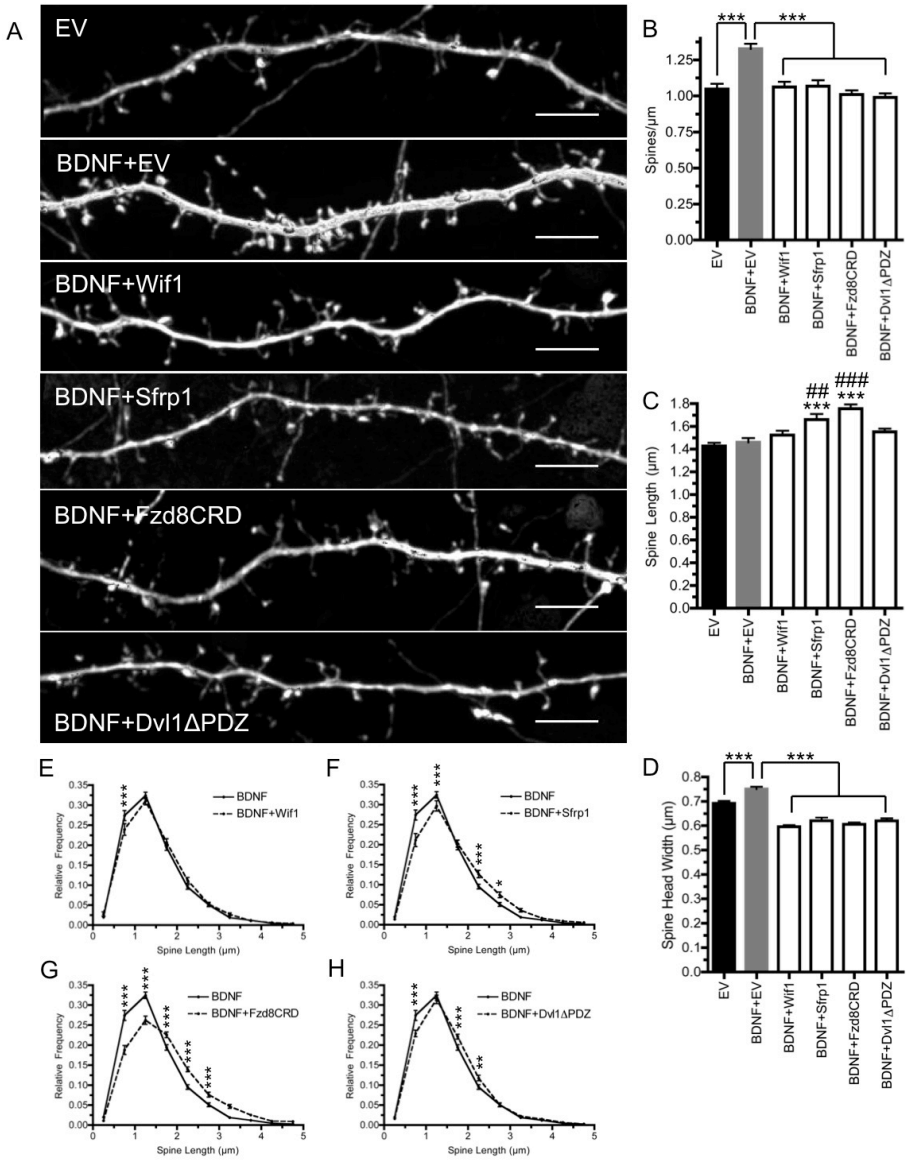

Figure 2.4. Wnt inhibition blocks BDNF-induced dendritic spine formation. (A) Representative dendritic of cortical neurons expressing EV, BDNF with EV, BDNF with Wif1, BDNF with Sfrp1, BDNF with Fzd8CRD and BDNF with Dvl1ΔPDZ. Quantification of dendritic spine density (B), average spine length (C) and average spine head width (D) for each treatment. (E-H) Relative frequency distributions comparing the distribution of spine length for expression of BDNF plus inhibitor to that of BDNF plus EV. Asterisks indicate comparisons to EV. Hash marks indicate comparisons to BDNF+EV. ***p<0.001, **p<0.01, *p<0.05. n=number of neurons: EV n=31, BDNF+EV n=30, BDNF+Wif1 n=29, BDNF+Sfrp1 n=25, BDNF+Fzd8CRD n=25, BDNF+Dvl1ΔPDZ n=30. Scale bar: 5μm.

spines and increased in the fraction of long spines when co-expressed with BDNF (Fig. 2.4E-H). In the absence of a clear effect of BDNF expression alone on dendritic spine length, these data are difficult to interpret. It should be noted, though, that expression of BDNF at an earlier time point (DIV7-11) was sufficient to decrease average spine length while also increasing dendritic spine density (Appendix Fig. 1B).

Despite the lack of an effect of BDNF on dendritic spine length when expressed from DIV10-14, BDNF expression alone significantly increased dendritic spine head width (Fig. 2.4D), suggesting that BDNF increased spine maturation. Interestingly, all four Wnt inhibitors blocked this increase (Fig. 2.4D). Further, the decrease in dendritic spine head width induced by all four Wnt inhibitors was significantly lower in comparison to EV control, indicating that BDNF co-expression with the Wnt inhibitors accentuated the dendritic spine maturation defect caused by inhibition of Wnt signaling alone (Fig. 2.4D). Together, these data suggest that Wnt signaling is required for BDNF-induced increases in dendritic spine density, perhaps because Wnt signaling regulates some aspect of BDNF-induced dendritic spine maturation.

2.3.6 Wnt signaling is not required for BDNF-induced primary dendrite formation

As previously mentioned, one possible reason for the increase in proximal dendritic complexity caused by BDNF may be a result of the ability of BDNF to induce the formation of primary dendrites. Indeed, previous work demonstrated

that BDNF expression rapidly and robustly increases primary dendrite formation in cortical neurons (Horch et al., 1999; McAllister et al., 1997; Wirth et al., 2003). I utilized this effect of BDNF to more succinctly determine whether inhibition of Wnt signaling affects the ability of BDNF to regulate dendrite growth. In order to determine whether Wnt signaling is required for BDNF-induced primary dendrite formation, I quantified the number of primary dendrites per neuron after 4DIV expression of BDNF in the presence of Wnt inhibition. Representative cell bodies displaying primary dendrites emanating from neuronal cell bodies are shown in Fig. 2.5A. BDNF expression caused a significant increase in primary dendrite formation (Fig. 2.5B). As previously mentioned, expression of any of the Wnt inhibitors did not have any effect on primary dendrite formation (Fig. 2.5B). Lastly, none of the four Wnt inhibitors blocked the ability of BDNF to increase primary dendrite formation (Fig. 2.5B). Together, these data indicate that Wnt signaling is not required for primary dendrite formation. Further, these data suggest that the mechanism underlying BDNF-mediated induction of primary dendrite formation is independent of Wnt signaling.

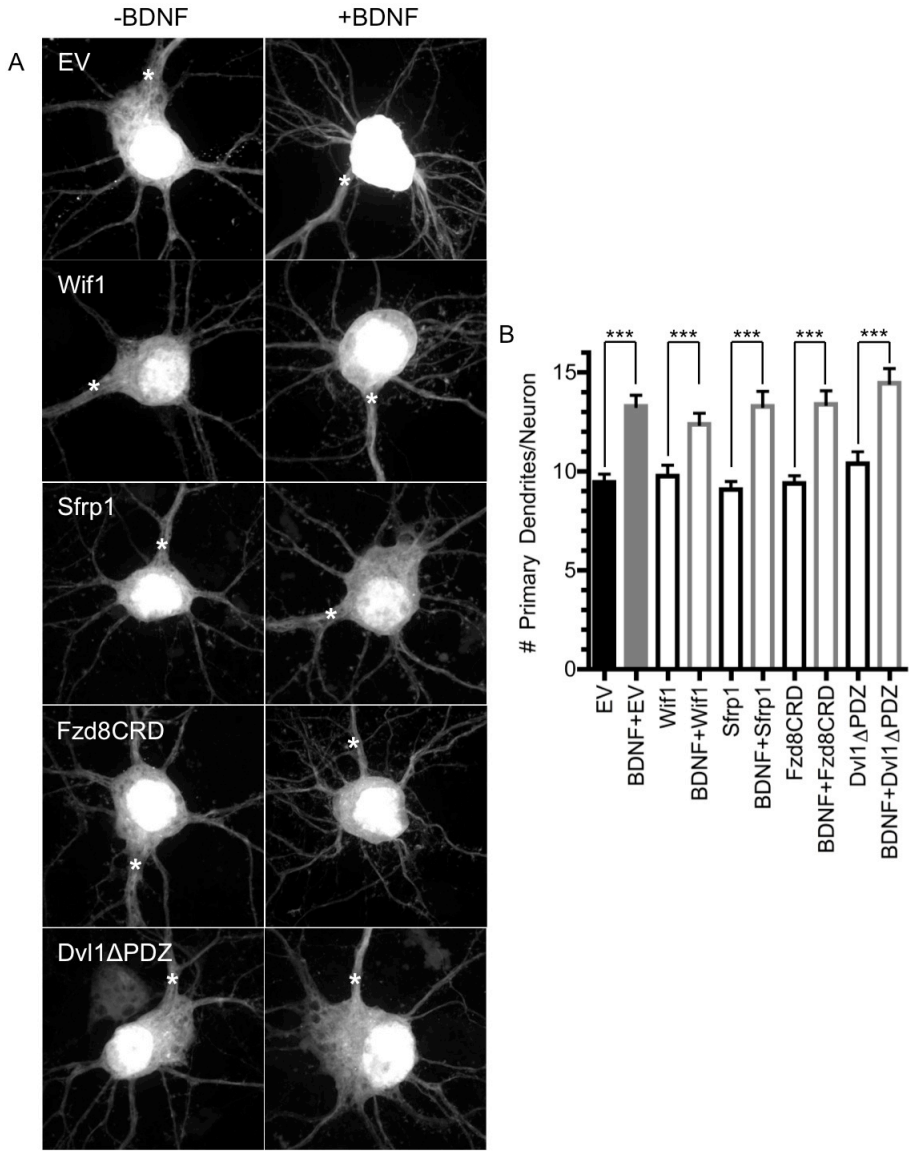

Figure 2.5. Wnt inhibition does not affect primary dendrite formation during baseline or BDNF-induced growth conditions. (A) Representative cell bodies of cortical neurons expressing EV, Wif1, Sfrp1, Fzd8CRD and Dvl1ΔPDZ either alone, or in combination with BDNF. (B) Quantification of the number of primary dendrites per neuron for each treatment. ***p<0.001. n=number of neurons: EV n=31, BDNF n=31, Wif1 n=34, BDNF+Wif1 n=29, Sfrp1 n=22, BDNF+Sfrp1 n=21, Fzd8CRD n=25, BDNF+Fzd8CRD n=25, Dvl1ΔPDZ n=25, BDNF+Dvl1ΔPDZ n=30. Scale Bar: 5μm.

2.3.7 Wnt signaling is not required for BDNF-induced c-Fos expression

One possible explanation for the reduced ability of BDNF to increase dendritic spine density during Wnt inhibition is that somehow the ability of BDNF to signal through its receptor TrkB is impaired. Although the data examining BDNF-induced primary dendrite formation suggest that at least some aspects of BDNF signaling are unaffected, I also examined the induction of the expression of a downstream BDNF signaling target, c-Fos. c-Fos is an immediate early gene whose transcription is rapidly upregulated by BDNF/TrkB signaling (Calella et al., 2007; Gaiddon et al., 1996). In this experiment I used a tetracycline-inducible BDNF expression plasmid to express BDNF in a temporally restricted manner. Transfected neurons expressed all four Wnt inhibitors for 2DIV prior to the induction of BDNF expression. Neurons were then treated with doxycycline in order to induce BDNF expression for 12 hours. After that time, neurons were fixed and stained for c-Fos protein. Representative cell bodies stained for c-Fos are shown in Fig 2.6A. Quantitation of nuclear c-Fos expression revealed that 12 hours of BDNF expression induced a significant increase in c-Fos induction (Fig 2.6B). 2DIV expression of any of the Wnt inhibitors did not block the ability of BDNF to induce c-Fos expression (Fig 2.6B), suggesting that Wnt inhibition did not impair the ability of BDNF to signal through TrkB.

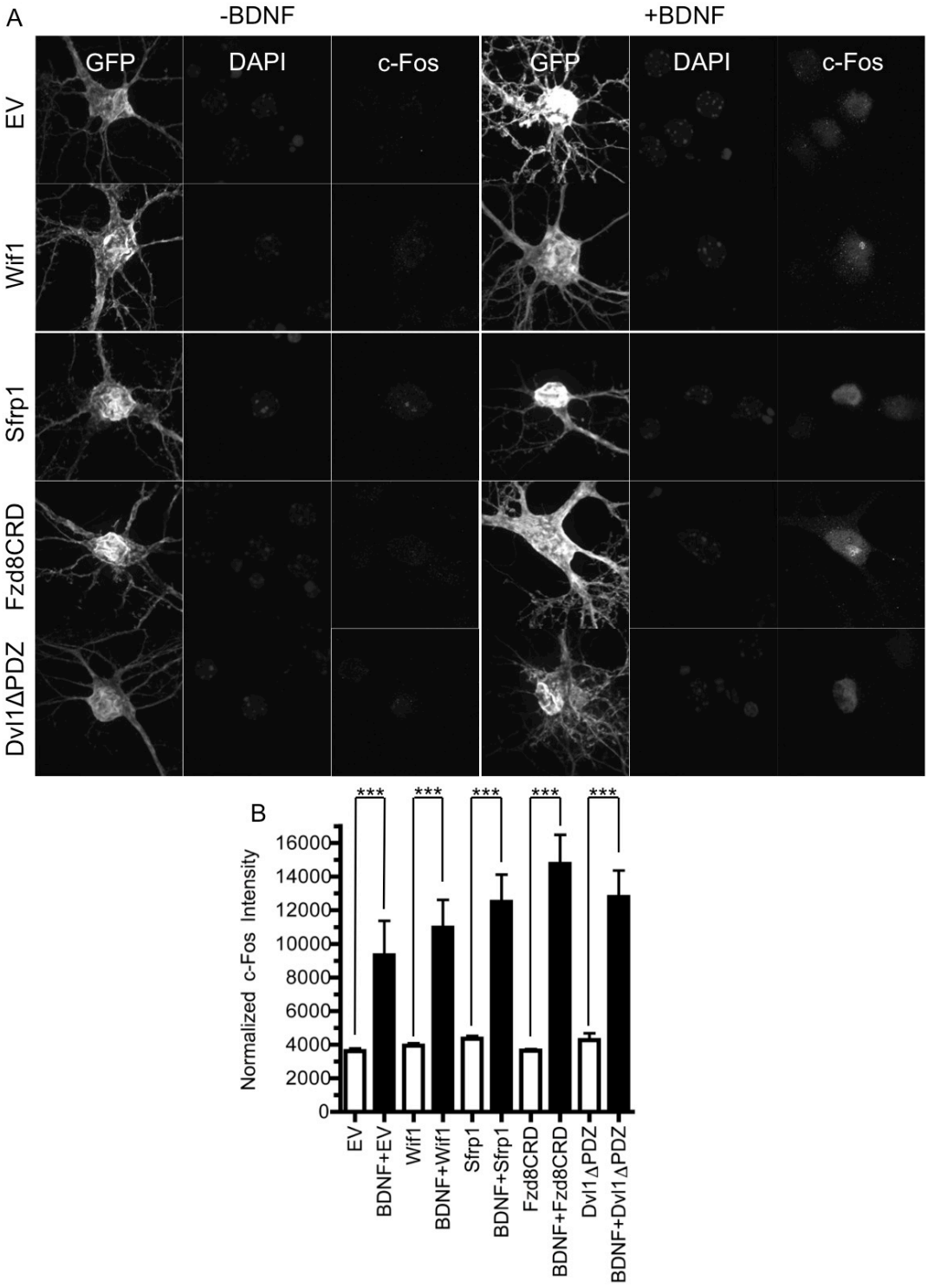

Figure 2.6. Wnt inhibition does not block BDNF-induced c-Fos expression. (A) Representative cell bodies of cortical neurons expressing EV, Wif1, Sfrp1, Fzd8CRD and Dvl1ΔPDZ either alone, or in combination with 12 hours of expression of BDNF, and stained for c-Fos. (B) Normalized nuclear c-Fos intensity for each treatment. ***p<0.001. n=10 neurons for each treatment.

2.4 CONCLUSIONS

The data presented in this chapter lead to several conclusions. First, inhibition of Wnt signaling impairs overall cortical dendrite growth and modestly impairs dendritic spine formation. Second, BDNF overexpression promotes cell autonomous increases in dendritic spine density and also promotes the maturation of dendritic spines. Importantly, the ability of BDNF to increase dendritic spine density and maturation is blocked by expressing all four Wnt inhibitors tested. Third, BDNF robustly increases the formation of primary dendrites in Wnt signaling-independent manner. In sum, the data I present in this chapter support a critical role for Wnt signaling in mediating the effects of BDNF on dendritic spine formation in cortical neurons and suggest that Wnt signaling is required for BDNF-induced dendritic spine formation.

CHAPTER 3: Examining the role of Wnt2 and Wnt4 during cortical dendrite growth and dendritic spine formation

3.1 INTRODUCTION

3.1.1 Wnt2: Initial discovery and generation of the $Wnt2^{KO}$ mouse

Wnt2 is a secreted glycoprotein in the Wnt family that is conserved throughout vertebrates. Wnt2 was cloned from a human lung cDNA library and found to encode a protein homologous to human Wnt1 (Wainwright et al., 1988). Murine Wnt2, originally termed m-irp (murine *int-1*-related protein), was cloned from 8.5-day mouse embryo due to its high sequence homology with human Wnt2 (McMahon and McMahon, 1989). Initial studies examining Wnt2 expression indicated that Wnt2 is highly expressed in the fetal pericardium and placental tissue, and also in the adult heart and lungs (McMahon and McMahon, 1989). Disruption of Wnt2 expression by homologous recombination results in perinatal lethality due to improper placentation in approximately 50% of $Wnt2^{-/-}$ pups (Monkley et al., 1996).

3.1.2 Wnt2 roles in cancer and development

Numerous studies examining Wnt2 function indicate that Wnt2 regulates a variety of biological processes using different signaling mechanisms. Similar to many of the other Wnt proteins, upregulation of Wnt2 expression is associated with the development of cancer. Wnt2 is upregulated in both gastrointestinal cancer (Katoh, 2003) and in colorectal cancer (Park et al., 2009). Additionally, Wnt2 upregulation in Esophageal squamous cell carcinoma results in increased canonical ß-catenin-dependent transcription (Wang et al., 2011). Wnt2

expression is upregulated in human Non-small cell lung carcinoma tissue (You et al., 2004). Interestingly, abrogation of Wnt2 signaling utilizing an antibody directed against Wnt2 induces apoptosis in cells derived from this tissue (You et al., 2004). Related, inhibition of Wnt2 through siRNA-induced knockdown decreases cytosolic ß-catenin levels and TCF-reporter activity, and induces apoptosis in human colorectal cancer cells (Shi et al., 2007). Wnt2-induced signaling is also implicated in cancer cell invasiveness, but this process is regulated by non-canonical activation of gene transcription through a signaling pathway involving JNK activation of AP-1 (Le Floch et al., 2005).

Wnt2 signaling is also implicated in regulation of normal development. Wnt2 promotes the specification of lung progenitors in a ß-catenin transcription-dependent manner (Goss et al., 2009). Additionally, Wnt2 coordinately regulates the development of organs derived from the early foregut with Wnt2b and Fzd5 (Poulain and Ober, 2011). Wnt2 also regulates the differentiation of cardiac myocytes derived from embryonic stem cells (Onizuka et al., 2012). Interestingly, this process appears to be dependent on non-canonical activation of transcription through JNK mediated regulation of AP-1.

3.1.3 Wnt2 roles in the CNS

Accumulating evidence from a number of studies implicates Wnt2 in the development and maintenance of the vertebrate nervous system. Wnt2 expression regulates cell progenitor proliferation in the developing midbrain, which gives rise to portions of the adult brainstem, in a canonical ß-catenin

dependent manner (Sousa et al., 2010). Additionally, in a model for neuronal differentiation, retinoic acid treatment of NT2 cells results in an increase of Wnt2 expression (Katoh, 2002).

Wnt2 also regulates the post-natal nervous system. Wnt2 is expressed in the developing hippocampus during the first two post-natal weeks and promotes dendritic growth of hippocampal neurons (Wayman et al., 2006). Further, Wnt2 expression in hippocampal neurons is regulated by neural activity (Wayman et al., 2006). Wnt2 is also expressed in the adult mouse striatum. Interestingly, a translational profiling study utilizing translating ribosome affinity purification (TRAP) of mRNAs indicates that Wnt2 mRNA is specifically expressed in Dopamine receptor-1 (Drd1) positive striatal medium spiny neurons and is enriched in a population of mRNAs that are highly associated with ribosomes (Doyle et al., 2008).

3.1.4 Wnt2 association with neuropathologies

Dysregulation of Wnt2 expression is associated with several different neuropathologies. Several studies have investigated the potential association of various Wnt2 SNPs with autism, a neurodevelopmental disease believed to arise due to improper development of neural connectivity (Geschwind and Levitt, 2007; LeBlanc and Fagiolini, 2011). Four population-based studies demonstrated positive correlations between different Wnt2 SNPs and autism (Chien et al., 2011; Lin et al., 2012; Marui et al., 2010; Wassink et al., 2001). However, two other family-based studies failed to find an association (Li et al., 2004; McCoy et

al., 2002). These discrepancies may be explained by the huge spectrum of causes of autism-like disorders. Nonetheless, these studies indicate that Wnt2 may play a role in the development of autism.

Wnt2 expression is also reduced in the cortex and hippocampus of $Fmr1^{KO}$ mice, a model for Fragile-X syndrome (Zhang et al., 2009). Fragile-X syndrome is a neurodevelopmental disorder related to autism. Like autism, Fragile-X syndrome may be caused by improper development of neural connectivity, and is characterized by an overabundance of long and immature dendritic spines (Irwin et al., 2001; Nimchinsky et al., 2001).

A small number of studies also implicate Wnt2 in depression. Several different classes of antidepressant drugs increase Wnt2 expression in the brain (Okamoto et al., 2010). Additionally, rats exposed to chronic electroconvulsive seizure treatment, which is a model of one therapeutic intervention used to treat depression, increases Wnt2 expression in the dentate gyrus of the hippocampus (Madsen et al., 2003). Importantly, increased proliferation of adult hippocampal neural progenitors and subsequent integration of those cells into pre-existing neural circuitry within the dentate gyrus may to play a role in the recovery from depression (Danzer, 2012). BDNF is also implicated in this process, which I will discuss in further detail in Chapter 4.

3.1.5 Wnt4 roles during mammalian development

Like Wnt2, Wnt4 is a member of the Wnt family of secreted glycoproteins and is conserved throughout vertebrates. Initial indications for the function of

Wnt4 in mammals came from examination of $Wnt4^{KO}$ mice in which Wnt4 exon 3 was replaced with a neomycin resistance cassette by homologous recombination (Stark et al., 1994). $Wnt4^{KO}$ mice die perinatally due to kidney failure and display severe deficits in kidney development. Subsequent studies revealed that Wnt4 is expressed in mesenchymal tissue within the developing kidney and is required for the mesenchymal to epithelial transformation during the formation of kidney nephrons (Kispert et al., 1998). Wnt4 loss-of-function in humans is associated with severe renal hypoplasia syndrome, indicating that the role for Wnt4 during kidney development is conserved throughout mammals (Malinow and Malenka, 2002).

In addition to its well-characterized role during nephron formation in the developing kidney, Wnt4 is also a critical regulator of female reproductive tract formation. Loss of Wnt4 leads to masculinization of female mouse embryos, as observed by the absence of Mullerian structures and the presence of the Wolffian duct (Vainio et al., 1999). Wnt4 was the first human gene identified to direct the development of the bipotential gonad toward ovaries (Biason-Lauber and Konrad, 2008). The mechanism of Wnt4 signaling during sex determination is unknown. However, Wnt4 is capable of transducing a canonical ß-catenin-dependent signal through the Fzd6 receptor in MDCK cells (Lyons et al., 2004).

3.1.6 Wnt4 roles in the mammalian CNS and PNS

Recent work implicates Wnt4 as a regulator of various functions within the mammalian CNS and PNS. First, a gradient of Wnt4 expression in the floorplate

of the developing spinal cord acts as an attractive signal for commissural axons after midline crossing (Lyuksyutova et al., 2003). A subsequent study demonstrated that Wnt4 regulates axon guidance through a signaling cascade involving Ca^{2+}-independent PI3K-mediated activation of atypical-Protein Kinase C (aPKC) (Wolf et al., 2008). Second, Wnt4 may also regulate neuronal differentiation. Treatment of NT2 cells with retinoic acid induces Wnt4 expression, and Wnt4 loss-of-function impairs retinoic acid-induced expression of several genes known to promote early neuronal differentiation (Elizalde et al., 2011). Third, Wnt4 is required for the proper formation of the vertebrate NMJ and may regulate this process by promoting postsynaptic acetylcholine receptor (AChR) clustering in the muscle by signaling through muscle-specific kinase (MuSK), a receptor tyrosine kinase (Strochlic et al., 2012).

3.2 EXPERIMENTAL PROCEDURES

For procedures relating to cortical neuron culture, neuron transfection, image acquisition and analysis, and statistical analysis, the reader is referred to the experimental procedures outlined in Chapter 2. The following procedures were used specifically during the experiments outlined in this chapter.

3.2.1 Plasmid Construction

pTRE-tight-Wnt2 was constructed by inserting the murine Wnt2 ORF into the pTRE-tight backbone (Clontech). pTRE-tight-UTR-Wnt2.3 was constructed

by inserting the endogenous murine Wnt2 3'UTR immediately downstream of the Wnt2 coding region in pTRE-tight-Wnt2. pTRE-tight-Wnt2 and pTRE-tight-UTR-Wnt2.3 were co-transfected with pCMV-rtTA in order to allow for inducible expression of Wnt2 as described in the experimental procedures for Chapter 2. pTRE-tight-Wnt2 was used in the experiments describing Wnt2 overexpression from DIV10-14. pTRE-tight-Wnt2UTR-GFP3, which was used to assay for the targeting ability of shWnt2.3, was constructed by inserting the same portion of the endogenous murine Wnt2 3'UTR as previously described immediately downstream of the coding region for GFP in pTRE-tight-GFP. pCMV-Wnt2Flag was constructed by inserting a coding sequence for c-terminally Flag-tagged murine Wnt2 in place of GFP in the p-CMV-EGFPN1 backbone. pCMV-Wnt4 was constructed by inserting the coding region for murine Wnt4 in place of GFP in the p-CMV-EGFPN1 backbone.

3.2.2 Quantitative Reverse Transcriptase PCR

DIV10 cultured neurons were treated for 4 hours with either 50ng/mL recombinant BDNF (Millipore, Billerica, MA), 4μm Tetrodotoxin (TTX, Sigma Aldrich), or both BDNF and TTX in combination. Total RNA was prepared using Trizol reagent (Life Technologies Corporation) according to manufacturer's instruction. RNA was converted to cDNA using the iScript cDNA synthesis kit (BIO-RAD, Hercules, CA) according to manufacturer's instruction. Quantitative real-time PCR analysis was performed using an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies Corporation). Wnt2 mRNA

abundance was normalized to 18s RNA. Reactions were performed in triplicate, in two separate experiments.

Primer sequences (all 5' to 3'):

Wnt2 F: GTCCTCCTCCGAAGTAGTCG
Wnt2 R: TCTTTGGATCACAGGAGCAG

Wnt4 F: AGACGTGCGAGAAACTCAAAG
Wnt4 R: GGAACTGGTATTGGCACTCCT

3.2.3 Wnt2^{KO} mouse breeding and genotyping

Wnt2^{KO} mice were generated by homologous recombination. Briefly, a neomycin resistance cassette was inserted into exon 2 of the murine Wnt2 gene in order to disrupt Wnt2 gene expression (Monkley et al., 1996). Wnt2^{KO} mice were obtained from the Richard Lang laboratory at the Cincinnati Children's Hospital Medical Center (Cincinnati, OH). Wnt2^{KO} mice were maintained in a CD1 strain background as heterozygotes. Homozygous Wnt2-null mice were generated by heterozygous timed matings. Wnt2KO mouse were genotyped by PCR amplification of three distinct amplicons from isolated genomic DNA. A diagram of the PCR amplification strategy and location of primers in relation to the wild type and Wnt2 null genomic locus is shown in Appendix Fig. 9.

Primer sequences (all 5' to 3'):

Wnt2.Geno F: CCAGGTACATGAGAGCTACAGG
Wnt2.Geno R: TCACTTCGGAGGAGGACC
Neo.Geno F: ATCTCCTGTCATCTCACCTTGC
Neo.Geno R: CAAGCTCTTCAGCAATATCACG
PGK1 R: GACTGCCTTAAAAGCGCCTCCC

3.2.4 Generation of Wnt2 shRNA constructs

Wnt2 shRNAs constructs were generated by cloning targeting sequences into the lentiviral vector pLentiLox 3.7 (pLL3.7) backbone (Rubinson et al., 2003). Wnt2 shRNA targets were chosen by cross-referencing target selections from the program pSicoliogomaker 1.5 (Jacks Lab, Massachusetts Institute of Technology, Cambridge, MA) with suggested shRNA target selections from software programs provided by Dharmacon and Clontech. Selected 19mer target sequences were then loaded into pSicoligomaker 1.5 in order to generate longer DNA oligomers to be used for subsequent cloning. DNA oligos were cloned into the pLL3.7 backbone according to protocols provided by the Jacks Lab (http://web.mit.edu/jacks-lab/protocols/pll37.htm).

Wnt2 shRNAs were tested for their ability to knockdown Wnt2 expression by co-expressing the shRNA plasmids either with a plasmid encoding Wnt2 (pCMV-Wnt2-Flag), or a plasmid encoding an shRNA sensitive GFP construct (p-TRE-Wnt2UTR-GFP.3). Details and results of these experiments are shown in Appendix Figs. 10-12. Prior to their final use, the CMV-GFP expression cassette was removed from all pLL3.7 based shRNA constructs. For the DIV7-10 shRNA experiment analyzing dendritic arbors, 500ng total plasmid DNA was transfected per well of a 12-well culture dish, which included 100ng of the appropriate shRNA plasmid. For the DIV10-14 experiment, 1.5µg of total plasmid DNA was transfected per well of a 12-well culture dish, which included 25ng of the appropriate shRNA plasmid.

19mer targeting sequences (All 5' to 3'):

shLuc: GATATGGGCTGAAGACAAA
shWnt2.1: GTAGCCAAGGAGAATTAAA
shWnt2.2: GCTTCACTGTAGCCAATAA
shWnt2.3: GAAAGATGGCTTCCAATAA
shWnt2.3.Scr: GAGAGTCAACGTGATATAA

3.3 RESULTS

3.3.1 Wnt2 expression analysis in the developing and adult brain

Our microarray analysis indicated that Wnt2 expression is decreased in the striatum of forebrain-specific BDNF conditional null mice (Strand et al., 2007). Additionally, Wnt2 expression is significantly decreased in both the anterior and posterior cortex (Table 1.1). Together, this suggested that BDNF regulates expression of Wnt2. I chose to more carefully examine Wnt2 as a candidate BDNF-regulated Wnt gene for several additional reasons. First, Wnt2 expression is increased by neural activity in hippocampal neurons (Wayman et al., 2006). Interestingly, BDNF expression is known to mediate some activity-dependent neuronal processes (Ghosh et al., 1994; Kuczewski et al., 2010). Second, Wnt2 is sufficient to increase dendrite length in developing hippocampal neurons (Wayman et al., 2006) and, similarly, BDNF influences dendrite growth in cortical neurons (Gorski et al., 2003; McAllister et al., 1997; Xu et al., 2000b). Third, antidepressant drug treatment can increase Wnt2 expression (Okamoto et al., 2010) and BDNF has been shown to be a crucial mediator of these antidepressants (Adachi et al., 2008; Shirayama et al., 2002).

I initially examined the expression pattern of Wnt2 in the developing brain using the Allen Developing Mouse Brain Atlas (Allen Developing Mouse Brain

Atlas, 2009). This analysis revealed that Wnt2 is expressed in several regions of the brain at P14, a developmental time period of active synapse and dendritic spine addition *in vivo* that is similar to the age of neurons used in our *in vitro* experiments. Notably, Wnt2 is expressed in the hippocampus, specifically in the CA1 and CA3 regions (Fig. 3.1A). Additionally, Wnt2 is expressed in the medial striatum (Fig. 3.1B). Lastly, Wnt2 is expressed in both the motor cortex and the visual cortex (Fig. 3.1C, D). In both cortical regions, Wnt2 expression is concentrated in layer II/III and layer V, suggesting that Wnt2 is primarily expressed in cortical excitatory pyramidal neurons. Importantly, dendritic spine formation on neurons in each of these brain regions is influenced by BDNF (Baquet et al., 2004; Chakravarthy et al., 2006; Rauskolb et al., 2010; Vigers et al., 2012). I also examined the expression pattern of Wnt2 at P56 using the adult Allen Brain Atlas in order to ascertain whether Wnt2 is expressed in the adult brain (Allen Mouse Brain Atlas, 2009). This analysis revealed that Wnt2 is expressed in the same structures in the adult brain as it is in the developing brain, albeit at lower levels (Appendix Fig. 5), and suggests that Wnt2 may regulate both nervous system development and maintenance.

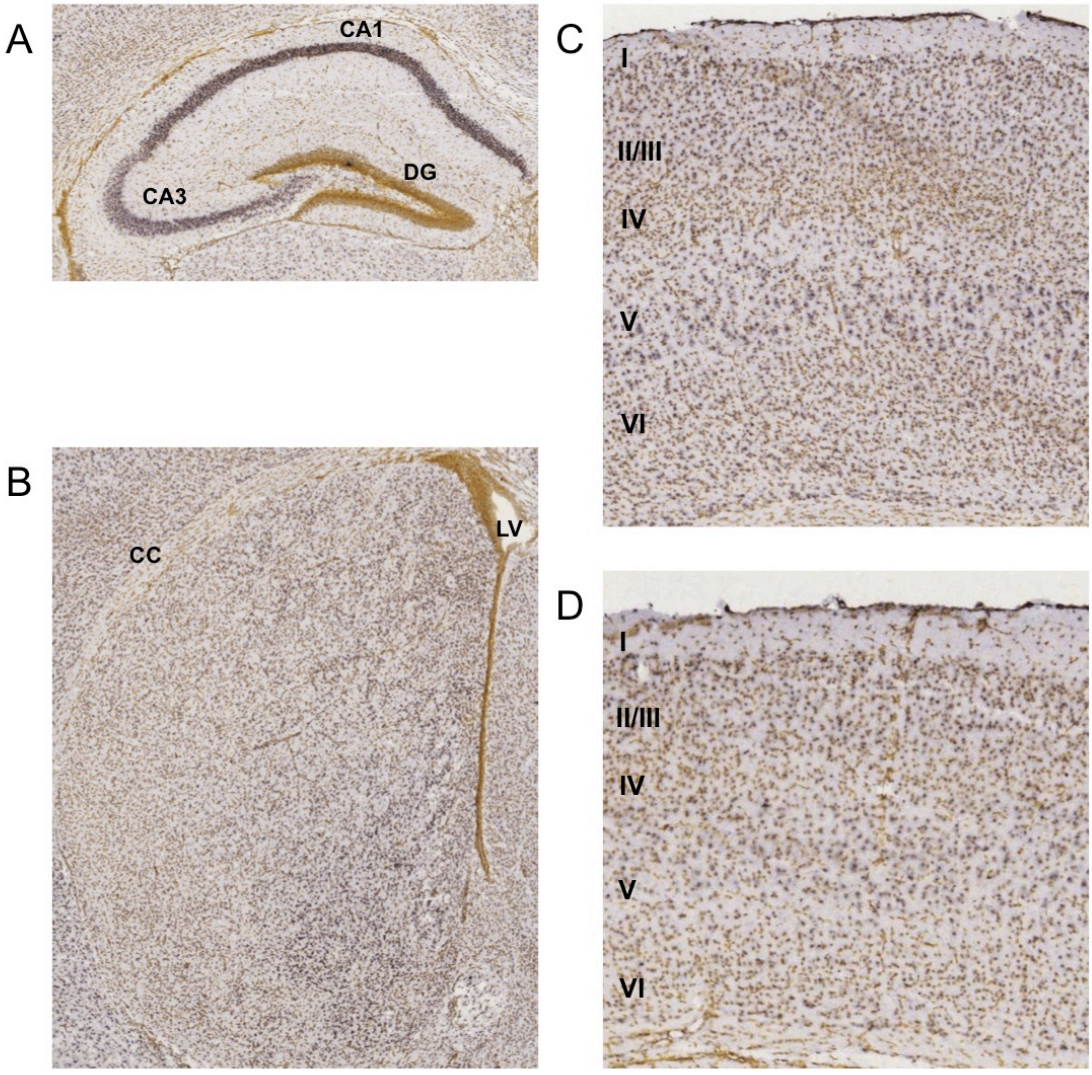

Figure 3.1. Wnt2 is expressed in the developing mouse brain. *in situ* hybridization images taken from the Allen Developing Mouse Brain Atlas (Allen Developing Mouse Brain Atlas, 2009) showing Wnt2 expression (purple color) in the (A) hippocampus, (B) striatum, (C) motor cortex, and (D) visual cortex.

3.3.2 BDNF treatment increases Wnt2 mRNA expression

To determine whether BDNF regulates Wnt2 expression, we treated DIV10 cultured cortical neurons with recombinant BDNF and used qRT-PCR to determine Wnt2 mRNA abundance (Fig. 3.2). Application of recombinant BDNF resulted in an approximate 3-fold increase in the level of Wnt2 mRNA. Additionally, co-treatment with tetrodotoxin (TTX), which blocks voltage-gated sodium channels, demonstrated that the BDNF-induced increase of Wnt2 mRNA abundance occurs in the absence of evoked neural activity. Four hour recombinant BDNF treatment also increased Wnt2 expression in cortical neurons treated on DIV16 (Appendix Fig. 14). These data are consistent with the possibility that Wnt2 is a target of BDNF regulation in cortical neurons and suggests that BDNF can regulate Wnt2 expression independently of neural activity.

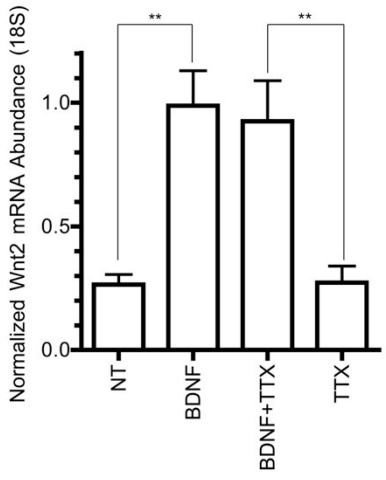

Figure 3.2. Wnt2 expression is regulated by BDNF. Quantification of Wnt2 mRNA abundance after 4-hour treatment of DIV10 cultured cortical neurons with recombinant BDNF (50 ng/mL), TTX (4μM) or both. **p<0.01. n=number of wells: NT n=5, BDNF n=4, BDNF+TTX n=5, TTX n=4.

3.3.3 Wnt2 promotes cortical dendrite growth

I next sought to determine whether Wnt2 overexpression by cortical neurons influences their dendritic morphology. A previous study indicated that Wnt2 overexpression in hippocampal neurons was increased dendrite growth and branching (Wayman et al., 2006). I first determined whether Wnt2 similarly affected cortical neuron by measuring three different aspects of dendritic arbors: total dendrite length, number of dendrite endpoints, and overall dendritic complexity. Representative images of cortical neurons expressing Wnt2 for 4DIV are shown in Figure 3.3A. I found that Wnt2 overexpression resulted in a small but significant increase in total dendrite length (Fig. 3.3B), consistent with what has been shown previously for Wnt2 in hippocampal neurons (Wayman et al., 2006). Additionally, Wnt2 expression resulted in a small but significant increase in the number of dendrite endpoints (Fig. 3.3C). However, Wnt2 did not significantly increase overall dendritic complexity as measured by Sholl analysis, nor did it increase the number of primary dendrites (Fig. 3.3D, E). Together, these data indicate that Wnt2 expression in cortical neurons is sufficient to increase dendrite growth, but that it only modestly increases dendrite branching.

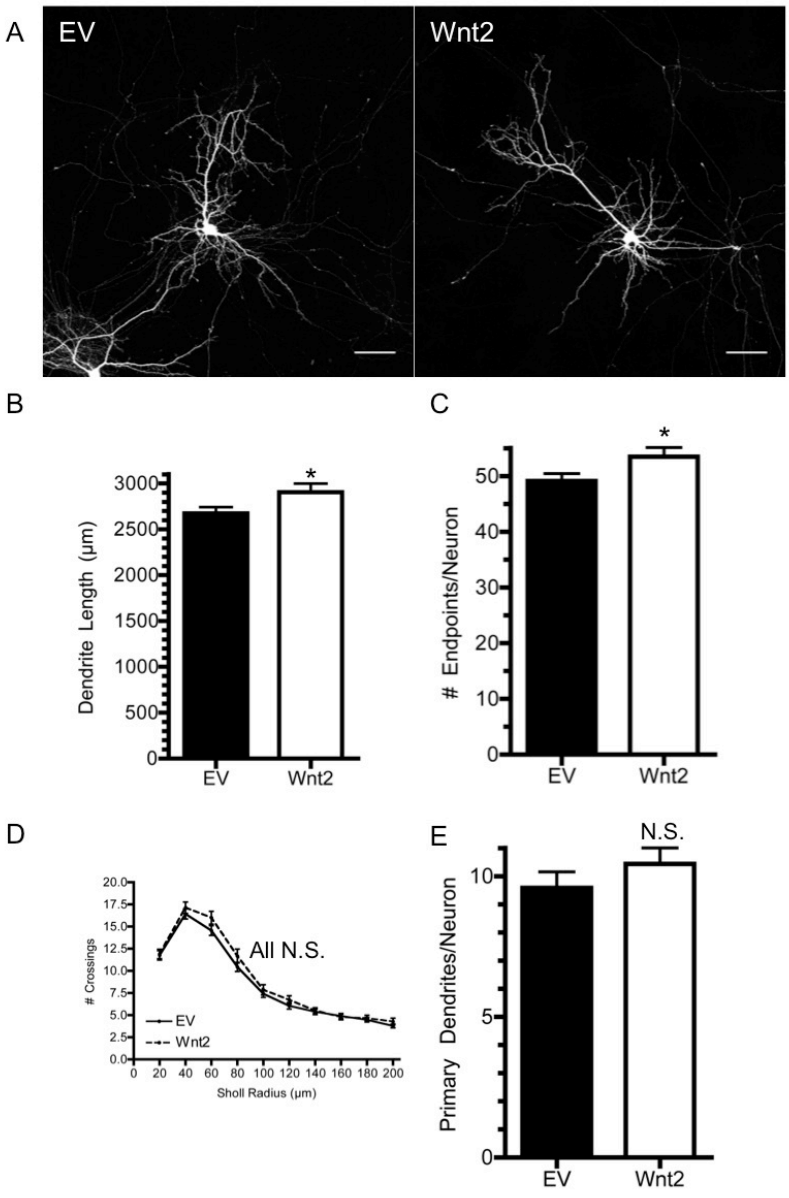

Figure 3.3. Wnt2 is sufficient to increase cortical dendrite length. (A) Representative cortical neurons expressing either EV or Wnt2 from DIV10-14. Quantification of (B) the total dendrite length per neuron and (C) the number of dendritic endpoints per neuron for each treatment. (D) Sholl analysis of dendritic complexity comparing Wnt2 expressing neurons to control. (E) Quantification of the number of primary dendrites per neuron for each treatment. *p<0.05. n=number of neurons: EV n=52, Wnt2 n=44. Scale Bar: 50µm.

3.3.4 Wnt2 promotes dendritic spine formation and maturation

Next, I determined whether Wnt2 expression is sufficient to increase dendritic spine density in cortical neurons. Using the same 4DIV expression paradigm as above, I quantified dendritic spines in the same manner as described in Chapter 2. Images of representative dendrite segments from cortical neurons expressing Wnt2 are shown in Figure 3.4A. Quantification revealed that Wnt2 caused a 17±4% increase in dendritic spine density (Fig. 3.4B). Additionally, Wnt2 caused a significant decrease in average dendritic spine length (Fig. 3.4C). The decrease in average spine length is mirrored by the relative frequency distribution plot of spine lengths, which reveals that Wnt2 expression significantly increased the fraction of short spines and significantly decreased the fraction of long spines (Fig. 3.4E). Lastly, Wnt2 expression significantly increased average dendritic spine head width (Fig. 3.4D). Together, these data indicate that Wnt2 is sufficient to increase cortical dendritic spine formation, and may play an important role in promoting dendritic spine maturation by possibly increasing the stability of dendritic spine contacts.

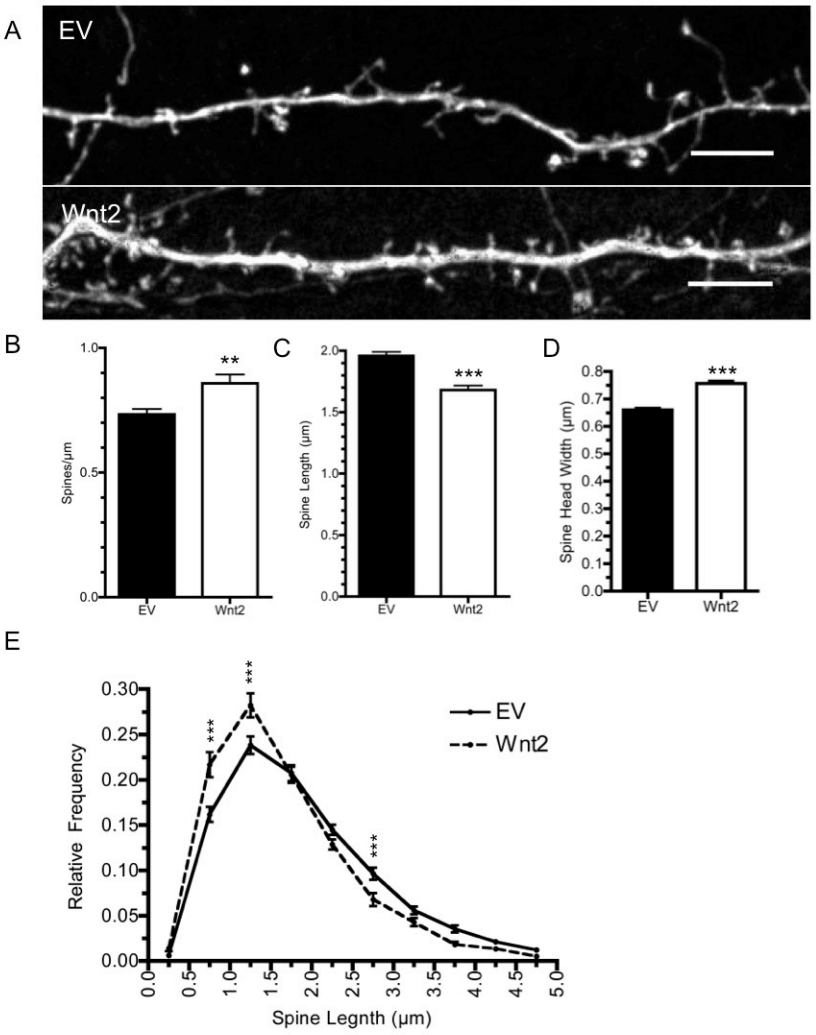

Figure 3.4. Wnt2 is sufficient to increase dendritic spine formation in cortical neurons. (A) Representative dendritic segments of cortical neurons expressing either EV or Wnt2 from DIV10-14. Quantification of (B) dendritic spine density, (C) average dendritic spine length, and (D) average spine head width for each treatment. (E) Relative frequency distribution comparing the distribution of spine length for Wnt2 to control. $^{**}p<0.01$, $^{***}p<0.001$. n=number of neurons: EV n=29, Wnt2 n=25. Scale Bar: 5µm.

3.3.5 Wnt2^{KO} dendritic spine analysis

In addition to determining the sufficiency of Wnt2 overexpression to increase dendritic spine formation, I attempted to determine whether Wnt2 expression is required for dendritic spine formation. I ultimately explored two different strategies to explore this question. The first strategy that I used was to quantify dendritic spine formation in cortical neurons cultured from Wnt2^{KO} mice. Wnt2 null mice were generated by inserting a neomycin resistance cassette into exon 2 of the Wnt2 gene (Monkley et al., 1996). Initial reports on the fertility and viability of this mouse strain indicated that approximately 50% of the Wnt2$^{-/-}$ mouse pups die perinatally, but that the remaining viable pups continue to develop to adulthood in a grossly normal fashion and are fertile (Monkley et al., 1996). However, in my hands, the viability of Wnt2$^{-/-}$ mouse was much lower. I was only able to successfully culture Wnt2$^{-/-}$ cortical neurons twice, as I suspect that the majority of Wnt2$^{-/-}$ died *in utero*.

Despite the low viability of Wnt2$^{-/-}$ offspring, I was able to perform an initial examination of dendritic spine formation in Wnt2$^{-/-}$ cortical neurons cultured from DIV7-11, quantifying both dendritic spine density and length. In this experiment, I compared Wnt2$^{-/-}$ neurons with neurons isolated from wild type littermates. Additionally, I overexpressed Wnt2 in neurons of each genotype in order to determine whether ectopic Wnt2 expression could rescue any observed phenotype. Representative dendritic segments from cortical neurons from each treatment are shown in Figure 3.5A. Quantification of dendritic spine density revealed no significant difference between any of the four treatments (Fig. 3.5B).

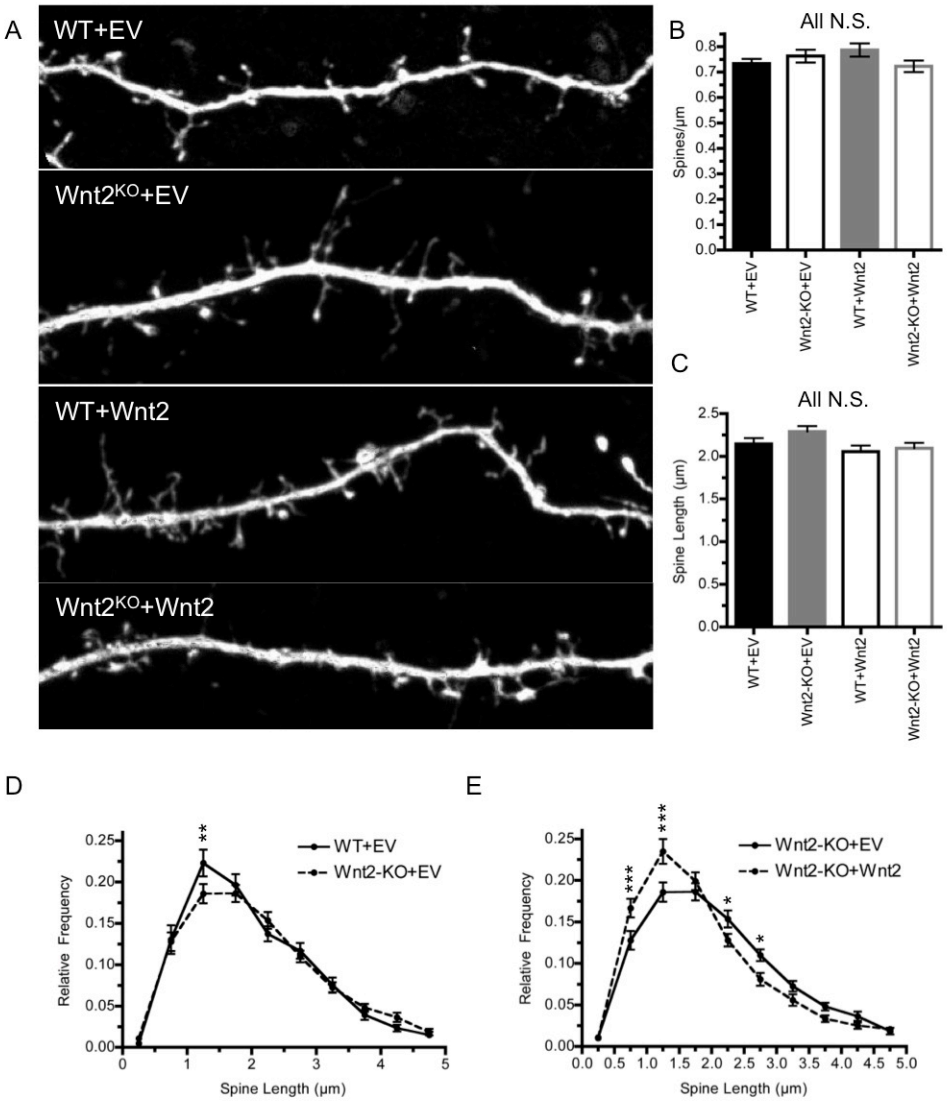

Figure 3.5. Wnt2KO neurons have longer dendritic spines. (A) Representative dendritic segments of cortical neurons from WT and Wnt^{KO} neurons expressing either EV or Wnt2 from DIV7-11. Quantification of (B) dendritic spine density and (C) average dendritic spine length for each treatment. (D) Relative frequency distribution comparing the distribution of spine length for WT and Wnt2^{KO} neurons expressing EV. (E) Relative frequency distribution comparing the distribution of spine length for Wnt2^{KO} neurons expressing EV or Wnt2. *p<0.05, **p<0.01, ***p<0.001. n=number of neurons: WT+EV n=26, Wnt2^{KO}+EV n=39, WT+Wnt2 n=19, Wnt2^{KO}+Wnt2 n=32.

Similarly, quantification of dendritic spine length revealed no significant difference between any of the four treatments (Fig. 3.5C), however, a trend toward an increase in average spine length of $Wnt2^{-/-}$ neurons suggested that spine length might have been affected. To further explore this trend, I examined relative frequency distributions of spine length. Interestingly, $Wnt2^{-/-}$ neurons displayed fewer short dendritic spines in comparison to WT (Fig. 3.5D). Importantly, ectopic Wnt2 expression in these neurons reversed this distribution (Fig. 3.5E), suggesting that the decreased proportion of short dendritic spines in $Wnt2^{-/-}$ is specifically due to the loss of Wnt2 expression.

3.3.6 Wnt2 shRNA knockdown analysis

The second strategy that I used to determine the requirement for Wnt2 during cortical dendritic spine formation utilized an shRNA knockdown approach. By transfecting plasmids that express different shRNAs targeting Wnt2, I aimed to reduce Wnt2 expression levels in isolated neurons, thus revealing a cell-autonomous requirement for Wnt2. To initially characterize the effects of Wnt2 shRNA knockdown, I observed and quantified dendrite growth parameters because these phenotypes were easier to identify and quantify than those associated with dendritic spines. I ultimately used a set of five different shRNA constructs in these experiments. I used three different shRNA constructs that targeted Wnt2 (shWnt2.1, shWnt2.2, shWnt2.3). The target site within the Wnt2 mRNA for each shRNA is shown in Appendix Fig. 10A. I also used two different negative control shRNA constructs. shLuc targets firefly luciferase, and

shWnt2.3.Scr consisted of a scrambled version of shWnt2.3. I confirmed that shWnt2.1 and shWnt2.2 properly targeted Wnt2 by co-expressing these constructs with a plasmid expressing a c-terminally Flag-tagged Wnt2 and assaying for levels of Wnt2-Flag by western blot. shWnt2.1 and shWnt2.2 nearly completely reduced Wnt2Flag expression when co-expressed at a 1:1 ratio (Appendix Fig. 10B). I confirmed this observation using immunofluorescence staining directed against Wnt2-Flag in a similar co-expression paradigm (Appendix Fig. 11). In order to test the ability of shWnt2.3 to reduce Wnt2 expression, I created a GFP expression construct that included the 3'UTR from mouse Wnt2. Co-expression of the GFP-Wnt2 3'UTR construct with shWnt2.3 significantly reduced GFP expression as assayed by western blot, while neither shLuc nor shWnt2.3.Scr had any effect (Appendix Fig. 10C). I confirmed the western blot by examining GFP fluorescence in cultured cells in a similar co-expression experiment (Appendix Fig. 12).

I first examined the effect of these shRNAs in cortical neurons when expressed from DIV7-10. In this experiment, I transfected 100ng of each shRNA plasmid per well of a 12-well culture dish. Interestingly, expression of all three targeting shRNA constructs reduced overall dendritic complexity as measured by Sholl analysis (Fig. 3.6A). In order to test whether the effects of the shRNA were due to specific knockdown of Wnt2 or to off-target effects, I ectopically co-expressed Wnt2 (which lacks the Wnt2 3'UTR) with shWnt2.3 (which targets the Wnt2 3'UTR). A previous study indicated that off target effects of shRNAs specifically in neurons are manifested by a decrease in dendrite growth and

branching, and a decrease in the formation of dendritic spines, presumably due to a cell-autonomous induction of the interferon response (Alvarez et al., 2006). Unfortunately, ectopic Wnt2 expression did not rescue the decrease in dendritic complexity (Fig. 3.6B), suggesting that this decrease was due to off-target shRNA effects.

I further examined the effect of shWnt2.3 when expressed from DIV10-14, quantifying both total dendrite length and dendritic complexity. In order to minimize the off target effects of the shRNAs, I reduced the amount of shRNA plasmid transfected per well to 25ng. This amount of plasmid was sufficient to decrease total dendrite length (Fig. 3.6C) and dendritic complexity (Fig. 3.6D). Once again, however, co-expression of a knockdown-resistant Wnt2 was not sufficient to rescue either the decrease in total dendritic length (Fig. 3.6C) or the decrease in dendritic complexity (Fig. 3.6D). However, it should be noted that in this experiment, Wnt2 co-expression with the negative control shRNA (shWnt2.3.Scr) was not sufficient to increase either dendrite parameter, which complicates the interpretation of the previous result. It may be necessary to utilize a more robust Wnt2-dependent phenotype such as dendritic spine length or dendritic spine head width in order to more fully characterize the effects of the shRNAs targeting Wnt2 in cortical neurons.

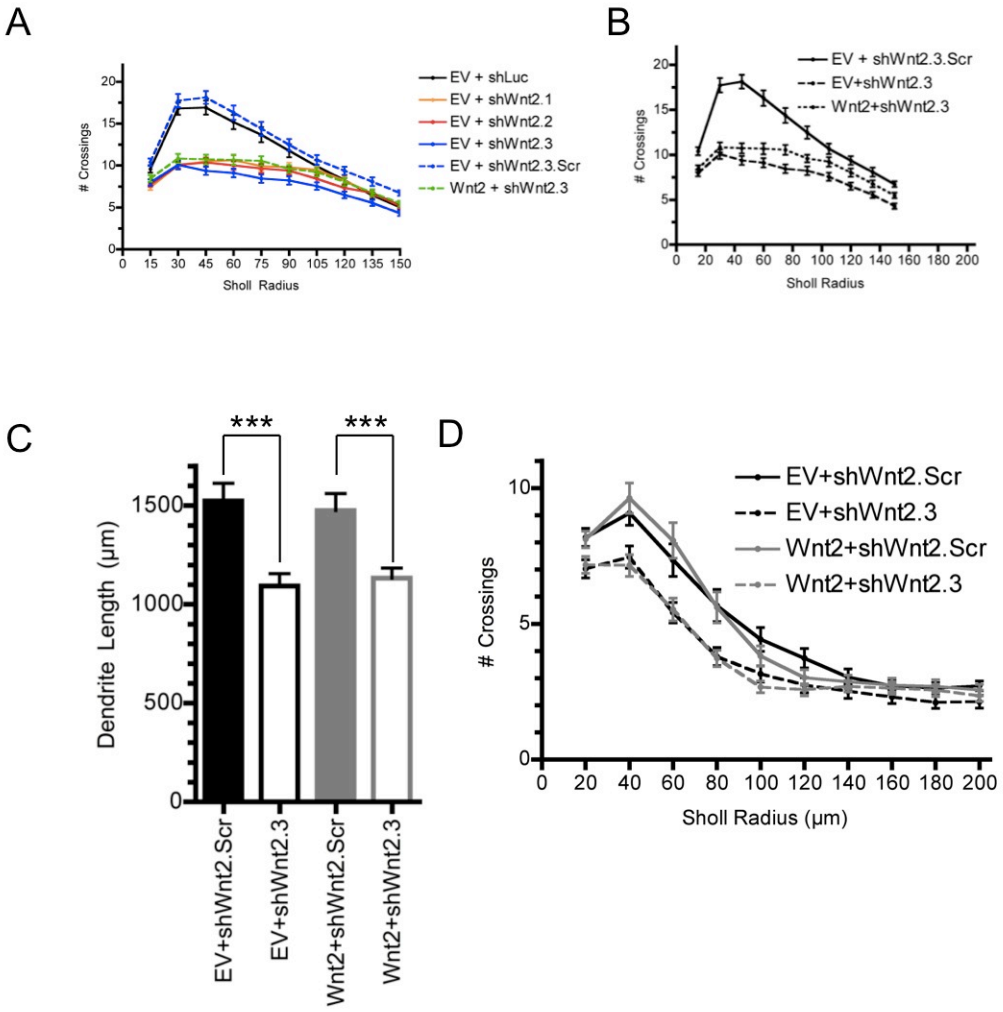

Figure 3.6. Examining the effect of shRNA-mediated knockdown of Wnt2. (A) Sholl analysis of dendritic complexity of cortical neurons expressing control and Wnt2 shRNAs from DIV7-10 with EV and neurons co-expressing shWnt2.3 with ectopic Wnt2. (B) The same diagram showing only neurons co-expressing either shWnt2.3 or shWnt2.3.Scr with EV, or shWnt2.3 with ectopic Wnt2. n=number of neurons: EV+shLuc n=34, EV+shWnt2.1 n=35, EV+shWnt2.3 n=38, EV+shWnt2.3 n=40, EV+shWnt2.3.Scr n=40, Wnt2+shWnt2.3 n-40. (C) Quantification of total dendrite length for cortical neurons co-expressing shWnt2.3 or shWnt2.3.Scr with either EV or Wnt2. (D) Sholl analysis of dendritic complexity for the same neurons in (C). ***p<0.001. n=number of neurons: EV+shWnt2.3.Scr n=49, EV+shWnt2.3 n=39, Wnt2+shWnt2.3.Scr n=43, Wnt2+shWnt2.3 n=40.

3.3.7 Wnt4 expression pattern in the developing and adult brain

Our microarray also indicated that another Wnt gene, Wnt4, is downregulated in the anterior cortex, posterior cortex and striatum of the forebrain-specific $BDNF^{KO}$ mouse, suggesting that Wnt4 may also be a regulatory target of BDNF. Using the Allen Developing Mouse Brain Atlas, I determined that Wnt4 expression is somewhat distinct from that of Wnt2 at P14 (Allen Developing Mouse Brain Atlas, 2009). Unlike Wnt2, Wnt4 is lowly expressed in the hippocampus (Fig. 3.7A) and does not appear to be expressed at all in the striatum (Fig. 3.7B). Similar to Wnt2, Wnt4 is expressed in both the motor and visual cortex, and its expression is enriched in cortical layers II/III and V, suggesting that Wnt4 is expressed in cortical excitatory pyramidal neurons (Fig. 3.7C,D). Generally, Wnt4 appeared to be more highly expressed in the cortex than Wnt2. Analysis of images from the adult Allen Brain Atlas revealed that Wnt4 is expressed in the adult cortex at P56, but that its expression levels are much lower than at P14 (Appendix Fig. 13).

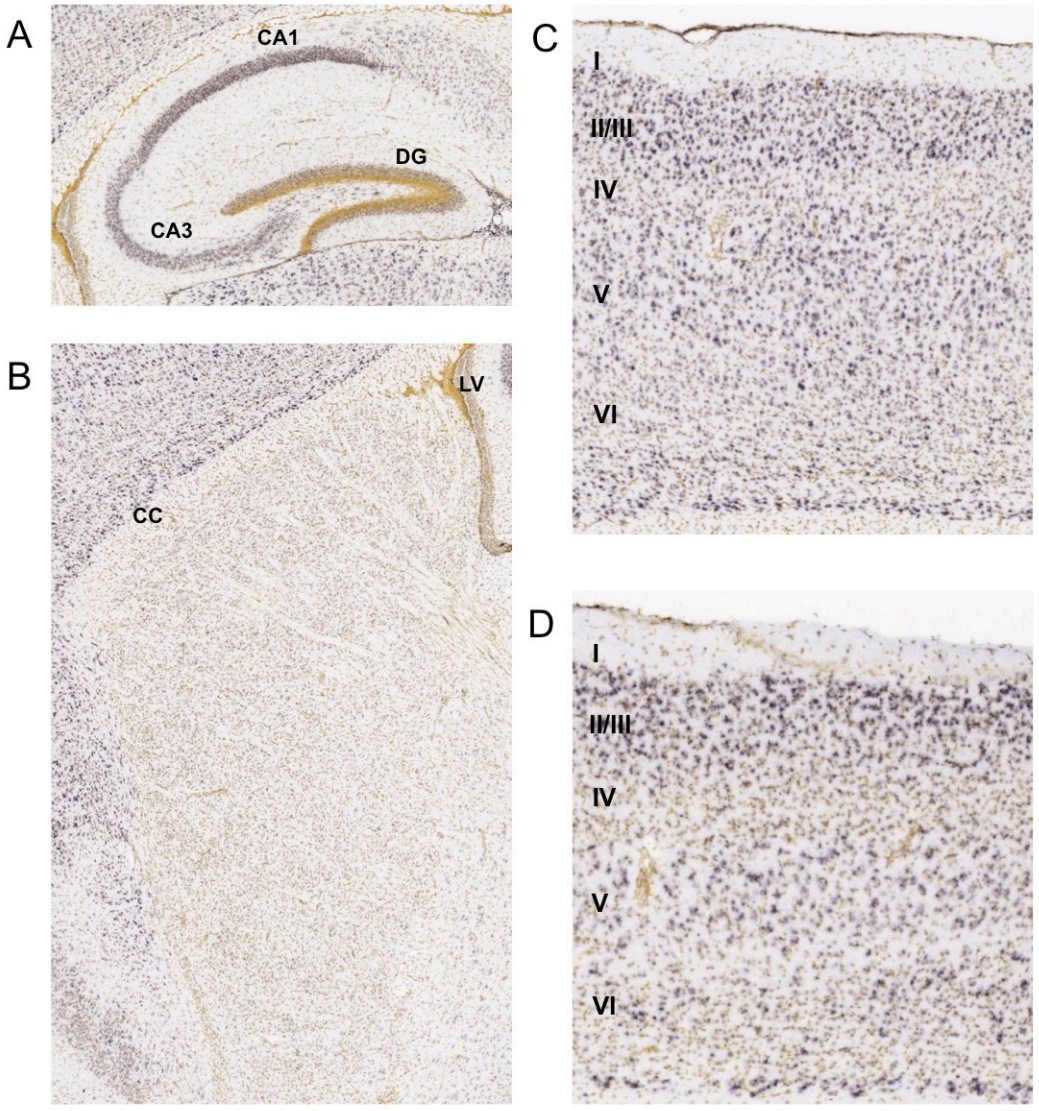

Figure 3.7. Wnt4 is expressed in the developing mouse brain. *in situ* hybridization images taken from the Allen Developing Mouse Brain Atlas (Allen Developing Mouse Brain Atlas, 2009) showing Wnt4 expression (purple color) in the (A) hippocampus, (B) striatum, (C) motor cortex, and (D) visual cortex.

3.3.8 BDNF treatment decreases Wnt4 mRNA expression

In order to investigate the potential role of BDNF in regulating Wnt4 expression, I treated DIV10 cortical neurons for four hours with recombinant BDNF and assayed for levels of Wnt4 mRNA. In addition, I treated neurons with KCl and TTX to determine whether Wnt4 expression is influenced by neural activity. Although this particular experiment lacked statistical significance, some interesting and unexpected trends emerged. Surprisingly, both BDNF and KCl treatment decreased Wnt4 mRNA abundance, while TTX treatment increased Wnt4 mRNA abundance (Fig. 3.8). A separate experiment performed at DIV16 using the same four-hour exposure to recombinant BDNF caused a significant reduction in Wnt4 mRNA abundance (Appendix Fig. 14). This indicates that, unlike Wnt2, Wnt4 is downregulated by BDNF, and suggests that BDNF may bi-directionally regulate Wnt gene expression.

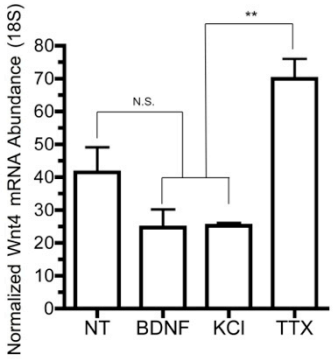

Figure 3.8. BDNF may decrease Wnt4 expression. Quantification of Wnt4 mRNA abundance after 4 hr. treatment of DIV10 cultured cortical neurons with recombinant BDNF (50 ng/mL), KCl (40 mM) or TTX (4μM). **p<0.01. n=number of wells: NT n=6, BDNF n=6, KCl n=3, TTX n=3.

3.3.9 Wnt4 promotes dendritic spine formation

Next, I determined whether Wnt4 overexpression is sufficient to increase dendritic spine formation in cortical neurons. Wnt4 expression from DIV7-11 significantly increased dendritic spine density (Fig. 3.9A) and significantly decreased dendritic spine length (Fig. 3.9B). Together, these data indicate that Wnt4 is sufficient to promote dendritic spine formation and may increase dendritic spine maturation.

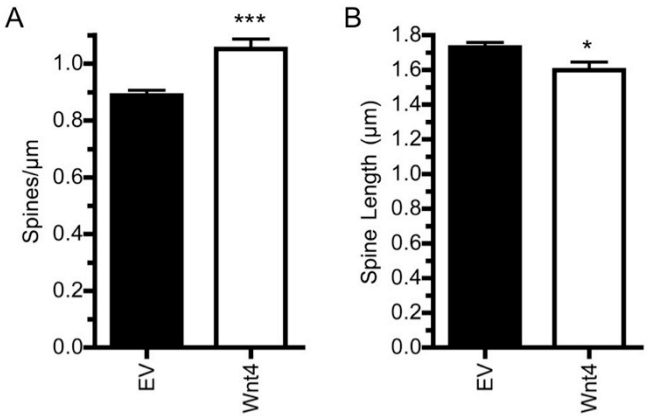

Figure 9. Wnt4 is sufficient to increase dendritic spine formation in cortical neurons. Quantification of (A) dendritic spine density of neurons expressing either EV or Wnt4 from DIV7-11. (B) average dendritic spine length of neurons from the same treatments. n=number of dendritic segments: EV n=120, Wnt4 n=78. *p<0.05, ****p*<0.001.

3.4 CONCLUSIONS

The data presented in this chapter support several conclusions. Analysis of Wnt2 expression indicates that Wnt2 is expressed in the developing brain. Additionally, BDNF is capable of regulating Wnt2 expression *in vitro*. Functional analysis of Wnt2 indicates that Wnt2 expression is sufficient to promote cortical neuron growth. Wnt2 expression increases dendrite growth and increases dendritic spine formation. Relatedly, Wnt2 may be required for the maturation of dendritic spines. Analysis of Wnt4 expression revealed that Wnt4 is expressed in the developing brain. Initial experiments aimed at elucidating a regulatory role for BDNF during Wnt4 expression suggest that BDNF may downregulate Wnt4 expression. Despite the possibility of BDNF-mediated downregulation of Wnt4, Wnt4 expression is sufficient to increase dendritic spine formation in cortical neurons.

CHAPTER 4: Discussion and Future Directions

My studies in cortical neurons are consistent with those in many other systems, indicating that Wnt signaling modulates dendrite growth and synapse formation. In addition, my observations argue that the Wnt signaling system is recruited by the neurotrophin BDNF as a necessary part of its mechanism of dendrite modulation. I present evidence that a specific Wnt, Wnt2, is regulated by BDNF, and its expression by cortical neurons is sufficient to cause increased dendrite growth and dendritic spine density. Additionally, I present preliminary findings that another Wnt, Wnt4, may be downregulated by BDNF but that Wnt4 may be sufficient to promote dendritic spine formation.

4.1 BDNF promotes dendritic spine formation in cultured cortical neurons

Several studies have shown that BDNF is sufficient to increase dendritic spine formation. Notably, BDNF increases dendritic spine density in both cerebellar and hippocampal neurons (Alonso et al., 2004; Shimada et al., 1998; Tyler and Pozzo-Miller, 2003). Similarly I demonstrate here that overexpression of BDNF in cultured cortical neurons is sufficient to increase dendritic spine density and to promote dendritic spine maturation. BDNF overexpression from DIV7-11 increases dendritic spine density and decreases dendritic spine length (Appendix Fig. 1). Additionally, BDNF overexpression from DIV10-14 increases dendritic spine density and increases dendritic spine head width (Fig. 2.4). While it is tempting to suggest that overexpressed BDNF is signaling through the

receptor TrkB in order to promote dendritic spine formation, the experiments outlined in this thesis do not directly address this possibility. Indeed, experiments utilizing some form of TrkB loss-of-function could be useful to determine whether TrkB is required for BDNF-induced dendritic spine formation in cortical neurons. Potential TrkB loss-of-function strategies include culturing neurons from TrkB null mice, or culturing neurons from a mouse strain expressing a mutant form of TrkB that can be antagonized by addition of a the chemical 1NMPP1 (Johnson et al., 2008).

Although the ability of overexpressed BDNF to promote dendritic spine formation in cortical neurons was both reproducible and robust, I did observe instances in which BDNF was not sufficient to increase dendritic spine density. Specifically, overexpression of BDNF in cultured cortical neurons from BDNF null mice from DIV7-11 was not sufficient to increase dendritic spine density (Appendix Fig. 15). Interestingly, BDNF was sufficient to increase primary dendrite formation in these neurons, suggesting that overexpressed BDNF was active in these neurons (Appendix Fig. 15). The lack of an increase in spine density in these neurons is certainly curious, as the rationale leading into this experiment was that the BDNF null neurons would be particularly sensitive to BDNF and may display a greater increase in dendritic spine density due to BDNF overexpression. One explanation as to why overexpression of BDNF did not increase spine density may relate to the expression of the receptor TrkB. It is possible that either the level of TrkB expression or the location of TrkB expression was altered in these neurons due to the lack of BDNF expression.

The fact that BDNF was able to increase primary dendrite formation suggests that TrkB was expressed, but it is possible that TrkB expression in more distal regions of the neurons, and specifically synaptic TrkB localization, was affected. Indeed, synaptic localization of TrkB protein increases during the development of cultured cortical neurons (Gomes et al., 2006), and it is possible that the development of BDNF null cortical neurons is delayed and that TrkB was not properly trafficked to proto-synaptic sites throughout the neuron. Additionally, it is possible that BDNF overexpression produced a subtle dendritic spine phenotype in this particular experiment. I did not analyze dendritic spine length or dendritic spine head width therefore I do not know whether BDNF promoted aspects of dendritic spine maturation. Further experiments examining the ability of BDNF overexpression to induce dendritic spine formation in BDNF null neurons will help to clarify the reproducibility of the result that I observed and could indicate an interesting requirement for BDNF expression during the early post-natal development of cortical neurons.

4.2 Wnt signaling is required for BDNF-induced dendritic spine formation

Recent evidence indicates that Wnt signaling regulates hippocampal dendritic spine formation. Both Wnt5a and Wnt7a are sufficient to increase dendritic spine density in hippocampal neurons (Ciani et al., 2011; Farias et al., 2009). Further, Wnt7a is sufficient to increase dendritic spine head width, suggesting that it promotes maturation of dendritic spines (Ciani et al., 2011). Additionally, application of recombinant Fzd2CRD, a soluble version of the Wnt

receptor Fzd2, reduces dendritic spine density in hippocampal neurons (Varela-Nallar et al., 2010), and cultured hippocampal neurons from Dvl1 knockout mice form fewer dendritic spines compared to wild-type control (Ciani et al., 2011).

The work I present in this thesis extends this line of research by demonstrating that Wnt signaling also regulates cortical dendritic spine formation and suggests that it is required for BDNF-induced dendritic spine formation. BDNF regulates the formation and development of dendritic spines (Yoshii and Constantine-Paton, 2010) and recent work has begun to elucidate some of the molecular mechanisms underlying this process. BDNF induces formation of hippocampal dendritic spines by regulating the surface expression of TRPC3 voltage-gated calcium channels (Amaral and Pozzo-Miller, 2007), by activating ERK1/2 signaling cascades (Alonso et al., 2004), and by increasing the expression of Ryanodine receptors (Adasme et al., 2011). In addition, BDNF regulates the trafficking and synaptic localization of PSD95 (Yoshii and Constantine-Paton, 2007), which is a major postsynaptic scaffolding protein that is sufficient to promote dendritic spine formation (El-Husseini et al., 2000). Notably, while each of these targets of regulation are cytoplasmic, our studies support a requirement for secreted Wnt signaling proteins during BDNF-induced dendritic spine formation.

This mechanism relies upon BDNF regulating Wnt expression, or other aspects of Wnt signaling, in order to regulate dendritic spine formation. Related, I report here that BDNF regulates the abundance of at least one Wnt mRNA, encoding Wnt2, and that Wnt2 is sufficient to promote dendritic spine formation.

Interestingly, activity-mediated regulation of Wnt signaling is required for activity-mediated synapse formation (Chen et al., 2006; Gogolla et al., 2009; Sahores et al., 2010). Neural activity not only regulates Wnt gene transcription (Wayman et al., 2006), but it regulates Wnt protein translation (Gogolla et al., 2009), Wnt protein transport (Tabatadze et al., 2011) and Wnt protein secretion (Ataman et al., 2008; Chen et al., 2006) as well. It is possible that BDNF may similarly regulate multiple aspects of Wnt signaling.

It is also possible that BDNF regulates Wnt signaling through modulation of neural activity. A key study investigating the interaction between BDNF and neural activity during hippocampal dendritic spine formation demonstrated that BDNF increases dendritic spine density and maturation when evoked neural activity is blocked, but only increases dendritic spine density when both evoked and spontaneous neural activity are blocked (Tyler and Pozzo-Miller, 2003). My work showed that BDNF was able to increase Wnt2 expression when evoked neural activity is blocked (Fig. 3.2, TTX treatment), but it did not investigate whether this increase may be due to the ability of BDNF to increase spontaneous neural activity through increased neurotransmitter release. Additional experiments testing the requirements for Wnt signaling during activity-independent BDNF regulation of dendritic spine formation will help to elucidate some of these details.

Although it will be important to attempt to isolate the activity-independent effect of BDNF on Wnt signaling, this may ultimately prove to be very difficult as BDNF signaling and neural activity are intimately linked. Neural activity

increases the secretion of BDNF (Kuczewski et al., 2010) and, in a reciprocal manner, BDNF signaling increases neural activity (Madara and Levine, 2008; Pozzo-Miller, 2006). Interestingly, BDNF signaling is required to induce long-lasting structural dendritic spine plasticity in a paradigm that pairs glutamate uncaging with post-synaptic neural activity (Tanaka et al., 2008), suggesting that neural activity and BDNF coordinately regulate dendritic spine formation. Together, these observations raise the possibility that BDNF and neural activity may coordinately regulate Wnt signaling in order to regulate dendritic spine formation. Further studies aimed at elucidating the full extent of the regulation of Wnt signaling by BDNF during dendritic spine formation will help to clarify this possibility.

4.3 Wnt inhibition alone modestly impairs dendritic spine formation

My observation that Wnt inhibition only modestly affected spine formation under standard “baseline” culture conditions may indicate that there is a unique population of BDNF-dependent spines that are not abundant under those conditions. Alternatively, it’s possible that under baseline culture conditions, BDNF is lowly expressed, thus the induction of Wnt signaling is concurrently low, meaning that inhibiting Wnt signaling has a relatively small effect on baseline dendritic spine formation. Indeed, the results that I show from experiments performed using $BDNF^{-/-}$ neurons indicate that cultured cortical neurons form dendritic spines at densities comparable to wild type controls (Appendix Fig. 15). It is also possible that cortical neurons express less BDNF than hippocampal

neurons at the same time point, which would explain why studies using hippocampal neurons demonstrated a greater requirement for Wnt signaling during normal growth conditions (Ciani et al., 2011; Varela-Nallar et al., 2010). Indeed, our lab has demonstrated that *in vivo* BDNF expression turns on earlier in the hippocampus than in the cortex, and that maximum cortical BDNF expression does not occur until after approximately 2-3 weeks of age (Baquet et al., 2004). Although my experiments were performed *in vitro*, this general timeline of BDNF expression correlates with a decreased requirement for cortical BDNF signaling in neurons less than 2 weeks old.

The modest impairment of dendritic spine formation due to Wnt inhibition in baseline culture conditions may also be a result of the Wnt inhibition strategy used in my experiments, and may specifically result from incomplete attenuation of Wnt signaling. The cytoplasmic Wnt inhibitor that I used, Dvl1ΔPDZ, is only predicted to act cell-autonomously, whereas Ciani et al. (2011) examined spine formation in a population of neurons that completely lacked Dvl1, meaning that both pre- and postsynaptic Wnt signaling was blocked. If, as it has been shown previously, Wnt signaling regulates dendritic spine synapses both pre- and postsynaptically, then inhibition of Wnt signaling on only one side of the synapse should have less of an effect than simultaneously inhibiting signaling on both sides of the synapse. The secreted inhibitors that I used in my experiments were predicted to do just that. However, it is possible that the three secreted Wnt inhibitors do not bind and inhibit all Wnt ligands with the same affinity and therefore incompletely inhibit signaling by multiple Wnts acting at some

synapses. To date, six of the 19 different mammalian Wnt proteins have been shown to be active on neurons, and more are likely to share this property. Previous studies have indicated that endogenous and non-endogenous secreted Wnt inhibitors vary in the affinity with which they bind and inhibit the activity of Wnt proteins (Carmon and Loose, 2010; Galli et al., 2006), which may explain why the Fzd2-CRD inhibitor used by Varela-Nallar et al. (2010) produced a stronger decrease in hippocampal dendritic spine formation. Additionally, it is possible that hippocampal dendritic spine formation requires a different subset of Wnts than in cortical neurons.

Future experiments utilizing simultaneous expression of multiple Wnt inhibitors should help to clarify whether Wnt inhibition in my experiments was incomplete or if, instead, there is a low requirement for Wnt signaling during baseline dendritic spine formation. Additionally, experiments pairing overexpression of individual Wnt genes with different Wnt inhibitors will indicate whether individual Wnt inhibitors are able to block the activity of specific Wnt proteins. Lastly, it may be helpful to investigate whether inhibiting Wnt signaling by expressing a Dickkopf (Dkk) protein. The Dkk-type inhibitors inhibit Wnt signaling by binding to the Fzd/LRP co-receptor complex, which may help to circumvent the problems associated with the varying affinities of the Wnt ligand-binding secreted Wnt inhibitors (e.g., Sfrp1, Wif1, Fzd8CRD).

4.4 Mechanisms of Wnt-mediated dendritic spine formation

Collectively, the results from experiments utilizing Wnt inhibition alone and in combination with BDNF overexpression indicate that Wnt signaling plays a key role during dendritic spine formation. The next important step is to identify the mechanisms by which Wnt signaling regulates cortical dendritic spine formation.

Previous studies examining the mechanisms of Wnt signaling-mediated synapse formation indicate that Wnt signaling is required for both pre- and postsynaptic assembly (Budnik and Salinas, 2011). In this context, it is possible that dendritic spine formation is impaired because the synapse itself is prevented from fully assembling. The results that I present pertaining to defects in spine maturation, and specifically dendritic spine head width, are consistent with a defect in postsynaptic assembly. Indeed, expression of PSD95, a major scaffolding component of the postsynaptic compartment, is tightly coupled with dendritic spine maturation (Ehrlich et al., 2007). Further, Wnt5a induces clustering of PSD95 (Farias et al., 2009; Varela-Nallar et al., 2010), and promotes increases in dendritic spine density (Farias et al., 2009) suggesting that regulation of postsynaptic assembly is a key step during dendritic spine formation. In this light, it would be important to determine if the individual Wnts used in my studies, Wnt2 and Wnt4, similarly affect assembly of synaptic components, whether pre- or postsynaptic. Initial studies that I performed aimed toward determining whether Wnt2 affects expression or localization of PSD95 were inconclusive (Data not shown). Additionally, it would be interesting to determine if the Wnt inhibitors used in my experiments preferentially affect pre-

or postsynaptic assembly. The fact that the cytoplasmic Wnt inhibitor Dvl1ΔPDZ blocks BDNF-induced dendritic spine formation to the same extent as the secreted inhibitors suggests that the primary requirement for Wnt signaling is postsynaptic. I will discuss this idea later in the chapter.

Alternatively, it is possible that defects in synaptic assembly indirectly impair dendritic spine formation by affecting excitatory neurotransmission. Treatment of hippocampal slices with Sfrp2 reduces both fEPSP and mEPSC amplitude (Varela-Nallar et al., 2010) while treatment with Sfrp1 decreases NMDAR-mediated neurotransmission and prevents paired-synaptic potentiation (Cerpa et al., 2011). Additionally, treatment of hippocampal slices with Fzd8-CRD decreases the magnitude of LTP (Chen et al., 2006). Importantly, dendritic spine formation is regulated by neural activity (Alvarez and Sabatini, 2007; Yuste and Bonhoeffer, 2001) so it is possible that the defects that I report in cortical dendritic spine formation during Wnt inhibition arise as a result of impaired neurotransmission due to improper excitatory synapse development. Electrophysiological studies examining various properties of neural activity during Wnt inhibition would help to indicate whether neurotransmission is affected in the experiments that I presented here. Further, because BDNF may, in part, regulate dendritic spine formation by modulating neural activity, it would be informative to determine if the changes in neural activity elicited by BDNF are altered in the absence of Wnt signaling. This could suggest that Wnt inhibition indirectly affects the ability of BDNF to regulate spine formation.

An alternative, but not mutually exclusive, possibility is that the changes in dendritic spine number and increases in dendritic spine length that we see during Wnt inhibition, either alone or during BDNF-induced dendritic spine formation, represent a failure of Wnt signaling to regulate the stabilization of dendritic spine contacts after the initiation of dendritic spine formation. Expression of a stabilized version of ß-catenin, a key downstream effector of Wnt signaling, promotes hippocampal dendrite growth and dendritic spine formation, presumably by mediating cell adhesion events via its interaction with N-cadherin, alpha-N-catenin and the actin cytoskeleton (Yu and Malenka, 2003, 2004). Further, disruption of either N-cadherin or alpha-N-catenin function in hippocampal neurons results in decreased spine stability and an increase in dendritic spine length (Abe et al., 2004; Togashi et al., 2002). Wnt-mediated stabilization of synaptic contacts through local regulation of the Cadherin-Catenin complex would prove to be an interesting means of promoting dendritic spine formation and maturation. Additionally, it may be interesting to determine whether cadherin-mediated cell adhesion is required for BDNF-induced dendritic spine formation. There is evidence that BDNF/TrkB signaling enhance excitatory synapse formation through mobilization of presynaptic neurotransmitter vesicles by disrupting the interaction between n-cadherin and ß-Catenin (Bamji et al., 2006). I envision a scenario in which a postsynaptic interaction between BDNF signaling and the cadherin/catenin complex mediated by a BDNF-induced Wnt signal acts to stabilize nascent synaptic contacts. Given the strong dendritic spine phenotypes associated with n-cadherin and alpha-catenin loss-of-function,

it may be possible to determine if BDNF rescues these defects. Additionally, a detailed analysis of dendritic spine turnover will help to clarify whether the impairment in dendritic spine formation due to Wnt inhibition is a result of failed stabilization of dendritic spine contacts. If dendritic spine contacts are not stabilizing properly, I would expect that the rate of dendritic spine initiation will remain unchanged in the absence of Wnt signaling, but that the proportion of dendritic spines that persist after initiation will decrease.

4.5 Identifying the role of specific Wnt signaling pathways during dendritic spine formation

Despite the dendrite arbor growth and dendritic spine formation phenotypes that I describe in regards to Wnt inhibition, both alone and in combination with BDNF overexpression, the identity of the specific signaling pathways required for Wnt signaling in these contexts is unknown. Similarly, despite the results that I describe suggesting that Wnt2 is sufficient to promote dendritic spine formation and maturation, the specific Wnt signaling pathway induced by Wnt2 is unknown. As previously described in Chapter 2, Wnt signaling is very complex and several different signaling cascades mediate the effects of Wnt signaling on the cell surface.

In an initial attempt to identify a potential downstream Wnt signaling cascade during BDNF-induced cortical neuron growth, I treated neurons with bath recombinant BDNF for 12 hours and determined whether ß-catenin protein translocated to the nucleus, which would indicate that BDNF could induce a

canonical Wnt signal that would subsequently cause ß-catenin-dependent transcriptional upregulation of a set of target genes. I found that BDNF treatment did not induce translocation of ß-catenin to the nucleus (Appendix Fig. 3). Interestingly, KCl treatment, which increases overall neural activity, appeared to induce ß-catenin translocation to the nucleus. This effect was blocked by co-treatment with TTX. Together, these data suggest that neural activity may be able to induce a canonical Wnt signal, while BDNF does not. This interpretation supports the speculation that BDNF-induced Wnt ligands signal in a non-canonical manner. In similar experiments in which I expressed either Wnt2 in cortical neurons and then examined ß-catenin localization, I also did not detect any nuclear translocation of ß-catenin to the nucleus (Data not shown). This observation is consistent with Wnt2 as a target of BDNF.

Although these experiments suggest a role for non-canonical Wnt signaling during cortical neuron growth and dendritic spine formation, more experiments need to be performed in order to further rule out a potential role for ß-catenin-dependent canonical Wnt signaling. The most rigorous means by which one can specifically determine a role for ß-catenin-dependent transcription is by using the TOPFlash reporter system, which consists of a plasmid that expresses Luciferase under the control of TCF/LEF transcription factor binding sites in its promoter. Transfection of this reporter plasmid into neurons followed by treatment BDNF should help to determine whether BDNF induces a canonical ß-catenin-dependent transcription signal. Additionally, co-transfection of this

reporter plasmid with a plasmid that expresses BDNF should indicate whether any such signal is cell-autonomous.

4.6 The primary requirement for Wnt signaling during BDNF-induced dendritic spine formation is cell-autonomous

The data I describe here provides insight into the directional requirements of Wnt signaling during BDNF-induced cortical dendritic spine formation. I found that the cytoplasmic Wnt inhibitor, Dvl1ΔPDZ, inhibits BDNF-induced increases in dendritic spine density to the same extent as the three secreted Wnt inhibitors, Wif1, Sfrp1 and Fzd8CRD (Fig. 3B). This indicates a cell-autonomous requirement for Wnt signaling, and suggests that an anterograde Wnt signal acts through a postsynaptic signaling mechanism to mediate BDNF-induced dendritic spine formation. Interestingly and related, Wnt7a has been suggested to promote hippocampal dendritic spine growth by regulating post-synaptic CamKII signaling (Ciani et al., 2011) while Wnt 5a is thought to promote hippocampal dendritic spine formation by regulating post-synaptic clustering of PSD-95 (Farias et al., 2009).

Alternatively, it may be possible that disruption of either pre- or postsynaptic regulation of dendritic spine formation by Wnt signaling is sufficient to block BDNF-induced dendritic spine formation. If this were indeed true, selective blockade of non-cell-autonomous Wnt signaling would be expected to abrogate dendritic spine function.

4.7 Cross-synaptic interaction between BDNF and Wnt signaling

My original hypothesis that guided me toward investigating Wnt signaling as a mediator of BDNF-induced dendritic spine formation proposed that BDNF signals across the synapse to regulate the expression of Wnts, which, in turn, signal back across the synapse. In this manner, BDNF and Wnt signaling could form a bi-directional signaling loop that functioned to regulate a synapse. The results that I present in this thesis neither contradict nor completely support this hypothesis, mainly because the experiments that I have performed lack specificity in the directional sense.

First, concerning the direction of BDNF signaling, although I expressed BDNF in isolated cells and demonstrated that this results in a cell autonomous increase in dendritic spine formation, I don't know whether BDNF is signaling in an autocrine or paracrine manner, or both. This means that BDNF could also be inducing a Wnt signal in any of these manners. Additionally, the experiments in which I demonstrate that BDNF increases expression of Wnt2 were accomplished using bath BDNF treatment, which provides no indication of whether BDNF can activate Wnt2 expression in a cross-synaptic manner. In order to begin testing whether BDNF is able to signal in an autocrine manner to induce a Wnt signal, it would be interesting to reduce or eliminate TrkB expression or function in a cell-autonomous manner, essentially forcing BDNF in isolated transfected neurons to signal in a paracrine manner. If BDNF-induced dendritic spine formation in this context were blocked by Wnt inhibition, it would suggest that BDNF is signaling across the synapse to regulate Wnt signaling.

Second, the Wnt signals that I examine in my experiments could similarly be signaling in an autocrine or paracrine manner. Indeed, when I express either Wnt2 or Wnt4 in isolated neurons, it involves the same caveats as when I express BDNF. While it would be incredibly informative to be able to force the direction of Wnt signals using receptor knockout or knockdown strategies, our lack of knowledge concerning the identity of specific Fzd receptors that mediate effects of individual Wnt proteins makes this a difficult proposition. While there are some indications in the literature of potential Wnt/Fzd interactions, as is the case for Wnt4 and Fzd3, a systematic analysis of which Fzd receptors that individual Wnt proteins bind has not been performed. Additionally, it is possible that Fzd expression context changes according to brain region and neural cell type. Combined, a detailed expression analysis of both Wnt ligands and Fzd receptors should begin to aid the process of identifying specific Wnt/Fzd interactions. Further, depending on the expression of ligand/receptor pairs within known neural circuits, this sort of analysis may suggest how the complex Wnt signaling system contributes to the establishment of ordered neural connectivity within and between brain regions.

4.8 Wnt signaling is required for cortical dendrite growth

Here, I present data indicating that normal growth and elaboration of cortical dendritic arbors requires endogenous Wnt signaling, consistent with what has been shown for hippocampal neurons (Rosso et al., 2005). The synaptotrophic hypothesis proposes that an essential function of synapses is to

stabilize dendritic arbors (Cline and Haas, 2008). Activation of postsynaptic signaling mechanisms promotes dendritic arbor stability (Lohmann et al., 2002; Niell et al., 2004; Rajan et al., 1999; Wu and Cline, 1998). In contrast, loss of synaptic inputs leads to reductions in dendrites, indicating that maintenance of synaptic input is critical for dendrite stability (Clark, 1957; Coleman and Riesen, 1968; Jones and Thomas, 1962; Matthews and Powell, 1962; Sfakianos et al., 2007). Previous work has established that there is a correlation between dendritic spine size and synaptic strength (Matsuzaki et al., 2001; Zito et al., 2009), and studies have shown that blocking Wnt signaling impairs excitatory neurotransmission (Cerpa et al., 2011; Varela-Nallar et al., 2010). I speculate that reduced excitatory synaptic input as a result of impaired dendritic spine formation may lead to the reduction in cortical dendrite growth with Wnt inhibition. Indeed, my data indicate that Sfrp1 and Dvl1ΔPDZ, which caused impairments in dendritic spine density and length (Fig. 1B and C), also caused significant reductions in total dendrite length (Fig. 2B).

As is the case with the dendritic spine formation results that I present in this thesis, the data pertaining to dendritic arbors represent only a snapshot of what is happening to dendrites during Wnt inhibition. In reality, neuronal dendrites, like dendritic spines, are dynamic structures. Dendrite growth is accomplished by iterative extension and stabilization of newly formed dendritic branches. In this context, it is possible that the dendrite growth impairments caused by Wnt inhibition are caused by a lack of new dendritic branch formation, rather than the failure of dendrite branches to stabilize. Additionally, in the case

of Sfrp1, which elicited the strongest negative effect on dendrite elaboration, it is possible that dendrites actually retract over the 4DIV expression period. Without a more detailed temporal analysis of dendrite growth, these distinctions are difficult to make.

4.9 BDNF increases proximal dendrite complexity in cortical neurons

A large portion of the research examining the role of BDNF in regulating neuron function demonstrates that BDNF regulates elaboration of cortical dendrites by signaling through TrkB (Horch et al., 1999; McAllister et al., 1996, 1997; Wirth et al., 2003). Interestingly, the effects of BDNF on cortical dendrites display cortical layer specificity. BDNF promotes the growth and branching of dendrites of neurons in layers II/III, IV and V (Horch et al., 1999; McAllister et al., 1996; Wirth et al., 2003), but inhibits the dendritic growth of neurons in layer VI (McAllister et al., 1997). Not only is BDNF sufficient to promote dendrite elaboration, but it is required for the maintenance of cortical dendrites as well (Gorski et al., 2003).

My experiments using BDNF overexpression in cultured cortical neurons recapitulated some, but not all, of the previously described effects of BDNF on dendritic arbors. I show here that BDNF overexpression results in a robust increase in the formation of primary dendrites, and that this leads to a small but significant increase in proximal dendritic complexity (Fig. 2.3.F). However, 4DIV expression of BDNF did not increase total dendrite length, which was unexpected

(Fig. 2.3.B). BDNF expression actually significantly decreased apical dendritic length (Fig. 2.3.C).

One possible explanation for the decrease in apical dendrite length is that strong BDNF expression for 4DIV causes a large increase in synaptic strength, which then results in a compensatory retraction of dendrites to scale back the total amount of excitatory input. Neurons have a remarkable ability, termed homeostatic plasticity, that allows them to maintain stable intrinsic excitability in the face of constantly changing external input (Turrigiano, 2011). While the mechanisms of homeostatic plasticity are unclear, there is evidence that dendritic arbor growth is regulated in a homeostatic manner by neural activity. A study examining the role of ß-catenin during dendrite growth demonstrated that there is an inverse correlation between synaptic strength and dendritic length, specifically that increased dendrite growth is associated with decreased amplitude of spontaneous neurotransmission events (Peng et al., 2009). In addition, there is evidence that an abnormal increase in neural activity results in decreased dendrite growth. Hippocampal neurons treated with the $GABA_A$ inhibitory neurotransmitter receptor blocker bicuculine for 4DIV prevented dendrite growth over that period (Nishimura et al., 2008), indicating that chronic increases in excitatory synaptic input limit dendrite growth. The data I present here suggest that BDNF increases the strength of presynaptic input in two ways. First, BDNF increases the number of synaptic inputs by increasing the number of dendritic spines. Second, BDNF increases the strength of synaptic input, as shown by the increase in dendritic spine head width. I speculate that BDNF overexpression in

cortical neurons causes a sufficiently large enough increase in excitatory synaptic input to elicit a compensatory decrease in dendrite length.

It is important to note that even though BDNF expression does not increase dendrite length, and even significantly decreases distal dendrite branching, overall neural connectivity as measured by the total number of dendritic spines per neuron is still increased by BDNF (Appendix Fig. 2). Additionally, Wnt inhibition in combination with BDNF expression decreases neural connectivity, further supporting a role for Wnt signaling during BDNF-mediated neural circuit formation.

4.10 Wnt2 promotes cortical dendrite growth and dendritic spine formation

My finding that Wnt2 overexpression in cortical neurons promotes dendritic spine formation and decreases dendritic spine length is consistent with studies that have examined the role of other Wnt proteins during dendritic spine formation by hippocampal neurons (Ciani et al., 2011; Farias et al., 2009; Varela-Nallar et al., 2010). The ability of Wnt2 to promote dendritic spine formation in cortical neurons suggests a potential cellular mechanism for its ability to promote dendrite growth in cultured hippocampal neurons (Wayman et al., 2006). We demonstrate here that Wnt2 is expressed in the developing mouse cortex and promotes dendrite growth in cortical neurons *in vitro*. It is possible that Wnt2 promotes dendrite growth primarily by enhancing neural connectivity through dendritic spine formation, stabilizing dendrites as predicted by the synaptotrophic hypothesis (Cline and Haas, 2008).

The increase in dendrite growth that we report in cortical neurons is smaller in comparison to the response demonstrated in hippocampal neurons but the discrepancy between the two results may be due to the difference in relative developmental stage of the neurons used. As neurons develop, the potential for increased dendrite growth decreases (Wu et al., 1999). Indeed, layer V pyramidal neurons in the rat cortex reach near maximal dendrite length by P14 (Romand et al., 2011), suggesting that the dendritic arbors of the DIV14 cortical neurons used in our experiments are nearly fully elaborated. Further studies using different developmental stages of cortical neurons and utilizing different modes of Wnt2 treatment strategies such as Wnt2 loss-of-function or short term application of recombinant Wnt2 protein should help to clarify whether or not dendritic spine formation is the primary neuronal function for Wnt2.

4.11 $Wnt2^{KO}$ dendritic spine phenotypes

My attempts to determine whether Wnt2 is required for dendritic spine formation were less conclusive than those testing Wnt2 sufficiency. Nonetheless, experiments using cultured cortical neurons from $Wnt2^{KO}$ mice suggest that Wnt2 may be required for dendritic spine maturation. Although dendritic spine density in $Wnt2^{KO}$ neurons cultured to DIV11 was unchanged compared to wild type control, there appeared to be an increase in spine length in those neurons. Importantly, ectopic Wnt2 expression in the $Wnt2^{KO}$ neurons for 4DIV was able to reverse the impairment in spine length. This specific effect on dendritic spine length may indicate that Wnt2 functions primarily to promote

dendritic spine maturation, rather than to promote the initiation of dendritic spine formation. Measuring average spine head width would help to support this conclusion.

Alternatively, it is possible that Wnt2 promotes the initiation of dendritic spine formation, but that expression of any of the other 18 Wnt proteins is able to compensate for the loss of Wnt2. Indeed, the experiments using neurons from $Wnt2^{KO}$ mice are complicated by the lack of knowledge regarding the role of Wnt2 in the prenatal mouse brain. Analysis of Wnt2 expression in the hippocampus indicates that Wnt2 expression begins near the end of the first postnatal week and is likely not expressed prenatally (Wayman et al., 2006). If Wnt2 expression initiates even later in the cortex, as is the case with BDNF, it is possible that I may observe more severe Wnt2 loss-of-function phenotypes in neurons grown to a later date *in vitro*.

One means of circumventing the problems associated with the full Wnt2 null mouse strain is to create a conditional Wnt2 allele. To my knowledge, such a mouse strain does not exist, but would prove to be invaluable in further examining the role for Wnt2 in the brain. By crossing a mouse strain harboring a conditional Wnt2 allele with a variety of different tissue-specific CRE expressing mouse lines, it would be possible to rigorously test the spatial and temporal requirements for Wnt2.

Ultimately, the key experiment to perform is to determine if the effect of BDNF on dendritic spine formation is altered by loss of Wnt2 expression. This is

an experiment that I tried on one occasion. Unfortunately, the samples on this attempt were not of sufficient quality to allow for quantitation.

4.12 Potential role for Wnt2-mediated dendritic spine formation in disease

Several different anti-depressant drugs increase the expression of Wnt2, and viral-mediated overexpression of Wnt2 in the hippocampus alleviates depressive-like symptoms in some animal models of depression (Okamoto et al., 2010). Loss of hippocampal dendritic spines has been reported in the learned helplessness model of depression (Hajszan et al., 2009), and anti-depressant drug treatment has been shown to increase dendritic spine formation in the hippocampus (Norrholm and Ouimet, 2001) and cortex (Ampuero et al., 2010). My results suggest that Wnt2 may alleviate depressive-like symptoms by promoting dendritic spine and synapse formation. Moreover, multiple studies have shown that BDNF is both necessary and sufficient for anti-depressant drug action (Banasr and Duman, 2008; Castren and Rantamaki, 2010; Duman and Monteggia, 2006; Schmidt et al., 2008; Yu and Chen, 2011), which leads to the interesting possibility that BDNF-mediated regulation of Wnt2 expression may serve as a mechanistic link between BDNF and the alleviation of symptoms during depression.

Wnt2 expression is reduced in the hippocampus and cortex of FMR1 knockout mice, a mouse model for Fragile-X syndrome (Zhang et al., 2009). Fragile-X syndrome is a neurodevelopmental disorder believed to be caused by improper development of cortical circuitry and is characterized by an

overabundance of immature dendritic spines (Antar et al., 2006; Irwin et al., 2001; Nimchinsky et al., 2001). Furthermore, it has been suggested that the specific dendritic spine defect seen in Fragile-X syndrome is a result of increased instability of dendritic spine contacts (Cruz-Martin et al., 2010; Pan et al., 2010). My result showing that Wnt2 increases spine number, decreases spine length and increases spine head width in cortical neurons implicates Wnt2 deficiency as a causal event in the dendritic spine pathology seen in Fragile-X syndrome, and suggests that Wnt2 may function to stabilize dendritic spines during their maturation. Further studies examining the effects of Wnt2 expression on the dynamics of dendritic spine formation and stabilization will provide insight into this possibility.

Fragile-X syndrome results from transcriptional silencing of the *Fmr1* gene, which causes a loss of Fragile-X mental retardation protein (FMRP). In neurons, FMRP functions as a synaptic RNA-binding protein and regulates neuron growth and plasticity (Bassell and Warren, 2008). One theory proposes that the underlying mechanistic cause of Fragile-X is that loss of FMRP leads to increases in LTD because of disruptions in local translation of proteins involved in synaptic plasticity (Bear et al., 2004). As previously mentioned, Wnt2 mRNA in the striatum is enriched in populations of mRNAs that are associated with ribosomes, suggesting that translational regulation of Wnt2 may be important. Although I was unable to find FMRP target sequences within the 3'UTR of Wnt2, there are a number of other neuronal RNA binding proteins thought to regulate local translation in neurons (Liu-Yesucevitz et al., 2011). In light of the ability of

BDNF to promote local translation of synaptic proteins, it would be interesting to determine whether BDNF regulates Wnt2 in such a manner.

One potential tool to use in experiments examining the post-transcriptional regulation of Wnt2 is a Wnt2 overexpression construct that includes the endogenous murine Wnt2 3'UTR. Initial experiments utilizing this construct indicate that overexpression of Wnt2 with its 3'UTR produces phenotypes similar to expression of the Wnt2 coding region alone. However, there were some subtle differences. The 3'UTR construct caused a stronger increase in cortical dendrite growth and branching (Appendix Fig. 6), but a smaller increase in dendritic spine density (Appendix Fig. 7). Wnt2 expression with its 3'UTR caused a similar decrease in dendritic spine length compared with expression of the coding region alone (Appendix Fig. 7). Although expression of the two different Wnt2 constructs produces similar phenotypes, the subtle differences suggest that the Wnt2 3'UTR might be important for Wnt2 function.

4.13 The role of Wnt4 during BDNF-induced dendritic spine formation

Although the results that I present concerning the regulation of Wnt4 by BDNF and the role of Wn4 during dendritic spine formation are preliminary, the implications of these results are interesting nonetheless. In contrast to the ability of BDNF to increase levels of Wnt2 mRNA, BDNF appears to decrease Wnt4 mRNA expression in a rapid manner (Fig. 3.8, Appendix Fig. 14). In the absence of data examining the rate of Wnt4 protein synthesis during BDNF treatment, it is difficult to formulate any concrete statements regarding overall Wnt4 expression.

Indeed, mRNA translation and mRNA stability are tightly coupled processes (Schoenberg and Maquat, 2012). However, the data presented here initially suggest that Wnt4 expression may be downregulated by BDNF.

The rapid BDNF-induced decrease in Wnt4 mRNA is complicated by the fact that microarray analysis of FS-BDNF^{KO} mice indicates that Wnt4 expression is decreased at P35 (Table 2.1). Admittedly, the timescale and nature of the two expression assays that I present here (e.g., 4 hr. BDNF treatment vs. embryonic knockout of BDNF) are vastly different. The downregulation of Wnt4 expression at P35 could reflect several indirect steps of regulation compensating for the initial loss of BDNF. Interestingly, this may involve regulation of overall neural activity. Indeed, increased neural activity (KCl treatment) appears to decrease levels Wnt4 mRNA while decreased neural activity (TTX treatment) appears to increase levels Wnt4 mRNA, suggesting that, at the very least, Wnt4 mRNA expression is sensitive to neural activity. Given the ability of BDNF to regulate the formation of inhibitory neural circuits (Baldelli et al., 2005; Frerking et al., 1998; Hong et al., 2008; Matsumoto et al., 2006; Ohba et al., 2005), it's possible that increased excitatory drive due to improper development of inhibitory synapses could lead to a decrease in Wnt4 expression. Future experiments aimed at examining the regulation of Wnt4 expression by both BDNF and neural activity in greater detail should help to clarify some of the conflicting initial results presented here.

Despite the conflicting nature of the results relating to BDNF-mediated regulation of Wnt4, I also present here that Wnt4 overexpression is sufficient to

increase dendritic spine density and decrease dendritic spine length in cortical neurons, suggesting that Wnt4 functions during dendritic spine formation. My results are consistent with what has been published previously on the role of Wnt4 in the CNS. Previous studies demonstrated that Wnt4 acts as an attractant for midline-crossing spinal cord axons during development (Lyuksyutova et al., 2003; Wolf et al., 2008). It is entirely possible that this role for Wnt4 is preserved within the developing and adult cortex, and that Wnt4 functions as a retrogradely secreted factor to influence excitatory synapse assembly in a presynaptic manner. Indeed, Wnt4 is expressed in the mouse cortex at P14 (Fig. 3.7) and at P56 (Appendix Fig. 13) in pyramidal excitatory neurons, all of which is consistent with the ability of Wnt4 to promote dendritic spine formation.

4.14 A potential role for Fzd3 in mediating Wnt4 signaling

Interestingly, the initial study demonstrating the role for Wnt4 during axon guidance suggested that Wnt4 might signal through the receptor Fzd3 to mediate this effect (Lyuksyutova et al., 2003). I utilized the Allen Developing Mouse Brain Atlas to examine expression of Fzd3 in the P14 mouse brain and found that Fzd3 is expressed in several regions of the brain at this time. Fzd3 also appears to be expressed in the brain at P56 (Appendix Fig. 18). Curiously, the expression of Fzd3 is different than that of Wnt4. Specifically in the cortex, Fzd3 is expressed highly in layers II/III and IV. Fzd3 expression is noticeably lower in layer V, where Wnt4 expression is highest. Cortical layer V receives presynaptic input from all layers of the cortex (Briggs and Callaway, 2005), including layers II/III

and IV. It is tempting to speculate that retrograde Wnt4 signaling from layer V pyramidal excitatory neurons signals through Fzd3 located on the presynaptic axons of layers II/III and IV in order to regulate dendritic spine formation within layer V. Future experiments first testing the role of Fzd3 during dendritic spine formation would be a good place to start to examine this possibility. Ultimately, Fzd3 loss-of-function should prevent the ability of Wnt4 to promote dendritic spine formation if this hypothesis is correct.

Finally, if BDNF does, in fact, downregulate Wnt4 expression while Wnt4 is able to promote dendritic spine formation, then this suggests that the functional requirement for Wnt signaling during dendritic spine formation may be more complex than initially thought. If these results prove to be consistent, the implication is that BDNF may bi-directionally regulate dendritic spine formation through Wnt signaling. Experiments examining the expression of different Wnt proteins (such as Wnt2 and Wnt4) at dendritic spines and the kinetics of BDNF-mediated regulation of those Wnts may help to determine why BDNF may differentially regulate multiple Wnts during dendritic spine formation.

4.15 Wnts and Neurotrophins reciprocally regulate each other

Neural synapses are an evolutionarily ancient structure thought to have evolved in early metazoans (Ryan and Grant, 2009). Similarly, the emergence of the Wnt signaling pathway coincides with the emergence of metazoan life (Croce and McClay, 2008), consistent with the availability of Wnt signaling to play a fundamental role during the communication events surrounding synapse

formation. In contrast, neurotrophin signaling pathways emerged more recently, with the ancestral neurotrophin believed to have arisen early in vertebrate evolution (Hallbook, 1999). The later evolution of neurotrophins is entirely consistent with the possibility that the newly evolved neurotrophin signaling system was able to recruit the pre-existing Wnt signaling system in regulating interactions at synapses. Indeed, NGF regulates Wnt5 expression during sympathetic nervous system development (Bodmer et al., 2009). Interestingly, there is evidence for reciprocal regulation of neurotrophins by Wnts; several Wnts regulate Neurotrophin-3 expression during sensory nervous system development (Patapoutian et al., 1999), and BDNF expression is regulated by Wnt signaling in a Muller glia cell line (Yi et al., 2012). I suggest that the neurotrophin and Wnt signaling systems mediate a communication dialog between neurons and their innervation targets that shapes the development and plasticity of neural circuitry in the cerebral cortex and elsewhere in the nervous system.

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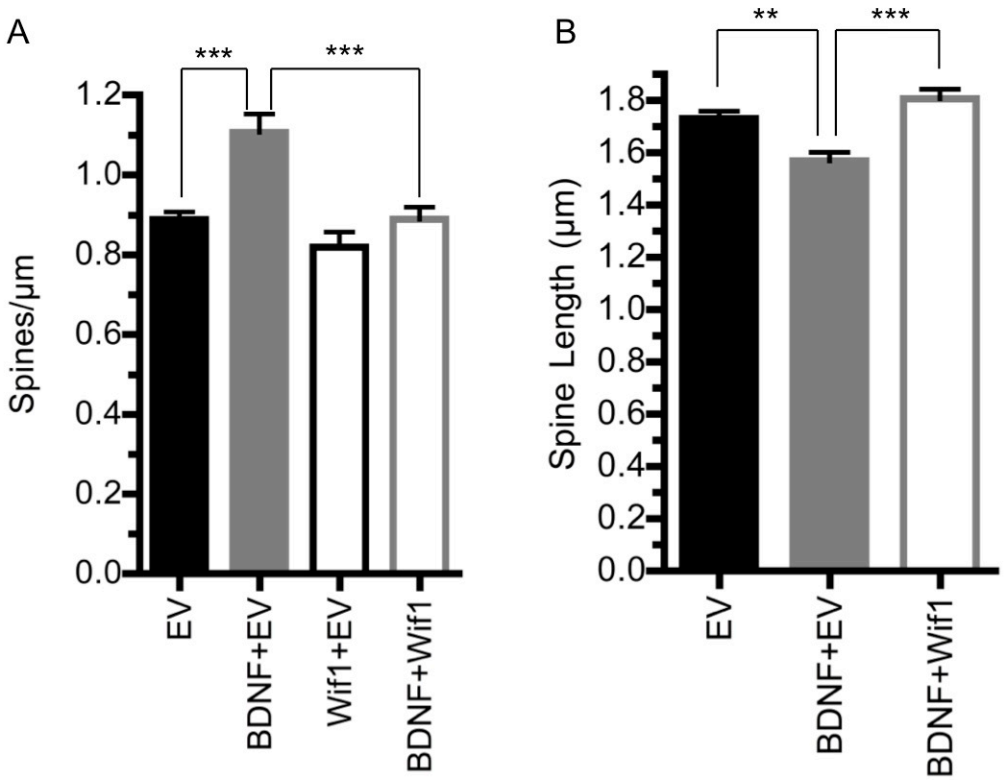
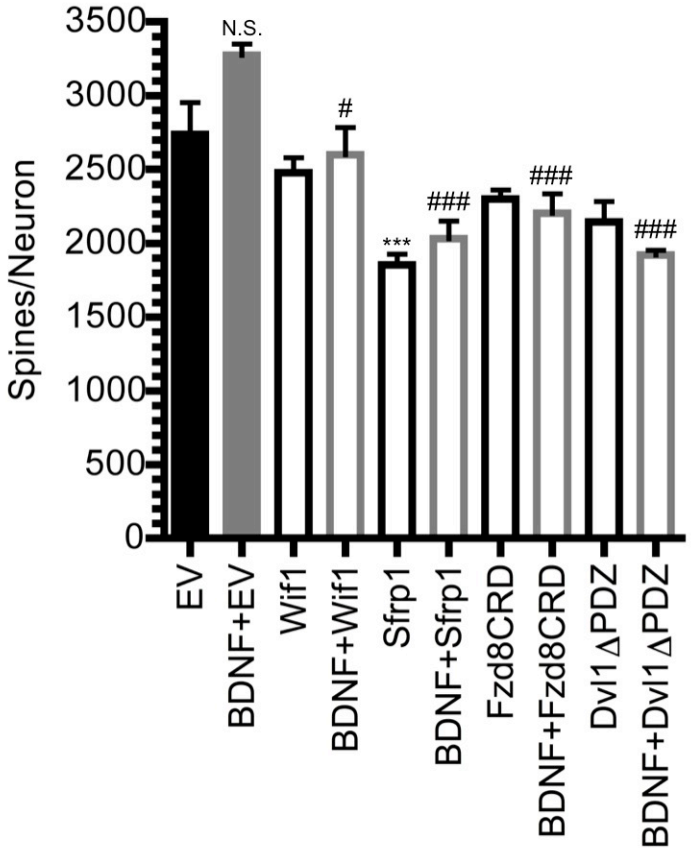
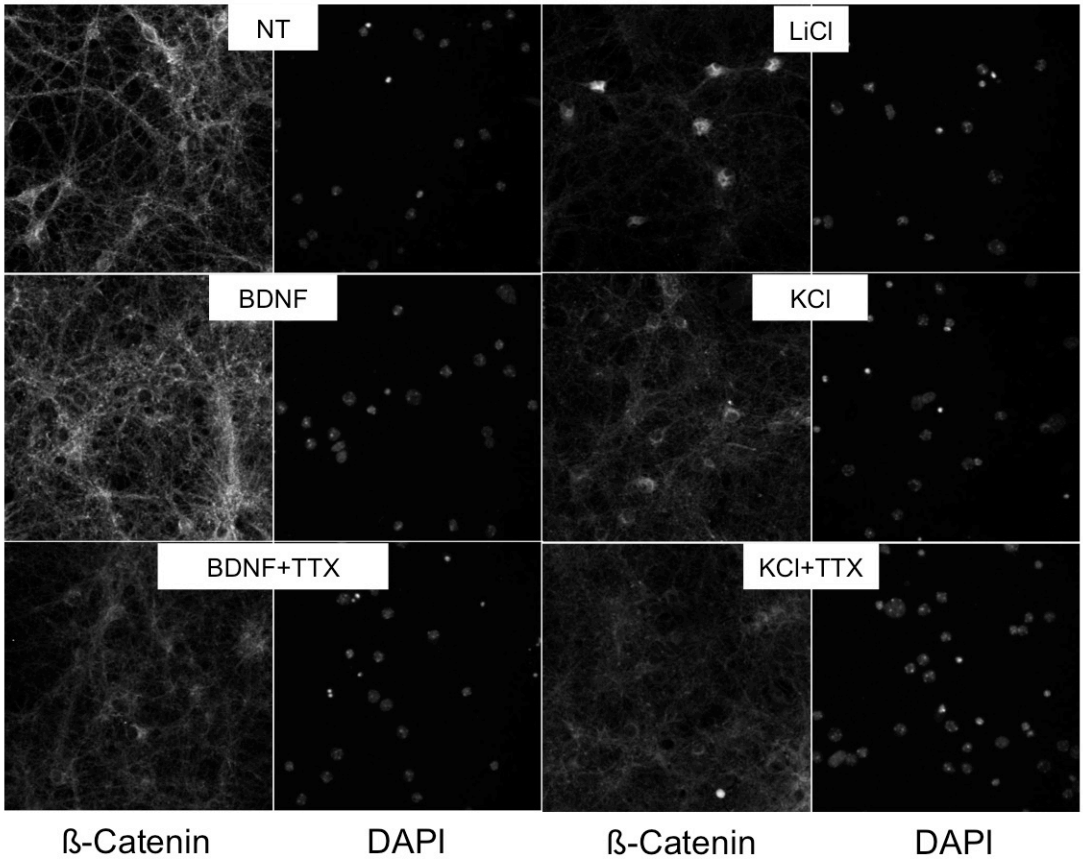
APPENDIX



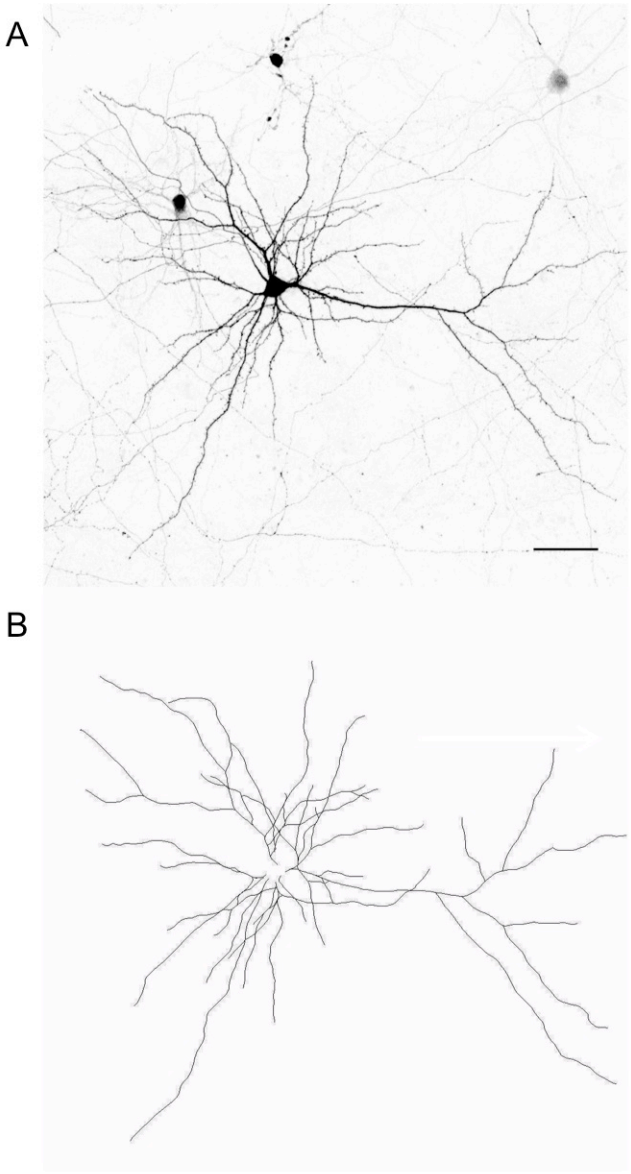
Appendix Figure 1. Wif1 blocks BDNF-induced dendritic spine formation. (A) Quantification of dendritic spine density from cortical neurons expressing EV, BDNF, Wif1 or BDNF+Wif1 from DIV7-11. (B) Quantification of average dendritic spine length of neurons expressing EV, BDNF or BDNF+Wif1. **p<0.01, ***p<0.001. n=number of neurons: EV n=37, BDNF+EV n=22, Wif1+EV n=24, BDNF+Wif1 n=30.



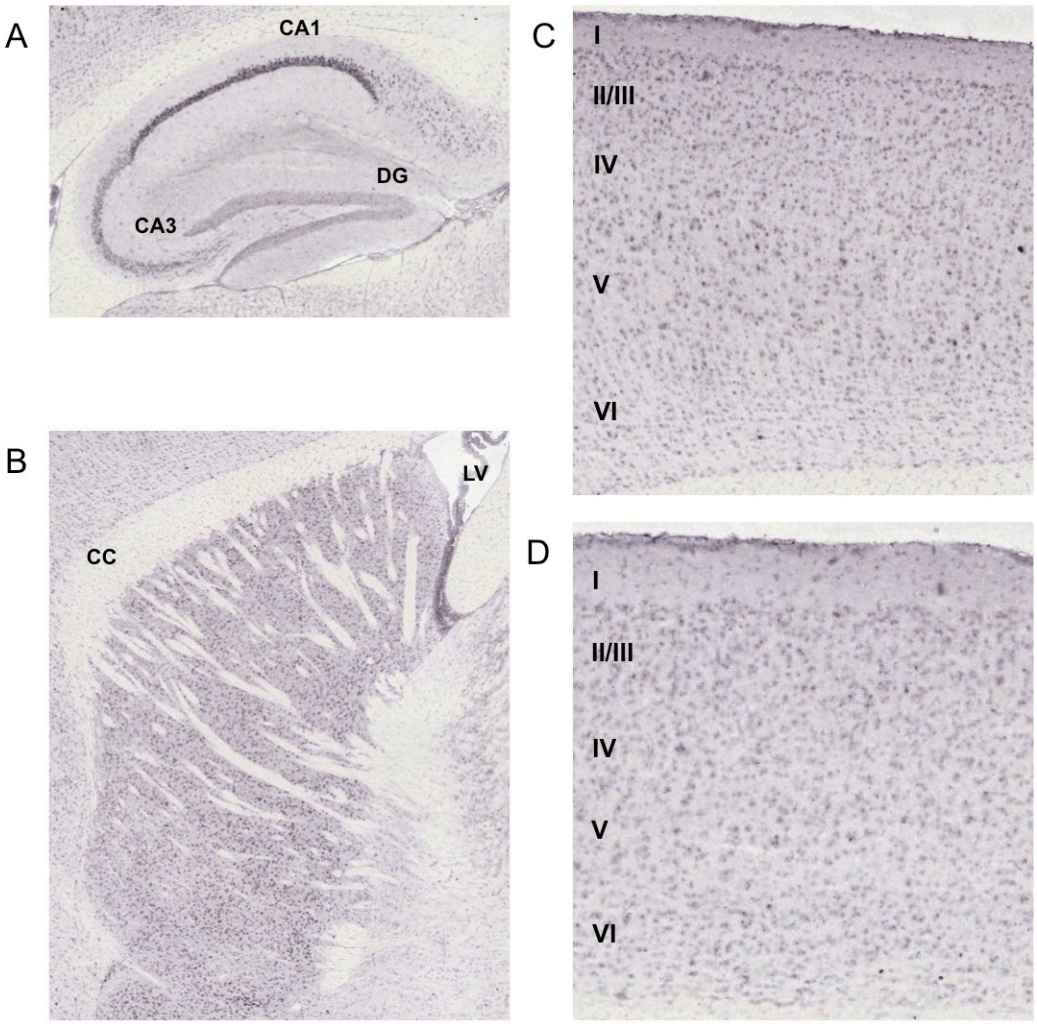
Appendix Figure 2. Wnt inhibition blocks BDNF-induced increases in neural connectivity. Quantification of the number of dendritic spines per neuron as calculated from the product of dendritic spine density and total dendrite length for cortical neurons co-expressing Wnt inhibitors (Wif1, Sfrp1, Fzd8CRD and Dvl1ΔPDZ) with either EV or BDNF from DIV10-14. Asterisks indicate comparisons to EV alone. Hash marks indicate comparison to BDNF+EV. *p<0.05, ***p<0.001. n=number of wells. EV n=4, BDNF+EV n=4, Wif1 n=4, BDNF+Wif1 n=3, Sfrp1 n=4, BDNF+Sfrp1 n=4, Fzd8CRD n=3, BDNF+Fzd8CRD n=3, Dvl1ΔPDZ n=3, BDNF+Dvl1ΔPDZ n=4.



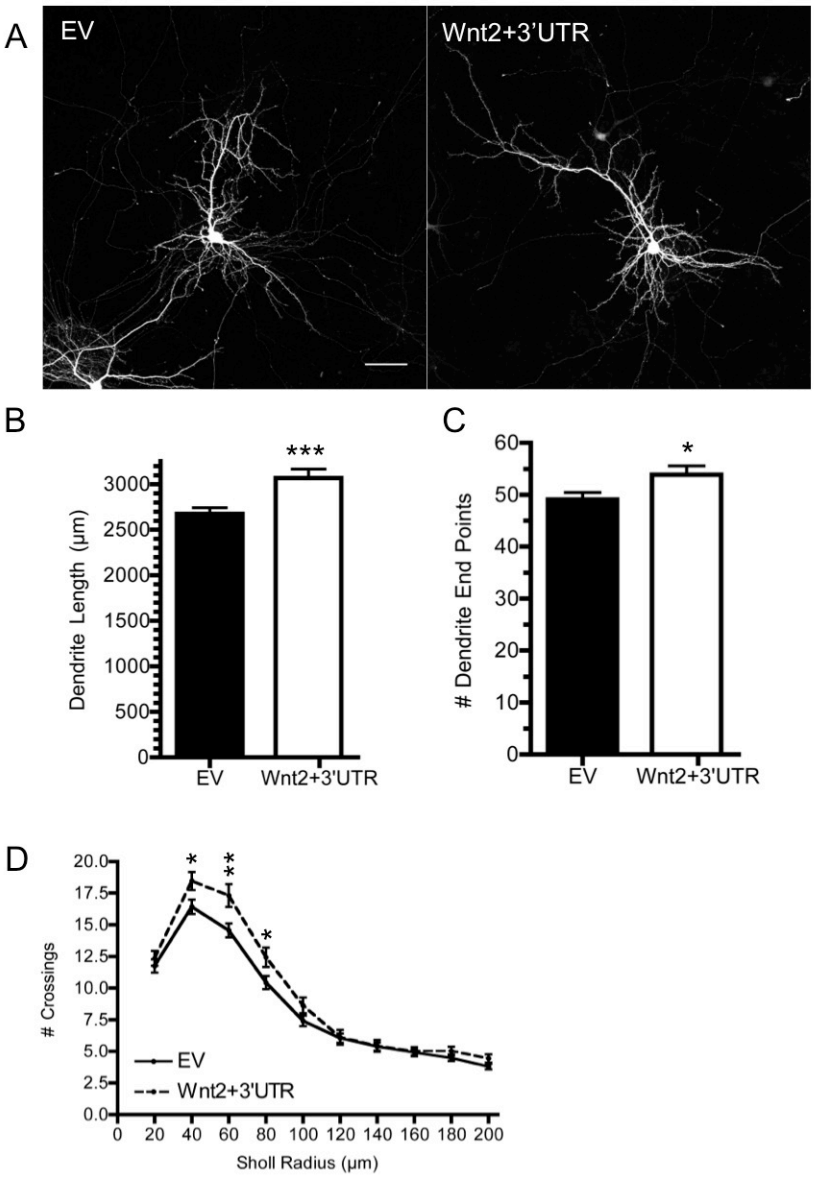
Appendix Figure 3. Nuclear accumulation of ß-Catenin in cultured cortical neurons. DIV10 neurons were treated with LiCl (10μM), BDNF (12.5 ng/mL), KCl (20mM), or with BDNF and KCl in combination with TTX (4μM) for 12 hours. Neurons were then fixed and stained for nuclear DNA (DAPI) and ß-Catenin. Images shown are compressed z-stacks (0.5μm step size) of neurons imaged at 20X.



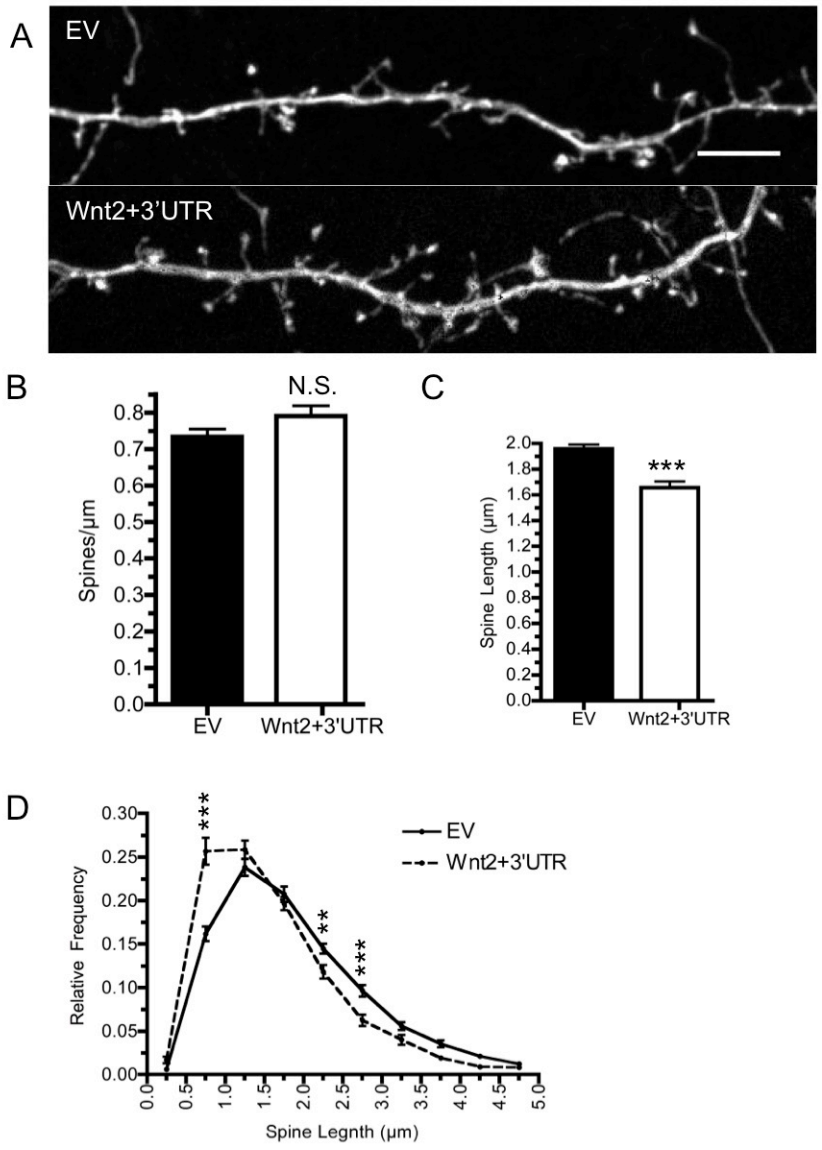
Appendix Figure 4. Example tracing of a cortical neuron using the ImageJ plugin NeuronJ. (A) Representative image of a cortical neuron imaged at 20X. scale bar=50μm. (B) Skeletonized tracing of the entire dendritic arbor that was used for subsequent dendritic arbor analysis.



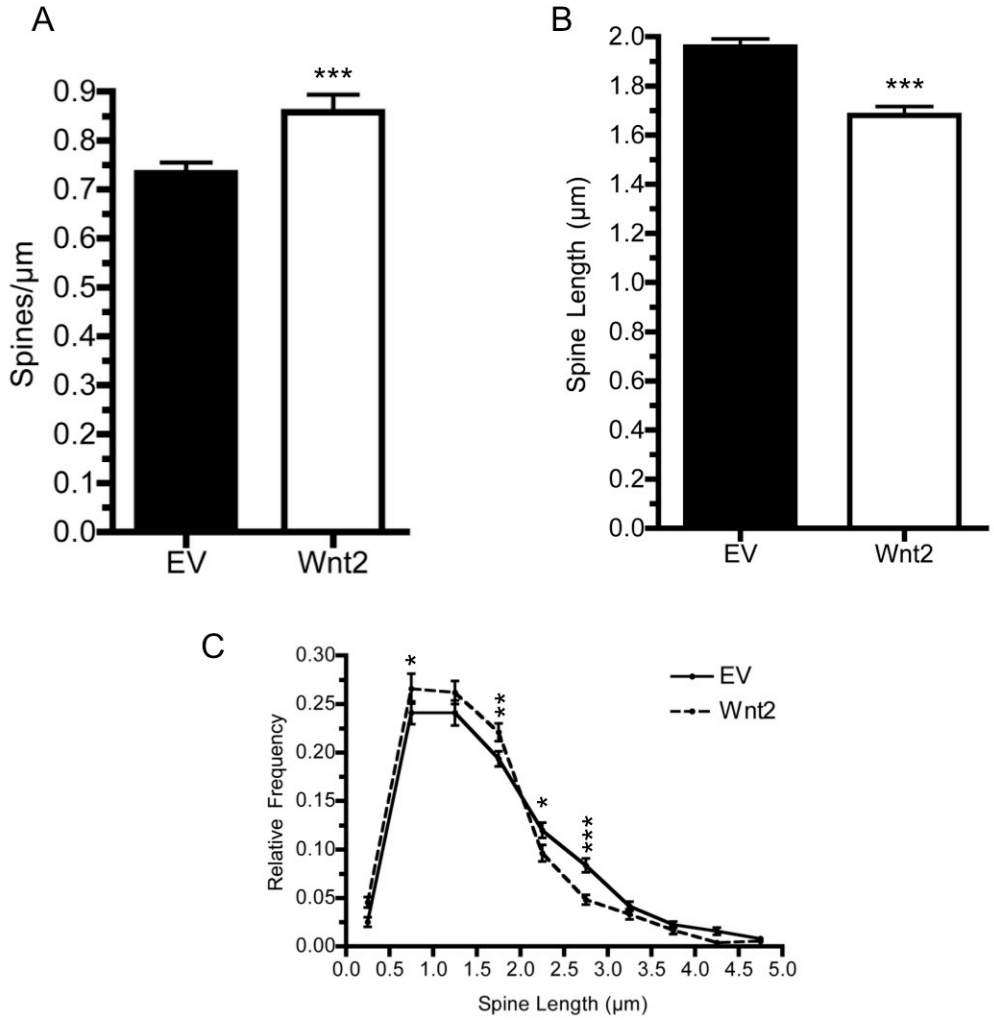
Appendix Figure 5. Wnt2 is expressed in the adult mouse brain at low levels. *in situ* hybridization images taken from the Allen Mouse Brain Atlas (Allen Mouse Brain Atlas, 2009) showing Wnt2 expression (purple color) in the (A) hippocampus, (B) striatum, (C) motor cortex, and (D) visual cortex at P56.



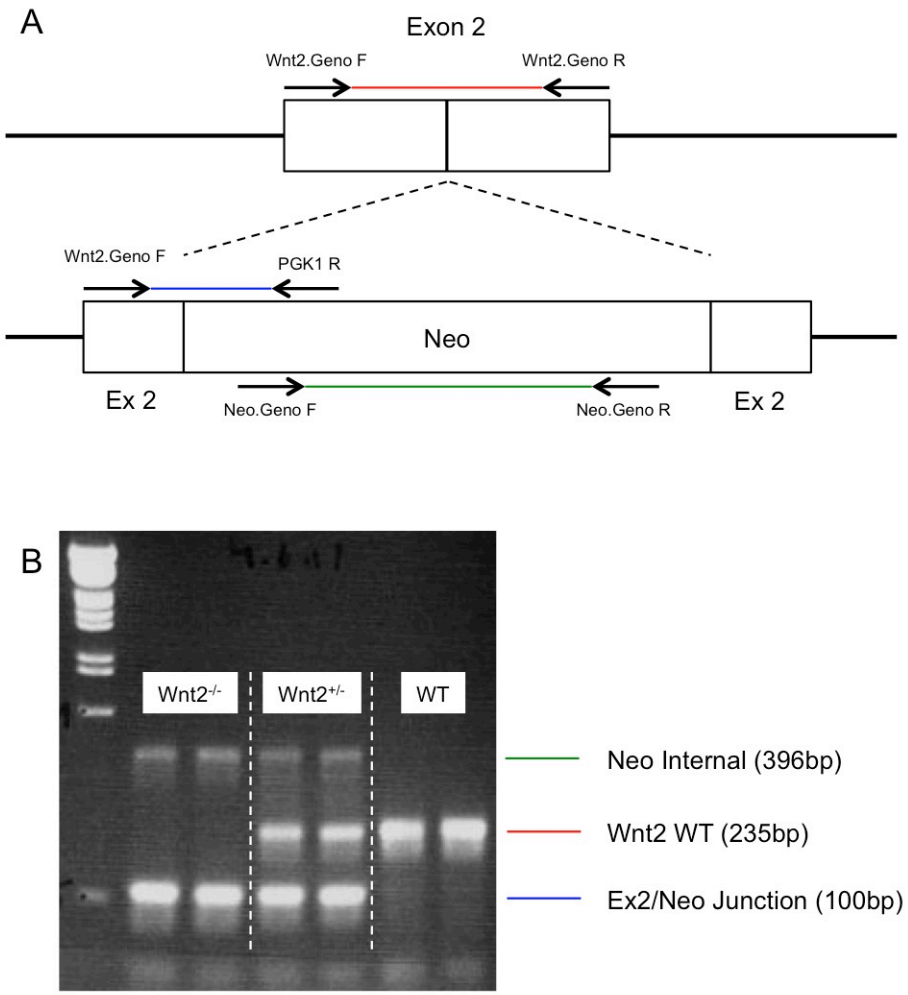
Appendix Figure 6. Expression of Wnt2 with its endogenous 3'UTR increases cortical dendrite growth and branching. (A) Representative cortical neurons expressing EV or Wnt2+3'UTR from DIV10-14. Quantification of (B) total dendrite length and (C) number of dendrite endpoints for each treatment. (D) Sholl analysis of dendritic complexity comparing neurons expressing EV with neurons expressing Wnt2+3'UTR. *p<0.05, *p<0.01, ***p<0.001. n=number of neurons: EV n=37, Wnt2+3'UTR n=22.



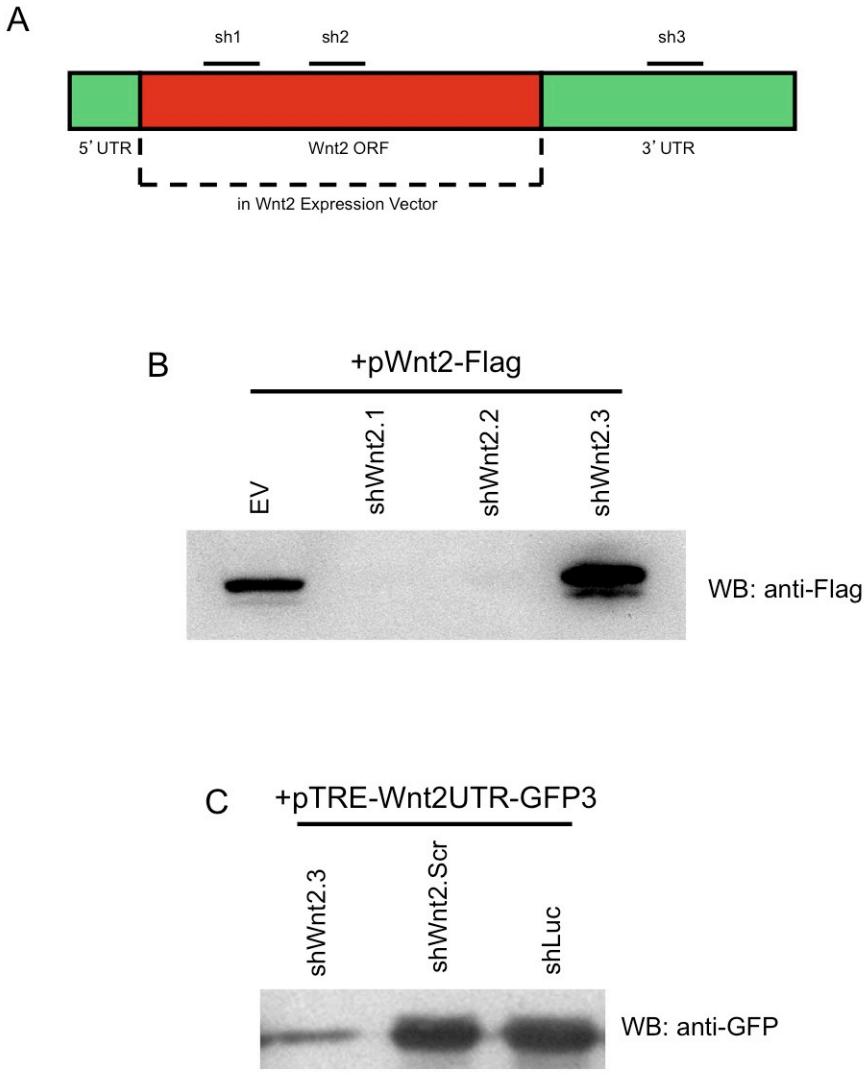
Appendix Figure 7. Expression of Wnt2 with its endogenous 3'UTR promotes dendritic spine formation. (A) Representative dendritic segments from cortical neurons expressing EV or Wnt2+3'UTR from DIV10-14. Quantification of (B) dendritic spine density and (C) average dendritic spine length of neurons from each treatment. (D) Relative frequency distribution comparing spine length for each treatment. **p<0.01, ***p<0.001. n=number of neurons: EV n=29, Wnt2+3'UTR n=28.



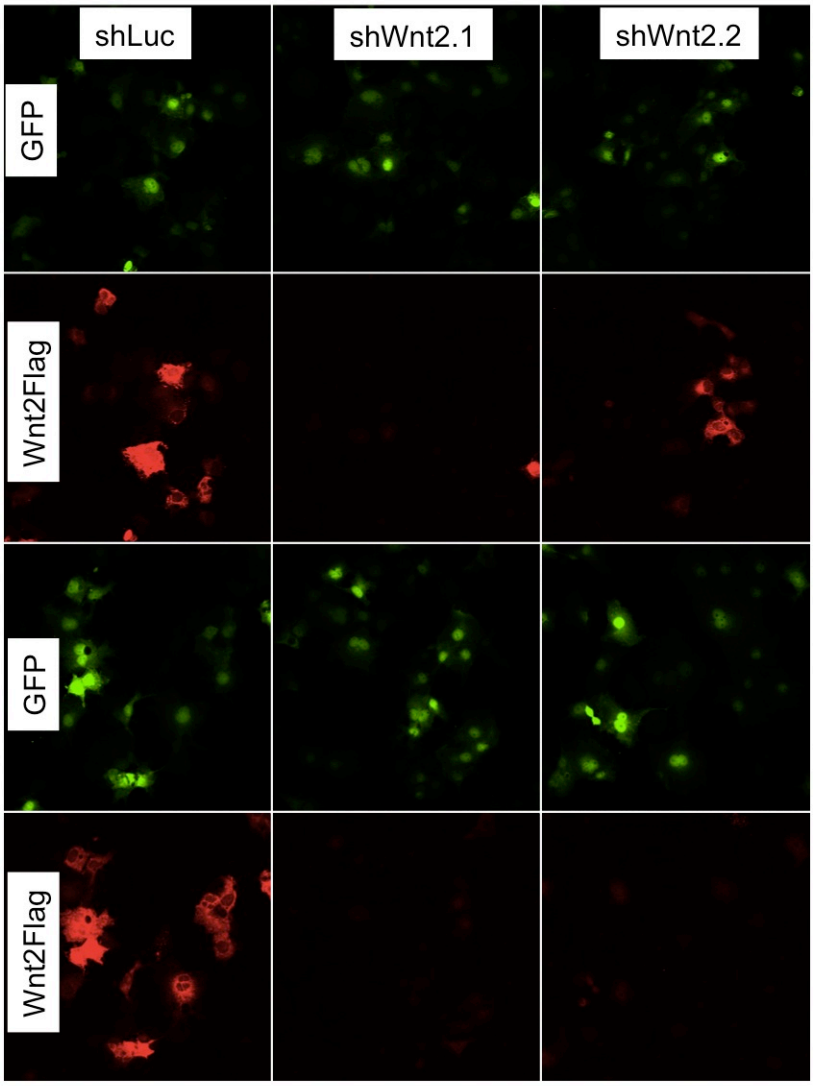
Appendix Figure 8. Wnt2 expression from DIV7-11 promotes dendritic spine formation. Quantification of (A) dendritic spine density and (B) average dendritic spine length of cortical neurons expressing EV or Wnt2. (C) Relative frequency distribution comparing spine length for each treatment. *p<0.05, **p<0.01, ***p<0.001. n=number of neurons: EV n=37, Wnt2 n=22.



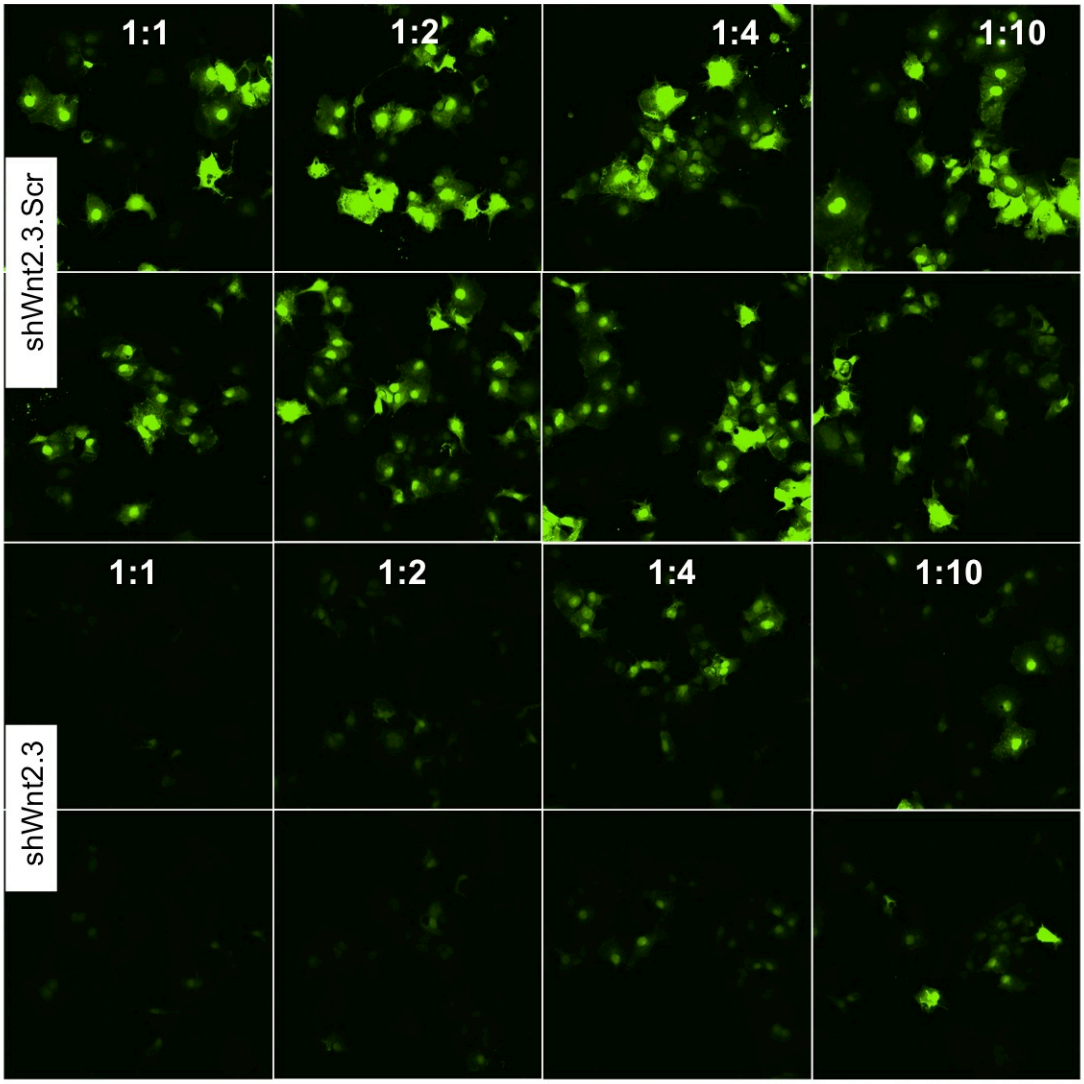
Appendix Figure 9. Genotyping strategy for $Wnt2^{KO}$ mice. (A) Schematic illustrating the location of insertion of the neomycin cassette within Exon 2 of the Wnt2 locus, position of the five different PCR primers used for genotyping, and the relative location and size of the products from each PCR reaction. (B) Example results from a genotyping assay showing the banding pattern generated from $Wnt2^{-/-}$, $Wnt2^{+/-}$ and WT mice. Identity of each of the bands is indicated on the right and is color-coded according to (A).



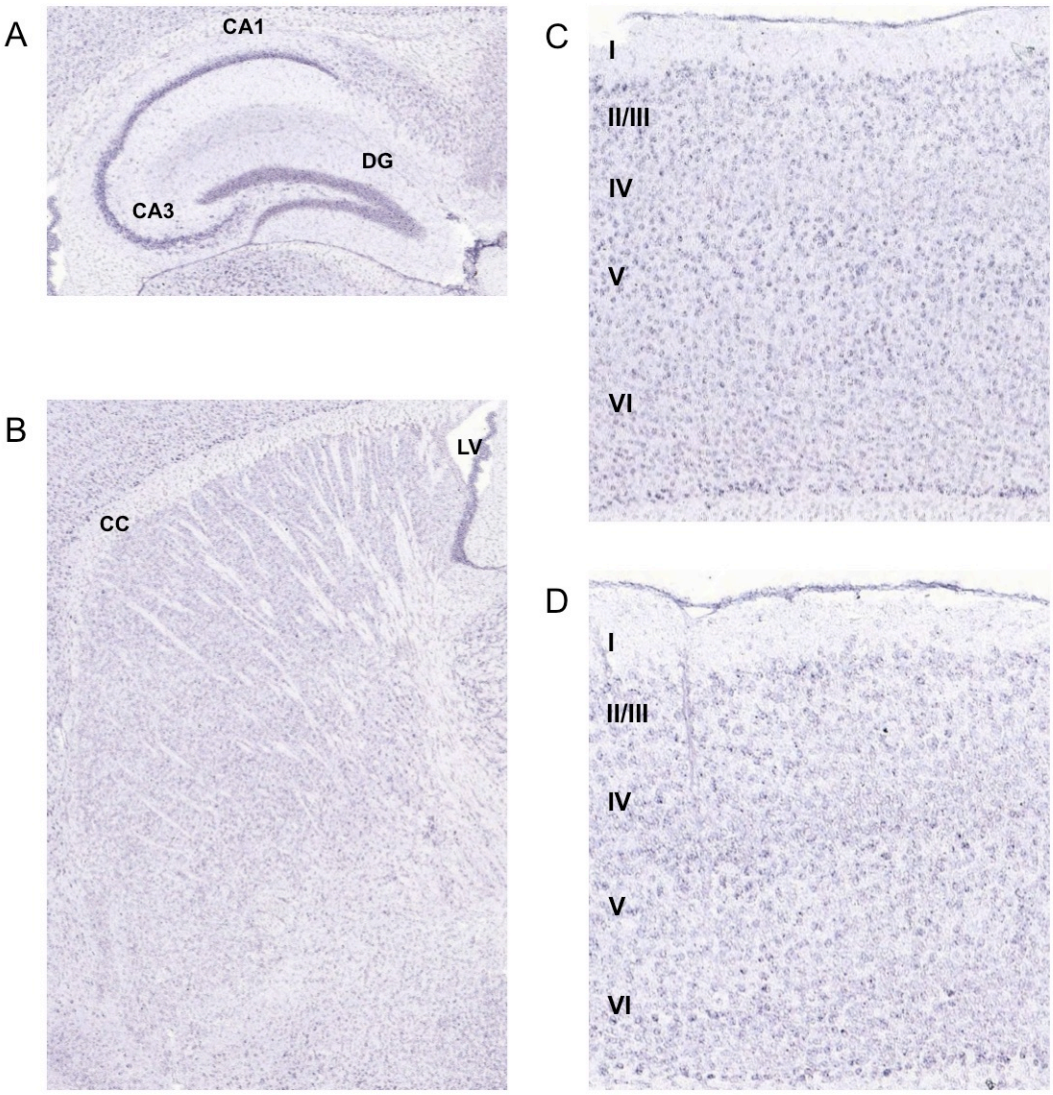
Appendix Figure 10. shRNA knockdown of Wnt2 expression. (A) Schematic indicating the location of the targets for each of the three Wnt2 shRNAs used for these experiments. (B) Western blot confirmation of the knockdown efficacy of shWnt2.1 and shWnt2.2. shRNAs were co-expressed with a plasmid encoding Wnt2-Flag at a 1:1 ratio in HEK293T cells for 48 hours. (C) Western blot confirmation of the knockdown efficacy of shWnt2.3. shRNAs were co-expressed with a plasmid encoding GFP fused with the endogenous Wnt2 3'UTR at a 1:1 ratio in HEK293-T cells for 48 hours.



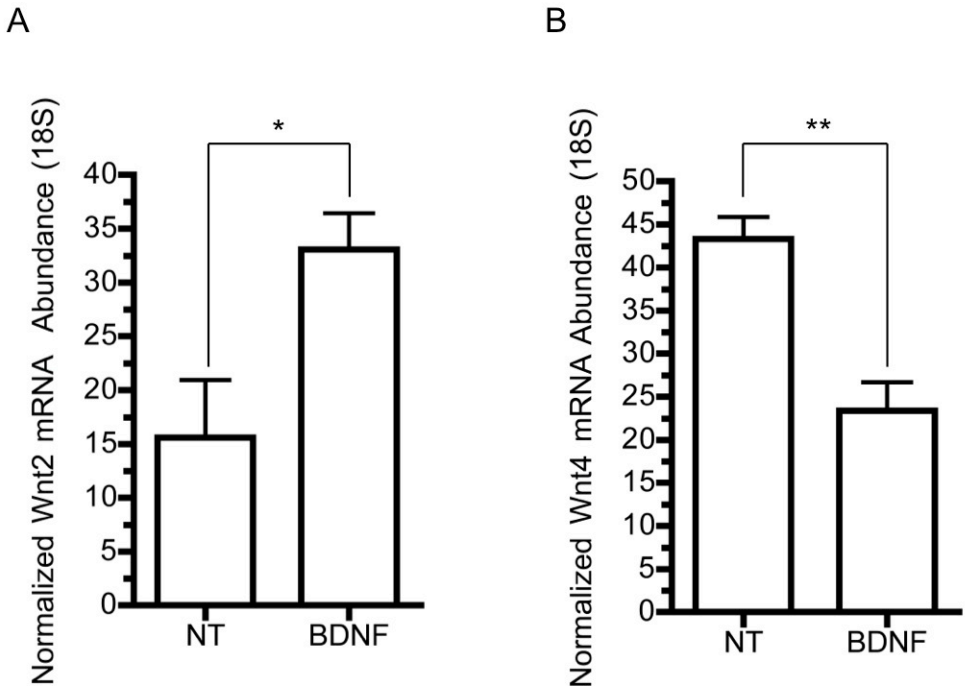
Appendix Figure 11. Immunofluorescent confirmation of the knockdown efficacy of shWnt2.1 and shWnt2.2. shRNAs were co-expressed with a plasmid encoding Wnt2-Flag and a plasmid encoding GFP in COS-7 cells for 48 hours and then subsequently stained using an antibody directed against the Flag epitope. Shown are two representative fields of view for each shRNA, imaged at 20X. Imaging settings were kept consistent throughout.



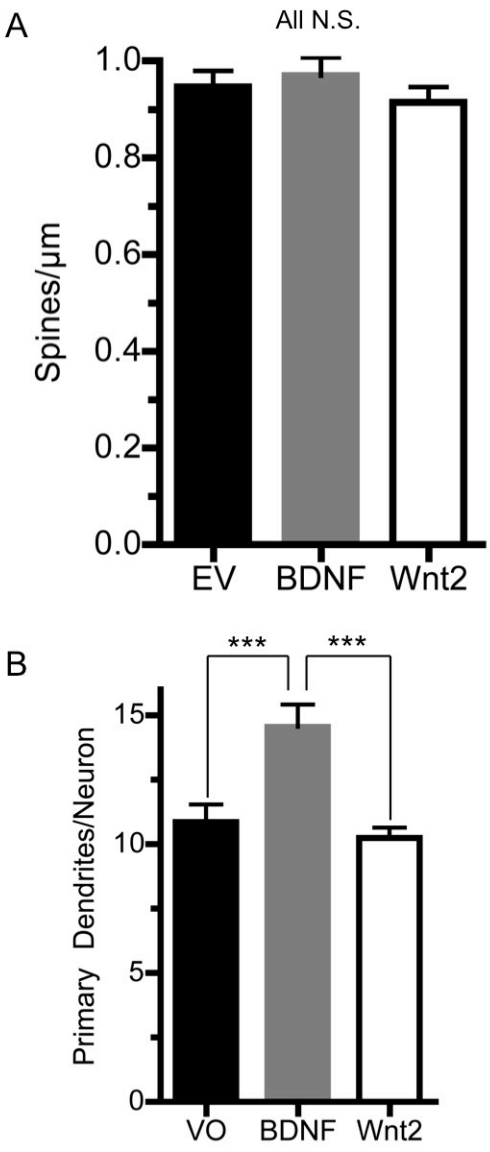
Appendix Figure 12. Fluorescent confirmation of the knockdown efficacy of shWnt2.3. shRNAs were co-expressed with a plasmid encoding GFP fused with the endogenous Wnt2 3'UTR at ratios of 1:1, 1:2, 1:4 and 1:10 for 48 hours in COS-7 cells. Shown are two representative fields of view of GFP fluorescence for each shRNA at each ratio, imaged at 20X. Imaging settings were kept consistent throughout.



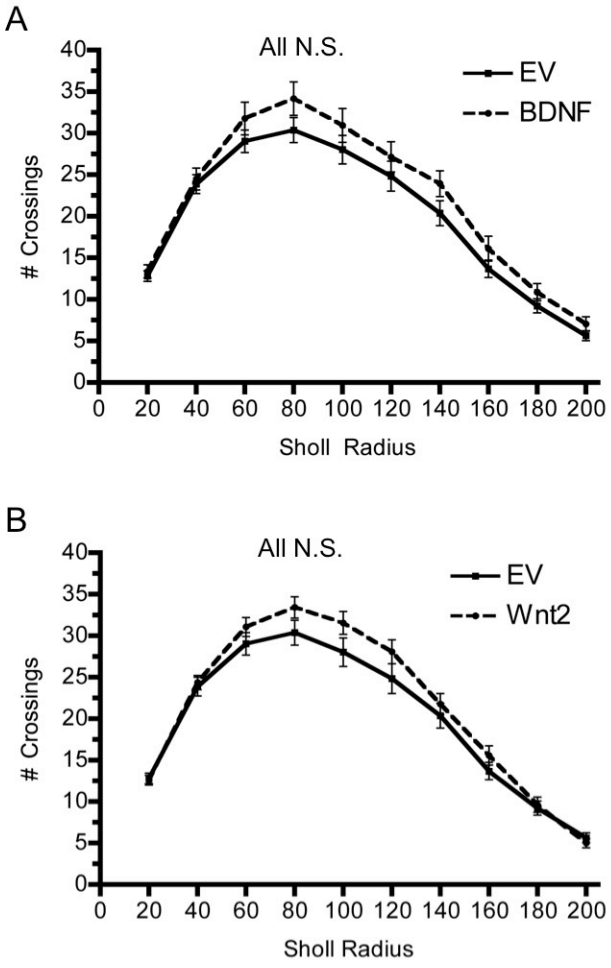
Appendix 13. Wnt4 is expressed in the adult mouse brain at low levels. *in situ* hybridization images taken from the Allen Mouse Brain Atlas (Allen Mouse Brain Atlas, 2009) showing Wnt4 expression (purple color) in the (A) hippocampus, (B) striatum, (C) motor cortex, and (D) visual cortex at P56.



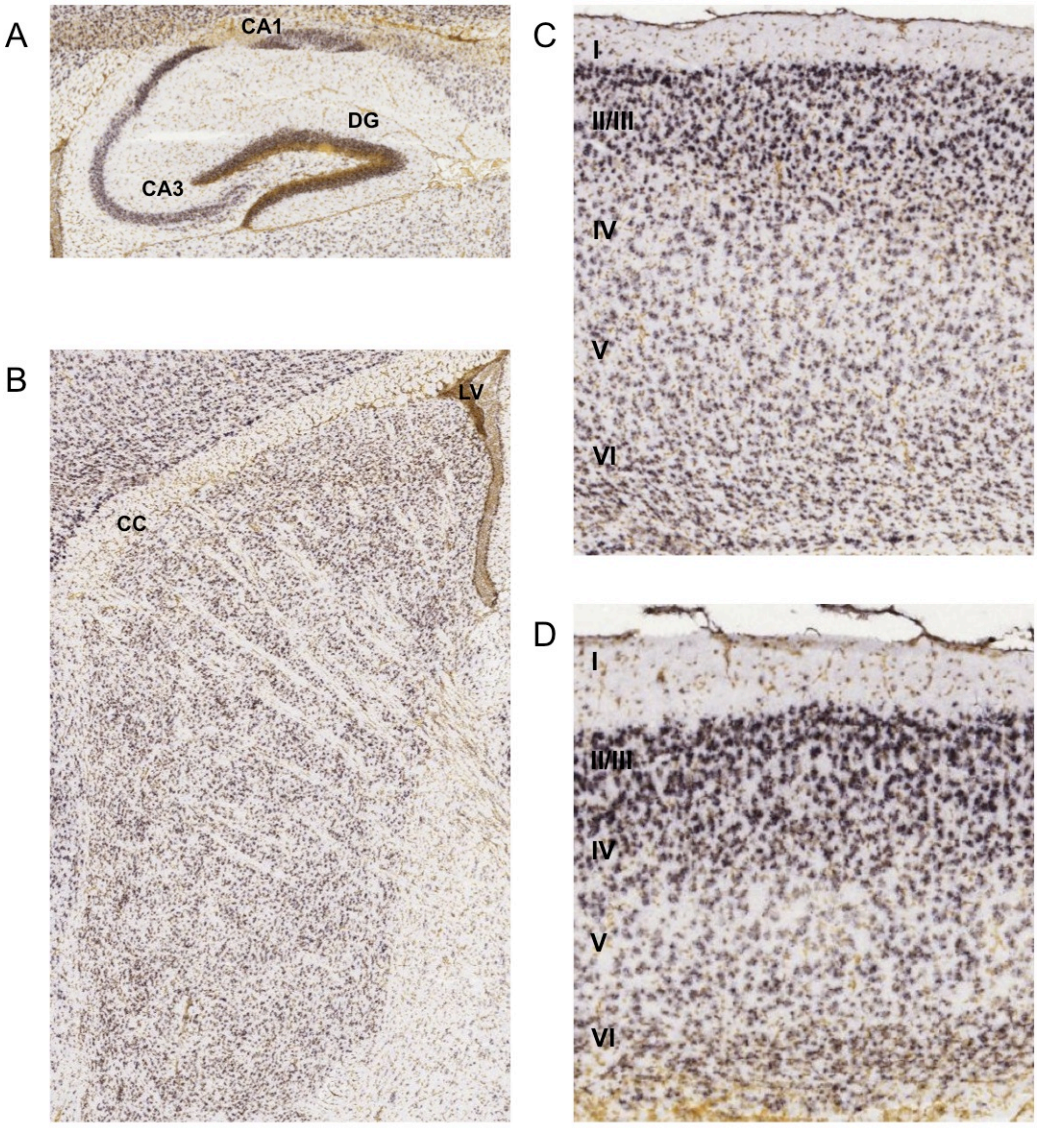
Appendix Figure 14. Regulation of Wnt2 and Wnt4 expression by BDNF at DIV16. Quantification of (A) Wnt2 and (B) Wnt4 mRNA abundance after 4 hr. treatment of DIV16 cortical neurons with recombinant BDNF (50 ng/mL). *p<0.05, **p<0.01. n=number of wells: (Wnt2) NT n=2, BDNF n=3, (Wnt4) NT n=3, BDNF n=3.



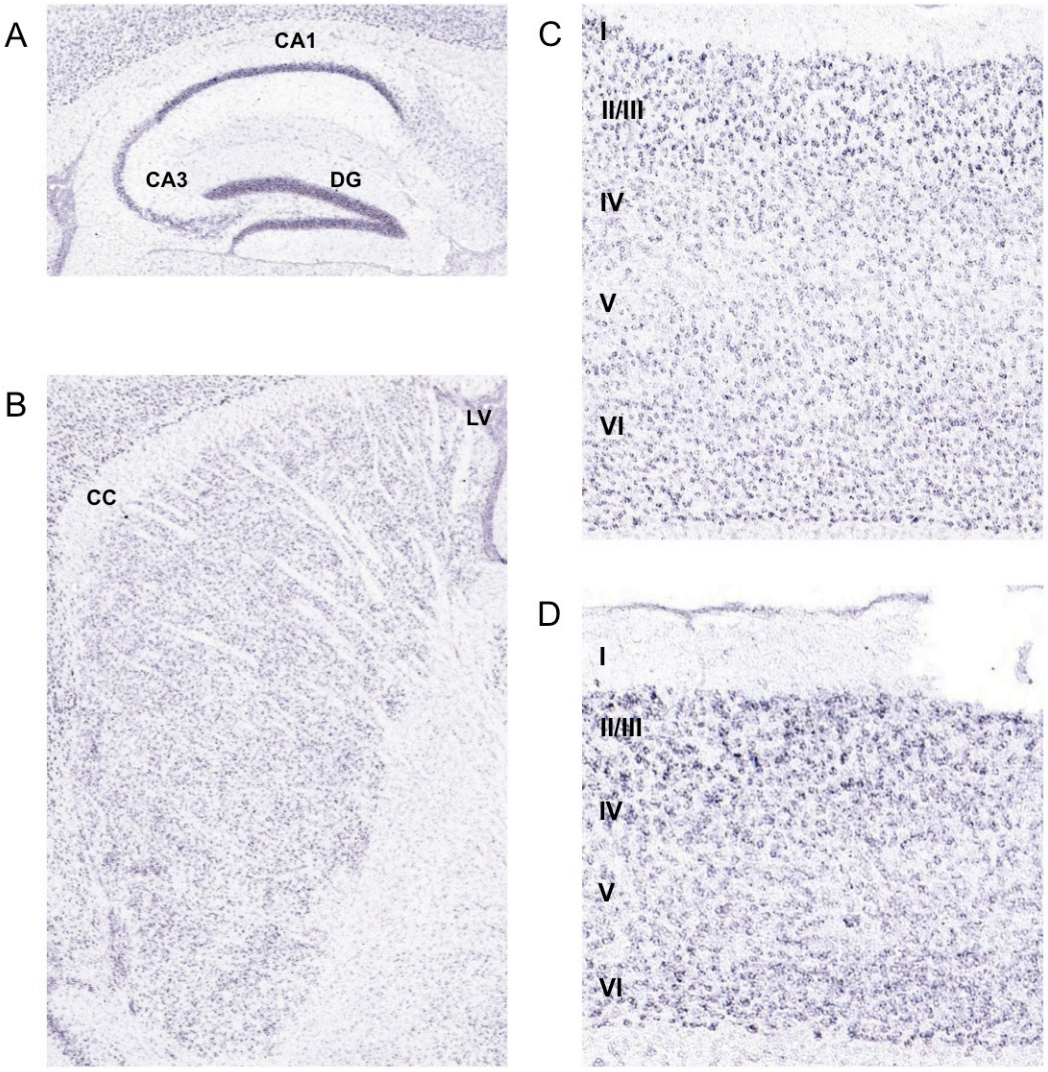
Appendix Figure 15. Quantification of (A) dendritic spine density and (B) number of primary dendrites in BDNF-/- cortical neurons expressing EV, BDNF or Wnt2 from DIV7-11. ***p<0.001. n=number of neurons: EV n=22, BDNF n=18, Wnt2 n=16.



Appendix Figure 16. Sholl analysis of dendritic complexity of hippocampal neurons comparing neurons expressing EV to neurons expressing (A) BDNF or (B) Wnt2 from DIV7-9. n=number of neurons: EV n=26, BDNF n=18, Wnt2 n=27.



Appendix Figure 17. Fzd3 is expressed in the developing mouse brain. *in situ* hybridization images taken from the Allen Developing Mouse Brain Atlas (Allen Developing Mouse Brain Atlas, 2009) showing Fzd3 expression (purple color) in the (A) hippocampus, (B) striatum, (C) motor cortex, and (D) visual cortex at P14.



Appendix Figure 18. Fzd3 is expressed in the adult mouse brain. *in situ* hybridization images taken from the Allen Mouse Brain Atlas (Allen Mouse Brain Atlas, 2009) showing Fzd3 expression (purple color) in the (A) hippocampus, (B) striatum, (C) motor cortex, and (D) visual cortex at P56.