The role of Wnt signalling in hippocampal synapse formation and function

Kieran A. Boyle

UCL

PhD

Supervisors:
Prof. Patrica C. Salinas
Dr. Alasdair J. Gibb

I, Kieran A. Boyle, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Wnt proteins are a large and diverse family of secreted signalling factors that play key roles in the development of the nervous system, including control of neuronal proliferation and differentiation, axon guidance, dendritogenesis and synaptogenesis. Despite recent advances in our understanding of Wnt function at synapses, key questions remain unanswered. For example the role of Wnt signalling in central postsynaptic development remains unclear, as does the specificity of Wnts for regulating different sub-types of synapse. The aim of this thesis was to investigate the role of Wnts in regulating the formation and function of central glutamatergic and GABAergic synapses in the rodent hippocampus, using complementary cell biological and electrophysiological approaches.

I find that Wnt7a specifically promotes the formation of excitatory glutamatergic synapses in cultured hippocampal neurons, with no effect on inhibitory GABAergic synapses. Furthermore, specific postsynaptic activation of Wnt signalling results in increased dendritic spine size, increased clustering of the postsynaptic protein PSD-95 and increased presynaptic innervation of dendritic spines. In contrast, GABAergic synapses are unaffected by Dishevelled-1 expression.

I also find that endogenous Wnt signalling regulates excitatory synaptic function. Acute blockade of endogenous Wnt signalling using the Wnt antagonists sFRP1, 2 and 3 results in a decrease in mEPSC frequency and evoked release probability at glutamatergic synapses, with no effect on GABAergic synapses. A similar decrease in evoked release probability is observed at glutamatergic Schaffer collateral-CA1 synapses in hippocampal slices from *Wnt7a-/-; Dvl1-/-* double knockout mice. Finally, I demonstrate that a prolonged reduction in glutamatergic release probability caused by chronic Wnt signalling blockade elicits a homeostatic increase in glutamatergic synapse number that acts to maintain normal levels of excitatory signalling.

In conclusion, the work presented in this thesis significantly advances our understanding of the role of Wnts at central synapses. Wnt signalling regulates multiple processes throughout the lifetime of an excitatory glutamatergic synapse. Wnt7a promotes the formation of excitatory synapses through the co-ordinated clustering of pre- and postsynaptic proteins. Postsynaptic Wnt signalling can directly regulate

excitatory postsynaptic formation at central synapses, and can also signal back to the presynaptic side. Endogenous Wnt signalling plays a role in maintaining normal levels of glutamate release, and chronic perturbation of this signalling results in compensatory changes in synapse density.

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AChR – Acetyl choline receptor

AD – Alzheimer's disease

AMPA - α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionic acid

AMPA-R – AMPA receptor

BDNF – Brain-derived neurotrophic factor

BSA – Bovine serum albumin

CaMK – Ca^{2+}/calmodulin-dependent kinase

CK1α - Casein kinase 1α

CNS – Central nervous system

CRD – Cysteine-rich domain

DFz2 – *Drosophila* Frizzled 2

DIV – Days *in vitro*

Dkk – Dikkopf

Dvl – Dishevelled

EJC – Endplate junctional current

EPSC – Excitatory postsynaptic current

ER – Endoplasmic reticulum

FGF – Fibroblast growth factor

FGF-R – FGF receptor

Fz – Frizzled

GABA - γ-aminobutyric acid

GAD – glutamic acid decarboxylase

GC – Granule cell

GSK3 – Glycogen synthase kinase 3

HBSS – Hank's balanced salt solution

HSPG – Heperan sulphate proteoglycan

I/O – Input/output

IPSC – Inhibitory postsynaptic current

JNK – c-Jun N-terminal kinase

Krm – Kremen

LDL-R – Low density lipoprotein receptor

LMT – Large mossy fibre terminal

MAP1B – Microtubule associated protein 1B

mEPSC – Miniature excitatory postsynaptic current

MF – Mossy fibre

mGluR – metabotropic glutamate receptor

mIPSC – Miniature inhibitory postsynaptic current

MIS – Multiply innervated spine

MT – Microtubule

NMDA – N-methyl-D-aspartate

NMDA-R – NMDA receptor

NMJ – Neuromuscular junction

PBS – Phosphate buffered saline

PCP – Planar cell polarity

PD – Parkinson's disease

PNS – Peripheral nervous system

PPR – Paired pulse ratio

PSD – Postsynaptic density

PTV – Piccolo transport vesicle

ROCK - Rho-associated coiled-coil containing protein kinase

sFRP – Secreted Frizzled-related protein

SNARE – Soluble NSF attachment protein receptors

STV – Synaptic vesicle protein transport vesicle

TSP – Thrombospondin

TTX – Tetrodotoxin

VAMP – Vesicle associated membrane protein

vGAT - Vesicular γ-aminobutyric acid transpoprter

vGlut – Vesicular glutamate transporter

Wg – Wingless

WIF – Wnt inhibitory factor

CHAPTER 1:

Introduction

The central nervous system of humans has been estimated to contain somewhere in the region of 85 billion neurons (Azevedo et al., 2009). These neurons communicate via highly specialised cellular junctions called synapses, which transmit information by transducing electrical signals in the presynaptic cell into electrical and/or biochemical signals in the postsynaptic cell. The average number of synapses in the human brain remains unclear, principally due to huge regional differences in synapse density. However a rough estimate often quoted in textbooks is that an average CNS neuron forms 1000 synapses with postsynaptic targets. This would mean that the average human brain contains somewhere in the order of 85 *trillion* synapses.

The sheer number of synapses in a fully developed human brain only hints at the complexity that these fascinating structures bestow. Synapses are not a homogenous population, but come in many different morphological and functional subtypes (Grant, 2007; Rizzoli and Betz, 2005). Furthermore, they are tightly controlled throughout life in terms of their formation (Waites et al.), connectivity (White, 2007), plasticity (Nelson and Turrigiano, 2008) and maintenance (McKinney, 2009). It is widely believed that this astonishing complexity is what allows us to display such a wide range of complex behaviour, including the capacity for abstract thought. Therefore a detailed understanding of how synapses form, function and adapt to different environmental stimuli is crucial to understanding normal brain function. Furthermore, defects in synapses have been implicated in a range of neurological diseases (Bennett, 2009; Dani et al., 2005; Helton et al., 2008; Tackenberg et al., 2009). Greater understanding of how synapses contribute and respond to these disease states will result in improvements in their diagnosis and treatment.

Our understanding of the formation, structure and function of synapses has improved dramatically over the past few decades. Much of this understanding has come from experiments performed in both vertebrates and invertebrates. Indeed, one theme that has emerged from these studies is the conservation of key molecules and signalling pathways from invertebrates all the way to humans (Ryan and Grant, 2009). The development of mutant animals that either lack or have modified genes has helped

identify molecules involved in synaptic development, and their mechanisms of action. Conditional mutant animals have been especially useful in investigating the actions of molecules at particular stages of synapse development, and in teasing apart pre- versus postsynaptic actions. *In vitro* cultures of neurons have also proven invaluable in elucidating the underlying cellular mechanisms that control synaptic development, due to the ease with which they can be pharmacologically and genetically manipulated. Finally, improvements in imaging synapses, particularly in live preparations, have provided an appreciation of just how dynamic a structure the synapse is.

1.1 Synaptic structure and function

As mentioned above, synapses are highly heterogeneous structures. They come in a wide range of shapes and sizes, based upon the particular function they perform. For example, typical central glutamatergic synapses between cortical pyramidal neurons are small and have a relatively low release probability (Branco and Staras, 2009). Their function is to transmit information about action potential firing in the presynaptic cell, which is then integrated in the postsynaptic cell with information coming from many other presynaptic neurons (Magee, 2000; Spruston, 2008). In contrast, synapses between motor neurons and muscle fibres ('neuromuscular junctions' or 'NMJs') are large and have a high release probability, and generally a single muscle fibre is innervated by a single motor neuron (Fagerlund and Eriksson, 2009). Their function is to reliably transduce an action potential in the presynaptic motor neuron into contraction of the postsynaptic muscle fibre (Fagerlund and Eriksson, 2009). A detailed description of all known synaptic subtypes is far beyond the scope of this introduction, and would indeed make for a weighty textbook. For the remainder of this introduction I will therefore focus on two examples: typical cortical glutamatergic excitatory synapses and GABAergic inhibitory synapses.

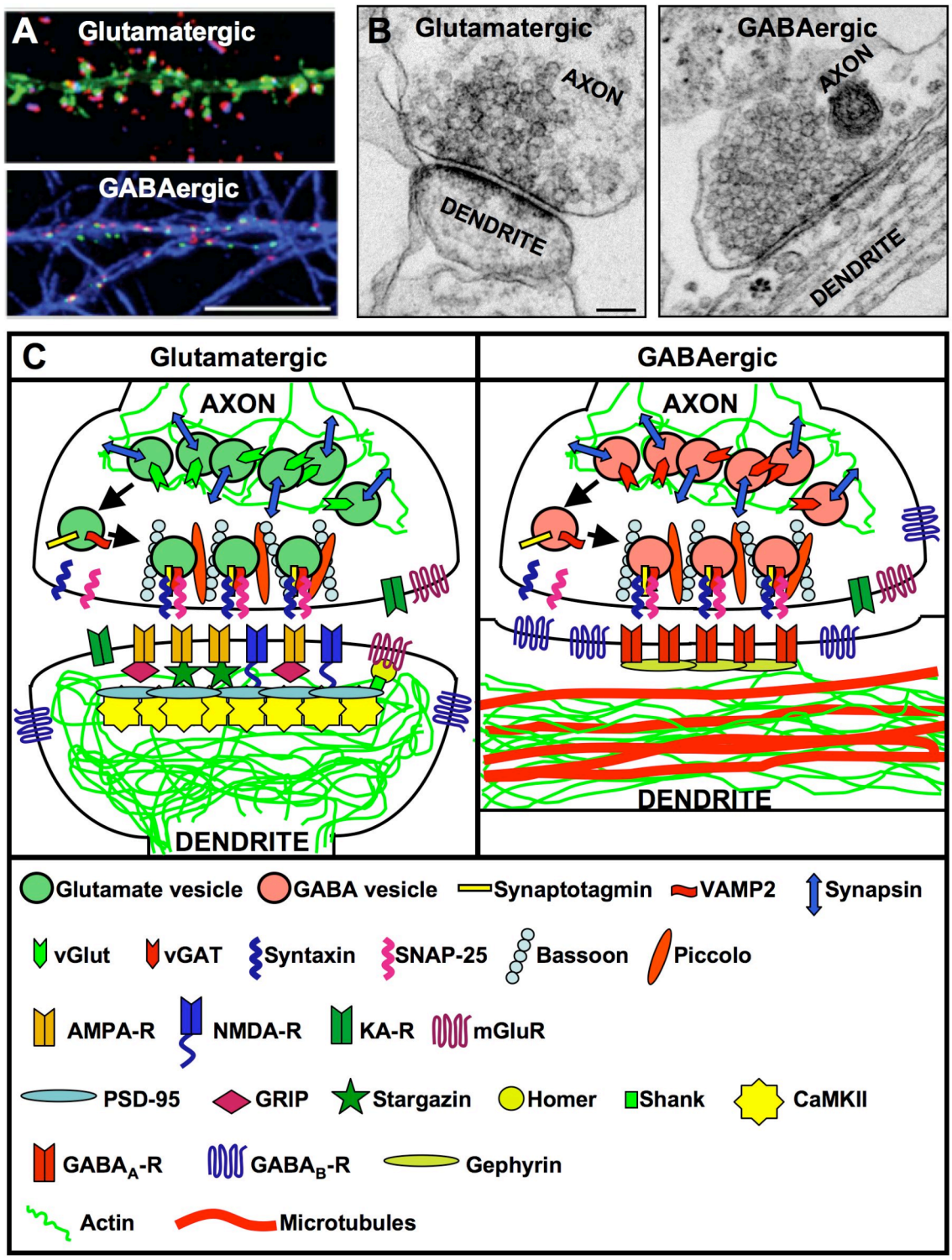
The choice of these two types of synapse is not arbitrary, as they are the focus of the experiments presented in this thesis. In the neocortex, which is believed to contribute to the advanced cognitive abilities of mammals, they account for the majority of synapses. Therefore they represent good models with which to study synaptic development, and a great deal can be learnt about cortical function and information processing through their study. Unsurprisingly then, these synapses (particularly cortical glutamatergic synapses) are amongst the best characterised synaptic subtypes. Glutamatergic synaptic signalling

generally results in postsynaptic excitation, whereas GABAergic signalling results in postsynaptic inhibition. Normal cortical function is believed to rely on an extremely complex and highly regulated interplay between excitation and inhibition. Accordingly, defects in this interplay have been implicated in a range of neuropathological disorders, including autism (Munoz-Yunta et al., 2008; Rubenstein and Merzenich, 2003), epilepsy (Leite et al., 2005), schizophrenia (Kehrer et al., 2008) and Rett syndrome (Dani et al., 2005). Therefore, in order to understand how the CNS functions in health and disease, it is crucial that we build a complete picture of the formation, structure and function of excitatory and inhibitory synapses, and the molecules and signalling pathways involved.

1.1.1 Common features of glutamatergic and GABAergic synapses.

Excitatory glutamatergic and inhibitory GABAergic cortical synapses share some common features, both at a gross morphological level and molecularly (Fig 1.1). Like all chemical synapses, they consist of a presynaptic terminal containing neurotransmitter loaded vesicles closely apposed to a postsynaptic specialisation containing neurotransmitter receptors and signal transduction machinery. Central glutamatergic and GABAergic presynaptic terminals are similar in size (~1μm in diameter), as are the number and size (~50nm diameter) of presynaptic vesicles, though terminal size and vesicle number can vary hugely from synapse to synapse (Bartlett and Banker, 1984; Eyre et al., 2007; Harris and Sultan, 1995; Schikorski and Stevens, 1997). Central excitatory and inhibitory synapses are therefore distinguished ultrastructurally based upon postsynaptic differences, as described below.

Glutamatergic and GABAergic synapses share core release machinery proteins, due to their fundamental roles in neurotransmitter release (Fig 1.1). These include the SNARE proteins VAMP2, SNAP-25 and syntaxin, the fast calcium sensor and docking protein synaptotagmin-1, and the priming factors Munc13 and Complexin-I/II. Genetic ablation of some of these core proteins has been shown to severely reduce action potential-evoked release at both glutamatergic and GABAergic synapses (Bronk et al., 2007; Geppert et al., 1994; Kerr et al., 2008; Varoqueaux et al., 2002), though the functions of many have only been studied at glutamatergic synapses. Interestingly, while several of these core molecules have been shown to be important in both glutamatergic and GABAergic transmission, differences have been observed in their importance and/or the



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isoforms involved between the two types of synapse. Synaptotagmin-1 knockout mice, for example, exhibit complete loss of action potential-evoked synchronous release at glutamatergic granule cell-basket cell synapses of the hippocampus, but only partial reduction at inhibitory basket cell-granule cell synapses (Kerr et al., 2008). This suggests that other isoforms of synaptotagmin (such as synaptotagmin-2) may play

Figure 1.1 Structure of central glutamatergic and GABAergic synapses. A) Glutamatergic and GABAergic synapses identified in hippocampal cultures by immunostaining. *Top panel*: Actin-rich dendritic spines (arrows) are clearly visible along a dendrite of a pyramidal cell transfected with EGFP-actin. Antibodies for PSD-95 label puncta within the spine heads (blue), which are contacted by presynaptic terminals labelled with antibodies against vGlut (red). Synapses onto non-transfected cells are also visible (purple). *Bottom panel:* Presynaptic clusters of vGAT (red) are found apposed to postsynaptic clusters of gephyrin (green) along the shafts of dendrites labelled with antibodies against the cytoskeletal marker Tuj-1 (blue). B) Glutamatergic and GABAergic synapses identified in hippocampal cultures by electron microscopy. *Left panel*: a presynaptic glutamatergic terminal apposed to a dendritic spine. Synaptic vesicles and active zone material are clearly visible presynaptically, and the dark postsynaptic density can be seen in the spine head, closely apposed to the active zone. *Right panel*: a GABAergic terminal synapses directly onto the dendritic shaft, in which microtubules are clearly observed. The presynaptic terminal again contains numerous vesicles and dark active zone material at the presynaptic membrane. However, a thick postsynaptic density is not present. Micrographs courtesy of Ellen Dickins. C) Diagram of key molecular components of glutamatergic and GABAergic synapses. The presynaptic terminals of both kinds of synapse share many proteins involved in active zone formation and vesicle exo-endocytosis. Postsynaptically, both types of synapse have several classes of neurotransmitter receptor that mediate and regulate synaptic transmission. These receptors are anchored by and signal through protein complexes at the postsynaptic membrane. Some of these receptors are also present presynaptically, where they act to regulate neurotransmitter release. Scale bars = 10μm in (A) and 200nm in (B).

a more crucial role at inhibitory synapses. This example serves as a reminder that, although excitatory and inhibitory synapses display general similarities in their release apparatus, they likely differ in the fine detail of the specific protein isoforms expressed, leading to functional differences.

Glutamatergic and GABAergic synapses also contain several common structural presynaptic proteins, such as Synapsin, Bassoon and Piccolo (Evergren et al., 2007; Schoch and Gundelfinger, 2006) (Fig 1.1). Antibodies to these proteins are often used as general synaptic markers, or in combination with antibodies to specific excitatory or inhibitory postsynaptic markers (Danglot et al., 2003; Goddard et al., 2007; Rumbaugh et al., 2006). Piccolo and Bassoon are large, structurally related active zone proteins that bind a wide range of core active zone proteins, and accordingly appear to play important roles in active zone formation, stabilisation and function (Schoch and Gundelfinger, 2006). Synapsin is a vesicle-associated protein that is believed to regulate the availability of synaptic vesicles in the reserve pool for exocytosis through its calcium-dependent binding to actin (Ceccaldi et al., 1995). It may also play a role in vesicle endocytosis, forming a 'Synapsin cycle' (Evergren et al., 2007). Synapsin is another

example of the principle that, though some molecular players are present at both glutamatergic and GABAergic synapses, this does not necessarily mean they perform exactly the same function. For example, Synapsin knockout produces defects in basal transmission at both types of synapse, but these are more pronounced at glutamatergic synapses (Gitler et al., 2004; Terada et al., 1999). The underlying reason for this may be differential expression of Synapsin isoforms – a recent study found that Synapsin-2a is the only isoform capable of rescuing the release defects seen in triple (Synapsin1-3) knockout mice (Gitler et al., 2008). Such subtle differences in the basic synaptic machinery of glutamatergic and GABAergic synapses are consistent with the idea that different synaptic subtypes diversified over evolutionary time from a common 'synaptic ancestor' (Ryan and Grant, 2009).

1.1.2 Structure and function of glutamatergic synapses

Glutamatergic synapses are so called because they utilise the amino acid neurotransmitter glutamate. Glutamate is formed in neurons both from glucose as a by-product of the Kreb's cycle, and from glutamine through the action of the enzyme glutaminase. Glutamate release from the presynaptic terminal results in the depolarisation of the postsynaptic neuron; hence glutamatergic synapses are also called 'excitatory' synapses. Following release, glutamatergic signalling is halted by the re-uptake of glutamate into glutamatergic terminals and glia. In glia, glutamate is converted to glutamine by the enzyme glutamine synthetase. This glutamine can then be secreted by glia, transported back into glutamatergic terminals and converted back to glutamate. Glutamate is packaged into synaptic vesicles through the action of the vesicular glutamate transporters (vGlut). The specificity of vGluts for glutamatergic presynaptic terminals has resulted in vGlut (in particular vGlut1) becoming the molecule of choice for identifying glutamatergic terminals (Takamori, 2006).

Upon release (either spontaneous or action potential-evoked), glutamate signals to the postsynaptic neuron through binding to several classes of receptors (Fig 1.1C). These can be broadly divided into two groups: ionotropic glutamate receptors and metabotropic glutamate receptors (mGluRs). The ionotropic receptors in turn include three separate receptor subtypes: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPA receptors or AMAP-Rs), N-methyl-D-aspartate receptors (NMDA receptors or NMDA-Rs) and kainate receptors (KA-Rs).

AMPA receptors are homo- or heterotetramers of the AMPA receptor subunits GluR1-GluR4 (Madden, 2002; Mayer, 2005; Mayer and Armstrong, 2004; Sobolevsky et al., 2009). Glutamate binding to AMPA-Rs results in rapid channel opening and depolarisation of the postsynaptic membrane through the influx of cations (principally Na^+), followed by rapid desensitisation and inactivation (Madden, 2002). The exact contribution of particular cations to the current underlying the depolarisation depends on the subunit composition of the receptor – for example, AMPA-Rs that lack a GluR2 subunit have a much higher calcium permeability than those that contain GluR2 (Brorson et al., 1999). AMPA-Rs also undergo post-translational modification at the C-terminal 'flip/flop' site, which acts to regulate kinetic properties such as the rate of desensitisation, resensitisation and channel closing (Mosbacher et al., 1994; Pei et al., 2007; Sommer et al., 1990). AMPA receptors are targeted to and anchored at the postsynaptic membrane through binding to Stargazin, which in turn binds the glutamatergic postsynaptic scaffolding protein PSD-95 (Chen et al., 2000), and also through binding to the PDZ domain of GRIP (Dong et al., 1997; Sheng and Pak, 1999) (Fig 1.1). AMPA-Rs are not static at the postsynaptic density however; they are rapidly trafficked in order to impart dynamic control over excitatory synaptic strength (Collingridge et al., 2004; Groc and Choquet, 2006; Heine et al., 2008).

NMDA receptors are heterotetrameric receptors formed from the NMDA receptor subunits, NR1, NR2(A-D) and NR3(A&B). Differential splicing gives rise to eight splice variants of the NR1 subunit from a single gene. It is believed that all functional NMDA receptors require the presence of the NR1 subunit, and that the majority of native receptors consist of two NR1 and two NR2 subunits, with glutamate binding sites on the NR2 subunits and binding sites for the co-agonists glycine an D-serine on the NR1 subunits (Chen and Wyllie, 2006; Furukawa et al., 2005). NMDA receptors are non-selective cation channels, with glutamate binding and channel opening resulting in depolarisation of the postsynaptic cell principally through an influx of Na^+ and Ca^{2+} (provided the post-synaptic cell is depolarised in order to reduce Mg^{2+} block of the NMDA-R channel – see below). The functional properties of NMDA-Rs are highly influenced by the NR subunits present. For example, NR1/NR2A receptors have a relatively quick deactivation time (though around 50 times slower than AMPA-Rs), NR1/NR2B or NR1/NR2C receptors are approximately four times slower and NR1/NR2D receptors deactivate extremely slowly, on the order of seconds (Cull-Candy

and Leszkiewicz, 2004; Vicini et al., 1998). Furthermore, the presence of NR3 subunits appears to have an inhibitory effect on NMDA receptor function, reducing Ca^{2+} permeability and Mg^{+} sensitivity (Cavara and Hollmann, 2008; Cull-Candy and Leszkiewicz, 2004). NMDA receptors also display the interesting property of voltage-dependent magnesium block; current can only flow through the channel when this block is removed at depolarised membrane voltages. This means NMDA-Rs can act as coincidence detectors, as they function when there is co-incident presynaptic glutamate release and postsynaptic depolarisation. Because of this unusual property, NMDA-Rs play a major role in activity-dependent changes in synaptic strength (see below). NMDA-Rs are anchored at the postsynaptic membrane through a direct interaction with PSD-95 (Niethammer et al., 1996; Sheng and Pak, 1999) and, like AMPA-Rs, are subject to complex and dynamic trafficking mechanisms (Collingridge et al., 2004; Groc and Choquet, 2006).

Kainate receptors (KA-Rs) are homo- or heterotetrameric receptors formed from GluR(5-7) and KA(1 and 2) subunits. GluR5-7 can form functional homomers and heteromers (Egebjerg et al., 1991; Schiffer et al., 1997; Sommer et al., 1992), whereas KA1 and KA2 must be incorporated with GluR5-7 subunits to form functional receptors (Herb et al., 1992; Sakimura et al., 1992). As for the other ionotropic receptors, the functional properties of KA-Rs are heavily influenced by the particular subunits that comprise them (Huettner, 2003). Activation of KA-Rs depolarises neurons, due to an inward current carried mainly by Na^{+} and with kinetics similar to, though somewhat slower than, AMPA-R-mediated currents (Savidge et al., 1999). Research into the function of KA-Rs has lagged behind that of AMAP-Rs and NMDA-Rs, as specific pharmacological tools have only relatively recently been developed. The emerging picture is that, although postsynaptic KA-Rs play a role in direct synaptic signalling at some synapses (Castillo et al., 1997; Vignes and Collingridge, 1997), the principal role of KA-Rs is regulation of presynaptic release. For example, activation of presynaptic KA-Rs has been shown to reduce transmitter release at both glutamatergic and GABAergic synapses (Chittajallu et al., 1996; Clarke et al., 1997; Rodriguez-Moreno et al., 1997). Therefore KA-Rs have emerged as important mediators of synaptic plasticity (Bortolotto et al., 1999). Unsurprisingly then, their trafficking is also tightly controlled, though less is known about this than for the other ionotropic glutamate receptors (Collingridge et al., 2004).

mGluRs are seven transmembrane domain G-protein-coupled receptors that transduce binding of glutamate into activation of relatively slow-acting second messenger signalling cascades. Eight mGluRs are known that are classed into three groups (Group I-III) based on the second messenger systems they activate. mGluRs are found both pre- and postsynaptically at glutamatergic synapses and presynaptically at GABAergic synapses, and have been implicated in the regulation of virtually every aspect of synaptic transmission (for review see (Ferraguti and Shigemoto, 2006)). Postsynaptically, mGluRs are anchored through binding to Homer, which is linked to the PSD-95 scaffold through the linker protein Shank (Tu et al., 1999). Again, the synaptic localisation of mGluRs is tightly controlled by complex trafficking mechanisms, with Homer playing a key role (Collingridge et al., 2004).

The majority of central glutamatergic presynaptic terminals form synapses onto specialised dendritic structures called spines (Bartlett and Banker, 1984; Bourne and Harris, 2008; Megias et al., 2001) (Fig 1.1). Spines are actin-rich protrusions of the dendrite that typically have an enlarged head region, which contains the glutamate receptors and associated scaffolding and signalling proteins described above, and a neck region, which connects the spine head to the dendritic shaft (Sorra and Harris, 2000). Spines show considerable variability in size and shape, even in adult brains, and they are commonly categorised based on shape (Harris et al., 1992; Sorra and Harris, 2000). The majority of spines in the adult are 'thin' spines, which have long, thin necks and rounded heads. Conversely 'stubby' spines are short and lack a discernable neck. 'Mushroom' spines have a relatively thin neck and a large, slightly flattened head and 'branched spines' are spines of any morphology that have multiple heads emerging from one neck. Formation of mushroom spines and branched spines from other spine types has been suggested to underlie activity-dependent increases in excitatory synapse strength and number, respectively (Harris et al., 1992; Matsuzaki et al., 2004; Sorra and Harris, 2000). Functionally, spines act to compartmentalise excitatory synapses from the dendritic shaft, allowing them to act as distinct electrical and biochemical units (Sorra and Harris, 2000; Spruston, 2008). These properties could allow individual excitatory synapses to independently regulate their strength in response to changes in activity at that synapse. In support of this theory, synaptic activity can result in changes in spine morphology that positively correlate with changes in excitatory synaptic strength (Bourne and Harris, 2007; Bourne and Harris, 2008; Dunaevsky and Mason, 2003; Nimchinsky et al., 2002), a relationship that has been demonstrated at single dendritic

spines (Matsuzaki et al., 2004). Such plastic changes require Rho GTPase activity and actin remodelling, and rely crucially on signalling through the calcium-calmodulin-dependent kinase CaMKII (Matsuzaki et al., 2004; Penzes et al., 2008; Saneyoshi et al., 2008). Indeed, the importance of CaMKII in excitatory synaptic plasticity is underscored by its abundance in the postsynaptic density, where it is estimated to make up 7.4% of the total protein content (Cheng et al., 2006) (Fig 1.1C).

In addition to, and related to the morphological plasticity described above, glutamatergic synapses exhibit a range of functional plasticity mechanisms that operate over both short and long timescales. Examples of short-term plasticity include facilitation and depression, in which the postsynaptic response following a presynaptic action potential increases or decreases, respectively, with successive action potentials. Facilitation and depression are due to a range of fast-acting mechanisms, including presynaptic calcium kinetics, depletion of vesicle pools and the intrinsic properties of postsynaptic receptors (e.g. desensitisation due to sustained presence of or repeated exposure to the ligand) (Thomson, 2000; Zucker and Regehr, 2002). Short-term plasticity thereby acts in an activity-dependent manner to tune synaptic transmission on a timescale of milliseconds to minutes.

Mechanisms also exist to alter glutamatergic transmission over timescales of hours to weeks, and perhaps even longer. The most well known of these long-term plasticity mechanisms is long-term potentiation (LTP), a long-lasting increase in synaptic strength in response to particular patterns of synaptic firing (Bliss and Lomo, 1973). LTP can be expressed through presynaptic mechanisms (for example nitric oxide acting retrogradely to increase release probability) or postsynaptic mechanisms (such as increased insertion of AMPA receptors), but appears to require NMDA-R signalling and CaMKII activation in both cases (Arancio et al., 1996; Bekkers and Stevens, 1990; Johnston and Morris, 1995; Lu and Hawkins, 2006; Lu et al., 2001). Slow, repetitive synaptic activity can result in long-term depression (LTD), which as the name suggests is the opposite of LTP i.e. a long-lasting decrease in synaptic strength. Interestingly, many of the same mechanisms and signalling pathways seem to be involved in both LTP and LTD (Feldman, 2009). Another form of long-term plasticity is homeostatic synaptic plasticity. This term describes a range of mechanisms including changes in presynaptic release, postsynaptic strength and synapse density that act to keep neuronal activity within a stable functional range in the face of changes in network activity (Turrigiano,

2007; Turrigiano, 2008; Turrigiano and Nelson, 2000). Overall, excitatory synapses utilise a wide range of mechanisms to adjust their functional state in response to their history of activity, a capability that is widely believed to underlie adaptive behaviour and information storage in the brain.

1.1.3 Structure and function of GABAergic synapses.

GABAergic synapses are so called because they utilise the amino acid neurotransmitter γ-aminobutyric acid (GABA). GABA is synthesised from neuronal glutamate by the actions of two glutamic acid decarboxlyases (GADs), GAD-65 and GAD-67. GABA release tends to result in the hyperpolarisation of the postsynaptic neuron; hence GABAergic synapses are also called 'inhibitory' synapses (but see below). Once released, GABA is inactivated by uptake into glia and nerve terminals, where it is broken down by the enzyme GABA transaminase (McIntire et al., 1997; Schousboe and Waagepetersen, 2007). GABA is loaded into presynaptic vesicles by the action of the vesicular GABA transporter (vGAT) (McIntire et al., 1997). GAD-65, GAD-67 and vGAT are all reliable and commonly used markers of GABAergic terminals.

Upon release (either spontaneous or action potential-evoked), GABA signals to the postsynaptic neuron through binding to two types of receptor (Fig 1.1C). The $GABA_A$ receptor is an ionotropic receptor that is analogous to AMPA-Rs at glutamatergic synapses, whereas the $GABA_B$ receptor is a metabotropic receptor analogous to mGluRs at glutamatergic receptors.

$GABA_A$ receptors are heteropentameric structures formed from a wide range of subunits (α1-6, β1-3, γ1-3, δ, ε1-3, θ and π) (Jacob et al., 2008) However it is believed the majority of $GABA_A$-Rs consist of two α, two β and one γ subunit, with GABA binding at the interfaces between the α, and β sites (Amin and Weiss, 1993; Rudolph and Mohler, 2004). GABA binding to $GABA_A$-Rs results in channel opening and hyperpolarisation of the postsynaptic cell through an influx of Cl^- ions (Curtis et al., 1970). However, it is important to note that this is only true postnatally as GABA undergoes a developmental switch from an excitatory to inhibitory transmitter due to changes in the expression of the K+/Cl- transporter KCC2. Indeed, in the developing nervous system, GABA is the principal excitatory neurotransmitter until this switch occurs during the neonatal period (Ben-Ari, 2002). Though both opening and

deactivation of $GABA_A$-Rs are relatively rapid, they are significantly slower than for AMPA-Rs. Like AMPA-Rs, the exact subunit composition of $GABA_A$-Rs determines their functional properties (Sieghart, 1992). $GABA_A$-Rs are targeted to and anchored at the postsynaptic membrane through the interaction of the α2 subunit with the postsynaptic scaffolding protein Gephyrin (Jacob et al., 2008; Tretter et al., 2008). Dynamic trafficking of $GABA_A$-Rs allows neurons to tune inhibition, and therefore network activity, in response to neuronal activity (Jacob et al., 2008; Tyagarajan and Fritschy, 2009). For example, chronic changes in neuronal activity levels regulate the targeting of $GABA_A$-Rs for destruction by ubiquitination (Saliba et al., 2007).

$GABA_B$ receptors are heterodimeric complexes of two seven transmembrane domain subunits, $GABA_{B(1)}$ and $GABA_{B(2)}$ (Marshall et al., 1999). They are metabotropic receptors, roughly analogous to mGluRs at excitatory synapses. They are found both pre- and postsynaptically at inhibitory synapses (Kulik et al., 2003) and regulate GABAergic transmission through a range of mechanisms (for review, see (Kornau, 2006)). Interestingly, $GABA_B$ receptors are also found extrasynaptically at excitatory postsynaptic sites (Kulik et al., 2003), where activation by GABA spillover can regulate glutamatergic transmission (Hirono et al., 2001; Huang et al., 2005; Scanziani, 2000).

Compared to the more intensively studied glutamatergic synapse, relatively little is known about plasticity at GABAergic synapses. Despite this, there is evidence that inhibitory synapses also display a wide range of plasticity mechanisms that allow activity-dependent tuning of inhibition. GABAergic synapses display many of the same short-term plasticity processes as glutamatergic synapses, such as facilitation or depression following pairs or short trains of action potentials (Ivanova et al., 2002; Jiang et al., 2000; Kaplan et al., 2003; Kravchenko et al., 2006). GABAergic synapses have recently been found to display both LTP and LTD, and these can be elicited by similar signalling mechanisms as at glutamatergic synapses (Feldman, 2009; McBain and Kauer, 2009). For example, a presynaptic form of LTD at GABAergic synapses has been found to rely on retrograde signalling of nitric oxide released from the postsynapse (Nugent et al., 2009; Nugent et al., 2007), similar to the signalling pathway that elicits LTP at some glutamatergic synapses (Arancio et al., 1996). Inhibitory synapses are also subject to synaptic homeostasis - adjustments in inhibitory synaptic number and strength that act to normalise network excitability have been demonstrated both *in vitro*

(Hartman et al., 2006; Ivanova et al., 2003; Rutherford et al., 1997) and *in vivo* (Foeller and Feldman, 2004; Knott et al., 2002; Lee et al., 2007; Maffei et al., 2004).

1.2 Formation of central glutamatergic and GABAergic synapses

Over the past few decades, our understanding of the morphological and molecular processes that underlie the formation of central synapses has improved dramatically. This is due to a combination of *in vitro* and *in vivo* studies that have used time-lapse imaging, genetically modified animals and/or molecular biology approaches to address the question of how synapses are assembled on many levels (Craig et al., 2006; McAllister, 2007). Despite this, many key questions remain. For example, the previous section of this introduction discussed the fact that, although all central synapses share some common features, they are a highly heterogeneous population. However the majority of studies that have looked at central synaptogenesis have focused on glutamatergic synapses. As a result, relatively little is known about the mechanisms that regulate the formation of different synapse subtypes. For example, are the same set of synaptogenic molecules responsible for driving excitatory and inhibitory synapse formation? As the number of studies that examine the formation of both excitatory and inhibitory synapses grows, the emerging picture is that there are both considerable overlaps and differences. Synapses are highly specialised cellular junctions, with the presynaptic active zone elements tightly apposed to the postsynaptic neurotransmitter receptor apparatus (Figs 1.1 and 1.2). Therefore a second key question is: do the same types of molecules regulate pre- and postsynaptic differentiation, and how are these processes co-ordinated to form a functional synapse? Again, recent work has shed much light on this question, with trans-synaptic adhesion molecules and secreted signalling factors emerging as key players in bi-directional signalling during synapse development.

1.2.1 Central synaptogenesis: cellular processes

Synaptogenesis involves distinct cellular processes acting over time in a co-ordinated manner to build a functional synapse (Fig 1.2). Axons and dendrites have to contact and recognise one other, recruit the pre- and postsynaptic protein complexes that mediate neurotransmission to nascent synaptic sites, and undergo the structural rearrangements that serve synaptic function.

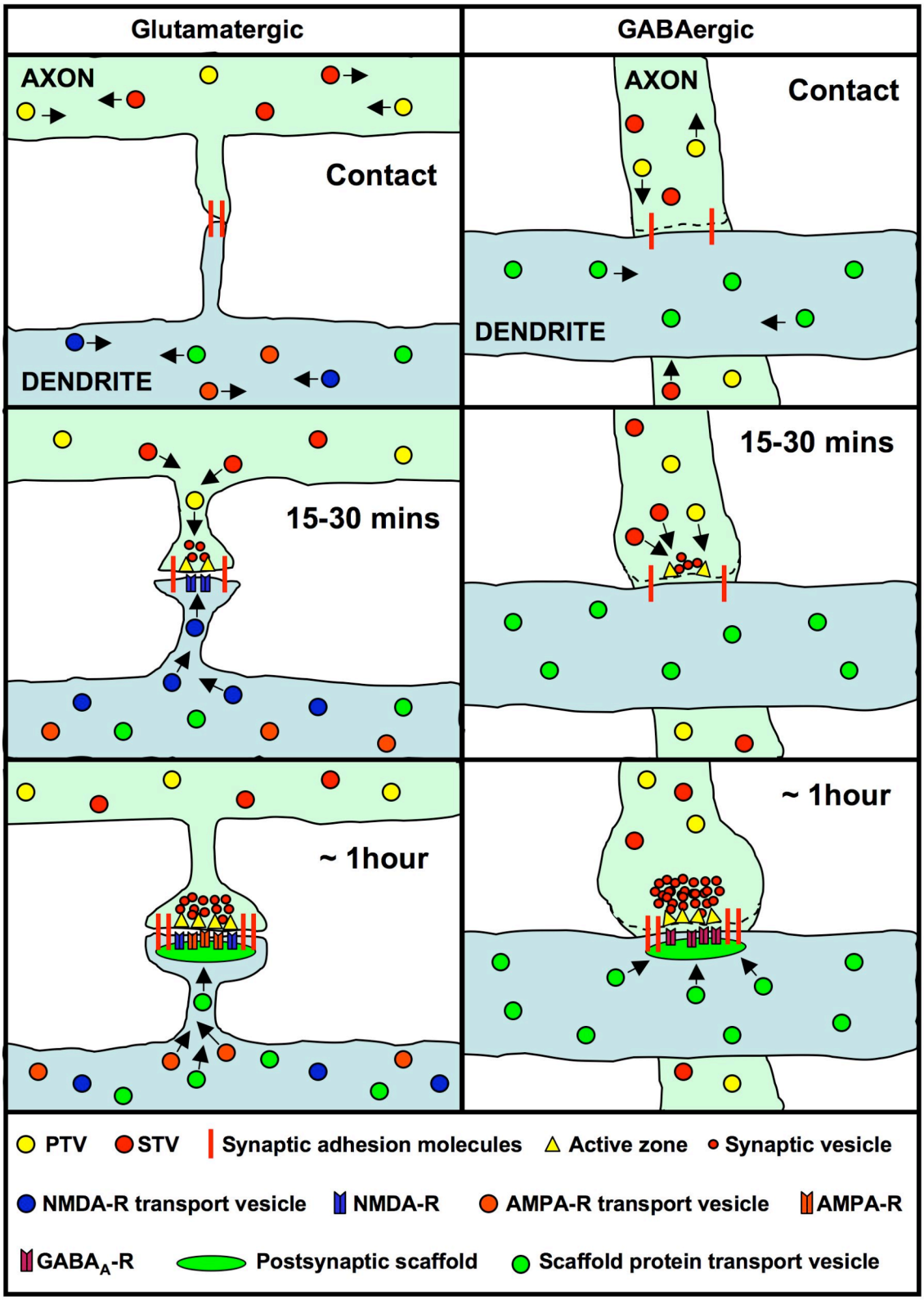

Figure 1.2 (Figure legend on next page)

Figure 1.2 Formation of central glutamatergic and GABAergic synapses. *Left-hand panels*: Key steps in glutamatergic synapse formation. Glutamatergic synapse formation can be initiated by contact through axonal and dendritic protrusions. Contact is stabilised by trans-synaptic adhesion molecules, and results in accumulation of active zone material and synaptic vesicles at nascent presynaptic sites through recruitment of PTVs and STVs. Postsynaptically, contact results in NMDA-R accumulation in the nascent postsynaptic membrane, which is shortly followed by the recruitment of AMPA-Rs and scaffolding proteins such as PSD-95, GKAP and Shank. The recruitment of synaptic proteins is accompanied by profound morphological changes, such as presynaptic terminal and postsynaptic spine formation, and a general increase in the size of the new synapse. *Right-hand panels*: Key steps in GABAergic synapse formation. Although GABAergic synapses appear to employ many of the same mechanisms as glutamatergic synapses in their formation, the details are much less clear. GABAergic synapses form at pre-existing crossings of axons and dendrites, and contact is again stabilised by adhesion molecules. Presynaptic vesicles accumulate at the site of contact with a similar timecourse to glutamatergic synapses. This is followed by the clustering of the key inhibitory postsynaptic scaffold Gephyrin, which acts to recruit $GABA_A$-Rs to the new synapse. The role of vesicular transport packets in these processes remains unclear however.

1.2.1.1 Morphological aspects of synaptogenesis

The initial contacts between axons and dendrites often happen at thin, actin-rich protrusions called filopodia, which are found at the growth cones and along the shafts of both axons and dendrites (Korobova and Svitkina, 2009; Sabo and McAllister, 2003) Interestingly, minimal synaptic release machinery is present along the axonal shaft and in filopodia, allowing depolarisation-dependent release of transmitter even before synapses have formed (Krueger et al., 2003; Sabo and McAllister, 2003). Therefore, neurotransmitters themselves may act as anterograde target recognition molecules in synaptogenesis. Unsurprisingly, neuronal cell adhesion molecules such as N-cadherins (Shapiro and Colman, 1999), SynCAMs (Biederer et al., 2002; Irie et al., 2004), Nectins (Irie et al., 2004) and neurexins/neuroligins (Nguyen and Sudhof, 1997) are very important in initial recognition and stabilisation of synaptic partners. Interestingly, some of these also appear to signal intracellularly to induce morphological changes and the recruitment of synaptic proteins (see below).

Time-lapse imaging studies have been extremely useful in elucidating the morphological changes that occur during the formation of excitatory and inhibitory synapses. Filopodia are highly motile and appear to ‘sample’ their immediate surroundings for appropriate synaptic partners (Dailey and Smith, 1996; Lohmann and Bonhoeffer, 2008). In the case of glutamatergic synapse formation, dendritic filopodia

appear to be particularly important. Dendritic filopodia are common during the major wave of postnatal cortical synaptogenesis, but are rare in the adult under normal conditions (Fiala et al., 1998; Miller and Peters, 1981; Petrak et al., 2005). Imaging studies have revealed that most filopodia are transient structures, with a life time of minutes (Dailey and Smith, 1996; Lohmann and Bonhoeffer, 2008; Ziv and Smith, 1996). However contact with an appropriate axonal target can stabilise dendritic filopodia, in a recognition step that involves dendritic calcium signalling (Lohmann and Bonhoeffer, 2008). Direct transformation of dendritic filopodia into spines has been demonstrated by time-lapse studies in developing hippocampal slice cultures (Marrs et al., 2001), and morphological synapses (with a presynaptic active zone and vesicles apposed to a PSD) are found on filopodia in the developing hippocampus (Fiala et al., 1998). However, many asymmetric synapses also form directly onto the dendritic shaft during development (Fiala et al., 1998) and the question of whether all dendritic spines form from filopodia is still a matter of considerable debate (Yuste and Bonhoeffer, 2004). It may be that the particular sequence of morphological changes that accompany glutamatergic synapse formation depends on developmental maturity and neuronal subtype. Technological advances mean that *in vivo* live imaging studies looking at synapse formation throughout the development of an animal are becoming possible (Knott et al., 2006), which should help shed light on this problem.

A recent live imaging study has also begun to elucidate the morphological changes that occur during GABAergic synapse formation (Wierenga et al., 2008). In this study, individual CA1 neurons were labelled by filling with Alexa-594 in hippocampal slice cultures from GAD65-GFP mice, which express GFP in around 50% of GABAergic interneurons in the hippocampus (Lopez-Bendito et al., 2004). This allowed the interaction of green GABAergic axons with the dendrites of red glutamatergic cells to be analysed by time-lapse microscopy. GABAergic boutons were often contacted by dendritic protrusions of CA1 cells, but these contacts were transient (usually <30 minutes), and never led to the formation of stable GABAergic synapses (Wierenga et al., 2008). This suggests these protrusions may represent nascent glutamatergic postsynaptic structures which fail to display calcium-dependent stabilisation upon contacting an incorrect presynaptic partner (GABAergic axons) (Lohmann and Bonhoeffer, 2008). Interestingly, stable synapses also failed to form when axonal protrusions from GABAergic axons contacted CA1 dendrites, though these contacts were slightly longer lasting than those formed by dendritic protrusions (~1 hour). In

fact, stable GABAergic formation was only observed at pre-existing crossings of GABAergic axons and CA1 dendrites. GABAergic synapse formation at such crossings manifested as the formation of axonal boutons and recruitment of pre- and postsynaptic inhibitory proteins over a period of a few hours. Interestingly, there were no detectable morphological changes in the dendrite during GABAergic synapse formation (Wierenga et al., 2008). Therefore it appears that there are fundamental differences in the morphological changes underlying central excitatory and inhibitory synapse formation. Glutamatergic synaptogenesis involves filopodial protrusions and extensive remodelling of the postsynaptic dendrite to form a spine, whereas GABAergic synaptogenesis requires neither of these processes (Fig 1.2).

1.2.1.2 Recruitment of pre- and postsynaptic machinery

As well as adopting the right morphological characteristics, developing excitatory and inhibitory synapses need to recruit the pre- and postsynaptic protein complexes that allow neurotransmission to occur. Again, the vast majority of our understanding of this process to date comes from studies on glutamatergic synapses (McAllister, 2007). As mentioned above, neurons are capable of vesicular neurotransmitter release before synapses have formed (Krueger et al., 2003; Sabo and McAllister, 2003). This is due to the presence and transport of synaptic vesicle protein transport vesicles (STVs) which contain vGlut as well as core vesicular proteins such as VAMP2, Synapsin-1 and SV2 and components of the exo- and endocytotic machinery (Ahmari et al., 2000; Kaether et al., 2000; Sabo et al., 2006; Sabo and McAllister, 2003). As well as STVs, another population of presynaptic proteins are shuttled within the axon as piccolo transport vesicles (PTVs). These are so-called as they contain the presynaptic scaffolding protein Piccolo, as well as other scaffolding proteins such as Bassoon and core active zone proteins such as Rab3, Munc13, Syntaxin and SNAP-25 (Shapira et al., 2003; Zhai et al., 2001). Interestingly, STVs and PTVs show some molecular overlap, as they both contain the N-type calcium channel subunit $\alpha 1$ (Ahmari et al., 2000; Shapira et al., 2003). STVs and PTVs are highly mobile in young neurons and move both anterogradely and retrogradely at varying speeds, implicating several different motor proteins in their transport along the cytoskeleton (Ahmari et al., 2000; Goldstein et al., 2008; Kaether et al., 2000; Nakata et al., 1998; Sabo et al., 2006; Shapira et al., 2003). Contact between axons and dendrites can lead to recruitment of STVs and accumulation of the proteins they transport within tens of minutes (Ahmari et al., 2000; Friedman et

al., 2000), suggesting that the presynaptic machinery may be assembled in a modular fashion from these pre-assembled transport packets (McAllister, 2007) (Fig 1.2). Indeed, it has been estimated that 2-3 PTVs could account for the total Piccolo and Bassoon protein at an average central synapse (Shapira et al., 2003). Stable presynaptic terminals are capable of functional release very shortly after they have formed (Ahmari et al., 2000; Friedman et al., 2000; Zhai et al., 2001), consistent with the idea that STVs are release-competent even in transit (Krueger et al., 2003; Sabo and McAllister, 2003).

The postsynaptic side of a synapse appears to utilise similar mechanisms as the presynaptic side during its assembly, as key postsynaptic proteins are also transported in vesicular structures. NMDA-Rs are transported in tubulovesicular structures that move bi-directionally in dendrites, and that also contain the scaffolding protein SAP-102, the synaptic adhesion molecule Neuroligin-1 and proteins involved in exo- and endocytosis (Sans et al., 2003; Washbourne et al., 2002; Washbourne et al., 2004). Like STVs, NMDA-R transport packets can be recruited to and accumulate at nascent synapses with a time course of tens of minutes (Washbourne et al., 2002) (Fig 1.2). A sub-set of NMDA-R transport packets also contain AMPA-Rs, but the majority of AMPA-Rs appear to be transported in separate transport vesicles with a speed around half that of NMDA-R transport vesicles (2μm/min as compared to 4μm/min) (Washbourne et al., 2002). This means that AMPA-R accumulation at new glutamatergic synapses lags slightly behind NMDA-R accumulation, suggesting these synapses are initially 'silent'. NMDA-R transport vesicles contain both NR1 and NR2B, whereas AMPA-R transport vesicles contain both GluR1 and GluR2, suggesting these receptors may exist in a functional conformation during transport (Washbourne et al., 2002). Furthermore, NMDA-R transport vesicles are capable of cycling into and out of the plasma membrane during transport, suggesting that they may be able to sense extracellular glutamate prior to (and perhaps leading to) accumulation at synaptic sites (Washbourne et al., 2004).

The abundant glutamatergic postsynaptic scaffolding protein PSD-95 has also been observed to be present in mobile clusters preceding synapse formation, and is transported in tandem with the scaffolding proteins Shank and GKAP (Gerrow et al., 2006; Okabe et al., 2001; Prange and Murphy, 2001). PSD-95 has been shown to accumulate at nascent synaptic sites within around 1 hour of axo-dendritic contact and appears that to lag behind glutamate receptor clustering, suggesting other scaffold

proteins such as SAP-102 may be important in the very early stages of excitatory synaptogenesis (Marrs et al., 2001; Okabe et al., 2001; Washbourne et al., 2002) (Fig 1.2). Interestingly, time-lapse studies have shown that PSD-95 clustering is closely correlated with spine formation (Marrs et al., 2001; Okabe et al., 2001), suggesting that the recruitment of scaffolding proteins plays an important role in morphological changes at newly forming glutamatergic synapses, or vice versa.

The prevailing view is that presynaptic differentiation precedes post synaptic differentiation during central synaptogenesis – however evidence exists both for (Friedman et al., 2000; Okabe et al., 2001) and against (Gerrow et al., 2006) this interpretation, producing considerable debate about this issue. These discrepancies most likely arise from the fact that conclusions regarding the precise timing of synaptogenesis have arisen from comparing studies that differ methodologically. It is likely that synaptogenesis can occur through a number of subtly different processes, and so the exact timing of key events may depend on factors such as neuronal subtype, maturity and whether contact is initiated by an axonal or dendritic protrusion (McAllister, 2007). Thus synaptogenic research will benefit from studies which look at the effect of these factors on the precise timing of pre- and postsynaptic processes during synaptogenesis.

Compared to glutamatergic synapses, very little is known about the recruitment of synaptic proteins during GABAergic synapse formation. The principal reason for this is that research into synaptic protein trafficking and recruitment has focused on excitatory synapses or has not discriminated between synaptic subtypes. Despite this, some studies have begun to shed light on how synaptic proteins accumulate at GABAergic synapses. As seen for glutamate at excitatory synapses, the neurotransmitter GABA appears to play an important role in inhibitory synapse formation. In hippocampal cultures, $GABA_A$ receptors and GAD-67 are amongst the first inhibitory synaptic proteins to cluster (Swanwick et al., 2006), and genetic depletion of GAD-67 results in decreased inhibitory synapse formation onto pyramidal cells of the visual cortex *in vivo* (Chattopadhyaya et al., 2007). A recent time-lapse study which looked at inhibitory synapse formation at identified axo-dendritic crossings revealed that vGAT and Gephyrin are progressively recruited to stable inhibitory contacts within around 1 hour, with Gephyrin accumulation lagging somewhat behind vGAT accumulation (Wierenga et al., 2008) (Fig 1.2). This suggests that presynaptic differentiation precedes

postsynaptic differentiation at GABAergic synapses. However, this was determined using *post-hoc* immunostaining following the live imaging period; therefore the precise dynamics of synaptic protein accumulation at inhibitory synapses remain to be determined.

1.2.2 Synapse organising molecules

Synapse organising molecules can be defined as molecules that play a role in the formation and/or maturation of synapses, and therefore regulate synaptic density and/or function. Recent years have seen an explosion in the discovery of such molecules, which both initiate and regulate the cellular processes described in the previous section. As such, a complete discussion of all synaptogenic molecules is beyond the scope of this introduction. Accordingly, in this section I will focus on the best characterised families of synapse organising molecules, with the emphasis again on their roles in excitatory and/or inhibitory and pre- and/or postsynaptic development.

1.2.2.1 Membrane-associated synapse organising molecules

Neurexins/Neuroligins

Neurexin and Neuroligin are heterophilic trans-synaptic adhesion proteins, with the presynaptic Neurexins binding to the postsynaptic Neuroligins (Ichtchenko et al., 1996). Interestingly, when Neuroligin or β-Neurexin are expressed in non-neuronal cells they promote clustering of key pre- and postsynaptic molecules, respectively, in contacting neurons (Graf et al., 2004; Sabo et al., 2006; Scheiffele et al., 2000). Furthermore, although perturbations of Neuroligin activity can affect both inhibitory and excitatory synapses (Chih et al., 2005), there are clear differences in the isoforms of Neuroligin involved. Neuroligin-1, -3, and -4 localise to glutamatergic sites, whereas Neuroligin-2 preferentially localises to GABAergic sites (Graf et al., 2004). Accordingly, Neuroligin-1 and Neuroligin-2 knockout mice show defects in excitatory and inhibitory neurotransmission, respectively (Chubykin et al., 2007). The specificity of Neuroligin-mediated postsynaptic protein clustering has in turn been shown to depend on the pre-synaptic Neurexin binding partner. β-Neurexin can promote the clustering of both excitatory and inhibitory postsynaptic proteins (Graf et al., 2004), but β-Neurexin containing an insert at splice site 4 has increased inhibitory synaptogenic activity

compared to β-Neurexin lacking the insert (Kang et al., 2008). In contrast, α-Neurexins exclusively promote GABAergic synapse formation (Kang et al., 2008). Therefore particular Neurexin-Neuroligin interactions appear to specify for excitatory or inhibitory synapses.

Interestingly, when Neuroligin-1 is overexpressed in neurons, the new glutamatergic synapses that are induced are postsynaptically silent (Sara et al., 2005). These findings suggest that Neuroligins/Neurexins control some aspects of synapse formation and specification, but must co-operate with other synaptogenic molecules to assemble fully functional synapses (see below). This interpretation is further strengthened by data from knockout animal studies. Triple Neuroligin 1-3 knockout mice die shortly after birth due to respiratory failure (Varoqueaux et al., 2006). Consistently, these animal show drastically reduced GABAergic/glycinergic and glutamatergic transmission in brainstem nuclei of the respiratory centre – additionally, this is more pronounced at GABAergic/glycinergic synapses, meaning there is a relative increase in the influence of glutamatergic synapses in the brain stem of the triple knockout mice. Despite this, there is no change in total synaptic density in these brainstem nuclei, though there is a subtle increase in the ratio of glutamatergic to GABAergic synapses (Varoqueaux et al., 2006). Similarly, double and triple knockout of α-Neurexins 1-3 results in respiratory dysfunction and greatly attenuated GABAergic and glutamatergic transmission, but only results in a relatively minor decrease in inhibitory synapse density in the brain stem (Dudanova et al., 2007; Missler et al., 2003). A study utilising transfection of Neuroligins in cultured neurons has demonstrated that the ability of Neuroligin-1 and Neuroligin-2 to increase the number and functionality of excitatory and inhibitory synapses, respectively, is dependent on synaptic activity (Chubykin et al., 2007). Therefore it seems that Neuroligins/Neurexins are involved in the specification and validation of glutamatergic and GABAergic synapses (rather than synapse formation *per se*), suggesting they play a crucial role in the correct patterning of excitatory and inhibitory innervation in the CNS.

SynCAMs and Nectins

SynCAMs and Nectins are two closely related families of heterophilic trans-synaptic cell adhesion molecules that have several members (SynCAM1-4 and Nectin1-4) that display distinct expression and adhesion profiles in the developing nervous system

(Fogel et al., 2007; Mizoguchi et al., 2002; Thomas et al., 2008). SynCAM1, like Neuroligin-1, can promote the formation of functionally releasing glutamatergic terminals in contacting axons when expressed in non-neuronal cells in co-cultures (Biederer et al., 2002). However, when overexpressed in immature neuronal cultures, SynCAM1 increased mEPSC frequency without increasing excitatory synapse number (Sara et al., 2005). This suggests that SynCAM1 promotes functional maturation of synapses in neurons rather than synapse formation, and may act synergistically with Neuroligin-1, which acts to increase excitatory synapse number (Sara et al., 2005). At present, SynCAMs have only been observed to promote excitatory synaptogenesis, based on studies looking at SynCAM1 and 2 (Biederer et al., 2002; Fogel et al., 2007). However SynCAMs are present at both excitatory and inhibitory synapses (Fogel et al., 2007; Thomas et al., 2008), and it is possible that particular SynCAMs or heterophilic pairings of SynCAMs could also play a role in inhibitory synapse development.

Nectins are transmembrane adhesion molecules that are linked to the actin cytoskeleton via the actin-binding protein Afadin (Mizoguchi et al., 2002). At the mossy fibre-CA3 synapse of the hippocampus, presynaptic Nectin-1 binds to postsynaptic Nectin-3, and this binding relationship appears to be retained in dissociated hippocampal cultures. Inhibition of Nectin binding in hippocampal cultures lead to a decrease in synaptic number, as assayed by Synaptophysin staining (Mizoguchi et al., 2002). As mossy fibre-CA3 synapses are glutamatergic, Nectins have been proposed to play a role in excitatory synapse formation, but it is unclear whether they also play a similar role at GABAergic synapses. In hippocampal cultures, Nectin-1 is initially found localised to both excitatory and inhibitory synapses, but as cultures mature the inhibitory synaptic localisation is lost (Lim et al., 2008). Therefore it is possible that Nectins could play a role in the initial formation of GABAergic synapses.

Ephs/Ephrins

Ephrins are GPI-anchored (EphrinA1-A5) or transmembrane (EphrinB1-B3) ligands that bind their transmembrane Eph receptor tyrosine kinases (EphA1-A8 and EphB1-B4 & -B6 in mammals). In neurons, Ephrins are found predominantly presynaptically, whereas Ephs are usually postsynaptic. Eph receptors can also act as ligands, mediating ‘reverse signalling’ through the transmembrane EphrinBs; therefore, Ephs/Ephrins can mediate bi-directional signalling (Kullander and Klein, 2002). Recent studies have

demonstrated that this bi-directional signalling allows Ephrins/Ephs to act as powerful regulators of pre- and postsynaptic development (Chen et al., 2008). Transgenic mice with disrupted EphrinA/EphA signalling (through overexpression of a soluble EphA receptor) display defects in hippocampal synapse formation, with a decrease in the number of synapses formed by projections from the entorhinal cortex and contralateral hippocampus (Martinez et al., 2005). Interestingly, mossy fibre projections displayed *increased* synapse number and eptotic connections under the same conditions (Martinez et al., 2005), raising the intriguing possibility that EphrinA/EphA signalling may act pro- or antisynaptogenically, depending on the cellular context. Reverse signalling through EphB/EphrinB3 also appears to have a synapse-limiting role in the hippocampus, as mice either lacking EphrinB3 or that express a mutant EphrinB3 that lacks the cytoplasmic signalling domain have an increased density of excitatory synapses (Rodenas-Ruano et al., 2006). In contrast reverse signalling through EphB/EphrinB2 can promote presynaptic differentiation. Expression of EphB2 in non-neuronal cells results in SV2 clustering in axons of co-cultured cortical neurons, even if the intracellular signalling domain is deleted. Conversely, knockdown of Eph2 results in decreased SV2 clustering and reduced mEPSC frequency (Kayser et al., 2006).

Forward EphrinB/EphB signalling, on the other hand, appears to be particularly important in postsynaptic development (Chen et al., 2008). In cortical neurons, EphB2 binds directly to NMDA-Rs via the NR1 subunit in an EphrinA-dependent manner (Dalva et al., 2000). Furthermore, treatment of cortical cultures with aggregated soluble EphrinB1 results in clustering of NR1 within 1 hour (Dalva et al., 2000), suggesting Ephrin/EphB signalling may play an important role in the recruitment of NMDA-R transport packets to nascent synapses. EphB2 also plays a role in spine formation as spine number is reduced in hippocampal neurons expressing a kinase-dead EphB2 receptor both *in vitro* and *in vivo* (Henkemeyer et al., 2003). In fact signalling through multiple EphB receptors appears to be important for normal postsynaptic development, as triple EphB1/2/3 knockouts display defects in spine number, morphology and postsynaptic protein clustering *in vivo* and *in vitro* that are more severe than in single knockouts (Henkemeyer et al., 2003). In conclusion, the role of Ephrin/Eph signalling at the synapse is of great interest due to their dual synaptogenic and anti-synaptogenic effects and ability to regulate pre- and postsynaptic development. Again, although the role of Ephrin/Eph signalling has been extensively investigated at excitatory synapses, it is unclear what role, if any, it plays in inhibitory synapse development.

Cadherins

The cadherins are an extremely large family of cell adhesion molecules that form homo- and heterophilic *cis* and *trans* interactions, and link intracellularly to the actin cytoskeleton through interaction with β- and α-catenin (Arikkath and Reichardt, 2008; Yagi and Takeichi, 2000). A number of cadherins have been implicated in synapse formation and maturation, perhaps most notably N-cadherin (Arikkath and Reichardt, 2008). Immunostaining for N-cadherin and key synaptic markers has demonstrated that it is synaptically localised during the major synaptogenic period (~6-16 DIV) in hippocampal cultures (Benson and Tanaka, 1998). Furthermore, N-cadherin is transported in transport packets presynaptically that resemble PTVs in terms of their dynamics and morphology, and these transport packets are recruited to nascent synapses (Jontes et al., 2004). Therefore, N-cadherin may be present in PTVs and involved in their recruitment to nascent synapses upon axo-dendritic contact. Blockade of cadherin function during the synaptogenic period in cultured hippocampal neurons induces a range of pre- and postsynaptic defects at glutamatergic synapses, including reduced presynaptic protein accumulation and vesicle recycling and retardation of spine development and postsynaptic protein accumulation. Furthermore, inhibitory presynaptic protein clustering is also reduced (Togashi et al., 2002). However, this study used a method that blocks the function of all classical cadherins, and the exact role of particular cadherins in excitatory versus inhibitory synaptogenesis remains unclear. N-cadherin is initially present at both types of synapse, but becomes restricted to glutamatergic synapses as neurons mature (Benson and Tanaka, 1998). Accordingly, it appears N-cadherin's main role is in the maintenance and plasticity of excitatory synapses (Arikkath and Reichardt, 2008). Conversely, cadherin-11 and -13 have been identified as inducers of GABAergic synapses (Paradis et al., 2007).

1.2.2.2 Secreted synapse organising molecules

Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) is well-characterised as a secreted synaptogenic molecule. BDNF signals predominantly through the receptor tyrosine kinase TrkB. Both BDNF and TrkB are found at a subset of excitatory and inhibitory

synapses in 14 DIV hippocampal cultures, as determined by co-localisation with appropriate pre- and postsynaptic markers (Gomes et al., 2006; Swanwick et al., 2004). Interestingly, TrkB receptors may be present in some STVs, as they show similar dynamics, and are often co-transported with VAMP2 in the axon (Gomes et al., 2006). Consistently, BDNF increases the frequency of mEPSCs and mIPSCs in hippocampal cultures, indicative of an increase in the number of functional glutamatergic and GABAergic synapses (Vicario-Abejon et al., 1998). However the role of BDNF in regulating inhibitory synapse number appears to be more complex than this. In one study, BDNF was overexpressed at low transfection efficiency in cultures from *BDNF-/-* mice, which were subsequently immunostained for excitatory or inhibitory presynaptic markers (Singh et al., 2006). This allowed the effect of postsynaptic BDNF on presynaptic terminal formation to be assessed in a BDNF-deficient background. Surprisingly, postsynaptic BDNF expression resulted in an increase in glutamatergic terminals contacting that neuron, but a *decrease* in inhibitory terminals (Singh et al., 2006). This suggests that BDNF acts retrogradely to increase excitatory innervation but suppress inhibitory innervation. BDNF may therefore play an important role in setting an appropriate excitatory/inhibitory balance. Genetic deletion of the TrkB receptor results in a range of synaptic defects at both mossy fibre and Schaffer collateral synapses in the hippocampus, including; decreased number and defective morphology of presynaptic terminals and dendritic spines, decreased accumulation of synaptic vesicles, decreased accumulation of pre- and postsynaptic proteins and decreased mEPSC frequency (Danzer et al., 2008; Luikart et al., 2005; Martinez et al., 1998; Otal et al., 2005). Therefore BDNF appears to play a crucial role in the formation of glutamatergic synapses in the hippocampus *in vivo*. However the exact role of BDNF in regulating a balance of excitatory and inhibitory synaptogenesis *in vivo* and the potentially differential roles of pre- and postsynaptic BDNF signalling in this process remain unclear.

Fibroblast growth factors

The fibroblast growth factors (FGFs) are a large family of heparin-binding growth factors that signal through four receptors (FGFR1-4) (Ornitz and Itoh, 2001; Reuss and von Bohlen und Halbach, 2003). As the name suggests, the first FGF was identified through its ability to promote fibroblast proliferation (Gospodarowicz, 1974). Since then, the FGFs have been found to have multiple roles in the embryonic and postnatal

development of virtually every body system (Bottcher and Niehrs, 2005; Ornitz and Itoh, 2001), including the CNS (Ozawa et al., 1996; Reuss and von Bohlen und Halbach, 2003). The first evidence for a synaptogenic role of FGF came from a study performed on cultured *Xenopus* spinal neurons (Dai and Peng, 1995). Local addition of FGF2 (also known as 'basic FGF' or 'bFGF') to axons via a micropipette or FGF2-coated beads resulted in a localised increase in intracellular calcium levels. FGF2-coated beads also elicited a calcium-dependent accumulation of synaptic vesicles at the site of contact with axons, as assayed by electron microscopy and immunostaining for Synaptotagmin-1, suggesting a role for FGF signalling in presynaptic development (Dai and Peng, 1995). A later study found similar results in cultured hippocampal neurons, as FGF2 increased the number of clusters of Synapsin-1 and Synaptophysin (Li et al., 2002a). Furthermore, these clusters of presynaptic proteins were apposed to clusters of PSD-95 and GluR1, and colocalised with depolarisation-dependent uptake of FM4-64 (Li et al., 2002a). Therefore, FGF2 promotes the co-ordinated differentiation of pre- and postsynaptic structures, and the formation of functional synapses. The presynaptic organising role of FGFs extends to several other family members, including FGF4, 6, 7, 9, 10 and 22, as evidenced by a screening experiment that assayed the ability of a range of FGFs to promote Synapsin clustering in cultured chick motorneurons (Umemori et al., 2004). Most importantly, this same study provided compelling evidence that FGF22 is secreted from granule cells of the developing mouse cerebellum *in vivo*, where it acts through FGFR2 on incoming pontine mossy fibres to promote presynaptic differentiation, as assayed through observing the clustering of a range of presynaptic proteins (Umemori et al., 2004). Thus the role of FGFs as target-derived presynaptic organisers is now fairly well established. However, it remains unclear whether these presynaptic effects indirectly lead to postsynaptic differentiation, or whether FGFs can also signal directly to the postsynaptic side. Also, it is not known whether FGFs affect only glutamatergic synapses, or whether they also regulate GABAergic synaptic development.

Thrombospondins

Thrombospondins are extracellular matrix glycoproteins with five family members, TSP1-5. TSP1 and TSP2 are secreted by astrocytes in the developing nervous system (Adams, 2001; Christopherson et al., 2005), where they have recently been found to have a synaptogenic function. Addition of TSP1 or TSP2 to cultured retinal ganglion

cells results in an increase in the number of glutamatergic synapses, as measured by Synapsin and PSD-95 co-localisation (Christopherson et al., 2005). Most importantly, TSP1 and TSP2 play a synaptogenic role *in vivo*, as P21 *TSP1/2* double knockout mice exhibit reduced synaptic density in the cortex (Christopherson et al., 2005). A later study identified Alpha2delta-1 as a thrombospondin receptor that is required for TSP-mediated synaptogenesis *in vitro* and *in vivo* (Eroglu et al., 2009). Alpha2delta-1 is required postsynaptically for TSPs to mediate their excitatory synaptogenic effect (Eroglu et al., 2009), suggesting TSPs act directly on the postsynaptic side which then leads to concomitant presynaptic differentiation through a retrograde signal. Interestingly, TSP promotes the formation of synapses that are presynaptically active but postsynaptically silent (Christopherson et al., 2005), and therefore mimics the effects of Neuroligin-1 (Sara et al., 2005). Consistent with this, TSP1 binds to Neuroligins, and disruption of TSP1-Neuroligin binding or knockdown of Neuroligin-1 attenuates the ability of TSP1 to induce excitatory synapse formation in hippocampal cultures (Xu et al., 2009). Therefore, TSPs may promote excitatory synaptogenesis through signalling to Neuroligins directly on the postsynaptic side, which in turn can signal to the presynaptic side via binding to Neurexins. TSPs appear to selectively increase glutamatergic synapses, having no effect on GABAergic synapse density (Hughes et al., 2009), despite the fact that TSP1 can also bind to Neuroligin-2 (Xu et al., 2009). Factors present in astrocyte-conditioned medium can promote GABAergic synaptogenesis, but this does not depend on TSPs, neurotrophins or cholesterol (see below), and these factors remain to be identified (Hughes et al., 2009).

Cholesterol

Cholesterol is another glia-derived molecule that has been implicated in synaptogenesis (Pfrieger, 2003). Cholesterol has a range of biological functions; it is an essential component of cellular membranes, a precursor of steroid hormones and a co-factor for a range of signalling molecules (Mann and Beachy, 2000; Yeagle, 1985). The role of cholesterol in synapse formation was first demonstrated in retinal ganglion cell cultures, where addition of cholesterol resulted in an increase in glutamatergic synapse number and efficacy, whereas the synaptogenic activity of glia-conditioned medium was reduced if cholesterol synthesis was blocked during conditioning (Mauch et al., 2001). Glial cholesterol is secreted in complex with apolipoprotien E (ApoE) and integrated into the neuronal membrane through the action of the low-density lipoprotein receptor

(LDL-R). Accordingly, antagonism of LDL-R resulted in a reduction in the synaptogenic activity of glia conditioned medium (Mauch et al., 2001). Cholesterol-induced synaptogenesis has also been observed in hippocampal slice cultures, and this study demonstrated that conversion of cholesterol to estradiol is critical for its synaptogenic activity (Fester et al., 2009). Consistent with its enrichment at presynaptic active zones, synaptic vesicles and postsynaptic membrane (Pfrieger, 2003), cholesterol appears to promote glutamatergic synapse formation and maturation through a range of mechanisms, including increased synaptic vesicle content of presynaptic terminals and increased AMPA-R clustering (Goritz et al., 2005). Additionally, cholesterol plays a crucial role in dendritic development, a prerequisite for efficient glutamatergic synaptic development (Goritz et al., 2005). Like TSPs, cholesterol is specifically important for excitatory synaptogenesis, as it does not promote GABAergic synapse formation in hippocampal cultures (Hughes et al., 2009).

Wnts

The Wnt family of secreted glycoproteins have also been identified as synaptogenic factors. Various members of this large protein family have been found to play multiple roles in synapse formation at a variety of synapses in both vertebrates and non-vertebrates. Accordingly, the next sections will give a description of Wnts and their associated signalling pathways, followed by a detailed discussion of their role in synapse formation and function.

1.3 Wnt signalling

Wnts are a large family (19 members have been described in humans) of secreted cysteine-rich glycoproteins that signal through a variety of receptors and intracellular signalling pathways (Angers and Moon, 2009; Chien et al., 2009; Kikuchi et al., 2007). The Wnts are so-called as a contraction of the *Drosophila* gene *wingless* and the human oncogene *int-1*, which were identified as homologs over 20 years ago (Cabrera et al., 1987; Rijsewijk et al., 1987). The role of *int-1* in cancer, along with the discovery that eptotic expression of *int-1* (now known as *wnt1*) in *Xenopus* embryos resulted in two-headed larvae due to body axis duplication (McMahon and Moon, 1989a, b), created considerable interest in the role of Wnt signalling in development and disease. As a result, the past two decades have seen an explosion in the study of Wnts and Wnt

signalling, and they are now firmly established as playing a crucial role in the embryonic development of every body system in both health and disease, including the CNS (Cadigan and Nusse, 1997; Chien et al., 2009; Ciani and Salinas, 2005; De Ferrari and Moon, 2006; Freese et al., 2009). Furthermore, Wnts and Wnt signalling-related proteins continue to be expressed in neural tissues postnatally and even in mature animals (Coyle-Rink et al., 2002; Davis et al., 2008; Gogolla et al., 2009; Henriquez et al., 2008; Lucas and Salinas, 1997; Rosso et al., 2005; Shimogori et al., 2004); consistently, they have also been found to regulate processes such as synaptic formation, function and plasticity (Ahmad-Annuar et al., 2006; Chen et al., 2006; Gogolla et al., 2009; Salinas, 2005). Before reviewing the role of Wnts in these processes, I will first give a brief overview of Wnt signalling, including how Wnts are secreted, how Wnt signals are transduced and the actions of endogenous Wnt antagonists.

1.3.1 Post-translational modification and secretion of Wnts

As mentioned above, Wnts are glycoproteins, being glycosylated at several asparginine residues within the endoplasmic reticulum (ER) (Komekado et al., 2007; Kurayoshi et al., 2007; Smolich et al., 1993). Glycosylation of Wnts appear to be important for Wnt secretion, but not necessarily for receptor binding and signalling, as *in vitro* deglycosylated Wnt5a is still able to elicit intracellular signalling (Kurayoshi et al., 2007). Instead, glycosylation appears to indirectly influence Wnt signalling through its crucial roles in Wnt secretion and palmitoylation (Komekado et al., 2007; Kurayoshi et al., 2007). Palmitoylation of Wnts at conserved cysteine residues occurs within the ER and is dependent upon prior glycosylation and the action of the transmembrane acyl-transferase Porcupine (Komekado et al., 2007; Takada et al., 2006; Zhai et al., 2004). Palmitoylation of Wnts appears to be crucial for both the secretion of and signalling by Wnts, as *porcupine* mutants retain Wnt within the ER (Tanaka et al., 2000; Zhai et al., 2004), and non-palmitoylated Wnt3a and Wnt5a are incapable of binding their Frizzled receptors to produce intracellular signalling (Komekado et al., 2007; Kurayoshi et al., 2007). Post-translational glycosylation and palmitoylation of Wnts also act to make them highly hydrophobic and ‘sticky’; they rapidly associate with cell membranes and ECM molecules such as heperan sulphate proteoglycans (HSPGs) upon release (Bradley and Brown, 1990; Reichsman et al., 1996). These properties of Wnts appear to be functionally important (Reichsman et al., 1996; Zhai et al., 2004), but they also make

the purification of Wnt proteins notoriously difficult. Despite this, recent years have seen the successful purification and commercial production of a number of Wnts, including Wnt3a, -5a and 7a.

Following modification in the ER, Wnt proteins are transported to the Golgi apparatus. Transport from the Golgi apparatus to the cell surface appears to rely crucially upon the conserved transmembrane protein Evenness interrupted (Evi; also known as Wntless (Wls)). Genetic disruption of *evi* in *Drosophila* results in morphological phenotypes that mimic those seen in *wingless* mutants, and knockdown of *evi* in cultured cells results in accumulation of Wg in the Golgi apparatus, reduced Wg secretion and a reduction in Wnt signalling activity in co-cultured cells (Banziger et al., 2006; Bartscherer et al., 2006). A recent study at the *Drosophila* NMJ further demonstrated that Evi is required for presynaptic release of Wingless at this synapse, and that Wg is in fact secreted in vesicular structures that contain Evi (Korkut et al., 2009). Interestingly, Evi was also required for postsynaptic reception and signalling by Wg in muscle cells (Korkut et al., 2009). However, whether this is a general feature of intercellular communication by Wnts or whether this mechanism is specific to the NMJ remains unclear.

1.3.2 Wnt reception and intracellular signalling

1.3.2.1 Wnt receptors

Following their secretion, Wnts bind to transmembrane receptors to trigger intracellular signalling through a number of distinct pathways. These include the 'canonical' β-catenin-dependent pathway and the 'non-canonical' Wnt/Ca^{2+}, planar cell polarity (PCP) and nuclear import pathways (Chien et al., 2009; Korkut et al., 2009; Veeman et al., 2003) (see below and Fig1.3). The principal Wnt receptor appears to be the seven transmembrane domain receptor Frizzled (Fz), of which there are 10 known isoforms in mammals, and which contain a highly conserved extracellular cysteine-rich domain (CRD) which is crucial for Wnt binding and signalling (Dann et al., 2001; Hsieh et al., 1999b; Wang et al., 2006). Wnt binding to Frizzled receptors can activate all known Wnt pathways, depending on the cellular context (van Amerongen et al., 2008).

The receptor tyrosine kinase Ror2 also has an extracellular CRD (Forrester, 2002; Xu and Nusse, 1998), and Wnt5a binding to Ror2 is able to activate the PCP pathway

(Mikels and Nusse, 2006; Oishi et al., 2003; Schambony and Wedlich, 2007). Interestingly, Wnt5a can also inhibit canonical Wnt signalling through Ror2, or activate it through Fz4 (Mikels and Nusse, 2006). This indicates that there is crosstalk between the different Wnt signalling pathways, and that the specific intracellular signalling elicited by a Wnt signal may depend on receptor expression and localisation.

Another recently characterised Wnt receptor is the atypical tyrosine kinase Ryk. Ryk does not contain a CRD, but rather binds Wnt through a domain similar to that found in the Wnt-sequestering protein Wnt inhibitory factor (WIF, see below) (Hsieh et al., 1999a; Kroiher et al., 2001). Wnt5a signalling through Ryk has been found to play a role in axon outgrowth and axon repulsion during development and inhibition of axon regeneration following injury in the corticospinal tract (Liu et al., 2005; Miyashita et al., 2009) Wnt5a/Ryk signalling also mediates axon repulsion in the corpus callosum (Keeble et al., 2006), whereas Wnt3 signals through Ryk to mediate axon repulsion during retinotectal mapping (Schmitt et al., 2006). Binding of Wnt5 to the *Drosophila* Ryk homolog *derailed* also mediates axon repulsion to guide anterior commissure crossing at the midline (Wouda et al., 2008; Yoshikawa et al., 2003). Wnt-Ryk signalling co-operates with other Wnt signalling pathways during development. Wnt3 repulses axons through Ryk and attracts axons through Fz signalling during retinotectal mapping (Schmitt et al., 2006), and Wnt5a-mediated corticospinal axonal outgrowth requires only Ryk, whereas axon repulsion requires both Ryk and Fz signalling (Li et al., 2009). Similarly, both Fz and Derailed signalling are required for proper development of the *Drosophila* salivary gland, with Wnt4-Fz signalling controlling cell positioning and Wnt5-Ryk controlling cell migration (Harris and Beckendorf, 2007).

Despite its obvious importance in development, little is known about how Wnt-Ryk binding is transduced intracellularly, other than it involves the Src family of tyrosine kinases (Wouda et al., 2008). However, a considerable amount of information has accumulated regarding intracellular Wnt signalling through the canonical, Wnt/Ca^{2+}, planar cell polarity and nuclear import pathways. Accordingly, I will briefly describe each of these pathways in turn.

1.3.2.2 Wnt signalling pathways

Canonical Wnt signalling

Canonical Wnt signalling occurs exclusively through binding of Wnts to Fz receptors and their single transmembrane domain co-receptor LRP5/6, resulting in stabilisation, accumulation and nuclear localisation of cytoplasmic β-catenin (Chien et al., 2009). In the absence of Wnt binding to Fz-LRP5/6, β-catenin is recruited to a 'destruction complex' consisting of Axin, APC and the kinases Casein kinase 1α (CK1α) and Glycogen synthase kinase 3β (GSK3β; Fig 1.3) (Angers and Moon, 2009). Upon recruitment to this complex, the N-terminal region of β-catenin is phosphorylated sequentially by CK1α at Ser45 and GSK3β at Thr41, Ser37 and Ser33. Phosphorylation of β-catenin leads to its ubiquitination by E2 ligase, which in turn marks it for removal and proteasomal degradation (Kimelman and Xu, 2006). Wnt binding to the Fz-LRP5/6 receptor complex results in the phosphorylation and subsequent recruitment of the cytoplasmic scaffolding protein Dishevelled (Dvl) to the complex (Rothbacher et al., 2000; Yanagawa et al., 1995), via a direct interaction of its PDZ domain with the C-terminal tail of Fz (Chen et al., 2003; Wong et al., 2003). Dvl recruitment in turn results in the binding of Axin and GSK3 to LRP5/6, thereby causing the dissociation of the destruction complex (Mao et al., 2001b; Zeng et al., 2008). This means that β-catenin, instead of being phosphorylated and degraded, is able to accumulate in the cytoplasm, translocate to the nucleus and activate the transcription of Wnt response genes through binding to the TCF/LEF family of transcription factors (Fig 1.3; Angers and Moon, 2009; Chien et al., 2009). The resulting changes in gene expression play a role in a wide variety of developmental processes such as cell proliferation, differentiation and migration (Chien et al., 2009).

Interestingly, recent work has demonstrated that recruitment of GSK3 to the Fz-LRP5/6 signalling complex does not only activate Wnt signalling through preventing GSK3β-dependent phosphorylation of β-catenin. The kinase activity of GSK3 is required at the Fz-LRP5/6 complex to phosphorylate LRP5/6, which in turn is important for subsequent phosphorylation by CK1 and Axin recruitment (Davidson et al., 2005; Tamai et al., 2004; Zeng et al., 2005). Therefore it has been proposed that the initial Axin-dependent phosphorylation of LRP5/6 by GSK3 may act as a feed-forward amplification system, leading to further sequestration of Axin and GSK3β from the destruction complex (Zeng et al., 2008). This is still consistent with the well-established observation that GSK3 inhibitors act as canonical Wnt signalling agonists

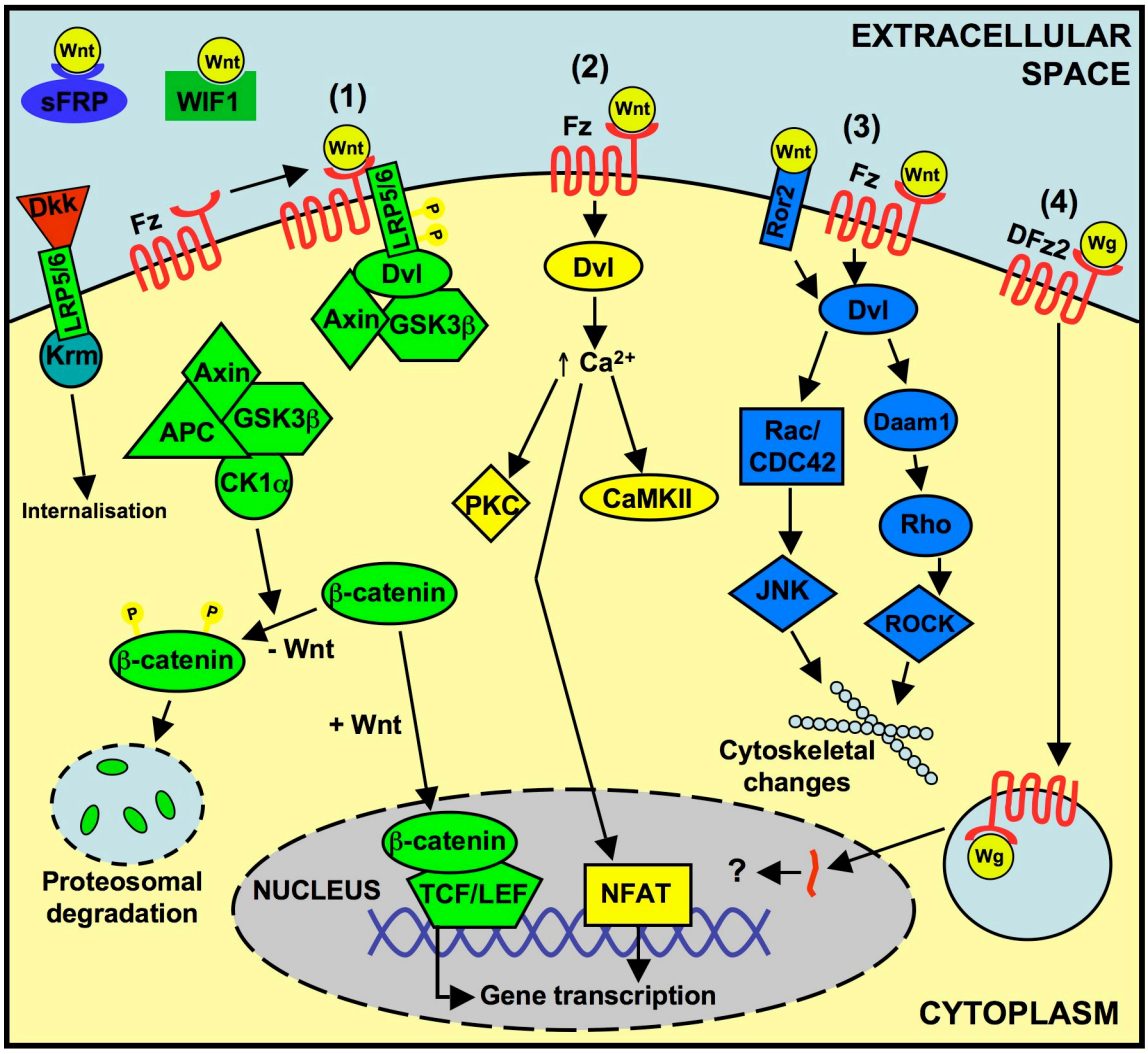

Figure 1.3 Wnt signalling pathways. The four main Wnt pathways are numbered and colour-coded for clarity. (1) In the canonical pathway (green) Wnt binding to Frizzled and LRP5/6 results in recruitment of Dishevelled, Axin and GSK3β, thereby promoting dissociation of the β-catenin destruction complex. This allows β-catenin to translocate to the nucleus where it regulates gene transcription through binding to TCF/LEF transcription factors. (2) In the Wnt/Ca^{2+} pathway (yellow) Wnt binding to Fz results in Dvl activation and an increase in intracellular calcium. This leads to the activation of PKC, CaMKII and the transcription factor NFAT. (3) In the planar cell polarity pathway, Wnt binding to Fz or Ror results in cytoskeletal reorganisation via the small GTPase-dependent activation of JNK and ROCK. (4) The nuclear import pathway has so far only been demonstrated at the *Drosophila* NMJ. Wingless binding to DFz2 results in internalisation and perinuclear transport of DFz2. The C-terminal region of DFz2 is then cleaved and transported into the nucleus, where it regulates NMJ formation through an as-of-yet undetermined mechanism. The actions of endogenous Wnt antagonists are also shown in the top left corner. sFRPs and WIF1 bind and sequester Wnts directly. Dkk binds to LRP5/6 and promotes its internalisation, thereby blocking canonical Wnt signalling.

(Hall et al., 2000; Hernandez et al., 2009), as they can act to directly inhibit GSK3β-mediated β-catenin phosphorylation in the destruction complex, downstream of the Fz-LRP5/6 complex.

It should also be noted that a transcription-independent 'divergent canonical pathway' has been described that directly regulates the cytoskeleton (Ciani et al., 2004; Salinas, 2007). In this pathway, Dvl and axin associated directly with microtubules (MTs) act to reduce GSK3β-mediated phosphorylation of microtubule-associated protein 1B (MAP1B), leading to increased MT stability (Ciani et al., 2004). Exactly how GSK3β is inhibited in the divergent canonical pathway is unknown – based on the actions of Dvl and axin in the regular canonical pathway, it is possible that Dvl/Axin act to bind and sequester GSK3β at MTs in such a way that it can no longer phosphorylate MTs. Although initially described in mammalian cell lines (Ciani et al., 2004), the divergent canonical pathway has also been described at the *Drosophila* NMJ, where it plays an important role in NMJ formation (Franco et al., 2004; Gogel et al., 2006; Miech et al., 2008), and in sensory neurons, where it mediates axonal and growth cone remodelling (Purro et al., 2008). Therefore this pathway, like the other Wnt signalling pathways, is conserved from invertebrates to vertebrates.

Wnt/Ca^{2+} signalling

The Wnt/Ca^{2+} pathway also involves signalling through Fzs and Dvl (Kuhl et al., 2000; Robitaille et al., 2002; Sheldahl et al., 2003; Slusarski et al., 1997a), and appears to be activated by a relatively small selection of Wnts including Wnt5a, Wnt5b and Wnt11 (Kuhl et al., 2000; Slusarski et al., 1997a; Slusarski et al., 1997b; Westfall et al., 2003). Downstream of Dvl, increases in intracellular calcium result in activation of PKC (Sheldahl et al., 2003), CaMKII (Kuhl et al., 2000; Robitaille et al., 2002; Sheldahl et al., 2003) and the calcium-dependent transcription factor NFAT (Saneyoshi et al., 2002) (Fig 1.3). The Wnt/Ca^{2+} pathway plays a crucial role in determining ventral cell fate and convergent extension during embryogenesis (Kuhl et al., 2000; Saneyoshi et al., 2002; Veeman et al., 2003). Again, Wnt/Ca^{2+} signalling interacts with other Wnt signalling pathways, as Wnt signalling through NFAT is able to inhibit Xwnt8-mediated dorsal axis formation through suppression of canonical Wnt signalling (Saneyoshi et al., 2002).

The planar cell polarity pathway

The planar cell polarity (PCP) pathway is so-called due to its crucial role in determining tissue polarity through the regulation of cellular polarity and movement (Fanto and McNeill, 2004), and again involves signalling through Fz (Adler et al., 1994; Adler et al., 2000; Park et al., 1994) and Dvl (Axelrod et al., 1998; Boutros et al., 1998; Tada and Smith, 2000). The PCP pathway exerts its cellular effects by regulating the cytoskeleton through two branches of the pathway that both involve GTPase activation. Dvl can activate c-Jun N-terminal kinase (JNK) through the small GTPases Cdc42 and Rac (Boutros et al., 1998; Moriguchi et al., 1999; Schlessinger et al., 2007). Dvl can also signal through the forming homology protein Daam1 to activate the small GTPase RhoA, leading to activation of Rho-associated coiled-coil containing protein kinase (ROCK) (Winter et al., 2001) (Fig1.3). Signalling in both pathways results in cytoskeletal changes that regulate cell polarity and movement (Fanto and McNeill, 2004). In vertebrates, the PCP pathway is especially important in convergent extension movements during gastrulation, and can be activated by Xwnt11 and Wnt5a (Schlessinger et al., 2007; Tada and Smith, 2000), therefore it is highly likely that there is considerable crosstalk between the PCP and Wnt/Ca^{2+} pathway.

The nuclear import pathway

A novel Wnt signalling pathway has recently been described at the *Drosophila* NMJ. In this pathway, Wg released from the presynaptic motor neuron crosses the synaptic cleft and binds to Dfz2 receptors on the postsynaptic muscle cell. Upon Wg binding, Dfz2 receptors are internalised and transported to the perinuclear region, where the C-terminal region of Dfz2 is cleaved and transported into the nucleus (Mathew et al., 2005) (Fig 1.3). Transport of Dfz2 to the perinuclear region is dependent upon the PDZ protein dGRIP, which is found at the postsynaptic membrane, Golgi apparatus and trafficking vesicles, where it co-localises with internalised Dfz2 (Ataman et al., 2006). Furthermore, proper postsynaptic localisation of dGRIP and endocytosis of Dfz2 upon Wg binding requires Evi, suggesting this protein can play a role in both Wnt secretion and reception (Korkut et al., 2009). Mutants lacking *dGRIP* or postsynaptic *evi* mimic the synaptic defects seen in *wg* and *dfz2* mutants (Ataman et al., 2006; Korkut et al., 2009; Mathew et al., 2005). Based on this, it was proposed that the cleaved nuclear Dfz2 fragment acts to transduce the Wg signal postsynaptically, presumably through

altering gene transcription. However it should be noted that, as of yet, there is no direct evidence for transcriptional activation/repression in this pathway. Also, this pathway has only been reported at the *Drosophila* NMJ, and whether it is a commonly used or highly specific mechanism is unknown.

1.3.3 Endogenous Wnt antagonists

The multiplicity of Wnts, Wnt receptors, and intracellular signalling pathways impart upon Wnt signalling an astonishing degree of diversity and complexity. In addition to this, Wnts are further regulated by several families of endogenous antagonists, including the secreted frizzled-related proteins (sFRPs), the Dikkopf proteins (Dkks) and the Wnt inhibitory factors (WIFs). Aside from their role in regulating Wnt signalling *in vivo*, the purification and commercial production of many of these proteins has provided invaluable tools for researchers investigating Wnt-regulated processes.

Secreted frizzled-related proteins

Secreted frizzled receptor proteins (sFRPs) are a family of 5 proteins in mammals (sFRP1-5) that all contain a CRD domain highly homologous to the CRD domain of Frizzled (Kawano and Kypta, 2003; Rattner et al., 1997). Consistently, direct binding has been demonstrated between multiple members of the Wnts and the sFRPs (Bafico et al., 1999; Dennis et al., 1999; Uren et al., 2000; Wawrzak et al., 2007). This binding is stabilised by sFRP binding to heperan sulphate proteoglycans (HSPGs) (Uren et al., 2000); as Wnts also bind HSPGs it is possible that they act as a scaffolding molecule for Wnt-sFRP interactions. Binding of Wnts by sFRPs is thought to sequester Wnts upstream of the receptor, and thereby inhibit all Wnt signalling pathways (Fig 1.3). Consistently, sFRPs have been demonstrated to inhibit both canonical and non-canonical Wnt signalling-dependent processes *in vitro* and *in vivo* (Leyns et al., 1997; Rosso et al., 2005; Satoh et al., 2008; Wang et al., 1997a; Wang et al., 1997b). However, different sFRP isoforms display differences in their abilities to bind and antagonise specific Wnts (Wang et al., 1997b; Wawrzak et al., 2007). As several Wnts show specificity in terms of the intracellular pathways they activate, this means sFRPs may also display specificity with regards to the Wnt pathways they inhibit, depending on the Wnt proteins present. Although this may be a useful property of sFRPs in some

experimental paradigms, it also means that efficient blockade of Wnt signalling through all pathways requires treatment with a cocktail of several sFRPs.

Though sequestration of Wnt is believed to be the primary mechanism of action of sFRPs, recent studies have provided evidence that they can act in other ways (Bovolenta et al., 2008). Direct binding of sFRP to Fz receptors has been observed (Bafico et al., 1999; Rodriguez et al., 2005), suggesting that sFRP may also be able to inhibit Wnt signalling through the formation of non-functional Fz-sFRP complexes, or even *activate* intracellular Wnt signalling in some circumstances (Rodriguez et al., 2005; Uren et al., 2000). Furthermore, there is evidence that sFRPs may be able to bind and antagonise each other under the right circumstances. In the developing kidney, Wnt4-dependent tubule formation is inhibited by sFRP1 but not sFRP2. However adding sFRP1 & -2 concomitantly rescues the defect, suggesting sFRP2 can inhibit sFRP1 in this system (Yoshino et al., 2001). Therefore the sFRPs appear to be capable of regulating Wnt signalling through multiple mechanisms.

Dikkopfs

Dikkopfs (Dkks) are a family of four proteins (Dkk1-4) which contain two CRD domains (CRD1 & 2) (Glinka et al., 1998). However, rather than binding Wnts, Dkks bind directly to LRP5/6 via their CRD2 domain (Bafico et al., 2001; Li et al., 2002b; Mao et al., 2001a). Dkk binding to LRP5/6 results in the recruitment of the transmembrane protein Kremen (Krm) to Dkk, resulting in the formation of a Dkk-LRP5/6-Krm ternary complex. This complex is then rapidly endocytosed, thereby removing LRP5/6 from the membrane and specifically inhibiting canonical Wnt signalling (Mao et al., 2002; Nakamura and Matsumoto, 2008; Yamamoto et al., 2008) (Fig 1.3). This inhibitory specificity of Dkks makes them a useful tool in examining the contribution of canonical Wnt signalling to a given cellular process (Nakamura and Matsumoto, 2008). However, this is somewhat complicated by the recent discovery in zebrafish that Dkk1 can simultaneously inhibit canonical signalling and *activate* the PCP pathway during gastrulation (Caneparo et al., 2007). This suggests that *in vivo* Dkks may play a role beyond simple Wnt inhibition, acting to control the relative activity in different Wnt pathways.

Wnt inhibitory factor 1

Wnt inhibitory factor 1 (WIF1) binds Wnts through its highly conserved WIF domain, and is therefore believed to inhibit Wnt signalling through the same mechanism as the sFRPs, namely sequestration of Wnt proteins (Hsieh et al., 1999a) (Fig 1.3). Relatively little is known about WIF-1 compared to the other secreted Wnt antagonists, but it appears to play a particularly important role in skeletal development (Cho et al., 2009; Surmann-Schmitt et al., 2009) and cancer (Batra et al., 2006; Kawakami et al., 2009; Tomm et al., 2009).

1.4 Wnts in synapse formation and function

As previously mentioned, Wnts are crucial in the early stages of nervous system development, regulating processes such as neuronal proliferation, differentiation and migration (Ciani and Salinas, 2005; Ille and Sommer, 2005). However, Wnts and key Wnt pathway components continue to be expressed throughout the nervous system into late embryonic development, postnatally, and even into adulthood (Coyle-Rink et al., 2002; Davis et al., 2008; Gogolla et al., 2009; Henriquez et al., 2008; Lucas and Salinas, 1997; Rosso et al., 2005; Shimogori et al., 2004). Consistently, research over the past decade has highlighted the importance of Wnts in later neural developmental processes.

One such process is the directed outgrowth of neurites. The role of Wnts in axon guidance and target recognition has already been discussed in some detail in the previous section, with Wnts directing axon growth in the corticospinal tract (Liu et al., 2005; Miyashita et al., 2009), corpus callosum (Keeble et al., 2006) and retinotectal projection (Schmitt et al., 2006) in vertebrates, and the midline in invertebrates (Wouda et al., 2008; Yoshikawa et al., 2003). Additionally, Wnt3a causes extensive axonal remodelling of sensory neurons in the peripheral nervous system (Krylova et al., 2002; Purro et al., 2008), whereas Wnt7a and Wnt7b have a similar effect on pontine mossy fibres and granule cell axons in the cerebellum (Ahmad-Annuar et al., 2006; Hall et al., 2000; Lucas and Salinas, 1997). Wnt7b, acting through the PCP pathway, is also important in early dendrite formation in hippocampal neurons (Rosso et al., 2005).

Once axons have found their proper postsynaptic partners, synapses form through complex structural rearrangements on both sides, as described in section 1.2. Furthermore, a functional synapse must be able to transmit information from the

presynaptic to postsynaptic cell, and alter how this information is transmitted based on previous activity ('plasticity'). Wnts have been found to play important roles in all of the above processes. I will therefore use the remainder of this introduction to describe the current state of knowledge of the role of Wnts in synapse formation and function.

1.4.1 Wnts in synapse formation

Since Wnts were first identified as presynaptic organisers over a decade ago (Lucas and Salinas, 1997), much has been learnt about their role in synaptogenesis from *in vitro* and *in vivo* studies in both vertebrates and invertebrates (Fig 1.4). The vast majority of these studies have concentrated on three regions; the NMJ in the peripheral nervous system, and the cerebellum and hippocampus in the CNS. Accordingly, I will describe the role of Wnts in synapse formation in each of the areas in turn.

1.4.1.1 Wnts in neuromuscular junction formation

The first direct evidence that Wnts were involved in NMJ formation came from studies in *Drosophila*. An initial study found that the Wnt1 homolog Wingless (Wg) is secreted from *Drosophila* motor neurons and acts bidirectionally to promote NMJ development, as both pre- and postsynaptic structures are highly defective at the NMJ of conditional mutants that fail to release Wg (Packard et al., 2002). These defects include a reduced number of boutons, misshapen boutons, microtubule unbundling and defective active zone structure, and enlarged and misshapen postsynaptic densities with defective glutamate receptor localisation. In addition, the precise apposition of pre- and postsynaptic structures is disrupted, consistent with the hypothesis that Wg acts bidirectionally at the developing NMJ to co-ordinate pre- and postsynaptic differentiation (Packard et al., 2002) (Fig 1.4).

Interestingly, further studies revealed that Wg appears to act through distinct signalling pathways pre- and postsynaptically. Presynaptically, Wg signals through the canonical pathway to promote bouton formation through cytoskeletal changes, including the formation of looped microtubules within boutons (Packard et al., 2002). Although this signalling pathway is 'canonical' in that it requires the *Drosophila* homologs of LRP5/6 (Arrow) and GSK3β (Shaggy), it is independent of transcription (Miech et al., 2008),

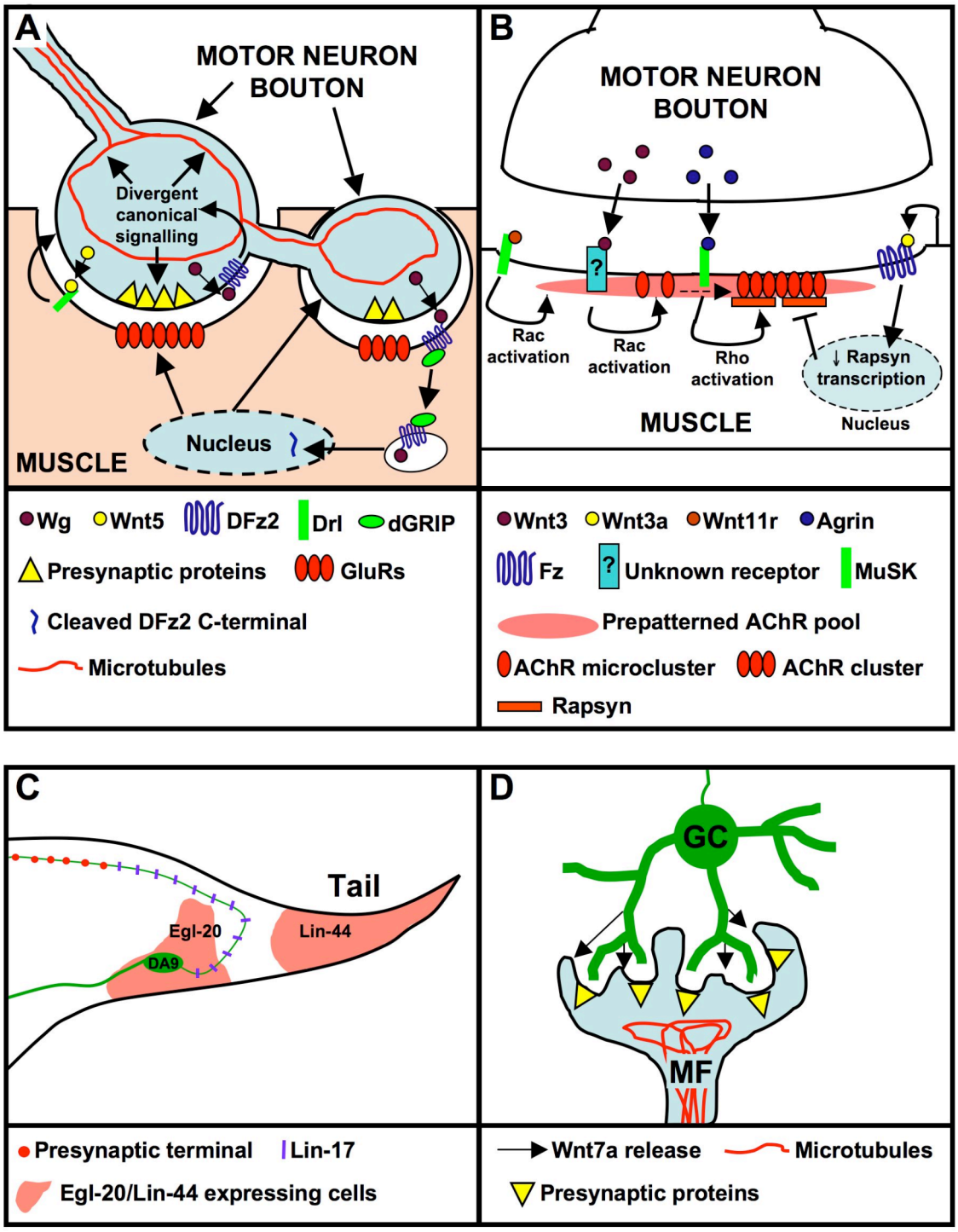

Figure 1.4 (Figure legend on next page)

and therefore closely resembles the 'divergent canonical pathway' also described in vertebrates (Ciani et al., 2004; Purro et al., 2008). Postsynaptically, Wg signals through the nuclear import pathway already described in detail in section 1.3, involving the internalisation and nuclear transport of the Wg receptor Dfz2 (Ataman et al., 2006; Korkut et al., 2009; Mathew et al., 2005). However disruption of this postsynaptic pathway results in both pre- and postsynaptic defects (Ataman et al., 2006; Korkut et

Figure 1.4 Wnts regulate the formation of a variety of synapses in invertebrates and vertebrates. A) The *Drosophila* neuromuscular junction. Wingless (Wg) released from motor neuron boutons binds to presynaptic D-Frizzled2 (DFz2) receptors to promote cytoskeletal rearrangement and presynaptic protein clustering through a divergent canonical pathway. Wg also binds to postsynaptic DFz2 receptors, which undergo internalisation and perinuclear transport, followed by cleavage and nuclear localisation of the DFz2 C-terminal. This nuclear transport pathway plays a role in glutamate receptor (GluR) localisation, and also promotes presynaptic bouton formation through an unknown retrograde signal. B) The vertebrate neuromuscular junction. In zebrafish, Wnt11r signals through MuSK to activate Rac, leading to pre-patterning of acetylcholine receptors (AChRs). In the chick, Wnt3 from the motor neuron terminal acts through an unknown receptor to activate Rac, leading to the formation of AChR microclusters. These microclusters are then aggregated in larger AChR clusters through Agrin-dependent Rho activation. In the mouse, Wnt3a signals through Frizzled (Fz) and the canonical pathway to decrease transcription of the AChR-clustering protein Rapsyn, thereby dispersing AChR clusters (presumably at non-synaptic sites). C) The DA9 motor neuron of *C. elegans*. The Wnt homologs Egl-20 and Lin-44 are released by groups of cells in close proximity to the proximal axonal segment of the DA9 neuron, which contains the Fz homolog Lin-17. Egl-20/Lin-44 signalling through Lin-17 prevents synaptic terminal formation in this area, and terminals instead form in the distal axon, which lacks Lin-17. D) The mossy fibre-granule cell (MF-GC) synapse of the mouse cerebellum. Wnt7a released from GCs acts retrogradely to activate divergent canonical signalling in the MF terminal, leading to cytoskeletal changes, synaptic terminal remodelling and clustering of presynaptic proteins.

al., 2009), suggesting that signalling through this pathway can produce a retrograde signal that regulates presynaptic differentiation.

Non-canonical signalling through Wnt5 binding to the *Drosophila* Ryk homolog Derailed (Drl) also regulates NMJ development (Fig 1.4), as genetic disruption of either *wnt5* or *drl* result in a decrease in presynaptic bouton number (Liebl et al., 2008). Wnt5 deficiency can only be rescued by presynaptic overexpression of *wnt5*, and conversely Drl deficiency can only be rescued by overexpression of *drl* in the muscle, suggesting that Wnt5-Drl signalling in the muscle fibre generates a retrograde signal that regulates presynaptic development. Surprisingly, postsynaptic development appeared largely normal in both *wnt5* and *drl* mutants (Liebl et al., 2008). However, this was only assessed by glutamate receptor staining, and it is possible more detailed examination of the postsynapse would reveal defects.

While discussing the role of Wnts in NMJ formation in invertebrates, special mention should be given to a study in *C. elegans* which describes a novel *anti*-synaptogenic action of Wnt (Klassen and Shen, 2007). In *C. elegans*, the axon of the DA9 motor neuron exhibits very specific patterning of its presynaptic terminals; terminals only

form in the distal portion of the axon, which innervates muscles in the posterior dorsal muscle wall. The more proximal segment of the axon is asynaptic. This patterning is controlled in part by the *C. elegans* Wnt proteins Lin-44 and Egl-20 acting to restrict presynaptic terminal formation, as eptotic terminals form in the asynaptic zone in *lin-44* and *egl-20* mutants. Lin-44 and Egl-20 act to regulate the axonal localisation of the Fz receptor Lin-17, which in turn signals through Dvl to restrict presynaptic terminal formation in the asynaptic zone (Fig 1.4). Interestingly, the total number of synapses remains the same in mutants in which the asynaptic zone is reduced or expanded in size, suggesting Wnt-Fz-Dvl signalling controls the subcellular localisation of a pre-determined number of synapses in DA9 neurons (Klassen and Shen, 2007). Subsequently, it was reported that Wnt5a reduces excitatory synapse number in cultured hippocampal neurons (Davis et al., 2008), suggesting that some Wnts may also play an anti-synaptogenic role in vertebrates..

Wnts have also been found to play important roles in NMJ formation in vertebrates (Fig 1.4), consistent with the idea that key synaptogenic roles of Wnts are conserved across evolutionary time. One of the key events in NMJ formation is the clustering of postsynaptic acetylcholine receptors (AChRs), which is already known to depend on the HSPG Agrin, secreted from neurons, acting upon its postsynaptic receptor MuSK to activate Rac1 and RhoA (Gautam et al., 1996; Weston et al., 2003; Weston et al., 2000). In the chick limb bud, Wnt3 is expressed by motorneurons as they innervate the developing limb muscles (Henriquez et al., 2008; Krylova et al., 2002). Transplantation of Wnt3-expressing cells into the developing wing increases AChR clustering during this period, whereas cells expressing sFRP1 decrease clustering, suggesting Wnts also play a role in AChR recruitment at the developing NMJ. Consistently, Wnt3 added to cultured myotubes enhances Agrin-dependent AChR clustering by promoting the formation of micro-clusters, which are then converted to larger clusters by Agrin. The formation of these microclusters by Wnt3 is dependent upon activation of Rac1 (Henriquez et al., 2008). Therefore in the chick Wnt3 co-operates with Agrin to promote efficient clustering of AChRs. Additionally, *Dvl1* knockout mice display a defect in the distribution of AChR clusters in the diaphragm (Henriquez et al., 2008), and Wnt11r acts through MuSK to regulate AChR pre-patterning in zebrafish (Jing et al., 2009), suggesting Wnt signalling regulates NMJ formation in a range of vertebrates. Another group has reported that Wnt3a acting through canonical signalling can *suppress* AChR clustering at the mouse NMJ by reducing the expression of the AChR-binding protein

Rapsyn (Wang and Luo, 2008; Wang et al., 2008). Therefore, Wnt3 and Wnt3a may promote simultaneous positive and negative effects on AChR clustering through separate pathways, which could be important for the subcellular localisation of AChRs.

1.4.1.2 Wnts and synaptogenesis in the cerebellum

The synaptogenic activity of Wnts was first discovered in cultured mouse cerebellar granule cell (GC) neurons, in which Wnt7a, in addition to inducing axonal branching, spreading and growth cone enlargement, also promoted clustering of the presynaptic protein Synapsin-1 (Lucas and Salinas, 1997). The effects of Wnt7a on GCs were mimicked by the GSK3β inhibitor lithium, suggesting that in vertebrates, as in invertebrates, Wnts act through the canonical pathway to regulate presynaptic differentiation. In a follow-up study, Wnt7a and lithium were found to have the same effect on mossy fibre axons growing from explants of the pontine nuclei, which *in vivo* form large terminal synapses with several GC dendrites at synaptic structures termed 'glomerular rosettes' (Hall et al., 2000). Furthermore, GCs express Wnt7a *in vivo* around the period in which mossy fibres are reaching their GC targets, and conditioned medium from GC cultures mimics the effects of Wnt7a on axonal remodelling and Synapsin-1 clustering. Conversely, P8 mutant mice lacking *wnt7a* displayed less complex glomerular rosettes with reduced levels of Synapsin-1 staining (Hall et al., 2000). Therefore, in the cerebellum, Wnt7a released by GCs acts as a retrograde presynaptic organiser, instructing incoming mossy fibres to form elaborate axon terminals and clustering key presynaptic proteins (Fig 1.4).

A later study found that Wnt7b is also capable of promoting the clustering of several presynaptic proteins in pontine explants, including Synapsin-1, VAMP2, Bassoon and SV2 (Ahmad-Annuar et al., 2006). This study also provided several pieces of evidence that Dvl1 is required for the presynaptic organising effects of Wnt7a/b in mossy fibres. Firstly, the density of presynaptic sites is reduced in pontine explants cultured from *Dvl1-/-* mutant mice, and these cells have reduced ability to respond to Wnt7a/b. Secondly, the decrease in Synapsin-1 clustering at glomerular rosettes is enhanced in double *Wnt7a-/-;Dvl1-/-* mutants compared to single *Wnt7a-/-* or *Dvl1-/-* mutants (Ahmad-Annuar et al., 2006). Therefore, in the cerebellum, Wnt7a acts through Dvl1 and the canonical pathway to promote presynaptic development.

Interestingly, Wnt7b added to cultured pontine neurons can cluster presynaptic proteins without a detectable change in β-catenin levels (Ahmad-Annuar et al., 2006), suggesting that, as is seen at the *Drosophila* NMJ, the presynaptic organising effect of Wnt in the CNS acts through a divergent canonical pathway – however, this requires further investigation (but see below). Although the effect of Wnts in presynaptic differentiation in the cerebellum is now well established, it is unknown whether Wnts play any role in postsynaptic development in this brain region. Finally, since GCs release Wnt7a *in vivo* and Wnt7a promotes axon remodelling and Synapsin-1 clustering in cultured GCs, it would be interesting to see if Wnt7a acts in an autocrine fashion to control the formation of synapses between GCs and their postsynaptic targets, such as Purkinje cells.

1.4.1.3 Wnts and synaptogenesis in the hippocampus

The study described in the previous paragraph (Ahmad-Annuar et al., 2006) was also the first to demonstrate a synaptogenic role for Wnts in the hippocampus. Cultured hippocampal neurons were transfected with Dvl1, which localised to and increased the density of presynaptic sites, as assessed by immunostaining for Synapsin-1 and Bassoon (Ahmad-Annuar et al., 2006). Since then, several Wnts (Wnt3a, Wnt7a and Wnt7b) have been shown to induce clustering of presynaptic proteins such as vGlut1 and Synaptophysin in cultured hippocampal neurons (Cerpa et al., 2008; Davis et al., 2008; Farias et al., 2009) (Ciani et al, submitted for publication). Wnt-mediated clustering of presynaptic proteins in hippocampal cultures is mimicked by lithium (Davis et al., 2008), blocked by Dkk1 and unaffected by transcriptional inhibitors (Dickins & Salinas, unpublished results), suggesting that this process is again under the control of a divergent canonical pathway.

Wnt7a/b is also important in activity-dependent synaptogenesis in relatively mature animals. 3 month old mice housed for 3 weeks under conditions of environmental enrichment show increases in Wnt7a/b expression in the hippocampal CA2-CA3 region and an increase in the size and number of bassoon puncta at GC large mossy fibre terminals (LMTs) onto CA3 dendrites. These changes are mimicked by Wnt7a and blocked by sFRP1 injected directly into the hippocampus *in vivo* (Gogolla et al., 2009). Therefore, in response to environmental enrichment, Wnt7a/b is released by CA3 neurons and acts retrogradely to promote terminal remodelling and presynaptic protein

clustering in contacting mossy fibres. This is strikingly similar to the actions of Wnt7a in the cerebellum during early postnatal development (Ahmad-Annuar et al., 2006; Hall et al., 2000) (Fig 1.4), suggesting that the same molecules and mechanisms that act to initially wire up the brain can be re-used for plasticity-related processes in the mature nervous system.

As in the cerebellum, investigations into the synaptogenic role of Wnts in the hippocampus have focused on presynaptic development. To date, the only published work on postsynaptic effects of Wnts in the hippocampus is a study reporting that Wnt5a promotes PSD-95 clustering in cultured hippocampal neurons via the PCP pathway (Farias et al., 2009). This raises the intriguing possibility that in the vertebrate CNS, as in the invertebrate NMJ, Wnts signal bidirectionally through distinct signalling pathways to co-ordinate pre- and postsynaptic development. As so little is currently known about the role of Wnts in postsynaptic development in the CNS, this is a key question which warrants further investigation.

1.4.2 Wnts in synaptic function and plasticity

At the NMJ, defects in synaptic function have been reported in mutant *Drosophila* larvae which lack *wnt5* (Liebl et al., 2008). However, the functional defects reported (reduced evoked EPSC amplitude and mEPSC frequency) could potentially be explained by the structural defects observed (reduced bouton and active zone number). Interestingly though, *drl* mutant larvae also exhibit reduced bouton number, but display normal synaptic function (Liebl et al., 2008). As Drl is expressed postsynaptically, this raises the possibility that Wnt5 may act presynaptically to regulate glutamate release at the *Drosophila* NMJ. mEPSC frequency is also reduced at cerebellar GCs of double *wnt7a;dvl1* knockout mice. Although Synapsin-1 clustering is reduced at MF-GC synapses of these animals, the number and ultrastructure of these synapses (as determined by electron microscopy) is largely normal, suggesting Wnt-Dvl signalling may play a role in glutamate release at this synapse (Ahmad-Annuar et al., 2006). As of yet, however, no-one has presented direct evidence that endogenous Wnts play a role in regulating neurotransmission. The mechanisms by which Wnts may regulate release also remain unclear. Research in this area will be guided by the knowledge that Dvl binds to several key proteins in the synaptic vesicle pathway, including Synaptotagmin

1, SNARE proteins, voltage-gated calcium channels and the endocytic mediator AP-2 (Kishida et al., 2007) (Ciani, Sahores & Salinas, unpublished results).

The capability of Wnts to regulate neuronal morphology, synapse formation and possibly synaptic function makes them ideal candidates for regulators of synaptic plasticity. To date however, very little research has been performed in this area. In hippocampal neurons, activity-dependent transcription of Wnt2 results in an increase in dendritic branching (Wayman et al., 2006), though it is unclear at this time what effect this has on synaptic formation and activity in these neurons. The functional consequence of the Wnt-dependent increase seen in mossy fibre-CA3 synapses in response to environmental enrichment (Gogolla et al., 2009) (discussed above) is also unclear. Finally, NMDA-dependent Wnt3a release has been shown to be involved in LTP at perforate path-GC synapses of hippocampal slices (Chen et al., 2006). The role of Wnts in synaptic plasticity is a new field that promises to produce many exciting results over the coming years.

In conclusion, it can be seen that Wnts have emerged over the past decade as versatile players in the life of a synapse, regulating various processes all the way from axon guidance and target recognition, through synapse formation and on to synaptic function and plasticity. Despite this, Wnt signalling at synapses is a young field, and many key questions remain unanswered. One limitation of research to date is that it has focused on excitatory synapses, or has not discriminated between different synaptic subtypes. Therefore, a crucial unanswered question is: do Wnts regulate inhibitory synapses as well? Also, research in the CNS has focused upon the presynaptic actions of Wnts. Do Wnts regulate postsynaptic form and function in the CNS, as they do in the PNS? Finally, the role of Wnts in regulating synaptic function and plasticity remains poorly characterised. Does endogenous Wnt signalling play a role in regulating neurotransmission, and if so, at which synapses?

1.5 Aims

My aim was to address the above questions by performing complementary cell biological and electrophysiological experiments in rodent cultured hippocampal neurons and acute hippocampal slices. More specifically, I wished to investigate:

a) Whether Wnts regulate the formation of both glutamatergic and GABAergic synapses
b) Whether Wnt signalling regulates postsynaptic formation, and how this relates to presynaptic innervation
c) Whether endogenous Wnt signalling regulates glutamatergic and GABAergic synaptic function
d) Whether acute and long-term alterations in Wnt signalling elicit different responses at hippocampal synapses

CHAPTER 2:

Methods and Materials

2.1 Hippocampal neuronal cell culture

All cultures were prepared from E18 Sprague-Dawley rat embryos using a modified version of the method first described by Banker and Cowan (Banker and Cowan, 1977). Pregnant females were killed by CO_2 overdose followed by cervical dislocation and the embryos were removed. The hippocampi were then carefully dissected from the embryonic brains in ice cold Hank's balanced salt solution (HBSS). Once dissected, the hippocampi were dissociated by 18 minutes incubation in 0.5% trypsin (diluted in HBSS) at 37°C, followed by trituration in plating medium (see section 2.6.1 for details of all solutions) through flame-polished glass Pasteurs. The density of the resulting single cell suspension was determined using a haemocytometer, and the cells were plated onto sterile acid-cleaned 13mm coverslips coated with poly-L-lysine (1μg/ml in borate buffer). Cells were initially plated in plating medium, which was replaced with serum-free culture medium after 2-4 hours. Cultures were treated with 5μM cytosine arabinoside (Sigma) at 3-5 DIV to halt the proliferation of glia. All cultures were maintained at 37°C in 95% O_2/5% CO_2 and received partial replacement of the culture medium with fresh medium once per week. Cultures were plated at 50 cells/mm^2 for recording evoked synaptic currents, 200 cells/mm^2 for recording mPSCs and for Lipofectamine transfection, and 100 cells/mm^2 for all other imaging experiments, unless otherwise stated.

2.2 Hippocampal culture transfection

Hippocampal cultures were transfected at 8 DIV with EGFP-actin and DVl1-HA constructs using Lipofectamine 2000 (Invitrogen). As a control, sister cultures were transfected with EGFP-actin and the empty vector used to express Dvl1-HA (PSC2+). For each P60 culture dish transfected (containing 10 coverslips), 2μl of Lipofectamine reagent was incubated for 5 minutes in 200μl of Opti-MEM (Invitrogen). This mixture was then added dropwise to another 200μl of Opti-MEM containing the DNA (5μg EGFP-actin + 3μg Dvl1-HA, or 5μg EGFP-actin + 3μg PCS2+ for control) and incubated for a further 30 minutes. The medium was removed from the culture dish,

retained at 37°C, and replaced with fresh medium. The Lipofectamine/DNA mixture was then added dropwise to the culture dish, which was subsequently placed in a 37°C incubator for 45 minutes. The cells were then washed twice in PBS and the old culture medium was returned to the culture dish, which was then placed back into the incubator. EGFP-actin was under control of the relatively weak SV40 promoter, and EGFP-transfected cultures were further labelled with an anti-GFP primary antibody followed by an Alexa-488-conjugated sencondary antibody. This amplification step allows bright labelling of actin in the cell body, neurites and dendritic spines with relatively low expression of EGFP-actin that does not appear to alter the morphology or function of neurons ((Fischer et al., 1998; Star et al., 2002) and unpublished observations).

2.3 Immunofluorescence, image acquisition and analyses

2.3.1 Immunofluorescence

Coverslips were fixed for 5 minutes in -20°C methanol (if PSD-95 antibodies were used) or for 18 minutes in 4% paraformaldehyde at room temperature. Cells were permeablised with 0.02% Triton, blocked with 5% bovine serum albumin (BSA) and then incubated with primary antibodies (diluted in 1% BSA) overnight at 4°C. Following three washes in PBS, the coverslips were incubated with fluorescence-conjugated secondary antibodies for one hour at room temperature. The coverslips were then washed again in PBS and mounted on glass coverslips using Fluoromount-G (Southern Biotech). All primary antibodies used are commercially available, and staining was compared to previously published studies suing these antibodies for similar purposes (see section 2.6.2 for a full list of all antibodies used and relevant references). All antibodies to pre- and postsynaptic proteins labelled punctuate structures along the neurites of cultured neurons, and co-labelling with pre- and postsynaptic antibodies resulted in apposed pre-and postsynaptic puncta, as expected. All secondary antibodies used are again from commercial sources, and their specificity was tested by labelling cultures in the absence of the primary antibody. For double- and triple-labelling experiments, coverslips were labelled with each primary antibody seperatley followed by the secondary antibodies for the other primary antibodies used. All secondary antibodies were found to be highly specific.

2.3.2 Image acquisition and analysis of synaptic puncta

Images were captured on an Olympus BX60 upright microscope using a 40x oil-immersion objective (Plan Apochromat, NA=1) (Figs 3.2, 3.4 and 3.5) or on a Leica TCS SP1 confocal microscope using a 63x oil-immersion objective (Plan Apochromat, NA=1.32) (all other fluorescent images). Final pixel sizes of images are as follows: Olympus BX60 – 1024x1280 at 161 nm/pixel; Leica TCS SP1 – 1024x1024 at 155 nm/pixel. Each confocal z-section was the average of 3 scans and the z-section interval was 0.3 μm (optimal step-size calculated by the software based on the Nyquist Theorem). Confocal scan head settings were altered between experiments to obtain optimal signal and contrast, but were kept constant between conditions within a given experiment. Each imaging experiment was performed at least 3 times on independent cultures, and imaging and analysis was peformed blind to the experimental conditions. 8-12 images were taken per condition and analysed using Volocity (Improvision). Objects of interest (puncta of synaptic proteins or Tuj-1 labelled neurites) were delineated using intensity thresholding. Thresholds were set visually for each experiment using images from control conditions. Once threshold values were chosen for each channel, the same thresholds were applied for all images from all conditions for a given experiment. Thresholded puncta were passed thorugh size filters to remove objects too small or too large to be considered synaptic puncta (Olympus BX60 - >0.1 and <xx μm^2; Leica TCS SP1 >0.1 and <10 μm^3). Puncta were then subjected to the 'Separate Touching Objects' function, based on the mean puntum size, and the minimum size filter was re-applied. Co-localisation or apposition of synaptic puncta was determined using custom-built protocols in Volocity.

2.3.3 Dendritic spine analysis

Images of EGFP-transfected spines were captured on a Leica TCS SP1 confocal microscope as described above. Spine analysis was performed manually using Volocity. For each image of EGFP-actin-transfected cells, 2-3 regions of interest containing ~50-100μm of dendrite each were cropped from maximum projections, with only the EGFP channel visible. The number of spines was then counted and the maximum head width of each spine determined by visual placement of a line tool on the maximally projected image of the EGFP-actin channel. Finally, the channels containing synaptic markers were switched back on and the number of spines containing PSD-95, vGlut and multiple

PSD-95 and/or vGlut puncta were counted. The 3D visualisation tool was used to confirm synaptic puncta were in the same focal plane as spines. For each condition, approximately 1000 spines were analysed in total from all three repeats of each experiment. 3D-rendered images were produced in Imaris (Bitplane).

2.4 Electrophysiology

2.4.1 Mouse breeding and genotyping

C57Bl/6J *Dvl-1* null mice were obtained from heterozygous crosses. All mutant mice were maintained on a C57BL/6 background. *Wnt7a; Dvl1* double mutant mice were obtained from crosses of *Wnt7a-/+*; *Dvl1-/-* mutant mice. Control mice were age-matched *wild-type* C57BL/6 animals. Genotypes were determined by three-primer PCR using ear clipping. For Wnt7a, the primers used were forward, 5′-T T C T C T T C G C T G G T A C T C T GG G T G -3′, reverse, 5`-C A G C G C T G A G C A G T T C C A A C G G -3′, and the Neo primer 5`-A G G C C T A C C C G C T T C C A T T G C T C A -3′. For Dvl1, the primers used were forward 5`-T C T G C C C A A T T C C A C C T G C T T C T T -3′, reverse 5`-C G C C G C C G A T C C C C T C T C -3′, and the Neo primer 5`-A G G C C T A C C C G C T T C C A T T G C T C A -3′.

2.4.2 Acute slice preparation

Slices were prepared from P10-14 mice for recording miniature postsynaptic currents and P18-23 mice for recording evoked postsynaptic currents. Mice were deeply anaesthetised using isoflurane before decapitation and removal of the brain into ice cold slicing solution. The cerebellum was removed and discarded, the hemispheres were divided and a thin slice (~1mm) of tissue was removed from the dorsal surface of each cortex. The resulting flat surface was used to glue the hemispheres onto the base of the slicing chamber using cyanoacrylate glue. The slicing chamber was then flooded with ice cold slicing solution bubbled with 95% O_2/5% CO_2. 300μm transverse slices were made using a Dosaka DTK 1000 tissue slicer at the level of the hippocampus. The hippocampal slices were then dissected from the surrounding tissue and stored initially in 34°C recording solution (supplemented with 4mM $MgCl_2$) continuously bubbled with 95% O_2/5% CO_2. This solution was then allowed to cool to room temperature, and the slices were allowed to recover for 1 hour before commencing recording.

2.4.3 Recording of postsynaptic currents

Coverslips or slices were placed in a chamber on an upright microscope and continuously perfused at room temperature with recording solution bubbled with 95% O_2/5% CO_2. Cells were patched in the whole cell voltage-clamp configuration using microelectrodes (resistance 5 – 8 MΩ) pulled from borosilicate glass (Harvard GC150F-7.5) and filled with sodium gluconate pipette solution. When recording miniature currents, 100nM TTX was included in the recording solution. Miniature or evoked EPSCs were recorded at -60mV in the presence of 10µM bicuculline and 1mM Mg^{2+}, whereas IPSCs were recorded at 0mV in the presence of 10µM CNQX and 50µM AP-5.

Evoked postsynaptic currents were recorded using the method described by Maximov and colleagues (Maximov et al., 2007). Briefly, currents were elicited using a bipolar concentric electrode (FHC) attached to a Grass S48 stimulator to depolarise axons close to the patched cell. For paired-pulse recordings, the stimulus intensity was altered from cell to cell to give the minimum reproducible response. For input-output analysis, stimuli were delivered at 3, 6, 9, 12 and 15V. 10mM QX-314 was included in the pipette solution to block action potential firing in the patched cell. In hippocampal slices the stimulating electrode was placed in the stratum radiatum approximately 100-200µm from the whole-cell patched neuron in the CA1 layer. In hippocampal cultures large cells with a pyramidal morphology were patched and the stimulating electrode was place within approximately 100-200µm of the patched cell. Paired-pulse stimuli were delivered at a rate of 0.2 Hz with an inter-stimulus interval of 50ms for EPSCs, and an interval of 100ms for IPSCs. Stimuli for input-output analysis were delivered at a rate of 0.1 Hz. All currents were recorded using an Axopatch 200A amplifier, filtered at 1 kHz and digitised onto computer at 10 kHz using WinEDR software.

2.4.4 Analysis of electrophysiological recordings

2.4.4.1 Analysis of miniature currents

mEPSCs and mIPSCs were analysed using a combination of WinEDR and WinWCP (freely available at http://spider.science.strath.ac.uk/sipbs/software_ses.htm). Currents were detected using the 'Template' function, based on the algorithm developed by Clements & Bekkers (1997). Briefly, an ideal waveform template is slid point-by point

along the recording and constantly scaled to obtain the optimal fit with the data. A detection threshold is set, which is based upon the quality of the fit. Therefore, lowering the threshold will allow detection of events which deviate further from the template (Clements and Bekkers, 1997). Since this method requires the experimenter to set the template and threshold, considerable effort was spent in choosing and testing these parameters. The parameters used to describe the template are rise time and decay time. Therefore, for a number of mEPSC and mIPSC recordings, events of >20pA were detected using the 'Threshold' detection function (which detects events based purely on an amplitude threshold) and confirmed visually. These events were then used to choose appropriate values for the template, based upon averages of synaptic currents and histograms of rise and decay times from a number of cells. The threshold of detection was chosen by running the template on recordings from several cells, and determining a threshold that detected events with a minimum of false positives and missed events (as determined by visual confirmation). A threshold was eventually chosen that resulted in slight over-detection, followed by removal of false positives by a series of filters. The values for these filters were again chosen based on histograms of visually confirmed events. I tested my detection protocols by: 1) running the mEPSC template on mIPSC recordings, and vice versa, 2) running the mEPSC template with a positive threshold, or the mIPSC template with a negative threshold and 3) running both templates on recordings in which both mEPSCs and mIPSCs were blocked. Under these conditions, a negligible amount of false positives were detected. By using these templates, mPSCs could be detected independently of human bias, and therefore experiments were not blinded. The details of the templates, thresholds and filters used for detection are given in Table 2.1 below:

	Template		**Threshold**	**Filters**			
	Rise time	Decay time		Rise time	Decay time	Area	Peak
mEPSCs	1ms	5ms	-8	<5ms	>1ms, <15ms	-ve	-ve
mIPSCs	1ms	25ms	+4	<10ms	>5ms, <100ms	+ve	+ve

Table 2.1 Parameters used to detect mEPSC and mIPSCs.

Detected events were exported to WinWCP. The events were averaged for each cell, and the amplitude of the average was determined using the 'Waveform Measurement' function. The rise and decay times of the average for each cell were determined using

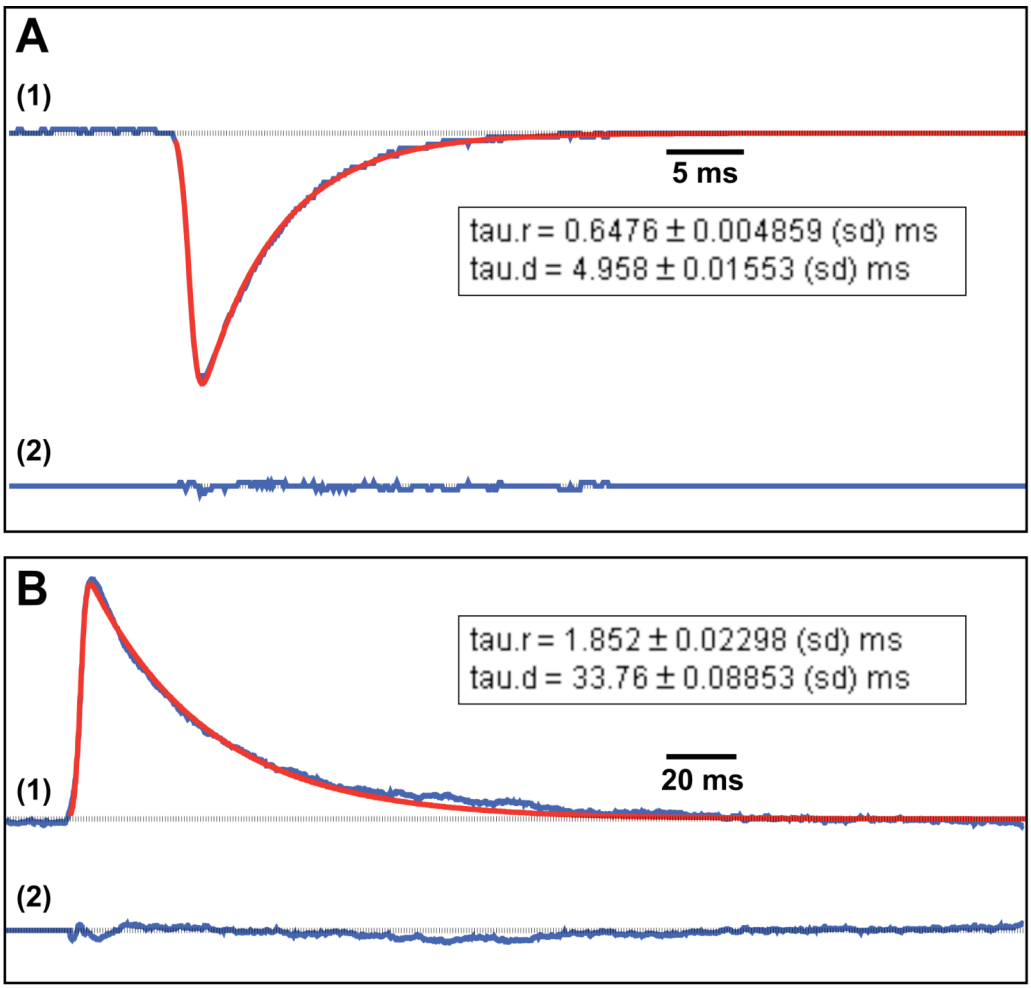

Figure 2.1 Examples of curve fitting using the 'EPC' function in WinWCP. Examples are given of curve fitting of the average mEPSC (A) and mIPSC (B) from representative cells. The upper traces in each panel (1) show the average response in blue with the overlaid fit in red. The insets show the tau rise and tau decay determined by the fit. The lower traces (2) sow the deviation of the actual data from the fit (deviation of the blue line from the dashed line).

the 'Fit Curves' function. The 'EPC' fit was used, which simulate an endplate current with a rising phase determined by a Gaussian function and an exponential decay (Fig 2.1). The quality of the space clamp was investigated for several cells by plotting the mEPSC rise time against amplitude (Fig 2.2).

2.4.4.2 Analysis of paired currents

Evoked currents were detected in WinEDR using the 'Rate of Rise' function, which easily and reliably detected the stimulus artefact preceding the current. Detected events

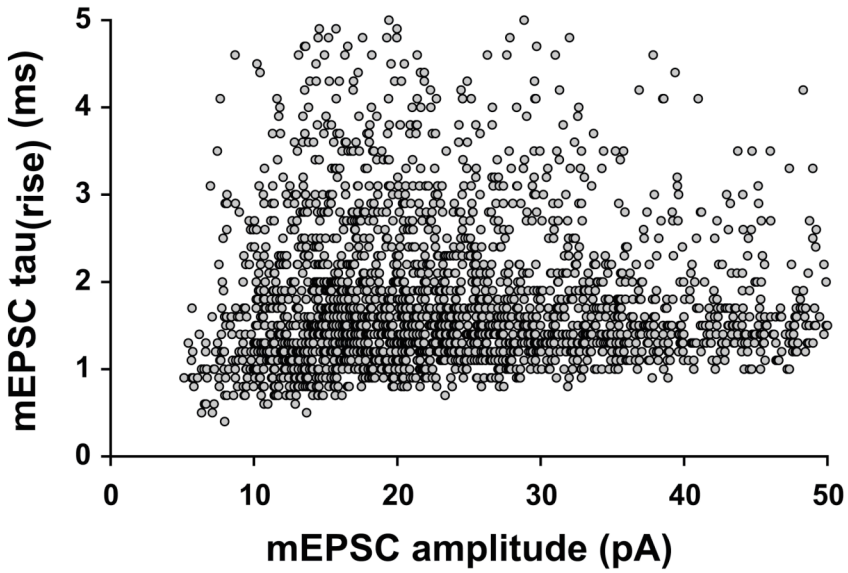

Figure 2.2 Patched pyramidal cells display good space clamp. mEPSC rise time vs. amplitude from a representative 21 DIV cultured pyramidal neuron. No correlation is seen between the two parameters, indicating good space clamp. 2591 events were plotted for this cell. Similar graphs were plotted for cells from each mPSC experiment, with similar results.

were exported to WinWCP and current amplitude was analysed as for miniature currents. The paired pulse ratio was calculated by dividing the peak amplitude of the second response by the peak amplitude of the first.

2.4.4.3 Input-output analysis

Evoked currents were detected in WinEDR using the 'Rate of Rise' function and exported to WinWCP. For each stimulus intensity, the average of 3-5 events was used to analyse the mean current amplitude, rise time and decay time as for miniature currents.

2.5 Statistical analysis

Statistical analysis was performed using a combination on InStat (GraphPad) and Excel (Microsoft). Normality of data was assessed using the Kolmogorov-Smirnov Test. For normally distributed data, the two-tailed Students t test was used when comparing two conditions, and ANOVA was used when making multiple comparisons. For non-normally distributed data, the Mann-Whitney test was used when comparing two conditions, whereas the Kruskal-Wallis test with Dunn post-test was used for multiple

comparisons. Statistical significance is denoted in all figures as follows: * = P<0.05, ** = P<0.01, *** = P<0.001.

2.6 Solutions and antibodies used

2.6.1 Solutions

Hippocampal plating medium – 50 ml

Neurobasal medium (Gibco)	45 ml
1 mM sodium pyruvate (Sigma)	5.5 mg
2 mM L-glutamine (Sigma)	14.5 mg
Horse serum (Gibco)	5 mls
Penicillin/streptomycin (final 20 μg/ml, Gibco)	200 μl

Hippocampal culture medium – 50 ml

Neurobasal medium (Gibco)	48.5 ml
1 mM sodium pyruvate (Sigma)	5.5 mg
2 mM L-glutamine (Sigma)	14.5 mg
B27 supplement (Invitrogen)	1 ml
N2 supplement (Invitrogen)	0.5 ml
Penicillin/streptomycin (final 20 μg/ml, Gibco)	200 μl

Borate buffer – 400 ml

50 mM boric acid (Sigma)	1.24 g
25 mM borax (Sigma)	1.9 g
Sterile filtered water	400 ml
pH to 8.5 with NaOH	

4% paraformaldehyde – 50 ml

4% paraformaldehyde (BDH)	2 g
4% sucrose (Sigma)	2 g
0.1 mM NaOH (BDH)	200 μl (of 25 mM)
2x PBS	25 ml
Distilled water	25 ml

Slicing solution – 500 ml

75 mM NaCl (Sigma)	2.19 g
25 mM $NaHCO_3$ (Sigma)	1.1 g
2.5 mM KCl (Fluka)	0.1 g
1.25 mM NaH_2PO_4 (Sigma)	0.1 g
100 mM sucrose (Sigma)	17 g
0.1 mM kynurenic acid (Sigma)	0.02 g
2 mM pyruvic acid (Sigma)	0.11 g
Distilled water	500 ml
1 mM $CaCl_2$ (Fluka)	0.5 ml (of 1 M)
4 mM $MgCl_2$ (Fluka)	2 ml (of 1 M)

Extracellular solution – 500 ml

125 mM NaCl	3.65 g
25 mM $NaHCO_3$ (Sigma)	1.1 g
2.5 mM KCl (Fluka)	0.1 g
1.25 mM NaH_2PO_4 (Sigma)	0.1 g
25 mM Glucose (Sigma)	2.25 g
1 mM $CaCl_2$ (Fluka)	0.5 ml (of 1 M)
1 mM $MgCl_2$ (Fluka)	0.5 ml (of 1 M)

Pipette solution – 100 ml

139 mM D-gluconic acid lactone (Sigma)	2.47 g
10 mM HEPES (Sigma)	0.24 g
10 mM EGTA (Sigma)	0.19 g
10 mM NaCl (Sigma)	0.02 g
0.5 mM $CaCl_2$ (Fluka)	50 μl (of 1M)
1 mM $MgCl_2$ (Fluka)	100 μl (of 1M)
1 mM ATP (Sigma)	0.1 g
1 mM GTP (Sigma)	0.1 g
pH to 7.4 with CsOH	

2.6.2 Antibodies

PRIMARY ANTIBODIES

Antibody (Supplier, catalogue #)	Dilution	Reference(s)
Chicken anti-Tuj-1 (Chemicon, AB9354)	1:1000	(Wu et al., 2006)
Mouse anti-PSD-95 (Affinity Bioreagents, MA1-046)	1:200	(Colledge et al., 2003)
Guinea pig anti-vGlut1 (Chemicon, AB5905)	1:5000	(Goddard et al., 2007; Hartman et al., 2006)
Rabbit anti-vGAT (Synaptic Systems, 131003)	1:1000	(Hartman et al., 2006)
Mouse anti-gephyrin (Synaptic Systems, 147011)	1:1000	(Hartman et al., 2006; Schneider Gasser et al., 2006)
Mouse anti-VAMP2 (Synaptic Systems, 104211)	1:1000	(Luthi et al., 2001)
Rat anti-HA (Roché, 1867423)	1:1000	(Gebhart et al.)
Chicken anti-GFP (Upstate, 06-896)	1:500	(Collins et al.)

SECONDARY ANTIBODIES

Antibody (Supplier, catalogue #)	Conjugate	Max exciation/ emission (nm)	Dilution
Goat anti-chicken IgG (Molecular Probes, A21449)	Alexa-647	650/665	1:600
Donkey anti-chicken IgG (Jackson Immunoresearch, 703-485-155)	DyLight-488	493/518	1:600
Goat anti-guinea pig IgG (Molecular Probes, A11073)	Alexa-488	495/519	1:600
Goat anti-guinea pig IgG (Molecular Probes, A11075)	Alexa-568	578/603	1:600
Donkey anti-mouse IgG (Molecular Probes, A21202)	Alexa-488	495/519	1:600
Donkey anti-mouse IgG (Molecular Probes, A10037)	Alexa-568	578/603	1:600
Donkey anti-mouse IgG (Molecular Probes, A31571)	Alexa-647	650/665	1:600
Donkey anti-rabbit IgG (Molecular Probes, A21207)	Alexa-594	590/617	1:600
Goat anti-rat IgG (Molecular Probes, A21247)	Alexa-647	650/665	1:600

CHAPTER 3:

Wnt7a promotes excitatory, but not inhibitory, pre- and postsynaptic differentiation in hippocampal cultures

3.1 Introduction

Wnt proteins are essential in regulating nervous system development in the embryo (Ciani and Salinas, 2005). However Wnts and Wnt signalling related proteins continue to be expressed in the CNS long after birth (Davis et al., 2008; Gogolla et al., 2009; Lucas and Salinas, 1997; Shimogori et al., 2004), suggesting they play a role in postnatal development of the nervous system, even into adulthood. The first demonstration that Wnts act as synaptogenic factors in the CNS was performed in cerebellar granule cell cultures, in which Wnt7a induced axonal remodelling and clustering of the presynaptic protein synapsin-1 (Lucas and Salinas, 1997). Later studies showed that pontine mossy fibres also displayed axonal remodelling and presynaptic protein clustering (synapsin-1, VAMP2, SV2 and bassoon) in response to Wnt7a or Wnt7b in a Dvl1-dependent manner. Furthermore, this occurs *in vivo*, where mossy fibre terminal remodelling and presynaptic differentiation is induced by Wnt7a released by cerebellar granule cells, the postsynaptic target of mossy fibres (Ahmad-Annuar et al., 2006; Hall et al., 2000; Lucas and Salinas, 1997). Interestingly, a similar mechanism seems to be employed in the hippocampus. Environmental enrichment promotes Wnt7a/b release from CA3 pyramidal cells, which signals retrogradely to induce remodelling and presynaptic protein clustering in contacting mossy fibre terminals (Gogolla et al., 2009). This suggests that Wnts play a general role in synaptogenesis throughout different areas of the CNS, including the hippocampus.

Several studies have provided evidence for a pro-synaptic role of Wnts in the hippocampus; Wnt7a, Wnt7b, and Wnt3a have all been shown to increase presynaptic protein clustering in hippocampal pyramidal neurons (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Davis et al., 2008; Gogolla et al., 2009). Despite these recent studies, the role of Wnt signalling in regulating central synaptogenesis remains poorly understood, and key questions remain to be answered. For example at the Drosophila neuromuscular junction (NMJ), where the role of the Wnt homolog Wingless (Wg) is well characterised, Wg acts to promote both pre- and postsynaptic differentiation

(Packard et al., 2002). Wnt signalling is also involved in postsynaptic development at the vertebrate NMJ, acting through the PCP pathway to promote clustering of acetylcholine receptors on muscle fibres (Henriquez et al., 2008; Jing et al., 2009). To date only one study has investigated the postsynaptic role of Wnts in the CNS, reporting an increase in clustering of the postsynaptic scaffold protein PSD-95 in response to Wnt5a in hippocampal cultures (Farias et al., 2009). Therefore the question of whether Wnts directly regulate postsynaptic development remains largely unanswered. Additionally, existing studies on the role of Wnt signalling in synaptogenesis focus on excitatory synapses, or utilise markers that do not discriminate between subtypes of synapse. Therefore it remains an open question as to whether Wnts affect all synapses equally, or whether they show some degree of specificity. For example, do Wnts promote the formation of both excitatory glutamatergic and inhibitory GABAergic synapses? This is an important question, as these two types of synapse account for the majority of vertebrate CNS synapses, and disruptions in the balance between the two have been implicated in a number of neurological disorders including autism (Munoz-Yunta et al., 2008; Rubenstein and Merzenich, 2003), epilepsy (Leite et al., 2005), schizophrenia (Kehrer et al., 2008) and Rett syndrome (Medrihan et al., 2008). Finally, although Wnts have classically been described as prosynaptogenic factors, there is emerging evidence that they can also have the opposite effect. The most convincing evidence for this comes from a study in *C. Elegans*, where the Wnt homologs Lin-44 and Egl-20 act through Lin-17 (frizzled) and Dvl-1 to exclude the formation of presynaptic specialisations in a segment of the DA9 motor neuron (Klassen and Shen, 2007). More recently, it has been claimed that Wnt5a decreases vGlut clustering in hippocampal cultures via a non-canonical pathway (Davis et al., 2008). However our understanding of which Wnts are actively involved in hippocampal synaptogenesis, and whether they mediate a pro- or antisynaptogenic effect remains poorly characterised.

In an attempt to address the questions raised above, the effects of gain and loss of Wnt signalling on excitatory and inhibitory synapses were assessed in 14 DIV hippocampal cultures, as at this stage a considerable degree of synaptogenesis is occurring. For gain of function studies, I focused on Wnt7a, which has previously been shown to promote presynaptic clustering in both cerebellum and hippocampus (Ahmad-Annuar et al., 2006; Davis et al., 2008; Hall et al., 2000; Lucas and Salinas, 1997). Specifically, I aimed to answer the following questions. Firstly, does Wnt7a also affect postsynaptic protein clustering, and how does this relate to the presynaptic effect? Secondly, does

Wnt7a affect both excitatory and inhibitory synapses, or does it display specificity? I found that treatment with purified Wnt7a increases synaptic density by promoting co-ordinated pre- and postsynaptic differentiation. This effect is restricted to excitatory synapses; GABAergic synapses are unaffected. The majority of existing studies on Wnt signalling in hippocampal synaptogenesis have utilised treatment with exogenous Wnts. Therefore I also addressed the following question; what is the effect of blocking endogenous Wnt signalling on excitatory and inhibitory synapse formation in hippocampal cultures? Surprisingly, treatment with the Wnt antagonist secreted frizzled-related protein (sFRP) does not affect the number of either excitatory or inhibitory synapses. However, electrophysiological recordings reveal a decrease in the frequency of mEPSCs, whereas mIPSCs are unaffected. Together, these results suggest that in the hippocampus Wnt signalling can regulate excitatory synapses through both the formation of pre- and postsynaptic specialisations and control of presynaptic glutamate release. Furthermore, this regulation is specific to excitatory synapses as inhibitory synapses are unaffected by perturbations of Wnt signalling. Therefore, Wnts may play a critical role in determining the balance between excitatory and inhibitory signalling in the hippocampus.

3.2 Results

3.2.1 Wnt7a promotes the formation of excitatory pre- and postsynaptic sites

Wnt7a and Wnt7b have previously been shown to induce clustering of the general presynaptic marker Bassoon and the excitatory presynaptic marker vGlut1 in 10-12 DIV hippocampal cultures (Ahmad-Annuar et al., 2006; Davis et al., 2008). To investigate how this relates to postsynaptic development, hippocampal cultures were treated with purified Wnt7a from 13-14 DIV and immunostained for excitatory pre- and postsynaptic markers (vGlut1 and PSD-95, respectively; Fig 3.1). Cultures were also stained with the neuronal cytoskeletal protein Tuj-1 to allow visualisation of neuronal morphology and normalisation of synaptic number to the density of processes. I found that Wnt7a treatment results in a 24% increase in the density of vGlut puncta (Fig 3.1A and B). Importantly, Wnt7a also induces a similar increase in the density of PSD-95 puncta (a 29% increase compared to vehicle treated cultures; Fig 3.1A and C). The size of both vGlut1 and PSD-95 puncta are unchanged by Wnt7a treatment (vGlut1: 0.56 ± 0.02μm^3 (vehicle) and 0.5 ± 0.02μm^3 (Wnt7a); PSD-95: 0.36 ± 0.01μm^3 (vehicle)

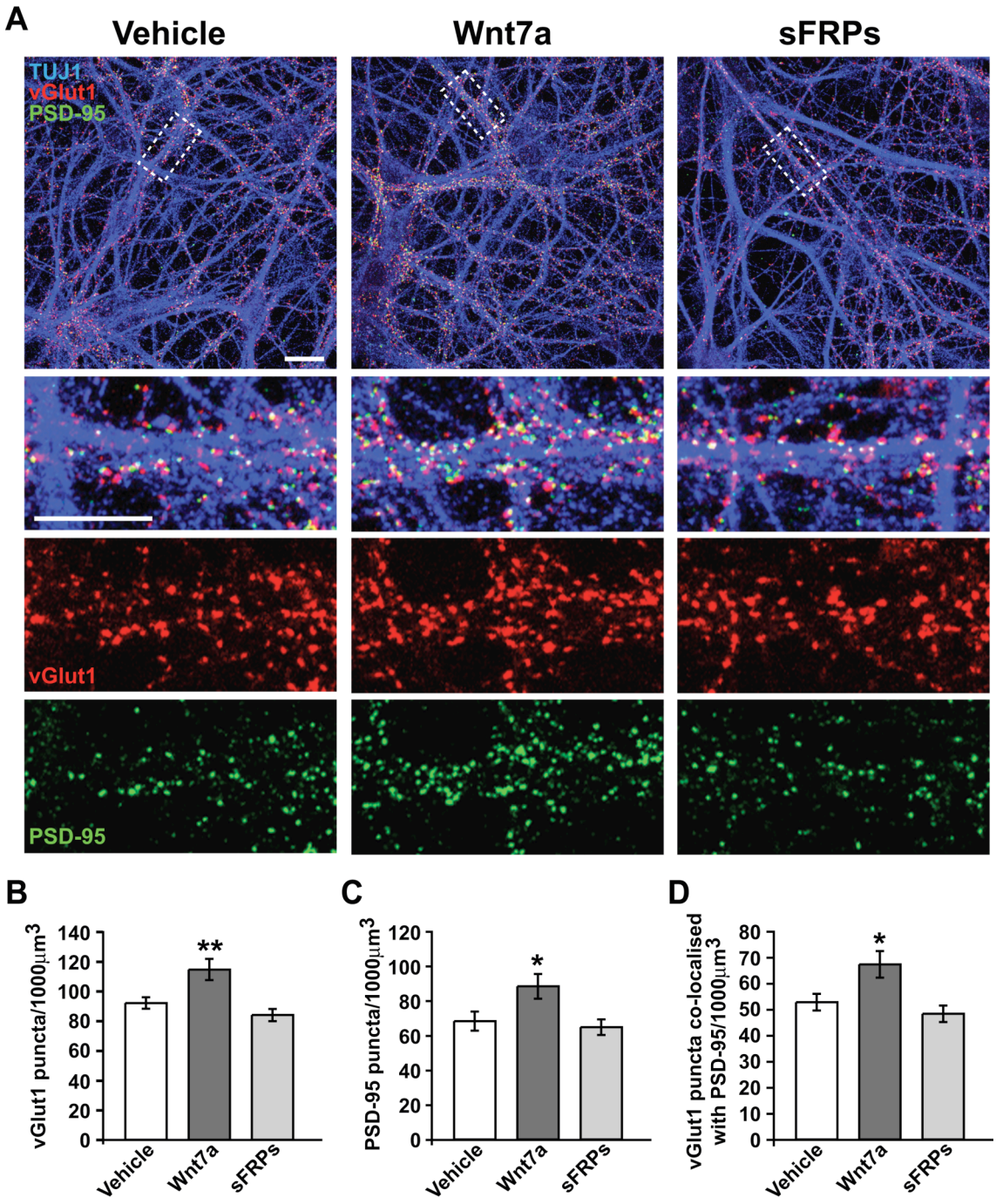

Figure 3.1 Purified Wnt7a can increase excitatory synapse number; however blockade of endogenous Wnt signalling with sFRP treatment does not affect excitatory synapse number. 14 DIV hippocampal cultures were treated with purified Wnt7a, a cocktail of sFRP1, 2 and 3 or vehicle for 20 hours. (A) Images of treated neurons show that exogenous Wnt7a increases the puncta density of the excitatory presynaptic marker vGlut1 (red) and postsynaptic marker PSD-95 (green), as normalised to the volume of the cytoskeletal marker TUJ1 (blue). However blockade of endogenous Wnt signalling with the sFRP cocktail does not affect these markers. White boxes indicate enlarged regions. Scale bars = 20μm in top panels, 10μm in enlarged panels. (B) and (C) Quantification shows that Wnt7a induces a 24% and 29% increase in vGlut1 (B) and PSD-95 (C) puncta density respectively. (D) Importantly, Wnt7a induces a 28% increase in the density of vGlut puncta that co-localise with PSD-95 (putative excitatory synapses). * = $P<0.05$, ** = $P<0.01$.

and 0.35 ± 0.01μm^3 (Wnt7a)). Where a presynaptic vGlut1 puncta is found apposed to a postsynaptic PSD-95 puncta, this is defined as an excitatory synapse. As the proportion of vGlut1 puncta apposed to PSD-95 puncta and vice versa remains constant between vehicle and Wnt7a treated cultures (vGlut1 co-localisation with PSD-95: 56.8 ± 2.1% (vehicle) and 57.5 ± 2.4% (Wnt7a); PSD-95 co-localisation with vGlut1: 79.4 ± 1.7% (vehicle) and 74.3 ± 1.6% (Wnt7a)), an increase in the density of excitatory synapses is observed in response to Wnt7a (28% increase compared to vehicle treated cultures; Fig 3.1 A and D). Therefore Wnt7a promotes co-ordinated pre- and postsynaptic differentiation in hippocampal cultures, leading to an increase in excitatory synapse number.

The effect of blocking endogenous Wnt signalling on excitatory synapse formation was also tested by treating with the Wnt antagonist secreted frizzled-related protein (sFRP), which blocks Wnt signalling by binding to and sequestering Wnts (Chien et al., 2009; Wang et al., 1997a). As several Wnts appear to be expressed by hippocampal neurons (Davis et al., 2008; Gogolla et al., 2009; Shimogori et al., 2004) which may differ in their affinity for any single sFRP, a cocktail of three sFRPs (sFRP1, 2 and 3; 'sFRP cocktail' or 'sFRPs') was utilised. Surprisingly, sFRP treatment does not affect the density of either vGlut1 or PSD-95 puncta in hippocampal cultures (Fig 3.1A, B and C). Co-localisation of vGlut1 and PSD-95 puncta is also unaffected (vGlut1 co-localisation with PSD-95: 56.8 ± 2.1% (vehicle) and 56.8 ± 2.1% (sFRPs); PSD-95 co-localisation with vGlut1: 79.4 ± 1.7% (vehicle) and 74.9 ± 1.6% (sFRPs)) and so the density of excitatory synapses remains the same between vehicle and sFRP treated cultures (Fig 3.1A and D). The size of PSD-95 puncta is unchanged by sFRP cocktail treatment (0.36 ± 0.01μm^3 (vehicle) and 0.35 ± 0.01μm^3 (sFRPs)); there is however a small (18%) but significant reduction in the size of vGlut1 puncta (0.56 ± 0.02μm^3 (vehicle) and 0.46 ± 0.01μm^3 (sFRPs); $p<0.01$ by ANOVA).

Blockade of endogenous Wnt signalling via the canonical pathway antagonist Dickkopf-1 (Dkk-1) decreases excitatory pre- and postsynaptic markers in hippocampal cultures (Dickins and Salinas, unpublished observations). Therefore the fact that sFRP cocktail does not have a similar effect is surprising, and could raise concerns about the activity of the purified sFRPs used. Co-treatment of Wnt7b and sFRP1 in hippocampal cultures results in sFRP1 abrogating Wnt7b-mediated clustering of bassoon or dendritogenesis (Ahmad-Annuar et al., 2006; Rosso et al., 2005). I therefore decided to

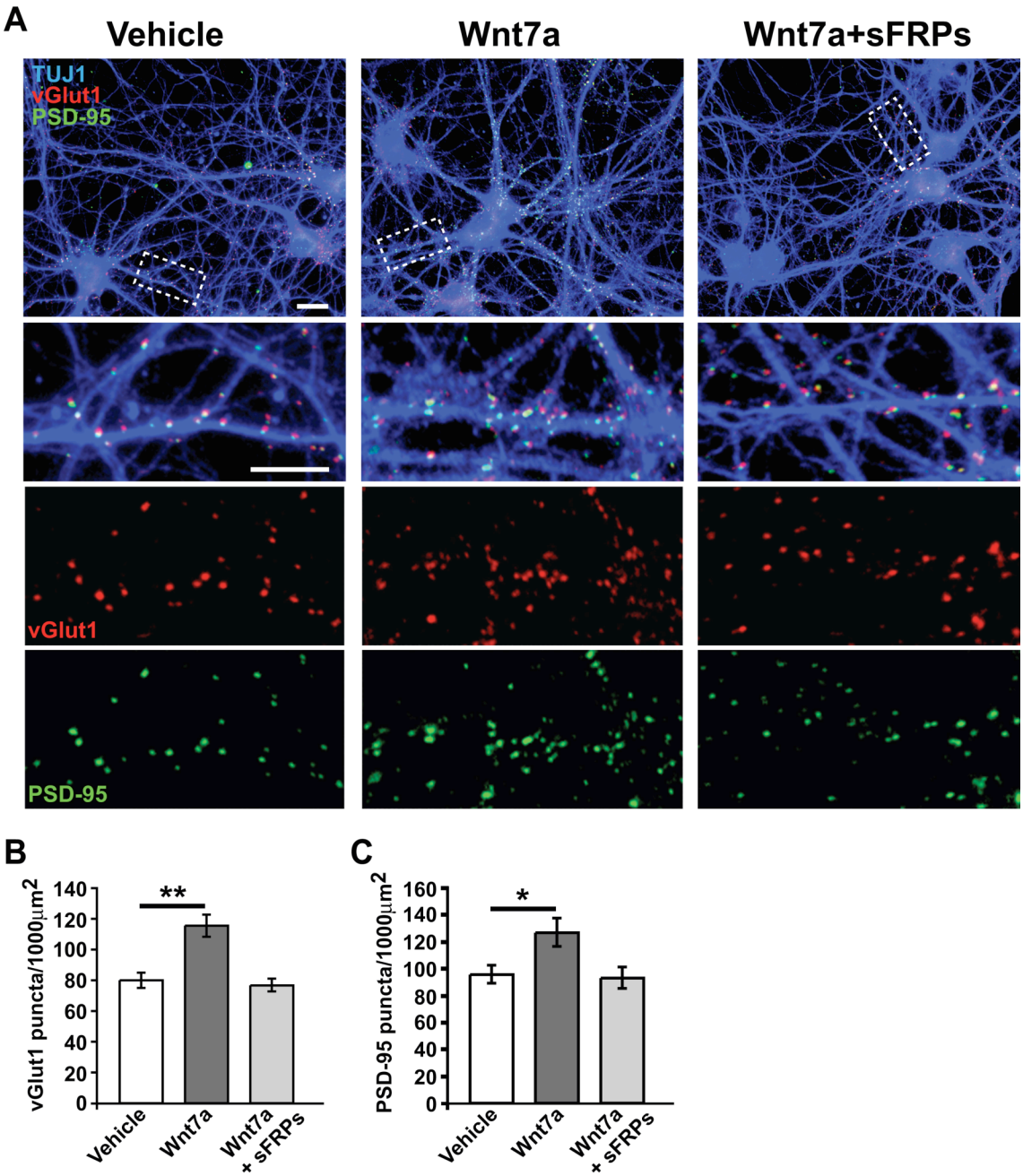

Figure 3.2 sFRP cocktail can block the Wnt7a-induced increase in excitatory synapse number. 14 DIV hippocampal cultures were treated with purified Wnt7a, Wnt7a plus a cocktail of sFRP1, 2 and 3 or vehicle for 20 hours. (A) Images of neurons immunostained for vGlut1 (red), PSD-95 (green) and TUJ1 (blue). Wnt7a treatment again results in an increase in both excitatory synaptic markers. However, this increase is blocked by co-treatment with the sFRP cocktail. White boxes indicate enlarged regions. Scale bars = 20μm in top panels, 10μm in enlarged panels. (B) and (C) Quantification shows that Wnt7a increases vGlut 1 (B) and PSD-95 (C) puncta density by a similar degree as shown in Figure 3.2 (44% and 33% increase in vGlut1 and PSD-95 puncta, respectively), and that this increase is completely abolished by co-treatment with sFRP cocktail. * = $P<0.05$, ** = $P<0.01$.

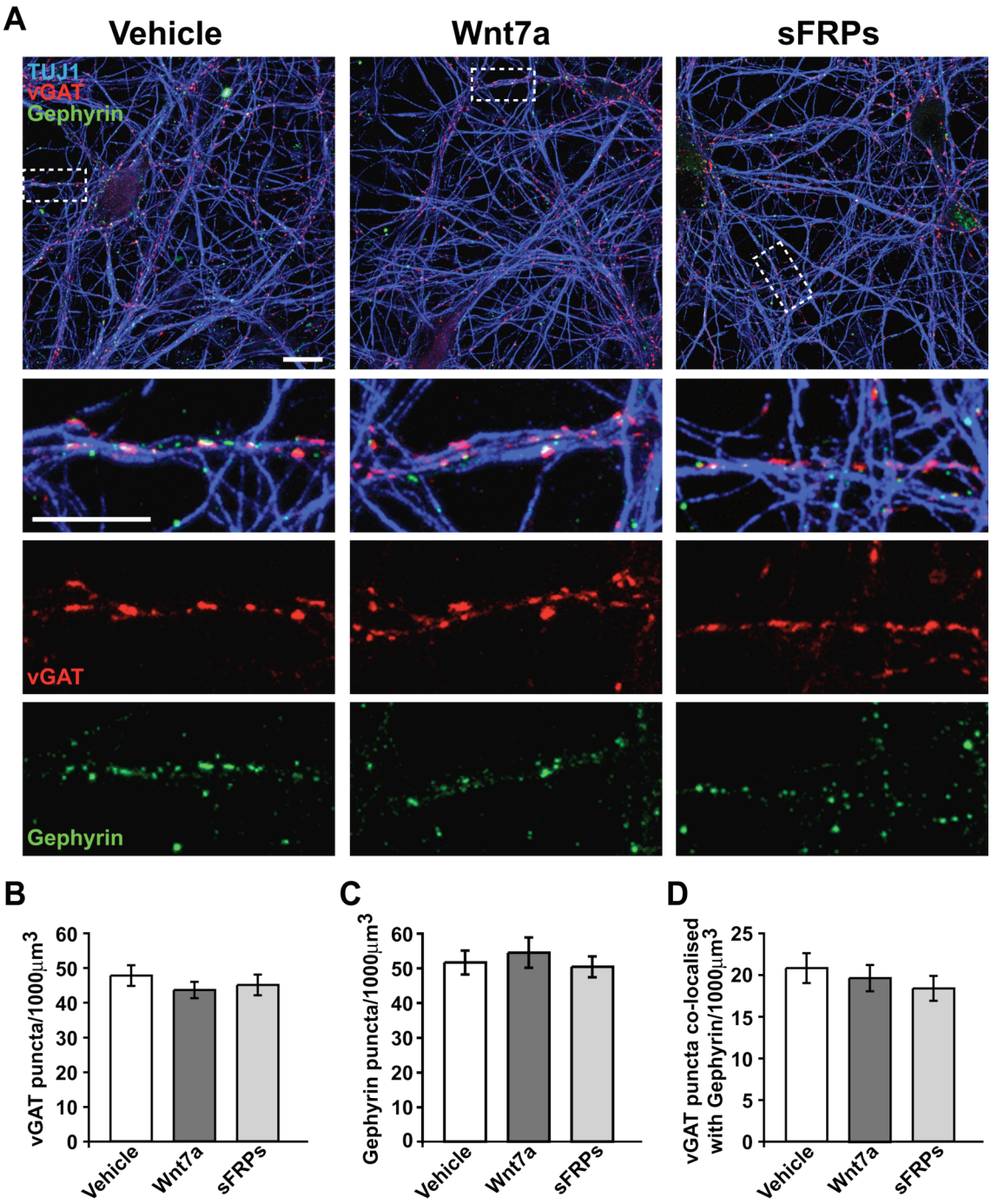

Figure 3.3 Inhibitory synapse number is unaffected by treatment with purified Wnt7a or sFRP cocktail. 14 DIV hippocampal cultures were treated with purified Wnt7a, a cocktail of sFRP1, 2 and 3 or vehicle for 20 hours. (A) Images of treated neurons immunostained for the inhibitory presynaptic marker vGAT (red) and postsynaptic marker Gephyrin (green), and TUJ1 (blue). The puncta density of both markers is unaffected by Wnt7a or sFRP treatment. White boxes indicate enlarged regions. Scale bars = 20μm in top panels, 10μm in enlarged panels. (B) and (C) Quantification shows no effect of Wnt7a or sFRP on vGAT (B) or Gephyrin (C) puncta density. (D) Quantification shows that the density of vGAT puncta that co-localise with Gephyrin (putative inhibitory synapses) is unaffected by Wnt7a or sFRP treatment.

test whether sFRP cocktail can block the Wnt7a-mediated increase in excitatory pre- and postsynaptic markers described in Figure 3.1. Cultures were treated with Wnt7a or Wnt7a plus sFRP cocktail from 13-14 DIV and immunostained for vGlut1 and PSD-95. Wnt7a again increases vGlut and PSD-95 puncta density to a degree similar to that described in Figure 3.1 (44% and 33% increase over vehicle treated cultures for vGlut1 and PSD-95, respectively; Fig 3.2A, B and C). However co-incubating Wnt7a with the sFRP cocktail completely blocks this increase, and Wnt7a plus sFRP treated cultures are indistinguishable from vehicle treated cultures (Fig 3.2A, B and C). Therefore, the sFRP cocktail is active and able to block Wnt signalling under my experimental conditions.

3.2.2 Wnt7a does not regulate inhibitory synapse number

To date, the role of Wnts in inhibitory synaptogenesis has not been investigated. To address this, hippocampal cultures were treated with purified Wnt7a or sFRP cocktail from 13-14 DIV and immunostained for inhibitory pre- and postsynaptic markers (vGAT and Gephyrin, respectively; Fig 3.3). The puncta density of both vGAT and Gephyrin remain unchanged by Wnt7a or sFRP treatment (Fig 3.3A, B and C). The co-localisation of vGAT with Gephyrin and vice versa are also unchanged in all conditions (vGAT co-localisation with Gephyrin: 42.3 ± 2.4% (vehicle), 44.2 ± 2.4% (Wnt7a) and 41.5 ± 2.5% (sFRPs); Gephyrin co-localisation with vGAT: 40.2 ± 2% (vehicle), 37.8 ± 1.5% (Wnt7a) and 38.2 ± 2.1% (sFRPs)). Therefore the density of inhibitory synapses (vGAT puncta apposed to Gephyrin puncta) remains constant (Fig 3.3A and D). vGAT and Gephyrin puncta size are also unaffected by Wnt7a or sFRP treatment (vGAT: 0.67 ± 0.05μm^3 (vehicle), 0.65 ± 0.06μm^3 (Wnt7a) and 0.63 ± 0.06μm^3 (sFRPs); Gephyrin: 0.41 ± 0.02μm^3 (vehicle), 0.4 ± 0.02μm^3 (Wnt7a) and 0.39 ± 0.03μm^3 (sFRPs)). These results demonstrate that Wnt7a is highly specific in promoting the formation of excitatory, but not inhibitory, synapses in hippocampal cultures.

3.2.3 sFRPs, alone or in combination, do not affect excitatory or inhibitory synapse number in 14 DIV hippocampal cultures

Evidence for an antisynaptogenic action of certain Wnts has been presented both *in vivo* and *in vitro* (Davis et al., 2008; Klassen and Shen, 2007) Therefore it is possible that in the experiments described thus far, treatment with a cocktail of sFRP1, 2 and 3 could be acting to block several endogenous Wnts with opposing pro- and anti-synaptogenic

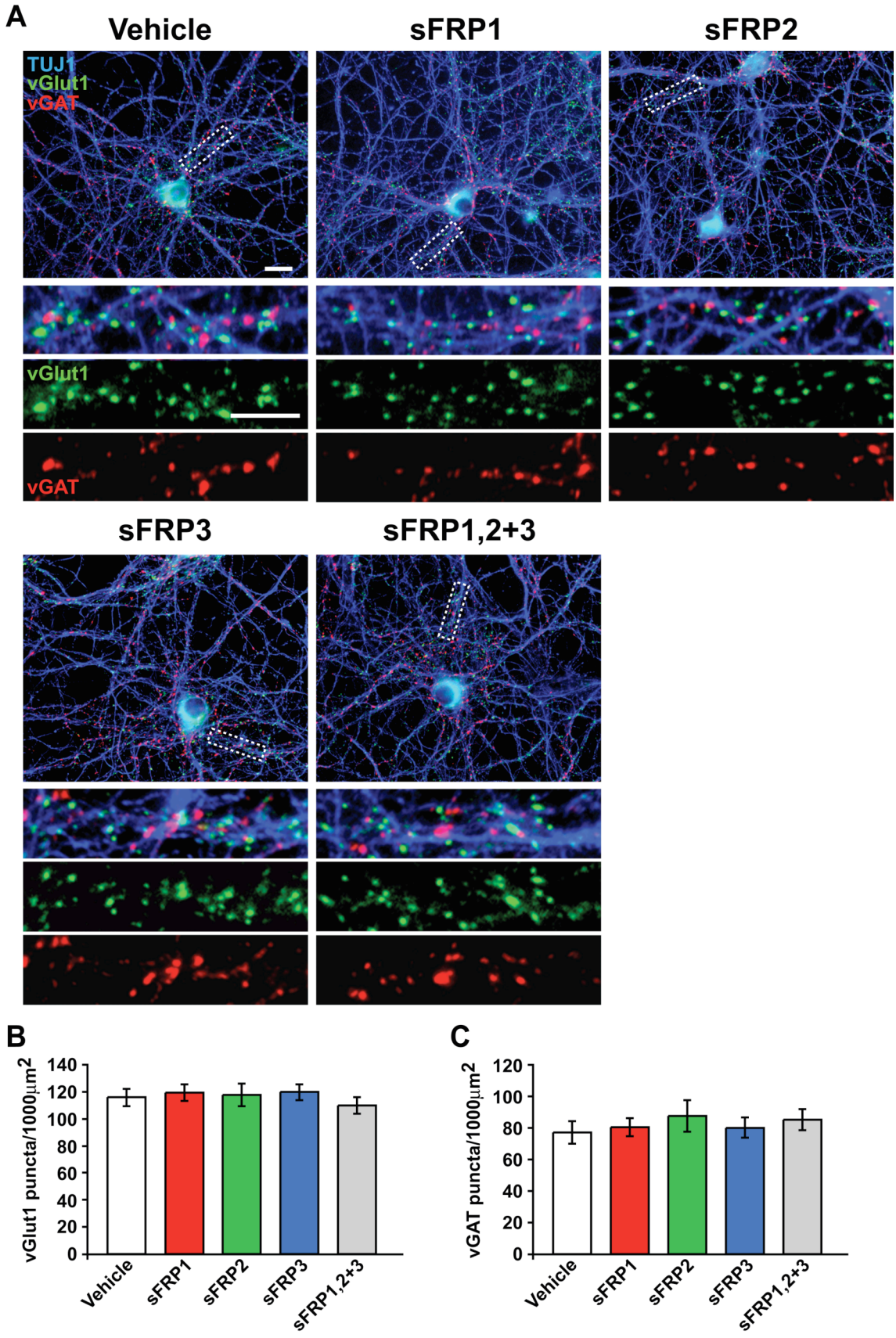

Fig 3.4 (Figure legend on next page)

Figure 3.4 sFRPs, alone or in combination, do not affect excitatory or inhibitory presynaptic markers in 14 DIV hippocampal neurons. Hippocampal cultures were treated with sFRP1, sFRP2, sFRP3, sFRP1, 2 and 3 or vehicle for 20 hours. (A) Images of neurons immunostained for vGlut1 (green), vGAT (red) and TUJ1 (blue). None of the treatments affected the puncta density of either marker, compared to vehicle treated cultures. White boxes indicate enlarged regions. Scale bars = 20μm in top panels, 10μm in enlarged panels. (B) and (C) Quantification shows that the density of vGlut1 (B) and vGAT (C) puncta is unaffected in all conditions.

activities, leading to no net change in synapse number. Furthermore, this could potentially be the case for both excitatory and inhibitory synapses. To address this possibility, 13 DIV hippocampal cultures were treated overnight (20 hours) with sFRP1, sFRP2 or sFRP3 alone or with all three in combination, then fixed at 14 DIV and immunostained for vGlut1 and vGAT (Fig 3.4A). None of these conditions resulted in any changes in the density of vGlut1 or vGAT puncta (Fig 3.4A, B and C), and puncta size was also unaffected (vGlut1: 0.75 ± 0.06μm^2 (vehicle), 0.72 ± 0.05μm^2 (sFRP1), 0.7 ± 0.04μm^2 (sFRP2), 072. ± 0.05μm^2 (sFRP3) and 0.69 ± 0.04μm^2 (sFRP cocktail); vGAT: 0.86 ± 0.05μm^2 (vehicle), 0.9 ± 0.05μm^2 (sFRP1), 0.89 ± 0.05μm^2 (sFRP2), 0.89 ±0 .06μm^2 (sFRP3) and 0.88 ± 0.05μm^2 (sFRP cocktail)). Therefore sFRP-mediated antagonism of Wnt signalling does not affect synapse formation under the experimental conditions used here (but see below).

3.2.4 The pro-synaptogenic effect of sFRP2 depends on neuronal maturity

sFRP2 has previously been reported to increase vGlut1 puncta density in hippocampal cultures (Davis et al., 2008), yet I found that sFRP2 had no effect on vGlut1 puncta (Fig 3.4A and B). However I noticed Davis et al used cultures of a considerably lower density (see Davis et al., 2008, Fig 8). Culture density and maturity are directly related, with denser cultures maturing more quickly (in terms of dendritic and axonal morphology and synapse formation), most likely due to the community effect (Fletcher et al., 1994). This raised the possibility that sFRP2 has a pro-synaptogenic effect in immature cultures that is lost as the cells mature. To test this possibility, hippocampal cultures plated at low density (50 cells/mm^2) or medium density (100 cells/mm^2, the density used in the experiments described in figures 3.1-3.4) were treated with vehicle or sFRP2 from 9-10 DIV before immunostaining with the general presynaptic marker VAMP2. VAMP2 was used in this case as it provides better presynaptic staining than vGlut1 in immature cultures. sFRP2 causes a robust increase in VAMP2 puncta number and a slight increase in puncta size in low density cultures; quantification shows puncta

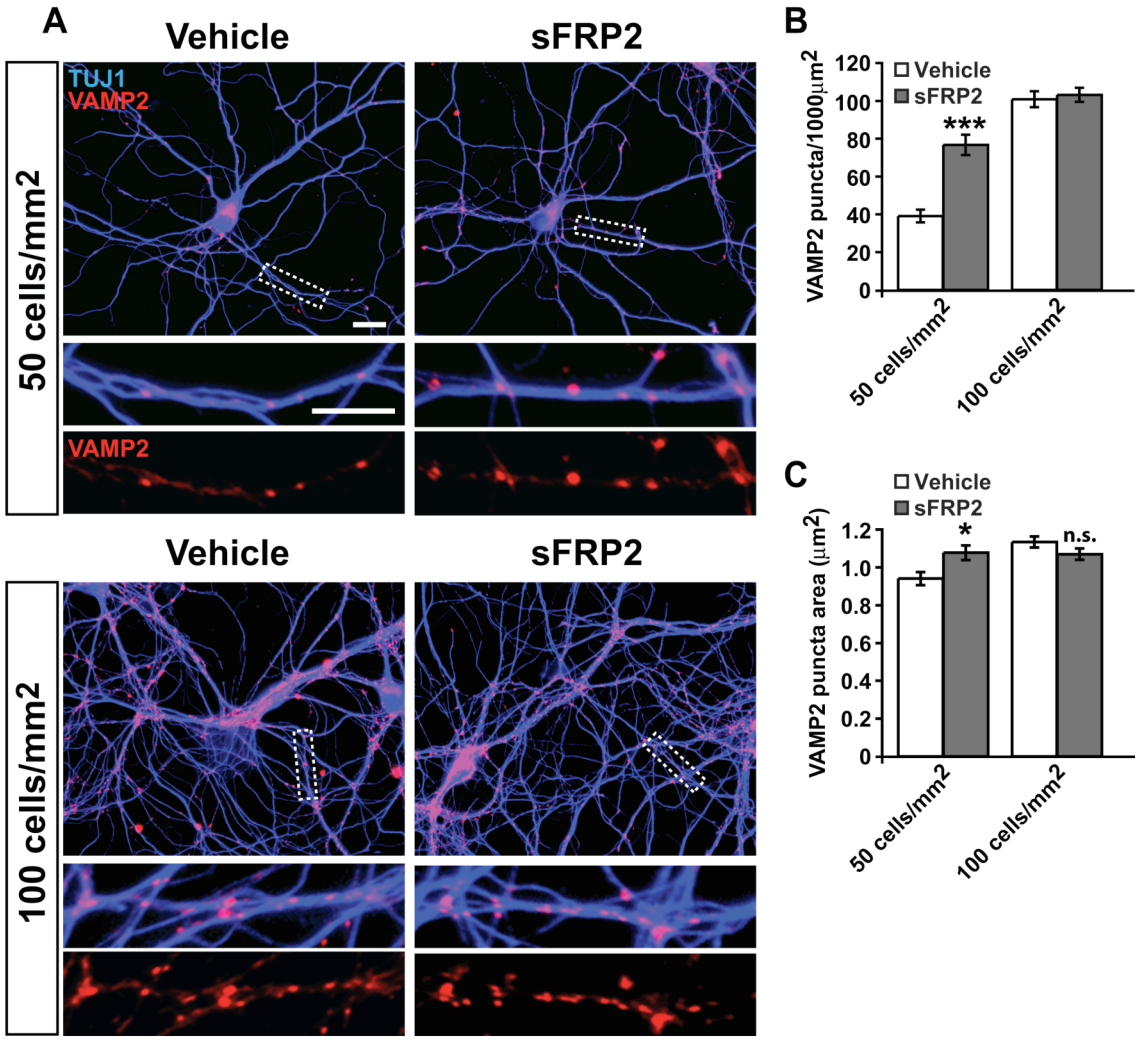

Fig 3.5 sFRP2 is only prosynaptogenic in young, low density cultures. Cultures plated at 50 cells/mm^2 (low density) or 100 cells/mm^2 (medium density) were treated with sFRP2 for 20 hours from 9-10 DIV. (A) Images of cultures immunostained for the presynaptic marker VAMP2 (red) and TUJ1 (blue) demonstrate that sFRP2 induces an increase in VAMP2 puncta density and size in low density cultures, but this effect is not present in medium density cultures. White boxes indicate enlarged regions. Scale bars = 20μm in top panels, 10μm in enlarged panels. (B) Quantification reveals sFRP2 induces a 96% increase in VAMP2 puncta density in low density cultures, whereas puncta density remains unchanged in medium density cultures. (C) Quantification shows that VAMP2 puncta size is increased by 15% by sFRP2 treatment in low density cultures, but this again remains unchanged in medium density cultures. * = P<0.05, *** = P<0.001.

density and area increase by 96% and 15% respectively (Fig3.5A, B and C). Medium density cultures have a higher VAMP2 puncta number and size than their low density counterparts under control conditions (Fig 3.5A-C). However, these cells do not respond to sFRP2, as VAMP2 puncta number and size remain unchanged in medium density cultures exposed to sFRP2, compared to vehicle treated controls (Fig 3.5A-C). This suggests that sFRP2 is only capable of promoting presynaptic differentiation in

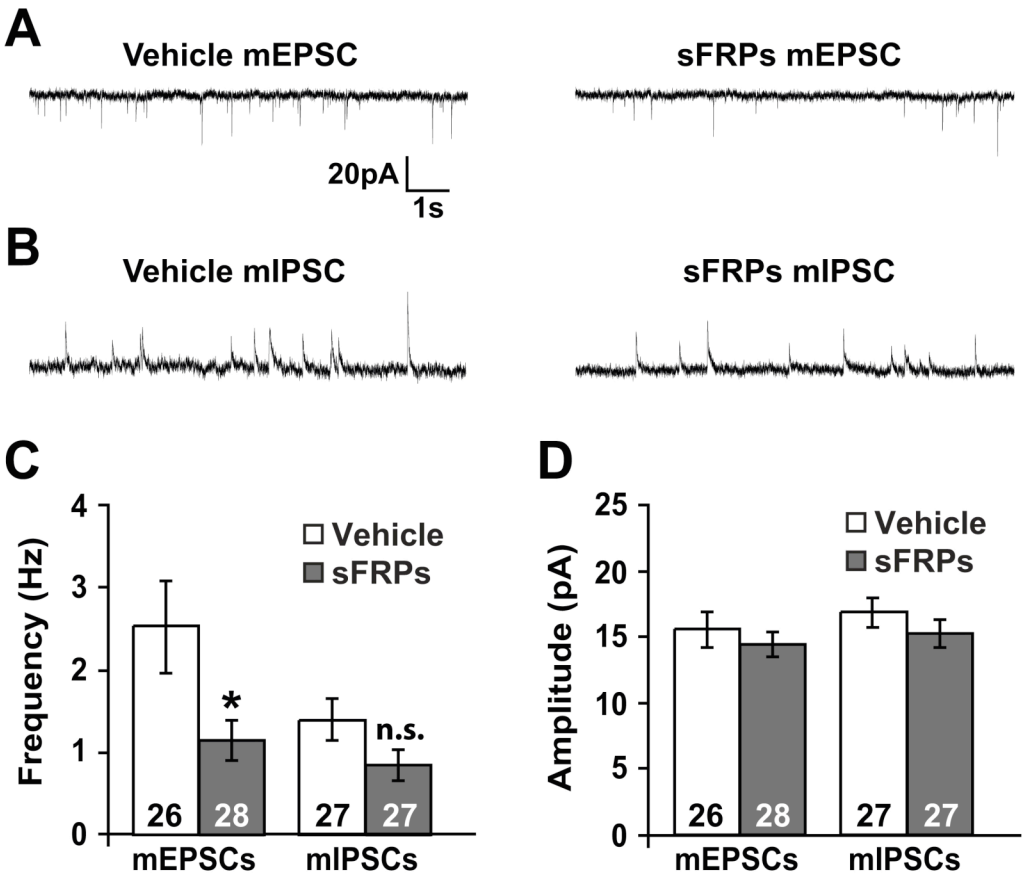

Figure 3.6 Blockade of Wnt signalling by sFRP treatment reduces excitatory, but not inhibitory, miniature transmission in 14 DIV hippocampal neurons. Hippocampal cultures were treated with a cocktail of sFRP1, 2 and 3 or vehicle for 20 hours. Pyramidal neurons were whole cell patch-clamped and mEPSCs and mIPSCs were recorded. (A) A clear reduction in mEPSC frequency is observed in response to sFRPs, while amplitude is unaffected. (B) sFRP treatment does not change either the frequency or amplitude of mIPSCs. (C) Quantification of mean mini frequency reveals a 55% decrease in mEPSC frequency in response to sFRP treatment, whereas mIPSC frequency does not change significantly. (D) Quantification of mean mini amplitude reveals no differences between vehicle and sFRP treatments for both mEPSCs and mIPSCs. The numbers at the base of bars show the number of cells recorded from. * = $P<0.05$.

immature cultures. However, an alternative explanantion is that sFRP2 may be able to promote presynaptic differentiation in cultures plated at low densities, regardless of the age of the culture.

3.2.5 sFRP-mediated blockade of Wnt signalling reduces excitatory, but not inhibitory, quantal synaptic transmission in 14 DIV hippocampal cultures

The experiments described in Figures 3.1 to 3.4 show that blockade of endogenous Wnt signalling with sFRPs does not affect synapse formation in 14 DIV hippocampal cultures. However the possibility remains that blocking endogenous Wnt signalling with sFRPs may affect synaptic function under these culture conditions, as Wnts have been

shown to regulate synaptic transmission as well as synaptic formation (Ahmad-Annuar et al., 2006; Beaumont et al., 2007; Cerpa et al., 2008; Chen et al., 2006; Kishida et al., 2007).

To determine whether sFRPs affect hippocampal synaptic function, I performed whole cell patch-clamp recordings of miniature post-synaptic currents (mPSCs or 'minis') in cultures treated from 13-14 DIV with the sFRP cocktail. Both excitatory AMPA receptor (AMPA-R) and inhibitory GABA receptor (GABA-R) mediated currents were recorded ('mEPSCs' and 'mIPSCs', respectively). Despite the fact that the same treatment does not affect excitatory synapse number (Figs 3.1 and 3.4), the sFRP cocktail significantly reduces mEPSC frequency by 55% compared to control without affecting mEPSC amplitude (Fig 3.6A, C and D), suggesting a decrease in spontaneous presynaptic release of glutamatergic vesicles. Interestingly, mIPSC frequency and amplitude were unaffected by sFRP treatment (Fig3.6B - D). These results demonstrate that blockade of endogenous Wnt signalling reduces synaptic transmission specifically at excitatory synapses.

3.3 Discussion

3.3.1 Wnt7a specifically promotes the formation of excitatory synapses

The results described in this chapter show that in hippocampal cultures Wnt7a promotes an increase in excitatory pre- and post synaptic specialisations, as assessed by antibody staining for vGlut1 and PSD-95. Furthermore, the increase in pre- and postsynaptic sites is co-ordinated, as the percentage co-localisation of the two markers remains constant, leading to an increase in the density of excitatory synapses. Interestingly, this effect was specific to excitatory synapses, as Wnt7a treatment had no effect on inhibitory synapses as assessed by antibody staining for vGAT and gephyrin. Blockade of endogenous Wnt signalling using the secreted Wnt antagonists sFRP1, 2 and 3, alone or in combination, does not affect the number of either excitatory or inhibitory synapses. Despite this, the sFRP cocktail reduced mEPSC frequency, suggesting that by this stage of maturity (14 DIV, medium density) endogenous Wnt signalling is regulating excitatory synaptic transmission rather than number. This effect was again specific to inhibitory synapses, as sFRP treatment had no effect on mIPSC frequency.

As mentioned above, a previous study has reported that Wnt5a induces PSD-95 clustering in hippocampal cultures (Farias et al., 2009). This study, and a previous paper from the same group, also reported that Wnt7a promotes clustering of the presynaptic protein synaptophysin, but not PSD-95 (Cerpa et al., 2008; Farias et al., 2009). This appears to directly contradict the result presented in this chapter that Wnt7a increases PSD-95 clustering in cultures at a similar stage of maturity. The most likely explanation for this apparent contradiction is the duration of Wnt7a treatment – Farias et al treat with Wnt7a for only 2 hours, whereas the experiments presented here utilise 20-24 hour Wnt7a treatment. This suggests that Wnt-induced increases in postsynaptic sites occur after increases in presynaptic sites, raising the possibility that the presynaptic changes may signal to the postsynaptic side in order to co-ordinate alterations in synaptic number. This issue will be examined in more detail in the next chapter.

To my knowledge, the results described in this chapter represent the first investigation into the effect of Wnt signalling on inhibitory synapse development. The results show that Wnt7a very specifically promotes excitatory synapse formation without affecting inhibitory synapses. Furthermore, blockade of endogenous Wnt signalling specifically decreases excitatory miniature synaptic transmission. This suggests that Wnts may selectively regulate excitatory synaptic form and function in the CNS, thereby influencing the balance between central excitatory and inhibitory transmission.

A correct balance between excitatory glutamatergic and inhibitory GABAergic signalling appears crucial for normal brain function; imbalances between the two have been implicated in a range of neurological disorders (Kehrer et al., 2008; Leite et al., 2005; Medrihan et al., 2008; Munoz-Yunta et al., 2008; Rubenstein and Merzenich, 2003). Despite this, relatively little is known about the mechanisms that regulate excitatory and inhibitory synapse formation. Perhaps the best-studied molecules in this regard are the Neurexins and Neuroligins. These two molecules form trans-synaptic adhesion complexes, with the presynaptic Neurexins binding to the postsynaptic Neuroligins (Ichtchenko et al., 1996). Interestingly, when Neuroligin or β-Neurexin are expressed in non-neuronal cells they promote clustering of pre- and postsynaptic molecules, respectively, in contacting neurons (Graf et al., 2004; Scheiffele et al., 2000). Furthermore, there is clear specificity between different isoforms of neuroligin. Neuroligin-1, -3, and -4 localise to glutamatergic sites, whereas neuroligin-2 preferentially localises to GABAergic sites (Graf et al., 2004). Accordingly, neuroligin-

1 and neuroligin-2 knockout mice show defects in excitatory and inhibitory neurotransmission, respectively (Chubykin et al., 2007). Another transynaptic adhesion molecule, SynCAM, has similar properties to neuroligin in that its expression in non-neuronal cells can elicit synapse formation on contacting neurons, but analysis of this effect has been restricted to excitatory synapses (Biederer et al., 2002; Fogel et al., 2007; Sara et al., 2005). Several classes of secreted proteins have also been shown to stimulate synapse formation, including the Wnts (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Davis et al., 2008; Gogolla et al., 2009; Hall et al., 2000), FGFs (Dai and Peng, 1995; Li et al., 2002a; Umemori et al., 2004), BDNF and NT-3 (Vicario-Abejon et al., 1998). The majority of these studies did not differentiate between different subtypes of synapse, or focused solely on excitatory synapses. In one study that did look at both excitatory and inhibitory synapses, in response to BDNF or NT-3, it was found that BDNF promoted the formation of both subtypes, whereas NT-3 specifically promoted excitatory synaptogenesis (Vicario-Abejon et al., 1998). Here I have shown that the secreted factor Wnt7a specifically regulates excitatory synapse formation in hippocampal cultures. Furthermore, blocking endogenous Wnt signalling with Sfrps specifically affects excitatory neurotransmission. These results add Wnt7a to a growing list of molecules implicated in the induction and maintenance of an appropriate balance of excitatory and inhibitory signalling in the CNS.

3.3.2 The effects of blocking Wnts with sFRPs on synapse number depends on culture maturity

Blockade of endogenous Wnt signalling with sFRPs had no effect on either excitatory or inhibitory synapse number in 14 DIV cultures. This was surprising considering the ability of Wnt7a to increase excitatory number, and raised concerns about the activity of the purified sFRPs used. sFRP1 has previously been shown to block both the presynaptic protein clustering activity and dendritogenic activity of Wnt7b in hippocampal neurons (Ahmad-Annuar et al., 2006; Rosso et al., 2005). Accordingly, I tested the ability of the sFRP cocktail to block Wnt7a-induced vGlut1 and PSD-95 clustering. Wnt7a was incapable of increasing puncta density in the presence of the sFRP cocktail. This shows that at least one of the sFRPs present was capable of binding to and antagonising Wnt7a, suggesting that endogenous Wnt7a is either no longer secreted by 14 DIV cultured hippocampal neurons or does not play a significant role in the regulation of synapse number at this stage.

Another possible reason for the inability of sFRP cocktail to reduce synapse number is that the different sFRPs may be blocking different Wnts that have opposing pro- and anti-synaptogenic effects. Therefore I tested whether sFRP1, 2 or 3 could affect vGlut1 or vGAT clustering when added to cultures alone, rather than in combination. None of the sFRPs tested affected either excitatory or inhibitory presynaptic protein clustering, further demonstrating that blocking endogenous Wnt signalling with sFRPs does not appear to affect synapse number in 14 DIV cultures. However it has been previously reported that sFRP2 increases vGlut1 puncta density in 14 DIV hippocampal cultures, and that this may be due to sFRP2 blocking the anti-synaptogenic action of Wnt5a (Davis et al., 2008). As I did not see any effect of sFRP2 on vGlut clustering, an explanation for this discrepancy was required. It was noticed that the cultures presented in Davis et al (2008) appear to be of lower density than those presented in Figure 3.4. The density at which cultures are plated is critical in determining the rate at which they mature; therefore, although the two experiments were performed around 14 DIV, the maturity of the cultures used could differ significantly, which in turn could affect their response to sFRP2. I found that sFRP2 is able to increase VAMP2 puncta number and size in low density cultures at 10 DIV, but this effect is completely lost in higher density cultures of the same age. This suggests the ability of sFRP2 to increase synapse number depends on culture maturity, with more mature cultures unable to respond to sFRP2 (perhaps due to a downregulation of the Wnts which sFRP2 can act upon). Asides from the interdependency of culture density and maturity, culture density alone will have a large impact on synaptogenesis in cultures, as it will effect the concentration of secreted synaptogenic factors within the culture medium. Therefore it would have been prefereable to compare 14 DIV low- and medium-density cultures treated with sFRP2 to the 10 DIV cultures, in order to determine the contribution of both culture density and maturity to the pro-synaptogenic effect of sFRP2. These issues highlight the fact that Wnt-mediated regulation of synaptogenesis is likely to be a dynamic process, with several Wnts playing different roles at different stages.

3.3.3 Blockade of Wnt signalling reduces spontaneous glutamatergic signalling without changing excitatory synaptic density

Although treatment with sFRP cocktail did not affect the density of excitatory synaptic contacts in 14 DIV hippocampal cultures, it was still able to reduce the frequency of AMPA-R-mediated mEPSCs. As miniature postsynaptic currents are caused by the

spontaneous fusion of single vesicles of neurotransmitter at presynaptic terminals (Bekkers and Stevens, 1995; Fatt and Katz, 1952), their frequency depends mainly on two factors – the number of release sites and the probability of a spontaneous fusion event occurring at any given release site. As the number of release sites (i.e. the number of excitatory synapses) appears to remain constant following treatment with sFRP cocktail, this suggests that endogenous Wnt signalling is regulating presynaptic glutamate release in 14 DIV cultures. Interestingly, mIPSC frequency is unaffected, demonstrating that the affect of sFRP treatment on miniature synaptic transmission, like the effect of Wnt7a on synapse number, is specific to excitatory synapses. The role of Wnt signalling in regulating excitatory and inhibitory synaptic transmission will be examined in more detail in Chapter 5.

CHAPTER 4:

Postsynaptic activation of Wnt signalling regulates dendritic spine morphogenesis and excitatory synaptic innervation

4.1 Introduction

In the previous chapter I demonstrated that Wnt7a promotes excitatory synaptogenesis through the co-ordinated formation of pre- and postsynaptic sites. The role of GSK-3β-dependent Wnt signalling in promoting presynaptic terminal formation is already relatively well characterised (Davis et al., 2008; Hall et al., 2000; Lucas and Salinas, 1997; Miech et al., 2008), but very little is known about the role of Wnts in postsynaptic differentiation in the CNS. This presents an interesting question: is the formation of new postsynaptic terminals in response to Wnt7a an indirect result of its presynaptic effects, or can Wnt signalling directly regulate the postsynaptic side of excitatory synapses? This will be the main focus of this chapter.

Although there is a paucity of studies directly investigating the role of Wnts in postsynaptic development in the CNS, previous studies do provide a precedent for such a role. Wnts signal directly to dendrites of hippocampal neurons, promoting dendritic arborisation by signalling through a non-canonical Rac/JNK pathway (Rosso et al., 2005). In addition to this role in early dendritic development, Wnts have also been shown to play a direct role in postsynaptic differentiation at the NMJ of both invertebrates and vertebrates. At the *Drosophila* NMJ the Wnt homolog Wingless (Wg) is secreted by motor neuron terminals and endocytosed into the postsynaptic myocytes. Ultrastructural analyses and localization of key postsynaptic markers demonstrates that *wg* mutant flies have severely disrupted endplate morphology (Packard et al., 2002). Interestingly, there is evidence that this postsynaptic organising function of Wg utilises a novel Wnt signalling pathway in which Wg binds its receptor DFrizzled2 (DFz2), resulting in internalisation and nuclear transport of DFz2 (Ataman et al., 2006; Mathew et al., 2005). At the vertebrate NMJ, Wnt3 is required for normal clustering of the acetylcholine receptor (AChR) at the endplate. Wnt3 acts to promote the formation of microclusters of AChRs, which are then aggregated into larger clusters by another crucial NMJ-organising protein, Agrin (Henriquez et al., 2008). Given the established role of Wnt signalling in postsynaptic development in the peripheral nervous system, it

seems a reasonable conjecture that it could play a similar role in the CNS. Indeed, Wnt5a has recently been shown to promote clustering of the postsynaptic protein PSD-95 in hippocampal cultures (Farias et al., 2009).

I therefore decided to investigate the effects of specifically manipulating postsynaptic Wnt signalling on the formation and function of hippocampal synapses. I have demonstrated in the previous chapter that Wnt7a promotes excitatory synaptogenesis through the clustering of key pre- and postsynaptic proteins, without affecting inhibitory synapses. However, it is possible that the postsynaptic effects observed are an indirect effect of presynaptic changes. Therefore a key question is; does perturbing Wnt signalling only in the postsynaptic compartment regulate postsynaptic development? In the CNS, the majority of excitatory inputs form onto specialised postsynaptic structures known as dendritic spines, which act to regulate the biochemical and electrical influence of individual excitatory synapses on the rest of the neuron (Bourne and Harris, 2008; Spruston, 2008). Therefore a second question is; does postsynaptic Wnt activity play a role in spine formation and/or morphogenesis, and how does this relate to synaptic activity in the postsynaptic cell? Finally, if postsynaptic Wnt signalling does play a role in central synapse development, is this again specific to excitatory synapses?

In answer to these questions, I find that postsynaptic activation of Wnt signalling through Dvl1 overexpression results in an increase in dendritic spine size, and a concomitant increase in excitatory synaptic strength (assessed by measuring mEPSC amplitude). Although spine number is unchanged, postsynaptic development and presynaptic innervation of existing spines is enhanced, resulting in an increased mEPSC frequency. Finally, these effects are again specific to excitatory synapses, as no changes are seen in inhibitory innervation or spontaneous transmission in Dvl1 overexpressing neurons.

4.2 Results

4.2.1 Postsynaptic activation of Wnt signalling enhances both dendritic spine size and excitatory synaptic strength

To study the effects of activating Wnt signalling specifically in the postsynaptic compartment I transfected hippocampal cultures at low efficiency with a construct

encoding HA-tagged Dvl1 (Dvl1-HA), or with empty vector as a control. Cells were co-transfected with EGFP-actin, allowing identification of transfected cells and visualisation of dendritic spines, as these are actin-rich structures. The proportion of EGFP-actin expressing cells that also express Dvl1-HA in co-transfected cultures was assessed using immunostaining with HA antibodies; the co-transfection rate was essentially 100% (data not shown). Transfecting cultures at low efficiency results in relatively few Dvl1 overexpressing cells scattered randomly across the coverslip. As these overexpressing cells are surrounded by non-transfected cells, the vast majority of (if not all) presynaptic inputs onto the Dvl1-HA transfected neurons come from neurons expressing normal levels of Dvl1. This approach allowed me to assess the effects of increasing Wnt signalling activity specifically at the postsynaptic side.

Using this technique, a colleague in the laboratory has already demonstrated that Dvl1 localises to dendritic spines. Furthermore, Dvl1 overexpression from 8-12 DIV results in a dramatic increase in mean spine size (50% larger than in control transfected cultures) without changing spine density (Ciani et al, submitted for publication). I firstly confirmed that I could repeat this result in my own transfected cultures. In good agreement with the previous results, I observed a 47% increase in spine width and no change in spine density in Dvl1-HA overexpressing neurons (Fig 4.1). I then investigated the functional effects of postsynaptic Dvl1 overexpression using electrophysiological methods (Fig 4.2A). Cultures were transfected at 8 DIV and recordings were made at 12 DIV. mEPSCs from Dvl1 overexpressing cells displayed a significant increase in amplitude compared to control transfected cells (Control = 11.6 ± 0.62pA; Dvl1-HA = 15.3 ± 0.77pA; Fig 4.2B, C & F). This is consistent with the spine enlargement observed in the same condition (Fig 4.1), as spine size and synaptic strength are known to be positively correlated (Bourne and Harris, 2008; Carlisle and Kennedy, 2005; Matsuzaki et al., 2004; Yuste and Bonhoeffer, 2001). Surprisingly, mEPSC frequency also increased in Dvl1-HA transfected neurons (Control = 0.82 ± 0.11Hz; Dvl1-HA = 1.59 ± 0.26Hz; Fig 4.2C & E). This effect was unexpected, as spine density does not change in Dvl1 overexpressing cells, yet mEPSC frequency relies partly on excitatory synapse number (see below). mEPSCs from Dvl-HA transfected cells also exhibited a change in their kinetics, as the rise time was significantly quicker than in control cells, though the $tau_{(decay)}$ was unchanged ($tau_{(rise)}$: 1.22 ± 0.06ms (control) and 0.92 ± 0.05ms (Dvl1-HA); $tau_{(decay)}$: 4.34 ± 0.14ms (control) and 4.74 ± 0.17ms (Dvl1-HA); Fig 4.3A, C & D).

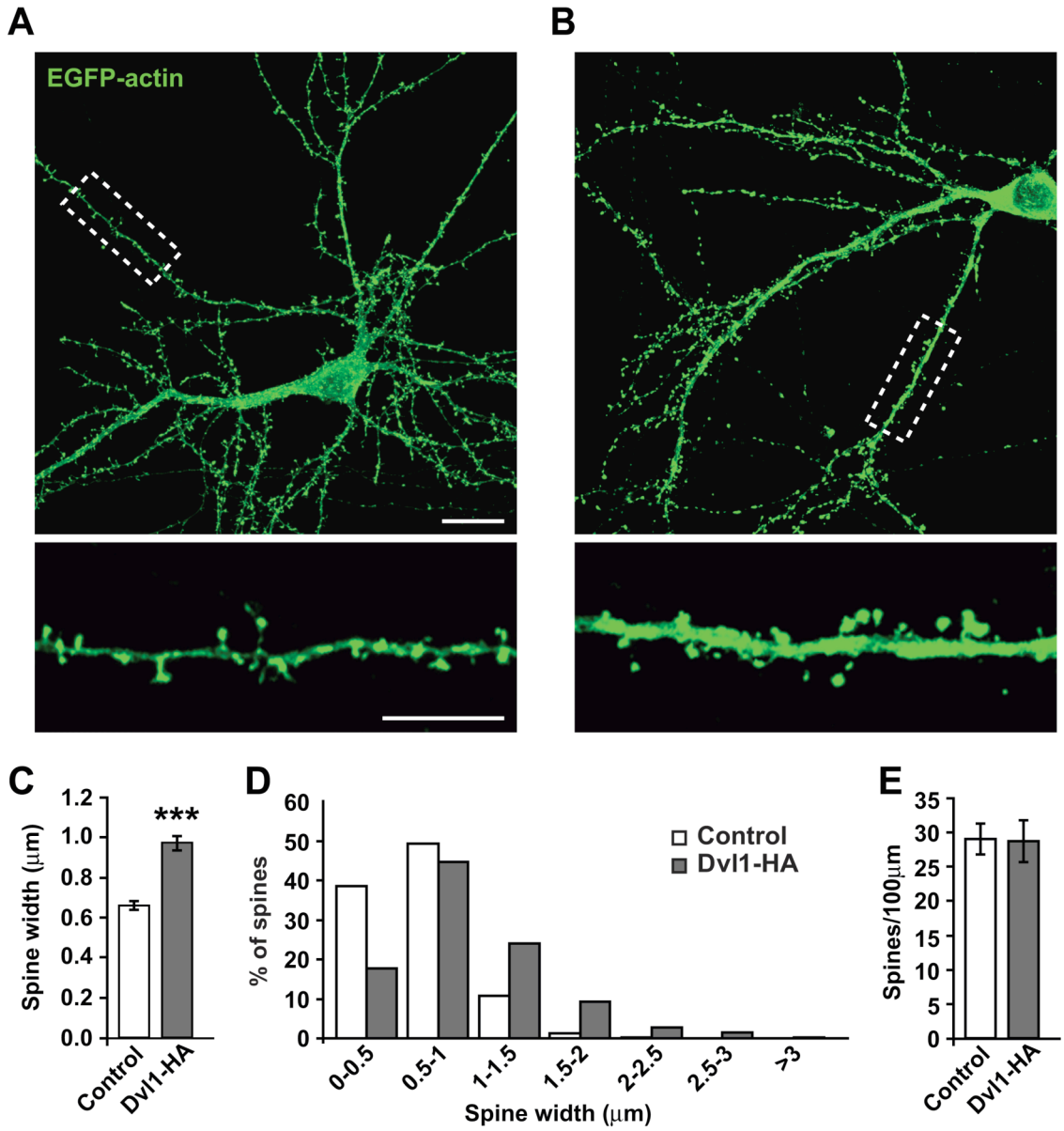

Figure 4.1 Increased spine size is observed in cultures overexpressing Dvl1. Hippocampal cultures were transfected with EGFP-actin and empty vector (control transfected) or EGFP-actin and Dvl1-HA at 8 DIV and imaged at 12 DIV. Representative images from (A) control transfected cells and (B) Dvl1-HA transfected cells demonstrate the enlargement of spines caused by Dvl1 overexpression. Scale bars = 20μm in top panels, 10μm in enlarged panels. (C) Quantification of mean spine width shows that Dvl1 overexpression causes a 47% increase compared to control transfected cells. (D) Histogram of spine width, demonstrating a clear rightwards shift towards larger spine size in Dvl1 overexpressing cells. (E) Quantification shows that spine density is unaffected by postsynaptic Dvl1 overexpression. *** = P<0.001.

Interestingly, mIPSC frequency and amplitude were unchanged in Dvl1 overexpressing cells (Fig 4.2 D - F). The kinetics of mIPSCs were also unaffected, as both mIPSC $tau_{(rise)}$ and $tau_{(decay)}$ were consistent between control and Dvl1-HA transfected cells ($tau_{(rise)}$: 1.94±0.11ms (control) and 1.80±0.09ms (Dvl1-HA); $tau_{(decay)}$: 30.04±1.38ms (control) and 32.74±1.37ms (Dvl1-HA); Fig 4.3B, E & F). Therefore increasing Wnt signalling activity in the postsynaptic compartment through overexpression of Dvl1

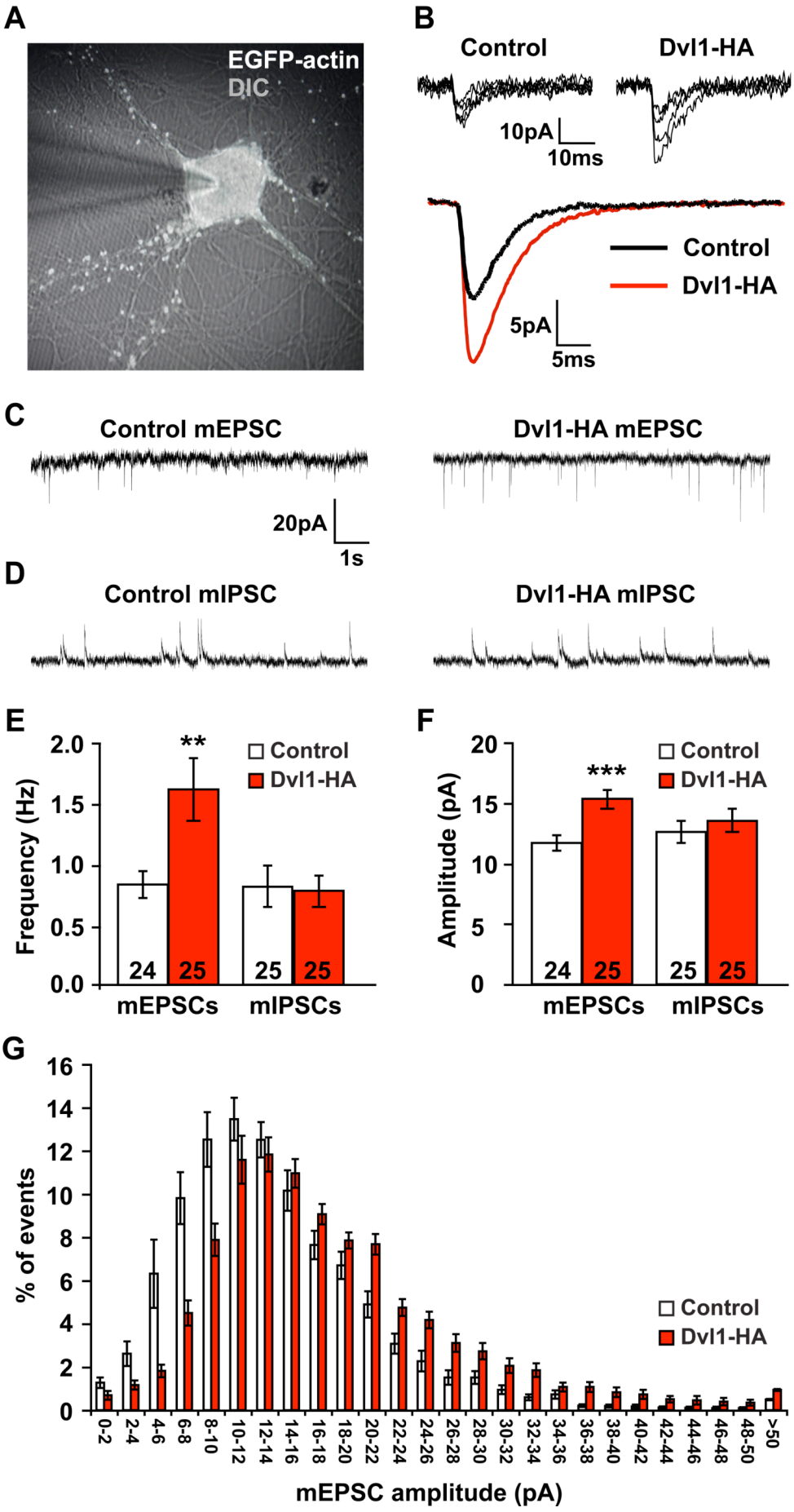

Figure 4.2 (Figure legend on next page)

Figure 4.2 Postsynaptic Dvl1 overexpression increases the frequency and amplitude of mEPCSs, but does not affect mIPSCs. Hippocampal neurons were transfected with EGFP-actin and empty vector (control transfected) or EGFP-actin and Dvl1-HA at 8 DIV and recorded from at 12 DIV. (A) A whole-cell patched EGFP-actin expressing neuron (white) is surrounded by non-transfected neurites (overlaid DIC image). (B) Overlays of 5 consecutive mEPSCs from control and Dvl1-HA transfected neurons show that Dvl1 increases mEPSC amplitude (top panels). Overlays of the averaged mEPSCs from the same cells further demonstrate the amplitude increase (bottom panel). Representative 10-second traces of mEPSCs (C) and mIPSCs (D) reveal a clear increase in mEPSC frequency and amplitude in response to Dvl1 expression, whereas mIPSCs remain unchanged. (E) Quantification of mini frequency reveals a 2-fold increase in mEPSCs in Dvl1-expressing neurons. (F) Quantification shows that Dvl1 induces a 33% increase in mEPSC amplitude. The numbers at the base of bars reflect the number of cells recorded from. (G) Frequency histogram of mEPSC amplitudes in control and Dvl-HA transfected cells. ** = $P<0.01$, *** = $P<0.001$.

specifically regulates excitatory neurotransmission.

4.2.2 Postsynaptic activation of Wnt signalling increases excitatory innervation of the postsynaptic neuron, without affecting inhibitory innervation

Although Dvl only increases spine head size, and not spine density, I observed an increase in both amplitude and frequency of mEPSCs. This poses the question as to why there is an increase in frequency when the number of spines remains unchanged. There are several possible explanations for this result. It could simply be the case that the increase in mEPSC amplitude greatly increases the number of mEPSC events that are capable of being detected above the level of the noise. However this appears unlikely. The threshold detection method employed is specifically designeed to be able to detect even small events close to the level of the noise (Clements and Bekkers, 1997). The amplitude histogram depicted in Figure 4G demonstrates that in both conditions, the vast majority of events are above the level of the noise (~4 - 5pA on average). Though there is a statistically significant difference in the proportion of events above 4pA between the two conditions, this difference is far too small to account for the two-fold increase in frequency observed (% events >4pA; Control = 96.1 ± 0.7; Dvl1-HA = 98.1 ± 0.4, P = 0.014); this is true even if the cut-off point is raised to events of greater than 6pA (% events >6pA; Control = 89.8 ± 2.2; Dvl1-HA = 96.3 ± 0.6, P = 0.006).

So what then could be responsible for the marked increase in mEPSC amplitude in Dvl1 transfected neurons? Firstly, Dvl1 overexpression may increase the release probability of the vesicle pool responsible for mEPSCs. Secondly, Dvl1 may result in the insertion

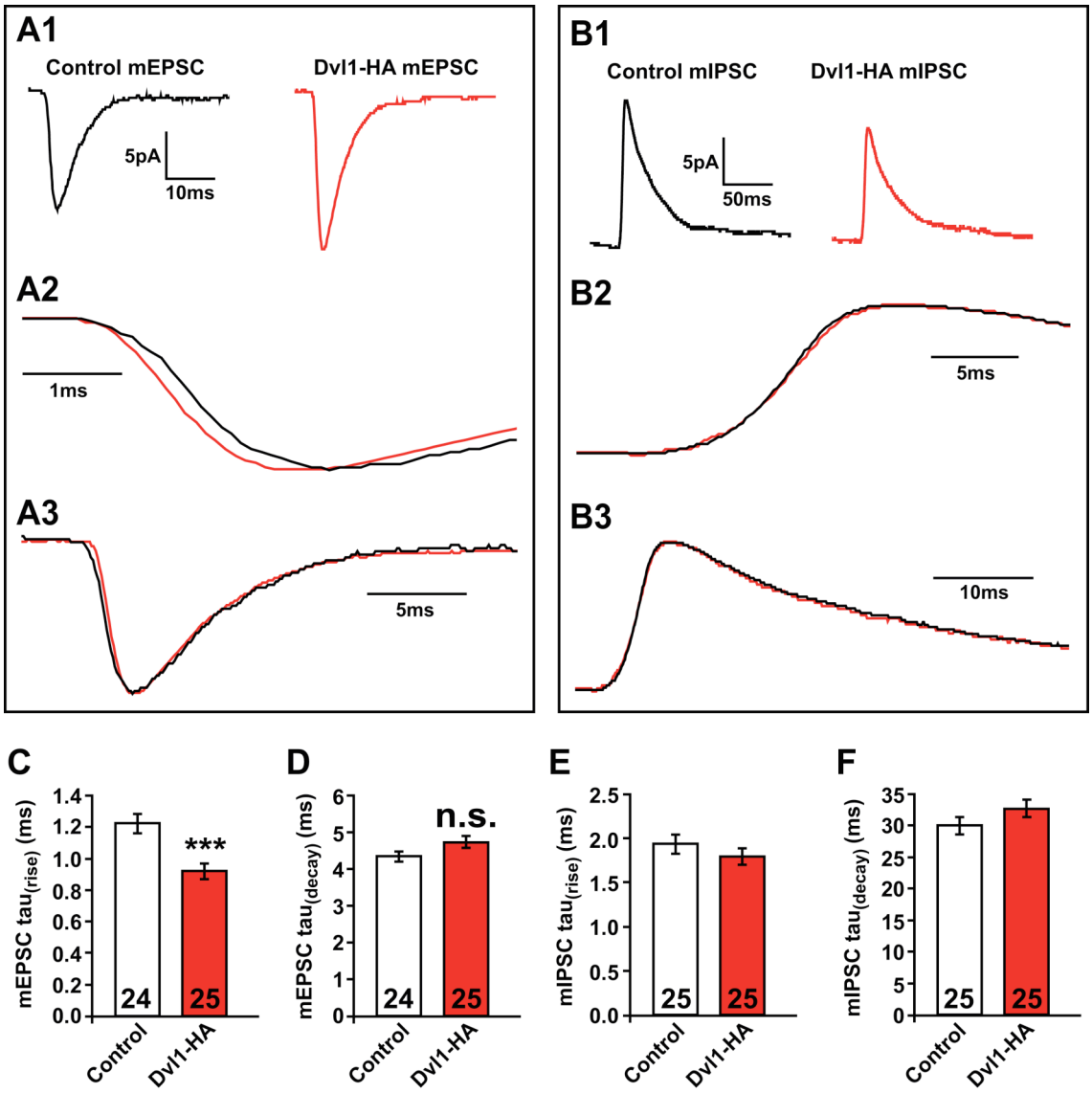

Figure 4.3 Postsynaptic Dvl1 overexpression decreases mEPSC rise time. (A) Averaged mEPSCs from example control (black trace) and Dvl1-HA transfected (red trace) cells. (A1) shows the unscaled averages. (A2) shows the averages scaled to the peak amplitude and aligned to the onset of the rise, highlighting the faster rise time in the Dvl1-HA transfected cell. (A3) shows the averages scaled and aligned to the peak amplitude, demonstrating no significant change in the slope of the decay in the Dvl1 overexpressing cell, compared to the control cell. (B) As for (A), except examples of mIPSCs are shown. The slopes of the mIPSC rise and decay are unchanged in the Dvl1-HA transfected cell as compared to the control transfected cell. (C) and (D) Quantification of the mean mEPSC $tau_{(rise)}$ and $tau_{(decay)}$, respectively. Dvl1 overexpressing cells exhibit a 25% decrease in $tau_{(rise)}$ compared to control transfected cells, whereas $tau_{(decay)}$ remains unchanged. (E) and (F) Quantification of the mean mIPSC $tau_{(rise)}$ and $tau_{(decay)}$, respectively. No differences in either parameter were observed between control and Dvl1-HA transfected cells. The numbers at the base of bars show the number of cells recorded from. *** = $P<0.001$.

of AMPA receptors into silent synapses, thereby unmasking them. Finally, in cultures at this stage of maturity, many spines appear to be 'orphan spines' as they lack key synaptic markers (Vazquez et al., 2004). Therefore, postsynaptic Dvl1 may promote the

formation of functional excitatory synapses onto orphan spines. As I had already observed that addition of Wnt7a to the culture medium promotes excitatory synapse formation (Fig 3.1), I decided to investigate the latter option.

In control transfected neurons, approximately 60% of dendritic spines contained PSD-95 puncta or were contacted by vGlut1 puncta, and around 50% displayed both markers in apposition (Fig 4.4A & D). By comparison, Dvl1 overexpressing cells exhibited a significant increase in the clustering of PSD-95 and vGlut1 at spines, with more than 70% displaying both markers in apposition (PSD-95-positive spines: 61.5 ± 3.4% (control) and 78.9 ± 2.8% (Dvl1-HA); vGlut1-positive spines: 55.6 ± 3.2% (control) and 79.9 ± 2.1% (Dvl1-HA); PSD-95+vGlut1-positive spines: 47.4 ± 3.4% (control) and 71.9 ± 2.8% (Dvl1-HA); Fig 4.4A-C). While analysing synaptic marker localisation, it became apparent that many Dvl1 transfected spines contained more than one discreet PSD-95 punctum (multiple PSDs), and/or were contacted by more than one vGlut punctum (multiply innervated spines (MIS)). Therefore these parameters were also quantitatively analysed. Dvl1 overexpression resulted in a significant increase in the proportion of MIS (control = 10.4 ± 1.4%; Dvl1-HA = 31.7 ± 1.9%: Fig 4.4A, B & D). A histogram of the number of vGlut1 puncta per spine reveals that there is a significant reduction in the percentage of non-innervated spines with a concomitant increase in the proportion of spines with 2 or more apposed vGlut1 puncta (Fig 4.4E). A similar pattern is observed with regards to the number of PSD-95 puncta within spine heads (% multiple PSD: control = 6.0 ± 0.9%; Dvl1-HA = 19.6 ± 2.0%; Fig 4.4F & G). Dvl1 overexpression also increased the mean PSD-95 punctum size, although the size of vGlut1 puncta was not significantly affected (Mean PSD-95 punctum size: 0.48 ± 0.05μm^3 (control) and 0.73 ± 0.12μm^3 (Dvl1-HA); Mean vGlut1 punctum size: 0.51 ± 0.03μm^3 (control) and 0.62 ± 0.05μm^3 (Dvl1-HA); Fig 4.4A, B & H). The combined effect of a decrease in orphan spines and an increase in the proportion of MIS results in a 69% increase in the density of excitatory synapses, defined again as vGlut1 puncta apposed to PSD-95 puncta (Fig4.4A, B & I). In conclusion, postsynaptic expression of Dvl1 acts to increase the excitatory input to the postsynaptic cell both by increasing the percentage of spines that are innervated by presynaptic neurons, and also increasing the proportion of spines that are multiply innervated. This strongly suggests that changes in postsynaptic Wnt signalling can signal retrogradely to the presynaptic side to bring about co-ordinated changes in pre- and postsynaptic morphology.

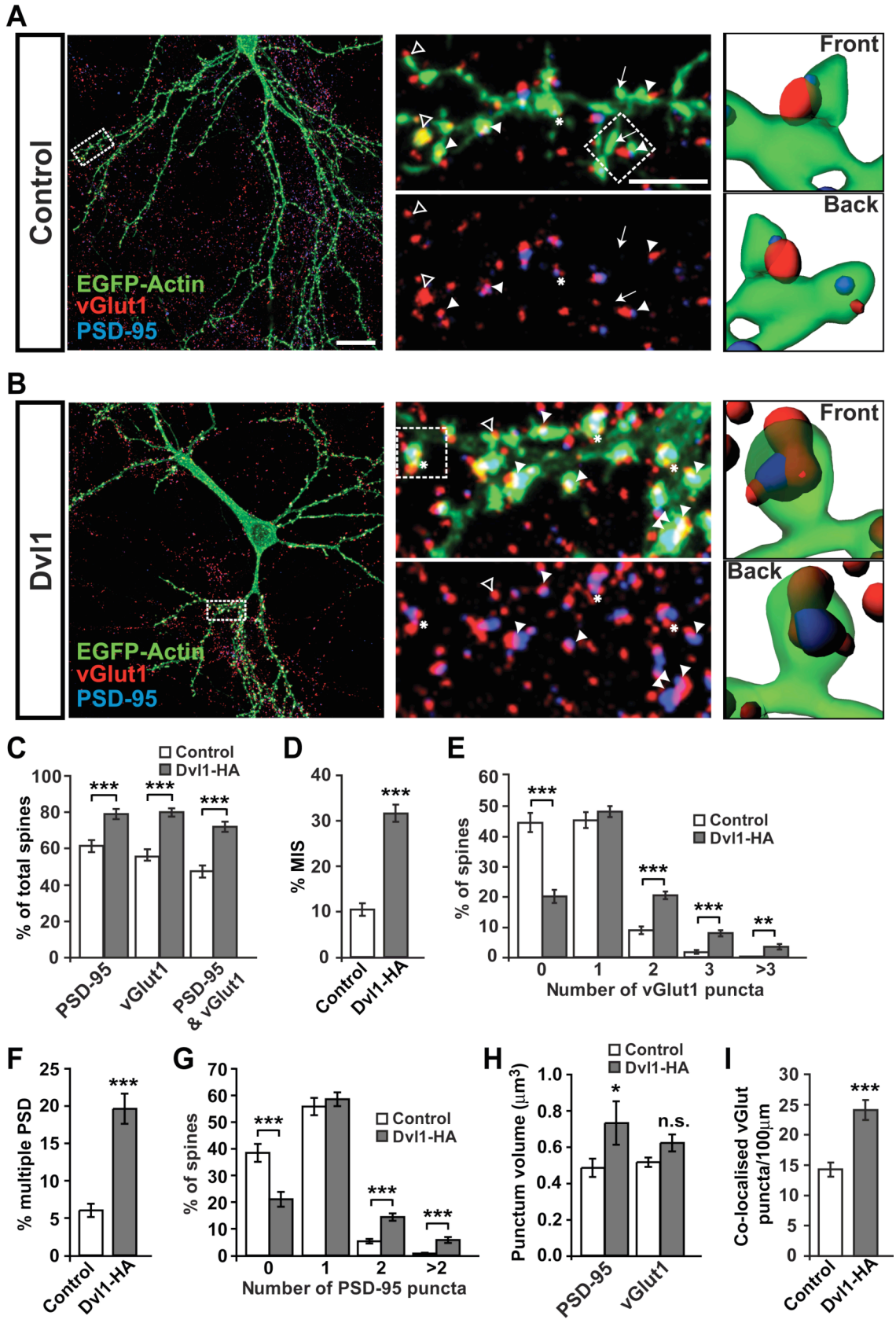

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Figure 4.4 Postsynaptic Dvl1 overexpression increases excitatory inputs onto transfected cells. (A) Control EGFP-actin expressing hippocampal neurons (green) were labelled with vGlut1 (red) and PSD-95 (blue). Low (left) and high (middle) magnification images show that half of the spines contain PSD-95 opposed to vGlut1 (filled arrowheads). The remaining spines co-localize with only one of the markers (open arrowheads) or lack both (arrows). Scale bars, 20μm (left panel) and 5μm (middle panels). Right panel is an Imaris 3D-rendered volume (corresponding to the boxed area in the middle panel) showing a spine from the control transfected cell. The spine contains a single PSD-95 punctum contacted by a single vGlut1 punctum. (B) As for (A), except a representative Dvl1-HA transfected cell is shown. Dvl1 overexpression increases the proportion of spines associated with PSD-95 and vGlut puncta. Additionally, Dvl1 overexpression increases the proportion of spines that are contacted by multiple vGlut puncta (multiply innervated spines (MIS), asterisks) and that contain more than one discreet PSD-95 punctum (double arrowhead). The 3D-rendered spine contains a single large PSD-95 punctum and is contacted by two vGlut1 puncta. (C) Dvl1 significantly increases the proportion of spines that contain PDS-95, have apposed vGlut1 puncta, or both. (D) Dvl1 overexpressing cells exhibit a 205% increase in the proportion of MIS compared to control cells. (E) Histogram of vGlut puncta number at spines demonstrating a reduction in non-innervated spines and shift towards multiply innervated spines in Dvl1-HA transfected neurons. (F) Dvl1 overexpression also increases the proportion of spines containing multiple PSD-95 puncta. (G) Histogram of PSD-95 puncta number at spines demonstrating a reduction in PSD-95-negative spines and shift towards spines containing multiple PSD-95 puncta. (H) Dvl1 induces a 65% increase in PSD-95 volume per spine compared to control cells, whereas vGlut1 puncta volume is unchanged. (I) Dvl1 increases by 69% the density of vGlut1 puncta associated with PSD-95 at spines (excitatory contacts). * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

Inhibitory innervation of control and Dvl1-HA transfected neurons was also investigated. Unfortunately in this case only vGAT staining could be analysed, as the Gephyrin antibody was found to cross-react with the HA tag of Dvl1-HA (data not shown). Consistent with the view that spines primarily receive excitatory inputs, the number of inhibitory presynaptic inputs (labelled with vGAT) onto spines was negligible. Instead, vGAT puncta were preferentially found along the shafts of proximal dendrites and at the cell body. No changes were observed in the density or size of vGAT puncta contacting Dvl1 overexpressing neurons as compared to control transfected neurons (Fig. 4.5), supporting the interpretation that postsynaptic Wnt signalling through Dvl1 specifically regulates excitatory synapse formation.

4.3 Discussion

4.3.1 Postsynaptic Wnt signalling regulates dendritic spine morphology and excitatory synaptic strength

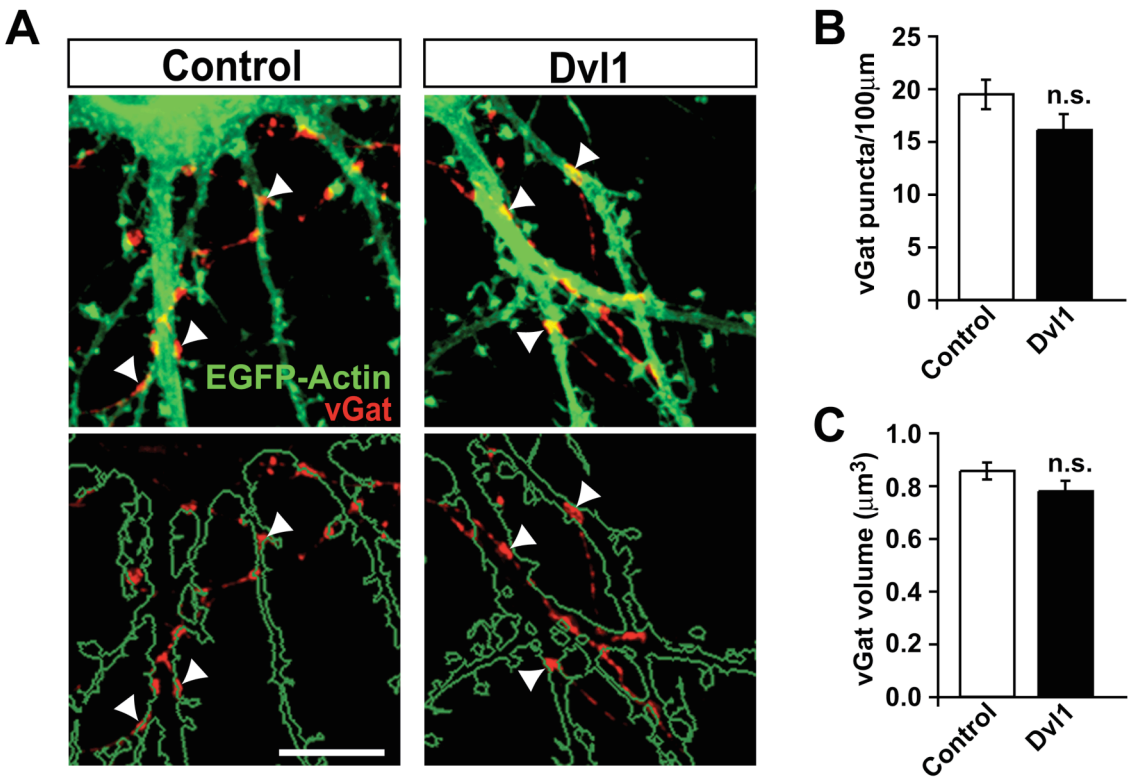


Figure 4.5 Postsynaptic Dvl1 expression does not affect the number of inhibitory inputs. Hippocampal cultures were transfected with EGFP-actin and empty vector (control transfected) or EGFP-actin and Dvl1-HA at 8 DIV and imaged at 12 DIV. (A) vGat puncta (red) are found mainly on the shafts of proximal dendrites (arrowheads) and the cell body. No difference in the number of contacting vGAT puncta is observed between control and Dvl1-HA transfected cells. Scale bar, 10μm. (B) and (C) Quantification reveals no significant differences in the density or volume of vGat puncta contacting control or Dvl1-transfected cells.

Postsynaptic activation of Wnt signalling through Dvl1 overexpression results in an approximately 50% increase in the mean size of dendritic spines, an effect that has been observed both by myself and another member of our laboratory (Figs 4.1& 4.2 andCiani et al., submitted for publication). Dendritic spine size has previously been shown to be correlated with glutamate receptor content and strength of glutamatergic synapses (Bourne and Harris, 2008; Carlisle and Kennedy, 2005; Matsuzaki et al., 2004; Yuste and Bonhoeffer, 2001). Consistently, I found that Dvl1 overexpression also increased the amplitude of AMPA receptor-mediated mEPSCs, demonstrating that excitatory synapses onto Dvl1-HA transfected cells were on average stronger than synapses onto control transfected cells. This effect was specific to excitatory synapses as mIPSC amplitude was similar between Dvl1-HA and control transfected cells. Therefore postsynaptic signalling through Dvl1 specifically regulates excitatory synaptic strength by promoting dendritic spine enlargement, presumably leading to increased AMPA receptor insertion into the enlarged spine heads. Interestingly, treatment of cultures with

Wnt7a for 3 hours or overnight results in increases in both spine size and density (Ciani et al., submitted for publication). This suggests that presynaptic activation of Wnt signalling results in changes in excitatory synapse number, whereas postsynaptic Wnt signalling regulates excitatory synaptic strength through changes in spine morphology.

4.3.2 Postsynaptic Wnt signalling regulates mEPSC kinetics

mEPSCs in Dvl1-HA overexpressing cells exhibited a significantly (25%) faster rise time than control transfected cells. The degree to which EPSCs temporally summate at the axon hillock, and therefore action potential output, depend crucially on EPSC kinetics (Magee, 2000). Therefore, Wnts may also be able to influence postsynaptic excitability through regulation of EPSC kinetics. I did not have time to investigate the underlying reasons for this, but mEPSC kinetics can be influenced by a wide range of factors including changes in AMPA receptor subunit composition (Dingledine et al., 1999), AMPA receptor regulatory proteins (Milstein and Nicoll, 2008) and dendritic morphology (Magee, 2000; Spruston, 2008). The latter factor seems a distinct possibility, as it was noted that dendrites of Dvl1-HA transfected neurons often appeared slightly shorter and thicker than dendrites of control transfected cells (see Figs 4.1 and 4.2), and Wnt signalling has an established role in regulating dendritic morphology (Rosso et al., 2005; Wayman et al., 2006). Larger calibre dendrites would result in reduced dendritic filtering of currents due to decreased axial resistance, which could account for the decreased rise time. Crucially, this could also account for the increased amplitude of mEPSCs in Dvl1 transfected cells, as synaptic currents would attenuate less as they travelled to the soma.

To investigate the hypothesis that local synaptic currents are unchanged by Dvl1 overexpression and changes in filtering alone account for the altered mEPSC rise time and amplitude, the following test was performed (see Appendix 1). An mEPSC was simulated to approximately match the response of hippocampal AMPA-Rs to a 1mM pulse of glutamate (0.2ms rise, 4.5ms decay). This mEPSC was then filtered at the average expected bandwidth for control recordings (265 Hz) or for the Dvl1 transfected (302 Hz) recordings, based upon the average membrane capacitance and series resistance for each condition. To determine whether additional filtering of the same original mEPSC could produce the 24% smaller amplitude (-11.6pA) and slower rise time (1.2ms) of control recordings, excess filtering was applied at 160 Hz which gives a

rise time of 1.2ms but only 13% decrease in amplitude. In order to reduce the amplitude by 24% a 100 Hz filter was applied which increased the risetime to 1.9ms. As filtering alone cannot produce the observed differences in amplitude and rise time between control and Dvl1 transfected recordings, these simulations suggest there is a real difference in the underlying synaptic mEPSCs in control and Dvl1 transfected recordings. As mentioned above though, the underlying mechanism(s) for these differences remain to be eluciadated.

4.3.3 Postsynaptic activation of Wnt signalling increases innervation of dendritic spines

In addition to the changes in mEPSC amplitude and rise time, Dvl1 overexpressing cells also displayed a 2-fold increase in mEPSC frequency. This increase could potentially be an artefact due to improved detection of the larger mEPSCs. This possibility should ideally have been test directly by recording mEPSCs within the same cell at two different holding potentials (i.e. -60mV and -80mV). Holding the cell at a more hyperpolarised potential would increase the driving force on sodium influx into the cell, and thereby increase the mEPSC amplitude. By doing this in a number of cells it would be possible to estimate the effect of increasing mEPSC amplitude on event detection. Although I cannot rule out the increased mEPSC amplitude having a slight effect on mEPSC frequency, it appears highly unlikely it could account for the 2-fold increase in frequency that is observed, as only a relatively small proportion of events are at or below the amplitude of the noise in both control and Dvl1-HA transfected cells. The increase in mEPSC frequency therefore suggested that the number of excitatory synapses onto Dvl1-HA transfected cells was increased, despite the fact that spine density was similar to that observed in control transfected cells. Accordingly, immunostaining experiments revealed that Dvl1 overexpression decreased the proportion of 'orphan spines' that lacked pre- and/or postsynaptic markers, thereby increasing the proportion of excitatory contacts (spines positive for both pre- and postsynaptic markers). Even more intriguingly, Dvl1 overexpression resulted in a three-fold increase in the proportion of multiply innervated spines and the proportion of spines displaying multiple PSDs. Therefore, postsynaptic activation of Wnt signalling significantly increases excitatory innervation of pre-existing spines on the postsynaptic cell.

Postsynaptic Dvl1-HA transfection results in an increase in presynaptic terminals contacting the transfected cell. This suggests that overexpression of Dvl1 leads to the generation of a retrograde signal that instructs axons to form new excitatory terminals onto spines of Dvl1-HA transfected cells. Although the mechanism by which Dvl signals back to the presynaptic site is currently unknown, a prime candidate is provided by a recent ultrastructural study which looked at the effects of PSD-95 overexpression on spine morphology and innervation in organotypic hippocampal slices. Neurons transfected with PSD-95-EGFP display a phenotype that is strikingly similar to that displayed by Dvl1-HA transfected neurons; spine size and PSD area are enhanced, and there is a drastic (~10-fold) increase in the proportion of spines displaying complex, perforated PSDs and innervation by multiple presynaptic terminals. Furthermore, blockade of nitric oxide (NO) signalling in PSD-95 overexpressing neurons blocked the increase in MIS without affecting PSD enlargement, whereas enhancement of NO signalling in control cells increased the proportion of MIS with no effect on PSD size. Therefore accumulation of PSD-95 leads to a downstream increase in NO production, which acts retrogradely on nearby axons to promote presynaptic differentiation and MIS formation (Nikonenko et al., 2008). Interestingly, Dvl1 overexpression also results in a significant increase in PSD-95 clustering at spines of transfected neurons. Thus, Dvl1 could promote increased spine innervation and MIS formation through increasing PSD-95 clustering and NO release at spines. It will be of great interest to determine if reducing NO signalling blocks the reduction in orphan spines, increase in MIS and/or increase in mEPSC frequency in Dvl1-HA transfected neurons.

4.3.4 Postsynaptic activation of Wnt signalling specifically regulates excitatory synapse density and function

The results of this chapter demonstrate that postsynaptic activation of Wnt signalling has a multitude of effects on excitatory synaptic form and function in hippocampal pyramidal neurons. Spines are enlarged, PSD-95 clustering is enhanced, and the proportion of spines receiving presynaptic input (including multiple inputs) increases. Accordingly, the amplitude and frequency of mEPSCs are also increased. Furthermore, the kinetics of mEPSCs are altered, as the rise time is significantly quicker. Strikingly, these effects are highly specific to excitatory synapses; Dvl1 overexpressing neurons exhibited no changes in the number of inhibitory presynaptic inputs, nor in the frequency, amplitude or kinetics of mIPSCs. These results are consistent with the results

presented in Chapter 3, which demonstrated that Wnt7a promotes the formation of excitatory, but not inhibitory synapses. The results with Wnt7a application do not preclude a role for other Wnts in regulating inhibitory synapses. Yet the fact that overexpression of Dvl1 also specifically regulates excitatory synapses argues more strongly that this specificity is a general feature of Wnts, as all known Wnt signalling involves Dvl (Chien et al., 2009). However it should be kept in mind that there are two other isoforms of Dvl in mammals (Dvl2 and Dvl3), which could signal through specific Wnts to affect inhibitory synapses. It would therefore be of great interest to investigate the effects of overexpression or knock-down of these other Dvl isoforms on excitatory and inhibitory synapses. Regardless of this, the results presented in this chapter demonstrate that postsynaptic activation of Wnt signalling through Dvl1 overexpression specifically regulates the morphology and function of glutamatergic synapses.

CHAPTER 5:

Endogenous Wnt signalling regulates release at glutamatergic hippocampal synapses

5.1 Introduction

In chapters 3 and 4 I have focused primarily on the role of Wnts in the formation of synapses, presenting evidence that Wnt signalling acts to promote the co-ordinated assembly of pre- and postsynaptic structures. This chapter addresses a key question that arises from these results; namely, do Wnts also act to regulate the function of synapses once they have formed?

Several studies have demonstrated that perturbation of Wnt signalling can affect synaptic function. For example *Drosophila* mutants lacking Wnt5 have a reduction in the evoked endplate junctional current (EJC) and mEJC frequency at the NMJ (Liebl et al., 2008), and cerebellar granule cells of mice null for both *Wnt7a* and *Dvl1* display a reduced mEPSC frequency (Ahmad-Annuar et al., 2006). However in these examples the functional defects may be secondary to the defects in synaptic morphology that are also apparent. Evidence has also been presented for a more direct role for Wnts in the regulation of neurotransmission. Application of Wnt7a or Wnt7b increases recycling of synaptic vesicles in hippocampal cultures (Ahmad-Annuar et al., 2006; Cerpa et al., 2008), and Wnt7a or synthetic Wnt agonists increase mEPSC frequency and excitatory presynaptic release probability in acute hippocampal slices (Beaumont et al., 2007; Cerpa et al., 2008). Synaptic vesicle recycling is also reduced in cerebellar mossy fibres in mice lacking the Dvl1 gene (Ahmad-Annuar et al., 2006). Taken together, these data suggest that Wnts act presynaptically to enhance neurotransmitter release.

The role of Wnts in regulating synaptic transmission is a relatively new field and key questions remain to be answered. Firstly, those studies that have provided direct evidence that Wnts regulate transmitter release from presynaptic terminals involved the addition of exogenous Wnts or synthetic activators of Wnt signalling (Ahmad-Annuar et al., 2006; Beaumont et al., 2007; Cerpa et al., 2008). Therefore it is crucial to investigate what effect, if any, perturbation of endogenous Wnt signalling has on synaptic transmission. Secondly, studies on Wnt signalling and synaptic transmission,

like studies on the role of Wnts in synapse formation, have so far focused on excitatory glutamatergic synapses. I have demonstrated in the previous two chapters that Wnt7a and signalling through postsynaptic Dvl1 specifically promote the formation of excitatory synapses onto hippocampal pyramidal neurons, without affecting inhibitory synapse formation. Does this specificity extend to the actions of Wnts on synaptic function, or can Wnts also regulate inhibitory synaptic transmission? In order to address these questions, I have performed electrophysiological analysis of excitatory and inhibitory synaptic transmission at hippocampal pyramidal neurons that either lack key Wnt signalling genes or have been exposed to Wnt antagonists for 3 hours. I find that in both cases the amplitudes of evoked EPSCs are reduced, but IPSCs are unaffected. Furthermore, by looking at a form of short-term plasticity that is related to presynaptic release probability (paired pulse ratio (PPR)), I present evidence that this is due to a reduction in release probability at excitatory synapses.

5.2 Results

5.2.1 mEPSCs, but not mIPSCs, have slower kinetics at CA1 cells of Wnt7a-/-;Dvl1-/- mice

As previously mentioned, mEPSCs are disrupted at cerebellar granule cells of acute slices taken from P15 *Wnt7a-/-;Dvl1-/-* mice, displaying a lower frequency than *wildtype* granule cells (Ahmad-Annuar et al., 2006). I have shown that blockade of Wnt signalling in 14 DIV hippocampal cultures using a cocktail of sFRPs reduces mEPSC frequency at pyramidal cells, whilst mIPSCs are unaffected (Fig 3.6). I therefore decided to investigate whether mEPSCs and mIPSCs were abnormal at CA1 cells in acute hippocampal slices taken from P10-P15 *Wnt7a-/-;Dvl1-/-* mice, compared to *wildtype* mice of the same age. Surprisingly, the frequency and amplitude of mEPSCc were similar between *Wnt7a-/-;Dvl1-/-* and *wildtype* slices (Fig 5.1A, C & D). The frequency and amplitude of mIPSCs was also unaffected (Fig 5.1B - D). However, mEPSCs from *Wnt7a-/-;Dvl1-/-* cells did exhibit altered kinetics compared to *wildtype* cells (Fig 5.2). The $tau_{(rise)}$ of mEPSCs was significantly slower in *Wnt7a-/-;Dvl1-/-* CA1 cells (*wildtype* = 0.96 ± 0.05ms; *Wnt7a-/-;Dvl1-/-* = 1.15 ± 0.08ms, P=0.04; Fig6.2A2 & C), whereas the $tau_{(decay)}$ was comparable to that seen in *wildtype* cells (*wildtype* = 5.38 ± 0.23ms; *Wnt7a-/-;Dvl1-/-* = 5.48 ± 0.26ms; Fig6.2A3 & D). Interestingly, this effect was restricted to mEPSCs, as mIPSC $tau_{(rise)}$ and $tau_{(decay)}$ were

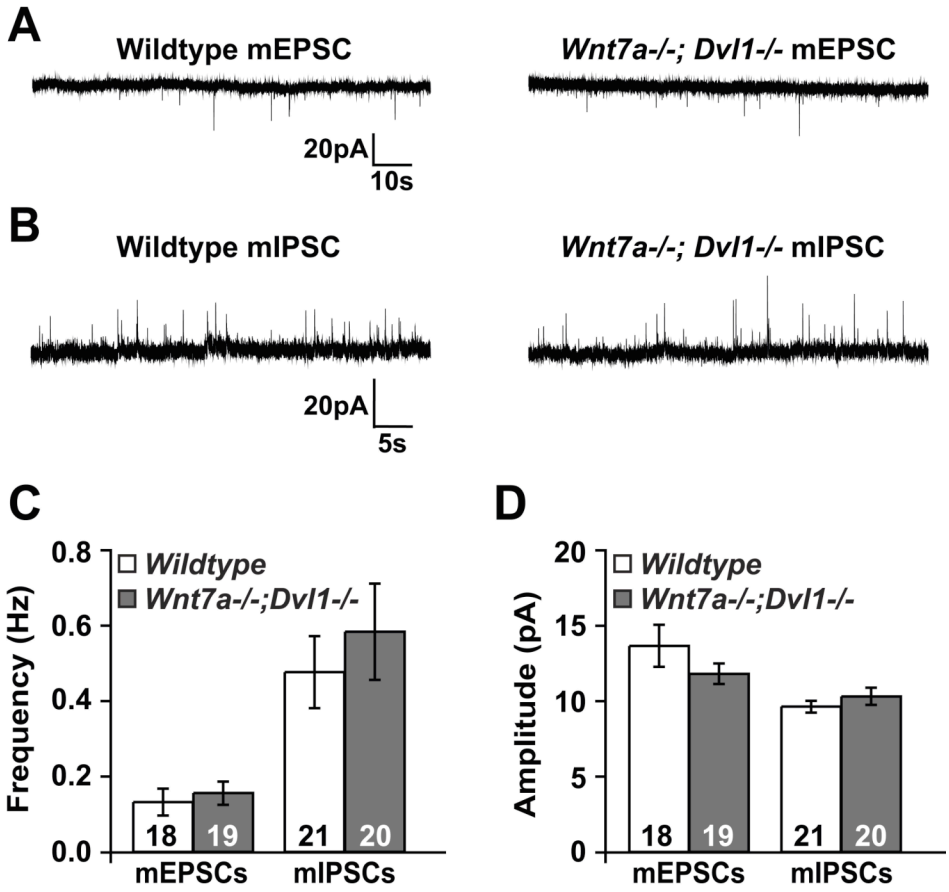


Figure 5.1 Miniature synaptic transmission is unaffected in CA1 cells of *Wnt7a-/-; Dvl1-/-* mice. CA1 cells in acute hippocampal slices from P10-P14 *wildtype* or *Wnt7a-/-; Dvl1-/-* mice were whole cell patch-clamped and mEPSCs and mIPSCs were recorded. Example 10s traces show no differences in mEPSC (A) or mIPSC (B) frequency or amplitude between *wildtype* and *Wnt7a-/-; Dvl1-/-* mice. (C) Quantification of mean mEPSC and mIPSC frequency. (D) Quantification of mean mEPSC and mIPSC amplitude. The numbers at the base of bars show the number of cells recorded from.

similar between *Wnt7a-/-;Dvl1-/-* and *wildtype* cells (*wildtype* $tau_{(rise)}$ = 1.82 ± 0.06ms; *Wnt7a-/-;Dvl1-/-* $tau_{(rise)}$ = 1.87 ± 0.06ms; *wildtype* $tau_{(decay)}$ = 25.69±1.32ms; *Wnt7a-/-;Dvl1-/-* $tau_{(decay)}$ = 23.06 ± 0.64ms; Fig 5.2B, E & F). Therefore, although the frequency and amplitude of spontaneous excitatory currents are normal at the CA3-CA1 synapse of *Wnt7a-/-;Dvl1-/-* mice, the kinetics of these currents are altered. This could have important implications for the dendritic integration of synaptic transmission at CA1 cells of *Wnt7a-/-;Dvl1-/-* mice.

5.2.2 Evoked excitatory, but not inhibitory, neurotransmitter release is disrupted at synapses onto CA1 cells of double Wnt7a-/-;Dvl1-/- mutant mice

As miniature synaptic transmission was largely normal in *Wnt7a-/-;Dvl1-/-* hippocampal slices, I next decided to look at evoked excitatory transmission. In these

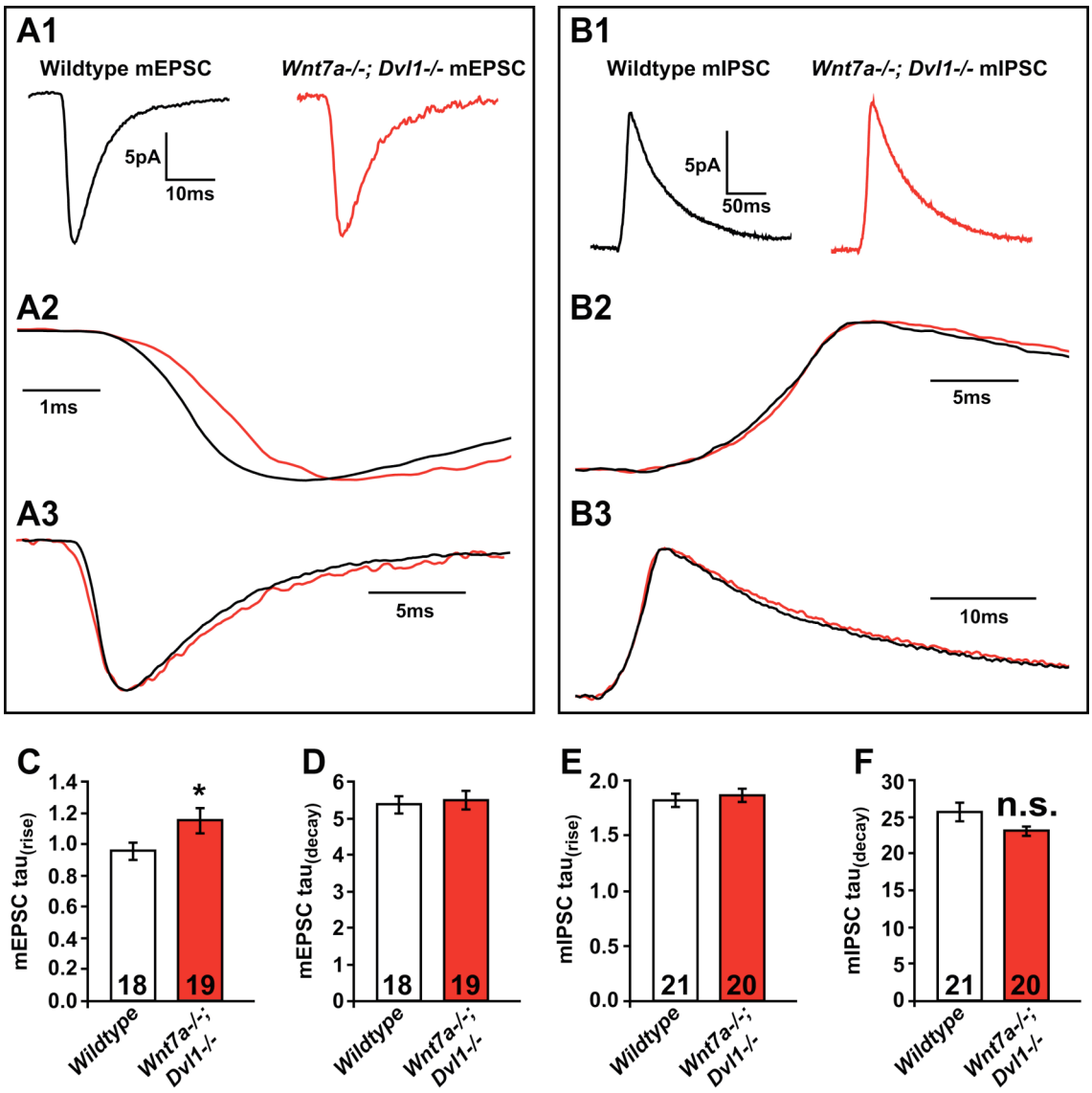


Figure 5.2 The rise time of CA1 mEPSCs is extended in *Wnt7a-/-;Dvl1-/-* mice. (A) Averaged mEPSCs from example *wildtype* (black trace) and *Wnt7a-/-;Dvl1-/-* (red trace) cells. (A1) shows the unscaled averages. (A2) shows the averages scaled to the peak amplitude and aligned to the onset of the rise, highlighting the slower rise time in the *Wnt7a-/-;Dvl1-/-* cell. (A3) shows the averages scaled and aligned to the peak amplitude, demonstrating no significant change in the slope of the decay in the *Wnt7a-/-;Dvl1-/-* cell. (B) As for (A), except examples of mIPSCs are shown. The slopes of the mIPSC rise and decay are unchanged in the *Wnt7a-/-;Dvl1-/-* as compared to the *wildtype* cell. (C) and (D) Quantification of the mean mEPSC $tau_{(rise)}$ and $tau_{(decay)}$, respectively. Cells from *Wnt7a-/-;Dvl1-/-* mice exhibit a 20% increase in $tau_{(rise)}$ compared to cells from *wildtype* mice, whereas $tau_{(decay)}$ remains unchanged. (E) and (F) Quantification of the mean mIPSC $tau_{(rise)}$ and $tau_{(decay)}$, respectively. No differences in either parameter were observed between *wildtype* and *Wnt7a-/-;Dvl1-/-* cells. The numbers at the base of bars show the number of cells recorded from. * = $P<0.05$.

experiments the slices were taken from P20-24 mice, after the major wave of postnatal synaptogenesis (Morys et al., 1998), in order to study transmission at relatively mature synapses. A concentric bipolar stimulating electrode was used to deliver stimuli of increasing intensity to elicit action potentials in axons in the straum radiatum close to

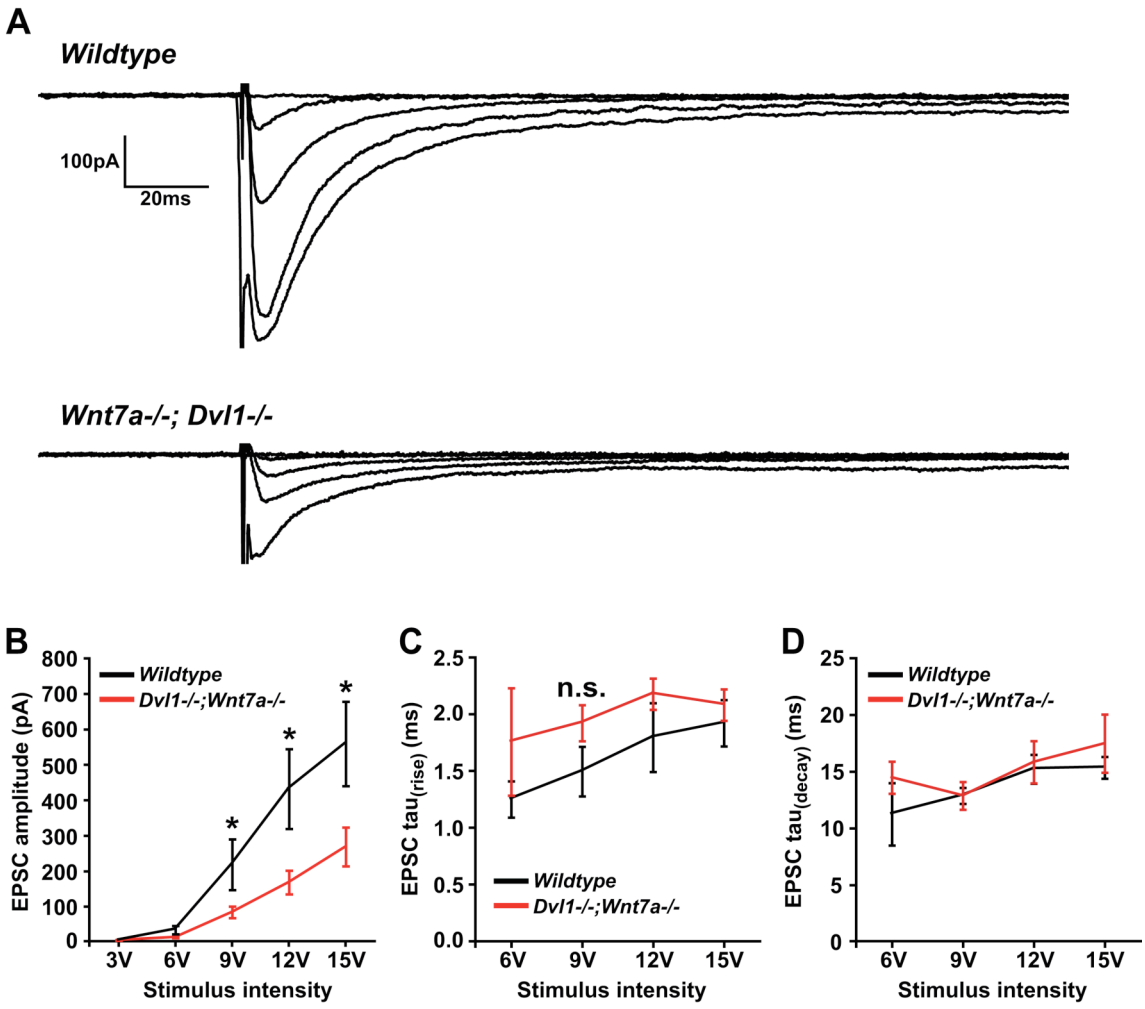
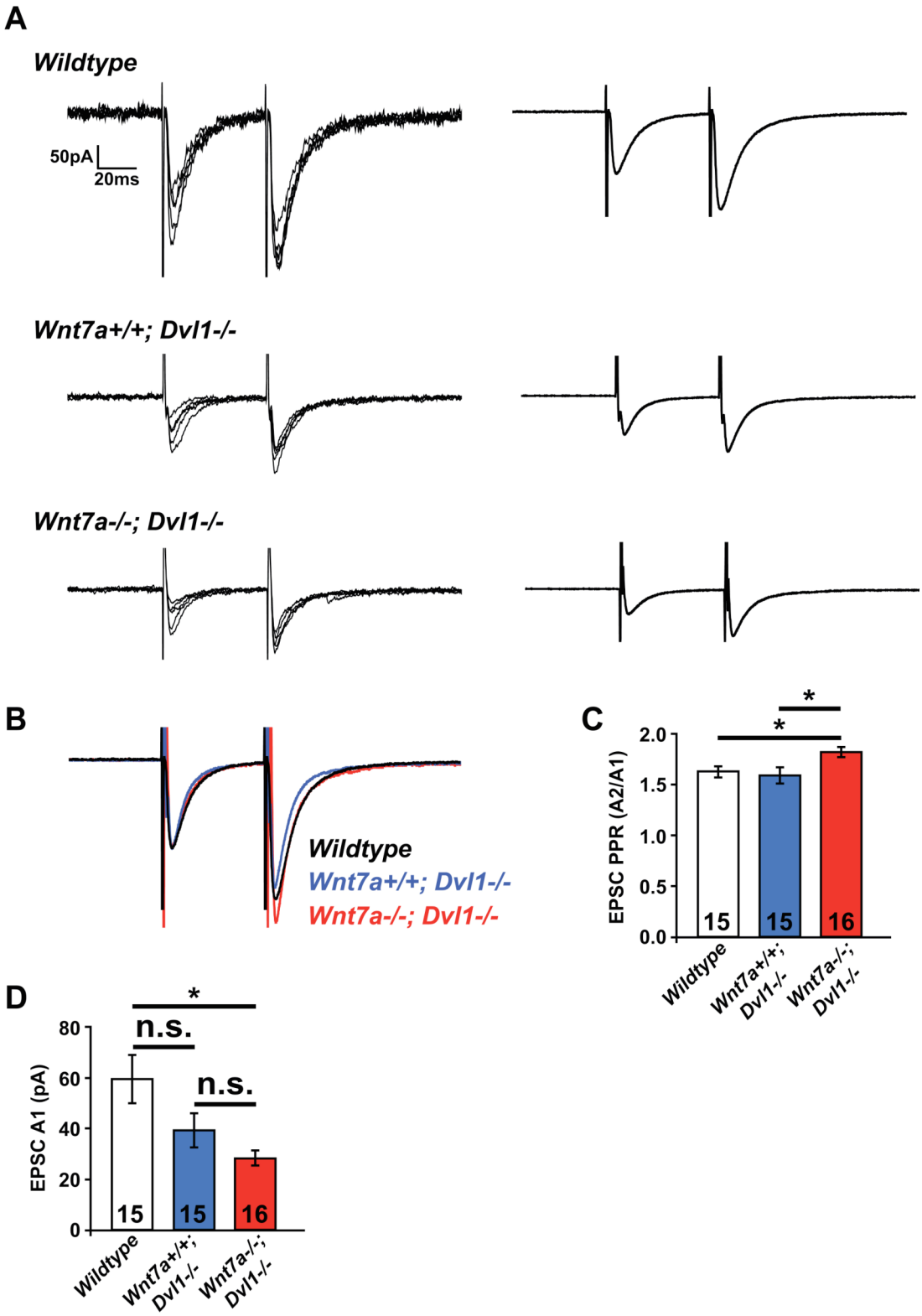


Figure 5.3 Basal excitatory transmission at CA3-CA1 synapses is impaired in *Wnt7a-/-;Dvl1-/-* mice. CA1 cells in acute hippocampal slices from P20-P24 *wildtype* or *Wnt7a-/-;Dvl1-/-* mice were whole cell patch-clamped and the EPSCs elicited by increasing Schaeffer collateral stimuli were recorded. (A) Overlays of EPSCs in response to increasing stimulus strength in *wildtype* or *Wnt7a-/-;Dvl1-/-* mice. Each trace is the average of 3-5 individual responses from a representative cell. A clear reduction in EPSC amplitude is seen in *Wnt7a-/-;Dvl1-/-* slices compared to *wildtype* slices at higher stimulus intensities. (B) Quantification of mean EPSC amplitude showing a 50-60% reduction in *Wnt7a-/-;Dvl1-/-* slices at stimulus intensities of 9, 12 and 15 volts. (C) and (D) Quantification of the mean EPSC $tau_{(rise)}$ and $tau_{(decay)}$, respectively. No significant differences in either parameter were observed between *wildtype* and *Wnt7a-/-;Dvl1-/-* cells. * = P<0.05.

the CA1 layer. The resulting AMPA receptor-mediated postsynaptic currents were measured in whole cell patch clamped CA1 cells and used to plot the input/output (I/O) relationship (Fig 5.3). The lowest stimulation voltage (3V) failed to elicit a measurable EPSC in any of the cells from either *wildtype* or *Wnt7a-/-;Dvl1-/-* slices. At all other stimulation intensities the proportion of cells which exhibited a measurable EPSC was similar between *wildtype* and *Wnt7a-/-;Dvl1-/-* slices (*wildtype*: 6V – 6/10 cells, 9V – 10/10 cells, 12V – 10/10 cells, 15V – 10/10 cells; *Wnt7a-/-;Dvl1-/-*: 6V – 11/14 cells,



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9V – 13/14 cells, 12V – 13/14 cells, 15V – 14/14 cells). However at higher stimulation intensities (9, 12 and 15V) the *Wnt7a-/-;Dvl1-/-* cells displayed a significantly reduced EPSC amplitude compared to *wildtype* cells (Fig 5.3A and B). The mean EPSC in response to 6V stimulation was also smaller in *Wnt7a-/-;Dvl1-/-* cells, but not

Figure 5.4 Excitatory synapses onto CA1 cells of *Wnt7a-/-;Dvl1-/-* mice exhibit impaired presynaptic release. CA1 cells in acute hippocampal slices from P20-P24 *wildtype, Wnt7a+/+;Dvl1-/-* or *Wnt7a-/-;Dvl1-/-* mice were whole cell patch-clamped and the EPSCs elicited by pairs of Schaeffer collateral stimuli were recorded. (A) Overlays of five consecutive responses (*left*) and averages of all responses (*right*) from example cells from *wildtype, Wnt7a+/+;Dvl1-/-* or *Wnt7a-/-;Dvl1-/-* slices. (B) Overlay of the average traces shown in (A), scaled and aligned to the peak of the first EPSC. The peak amplitude of the second EPSC (A2) relative to the first (A1), and therefore the paired-pulse ratio (PPR=A2/A1), is larger in *Wnt7a-/-;Dvl1-/-* slices than in *wildtype* or *Wnt7a+/+;Dvl1-/-* slices. (C) Quantification of mean PPR shows a 12% increase in *Wnt7a-/-;Dvl1-/-* slices compared to *wildtype* slices. (D) Quantification of the mean peak amplitude of the first EPSC shows a 52% decrease in *Wnt7a-/-;Dvl1-/-* slices compared to *wildtype* slices. The numbers at the base of bars show the number of cells recorded from. * = $P<0.05$.

significantly so (*wildtype* = 31.52 ± 11.62pA; *Wnt7a-/-;Dvl1-/-* = 11.36 ± 2.27pA. P=0.054). The rise and decay times of the EPSCs displayed a tendency to increase with increasing stimulus intensity (Fig 5.3C & D), presumably due to the recruitment of increasing numbers of synapses that release at slightly different times, leading to a spreading of the waveform; additionally, there may be glutamate spillover occurring at higher stimulus intensities. Although the $tau_{(rise)}$ showed a tendency to be higher in *Wnt7a-/-;Dvl1-/-* cells, as might be expected given the effect seen in the mEPSC $tau_{(rise)}$, this was not statistically significant at any of the stimulus intensities used (Fig 5.3C). No differences were detected in the $tau_{(decay)}$ at any of the stimulus intensities (Fig 5.3D).

The reduction in EPSC amplitude seen in *Wnt7a-/-;Dvl1-/-* mice could be explained by a number of factors, including a decrease in postsynaptic AMPA receptor number or sensitivity, decreased Schaeffer collateral number or excitability or a decrease in presynaptic release probability. As application of Wnt7a has previously been shown to increase EPSC amplitude at CA1-CA3 synapses by increasing release probability (Cerpa et al., 2008), I decided to investigate the latter possibility. To do this I measured the EPSC paired pulse ratio (PPR) at CA3-CA1 synapses by administering two stimuli separated by a 50ms interval to the Schaeffer collaterals and dividing the amplitude of the second CA1 EPSC by the first. The PPR reflects changes in presynaptic release probability, with an increase in the PPR reflecting a decrease in release probability, and vice versa (Cerpa et al., 2008; Dobrunz and Stevens, 1997; Schulz et al., 1995; Zucker and Regehr, 2002). In this experiment the stimulus intensity was altered for each individual cell in order to produce the minimal reproducible response, thereby minimising the variability that could be produced by stimulating differing numbers of

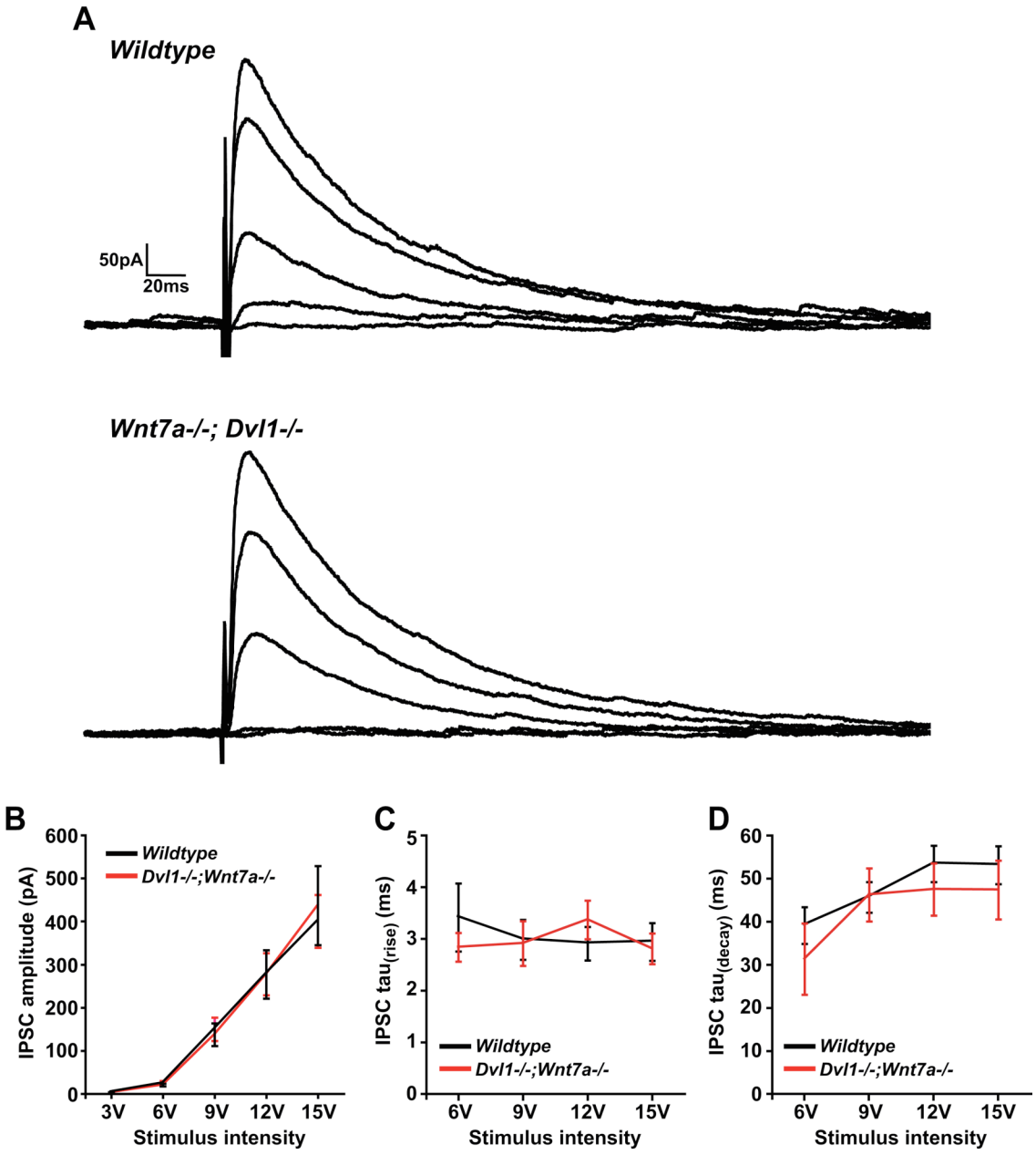
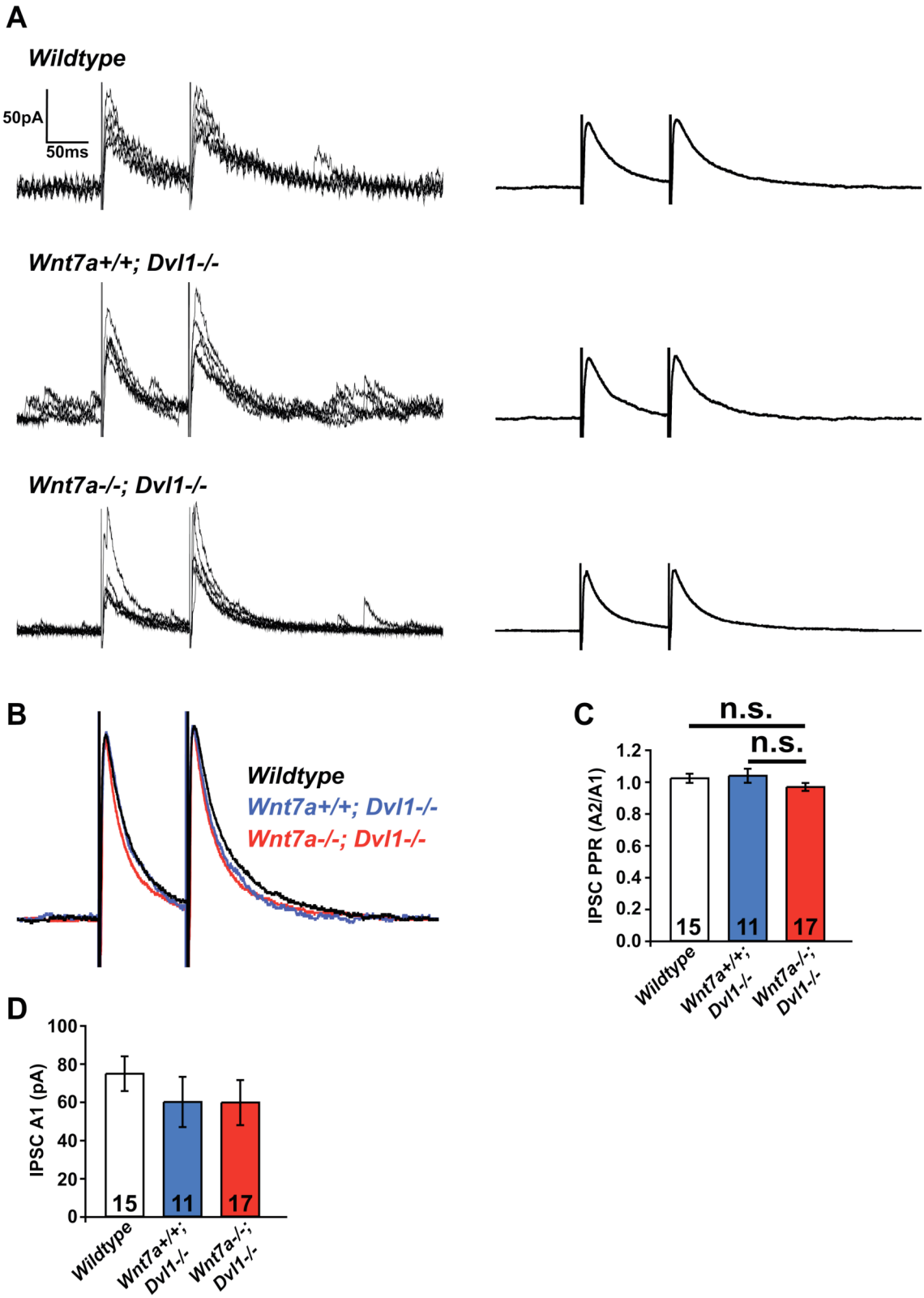


Figure 5.5 Basal inhibitory transmission onto CA1 cells is normal in *Wnt7a-/-;Dvl1-/-* mice. CA1 cells in acute hippocampal slices from P20-P24 *wildtype* or *Wnt7a-/-;Dvl1-/-* mice were whole cell patch-clamped and the IPSCs elicited by stimuli of increasing strength were recorded. (A) Overlays of IPSCs in response to increasing stimulus strength in *wildtype* or *Wnt7a-/-;Dvl1-/-* slices. Each trace is the average of 3-5 individual responses from a representative cell. The responses to a given stimulus intensity are similar between the *wildtype* and *Wnt7a-/-;Dvl1-/-* cell (although notice that in the examples given 6-volt stimulation elicited a small IPSC in the *wildtype* cell, but no response in the *Wnt7a-/-;Dvl1-/-* cell). (B) Quantification of mean IPSC amplitude shows no differences between *wildtype* and *Wnt7a-/-;Dvl1-/-* slices at any of the stimulus intensities. (C) and (D) Quantification of the mean IPSC $tau_{(rise)}$ and $tau_{(decay)}$, respectively. No differences in either parameter were observed between *wildtype* and *Wnt7a-/-;Dvl1-/-* cells.

inputs between cells (see materials and methods for more details).

EPSCs of *wildtype* CA1 cells displayed paired-pulse facilitation with a mean PPR of 1.63±0.05 (Fig 5.4A, B & C), showing good accordance with previous studies of PPR at CA3-CA1 synapses (Cerpa et al., 2008; Kang and Schuman, 1995; Maruki et al., 2001; Santschi and Stanton, 2003). Crucially, EPSCs of *Wnt7a-/-;Dvl1-/-* CA1 cells displayed a significant increase in the PPR (1.82 ± 0.05, P=0.015; Fig 5.4A, B & C),demonstrating a decrease in release probability at Schaffer collateral-CA1 synapses of *Wnt7a-/-;Dvl1-/-* mice. The mean amplitude of the first EPSC response was also significantly smaller in *Wnt7a-/-;Dvl1-/-* cells (*wildtype* = 59.4 ± 9.6pA, *Wnt7a-/-;Dvl1-/-* = 28.3 ± 3.1pA, P=0.007; Fig5.4A & D), though the caveat that the stimulus intensity was varied between cells should be taken into consideration here, and EPSC amplitude varied much more widely from cell to cell than PPR in *wildtype* cells (see error bars in Fig 5.4C & D). Interestingly, EPSC amplitude was much less varied in *Wnt7a-/-;Dvl1-/-* cells (range of 12.7 - 52.7pA), with the majority of the cells within the range of 1-3 mEPSCs (based on the mean mEPSC amplitude measured from *Wnt7a-/-;Dvl1-/-* slices). The PPR was also measured in CA1 cells of slices from single *Dvl1-/-* mutant mice. In these cells PPR and peak EPSC amplitude were not significantly different from *wildtype* cells, though there was a trend for reduced amplitude (PPR = 1.59 ± 0.08, A1 = 39.1 ± 6.6pA, P=0.71 and 0.09 respectively; Fig 5.4A-D). These results indicate that release probability is normal at CA3-CA1 synapses of *Dvl1-/-* mice, but is reduced in *Wnt7a-/-;Dvl1-/-* mice.

I also measured IPSCs in CA1 cells in response to stimuli of increasing intensity. For these experiments the stimulating electrode was placed in the same area as for evoking EPSCs; indeed for the majority of recordings EPSC and IPSC responses were measured from the same cell (see materials and methods for details). As CA3-CA1 synapses are purely glutamatergic, the IPSC responses recorded represent GABA release from axons of local interneurons. The proportion of cells exhibiting a response at each stimulus intensity was very similar to that seen for EPSCs, and did not differ between *wildtype* and *Wnt7a-/-;Dvl1-/-* slices (*wildtype*: 3V – 0/12 cells, 6V – 8/12 cells, 9V – 12/12 cells, 12V – 12/12 cells, 15V – 12/12 cells; *Wnt7a-/-;Dvl1-/-*: 3V – 0/10 cells, 6V – 8/10 cells, 9V – 10/10 cells, 12V – 10/10 cells, 15V – 10/10 cells). Unlike the EPSC responses however, there was no difference in IPSC amplitude between *wildtype* and *Wnt7a-/-;Dvl1-/-* cells at any of the stimulus intensities used (Fig 5.5A & B). There



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were also no differences in either the $tau_{(rise)}$ or the $tau_{(decay)}$ at any of the stimulus intensities (Fig 5.5C & D). The IPSC PPR was also measured for slices from *wildtype*, *Dvl1-/-* and *Wnt7a-/-;Dvl1-/-* mice, using an interstimulus interval of 100ms. The mean *wildtype* IPSC PPR was 1.02±0.03, with most individual cells exhibiting either slight

Figure 5.6 Inhibitory synapses onto CA1 cells of *Wnt7a-/-;Dvl1-/-* mice exhibit normal presynaptic release. CA1 cells in acute hippocampal slices from P20-P24 *wildtype, Wnt7a+/+;Dvl1-/-* or *Wnt7a-/-;Dvl1-/-* mice were whole cell patch-clamped and the IPSCs elicited by pairs of stimuli were recorded. (A) Overlays of five consecutive responses (*left*) and averages of all responses (*right*) from example cells from *wildtype, Wnt7a+/+;Dvl1-/-* or *Wnt7a-/-;Dvl1-/-* slices. (B) Overlay of the average traces shown in (A), scaled and aligned to the peak of the first IPSC. The peak amplitude of the second response (A2) relative to the first (A1), and therefore the paired-pulse ratio (PPR=A2/A1), is unchanged between genotypes. (C) Quantification of mean PPR reveals no differences between genotypes. (D) Quantification of the mean peak amplitude of the first IPSC also reveals no differences between genotypes. The numbers at the base of bars show the number of cells recorded from.

facilitation or depression (PPR range of 0.89 – 1.19), in agreement with previous studies(Jiang et al., 2000). No significant differences were observed between any of the genotypes in either the PPR (*wildtype* = 1.02 ± 0.03, *Dvl1-/-* =1.04 ± 0.04, *Wnt7a-/; Dvl1-/-* = 0.96 ± 0.02) or peak IPSC amplitude (*wildtype* = 74.8 ± 35.3pA, *Dvl1-/* -= 60.0 ± 13.2pA, *Wnt7a-/-;Dvl1-/-* = 59.7±11.8pA) (Fig 5.6). Therefore the reduction in release probability at hippocampal synapses of *Wnt7a-/-;Dvl1-/-* mice is specific to excitatory synapses.

5.2.3 Blockade of Wnt signalling disrupts evoked release specifically at excitatory synapses in hippocampal cultures

Acute slices from *Wnt7a-/-;Dvl1-/-* mice exhibit a defect in release probability at glutamatergic synapses, providing evidence that Wnt signalling plays a role in regulating neurotransmitter release *in vivo*. However a possible criticism of this interpretation is that these mice are not conditional knockouts, and the defects in synaptic function observed could be secondary to developmental defects. In order to address this, I performed complementary *in vitro* experiments, in which mature (21 DIV) hippocampal cultures were treated acutely (for 3 hours) with the sFRP cocktail or with vehicle. The I/O relationship and PPR of EPSCs and IPSCs were then measured in whole-cell patch-clamped pyramidal neurons using the same methods that were employed in the acute hippocampal slice experiments described above.

Evoked EPSCs in control cultures were similar to EPSCs in slices in terms of their sensitivity to stimulus intensity. The proportion of cells exhibiting a measurable EPSC at each intensity was also similar between vehicle and sFRP treated cells (vehicle: 3V – 0/14 cells, 6V – 4/14 cells, 9V – 13/14 cells, 12V – 14/14 cells, 15V – 14/14 cells;

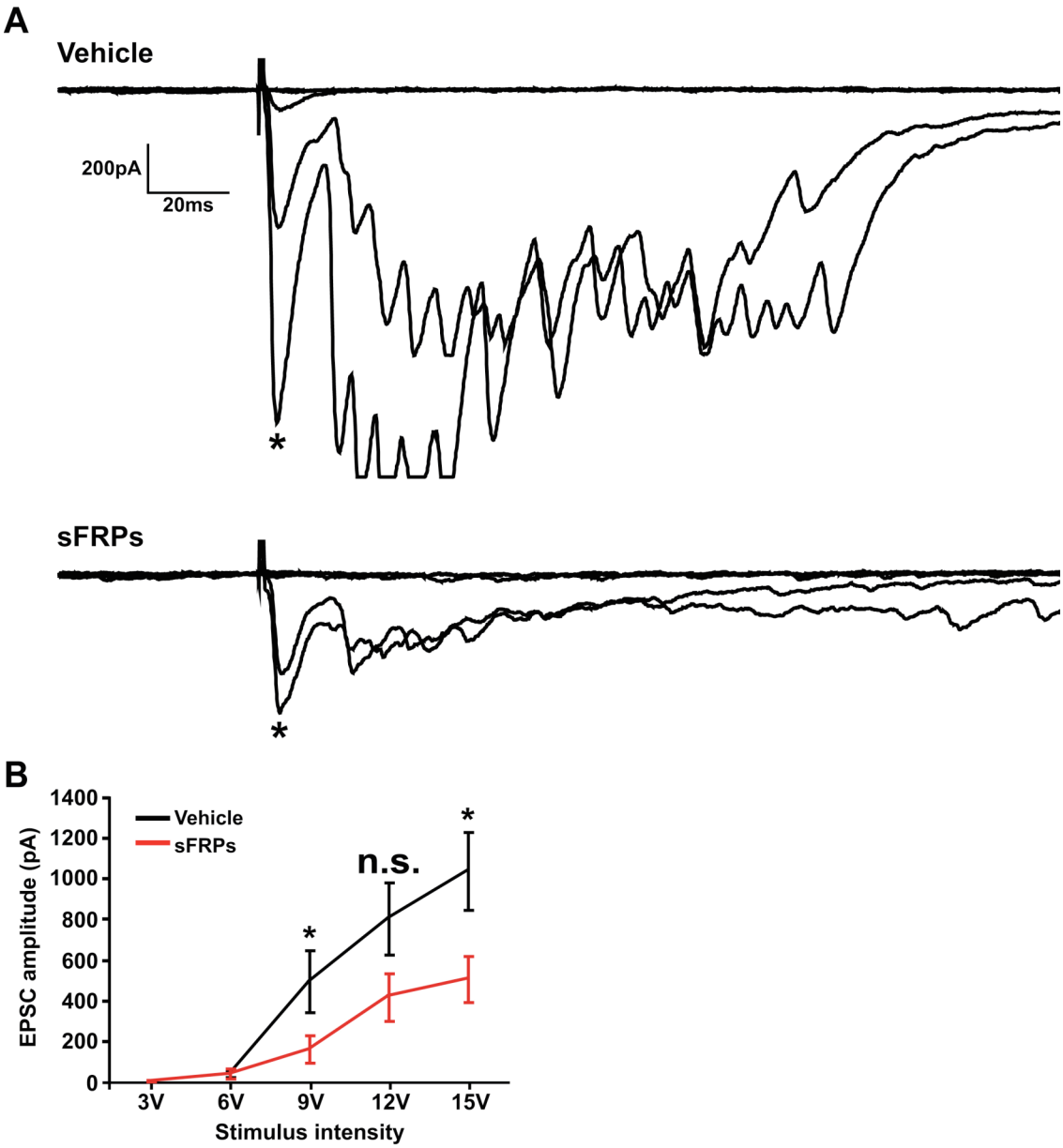


Figure 5.7 Basal excitatory transmission is impaired in mature hippocampal cultures following acute exposure to sFRPs. 21 DIV hippocampal cultures were treated with a cocktail of sFRP1, 2 and 3 or vehicle for 3 hours. Pyramidal neurons were whole cell patch-clamped and the EPSCs elicited by stimuli of increasing strength were recorded. (A) Overlays of EPSCs in response to increasing stimulus strength in vehicle or sFRP treated cells. Each trace is the average of 3-5 individual responses from a representative cell. Recurrent activity can be seen following the initial EPSC response (marked by an asterisk) – this recurrent activity is much more pronounced in the vehicle treated example. A clear reduction in amplitude in the initial EPSC amplitude is seen in response to the sFRP cocktail at higher stimulus intensities. (B) Quantification of mean EPSC amplitude showing a reduction in with sFRP treatment at stimulus intensities of 9 (68% reduction) and 15 (51% reduction) volts, compared to vehicle treated cultures. The reduction at 12 volts was not statistically significant (P=0.08). * = P<0.05.

sFRP: 3V – 0/15 cells, 6V – 3/15 cells, 9V – 9/15 cells, 12V – 15/15 cells, 15V – 15/15 cells). However, EPSCs in cultures tended to be much larger than EPSCs in slices (mean EPSC amplitude at 15V in vehicle treated cells = 1037.3±190pA, range = 131.1 –

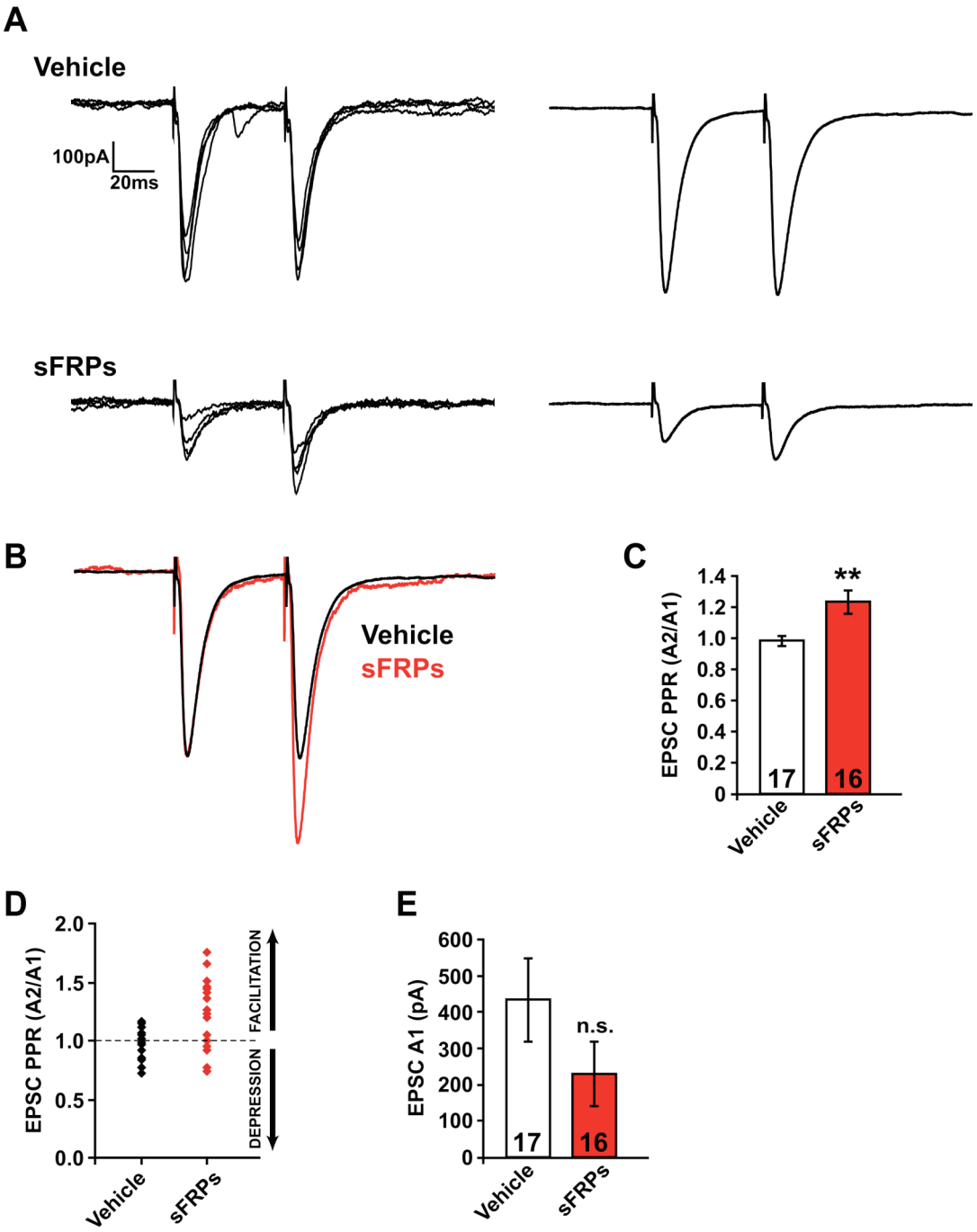


Figure 5.8 (Figure legend on next page)

2496pA; compare with *wildtype* slices, mean amplitude = 556.6±119.1, range = 120.9 – 997.5pA). Evoked EPSCs in cultures also tended to exhibit striking polysynaptic responses at higher stimulus intensities (Fig 5.7A), presumably due to the loss of the stereotypical *in vivo* cytoarchitecture that is largely retained in slice preparations. The incidence of this polysynaptic behaviour was similar between vehicle and sFRP treated cultures. Both of these differences between EPSCs in hippocampal slices and cultures have been observed previously (Maximov et al., 2007).

Figure 5.8 Excitatory synapses onto pyramidal neurons of hippocampal cultures display impaired presynaptic release following acute exposure to sFRPs. 21 DIV hippocampal cultures were treated with a cocktail of sFRP1, 2 and 3 or vehicle for 3 hours. Pyramidal neurons were whole cell patch-clamped and the EPSCs elicited by pairs of stimuli were recorded. (A) Overlays of five consecutive responses (*left*) and averages of all responses (*right*) from example cells from vehicle or sFRP treated cultures. (B) Overlay of the average traces shown in (A), scaled and aligned to the peak of the first EPSC. The peak amplitude of the second EPSC (A2) relative to the first (A1), and therefore the paired-pulse ratio (PPR=A2/A1), is larger in cultures treated with sFRPs than in vehicle treated cultures. (C) Quantification of mean PPR shows a 26% increase in response to the sFRP cocktail. (D) Scatter-plot showing the mean PPR for each cell recorded from vehicle and sFRP treated cultures. sFRP treatment results in a clear shift towards increasing PPR and facilitation of the second EPSC. (E) Quantification of the mean peak amplitude of the first EPSC reveals no significant difference between vehicle and sFRP treated cultures (but see text and Fig 5.7). The numbers at the base of bars show the number of cells recorded from. ** = $P<0.01$.

Crucially, the sFRP treated cultures display a significant reduction in mean EPSC amplitude at higher stimulus intensities compared to control cultures (Fig 5.7), mirroring the reduction seen in *Wnt7a-/-;Dvl1-/-* mice (Fig 5.3). Though only the amplitude of the EPSC immediately following the stimulus artefact was measured (asterisks in Fig 5.7A), it was noted that the amplitudes of the polysynaptic EPSCs were also consistently lower in sFRP treated cultures (Fig 5.7A). Therefore acute blockade of Wnt signalling in hippocampal cultures mimics the reduction in basal excitatory transmission observed in Wnt signalling-deficient hippocampal slices.

I also investigated the paired-pulse ratio in cultured cells to determine if release probability is also altered following acute sFRP treatment. EPSCs in vehicle treated cultures exhibited a PPR of approximately 1 (0.98±0.03; Fig 5.8A –C)), with around half of the cells exhibiting PPD (53%; Fig 5.8D). This suggests that excitatory synapses onto hippocampal pyramidal cells in culture have a significantly higher release probability than in slices (the majority of *wildtype* cells displayed facilitating behaviour, with a mean PPR of 1.63), as has been previously observed (Deuchars and Thomson, 1996; Heine et al., 2008; Kaplan et al., 2003). Importantly, acute sFRP treatment resulted in a striking change in short term plasticity at excitatory synapses, with the majority of cells now exhibiting PPF (69%, compared to 43% in vehicle treated cultures; Fig5.8D) resulting in a significant increase in the PPR compared to vehicle treated cells (vehicle: PPR = 0.98±0.03; sFRP: PPR = 1.23±0.08; Fig 5.8A - D). Therefore acute blockade of Wnt signalling in cultures produces defects in excitatory presynaptic release probability similar to those observed in *Wnt7a-/-;Dvl1-/-* mice.

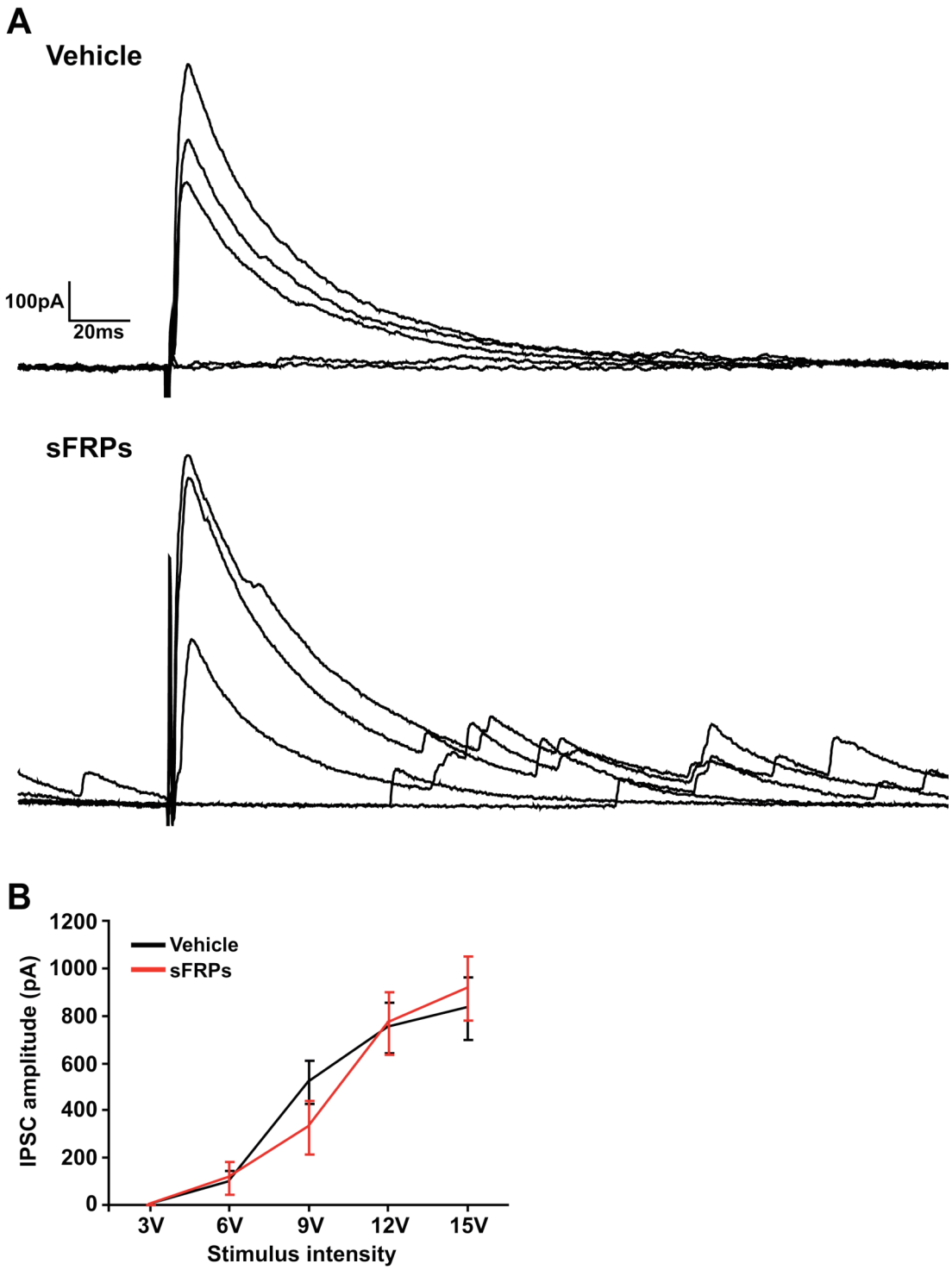


Figure 5.9 Basal inhibitory transmission is normal in mature hippocampal cultures following acute exposure to sFRPs. 21 DIV hippocampal cultures were treated with a cocktail of sFRP1, 2 and 3 or vehicle for 3 hours. Pyramidal neurons were whole cell patch-clamped and the IPSCs elicited by stimuli of increasing strength were recorded. (A) Overlays of IPSCs in response to increasing stimulus strength in vehicle or sFRP treated cells. Each trace is the average of 3-5 individual responses from a representative cell. The responses to a given stimulus intensity are similar between the vehicle and sFRP treated cells. Note that the particular example shown for sFRP treatment also displayed a high degree of spontaneous activity; there was no obvious difference in spontaneous inhibitory activity overall between the two conditions. (B) Quantification of mean IPSC amplitude shows no differences between vehicle and control treated cultures at any of the stimulus intensities.

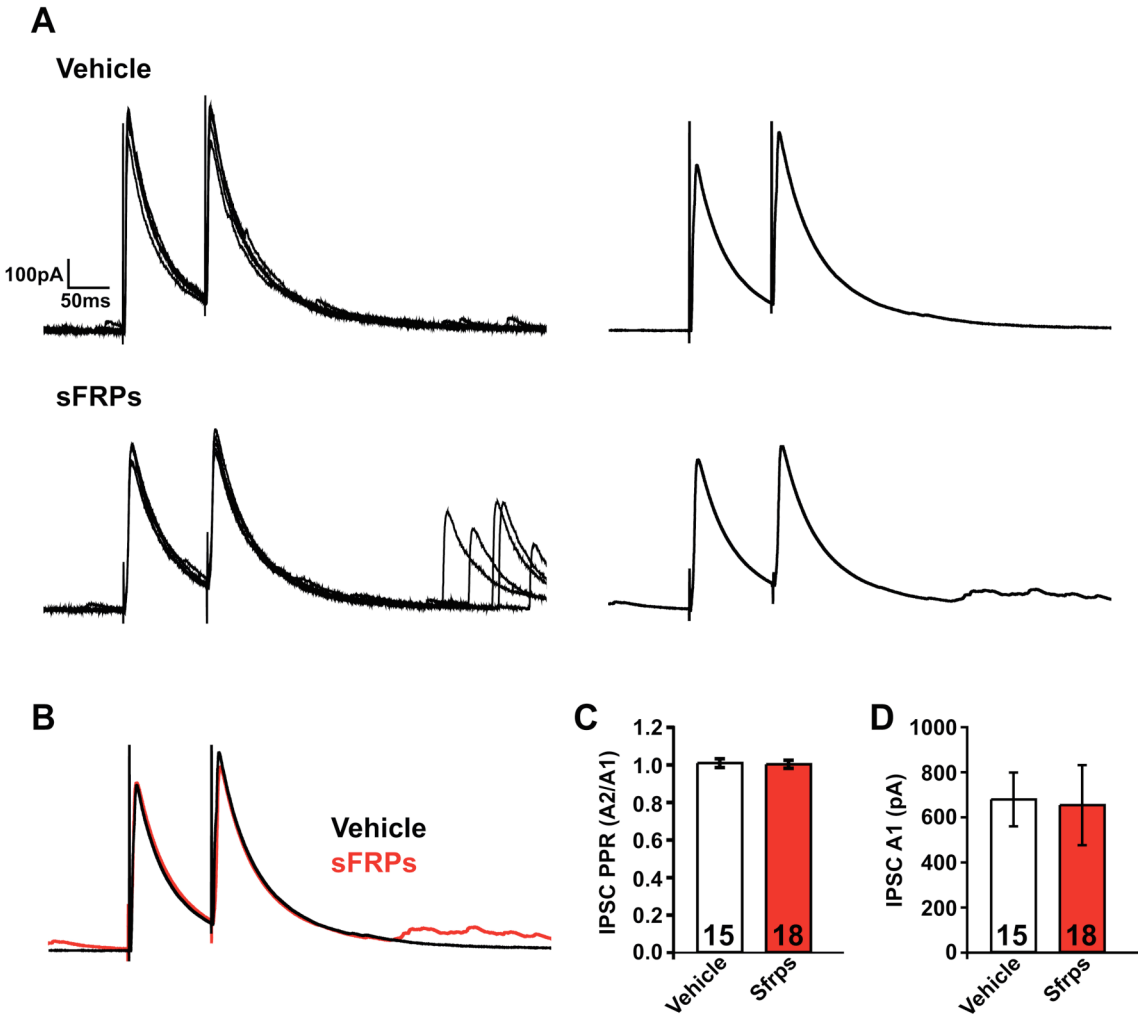


Figure 5.10 Inhibitory synapses onto pyramidal neurons of hippocampal cultures display normal presynaptic release following acute exposure to sFRPs. 21 DIV hippocampal cultures were treated with a cocktail of sFRP1, 2 and 3 or vehicle for 3 hours. Pyramidal neurons were whole cell patch-clamped and the IPSCs elicited by pairs of stimuli were recorded. (A) Overlays of five consecutive responses (*left*) and averages of all responses (*right*) from example cells from vehicle or sFRP treated cultures. Note that the particular example shown for sFRP treatment also displayed a high degree of spontaneous activity; there was no obvious difference in spontaneous inhibitory activity overall between the two conditions. (B) Overlay of the average traces shown in (A), scaled and aligned to the peak of the first IPSC. The peak amplitude of the second IPSC (A2) relative to the first (A1), and therefore the paired-pulse ratio (PPR=A2/A1), is unchanged between vehicle and sFRP treated cultures. (C) Quantification of mean PPR. (D) Quantification of the mean peak amplitude of the first IPSC reveals no differences between vehicle and sFRP treated cultures. The numbers at the base of bars show the number of cells recorded from.

I next analysed inhibitory transmission in control and sFRP treated cultures. Like EPSCs, IPSCs in cultures were similar to IPSCs in slices in terms of their sensitivity to stimulus intensity. The proportion of cells exhibiting a measurable IPSC at each intensity was similar between vehicle and sFRP treated cells in culture (vehicle: 3V - 0/15 cells, 6V – 4/15 cells, 9V – 14/15 cells, 12V – 15/15 cells, 15V – 15/15 cells;

sFRP: 3V – 0/18 cells, 6V – 4/18 cells, 9V – 11/18 cells, 12V – 18/18 cells, 15V – 18/18 cells). IPSCs evoked in cultures also tended to be larger than IPSCs evoked in slices (mean IPSC amplitude at 15V in vehicle treated cells = 830.2±134.1pA, range = 194 – 2208pA; compare with *wildtype* slices, mean amplitude = 398.8±61.5, range = 169.1 – 899.2pA), as has previously been observed (Ivanova et al., 2002; Jiang et al., 2000).

Importantly, the amplitude of IPSCs did not differ significantly between vehicle and sFRP treated cultures at any of the stimulus intensities used (Fig 5.9), demonstrating that the sFRP-induced reduction in basal synaptic transmission in cultures is specific to excitatory synapses and mimics the defect seen in *Wnt7a-/-;Dvl1-/-* mice. Furthermore, when IPSC PPR was measured there were no detectable differences between vehicle and sFRP treated cultures (vehicle: PPR = 1.01±0.02; sFRP: PPR = 1.00±0.02, P=0.85; Fig 5.10A-C), and the mean peak amplitudes of the first IPSC responses were also similar (vehicle: A1 = 677.1±119.5pA; sFRP: A1 = 651.6±177.6pA, P=0.91; Fig 5.10A & D), demonstrating that release probability is normal at inhibitory synapses of sFRP treated pyramidal cells in culture. Therefore acute blockade of Wnt signalling with the sFRP cocktail in hippocampal cultures recapitulates the decrease in release probability observed specifically at excitatory synapses of *Wnt7a-/-;Dvl1-/-* mice. These data show that Wnt signalling plays an important role in maintaining normal neurotransmission at mature glutamatergic synapses of the hippocampus.

5.2.4 Miniature neurotransmission is also disrupted at excitatory, but not inhibitory, synapses in hippocampal cultures treated with a cocktail of sFRPs

In hippocampal slices it was seen that loss of both Wnt7a and Dvl1 decreased evoked excitatory release probability, yet mEPSC frequency was normal. A possible explanation of this is that some compensatory mechanism acts to normalise mEPSC frequency during the development of *Wnt7a-/-;Dvl1-/-* mice. If this is the case, one might detect changes in mEPSCs when Wnt signalling is instead blocked acutely. Accordingly, I looked at excitatory and inhibitory miniature neurotransmission in 21 DIV cultures treated for 3 hours with vehicle or sFRP cocktail. Indeed this is what I observed, with mEPSC frequency decreasing almost two-fold in response to the sFRP cocktail (vehicle = 3.89±0.62Hz, sFRP = 2.23±0.33Hz, P=0.04; Fig 5.11A & C).

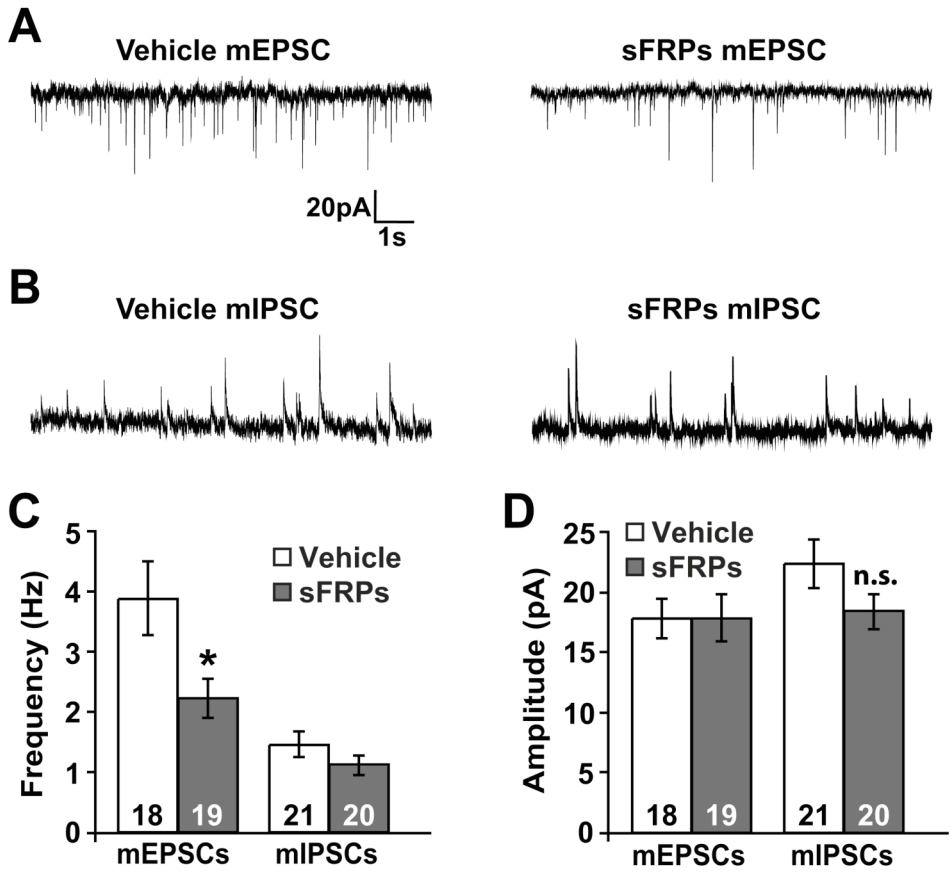


Figure 5.11 Acute blockade of Wnt signalling by sFRP treatment reduces excitatory, but not inhibitory, miniature transmission in mature hippocampal neurons. 21 DIV hippocampal cultures were treated with a cocktail of sFRP1, 2 and 3 or vehicle for 3 hours. Pyramidal neurons were whole cell patch-clamped and mEPSCs and mIPSCs were recorded. (A) sFRP treatment results in a decrease in mEPSC frequency compared to vehicle treated controls, without affecting mEPSC amplitude. (B) sFRP treatment does not change either the frequency or amplitude of mIPSCs. (C) Quantification of mean mini frequency reveals a 43% decrease in mEPSC frequency in response to sFRP treatment, whereas mIPSC frequency is unchanged. (D) Quantification of mean mini amplitude reveals no significant differences between vehicle and sFRP treatments for both mEPSCs and mIPSCs. The numbers at the base of bars show the number of cells recorded from. * = $P<0.05$.

mEPSC amplitude was similar between vehicle and sFRP treated cultures however (Fig 5.11A & D), arguing for a presynaptic action of the sFRP cocktail on mEPSCs. Importantly, inhibitory currents were once again unaffected by sFRP treatment; no significant differences were observed in either mIPSC frequency or amplitude in response to sFRP treatment (Fig 5.11B - D). Therefore acute blockade of Wnt signalling in mature hippocampal cultures specifically reduces both evoked and miniature excitatory neurotransmission.

I have already demonstrated that in less mature cultures (12-14 DIV) Wnt signalling acts to promote formation of excitatory synapses (see Chapters 3 and 4). mPSC

frequency is also dependent on synapse number; therefore it is possible that the decrease in mEPSC frequency observed n 21 DIV cultures in response to sFRPs is due to a decrease in synapse number rather than changes in presynaptic glutamate release. To investigate this, I examined the number of synaptic sites in these cultures by immunostaining for vGlut and PSD-95 or vGAT and Gephyrin. I also examined the effect of Wnt7a for 3 hours, to assess whether mature cultures (21 DIV) respond to Wnt7a in a similar manner to 14 DIV cultures.

In contrast to its effect at 14 DIV, Wnt7a did not increase excitatory synapse number in 21 DIV cultures (Fig 5.12 – compare with Fig 3.1), suggesting the synaptogenic action of Wnt7a is lost as cultures mature. However, sFRP treatment led to a significant 23% decrease in the density of excitatory synapses (defined as vGlut puncta apposed to PSD-95 puncta) (Fig 5.12A & D). This appears to be principally due to a decrease in the density of vGlut puncta (Fig 5.12B), resulting in a decrease in the percentage of PSD-95 puncta that co-localise with vGlut puncta (Vehicle = 67.6%; Sfrp = 54.9%: $P < 0.01$). There were no differences in vGlut or PSD-95 puncta volume between vehicle and Wnt7a or sFRP treated cultures (vGlut1: 0.38±0.02μm^3 (vehicle), 0.43±0.03μm^3 (Wnt7a) and 0.35±0.02μm^3 (sFRPs); PSD-95: 0.37±0.01μm^3 (vehicle), 0.36±0.01μm^3 (Wnt7a) and 0.37±0.02μm^3 (sFRPs)). The fact that excitatory synapse number is decreased in response to acute sFRP treatment in 21 DIV cultures could potentially explain the decrease in mEPSC frequency observed under the same conditions. However there are two caveats to this. Firstly, the quantification shown in Figure 5.12 is the pooled data from three experiments. However, the decrease in vGlut density was seen in only one of the three experiments (by comparison the increase in excitatory synapse number in response to Wnt7a in 14 DIV cultures was seen consistently across three experiments), and so this result should be regarded with some caution. Secondly, all other things being equal, changes in mEPSC frequency should be directly proportional to changes in excitatory synapse number. However acute sFRP treatment results in a 43% decrease in mEPSC frequency, but only a 23% decrease in excitatory synapse number. Therefore even if the decrease in excitatory synapse number is a real affect of sFRP treatment, it would appear insufficient to account fully for the defect in mEPSC frequency. Taking these points into account, the results presented in Figure 5.12 are consistent with the idea that blockade of Wnt signalling in mature cultures decreases release probability at glutamatergic synapses.

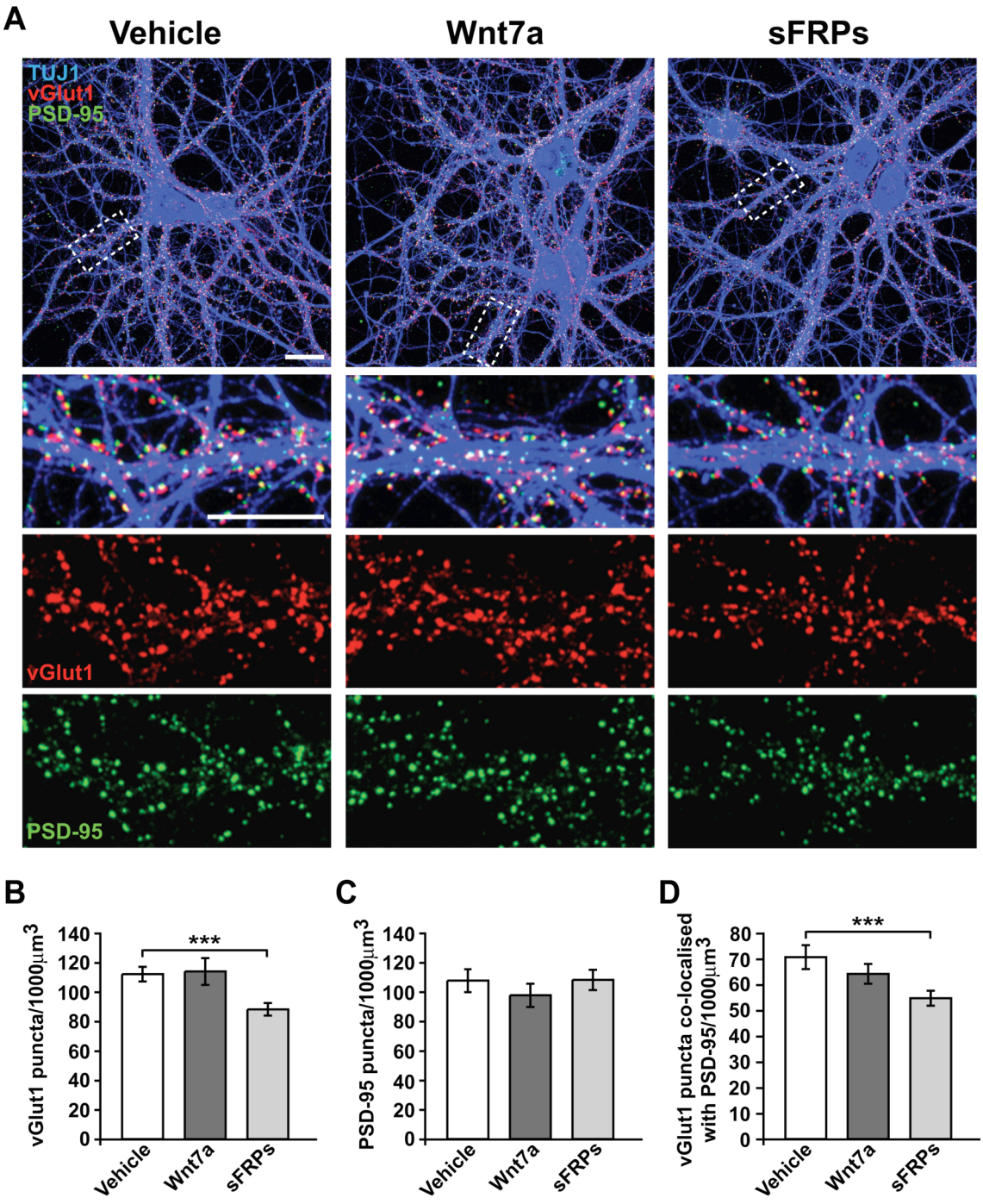


Figure 5.12 Acute blockade of Wnt signalling by sFRP treatment reduces excitatory synapse number in mature hippocampal cultures. 21 DIV hippocampal cultures were treated with purified Wnt7a, a cocktail of sFRP1, 2 and 3 or vehicle for 3 hours. (A) Images of treated neurons immunostained for the excitatory presynaptic marker vGlut1 (red) the postsynaptic marker PSD-95 (green), and the cytoskeletal marker TUJ1 (blue). White boxes indicate enlarged regions. Scale bars = 20μm in top panels, 10μm in enlarged panels. (B) Quantification of vGlut1 puncta density reveals no change in response to Wnt7a, but a 24% decrease in response to sFRPs, compared to vehicle treated cultures. (C) Quantification of PSD-95 puncta density reveals no difference between any of the conditions. (D) sFRP treatment induces a 23% decrease in the density of vGlut puncta that co-localise with PSD-95 (putative excitatory synapses) compared to vehicle treated cultures; Wnt7a treatment has no effect. *** = P<0.001.

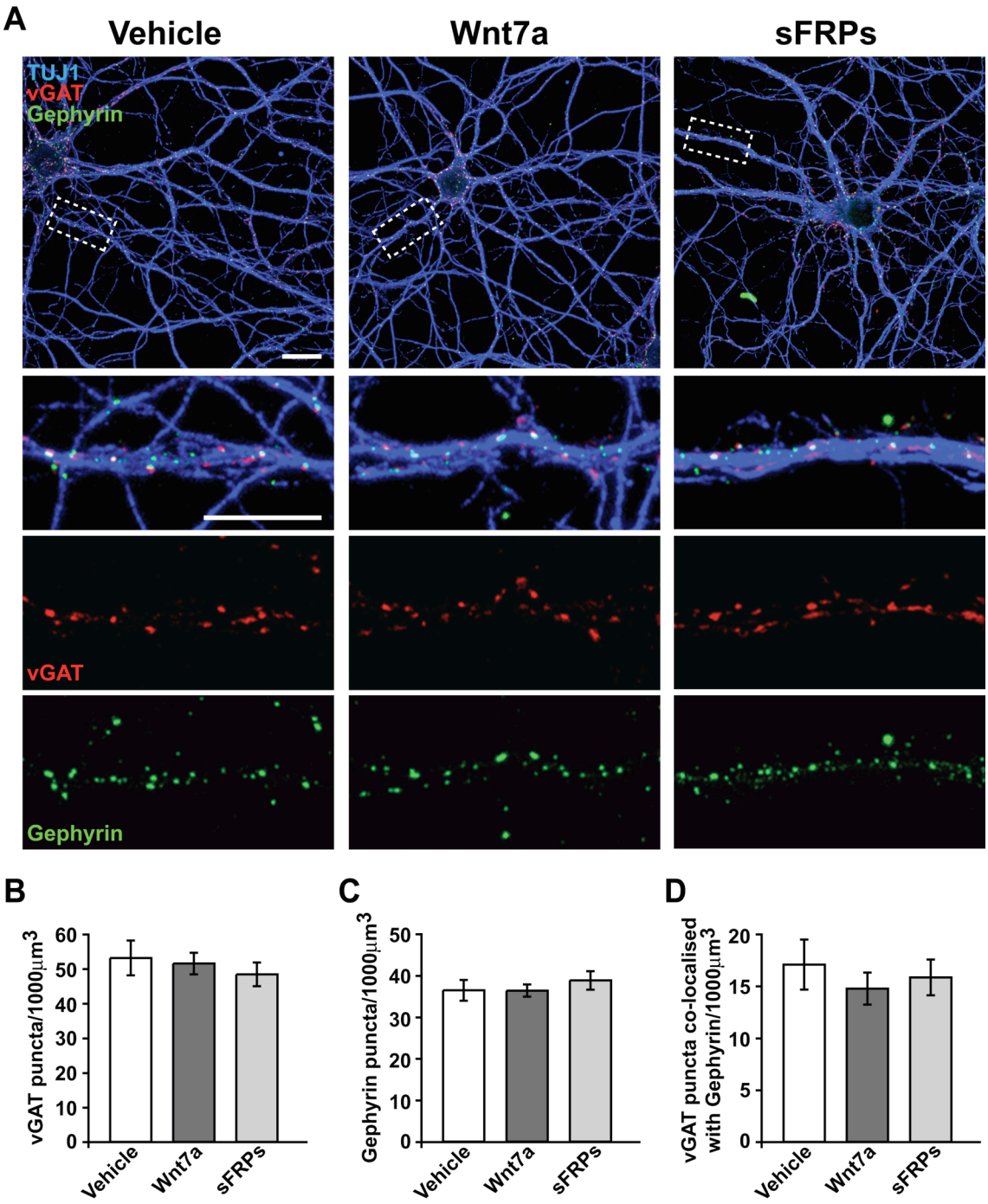


Figure 5.13 Inhibitory synapse number in mature hippocampal cultures is unaffected by acute treatment with purified Wnt7a or sFRP cocktail. 21 DIV hippocampal cultures were treated with purified Wnt7a, a cocktail of sFRP1, 2 and 3 or vehicle for 3 hours. (A) Images of treated neurons immunostained for the inhibitory presynaptic marker vGAT (red) and postsynaptic marker gephyrin (green), and TUJ1 (blue). White boxes indicate enlarged regions. Scale bars = 20μm in top panels, 10μm in enlarged panels. (B) and (C) Quantification reveals no effect of Wnt7a or sFRP on vGAT (B) or gephyrin (C) puncta density. (D) Quantification shows that the density of vGAT puncta that co-localise with gephyrin (putative inhibitory synapses) is unaffected by Wnt7a or sFRP treatment.

Consistent with the electrophysiological data presented so far in this chapter, inhibitory synapses were unaffected by acute Wnt7a or sFRP cocktail treatment in 21 DIV cultures in terms of vGAT and Gephyrin puncta density and inhibitory synapse number (Fig5.13). The percentage co-localisation between and volume of vGAT and Gephyrin puncta were also unchanged between vehicle and Wnt7a or sFRP treated cultures. These results again demonstrate that blockade of Wnt signalling with the sFRP cocktail specifically affects glutamatergic synapses, whereas GABAergic synapses are unaffected.

5.3 Discussion

5.3.1 Miniature neurotransmission is largely unaffected at CA1 cells of Wnt7a-/-;Dvl1-/- mice

The frequency and amplitude of mEPSCs and mIPSCs was normal at CA1 cells of acute hippocampal slices taken from *Wnt7a-/-;Dvl1-/-* mice. This was surprising given the established role of Wnts in excitatory synapse formation. A possible explanation for the lack of an mEPSC phenotype is provided by a recent study, published after the experiment shown in Figure 5.1 was performed (Gogolla et al., 2009). In this study it was found that Wnt7a applied directly to the CA3 *in vivo* increased the number of synapses between mossy fibres of dentate granule cells and CA3 cells, whereas sFRP-1 application reduced synapse number. However when Wnt7a or sFRP-1 were applied to the CA1 region, no changes in synapse number were observed. Furthermore, pharmacologically manipulating activity levels in the hippocampus resulted in changes in Wnt7a/b expression in the CA3 but not CA1 region (Gogolla et al., 2009). Therefore Wnt7a may not only selectively regulate formation of glutamatergic synapses in the hippocampus but, even more specifically, excitatory synapses in certain regions. This raises the intriguing possibility that Wnts show great specificity with regards to the subtypes of synapse they regulate. Taking this into account, it will be of interest to record mPSCs from CA3 neurons of *Wnt7a-/-* and *Wnt7a-/-;Dvl1-/-* mice, as the results of Gogolla et al predict that here a decrease in mEPSC frequency would be observed.

mEPSCs at CA1 cells of *Wnt7a-/-;Dvl1-/-* mice were not completely normal however – they did display a significant slowing of the mEPSC rise time. This fits well with the Dvl1 gain of function experiment presented in Chapter 4, as Dvl1 overexpression

resulted in a quickening of the rise time (Fig 4.2). The underlying cause of the change in mEPSC rise time in *Wnt7a-/-;Dvl1-/-* CA1 cells is unknown and will require further experimentation to elucidate. As was previously outlined in Chapter 4, factors worth investigating include potential changes in AMPA receptor subunit composition (Dingledine et al., 1999), AMPA receptor regulatory proteins (Milstein and Nicoll, 2008) and dendritic morphology (Spruston, 2008) of hippocampal pyramidal cells of *Wnt7a-/-;Dvl1-/-* mice. Regardless of the mechanism, the fact that Wnt signalling is able to regulate the waveform of excitatory synaptic currents provides another mechanism by which they may regulate glutamatergic transmission.

5.3.2 Wnt7a acts to maintain normal glutamatergic release

In acute hippocampal slices taken from ~P21 *Wnt7a-/-;Dvl1-/-* mice, glutamatergic Schaffer collateral-CA1 synapses exhibited a decreased input-output relationship and increased PPR compared to slices from *wildtype* mice, demonstrating that release probability is reduced at this synapse. Note that this is a presynaptic defect; i.e. a defect in glutamate release from boutons of Schaeffer collaterals of CA3 cells, consistent with the proposal that Wnt7a signalling plays more of a role in regulating CA3 than CA1 cell function (Gogolla et al., 2009). However this does not rule out the possibility that CA1 cells of Wnt7a-deficient mice display postsynaptic defects. Although a possible effect of Wnt7a signalling on CA1 postsynaptic function needs to be further examined, my results demonstrate that Wnt7a signalling through Dvl1 is required for proper neurotransmitter release at the CA3-CA1 synapse.

Dvl1-/- mice did not display an increase in PPR (Fig 5.4). This might be considered surprising, as Dvl is downstream of Wnt7a and therefore one might expect the *Dvl1-/-* mutant to phenocopy the *Wnt7a-/-;Dvl1-/-* mutant. However it should be remembered that the *Dvl1-/-* and *Wnt7a-/-;Dvl1-/-* mice are hypomorphs in some respects, in that they will continue to express other Wnt and Dvl family members. Therefore it is possible that in the *Dvl1-/-* mutant Wnt7a is still able to signal through Dvl2 and/or Dvl3 to regulate release probability. The fact that *Wnt7a-/-;Dvl1-/-* mice, but not *Dvl1-/-* mice, display a defect in release at CA3-CA1 synapses does on the other hand argue strongly for a crucial role of Wnt7a in this process. This is in agreement with a previous study that found that application of exogenous Wnt7a in hippocampal cultures reduces PPR and increases synaptic vesicle cycling and mEPSC frequency, indicating an

increase in release probability (Cerpa et al., 2008). Crucially, I found that acute blockade of Wnt signalling in hippocampal cultures phenocopied the defect seen in the *Wnt7a-/-;Dvl1-/-* mutant, as sFRP treated pyramidal cells exhibited a decreased input-output relationship and increased PPR. Together, these data represent the first reports on the effects of decreasing endogenous levels of Wnt activity on synaptic transmission in the vertebrate CNS, and argue strongly that Wnt7a acts to maintain the basal release probability at glutamatergic synapses onto hippocampal pyramidal neurons.

This raises the intriguing question; how general is this role of Wnts in regulating hippocampal excitatory synaptic function? Do all Wnts expressed in the postnatal hippocampus share this role? Interestingly, the 'non-canonical' Wnt5a, unlike Wnt7a, does not increase synaptic vesicle recycling, nor does it have any effect on EPSC PPR in hippocampal cultures (Cerpa et al., 2008). Furthermore, both Wnt5a and Wnt3a have been shown to have no effect on spontaneous EPSC frequency in hippocampal cultures (Beaumont et al., 2007), whereas Wnt7a increases mEPSC frequency (Cerpa et al., 2008). Therefore, it seems that Wnts expressed in the postnatal hippocampus may differ in their ability to regulate excitatory neurotransmission. This could potentially be achieved by distinct populations of hippocampal neurons differing in their abilities to respond to a given Wnt, due to the particular Wnt receptors they express and/or the endogenous Wnt antagonists present. Indeed, different Fz receptors and sFRPs show specific expression patterns within the CA region of the postnatal hippocampus (Shimogori et al., 2004).

Wnt7a treatment at 21 DIV had no effect on the number of excitatory synapses, compared to the increase observed at 14 DIV (Fig 3.1). However it should be noted that the duration of treatment varies between these two experiments i.e. 3 hours of Wnt7a exposure at 21 DIV compared to 20 hours at 14 DIV. The effects of overnight treatment with Wnt7a and sFRPs in mature cultures will be dealt with in the next chapter. Acute treatment with the sFRP cocktail at 21 DIV did however result in a significant decrease in excitatory synapse number. As mentioned in the results section, this should be regarded with some caution, as this effect was only observed in one out of the three cultures used for the experiment. If this effect is genuine however, it would suggest that Wnts play a role either in the formation of new synapses or the maintenance of existing synapses in mature cultures. As the synaptogenic rate in mature cultures is relatively low, the second option appears more likely. Indeed there is evidence that endogenous

Wnts play a role in synaptic maintenance, as treatment with the canonical Wnt antagonist Dikkopf-1 results in synaptic disassembly in 14 DIV and 21 DIV hippocampal cultures (Dickins & Salinas, unpublished results). Therefore it appears Wnt signalling may play a progressive role in synapse development, promoting synapse formation at early stages and regulating synaptic function and maintenance as synapses mature.

5.3.3 Wnt7a does not regulate GABA release at inhibitory synapses

As was seen for the effects of Wnt7a on synapse formation in chapter 2, the effect of Wnt7a on regulating presynaptic release probability appears to be specific to glutamatergic synapses, as the PPR was not altered at GABAergic synapses of *Dvl1-/-* and *Wnt7a-/-; Dvl1-/-* mice or hippocampal neurons treated with sFRP cocktail. However, a potential concern with this interpretation is that the inter-stimulus interval used for recording paired-pulse responses at GABAergic synapses was twice that used for glutamatergic response (100ms compared to 50ms). As the PPR in wild-type mice and control treated neurons was on average approximately 1, and as no change was seen in any of the experimental conditions examined, it is possible that 100ms is too long an interval to result in paired-pulse interaction in the first place. However, it is important to note that although the *average* PPR of IPSCs was ~1, individual cells tended to display either slight facilitation or depression (PPR range: 0.76 – 1.31 in hippocampal slices and 0.79 – 1.21 in hippocampal cultures; see Appendix 1). Note that this is very similar to the behaviour of paired EPSCs in cultured cells, which do display changes in PPR following sFRP treatment (Fig. 5.8), and is similar to previous reports of IPSC paired-pulse responses in hippocampal preparations that were used to choose the inter-stimulus interval (Ivanova et al., 2002; Jiang et al., 2000; Kravchenko et al., 2006). Therefore it would appear that hippocampal GABAergic synapses are capable of displaying paired-pulse interactions at an inter-stimulus interval of 100ms. Furthermore, a decrease in current amplitude following a single stimulation was only seen in EPSCs, not IPSCs (Figs. 5.3, 5.5, 5.7 & 5.9), further arguing for a specific action of Wnt7a on glutamatergic transmission. Ideally though, a range of inter-stimulus intervals would have been tested for both EPSCs and IPSCs to determine the relationship between inter-stimulus interval and PPR.

CHAPTER 6:

Prolonged perturbation of Wnt signalling results in a homeostatic response that compensates for changes in release probability

6.1 Introduction

In the previous chapter I presented evidence that Wnts act to maintain a normal level of excitatory activity, as release probability is decreased at glutamatergic synapses when Wnt signalling is compromised both *in vitro* and *in vivo*. However, the *in vitro* experiments were performed over a relatively short time period, examining the effect of 3 hours of Wnt blockade with the sFRP cocktail. This leaves open the question of whether excitatory synapses will continue to display defects in release in the presence of sustained Wnt signalling blockade, or whether normal release will recover. Furthermore, if prolonged Wnt blockade *does* result in a prolonged decrease in glutamatergic signalling, what downstream effect does this have on the network as a whole?

This is an important question, as it has become clear over the last decade that neuronal networks respond dynamically to prolonged changes in overall activity. This was first demonstrated in cultured neurons of the rat visual cortex, where prolonged activity blockade (48 hour treatment with TTX) resulted in a compensatory increase in mEPSC amplitude, whereas increasing network activity (48 hour treatment with bicuculline) had the opposite effect (Turrigiano et al., 1998). The mEPSCs were found to scale multiplicatively (i.e. synaptic strength changed by a factor of the original strength at individual synapses). The authors named this phenomenon 'synaptic scaling', and proposed that it could act to maintain neuronal activity within a stable range, whilst still allowing the relative changes in individual synaptic strength necessary for network development and plasticity (Turrigiano et al., 1998; Turrigiano and Nelson, 2004). This form of homeostatic control of synaptic activity was subsequently found to operate in a variety of preparations, including cultured spinal neurons (O'Brien et al., 1998), cultured hippocampal neurons (Cingolani et al., 2008; Hou et al., 2008; Lissin et al., 1998; Sutton et al., 2006; Thiagarajan et al., 2005) and the visual cortex *in vivo* (Desai et al., 2002; Maffei et al., 2004).

Activity-dependent scaling of synaptic strength appears to depend principally on changes in the AMPA receptor content of excitatory synapses, with changes in the levels of Ca^{2+}-permeable GluR1 homomers especially important in the initial stages (Cingolani et al., 2008; Hou et al., 2008; Lissin et al., 1998; O'Brien et al., 1998; Sutton et al., 2006; Thiagarajan et al., 2005; Wierenga et al., 2005). Various signalling molecules have been implicated in the appearance of synaptic scaling including Ca^{2+} (Cingolani et al., 2008; Thiagarajan et al., 2005), PI3-kinase (Hou et al., 2008), β3 integrins (Cingolani and Goda, 2008; Cingolani et al., 2008) and glia-derived TNF-α (Stellwagen and Malenka, 2006). However it remains unclear to what degree these different signalling pathways may interact or operate redundantly in the expression of synaptic scaling.

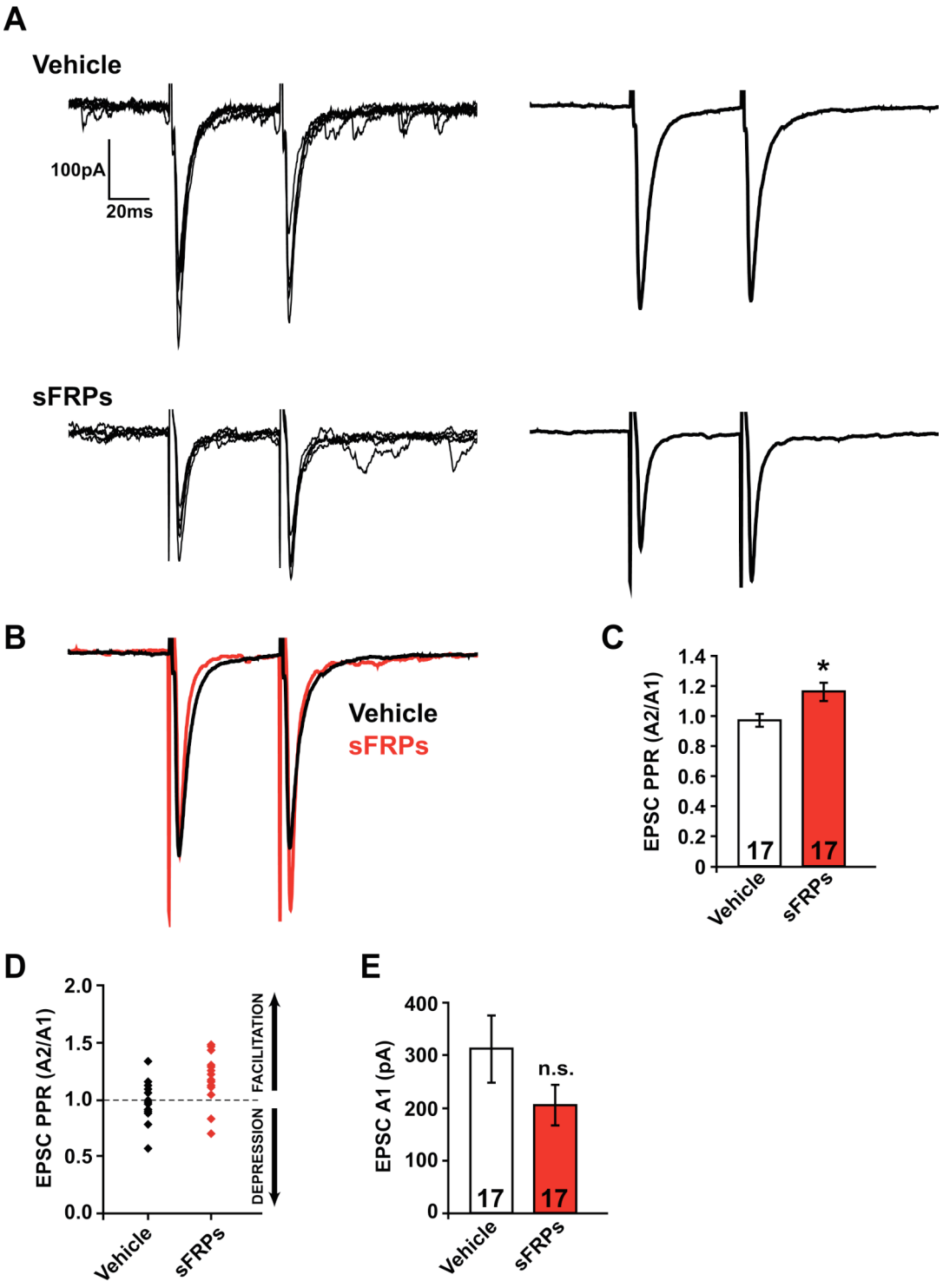
Synaptic homeostasis does not only manifest itself as postsynaptic changes resulting in altered synaptic strength. At the *Drosophila* NMJ, reducing postsynaptic activity results in a compensatory increase in presynaptic bouton size and release probability, and this correlates with changes in postsynaptic CaMKII activity (Haghighi et al., 2003; Paradis et al., 2001). A similar increase is observed in the size of and release probability at excitatory synapses of mature hippocampal cultures subjected to prolonged activity blockade (Bacci et al., 2001; Murthy et al., 2001; Thiagarajan et al., 2005). Prolonged suppression of activity also results in an increase in excitatory synapse density in mature hippocampal cultures (Burrone et al., 2002; Han and Stevens, 2009; Wierenga et al., 2006). Consequently, changes in mEPSC frequency (as well as changes in mEPSC amplitude) are observed in mature cultures in response to prolonged manipulations of activity (Bacci et al., 2001; Burrone et al., 2002; Han and Stevens, 2009; Thiagarajan et al., 2005; Thiagarajan et al., 2002; Wierenga et al., 2006). This is in contrast to younger cultures, where only synaptic scaling of mEPSC amplitude is observed (Han and Stevens, 2009; Wierenga et al., 2006). The multiplicity of mechanisms by which older cultures produce homeostatic changes in release complicates efforts to elucidate the underlying signalling involved. However it seems likely that Ca^{2+} signalling through CaMKs is involved, as levels of βCaMKII are increased following chronic TTX treatment, and overexpression of βCaMKII mimics the effect of chronic TTX treatment on mEPSC frequency and decay time (Thiagarajan et al., 2002). In summary, it can be seen that homeostatic responses to changes in overall synaptic activity are complex, with the particular response observed depending on the method used to alter activity, the preparation used, and the developmental stage of the neurons.

All of the studies discussed above involve drastic manipulations of activity levels such as complete blockade of action potential generation (e.g. TTX, overexpression of an inward-rectifying potassium channel) or global blockade of GABAergic inhibition (e.g. bicuculline or picrotoxin). Since acute blockade of Wnt signalling resulted in a relatively mild decrease in glutamatergic release probability (Fig 5.8), I was interested to see if this decrease persisted with prolonged Wnt blockade, and whether this recruited any homeostatic compensatory mechanisms. Accordingly, I exposed mature (21 DIV) hippocampal cultures to the sFRP cocktail for a prolonged period (20 hours). I find that the release probability at excitatory synapses is reduced to a similar degree as observed with acute Wnt blockade; this effect is again specific to excitatory synapses. However, in contrast to acute sFRP treatment, cultures subjected to prolonged sFRP treatment have an mEPSC frequency comparable to control cultures. This is due to a homeostatic increase in the density of excitatory synapses, which offsets the decrease in release probability at individual synapses. Conversely, prolonged Wnt7a treatment causes a reduction in excitatory synapse density. mIPSCs and inhibitory synapse density are unaffected by prolonged sFRP or Wnt7a treatment. Finally, I find that younger (12-14 DIV) cultures display a similar increase in excitatory synapses in response to prolonged sFRP treatment, but this requires a longer treatment time (48 hours) to manifest.

6.2 Results

6.2.1 Glutamatergic release probability continues to be disrupted in hippocampal cultures subjected to prolonged blockade of Wnt signalling

The *in vitro* experiments described in Chapter 6 revealed a decrease in glutamatergic release probability following relatively short periods (3 hours) of Wnt signalling blockade in mature (21 DIV) hippocampal cultures. I was therefore interested to see if more prolonged treatment (20 hours) with the sFRP cocktail would result in a similar decrease in excitatory release probability, or whether recovery of normal release would be observed. The effect of prolonged sFRP treatment on excitatory release probability was very similar to that seen following 3 hours treatment (compare Figs 5.8 and 6.1). 20 hours exposure to the sFRP cocktail resulted in a shift towards facilitating responses (% of cells exhibiting PPF: Vehicle = 31%; sFRPs = 81%) and a significant increase in the



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mean EPSC PPR (Vehicle: PPR = 0.97±0.04; sFRPs: PPR = 1.16±0.06; Fig 6.1A – D). The mean amplitude of the first EPSC response did not differ significantly betweenvehicle and sFRP treated cultures, although there was a trend for a reduction in amplitude in sFRP treated cells (Fig 6.1E). The fact that prolonged exposure to a

Figure 6.1 Excitatory synapses onto pyramidal neurons of hippocampal cultures display impaired presynaptic release following prolonged exposure to sFRPs. 21 DIV hippocampal cultures were treated with a cocktail of sFRP1, 2 and 3 or vehicle for 20 hours. Pyramidal neurons were whole cell patch-clamped and the EPSCs elicited by pairs of stimuli were recorded. (A) Overlays of five consecutive responses (*left*) and averages of all responses (*right*) from example cells from vehicle or sFRP treated cultures. (B) Overlay of the average traces shown in (A), scaled and aligned to the peak of the first EPSC. The paired-pulse ratio is increased in cultures treated with sFRPs compared to vehicle treated cultures. (C) Quantification of mean PPR shows a 19% increase in response to the sFRP cocktail. (D) Scatter-plot showing the mean PPR for each cell recorded from vehicle and sFRP treated cultures. sFRP treatment results in a clear shift towards increasing PPR and facilitation of the second EPSC. (E) Quantification of the mean peak amplitude of the first EPSC reveals no significant difference between vehicle and sFRP treated cultures. The numbers at the base of bars show the number of cells recorded from. * = $P<0.05$.

cocktail of Wnt antagonists results in a sustained defect in glutamatergic release argues strongly that endogenous Wnts act to maintain release at excitatory synapses.

Despite its effect on glutamatergic release, acute treatment with the sFRP cocktail did not affect inhibitory GABAergic release in mature hippocampal cultures (Figs 5.9 and 5.10). This is consistent with the general observation throughout Chapters 3-5 that perturbations of Wnt signalling specifically regulate excitatory synapses, whereas inhibitory synapses are unaffected. However, the possibility remained that chronic blockade of Wnt signalling in mature cultures could affect inhibitory transmission, either directly or via compensatory mechanisms in response to prolonged changes in excitatory transmission. Accordingly, I also measured the PPR of IPSCs following 20 hours treatment with the sFRP cocktail or vehicle. As was observed for 3 hours treatment, 20 hours exposure to the sFRP cocktail had no significant effect on IPSC PPR or amplitude (Vehicle: PPR = 0.99±0.02; sFRPs: PPR = 0.98±0.02; Fig 6.1A – D). Therefore, prolonged blockade of endogenous Wnt signalling in mature hippocampal cultures results in a sustained decrease in release probability at glutamatergic synapses, with no discernable effect on GABAergic release.

6.2.2 Prolonged blockade of Wnt signalling results in a homeostatic increase in excitatory synapses that normalises mEPSC frequency

Acute blockade of Wnt signalling does not only decrease evoked release at excitatory synapses but also reduces the frequency of mEPSCs in mature hippocampal cultures (Fig 5.11). This effect appears to be at least partly due to a decrease in the probability of

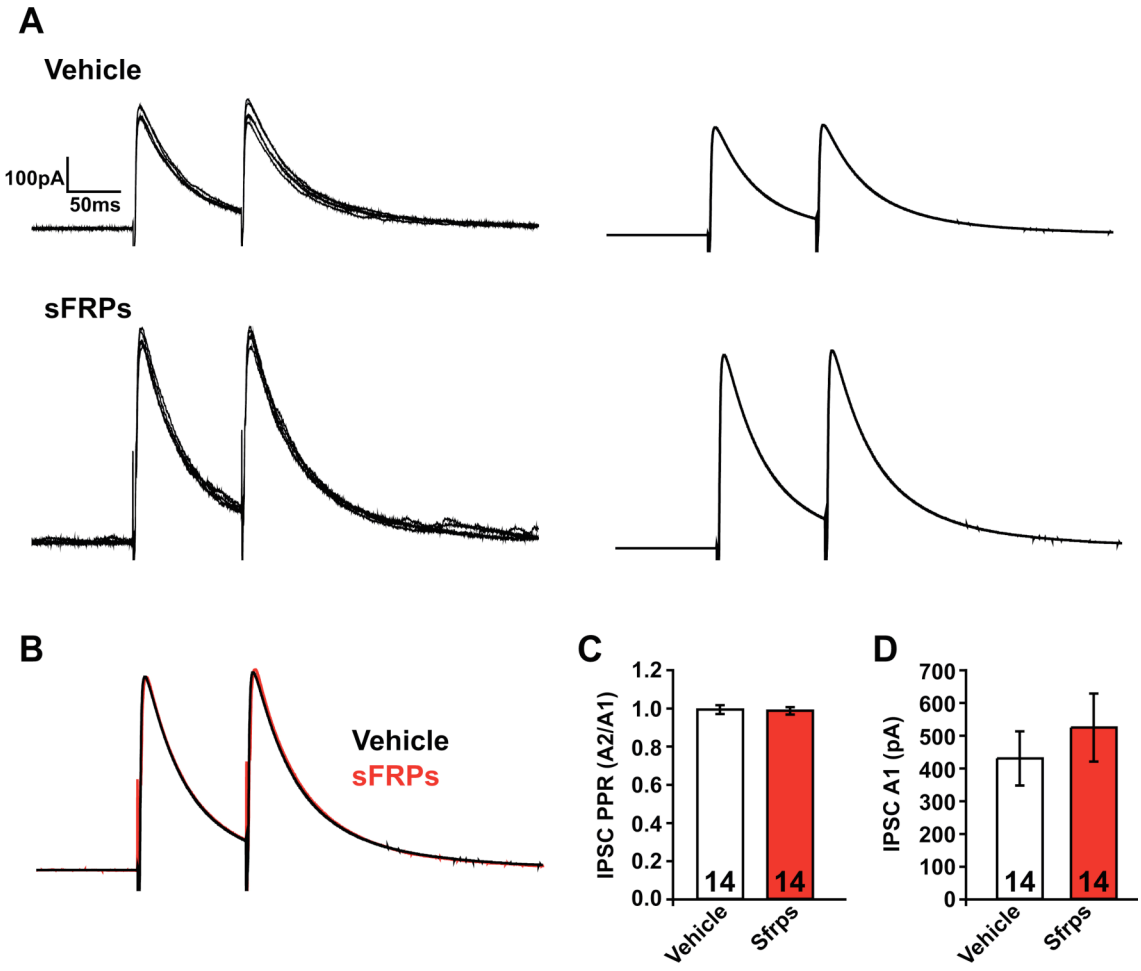

Figure 6.2 Inhibitory synapses onto pyramidal neurons of hippocampal cultures display normal presynaptic release following prolonged exposure to sFRPs. 21 DIV hippocampal cultures were treated with a cocktail of sFRP1, 2 and 3 or vehicle for 20 hours. Pyramidal neurons were whole cell patch-clamped and the IPSCs elicited by pairs of stimuli were recorded. (A) Overlays of five consecutive responses (*left*) and averages of all responses (*right*) from example cells from vehicle or sFRP treated cultures. Note that although in the examples given here the currents from the sFRP cocktail treated cell are larger than from the vehicle treated cell, on average there was no difference in IPSC amplitude (see (D)). (B) Overlay of the average traces shown in (A), scaled and aligned to the peak of the first IPSC. The paired-pulse ratio is unchanged in cultures treated with sFRPs compared to vehicle treated cultures. (C) Quantification of mean PPR. (D) Quantification of the mean peak amplitude of the first IPSC reveals no differences between vehicle and sFRP treated cultures. The numbers at the base of bars show the number of cells recorded from.

spontaneous events. To test whether this decrease in mEPSC frequency is still observed following prolonged Wnt blockade, I recorded mEPSCs from 21 DIV cultures treated with sFRPs for 20 hours. Surprisingly, mEPSC frequency was found to be similar in cultures treated with vehicle or sFRP cocktail for 20 hours (vehicle = 2.75±0.63 Hz, sFRP = 2.58±0.37 Hz; Fig 6.3A & C). mEPSC amplitude was also unchanged (vehicle = 19.6±1.5 pA, sFRP = 18.9±1.4 pA; Fig 6.3A & D), and neither the frequency nor

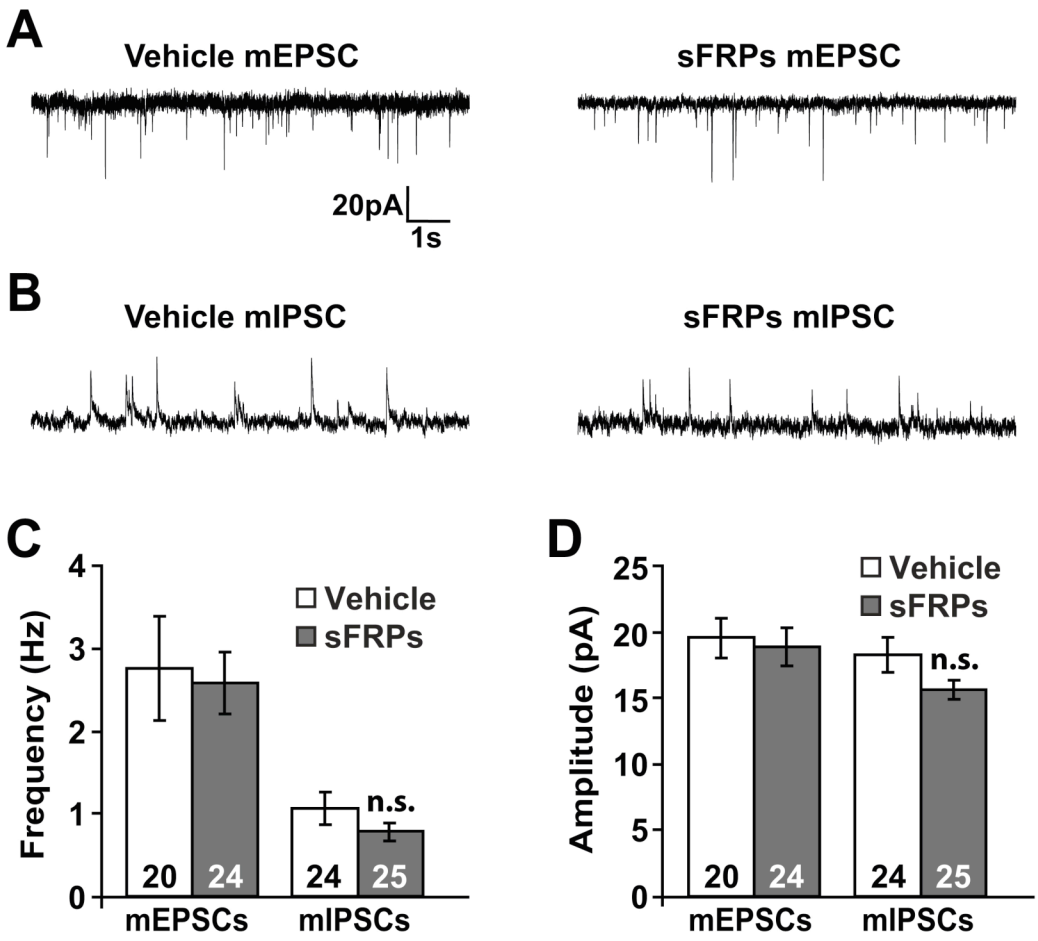

Figure 6.3 Miniature neurotransmission in mature hippocampal neurons exposed to sFRPs for 20 hours is indistinguishable from control treated neurons. 21 DIV hippocampal cultures were treated with a cocktail of sFRP1, 2 and 3 or vehicle for 20 hours. Pyramidal neurons were whole cell patch-clamped and mEPSCs and mIPSCs were recorded. Representative 10 second traces demonstrate that mEPSC (A) and mIPSC (B) frequency and amplitude are similar in sFRP and vehicle treated cells. Quantification of mean mini frequency (C) and amplitude (D) reveals no significant differences between vehicle and sFRP treatments for both mEPSCs and mIPSCs. The numbers at the base of bars show the number of cells recorded from.

amplitude of mIPSCs were significantly affected by prolonged sFRP treatment (mIPSC frequency: vehicle = 1.06±0.19 Hz, sFRP = 0.78±0.11 Hz; mIPSC amplitude: vehicle = 18.3±1.3 pA, sFRP = 15.7±0.8 pA; Fig 6.3B - D). Finally the rise and decay times of both mEPSCs and mIPSCs were similar between sFRP cocktail and vehicle treated cultures (data not shown). Therefore, despite the sustained reduction in evoked release probability at excitatory synapses, both excitatory and inhibitory miniature synaptic currents are normal in mature hippocampal cultures subjected to prolonged blockade of Wnt signalling.

Acute treatment of 21 DIV hippocampal cultures with a cocktail of sFRP-1, 2 and 3 results in both a decrease in evoked release probability and mEPSC frequency at

excitatory synapses (Figs 5.8 and 5.11), consistent with the idea that evoked and spontaneous release probability are correlated at central synapses (Prange and Murphy, 1999). Following 20 hour exposure to sFRP cocktail, mEPSC frequency is normal whereas evoked glutamate release probability is still decreased. This suggests that excitatory synapse number may be increased in these cultures to compensate for reduced glutamate release at individual synapses, as has been observed in response to prolonged TTX treatment in mature hippocampal cultures (Wierenga et al., 2006). If this is the case, prolonged Wnt7a treatment in mature cultures might result in opposite effects on excitatory synapse number compared to sFRP treatment, as acute application of Wnt7a has been shown previously to increase glutamatergic release probability and mEPSC frequency (Cerpa et al., 2008) (Anane & Salinas, unpublished results). I therefore immunostained 21 DIV cultures treated for 20 hours with vehicle, Wnt7a or sFRP cocktail with antibodies to vGlut1 and PSD-95. Chronic sFRP treatment led to a 37% increase in the density of vGlut1 puncta and a similar increase (35%) in PSD-95 puncta density, compared to vehicle treated cells (Fig 6.4A –C). The opposite effect was observed on vGlut1 puncta density following chronic Wnt7a treatment (25% decrease compared to vehicle treated cells), although the density of PSD-95 puncta was not significantly altered (Fig 6.4A –C). The proportion of vGlut1 puncta apposed to PSD-95 puncta and vice versa did not significantly differ between conditions (vGlut1 co-localisation with PSD-95: vehicle = 61.7±3.7%, Wnt7a = 56.0±3.7%, sFRPs = 65.4±2.0%; PSD-95 co-localisation with vGlut1: vehicle = 68.4±2.6%, Wnt7a = 61.0±4.1%, sFRPs = 70.5±1.7%). Accordingly, the density of excitatory synapses (defined as vGlut1 puncta apposed to PSD-95 puncta) is decreased following chronic Wnt7a treatment and increased following chronic sFRP treatment (by 32% and 41%, respectively; Fig 6.4D). No significant changes were observed in the mean volume of vGlut1 or PSD-95 puncta following chronic exposure to Wnt7a or sFRP cocktail (vGlut1: vehicle = 0.37±0.02μm^3, Wnt7a = 0.34±0.02μm^3, sFRPs = 0.37±0.01μm^3; PSD-95: vehicle = 0.32±0.02μm^3, Wnt7a = 0.31±0.01μm^3, sFRPs = 0.35±0.02μm^3). These results are consistent with the hypothesis that long-term alterations in glutamatergic release probability caused by altered Wnt signalling promote compensatory changes in excitatory synapse number in mature cultures.

It is conceivable that hippocampal cultures could also compensate for Wnt signalling-mediated changes in glutamatergic release through altered inhibitory signalling, as this

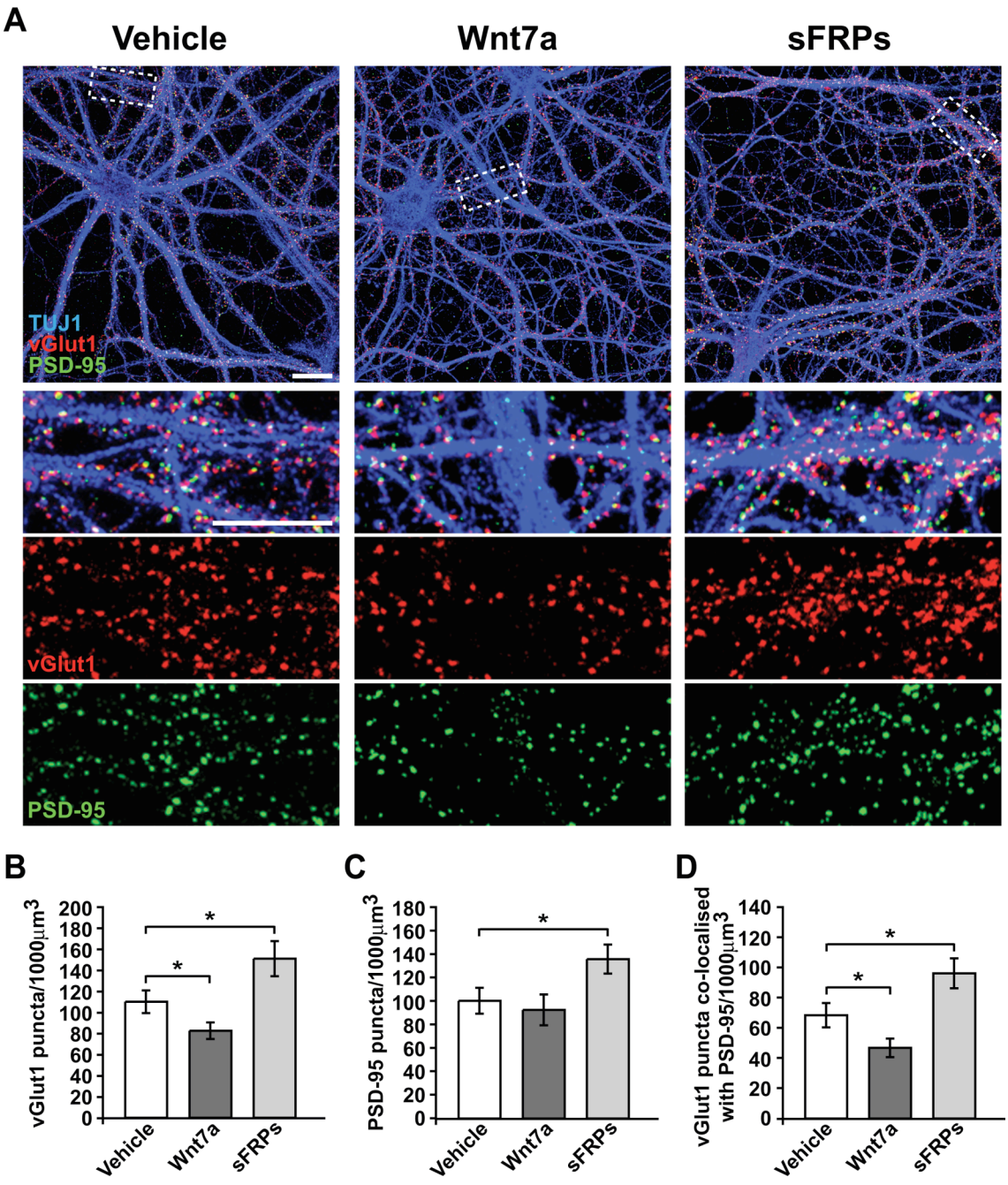

Figure 6.4 Prolonged blockade of Wnt signalling by sFRP treatment increases, whereas prolonged Wnt7a treatment decreases, excitatory synapse number in mature hippocampal cultures. 21 DIV hippocampal cultures were treated with purified Wnt7a, a cocktail of sFRP1, 2 and 3 or vehicle for 20 hours. (A) Images of treated neurons immunostained for vGlut1 (red), PSD-95 (green), and TUJ1 (blue). White boxes indicate enlarged regions. Scale bars = 20μm in top panels, 10μm in enlarged panels. (B) Quantification of vGlut1 puncta density reveals a 25% decrease in response to Wnt7a, and a 37% increase in response to sFRPs, compared to vehicle treated cultures. (C) Quantification of PSD-95 puncta density reveals no significant change in response to Wnt7a, but a 35% increase in response to sFRPs, compared to vehicle treated cultures. (D) Wnt7a treatment induces a 32% decrease in the density of vGlut puncta that co-localise with PSD-95 (putative excitatory synapses), whereas the sFRP cocktail induces a 41% increase, compared to vehicle treated cultures. * = $P<0.05$.

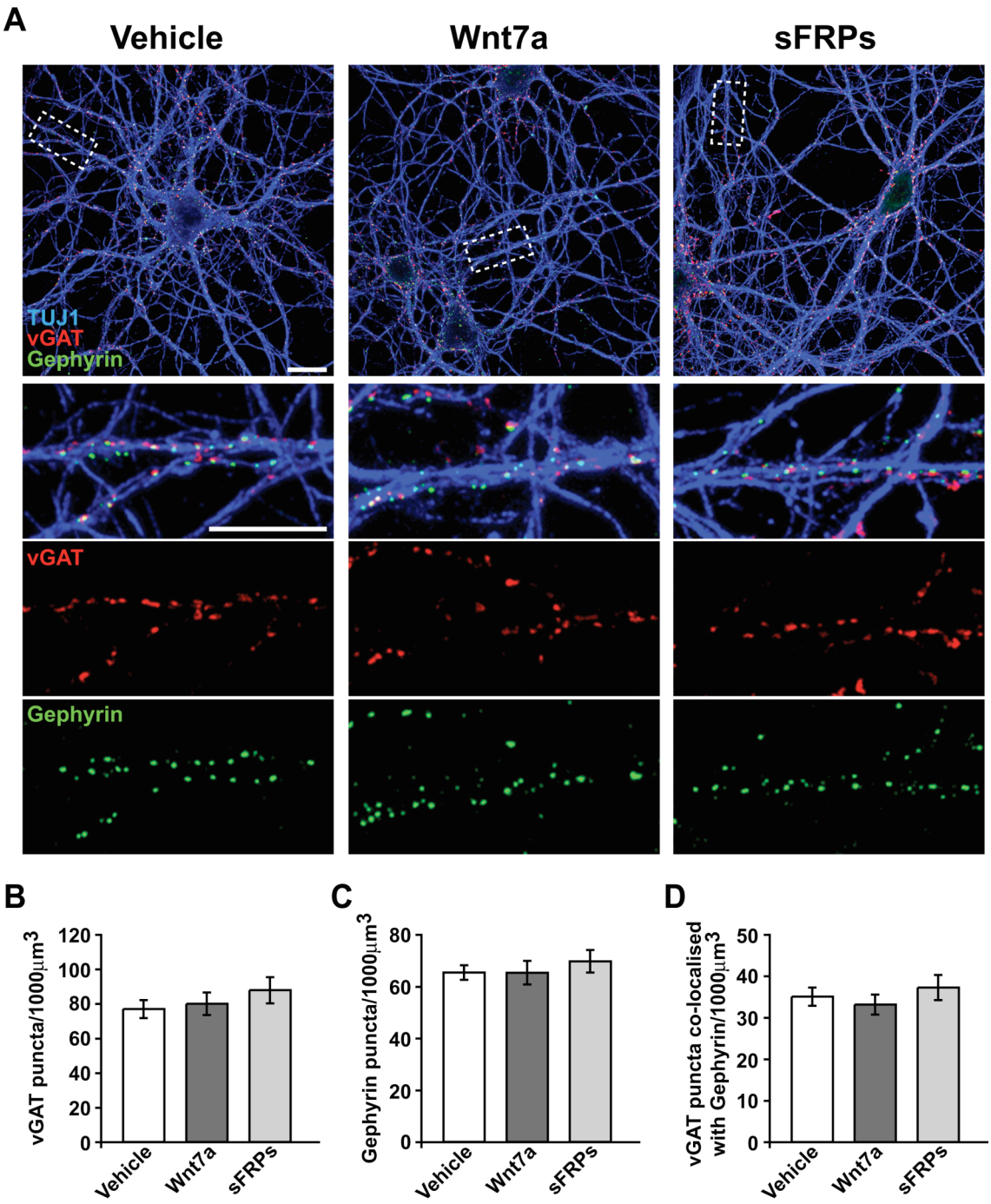

Figure 6.5 Inhibitory synapse number is unaffected by prolonged treatment with purified Wnt7a or sFRP cocktail in mature hippocampal cultures. 21 DIV hippocampal cultures were treated with purified Wnt7a, a cocktail of sFRP1, 2 and 3 or vehicle for 20 hours. (A) Images of treated neurons immunostained for vGAT (red), Gephyrin (green) and TUJ1 (blue). White boxes indicate enlarged regions. Scale bars = 20μm in top panels, 10μm in enlarged panels. (B) and (C) Quantification reveals no effect of Wnt7a or sFRP on vGAT (B) or Gephyrin (C) puncta density. (D) Quantification shows that the density of vGAT puncta that co-localise with Gephyrin (putative inhibitory synapses) is unaffected by Wnt7a or sFRP treatment.

has been reported following other methods of activity blockade (Hartman et al., 2006; Ivanova et al., 2003; Rutherford et al., 1997; Zhang et al., 2009). I therefore examined the effect of prolonged Wnt7a or sFRP cocktail treatment on inhibitory synapse density by looking at the distribution of vGAT and Gephyrin puncta. No significant changes were found in the density or co-localisation of vGAT or Gephyrin puncta following 20 hours exposure to Wnt7a or sFRP cocktail; accordingly, the density of inhibitory synapses (defined as vGAT puncta apposed to Gephyrin puncta) was similar between all three conditions (Fig 6.5). The volume of vGAT and Gephyrin puncta did not differ significantly between vehicle and Wnt7a or sFRP treated cultures (vGAT: vehicle = 0.65±0.02μm^3, Wnt7a = 0.58±0.02μm^3, sFRPs = 0.65±0.01μm^3; Gephyrin: vehicle = 0.35±0.01μm^3, Wnt7a = 0.36±0.01μm^3, sFRPs = 0.35±0.01μm^3). Thus, prolonged changes in Wnt signalling do not affect the number of inhibitory synapses. These results demonstrate that the compensatory mechanisms recruited in response to Wnt signalling-mediated alterations in glutamate release specifically target excitatory synapses, at least over the timescale studied here.

6.2.3 Homeostatic increases in excitatory synapse number require longer to develop in younger (12-14 DIV) cultures

I have described how, in mature (21 DIV) hippocampal cultures, prolonged (20 hours) blockade of Wnt signalling results in a compensatory increase in excitatory synapse number that counteracts the decrease in release probability at individual excitatory synapses. However, in Chapter 3 I provided evidence that in younger cultures (14 DIV) the same treatment (20 hours exposure to sFRP-1, 2 and 3) results in a decrease in mEPSC frequency, with no significant change in excitatory synapse number (Figs 3.1 and 3.6). This suggests that in these younger cultures, 20 hours of Wnt signalling blockade again results in a decrease in release probability at glutamatergic synapses, but no compensatory increase in excitatory synapse number occurs within this time period. This raises the question of whether younger cultures are incapable of this form of homeostatic response, or whether they may take longer to express such a response. In order to investigate this question I immunostained hippocampal cultures for vGlut1 and PSD-95 at 14 DIV, following treatment with vehicle or the sFRP cocktail for either 24 or 48 hours. I performed this experiment on cultures transfected with EGFP-actin, as this also allowed me to assess any changes in dendritic spine density, morphology and innervation. Consistent with my previous observation that 20 hours treatment with the

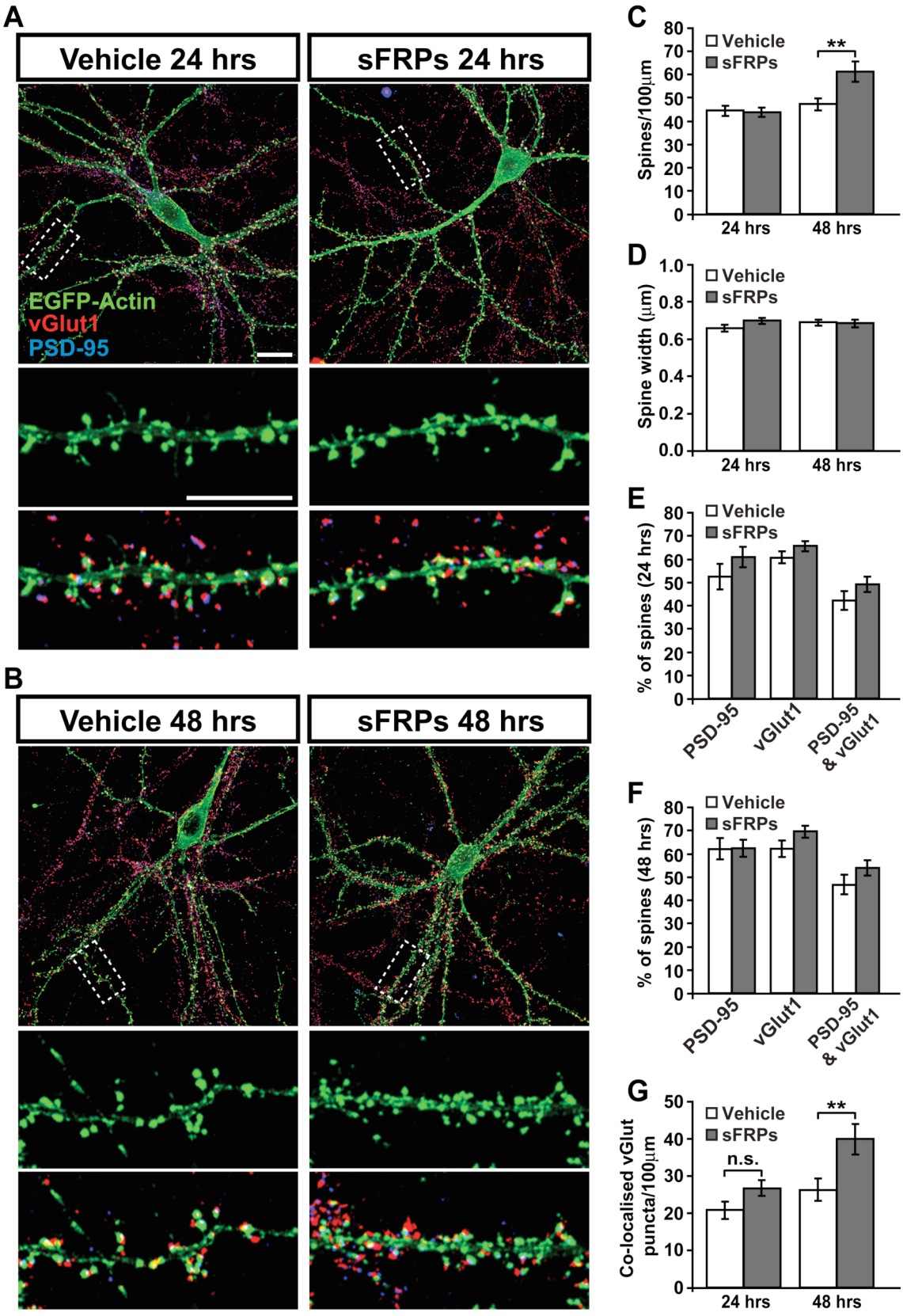

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Figure 6.6 48 hour, but not 24 hour, treatment of younger cultures with sFRP cocktail results in increased dendritic spine and excitatory synapse number. Hippocampal cultures transfected with EGFP-actin at 8 DIV were treated with a cocktail of sFRP1, 2 and 3 or vehicle from either 13-14 DIV (24 hours) or 12-14 DIV (48 hours). (A) Example EGFP-actin transfected neurons (green) treated for 24 hours and immunostained for vGlut (red) and PSD-95 (blue). White boxes indicate enlarged regions. Scale bars = 20μm in top panels, 10μm in enlarged panels. (B) As for (A), except cultures were treated for 48 hours. Note the clear increase in spine density in response to 48 hours exposure to sFRP cocktail. (C) Quantification shows that 24 hour sFRP treatment does not affect spine density, whereas 48 hour sFRP treatment results in a 30% increase, compared to vehicle treated cells. (D) Mean spine width is unaffected by both 24 and 48 hour sFRP cocktail treatment. Quantification also reveals that the proportion of spines that contain PSD-95, that are contacted by vGlut, or both, remains constant following 24 hour (E) or 48 hour (F) sFRP cocktail treatment. (G) The density of vGlut puncta that co-localise with PSD-95 (putative excitatory synapses) is unchanged following 24 hours of sFRP treatment. However, 48 hours of sFRP treatment results in a 52% increase, compared to vehicle treated cells. ** = $P<0.01$.

sFRP cocktail from 13-14 DIV results in no changes in excitatory synapse number (Fig 3.1), I observed that 24 hours treatment had no effect on the density of dendritic spines, nor did it affect the proportion of spines that contain PSD-95, that are contacted by vGlut1, or both (Fig 6.6A, C and E). As a result, the density of excitatory synapses (defined as vGlut1 puncta apposed to PSD-95 puncta within spines) was unchanged in cultures treated with sFRPs for 24 hours, compared to cultures treated with vehicle for the same time period (Fig 6.6G).

In contrast, when neurons were exposed to the sFRP cocktail for 48 hours (from 12-14 DIV), a significant 30% increase in spine density was observed (Fig 6.6B and C). As the proportion of spines containing PSD-95, contacted by vGlut1, or both was again comparable to vehicle treated cultures (Fig 6.6F), this resulted in a significant 52% increase in excitatory synapse density following 48 hours of sFRP treatment (Fig 6.6G). Interestingly, the proportion of multiple PSDs and multiply innervated spines did not differ significantly from control at either timepoint, suggesting the mechanism employed to increase excitatory density is different from that observed following postsynaptic Dvl1 overexpression (% spines with multiple PSDs: vehicle (24 hours) = 2.8 ±0.6%, sFRPs (24 hours) = 4.7±1.0%; vehicle (48 hours) = 5.0 ±1.1%, sFRPs (48 hours) = 5.1±0.8%; % spines with MIS: vehicle (24 hours) = 8.5 ±1.3%, sFRPs (24 hours) = 12.1±1.4%; vehicle (48 hours) = 9.9 ±1.8%, sFRPs (48 hours) = 14.8±2.2%). The mean spine width was also unaffected by both periods of treatment with the sFRP cocktail (Fig6.6D), as was the mean volume of vGlut1 and PSD-95 puncta (data not shown). This suggests that the cultures compensate for the reduction in glutamatergic

signalling principally through an increase in excitatory synaptic number, rather than through any changes in synaptic morphology. Therefore 14 DIV cultures show a similar homeostatic response to prolonged blockade of Wnt signalling as do 21 DIV cultures; however this response takes longer to manifest itself in the younger cultures.

6.3 Discussion

6.3.1 Prolonged blockade of Wnt signalling results in a homeostatic response in hippocampal cultures

In 21 DIV hippocampal cultures, acute (3 hours) blockade of Wnt signalling with a cocktail of sFRP-1, 2 and 3 results in a decrease in release probability at excitatory synapses, a slight decrease in excitatory synapse number and a concomitant decrease in mEPSC frequency (see Chapter 5). Prolonged (20 hour) blockade of Wnt signalling also results in a decrease in release probability at excitatory synapses that is similar in degree to the defect observed following 3 hours blockade. However, following prolonged Wnt blockade, the density of excitatory synapses actually *increases* and mEPSC frequency is indistinguishable from that observed in control cultures. Furthermore, the same length of treatment with purified Wnt7a, which has previously been shown to produce an increase in excitatory release probability (Cerpa et al., 2008), has the opposite effect on excitatory synapse density. Therefore prolonged changes in glutamatergic transmission caused by perturbing Wnt signalling result in homeostatic alterations of excitatory synapse number.

Consistently, previous studies have demonstrated that prolonged activity deprivation induces an increase in synaptic density in mature hippocampal cultures (Burrone et al., 2002; Han and Stevens, 2009; Wierenga et al., 2006). However, these studies used TTX application or transfection with inward-rectifying potassium channels, manipulations which result in complete loss of action potential generation. Even the few studies which have demonstrated *in vivo* synaptic homeostasis have used relatively drastic measures to alter activity, such as monocular deprivation (Kaneko et al., 2008; Maffei and Turrigiano, 2008) or postsynaptic silencing (Haghighi et al., 2003; Paradis et al., 2001). In contrast, the manipulations I performed produced a significant but relatively mild defect in excitatory signalling, yet still resulted in a robust homeostatic response (given adequate time). To my knowledge, this is the first report that mild changes in overall

network activity induce a homeostatic response. This finding is significant, as *in vivo* networks are likely to experience subtle changes in activity under normal physiological conditions. Therefore, manipulations which result in relatively small changes in network activity may allow us to study synaptic homeostasis of mature networks in a more physiologically relevant manner.

Prolonged blockade of Wnt signalling also led to a homeostatic increase in excitatory synapse number in younger (14 DIV) cultures. However, these younger cultures require 48 hours of Wnt blockade to display this effect, with no changes in excitatory synapse number observed after 24 hours of treatment. Consistent with this finding, 24 hrs blockade of neuronal activity with TTX does not affect synapse density in young hippocampal cultures (16 DIV) whereas 48 hours treatment results in a 27% increase in excitatory synapse density (Han and Stevens, 2009). Interestingly, 48 hours treatment with TTX in even younger cultures (7-10 DIV visual cortical cultures) does not affect synapse density, and synaptic homeostasis instead relies on purely postsynaptic mechanisms such as increased AMPA-R clustering and boosting of dendritic AMPA-R-mediated currents (Wierenga et al., 2005). Therefore it appears that the developmental maturity of neurons is one of the crucial factors determining the homeostatic mechanisms recruited by changes in activity levels.

6.3.2 Possible mechanisms underlying synaptic homeostasis in response to alterations in Wnt signalling

Recent studies have highlighted the fact that synaptic homeostasis can be achieved by a number of distinct but often overlapping mechanisms (Turrigiano, 2007). Unfortunately I did not have time to investigate in detail the cellular mechanisms that underlie the homeostatic response observed following prolonged blockade of Wnt signalling. However consideration of the changes observed, combined with previously published data, allows for some reasonable speculation concerning possible mechanisms. One interesting aspect of the homeostatic response I observe is that it does not appear to involve postsynaptic changes that alter the strength of individual synapses ('synaptic scaling'), as mEPSC amplitude was unaffected following 20 hours treatment with the sFRP cocktail in mature cultures, and dendritic spine size was unaffected by 24 or 48 hours treatment in younger cultures. Synaptic scaling appears to be particularly

important in synaptic homeostasis in immature networks; as neurons mature presynaptic alterations and changes in synapse density become more important, although postsynaptic modifications can persist (Burrone et al., 2002; Han and Stevens, 2009; Wierenga et al., 2006). The mechanisms underlying synaptic scaling are starting to become apparent. The best characterised pathway involves TNFα released by glia acting to increase levels of synaptic β3 integrin, which in turn promotes the insertion of GluR2 subunits postsynaptically and therefore increases excitatory synaptic strength (Cingolani et al., 2008; Kaneko et al., 2008; Stellwagen and Malenka, 2006). This provides a possible explanation of why I did not observe changes in synaptic strength – I cultured my hippocampal neurons in the absence of a glial feeder layer (see material and methods). In addition, it has been demonstrated that the degree of GluR accumulation in young cultures depends on the particular method employed to alter activity levels (O'Brien et al., 1998). Therefore it is perhaps not surprising that the relatively mild changes in activity produced by manipulating Wnt signalling result in homeostatic changes that differ from those produced by TTX treatment.

Another mechanism through which neuronal networks (particularly mature networks) can compensate for reductions in activity is through an increase in presynaptic release probability (Bacci et al., 2001; Han and Stevens, 2009; Murthy et al., 2001; Paradis et al., 2001; Thiagarajan et al., 2005; Wierenga et al., 2006). The mechanisms underlying this activity-dependent change in release probability are far from clear at present, but calcium signalling in both the pre- and postsynaptic compartments seems to be important (Frank et al., 2009; Haghighi et al., 2003; Jensen et al., 2009; Thiagarajan et al., 2002). An increase in release probability is not involved in the homeostatic compensation I observe in mature cultures however, as release probability is still *decreased* following 20 hours treatment with the sFRP cocktail, to a similar degree as seen at 3 hours. This suggests that the prevailing compensatory mechanism is the increase in excitatory synapse number, and this alone is responsible for normalising mEPSC frequency to control levels following prolonged Wnt blockade. Indeed it is interesting to note that 3 hours sFRP treatment results in a 43% decrease in mEPSC frequency, and 20 hours sFRP treatment results in a 41% increase in excitatory synapse density. Therefore this increase in excitatory synapse number can fully account for the normalisation of mEPSC frequency also observed.

Almost nothing is known concerning the mechanisms underlying homeostatic changes in synapse number, as most studies thus far have focused on synaptic scaling. As the homeostatic increase in synaptic density I observe is specific for excitatory synapses, a good place to start would be with molecules already known to specifically promote glutamatergic synapse formation, such as neuroligin-1 (Chubykin et al., 2007), SynCAM (Biederer et al., 2002) and MeCP2 (Chao et al., 2007). In summary, the results presented in this chapter demonstrate that hippocampal neurons compensate for changes in glutamatergic release in response to altered Wnt signalling through changes in excitatory synaptic density. Furthermore, the timescale over which this compensatory mechanism is recruited depends upon the maturity of hippocampal neurons.

CHAPTER 7:

Discussion

7.1 Summary of results

Complementary cell biological and electrophysiological approaches were used to investigate the role of Wnt signalling in the formation and function of excitatory and inhibitory synapses in the hippocampus. My studies demonstrate that Wnt signalling regulates multiple aspects of excitatory synapse development, including pre- and postsynaptic formation, functional release of neurotransmitter and synaptic plasticity.

Wnt7a promotes the formation of excitatory synapses in 14 DIV cultured hippocampal neurons, as evidenced by an increase in the number of apposed clusters of vGlut1 and PSD-95. Postsynaptic expression of Dvl1 also results in an increase in excitatory synapse density, both through the formation of new synapses and the formation of multiply innervated spines. Furthermore, postsynaptic Dvl1 expression causes an increase in the average size of dendritic spines, highlighting a role for Wnt signalling in spine morphogenesis. In agreement with these morphological changes, postsynaptic expression of Dvl1 results in an increase in mEPSC frequency and amplitude. Therefore Wnt signalling regulates both the formation and postsynaptic strength of excitatory synapses in the hippocampus. Interestingly, the effects of Wnt7a and postsynaptic Dvl1 overexpression were specific to excitatory synapses; inhibitory synapse density and mIPSC frequency and amplitude were unaffected.

I also present evidence that endogenous Wnt signalling regulates neurotransmitter release at hippocampal synapses. 3 hours blockade of Wnts with a cocktail of sFRPs in 14 DIV or 21 DIV hippocampal cultures results in a decrease in mEPSC frequency. In 21 DIV cultures, 3 or 20 hour sFRP cocktail treatment also increases the EPSC paired pulse ratio, direct evidence of a reduction in presynaptic release probability. This defect is also observed in hippocampal slices from double *Wnt7a;Dvl1* knockout mice. Therefore endogenous Wnt signalling facilitates glutamate release at hippocampal excitatory synapses. Again, these effects are specific to excitatory synapses, as mIPSCs and evoked IPSCs are unaffected.

Finally, I show that prolonged reduction of glutamate release by Wnt signalling blockade induces homeostatic mechanisms that act to normalise excitatory synaptic activity. Although the PPR remains elevated in mature (21 DIV) cultured hippocampal neurons treated with sFRP cocktail for 20 hours, mEPSC frequency is normal. This is due to a homeostatic increase in excitatory synapse density. A similar response is observed in younger (14 DIV) cultures, but this requires a longer period of Wnt blockade to manifest.

7.2 Limitations of methods

The interpretation of any scientific data is dependent upon the methods used, and their limitations. The imaging experiments performed in this thesis involve detection of very small structures which are close to the resolution limit of optical microscopy. Therefore, before discussing the results and their implications, it would be beneficial to identify and discuss the limitations of the methods used, and how these limitations might affect interpretation of the data.

The resolution of an optical system is fundamentally limited due to the wave-like nature of light, which acts to blur point-like light sources due to diffraction. This 'diffraction limit' is therefore the smallest resolvable distance in an optical system, and is related to the wavelength of light used and the numerical aperture of the lens used to collect and focus the light; shorter wavelengths and higher numerical apertures allow greater resolution. The majority of imaging experiments in this thesis were performed on a laser-scanning confocal microscope using a 63x objective with a numerical aperture of 1.32 and fluorescent molecules that emit maximally at 519, 603, 617 and 665 nm. The theoretical limit of resolution using each of these fluorophores is given in the table below, calculated using the following equation which uses the Rayleigh criterion to define maximum resolution:

$$\mathbf{r = 0.61\lambda / NA}$$

Where **r** = resolution, **λ** = wavelength of emission and **NA** = numerical aperture.

Fluorophore	DyLight/Alexa-488	Alexa-568	Alexa-594	Alexa-647
Max emission (nm)	519	603	617	665
Max resolution (nm)	236	274	280	302

Table 7.1 Theoretical maximum resolution obtainable for fluorophores used, with an objective with a numerical aperture of 1.32.

It should be kept in mind that these are theoretical limits, and a number of practical factors mean that the true resolution obtained will be slightly lower than these figures. Taking these numbers as a rough guide however, I should be capable of resolving structures as small as 0.25 - 0.3 μm (depending on the fluorophore used). When analysing puncta of synaptic of synaptic proteins, I rejected any thresholded objects smaller than $0.1\mu m^3$. Assuming the puncta to be perfect spheres (which is not the case, but is a reasonable approximation), the smallest objects included in the analysis would therefore have a diameter of 0.56μm, which is above the limit of resolution of the system even using the longest-wavelength fluorophore (Alexa-647). Furthermore, the pixel size of the resulting images was 0.155μm, meaning even the smallest puncta are 3-4 pixels in diameter, satisfying the Nyquist criterion of sampling the smallest resolvable element at least twice. Note that this is in the lateral (x-y) plane. In the z-plane, the interval between sections was set as 0.3μm. Again, this means that most objects included in the analysis should be sampled twice (though the decreased resolution in the z-axis inherent to laser-scanning confocal systems means the smallest objects may be slightly under-sampled). Therefore the parameters of the confocal imaging would appear to be suitable to detect synaptic puncta of the size range reported in this thesis.

Of more concern are the experiments imaged using a regular wide-field fluorescence microscope (Figs 3.2, 3.4 and 3.5), as here a 40x objective with a numerical aperture of 1 was used. In these experiments, thresholded puncta smaller than $0.1\mu m^2$ were rejected, equating to a minimum puncta diameter of 0.36μm (in hindsight, it would have been preferable to match this to the minimum size of the confocal experiments by setting the cut-off at $0.3\mu m^2$). Using the longest wavelength fluorophore (Alexa-647) with this objective gives a maximum resolution of 0.4μm, which is slightly below the minimum puncta diameter of 0.36μm. However Alexa-647 was used in these experiments to outline neuronal morphology (and therefore larger structures) by labelling Tuj-1. The longest wavelength used to label puncta of synaptic proteins in these experiments was Alexa-594, which gives a theoretical maximum resolution of 0.37μm with the objective used. Note this is therefore approximately equal to the

minimum puncta size included in the analysis. An important point to bear in mind with respect to these size measurements of synaptic is that they should not be taken as absolute values – the puncta represent the labelling of a population of the molecules of interest spread throughout a structure or structures of interest (i.e. synaptic vesicles, active zones or the post-synaptic density). Rather it is the relative changes between experimental treatments that are of interest, as all other factors have been kept constant. For measuring the absolute size of synapses and synaptic ultrastructures, electron microscopy is a far more suitable method.

While discussing the limits of resolution, it is important to note that the diffraction limit not only determines the smallest resolvable structure, but is also the smallest distance between two structures that allows them to be resolved as two separate objects (indeed, strictly speaking this is the correct definition of resolution). This has obvious implications for the determination of both the number and size of synaptic puncta, as two puncta that are separated by a distance below this limit will be detected as a single, larger punctum. It is in this context that the detection of puncta by intensity thresholding is particularly important. A point-like light source displays a typical intensity profile known as a point-spread function, with a central Airy disc that has a maximum intensity at its centre and which decays with decreasing distance from that centre. Therefore the intensity thresholds chosen for detecting objects will influence both the size of the objects, and the degree to which nearby thresholded objects overlap. In practice this means there is a balance in choosing a threshold – high thresholding results in the loss of smaller, dimmer puncta, whereas low thresholding results in the merging of closely-spaced puncta. An appropriate threshold should aim to minimise both of these errors. While performing these analyses, it was clear that some puncta in close proximity were detected as single objects, even with the use of the 'Separate Touching Objects' function. However, this represented such a low proportion of the total puncta detected in any given image that it would not be expected to significantly affect the data. Again, it should be stressed that the values given are estimates of synaptic number and size, and it is the relative changes between experimental conditions that are of primary interest.

Finally, some discussion of the measurement of spine size is warranted. Any method of measuring spine width from fluorescently labelled spines requires a way of determining the edge of the spine, as the signal within the spine will not have a clear edge, but will rather decrease gradually with distance from the centre of the spine. In the experiments

described in this thesis, spine width was measured by visual placement of a line tool across the widest point of a maximum projection of the spine from a z-stack. This means that edge detection is somewhat subjective using this method, and depends upon the visual system of the experimenter performing the analysis. Analysis was performed blind to avoid bias, and the contrast and brightness of the fluorescent signal (which would greatly influence the width measured) was not altered during analysis. However, it is important to realise that the spine sizes measured by this method are reliant upon a number of factors, including the angle of the spine measured and the concentration of EGFP-actin within a given spine. In terms of spine angle, one would expect the spines to be randomly orientated with respect to the dendrite, and therefore this should not be an issue if a large number of spines are analysed (approximately 1000 spines were analysed per condition per experiment here). The concentration of EGFP-actin within a given spine is an important factor, as the analysis performed here assumes 1) that EGFP-actin concentration is constant from spine to spine (i.e. spines of the same size would have the same brightness) and 2) the experimental treatments performed do not affect the EGFP-actin concentration within spines. EGFP-actin expression did not appear to be affected by any of the experimental treatments used, and similar spine head widths (and changes in spine head width) are obtained if soluble GFP is used to label spines instead of EGFP-actin (Ciani and Salinas, unpublished results). However, these issues could be avoided by using a method of spine width analysis that produces a value that is independent of the maximum intensity of the signal within the spine. A line scan of fluorescence intensity can be taken through the widest point of the spine head and a Gaussian distribution fit to the data. The spine head width is then measured as the width of some fixed proportion of this distribution i.e. a set number of standard deviations from the maximum intensity or a percentage drop from the maximum intensity (Busetto et al., 2008; De Simoni et al., 2004). It is again important to realise that, whatever the method used, the value produced is an estimate of the true spine size, and it is the relative changes between experimental conditions that is usually of primary interest.

7.3 Pre- and postsynaptic actions of Wnts during synaptogenesis

The actions of Wnts as presynaptic organisers in the CNS are already well established, but their role in postsynaptic development is less clear. I found that treatment with purified Wnt7a in 14 DIV hippocampal cultures resulted in an increase in vGlut1 puncta, in agreement with previously published data (Davis et al., 2008). In addition, I

found that Wnt7a increases the density of PSD-95 puncta (Fig 7.1A). A similar PSD-95 clustering effect of Wnt5a has recently been reported in hippocampal cultures (Farias et al., 2009). Interestingly, this same study reported that Wnt7a increased the number of puncta of the presynaptic protein Synaptophysin, but did not increase PSD-95 puncta density. However this study used short-term (1 hour) Wnt7a treatment compared to the 20 hour treatment used in my studies, suggesting that the effect of Wnt7a on postsynaptic development occurs more slowly than its presynaptic effect.. This is consistent with the generally accepted hypothesis that presynaptic development precedes postsynaptic development during the initial wave of synaptogenesis in the CNS (McAllister, 2007).

Wnt7a also increases the density of vGlut1 puncta apposed to PSD-95 puncta, taken to represent excitatory synapses, though the percentage co-localisation remained constant (Fig 7.1A). This means that a large proportion of the newly formed vGlut1 and PSD-95 formed in apposition to each other. Such co-ordinated pre- and postsynaptic development could be explained in two ways. Firstly, Wnt7a could act directly to promote presynaptic differentiation, which could then elicit postsynaptic differentiation through Wnt-independent mechanisms (i.e. transynaptic adhesion molecules and/or secreted synaptogenic factors other than Wnts). Secondly, Wnts may act directly at both sides of the nascent synapse to bring about co-ordinated pre- and postsynaptic development. I investigated the ability of Wnt signalling to directly regulate postsynaptic development by sparsely transfecting hippocampal cultures with Dvl1, resulting in Dvl1 overexpressing dendrites that were contacted by non-transfected axons.

Postsynaptic Dvl1 overexpression had multiple effects on postsynaptic development (Fig 7.1B). The percentage of spines containing PSD-95 puncta increased, consistent with a direct postsynaptic role for Wnt signalling in PSD-95 clustering. PSD-95 puncta were also larger and a higher percentage of spines displayed multiple PSDs. Dendritic spines themselves were also larger on average, demonstrating a novel role of Wnt signalling in spine morphogenesis. Finally, postsynaptic Dvl1 overexpression also resulted in an increase in the presynaptic innervation of dendritic spines; both the percentage of spines contacted by vGlut1 puncta and the percentage of multiply innervated spines increased dramatically. Interestingly, though postsynaptic Dvl1 overexpression increases excitatory synapse number through the mechanisms described

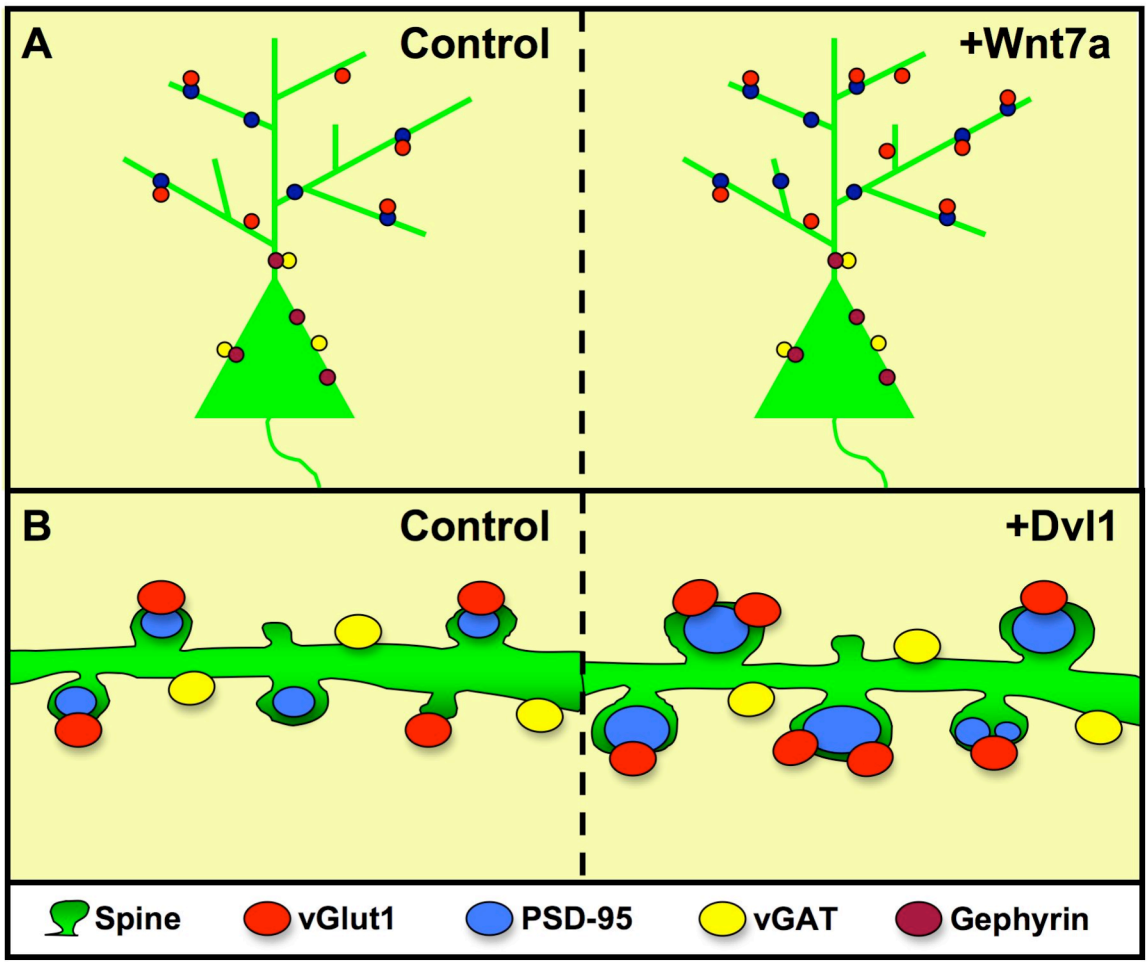

Figure 7.1 Wnt signalling regulates the formation of excitatory pre- and postsynaptic specialisations. A) Wnt7a increases vGlut (red) and PSD-95 (blue) puncta density. The percentage of co-localised vGlut and PSD-95 puncta (putative excitatory synapses) remains constant, resulting in an increase in excitatory synapse density. In contrast, the number of vGAT (yellow) and Gephyrin (purple) puncta is unchanged by Wnt7a, as is the number of inhibitory synapses (vGAT puncta apposed to Gephyrin puncta). B) The diagram outlines the effects of postsynaptic Dvl1 overexpression in cultured hippocampal neurons. Compared to control transfected cultures, Dvl1 overexpression results in an increase in the percentage of spines associated with pre- and postsynaptic markers, and in the proportion of multiply innervated spines and multiple PSDs. Additionally, Dvl1 expression causes an increase in the average size of dendritic spines. Inhibitory innervation is unaffected by Dvl1 overexpression, as the number of vGAT puncta contacting Dvl1-transfected cells is indistinguishable from control cells.

above, it does not increase dendritic spine number. However, experiments by a colleague in the laboratory have shown that addition of Wnt7a to cultured hippocampal neurons increases both spine number and size (Ciani et al, submitted for publication). A possible explanation for this is that Wnt7a signals to the axon to form new excitatory terminals, which then elicit the formation of new spines by anterograde signalling. At the same time, Wnt7a can also signal postsynaptically to regulate spine size. In contrast, when Wnt signalling is specifically activated postsynaptically spine size increases, but

spine density is unaffected. These findings suggest that Wnt signalling can influence postsynaptic development both directly, and indirectly through presynaptic changes.

Due to time constrains, I personally did not investigate the molecular pathways activated by Wnt-Dvl signalling that regulate pre- and postsynaptic development. However published and unpublished data from our laboratory suggest that Wnts signal through different pathways pre- and postsynaptically to co-ordinate synaptic development in the CNS. The presynaptic organising actions of Wnts rely upon signalling through the canonical pathway, as axonal remodelling and presynaptic protein clustering are mimicked by GSK3β inhibition or β-catenin overexpression (Hall et al., 2000; Lucas et al., 1998; Lucas and Salinas, 1997; Purro et al., 2008). Conversely, Wnt-mediated presynaptic protein clustering is reduced in the presence of the canonical Wnt antagonist Dkk1 (Dickins & Salinas, unpublished results). Interestingly, both the axonal remodelling and presynaptic protein clustering effects of Wnts are unaffected by transcriptional blockers (Purro et al., 2008) (Dickins & Salinas, unpublished results). This suggests that the presynaptic effects of Wnts during synaptogenesis depend upon the divergent canonical pathway, which has been shown to regulate microtubule dynamics (Ciani et al., 2004; Purro et al., 2008), implicating cytoskeletal rearrangement as an important mechanism in Wnt-mediated synaptogenesis.

Non-canonical Wnt signalling appears to be important for postsynaptic development. Both the dendritogenic affect of Wnt7b and the PSD-95 clustering activity of Wnt5a are dependent upon signalling through JNK, implicating the PCP pathway in these processes (Farias et al., 2009; Rosso et al., 2005). However, the spine-enlarging effect of Wnt7a or postsynaptic Dvl1 is dependent on CaMKII activation, implicating the Wnt/Ca^{2+} pathway in this process (Ciani et al, submitted for publication). This is consistent with my observation that postsynaptic Dvl1 overexpression results in both spine enlargement and PSD-95 clustering, as Dvl activation is required for both PCP and Wnt/Ca^{2+} signalling. Therefore, it seems that co-ordinated signalling through separate non-canonical Wnt pathways is required for normal postsynaptic development. Taken together with the results of studies into Wnt signalling at the presynaptic side, the emerging picture is that Wnts signal bidirectionally during central synaptogenesis, promoting presynaptic development through a divergent canonical pathway and postsynaptic development through non-canonical pathways.

In this context, it is interesting to compare Wnt regulation of CNS synaptogenesis with what is known about Wnts and NMJ formation. Wg also signals bidirectionally at the *Drosophila* NMJ to regulate processes such as pre- and postsynaptic morphology and protein clustering (Ataman et al., 2008; Packard et al., 2002). The presynaptic function of Wg at the *Drosophila* NMJ involves activation of canonical Wnt signalling, yet is independent of transcriptional activity (Ataman et al., 2008; Miech et al., 2008). Postsynaptically, NMJ development relies on signalling through postsynaptic DFz2 in the nuclear import pathway (Ataman et al., 2008; Korkut et al., 2009; Mathew et al., 2005). Therefore, at the invertebrate NMJ, as in the vertebrate CNS, different Wnt signalling pathways are involved in pre- and postsynaptic development. Interestingly, it appears that signalling through the postsynaptic nuclear import pathway can also signal retrogradely to regulate presynaptic development, as mutants lacking postsynaptic *dfz2* or *evi* (required for DFz2 internalisation) display defects in presynaptic morphology (Ataman et al., 2006; Korkut et al., 2009). This is reminiscent of the results I obtained with postsynaptic Dvl1 overexpression, which resulted in presynaptic effects, presumably through the generation of a retrograde signal. Therefore, the functions and mechanisms of Wnt signalling at developing synapses display considerable evolutionary conservation, highlighting the usefulness of studies in simpler as well as higher organisms in elucidating the molecular mechanisms of synapse formation.

7.4 Wnt signalling regulates excitatory synapse development: implications for disease

The results presented in this thesis implicate Wnt7a/Dvl1 signalling as an important regulator of excitatory transmission in the developing brain. As changes in the relative levels of excitatory and inhibitory activity have been implicated in a range of neurological disorders (Kehrer et al., 2008; Leite et al., 2005; Medrihan et al., 2008; Munoz-Yunta et al., 2008; Rubenstein and Merzenich, 2003), this highlights Wnt signalling as an area of exciting potential in research into the development and/or treatment of disease in the CNS.

In contrast to the well-established role of Wnt signalling in cancer and various developmental diseases (particularly canonical signalling, as this is the best-studied pathway; for review see (MacDonald et al., 2009)), the contribution of Wnt signalling to disease in the postnatal CNS is only just beginning to emerge (for reviews, see (De

Ferrari and Moon, 2006; Freese et al., 2009). The best established links so far are for neurodegenerative diseases. For example, a direct link has recently been made between canonical Wnt signalling and Alzheimer's disease (AD) with a study demonstrating that a genetic variant of the Wnt co-receptor LRP6 that results in reduced β-catenin signalling is associated with late-onset AD (De Ferrari et al., 2007). GSK3β, a key kinase in the canonical Wnt signalling pathway, has also been implicated in AD, due to its ability to hyperphosphorylate tau (Kosik, 1992; Lucas et al., 2001). Tau hyperphosphorylation is believed to participate in the formation of neurofibrillary tangles, one of the key cell biological hallmarks of the disease. Pharmacological inhibition of GSK3β in mouse models of AD reduces tau phosphorylation and amyloid plaque formation, increases neuronal survival and rescues deficits in performance of the Morris water maze test (De Ferrari et al., 2003; Sereno et al., 2009). Wnts act to inhibit GSK3β through the canonical pathway, so there is considerable interest in modulators of Wnt signalling as potential treatments for AD.

Wnt signalling has also recently been linked to Parkinson's disease (PD; (Sancho et al., 2009). One of the key pathogenic stages in both AD and PD is believed to be synapse loss and dysfunction (Hashimoto et al., 2003; Jellinger, 1996; LaFerla and Oddo, 2005; Shankar and Walsh, 2009). Therefore the actions of Wnts in synaptic formation, function and maintenance (Fig 7.2) may have a role to play in protecting against these disorders. As AD and PD are both ageing-related disorders, it is interesting to note that Wnt7a/b levels in the hippocampus have been to shown to display a steep decline with age (Gogolla et al., 2009). It would be of great interest to search for behavioural phenotypes in the *Wnt7a-/-;Dvl1-/-* mice that mimic disease states, particularly diseases that are believed to involve imbalances in excitatory and inhibitory activity. Unfortunately *Wnt7a-/-* mice suffer from pronounced limb deformities (Parr and McMahon, 1995) which are compounded in the *Wnt7a-/-;Dvl1-/-* double knockout mouse, severely restricting the behavioural tests that can be performed due to impaired locomotion. One test that could be performed however is to see if *Wnt7a-/-;Dvl1-/-* mice are more resistant to experimentally induced epileptic seizures than *wildtype* mice. As *Wnt7a/Dvl1* deficiency leads to a selective reduction in excitatory activity in *Wnt7a-/-;Dvl1-/-* mice, one would predict this to be the case.

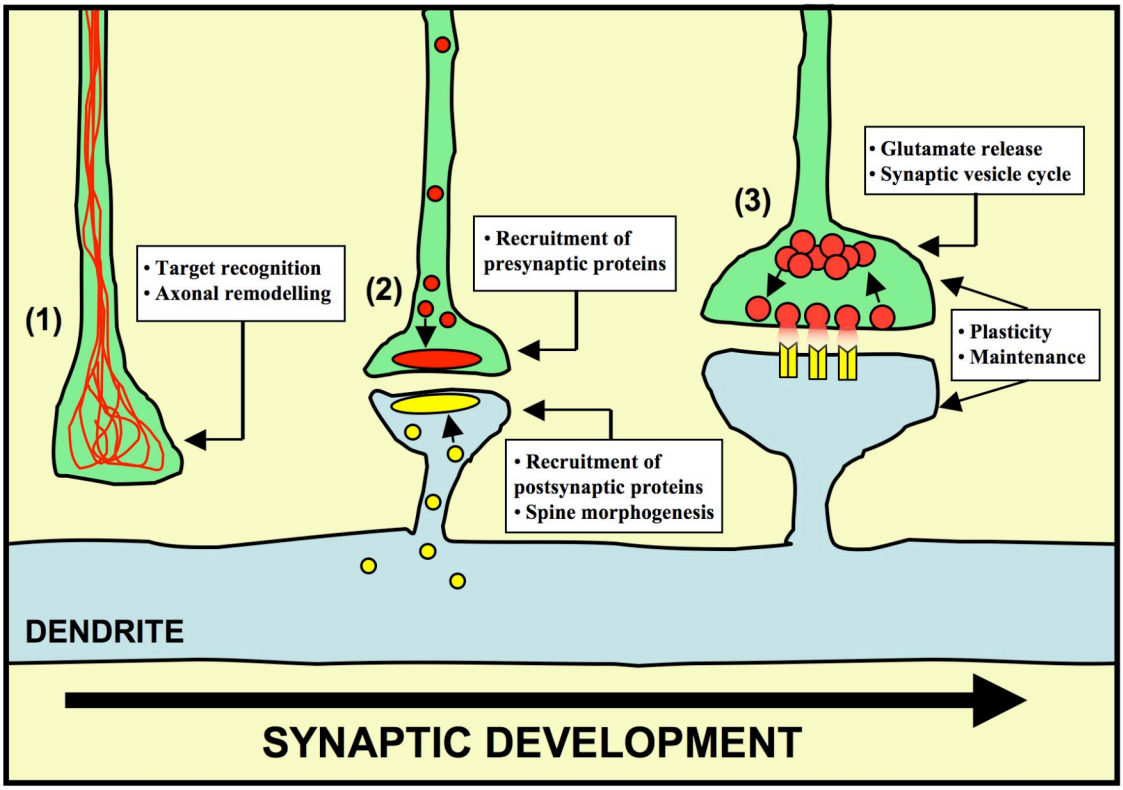

Figure 7.2 Wnt signalling regulates multiple processes throughout the developmental life time of a synapse. The diagram outlines the actions of Wnts during key steps in excitatory synaptic development. (1) As axons grow towards their targets, target-derived Wnts signal to the axon, eliciting axonal remodelling through cytoskeletal rearrangement. (2) During synaptogenesis, Wnts signal to both sides of the synapse to promote recruitment of pre- and postsynaptic proteins and dendritic spine morphogenesis. (3) As the synapse matures, Wnts regulate presynaptic vesicle cycling and glutamate release. Emerging evidence also points to a role for Wnts in synaptic maintenance and plasticity; though little is known regarding the mechanisms, this is likely to involve Wnt signalling at both sides of the synapse.

7.5 Can Wnts regulate inhibitory synapse development?

As mentioned throughout this thesis, previous studies of the role of Wnt signalling in synapse development have focused on excitatory synapses, or have not differentiated between different synaptic subtypes. The results presented in this thesis demonstrate that Wnt signalling regulates the development of excitatory synapses through multiple mechanisms (Fig 7.1 & 2), but no effects were observed on inhibitory synapses. I found that Wnt7a and postsynaptic Dvl1 promote excitatory synapse formation without affecting inhibitory synapse number. Furthermore, mice that lack both *Wnt7a* and *Dvl1* have a reduction in release probability at glutamatergic, but not GABAergic synapses, and a similar defect is seen following Wnt blockade with sFRPs in cultured neurons.

However, it is important to realise that these results do not exclude a role for Wnts in inhibitory synapse development. Wnt7a is just one of several Wnts expressed in the postnatal hippocampus, including Wnt3a,Wnt5a and Wnt7b (Davis et al., 2008; Gogolla et al., 2009; Lucas and Salinas, 1997; Rosso et al., 2005; Shimogori et al., 2004). It is therefore possible that these other Wnt factors could directly affect GABAergic synapse formation, and this should be investigated in the future. Similarly, Dvl exists as three isoforms (Dvl1-3), and it is possible that Dvl1 may be specifically localised to excitatory synapses, whereas other isoforms could localise to and regulate the development of inhibitory synapses. An example of this principle is provided by the Neurexins/Neuroligins. Neuroligins-1, 3 and 4 are found principally at glutamatergic synapses and can promote excitatory synapse formation, whereas Neuroligin-2 is preferentially found at GABAergic synapses and promotes inhibitory synapse formation (Graf et al., 2004). Furthermore, β-neurexins can promote both excitatory and inhibitory synapse formation, whereas α-neurexins specifically promote the formation of GABAergic synapses (Kang et al., 2008).

The fact that the sFRP cocktail reduces release probability at glutamatergic but not GABAergic synapses argues more strongly for a highly specific action of Wnts, as the sFRP cocktail would be expected to block several Wnts. However it should be kept in mind that relatively little is known regarding the binding of specific Wnts to specific sFRPs, and there is no guarantee that the sFRP cocktail blocks all Wnt signalling; indeed, this seems somewhat unlikely. Therefore it is possible that a Wnt other than Wnt7a that is not antagonised by the sFRP cocktail could play a role in GABA release at inhibitory synapses. The possibility that different Wnts or Wnt signalling proteins could play a role at inhibitory synapses is an intriguing idea that warrants further investigation. For example, the abilities of all Wnts expressed in the hippocampus to regulate excitatory and inhibitory synapse development could be tested systematically by gain and loss of function experiments.

7.6 Wnts and presynaptic function

Application of exogenous Wnt7a or synthetic Wnt agonists has previously been shown to increase release probability in hippocampal cultures and slices (Beaumont et al., 2007; Cerpa et al., 2008), suggesting Wnt signalling may play a role in regulation of neurotransmitter release *in vivo*. However, evidence that endogenous Wnt signalling

regulates transmitter release was lacking. Here I have presented such evidence by demonstrating that blockade of endogenous Wnt signalling using a cocktail of sFRPs reduces release probability at glutamatergic synapses in hippocampal cultures. Importantly, this effect is mimicked by genetic loss of Wnt7a and Dvl1 function in acute hippocampal slices. These data argue strongly that endogenous Wnt signalling acts to facilitate glutamate release at hippocampal excitatory synapses, and suggests that Wnt signalling could play a potential role in plasticity through modulation of release probability.

Though I did not have time to investigate the mechanism of Wnt regulation of release probability at glutamatergic synapses, previously published data and unpublished data from our laboratory allows me to propose a potential mechanism. Vesicle fusion depends critically upon docking of vesicles to the presynaptic active zone and formation of the SNARE complex, consisting of the presynaptic membrane SNAREs syntaxin-1 and SNAP-25, and the vesicular proteins VAMP2 and synaptotagmin-1. VAMP2 is the vesicular SNARE protein, whereas synaptotagmin-1 acts as the calcium sensor for action potential-elicited fast synchronous release (Verhage and Sorensen, 2008). In addition to its role in coupling Ca^{2+} influx with vesicle fusion, synaptotagmin-1 has recently been identified as the vesicular docking protein through its binding to syntaxin-1/SNAP-25 complexes (de Wit et al., 2009). Interestingly, Dvl1 binds to synaptotagmin-1, syntaxin-1 and SNAP-25, but not VAMP2 ((Kishida et al., 2007) and Ciani, Sahores and Salinas, unpublished results), indicating that it is present in the docking complex, but not the fusion complex. Dvl is well characterised as a scaffolding protein in Wnt signalling pathways (Chien et al., 2009), therefore it is tempting to speculate that Dvl could regulate release by stabilising the docking complex. The number of docked vesicles at hippocampal synapses correlates strongly with the size of the readily releasable pool of vesicles (RRP) (Rizzoli and Betz, 2005), which in turn is one of the factors affecting release probability (Dobrunz and Stevens, 1997; Stanton et al., 2005; Tyler et al., 2006). Therefore, Wnt7a may act through Dvl1 to control release probability by regulating vesicle docking, and therefore the size of the RRP. Future studies focusing on the presynaptic binding partners of Dvl will unravel the mechanism by which Wnt signalling regulates release of glutamate at central synapses.

7.7 Wnts and postsynaptic function

I also presented evidence that Wnts can regulate synaptic neurotransmission through postsynaptic mechanisms. Postsynaptic overexpression of Dvl1 resulted in an increase in the AMPA-R-mediated mEPSC amplitude, indicative of an increase in excitatory synaptic strength. mEPSC rise time was also reduced by postsynaptic Dvl1 overexpression, and the opposite (increased mEPSC rise time) was observed in hippocampal slices of *Wnt7a-/-; Dvl1-/-* mice. These data suggest that Wnt signalling is able to regulate the number, and perhaps the subunit composition, of postsynaptic AMPA receptors.

The functional effect of Dvl1 overexpression (increased AMPA-R-mediated mEPSC amplitude) correlates well with the postsynaptic morphological changes observed (spine enlargement and increased PSD-95 clustering), as spine size and synaptic strength are known to be correlated (Matsuzaki et al., 2004; von Bohlen Und Halbach, 2009) and PSD-95 helps anchor AMPA-Rs at the synapse, through its binding to Stargazin (Chen et al., 2000). As previously mentioned, Wnt signalling-dependent enlargement of spines requires CaMKII activation (Ciani et al, submitted for publication), which correlates well with previous studies into the role of CaMKII at dendritic spines (Asrican et al., 2007; Matsuzaki et al., 2004; Okamoto et al., 2007). Although it remains to be tested whether the Dvl1-dependent increase in mEPSC amplitude is also dependent on CaMKII activation, this seems highly likely. Interestingly, CaMKII activity and PSD-95 dynamics are also crucial for changes in spine morphology and AMPA-R localisation in some forms of LTP (Carlisle et al., 2008; Ehrlich et al., 2007; Johnston and Morris, 1995; Lu and Hawkins, 2006; Lu et al., 2001), a type of plasticity that Wnt signalling has also been implicated in (Chen et al., 2006). Therefore Wnt signalling through postsynaptic Dvl1 could also play a role in this process by increasing spine size and/or PSD-95 clustering, leading to increased synaptic strength.

Another potential mechanism by which Wnt signalling could affect synaptic AMPA-R levels is through regulation of AMPA-R internalisation via endocytosis. Dvl1 has been shown to bind directly in synaptosomal preparations to μ2-Adaptin of the AP-2 complex (Kishida et al., 2007), which is crucially required for efficient clathrin-dependent endocytosis (Smythe et al., 1992). AMPA-R internalisation has been implicated in synaptic plasticity, in this case LTD (Lee et al., 2002; Man et al., 2000).

Therefore, Wnts may also be able to regulate synaptic AMPA-R levels through altering the rate of AMPA-R internalisation at synapses. Alternatively, Wnts may act to reduce AMPA-R diffusion away from the synapse (Groc and Choquet, 2006). The potential role of Wnt signalling in regulating synaptic strength and plasticity is emerging as a fascinating area of research that promises exciting results over the coming years.

7.8 Wnts and synaptic homeostasis

Synaptic homeostasis is the term for a range of mechanisms by which neurons can bidirectionally regulate synaptic efficacy to maintain action potential firing rates within a stable range, whilst still allowing for the input-specific Hebbian changes in synaptic strength that are believed to underlie information storage in the brain (Turrigiano, 2007; Turrigiano, 2008; Yu and Goda, 2009). Synaptic homeostatic mechanisms include changes in postsynaptic strength ('synaptic scaling') (Cingolani et al., 2008; Turrigiano, 2008; Turrigiano et al., 1998), presynaptic release probability (Murthy et al., 2001; Paradis et al., 2001), synapse number (Han and Stevens, 2009; Wierenga et al., 2006) and intrinsic neuronal excitability (Desai et al., 1999; Turrigiano et al., 1995). Although initially believed to be a 'global' phenomenon (i.e. all synapses on a neuron are homeostatically altered) (Turrigiano et al., 1998), recent evidence suggests that homeostatic changes in synaptic signalling can occur more locally within the dendritic tree of a given neuron (Sutton et al., 2006; Yu and Goda, 2009). Although the exact signalling pathways through which synaptic homeostasis is achieved remain unclear, progress has been made over the last decade. Signalling molecules implicated in synaptic homeostatsis include secreted molecules such as TNFα (Stellwagen and Malenka, 2006) and BDNF (Rutherford et al., 1997); trans-synaptic adhesion proteins including β3-integrin (Cingolani and Goda, 2008; Cingolani et al., 2008) and N-cadherin (Okuda et al., 2007), and calcium signalling (Thiagarajan et al., 2005; Thiagarajan et al., 2002; Yu and Goda, 2009).

Studies into synaptic homeostasis have traditionally used techniques that drastically alter synaptic transmission, such as TTX treatment (Han and Stevens, 2009; Turrigiano et al., 1998), overexpression of an outward-rectifying potassium channel (Burrone et al., 2002) or treatment with neurotransmitter receptor antagonists (O'Brien et al., 1998; Thiagarajan et al., 2005). By comparison, the reduction in release probability I observed following sFRP treatment is relatively mild, yet it still resulted in a robust homeostatic response. Short-term Wnt blockade (using a cocktail of sFRPs) in 21 DIV hippocampal

cultures decreased presynaptic release probability, as evidenced by an increase in the EPSC PPR and a decrease in mEPSC frequency. Longer-term (20 hour) blockade of Wnt signalling resulted in a prolonged reduction in release probability, as the EPSC PPR remained elevated. This prolonged reduction in glutamate release in turn led to a homeostatic increase in glutamatergic synapse number which acted to normalise excitatory activity, as mEPSC frequency was indistinguishable from control (Fig 7.3). Furthermore, I found that long-term treatment with Wnt7a, which has previously been shown to increase glutamatergic release probability (Cerpa et al., 2008), decreases excitatory synapse number. Therefore changes in glutamate release probability caused by prolonged gain and loss of Wnt function result in bidirectional homeostatic changes in excitatory synapse number (Fig 7.3).

An increase in excitatory synapse number in response to long-term sFRP treatment was also observed in younger (14 DIV) cultures; however, in this case a longer period of Wnt blockade (48 hours) was required. These results are consistent with previous studies which have used TTX to block activity in hippocampal neurons for extended periods of time. TTX incubation can cause a homeostatic increase in synapse number in both young (9 DIV) and older (~16 DIV) cultures (Han and Stevens, 2009; Wierenga et al., 2006). However, younger cultures require 48 hours TTX treatment to display this response, whereas 24 hour TTX treatment does not affect synapse number (Han and Stevens, 2009). Surprisingly, these studies did not investigate whether 24 hour TTX treatment could also increase synapse number in older cultures – only 48 hour treatment was used. My results suggest that, as hippocampal neurons mature, they become more competent to utilise changes in synapse number as a homeostatic mechanism.

Wnts are perhaps unlikely to directly mediate the homeostatic increase in synapse number I observed in mature cultures, given that these experiments involved treatment with a cocktail of Wnt antagonists. However, raises the question of whether Wnts may participate in homeostatic mechanisms under different experimental conditions, or even *in vivo*. Indeed it should be noted that many of the mechanisms utilised by neurons to maintain synaptic homeostasis, including changes in release probability, synaptic strength and synaptic number, are processes Wnts themselves are involved in. Furthermore, Wnt expression and release can be altered in response to activity (Chen et al., 2006; Gogolla et al., 2009; Wayman et al., 2006) and can signal across synapses

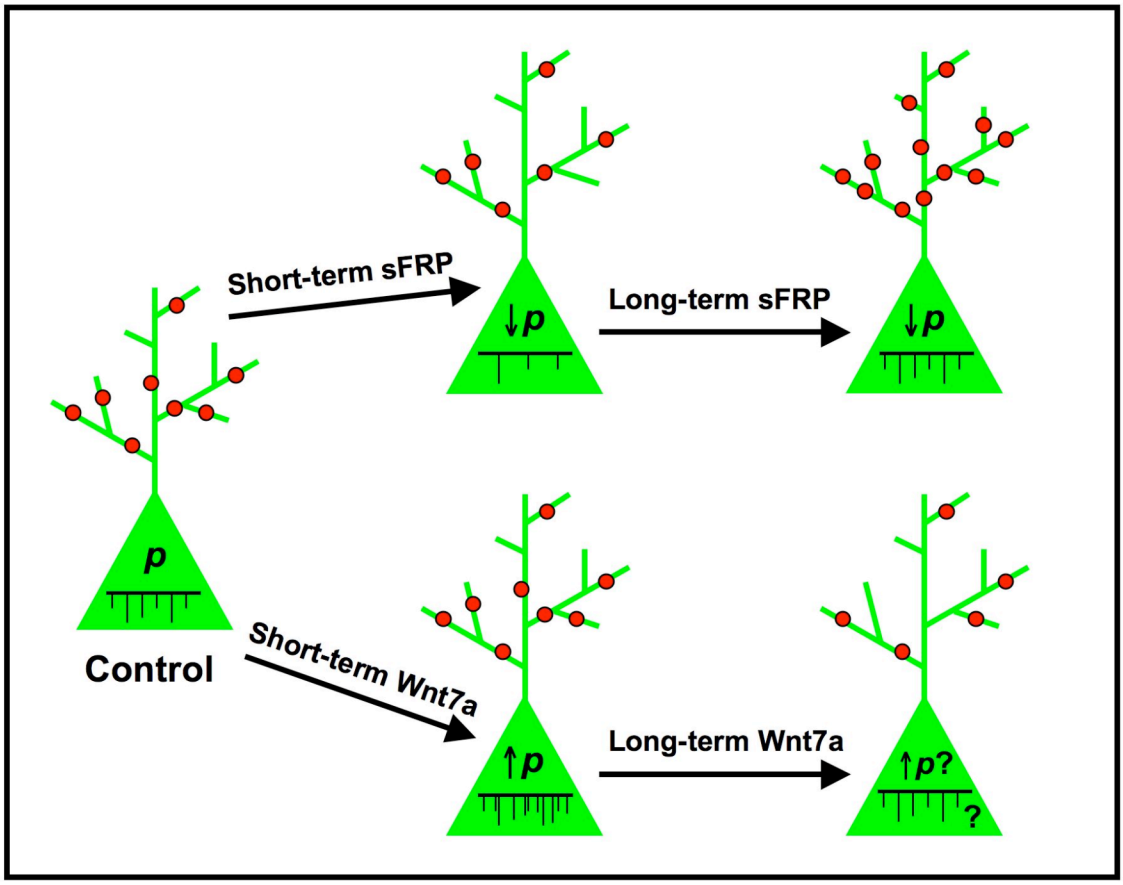

Figure 7.3 Summary of homeostatic responses to prolonged perturbation of Wnt signalling. On the left-hand side is a representation of a neuron from a control treated culture. The neuron has a certain number of excitatory synapses (red circles) onto its dendritic tree, each of which has a particular release probability (*p*). The mEPSC frequency of the cell is depicted by the trace in the cell body. Short-term sFRP treatment lowers the average release probability and mEPSC frequency, with a mild decrease in synaptic number. In response to longer term sFRP treatment, synaptic number increases, balancing out the decrease in *p* and restoring the mEPSC frequency. Conversely, short-term Wnt7a treatment increases *p* and mEPSC frequency (Cerpa et al 2008; Anane & Salinas, unpublished results), but does not affect synapse number in mature cultures. Longer-term Wnt7a treatment results in a decrease in synaptic density. This presumably counteracts a sustained increase in *p* and also acts to normalise mEPSC frequency; however, this remains to be tested directly (question marks).

(Ahmad-Annuar et al., 2006; Korkut et al., 2009; Packard et al., 2002). Therefore, Wnt signalling would seem to be ideally placed to play a role in synaptic homeostasis. This possibility could be explored by investigating whether the levels, activity or localisation of key Wnt signalling molecules are altered by prolonged manipulations of global activity, such as TTX treatment. It will also be of interest to systematically investigate how gain and loss of Wnt function affects the expression of synaptic homeostatic responses.

7.9 Conclusions

This thesis has examined the role of Wnt signalling in the formation and function of hippocampal synapses and has made several significant advances in our knowledge in this area. Firstly, Wnt7a promotes the formation of excitatory synapses through the co-ordinated clustering of pre- and postsynaptic proteins. This effect is specific to excitatory synapses, as inhibitory pre- and postsynaptic markers are unaffected. Secondly, activation of postsynaptic Wnt signalling regulates several aspects of excitatory postsynaptic development, including PSD-95 clustering, spine size, presynaptic innervation of spines and synaptic strength. Thirdly, endogenous Wnt signalling regulates presynaptic release probability at excitatory synapses. Finally, prolonged perturbation of Wnt signalling leads to homeostatic changes in synapse number that act to off-set the changes in release probability. These results provide novel insights into the role of Wnts as modulators of synaptic formation and function.

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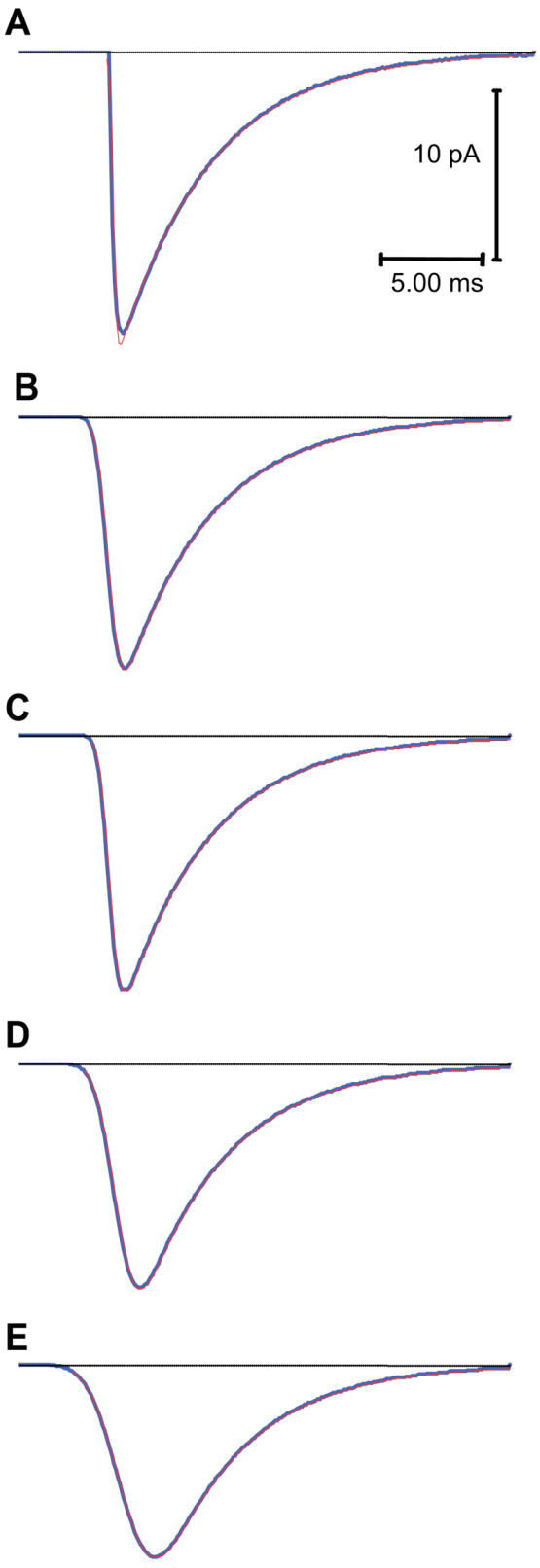
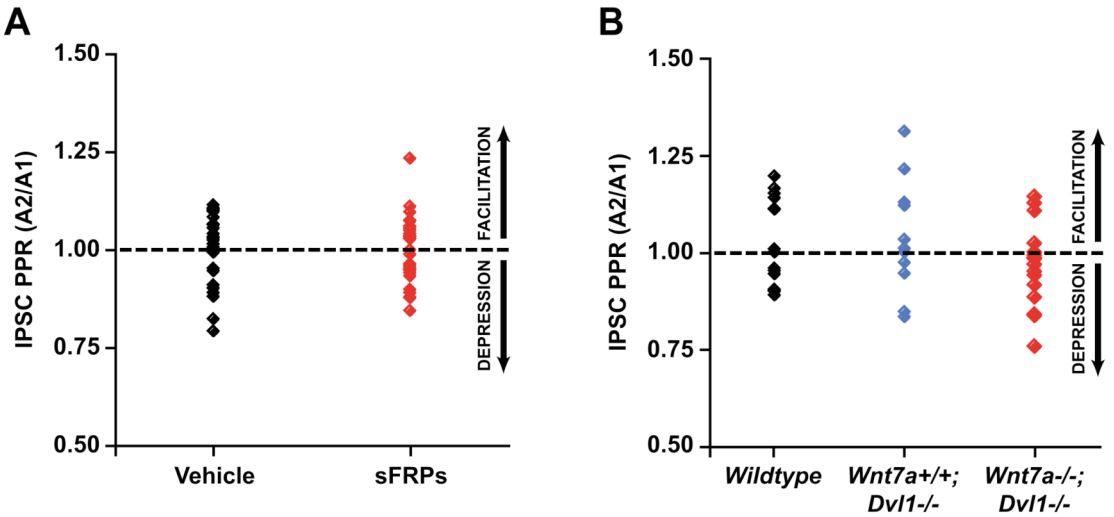
Appendices



Appendix 1 (Legend on next page)

Appendix 1 Changes in dendritic filtering alone cannot account for the effects of postsynaptic Dvl1 overexpression on mEPSC amplitude and rise time. (A) Simulated local synaptic mEPSC, with a $tau_{(rise)}$ of 0.2ms and a $tau_{(decay)}$ of 4.5ms. This mEPSC was then filtered at the average expected bandwidth (B) for control recordings (265 Hz) or (C) for Dvl1 transfected recordings (302 Hz). Increased filtering of the synaptic mEPSC can (D) increase the rise time to the control level, but with an amplitude higher than is observed or (E) attenuate the amplitude to control level, but with a longer rise time than is observed. Therefore the differences in mEPSC amplitude and rise time between control and Dvl1 transfected cells cannot be recreated using the assumptions that the underlying synaptic currents are the same, and dendritic filtering is higher in control transfected cells. This suggests the amplitude and kinetics of local excitatory synaptic currents are different in the two conditions.



Appendix 2. Distribution of IPSC paired-pulse ratios from individual cells. Scatter-plots showing the mean PPR for each cell recorded from (A) cultured hippocampal neurons and (B) CA1 cells in acute hippocampal slices. In all conditions the majority of cells show some degree of paired-pulse facilitation or depression, with the resulting average PPRs being approximately 1. Note that in (A) data from 3 hour and overnight conditions were pooled, as there were no significant differences in mean PPR values between the two treatment times.

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