The role of Wnt signalling in excitatory hippocampal synapse formation and function

Derek Anane

UCL

PhD Thesis

Supervisors:

Prof. Patricia C. Salinas

Dr. Alasdair Gibb

I, Derek Anane 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

The formation of a functional neural network is dependent on the correct assembly of cell-cell contacts. Apposition of pre and post synaptic terminals is a highly regulated process culminating in the formation of the functional junction points called synapses. Whilst much is understood of the processes involved in bringing axon and target together less is understood of the mechanisms controlling synapse assembly and maintenance.

Wnts are highly glycosylated secretary proteins which have been demonstrated to be involved at several stages of the developing nervous system. Through a range of signalling pathways Wnts are able to produce cellular effects including embryonic patterning, fate and movement. Much recent research has been focused on the role of Wnts in synapse formation and function.

In this thesis I present data from hippocampal cultures showing Wnt7a regulation of excitatory synapses. Exposure of developing hippocampal neurons to Wnt7a results in an increase in the density of surface GluA1, GluA2 and GluN1 puncta on dendritic spines. Wnt7a also regulates the co-localisation of postsynaptic glutamate receptor puncta with presynaptic sites labelled with vesicular glutamate transporter protein (vGlut). Interestingly the Wnt7a mediated increase in excitatory synapse formation is no longer present on neurons from mature cultures.

At the main postsynaptic site of excitatory synaptic transmission I identified Wnt7a and Dvl mediated maturation of glutamatergic receptor localisation. Both exogenous Wnt7a and overexpression of Dvl in 14DIV hippocampal cultures caused an increase in the size and number of receptor puncta located on spines and the proportion of spines containing GluA1, GluA2 and GluN1 puncta. In my thesis I also present *in vivo* functional data, using animals null for both Wnt7a and Dvl expression. These animals demonstrate defects in evoked post synaptic currents and paired pulse ratio at the CA3-CA1 hippocampal synapse.

In conclusion this report demonstrates the crucial role of Wnt7a – Dvl signalling in the regulation of excitatory synapse formation in the hippocampus. Furthermore amongst the myriad ways in which Wnt7a-Dvl signalling affects development of the CNS, Wnt7a acts directly at postsynaptic sites to increase synaptic strength.

Thesis Contents

List of Abbreviations

AChR – Acetyl choline receptor

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

AMPA-R – AMPA receptor

BDNF – Brain-derived neurotrophic factor

BSA – Bovine serum albumin

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

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

CK1α - Casein kinase 1α

CNS – Central nervous system

CRD – Cysteine-rich domain

Daam1- Dishevelled associated activator of morphogenesis 1

DFz2 – *Drosophila* Frizzled 2

DIV – Days *in vitro*

Dkk1 – Dikkopf-1

Dvl1 – Dishevelled-1

EPSC – Excitatory postsynaptic current

ER – Endoplasmic reticulum

Evi- Eveness interrupted

FGF – Fibroblast growth factor

FGF-R – FGF receptor

Fz – Frizzled

GABA - γ-aminobutyric acid

GC – Granule cell

dGRIP- Drosophila glutamate receptor interacting protein

GSK3β – Glycogen synthase kinase 3β

GTPase- Guanosine Triphosphate hydroxase

HBSS – Hank's balanced salt solution

HSPG – Heperan sulfate proteoglycan

IPSC – Inhibitory postsynaptic current

JNK – c-Jun N-terminal kinase

KO- Knockout

LMT – Large mossy fibre terminal

MAP1B – Microtubule associated protein 1B

mEPSC – Miniature excitatory postsynaptic current

mEJP- Miniature excitatory junction potential

MF – Mossy fibre

mGluR – metabotropic glutamate receptor

mIPSC – Miniature inhibitory postsynaptic current

MIS – Multiply innervated spine

NMDA – N-methyl-D-aspartate

NMDAR – NMDA receptor

NMJ – Neuromuscular junction

nNOS- Neuronal nitric oxide synthase

PBS – Phosphate buffered saline

PCP – Planar cell polarity

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

TTX – Tetrodotoxin

VAMP – Vesicle associated membrane protein

vGAT - Vesicular γ-aminobutyric acid transpoprter

vGlut – Vesicular glutamate transporter

Wg – Wingless

Wls- Wntless

Chapter 1:

Introduction

The ability of complex multicellular organisms to perceive their environment, to sense different cues and to make voluntary and reflex movements in response to sensory input or conscious decisions is the direct result of the development and maintenance of a functional nervous system. Across living species the complexity of the nervous system varies by orders of magnitude. The nervous system of *C.elegans* consists of a total of 302 neurons, drosophila have about 300,000, whilst current estimates suggest that the human brain contains around 10^{11} neurons (Williams and Herrup, 1988). However the complexity of these nervous systems can be observed at more levels than just the number of neurons involved. A key characteristic of nervous systems is the organisation of neurons into functional neural networks. These neural networks are defined by tightly regulated communication between specific groups of neurons. The complexity of a fully functional neural network is further increased by the variety of types of connection between neuron and communicating partner (excitatory glutamatergic, inhibitory GABAergic, modulatory dopaminergic, cholinergic, serotonergic or adrenergic for example).

These connecting sites were termed 'synapsis' as early as 1897 by Sir Charles Sherrington (Foster and Sherrington, 1897). In the intervening period since their discovery much research has been conducted in understanding their function and the molecular processes underlying this. It has been estimated that on average in the human brain each neuron forms approximately 600 synapses suggesting a total of approximately 6×10^{13} synapses in the fully developed adult (Cowan et al., 2001).

The structure of a chemical synapse is the apposition of specialised regions from two communicating cells. Whilst these connections may be between neurons, glands and muscles, in the central nervous system the communicating partners are neurons. Neuronal signalling begins in the presynaptic neuron where an action potential or spike is generated. This signal is the result of rapid movement of positively charged ions across the membrane generating an electrical signal which propagates from the soma down the axon. This electrical signal is transferred, cell to cell via chemical (neurotransmitter) messengers at the synapse. The action potential in the presynaptic neuron arriving at the synaptic specialisation results in release of

neurotransmitter into the synaptic cleft and activation of postsynaptic receptors. Sufficient activation of these receptors generates an electrical signal which is propagated throughout the postsynaptic cell. Generally the direction of synaptic transmission is uni-directional, one notable exception to this is observed in the *cyanea* jellyfish (Anderson, 1985). Commonly synaptic transmission generates a similar action potential in the postsynaptic cell as was present in the presynaptic neuron. The presence of synapses maintains the cell to cell electrical signalling fidelity whilst also providing a site for modulation of the signal. As such the location of synapses in relation to the structure of the neuron is tightly regulated. Presynaptic sites are generally found on axons whilst postsynaptic sites may be found across the cell usually concentrated on dendrites and soma but also on axons. Synapses can therefore be described in terms of the direction and sites of transmission e.g., axodendritic, axosomatic or dendrodendritic.

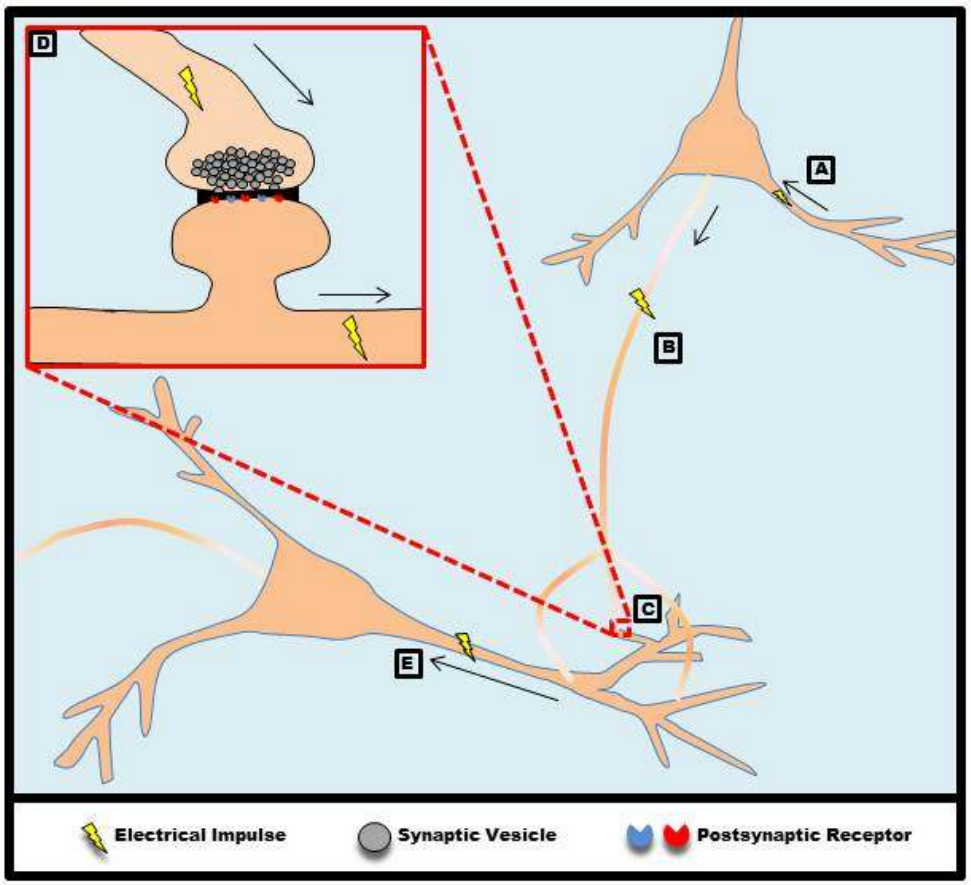

Figure 1.1 Electrochemical neuronal transmission. Neuron to neuron signalling involves the generation of an action potential in a presynaptic neuron. A) This action potential is

initiated in the dendrites of the neuron and is integrated in the soma. B) An action potential is subsequently generated in the axon initial segment of the axon (proximal to the soma) and propagates all the length of the axon. C) The axon may form synaptic sites with a postsynaptic partner in terminal structures or *en passant* formations. The majority of these synapses are made between axon and dendrite. D) An enlarged view of the pre and postsynaptic junction site reveals the structure of a typical synaptic site. Here the action potential enters the presynaptic bouton and initiates synaptic vesicle exocytosis causing neurotransmitter release into the synaptic cleft. Receptors on the postsynaptic surface bind neurotransmitter and generate electrical currents which propagate into and along the dendrite. E) Sufficient postsynaptic activation generates a new action potential which propagates along the dendrite in the direction of the soma.

Central synapses are generally classified as either Type I asymmetric or Type II symmetric (Colonnier, 1968; Gray, 1959). These descriptions refer to their appearance under electron microscopy (EM) and broadly describe whether these synapses are excitatory or inhibitory. The overwhelming majority of excitatory asymmetric synapses in the CNS release glutamate to generate depolarising excitatory postsynaptic potentials (EPSPs) and action potentials in the postsynaptic neuron via the presence of specific ionotropic and metabotropic receptors. Inhibitory symmetrical synapses generally act to prevent the formation of an AP in the post synaptic neuron. The number and strength of these different synapses within neural networks are tightly regulated to ensure appropriate levels of electrical signalling through these pathways. Defects in the formation or function of the excitatory and inhibitory synaptic networks have been linked with neurological disorders such as epilepsy, autism and schizophrenia (Kehrer et al., 2008; Leite et al., 2005; Rubenstein and Merzenich, 2003). Whilst these synapses have different functions many of the morphological and molecular components are common to both types. The next sections address the molecular components and structures common and unique to both excitatory and inhibitory synapses.

1.1 Synapses: Presynaptic terminus

Presynaptic and postsynaptic sites contain an array of protein machinery, allowing the translation of electrical signal to chemical and back to electrical. These proteins may also play roles in the structural and regulatory aspects of synaptic transmission. The presynaptic terminal is ultrastructurally characterized by the accumulation of neurotransmitter filled

vesicles. Often larger organelles such as mitochondria are also found associated with this region (Ly and Verstreken, 2006). Mitochondria provide ATP for many of the energy dependent processes which occur at this specialisation. This generalised description of the presynaptic terminal ignores a plethora of proteins found at the terminal.

A key feature of any presynaptic terminal is the presence of a number of spherical vesicles containing neurotransmitter ranging in size from 35-50nm (Jahn et al., 1990).Whilst ultrastructural observations suggest synaptic vesicles form large continuous clusters in synaptic terminals, fluorescence and electrophysiological data suggests more discrete "pools" of organisation (Rizzoli and Betz, 2005). Experiments looking at the depletion of vesicles in response to high frequency electrical stimulation or hypotonic shock have allowed grouping of vesicles into readily releasable (RRP), recycling or reserve pools. Maintenance of these various pools is important for sustained presynaptic release (Rizzoli and Betz, 2005). Alongside the small synaptic vesicles larger dense core vesicles (70-200nm) are often observed at synapses across the nervous system (Thureson-Klein and Klein, 1990). These neuropeptide containing vesicles are released with lower frequency than neurotransmitter containing vesicles (Hokfelt et al., 2000).

Synaptic vesicles mediate the chemical signalling at these sites by exocytosis of a range of neurotransmitters and neuropeptides into the synaptic cleft. The neurotransmitters present at a synapse are specific to the function of the synapse. In the central nervous system (CNS) the most common synaptic neurotransmitters are glutamate, glycine and gamma-aminobutyric acid (GABA). Glutamate is the most abundant neurotransmitter in the CNS and found exclusively at excitatory synapses (Cowan et al., 2001). Glycinergic synapses are mainly found in the brainstem and spinal cord but have also been identified in the retina, forebrain and cerebellar cortex (Kirsch, 2006). Glycine primarily plays an inhibitory role in the CNS. This neurotransmitter however is also a co-agonist in activation of the NMDA receptor (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). Both glycine and GABA in early development are excitatory neurotransmitters and become inhibitory as the nervous system develops (Kirsch, 2006; Li and Xu, 2008).

Vesicle exocytosis and endocytosis occur in the presynaptic terminal in a cyclic manner. Vesicles travel from reserve pool to RRP adjacent to the active zone (Sudhof, 2004). These docked vesicles then fuse with the active zone membrane, release neurotransmitter into the

synaptic cleft and are then endocytosed back into the cytoplasm. These vesicles are ready to again be loaded with neurotransmitter by the action of specific vesicle associated uptake transporters. At central synapses vesicle loading of glutamate, glycine and GABA is driven by the proton gradient and indirectly requires ATP hydrolysis to drive the proton pump (Gasnier, 2000). Interestingly whilst glutamate loading is mediated by the protein vesicular glutamate transporter-1 (vGlut) both the inhibitory neurotransmitters glycine and GABA are loaded by the same protein, vesicular GABA transporter (vGat). In fact at synapses where both glycine and GABA are expressed vesicles may be double loaded with a combination of the two neurotransmitters (Gasnier, 2000). Because vGlut and vGat segregate to excitatory and inhibitory presynaptic sites respectively, antibodies to these proteins are useful for identifying excitatory and inhibitory terminals. Besides the distinct vesicle pools an array of proteins essential to the regulation of vesicle transport underlies the presynaptic terminal.

Synapsins are vesicle associated proteins making up approximately 9% of the vesicle surface (Huttner et al., 1983). Synapsins are generally believed to play a role in tethering vesicles to the actin cytoskeleton maintaining a pool for efficient release (Dresbach et al., 2001). Knockout of various combinations of the synapsin I, II and/or III genes in mice causes an enhancement in the frequency of seizures and defects in learning (Rosahl et al., 1993; Rosahl et al., 1995).

Proteins such as bassoon and piccolo are large cytoskeletal proteins important in the structural maintenance of both exocytic and endocytic machinery in close proximity to the synaptic cleft (Dresbach et al., 2001). Studies by Mukherjee and colleagues using Piccolo and Bassoon KO mice have revealed these proteins are primarily involved in the regulation of synaptic vesicle clustering at the active zone (Mukherjee et al., 2010). Actin fibres are key structural components of the presynaptic terminal, supporting both the morphology of the terminal and proteins within the structure. The specific role of actin at synapses in relation to transmission is mired in debate in the field. Actin depolymerisation via latrunculin A resulted in a short lived enhancement of vesicle release (Morales et al., 2000). In investigations by Cole and colleagues latrunculin A inhibited vesicle release as observed by FM staining in Garter snake nerve terminal (Cole et al., 2000) In contrast f-actin disruption with cytochalasin D had no effect on FM1-41 staining suggesting no role for actin in vesicle exocytosis/endocytosis (Job and Lagnado, 1998). The discrepancies in results may be explained by differences in both experiment design and experimental system. Closer to the

plasma membrane several proteins are involved in the process of both action potential and non-action potential stimulated release.

Action potential evoked release is coordinated by the Ca^{2+} sensitive SNARE protein complex. This complex consists primarily of synaptobrevin/VAMP, syntaxin and Snap-25 (Ungar and Hughson, 2003). Calcium sensitivity of both AP evoked and spontaneous release is conferred by the non-core SNARE protein synaptotagmin (Geppert et al., 1994; Xu et al., 2009). AP evoked release occurs in several stages mediated by both the vesicle and plasma associated proteins combining to form the active snare (Sudhof and Rothman, 2009). The snare protein mechanically draws synaptic vesicles into close apposition with the plasma membrane initiating the fusion process (Sudhof, 2004).

Munc-13, Munc-18 and Rim proteins are further examples of active zone proteins essential in presynaptic function (Dresbach et al., 2001).RIM proteins impact vesicle release in a number of ways leading to defects in basic synaptic transmission. RIM1/2 isoform knockout reduced Ca^{2+} channel density at the calyx of Held in the auditory brain stem (Han et al., 2011). Schoch and colleagues observed severe defects in neuromuscular transmission of E18 mice with the double knockout of RIM1α/2α. Evoked release amplitudes decreased in size whilst synaptic failure rates were increased (Schoch et al., 2006). These effects were complemented by an inability to modulate mEPSC frequency by Ca^{2+} concentration increase again demonstrating RIM mediated regulation of the link between vesicle release and Ca^{2+}. This removal of RIM also resulted in a decrease in the number of readily releasable pool vesicles and docked vesicles. Munc-13 KO mice exhibit hippocampal defects in readily releasable vesicle pool and also display severe impairment of presynaptically generated EPSCs (Augustin et al., 1999). Deletion of Munc-18 prevents vesicle exocytosis without affecting synaptic ultrastructure (Verhage et al., 2000). This is most likely because munc-18 is directly involved in vesicle exocytosis via interactions with syntaxin and SNAP25 (Jahn and Sudhof, 1999).

Voltage gated Ca^{2+} channels are also present clustered adjacent to release sites in the active zone. Calcium influx mediated by these channels triggers the rapid exocytosis of vesicles at the active zone. Indeed direct binding between the SNARE associated protein synaptotagmin and Ca^{2+} channels further strengthens this idea (Catterall, 1999). It is clear that whilst presynaptic sites have many common features several proteins and processes are distinct to

the type of synapse. These differences are further accentuated on the opposing side of the synaptic cleft.

1.2 Synapses: Postsynaptic terminal

Postsynaptic specialisations of the CNS are generally observed ultrastructurally as regions with morphology varying from dendrite to exaggerated dendritic protrusions. These structures are generally crowned by one or several regions of high electron density described as the postsynaptic density. In order to further examine the postsynaptic density the next section will be divided in to sections focusing on inhibitory and excitatory specialisations.

1.2.1 GABAergic postsynaptic specialisation

Inhibitory synapses in the CNS generally form as axosomatic or axodendritic specialisations with little or no distinctive postsynaptic protrusions. GABAergic presynaptic terminals have however been observed synapsing directly onto dendritic spines of excitatory cortical synapses (Kubota et al., 2007). This type of integrated excitatory/inhibitory postsynaptic structure is believed to allow greater inhibitory regulation by the GABAergic neuron.

At the molecular level the postsynaptic density of central inhibitory synapses differs fundamentally from that of excitatory synapses. Inhibitory synaptic transmission in the CNS is mediated by GABA and glycine ionotropic receptors. A lack of glycinergic transmission and the presence of glycine receptors only at extra-synaptic sites suggest that GABAergic transmission is dominant in the hippocampus, cortex and entire CNS with the exception of the spinal cord (Lynch, 2009). The data presented in this thesis is primarily concerned with the synapses of the hippocampus and so I will focus my discussion in the following section specifically on GABAergic postsynaptic specialisations.

$GABA_{(A/C)}$ receptors are chloride ion channels which upon activation by GABA in mature neurons allow a flow of Cl^- ions across the membrane, hyperpolarising the neuron. Pharmacologically these receptors are characterised by inhibition by bicuculine or picrotoxin. As mentioned previously during the first postnatal week these receptors mediate depolarising effects on neurons. This developmental effect is due to the high $[Cl^-]_i$ in postnatal neurons generated by reversed operation of the membrane Cl^- pump (Misgeld et al., 1986). This is

because the GABA receptor ion flow is reversed due to the Cl^- reversal potential of approximately -52mv generating a depolarising effect (Cherubini et al., 1991). This shift in Chloride ion reversal potential between adults and neonates coincides with the expression of chloride transporters (Blaesse et al., 2009). Interestingly whilst these receptors depolarise postsynaptic neurons the summation of these synaptic receptors may not lead to the generation of an excitatory current in the postsynaptic neuron in the same way as glutamatergic transmission in mature systems.

Functional channel forming $GABA_A$ receptors, based on biochemical analysis, atomic force microscopy and comparison with heterologous expression studies, are composed of five subunits (Sieghart et al., 1999). However the crystal structure of the receptor has not been resolved. Homology between $GABA_A$ receptors and other cys-loop membrane proteins such as the glycine receptor, 5HT3R and the nAChR have led to clues about the membrane structure of the functional receptor. In humans several subunit isoforms exist many of which also have splice variants. Six α subunits: three β: three γ: alongside π, θ, δ and ε classes of subunit have been identified (Hevers and Luddens, 1998). Any two α, two β and one γ subunit may comprise the functional channel. Despite the large potential variety of subunit combinations it appears that only a fraction have been observed expressed in humans (Whiting et al., 1995). Differences in agonist and antagonist binding affinity between subunits, confers a range of channel properties on the various functional channels. Differences in the expression of subunit types in various brain regions have also been observed demonstrating a subtle regulation of $GABA_A$ receptors in the CNS. This regulation is further accentuated at the synapse where receptors containing α1,2,3 or 5 with β and γ subunits are predominantly located centrally as opposed to the α5 containing receptors which are found extra-synaptically (Jacob et al., 2008) . Interestingly $GABA_C$ receptors are almost exclusively expressed at the retina where they function alongside the other subtypes (Passafaro and Sheng, 1999). The above data clearly indicates spatial segregation of the various $GABA_A$ and $GABA_C$ subtypes suggesting both specialised regulation of expression and trafficking alongside unique roles for each subtype in the CNS.

Insertion and removal of $GABA_A$ receptors at synapses is regulated by a variety of receptor associated proteins. Gephyrin is a scaffolding protein found associated with inhibitory receptors, binding directly to glycine receptors via their β subunits (Meyer et al., 1995). Gephyrin proteins are highly enriched at inhibitory synaptic sites and are believed to be

important in the stabilisation of GABA receptor at these sites. Indeed reduction of gephyrin in neurons leads to the loss of α2 and γ2 subunits at synapses (Jacob et al., 2005; Kneussel et al., 1999). In addition synaptic $GABA_A$ receptors are significantly more mobile in neurons where gephyrin has been knocked down (Jacob 2005). In spinal cord, loss of gephyrin destabilises α3 and β2/3 containing receptor pools but has minimal effect on α1/5 containing receptors suggesting a degree of subunit specificity in the action of gephyrin. Indeed, on neurons where Gephyrin expression has been knocked down, surface receptor number was increased but the larger $GABA_A$ clusters were significantly decreased (Jacob et al., 2005).

GABA receptor associated protein (GABARAP) binds GABA receptors in the cytosol and ER but not at cell membranes (Wang et al., 1999). $GABA_A$ receptors bind GABARAP via the intracellular loop of the γ2 subunit, which is the most abundant in the CNS suggesting most $GABA_A$ receptors are associated with GABARAP in the CNS. Overexpression studies of this protein result in an increase in surface $GABA_A$ receptor possibly via addition of phospholipid groups to the immature receptor (Mohrluder et al., 2009). GABARAP expression is essential for the activity dependent synaptic localisation of GABA receptor following NMDAR activation (Marsden et al., 2007; Muir et al., 2010).

Microtubule associated proteins (MAP1A, MAP1B and MAP1C) as the name suggests are scaffolding and linker proteins which associate other proteins with both the microtubule and actin cytoskeletons. In accordance with their function these proteins are found in neurites particularly in dendrites. MAP1B plays a key role in the stabilisation of $GABA_C$ receptors at synaptic sites (Passafaro and Sheng, 1999). MAP1B was discovered as a binding partner of ρ1 subunits which are exclusive components of $GABA_C$ receptors (Billups et al., 2000). Interestingly the similarity between the MAP1A/1B light chain and GABARAP protein structures suggest that these proteins may use similar mechanisms to stabilise $GABA_C$ receptors at synapses (Passafaro and Sheng, 1999).

Whilst initial fast inhibitory signalling is mediated by $GABA_A$ receptors, slow long acting inhibitory control occurs via $GABA_B$ receptors. $GABA_B$ receptors are metabotropic receptors linking synaptic GABA activity to membrane potassium channels. Activation of these synaptic receptors causes opening of synapse adjacent K^+ channels via G-protein α/β subunit signalling (Luscher et al., 1997). Potassium ion flow hyperpolarises the neuron, inhibiting action potential duration. Interestingly both $GABA_A$ and $_B$ receptors are found both pre and

postsynaptically (Kasugai et al., 2010; Vigot et al., 2006). Depolarisation in the presynaptic neuron inactivates Na^+ and Ca^{2+} channels, thereby reducing AP amplitude and subsequent presynaptic release (Kullmann et al., 2005). In comparison to excitatory synapses, inhibitory synapses are still poorly understood particularly with respect to the mechanisms which actively regulate receptor movement in and out of the synapse. Interestingly the variety of subtypes and division of inhibitory synaptic receptors into both metabotropic and ionotropic is mirrored at excitatory synapses.

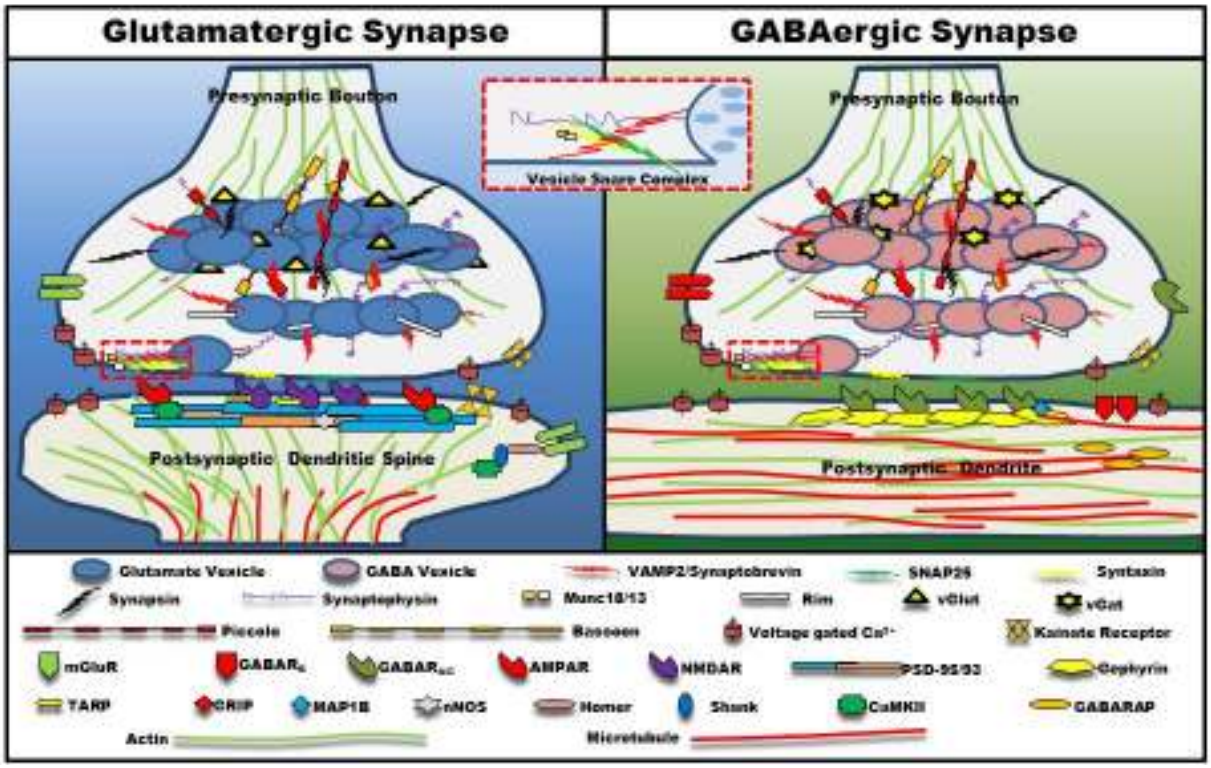

Figure 1.2 The Excitatory Glutamatergic and Inhibitory GABAergic specialisation. Left and right mirror panels demonstrate the complexity, similarities and differences pre and postsynaptically at synaptic sites. Both glutamatergic and GABAergic presynaptic sites contain many of the same proteins essential for vesicle release. These include both vesicle associated proteins such as Rims and proteins which form part of the snare complex. These proteins are displayed in greater detail in the inset where the cell membrane associated proteins syntaxin and SNAP25 and VAMP2 and synaptophysin appear in the complete complex. Associated with the SNARE complex, Munc 18 and 13 are present associating with syntaxin. The major differences between glutamatergic and GABAergic presynaptic boutons are visible in the neurotransmitters and vesicle loading proteins. Postsynaptically the most striking difference is the morphologies of the sites. Where the postsynaptic glutamatergic

synapse is a specialised dendritic spine, the GABAergic site is directly on the surface of the dendrite. Microtubules are present throughout dendrites but are only present in the dendritic initial segment of spines, whilst Actin is polymers are found both throughout the synapse. Both types of postsynaptic site contain an array of receptors stabilised by scaffolding proteins. Associated with both scaffolding arrays are a number of signalling proteins which link receptor activation to intracellular signalling cascades.

1.2.2 Excitatory postsynaptic specialisation: Dendritic spines

Typically excitatory synapses (in contrast to the axosomatic synapses of inhibitory synapses) form almost exclusively on morphogically specialised dendritic protrusions called spines (Cowan et al., 2001; Luscher et al., 2000; Segal, 2010). Variety in spine morphology, number, location and regulation has significant consequences on the ability of a neuron to function within a neural network.

At the most simple level these protrusions act to increase the surface area for synaptic contact however there are various theories for the biological significance of dendritic spines. Generally spines are thought of as areas of separate synaptic compartment. This idea stems from both computational and immunofluorescence studies describing spines as discrete regions that allow the neuron to better discriminate single synaptic activation and subsequently integrate this information in the neuron. Diffusion between a spine and the adjacent dendritic shaft has been observed to be approximately 100 times slower than expected in a 'freely diffusible' system (Nevian and Sakmann, 2004; Svoboda et al., 1996). This data suggests larger signalling proteins and other essential species would exhibit similar or slower diffusion rates making a continuous flow of signalling with the dendritic shaft extremely inefficient. This compartmentalisation allows greater sensitivity of signalling proteins at the postsynaptic density due to small changes in cytoplasmic protein concentrations (Cowan et al., 2001). In particular Ca^{2+}signalling is well served by the microenvironment of spines which will be discussed later in further detail.

At the ultrastructural level excitatory spines are very distinctive structures. A typical spine consists of a spherical head connected to the dendritic shaft by a relatively thin stalk. Generally spines may range from 0.01 μm^3 to 0.8μm^3 in volume (Harris, 1999). Other types of spine may vary in the relative proportion of head volume to spine volume and/or the total

length of the protrusion. Spine morphology can be loosely grouped into 3 types; stubby (I), thin (II) and mushroom (III) (Harris et al., 1992).

As important structures within the CNS it is not surprising that dendritic spines also undergo developmental regulation. Generally spines are believed to develop from filopodia (type I) to more mature mushroom (type III) spine. Dendrites of early neonatal pyramidal cells are relatively bare and this situation is rapidly modified over the first and second weeks. Coinciding with the generally perceived peak of synaptogenesis (P14-18) there is a rapid expansion in the density of dendritic protrusions on neurons (Nimchinsky et al., 2002). The vast majority of spines in the 1-2 week postnatal period consist of stubby and thin (type I/II) or filopodia morphology (Cowan et al., 2001). Spine motility in early postnatal neurons has been suggested as the key mechanism in the coming together of pre and postsynaptic sites. Time lapse microscopy has led to the proposal of three mechanisms by which spines find presynaptic partners and mature, reviewed in (Yuste and Bonhoeffer, 2004). Most studies identify the high motility of filopodia and long thin spines as the active structures in searching out axons for nascent synaptic sites. Model 1 suggests these early spines come into contact with presynaptic partners and then change into mature mushroom like spines. The observation that a large number of shaft synapses are present on early neurons led to model 2. In this mechanism once the pre synaptic site and immature filopodia come into contact the filopodia retracts to form a shaft synapse and subsequently develops a mature spine from this site. The third model presents an idea of spines as constantly fluctuating in terms of morphology. Here spines may form nascent synapses, retract from the presynaptic partner and subsequently reposition with the same or a different partner as a mature mushroom shaped spine. Far from being competing theories of synapse development it is likely that all three models can describe spines in the formation, elimination and maintenance of their structures. After the rapid formation of synapses a period of synapse elimination occurs in development. This is marked at the spine level by decreases in spine density and shifts in spine morphology. The number of spines decreases until there is a balance of spine removal and formation resulting in a net zero change of spine number (Dunaevsky et al., 1999). The composition of mostly filopodia, stubby and thin spines shifts to incorporate a large proportion of mushroom spines and spines with relatively large head to neck ratio morphology.

Importantly the dynamic nature of dendritic spines remains in the mature CNS allowing these structures to respond to intrinsic and extrinsic stimuli (Fischer et al., 1998). Presynaptic activity amongst other stimuli is crucial for both the formation and stabilisation of spines into nascent synaptic sites. Local application of glutamate to dendrites encouraged formation of new spines whilst uncaging of glutamate at established spines caused an increase in spine head volume (Matsuzaki et al., 2004). These observations have also been recorded by afferent stimulation of presynaptic neurons resulting in an increase in presynaptic release. The activity dependent stabilisation of spine synapses is a highly detailed process observed in regions as diverse as the retino-tectal system to the neuromuscular junction (Nimchinsky et al., 2002). The most clear example of this is the loss of spines upon deafferentation of postsynaptic neurons (Cheng et al., 1997). Cheng and colleagues studied morphological changes to spines of medium spiny striatal neurons in 6 month old mice. By creating lesions in the cortex these neurons were deprived of presynaptic partners from corticostriatal terminals, the result being a decrease in spine density with no observed defect in overall dendrite structure. Interestingly after 10 days, spine density began to recover to control levels coinciding with clearance and replacement of degenerated cortical afferent fibres by the contralateral cortex. Similarly Parnevelas and colleagues (1974) were able to show defects in spine density on dentate granule neurons as a result of entorhinal cortex lesions. Spine density again returned to control levels after a period as a result of reafferentation from nearby axons (Parnavelas et al., 1974).

The variety in spine morphology is largely maintained by the rich F-actin cytoskeleton found in these structures. Interestingly, in contrast to the rest of the dendrite, microtubules and microtubule associated proteins are largely excluded from spines helping to generate a specialised compartment within these structures (Harris and Kater, 1994). This accumulation of actin in spines allows use of antibodies to actin and actin associated proteins in the identification of dendritic spines. In confirmation of actins role in the regulation of spine shape, experiments looking at small actin GTPases such as Rac, Rho and CDC42 (which themselves regulate actin structure) revealed some interesting results. Schubert and colleagues identified RhoA kinase inactivation as key in the maintenance of spine size (Schubert et al., 2006), whilst when using a transgenic mouse model expressing constitutively active Rac1, Luo and colleagues observed development of smaller spines on Purkinje cells (Luo et al., 1996).

Defects in the formation and maintenance of dendritic spines have been linked with several neurological disorders. Spine and synapse loss has been identified in mouse models for Alzheimer's disease (D'Amelio et al., 2011). Upon comparison of post-mortem hippocampi from Alzheimer's patients with age matched non-sufferers, defects in spine number were observed (Ferrer et al., 1990). Dysfunction of spine morphogenesis is also associated with Fragile X syndrome, one the most common forms of single gene mutation associated with mental retardation. Cortical spines of neurons in which Fragile X gene expression is removed appear greater in density but have long, thin immature morphology (Comery et al., 1997). Similarly to Fragile X syndrome, autism spectrum disorders are associated with both dysfunction in spine morphology and significant increase in the density of spines throughout the brain in comparison to 'normal' specimens (Penzes et al., 2011). Spine loss has also been observed in caudal putamen of neostriatal medium spiny neurons in late stage Parkinson disease sufferers (Zaja-Milatovic et al., 2005). The work of Garey and colleagues revealed details of spine defects in the brains of schizophrenia sufferers. Looking at post-mortem temporal and frontal cortex brain samples of schizophrenia sufferers showed respectively 59% and 66% decreases in spine density observed (Garey et al., 1998).

In comparison to inhibitory synapses the postsynaptic density is defined by an area of very strong electron density. Postsynaptic densities are found at virtually every excitatory synaptic site and generally the structures are 40-50nm thick and a few hundred nanometres wide. Three dimensional EM reconstructions reveal the region to be disc-like occasionally perforated suggesting discontinuity of the structure (Harris and Kater, 1994) Interestingly across all brain regions PSD area is proportional to the total size of the spine suggesting a relationship between spine size and synapse content (Harris and Stevens, 1988; Peters and Kaiserman-Abramof, 1970; Westrum and Blackstad, 1962).

More than a single structure, this region contains a vast number of proteins essential for and associated with excitatory postsynaptic transmission and downstream signalling. Several hundreds of these proteins were identified by the various proteomic studies conducted by groups headed by Morgan Sheng and Seth Grant (Husi and Grant, 2001; Sheng and Hoogenraad, 2007). In particular proteomic studies conducted by the aforementioned groups allowed analysis of synaptic sites and the identification of large proteins associated with PSD proteins. One of the earliest identified PSD interactions was the link PSD-95 and NMDARs (Husi and Grant, 2001; Kornau et al., 1995). These proteins include; postsynaptic

neuroreceptor proteins, protein kinases, structural protein, proteins involved in endocytosis and glycolytic proteins. The above list does not begin to account for the number of proteins loosely associated with the PSD or whose localisation may be dependent on synaptic activity or activation of specific cell signalling pathways.

The postsynaptic density is populated by groups of scaffolding proteins which are essential for the assembly and maintenance of machinery at the excitatory synapse. PSD-95/SAP90, PSD-93/Chapsyn-110, SAP97 and SAP102 proteins are prominent features of excitatory synapses and associate with many of the receptors, scaffolding proteins and signalling molecules that localise in the region (Boeckers, 2006). Members of the MAGUK superfamily, these proteins contain several protein-protein interacting domains. In particular this family of proteins are characterised by the presence of PDZ, SH3 and GK domains (Anderson, 1996). PSD-95 proteins in particular have been studied in detail because of their relationship with glutamate receptors in the PSD. PSD-95 contain three PDZ domains in the N-terminal domain and through the first two (PDZ1 and PDZ2), PSD-95 binds NMDARs (Kornau et al., 1995).

Neuronal nitric oxide synthase (nNOS) are important biological catalysts involved in the conversion of L-arginine and oxygen to nitric oxide (Alderton et al., 2001; Bredt, 1999; Luo and Zhu, 2011). In the CNS, Nitric oxide (NO) has been identified as a retrograde signalling molecule in presynaptic LTP (Schuman and Madison, 1991). The presence of nNOS at the synapse and the regulation of the enzyme is therefore important in synaptic function. Alternative splicing generates various nNOS isoforms which contain a range of PSD interacting domains. More specifically the α and μ variants contain N-terminal PDZ domains localising these proteins to the synaptic PSD whilst β and γ isoforms lack these domains and are found in the cytoplasm (Alderton et al., 2001). Synaptic localisation of nNOS is mediated via direct binding to PSD-95 and PSD-93 (Brenman et al., 1996). Interestingly overexpression of PSD-95 results in both an increase in nNOS expression and the formation of multiple innervated dendritic spines (MIS) (Nikonenko et al., 2008). In the same investigation pharmacological inhibition of nNOS decreases MIS formation suggesting NO acts as the retrograde signal at sites where PSD-95 accumulation occurs.

The remainder of this section will take a more detailed look at the key postsynaptic proteins involved in transduction of excitatory signalling. Of the range of proteins present at the PSD

the postsynaptic neurotransmitter receptors are the most essential in excitatory transmission. As with inhibitory receptors, glutamatergic excitatory receptors exist as a similarly diverse group. This similarity extends to the presence of both ionotropic and metabotropic glutamate receptors.

1.2.3 Metabotropic glutamate receptors

The metabotropic glutamate receptors or mGluRs are a family of G-protein coupled glutamate binding receptors primarily derived from 8 gene sequences with several splice variants (Niswender and Conn, 2010). $mGluR_1$ and $mGluR_5$ are classed as Group I mGluRs. Classical group 1 mGluR signalling acts to enhance Ca^{2+} concentration postsynaptically by signalling to intracellular stores using G_q/G_{11} (Ferraguti et al., 2008). Activation of these receptors activates phospholipase C which generates IP_3 via hydrolysis of phosphoinositides. IP_3 translocates to the ER and affects calcium exocytosis from internal stores as well as PKC activation. However depending on the cell type or neuronal population a range of pathways involving phospholipase D, casein kinase 1 and Jun kinase may become active (Niswender and Conn, 2010). Group 1 mGluRs are predominantly, postsynaptically located and tend to be found around the periphery of the PSD (Lujan et al., 1996).

Group II and Group III mGluRs are generally coupled to $G_{i/o}$ and as a result activate similar pathways (Niswender and Conn, 2010). The most well studied pathways of Group I and II mGluRs involve inhibition of adenylate cyclase reducing the formation of cAMP. At the synapse these receptors are found both pre and postsynaptically with seemingly inhibitory effects on transmission. Presynaptically these receptors are associated with ion channels and inhibition of release. Postsynaptically these receptors decrease NMDA activity and act to prevent excitotoxicity (Ambrosini et al., 1995).

1.2.4 Kainate receptors

A second group of glutamate binding receptors are those of the kainate (KAR) family. These ionotropic receptors are mediators, postsynaptically of excitatory effects and inhibitory effects presynaptically (Ozawa et al., 1998). Kainate receptors are derived from a combination of 5 subunits GluR5, GluR6, GluR7 and KA1 and KA2. The functional receptor tetramer may be composed of homomers or heteromers of GluR5-7, KA1/2 containing

receptors must contain any of the GluR5-7 subunits (Bettler et al., 1990; Egebjerg et al., 1991; Herb et al., 1992; Schiffer et al., 1997). These ionotropic receptors once activated by glutamate are permeable to Na^+, K^+ and dependent on the presence of Q/R edited subunits are permeable to Ca^{2+} (Huettner, 2003). These characteristics combined with AMPAs ability to act as an agonist (and the ability of kainate to activate AMPARs) initially made identification of these receptors quite difficult. Kainate receptors are expressed strongly throughout the cerebellum with lower levels in the hippocampus and caudate putamen (Ozawa et al., 1998). Postsynaptically, antibodies for GluR5-7 suggest these receptors are present both in dendrites and PSDs. Presynaptic localisation has been identified with antibodies for GluR6 and GluR7 in unmyelinated axons of the CA3 region in the hippocampus. The role of KA receptors both pre and postsynaptically is still under much debate. Experimenters have observed both enhancement and depression of neurotransmitter release as a result of KAR activation (Huettner, 2003). The discrepancy in these experiments lies in the synapses under observation suggesting KAR activation presynaptically may have different effects depending on the receptor type. Postsynaptically KA receptors contribute a slow current with low (relative to AMPAR) amplitude (Castillo et al., 1997; Kidd and Isaac, 1999; Li et al., 1999; Mayer and Westbrook, 1987; Vignes and Collingridge, 1997).

1.2.5 AMPA receptors

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are primarily responsible for fast synaptic current generation in postsynaptic neurons. In combination with NMDARs they mediate most of the basic transmission and plasticity associated with excitatory synaptic transmission. In addition, these receptors are the most commonly expressed glutamate receptors in the CNS and whilst these patterns vary throughout development these receptors are fundamental in synaptic transmission across vertebrates.

As is true for many of the receptors of the CNS functional pore forming receptors are composed of various AMPA subunits. *In vivo* these receptors are formed as hetero-tetramers consisting of pairs of subunits or ‘dimers of dimers (Mayer, 2005). Currently 4 subunits have been isolated; GluA1, GluA2, GluR3 and GluR4. All four subunits share high homology and are predicted to have similar tertiary structure with the crystal structure of the GluA2 subunit being one of the few ionotropic receptors to have been resolved (Greger et al., 2007). Each subunit contains a large extracellular N-terminal domain adjacent to an S1 domain which forms part of the glutamate binding domain (Ayalon and Stern-Bach, 2001). All subunits

contain four transmembrane regions, one of which forms a re-entrant loop within the membrane and the third of which is joined to an extracellular region which forms the second part of the glutamate binding domain. Much of the diversity between the 4 receptor subunits and other receptors is derived from differences in the sequences of the intracellular C-terminal tails. Subunits GluA1, 4 and an alternately spliced $GluA2_{(l)}$ have long C-terminal tails containing unique interaction sites for CaMKII, PKC and PKA (McDonald et al., 2001). Whereas the short sequence C-terminal tail containing subunits, GluA2, GluR3 and alternately spliced $GluR4_{(c)}$ contain binding sequences for GRIP and PICK1 (Dong et al., 1997; Xia et al., 1999).

AMPAR gating is regulated by the binding of glutamate to sites in each of the four subunits in the heterotetramer (Rosenmund et al., 1998). Once two sites are occupied channel opening occurs with the single channel conductance increasing upon further glutamate binding. AMPA receptors, dependent on the complement of subunits, have varying gating properties. Ca^{2+} permeability in AMPA receptors is dependent on the presence of the GluA2 subunit. AMPA receptors lacking GluA2 subunits are largely impermeable to Ca^{2+} due to post-translation mRNA editing by adenosine deaminase of the Q/R site in the subunits transcript (Hume et al., 1991; Verdoorn et al., 1991). This changes an amino acid from the uncharged glutamine to the positively charged arginine and alters the otherwise energetically favourable flow of Ca^{2+} through the channel. Interestingly, this form of editing occurs in greater than 99% of expressed GluA2 transcripts rendering the vast majority of GluA2 subunit-containing protein with these characteristics (Schmauss and Howe, 2002; Seeburg, 2002).

A second channel property of GluA2 lacking receptors results in decreased ion conductance at polarized potentials. These receptors are described as inwardly rectifying stemming from channel blockade at depolarised potentials by intracellular Mg^{2+} and polyamines (Donevan and Rogawski, 1995). GluA2 containing receptors are unaffected by this blockade and as such have a non- rectified I/V relationship (Seeburg et al., 2001).

The presence of a 38 amino acid sequence in the extracellular space, adjacent to the fourth transmembrane region confers a variety of channel properties on all members of the AMPAR family. Generated by alternative splicing, point mutations in the glutamate binding domain results in either ‘flip’ or ‘flop’ subunit varieties (Sommer et al., 1990). Specifically ‘flop’ versions desensitize more rapidly to glutamate than ‘flip’ versions. These differences also

result in differences in re-sensitisation and inhibition of desensitisation by allosteric compounds benzothiadiazides (Partin et al., 1995). Alterations in the expression of 'flip' or 'flop' containing receptors are observed in disorders such as schizophrenia and epilepsy (Eastwood et al., 1997; Rosa et al., 1999).

Transmembrane AMPA receptor regulator proteins (TARPS) are recently identified ionotropic AMPAR auxiliary subunits influencing receptor trafficking and gating (Jackson and Nicoll, 2011; Payne, 2008). Until quite recently ionotropic ligand binding channels were stood apart from many other membrane channels due to the lack of evidence of accessory β subunits. These subunits form central pieces of the channel complex and affect channel processing and localisation (Arikkath and Campbell, 2003). The γ-2 subunit, also referred to as stargazin, was identified in 1998 (Letts et al., 1998). Work by Chen and colleagues later identified the ability of stargazing to interact with both AMPAR and PSD-95 and mediate the surface synaptic localisation of receptor (Chen et al., 2000). Further investigation into receptor gating revealed stargazin slows both desensitization and deactivation by modulating the rate of channel opening (Tomita et al., 2005). Gene comparison studies have revealed a number of other TARPs which are expressed throughout the CNS and demonstrate a variety of effects on channel gating (Burgess et al., 2001; Klugbauer et al., 2000; Milstein et al., 2007).

1.2.6 NMDA receptors

N-Methyl D-Aspartate receptors are mediators of 'slow' excitatory currents and are crucial to many synaptic plasticity mechanism including those likely to be important for learning and memory functions in the brain (Bliss and Collingridge, 1993; Cowan et al., 2001; Li and Tsien, 2009; Lynch, 2004; Malenka and Nicoll, 1999). These ionotropic neurotransmitter receptors are unique in that they require the binding of two independent ligands, glutamate and glycine and their channel has a voltage dependent block by Mg^{2+} (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). This means that following receptor activation membrane depolarisation is necessary before significant synaptic current and calcium and other ion influx can occur through the channel (Collingridge et al., 1988).

Several lines of evidence suggest the pore forming receptor is composed of a heteromeric composition of two obligate GluN1 and either homo or heteromeric combinations of GluN2/GluN3 gene products (Behe et al., 1995; Benveniste and Mayer, 1991; Clements and

Westbrook, 1991). GluN1 subunits are derived from alternative splicing of a single gene whilst NR2 and NR3 subtypes are the result of alternative splicing of these 2 genes (Stephenson, 2006). These subtypes comprise; GluN1 1a, 1b 2a, 2b, 3a, 3c, 4a, 4b, GluN2a, b, c, d, GluN3a, b.

Tertiary structure predictions suggest these subunits have similar structures to other cys-loop ionotropic glutamate receptors with key differences in agonist binding and pore forming domains (Mayer and Armstrong, 2004). Similar to the glutamate binding domain of AMPA subunits, NMDA subunits bind glycine (GluN1, GluN3) or Glutamate (GluN2) between modified extracellular S1 and S2 domains (Anson et al., 1998; Hirai et al., 1996; Kuryatov et al., 1994; Laube et al., 1997; Wafford et al., 1993). Residue differences in the pore forming region confer the unique properties of voltage dependent Mg^{2+} blockade and lack of specificity in ion permeability (Na^{+}, K^{+} and Ca^{2+}) through the channel. Mg^{2+} blocks flow through the channel at resting membrane potentials requiring depolarisation of the neuron before the channel can pass significant inward current (Mayer et al., 1984; Nowak et al., 1984). For this property NMDARs are termed 'coincidence detectors' as they are able to integrate electrical activity in the cell with the action of receptors such as AMPARs, with their own binding of glutamate (Sjostrom et al., 2008). These characteristics combined with the ability to permit 'efficient' Ca^{2+} flow into neurons led to NMDARs identified as the key mediators of synaptic potentiation in most brain regions. Indeed experiments where NMDAR function is blocked or receptor expression is ablated, severe defects in the formation of both LTP and LTD are observed (Kirkwood and Bear, 1995; Niewoehner et al., 2007; Tsien et al., 1996). Interestingly the presence of the various subunits is crucial for the mechanisms of different kinds of memory/potentiation. Mice unable to produce the GluN2A subunit suffered defects in both CA1 LTP and spatial learning (Kiyama et al., 1998; Sakimura et al., 1995). Deletion of the GluN2B gene causes defects in LTP formation in both the hippocampus and neuronal barrelette of the trigeminal complex (Kutsuwada et al., 1996). This was mirrored by GluN2B overexpression experiments resulting in enhancement of LTP (Tang et al., 1999). The resultant transgenic mouse line termed the "smart" mouse performed significantly better than wild-type animals in learning and memory experiments these included water maze and contextual and fear conditioned tests.

Calcium signalling, common to most cell types is essential in both the potentiation and depression of neuronal synapses. In particular calcium, derived from the extracellular space

via NMDARs, is the key mediator of synaptic plasticity (Gnegy, 2000). Long term potentiation and depression are generated at molecular level, postsynaptically by changes in the activity and number of AMPA receptors (Kessels and Malinow, 2009). LTP and Ca^{2+} influx via NMDA receptors are directly linked by the Ca^{2+}/Calmodulin-dependent protein kinase (CaMKII) (Lisman et al., 2002). This multimeric holoenzyme translates the fast Ca^{2+} influx event into a more sustained phosphorylation based signal. CaMKII holoenzymes are composed of subunits arranged into dodecamers, which in the presence of Ca^{2+}/calmodulin, autophosphorylate themselves and subsequently proceed to phosphorylate synaptic proteins (Lou et al., 1986; Miller and Kennedy, 1986; Schworer et al., 1986). Over 30 targets of CaMKII phosphorylation have been identified. These include proteins such as receptors and channel proteins, scaffold proteins, cytoskeletal proteins, motor proteins and enzyme (Fink and Meyer, 2002; Soderling, 2000; Wayman et al., 2008). Phosphorylation of S831 on GluA1 subunits by CaMKII increases single channel conductance whilst impairment of this phosphorylation causes defects in behavioural learning (Crombag et al., 2008; Derkach et al., 1999). CaMKII mediated phosphorylation of GEFs has been directly linked to activity dependant spine morphogenesis (Saneyoshi et al., 2008).

1.2.7 Developmental regulation of glutamate receptor expression

Both AMPA and NMDA receptor expression at synaptic sites is dynamic throughout the development of the nervous system (Hall and Ghosh, 2008; Monyer et al., 1994). Subunit expression varies both in brain regions and during developmental time. In virtually every neuron in the CNS the obligatory GluN1 gene is expressed at all stages whereas there are various differences in the expression of the four NR2 transcripts. GluN2a and GluN2c are poorly expressed at prenatal stages and become significantly expressed in discrete brain regions such as the cerebellum by P7 (Monyer et al., 1994). GluN2b and GluN2d are strongly expressed at E17 and E19 and postnatally the strength of expression decreases into adulthood. For all four subtypes these developmental differences are particularly striking in the cortex and hippocampi of rat forebrains (McCarthy, 2006). These changes coincide with major periods of synapse formation in the rodent brain (Hall et al., 2007).

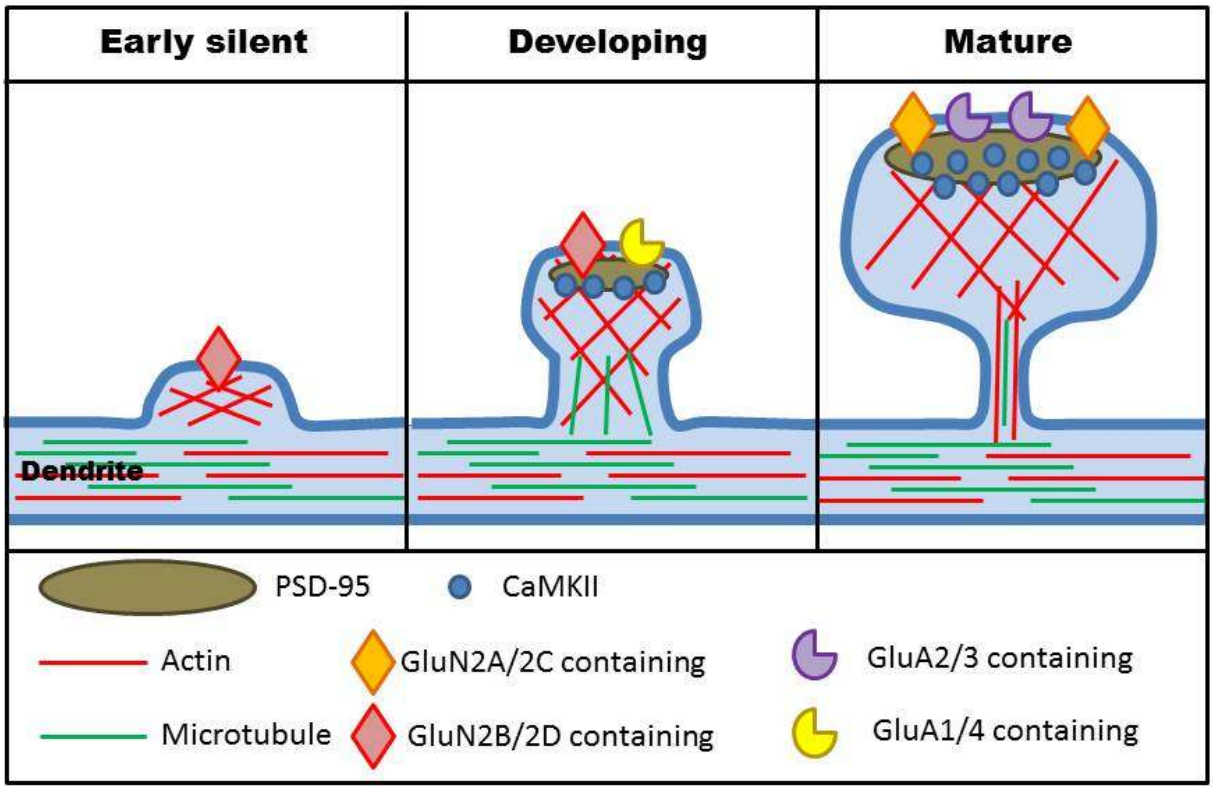

Figure 1.3 Developmental regulation of dendritic spines and glutamate receptors. In early postnatal brain postsynaptic structures are generally minor dendritic protrusions. Functional synaptic sites lack AMPAR but contain functional NMDAR. Developing synaptic sites feature structurally better defined spine, filopodia and spine like morphology. Actin accumulation underlies increased motility of these sites. Both NMDAR and AMPAR are present at these sites with receptors containing GluN2B, GluN2D, GluA1 and GluA4 subunits particularly prevalent. These receptors are associated with structural PSD proteins such as PSD-95 and signalling proteins such as CaMKII. More mature postsynaptic spines tend to morphologically resemble mushrooms with spine necks decreasing in diameter. PSD-95 volume increases in proportion with spine volume alongside associated signalling proteins such as CaMKII. Both the number and subunits of glutamate receptor change as synapses mature. Ca2+ impermeable AMPARs such as GluA2/3 containing increase in proportion whilst GluN2A and GluN2C containing NMDARs are more prevalent at synapses.

AMPA receptor expression is similarly developmentally regulated with changes in synaptic subunit composition as the neural network matures. The presence of GluA4 in particular is highly indicative of an immature hippocampus (Zhu et al., 2000). In contrast, the presence of GluA2/3 containing synapses are more prevalent in postnatal brains where the majority of synapse development has already occurred (Hall and Ghosh, 2008; Zhu et al., 2000). GluA1

expression in the hippocampus seems to follow a similar profile to that of GluA2, increasing after birth of the organism. In contrast to GluN1, the GluA1 subunits of AMPARs are non-obligate. However the majority of receptors found in brain tend to contain this subunit. It is therefore of significance that a majority of synapses in early postnatal brain whilst containing GluN1 receptors, lack GluA1 subunits. These synapses are rendered inactive under 'normal' conditions and as such are termed 'silent synapses' (Isaac et al., 1995). In the hippocampus, virtually all CA3-CA1 synapses are silent during the first postnatal week rising to approximately 50% functional by the second and third weeks (Kerchner and Nicoll, 2008). Classically, silent synapses have been studied by analysis of differences between AMPA and NMDA synaptic failure rates during development and after unsilencing (Isaac et al., 1995). These studies assume the likelihood of release at a non AMPA synapse is the same as that of an AMPA-NMDA synapse. Similarly differences in the amplitude of mEPSCs between NMDA only and AMPA-NMDA synapses could suggest changes to the number of silent synapses or simply synapse number. However it has been postulated that during paring or unsilencing protocols only a fraction of synapses involved in asynchronous release become unsilenced making changes difficult to observe via mEPSCs (Isaac et al., 1995). Interestingly, the formation of silent synapses early in network development seems to be a product of the regulation by presynaptic activity and NMDAR activation on both expression and synaptic localisation of AMPARs.

Much data has been accrued looking at the specific developmental surface expression of AMPA receptor subunits and the relationship this has with NMDAR function. Indeed localisation of AMPARs at synaptic sites is regulated by NMDARs at transcriptional, translational, protein degradation and synaptic insertion levels. As previously described AMPARs are derived from 4 gene products and as such are transcribed in neuronal ER. In response to NMDAR activation both GluA1 and GluA2 mRNA transcript levels are decreased via ERK/MAPK and Ca^{2+} signalling (Grooms et al., 2006). This activation using NMDA decreases the colocalisation of GluA2 puncta with synapsin-1 puncta in hippocampal cultures suggesting this down regulation ultimately affects synaptic localisation of receptor at synaptic sites (Grooms et al., 2006). Interestingly by use of bath applied NMDA the results of this investigation were unable to distinguish the effects of activation of synaptic vs. extra-synaptic NMDAR activation. It has been established that these distinct pools often promote antagonistic cell signalling pathways e.g., BDNF/CREB signalling in cell survival

(Vanhoutte and Bading, 2003). Further strengthening this idea NMDAR deletion both *in vivo* and *in vitro* enhances synapse unsilencing (Adesnik et al., 2008).

Due to the relatively large distance separating distal dendritic compartments from the soma of neurons, the presence of protein synthesis machinery in dendrites is not surprising (Steward and Schuman, 2001; Waung and Huber, 2009). Receptor subunit mRNA has been observed in dendrites adjacent to synaptic sites providing a rapid means for receptor replenishment (Bilak et al., 1995). Indeed dendritically located receptor appears to be important in the process of synaptic scaling (Sutton et al., 2006). Synaptic scaling describes the homeostatic up-regulation of synaptic transmission in response to prolonged or chronic inhibition of AP firing. Sutton and colleagues were able to prevent homeostatic up-regulation of AMPAR mEPSC amplitude by blockade of local protein synthesis (Sutton et al., 2006). This data was strengthened by observations of the inhibition of GluA1 receptor insertion, post chronic APV and TTX treatment by local application of anisomicin in dendrites. Interestingly whilst GluA1 subunits are believed to be the mediators of the synaptic scaling events, later analysis of these neurons gradually saw an incorporation of GluA2 subunits at synaptic sites (Sutton et al., 2006).

Regulation of synaptic levels of AMPAR by ubiquitin proteasome degradation are mediated by the removal of PSD-95 and the glutamate receptor interacting protein (GRIP1). NMDAR activation causes ubiquitination of PSD-95 resulting in the removal of GluA1 subunits from synaptic sites (Colledge et al., 2003).GRIP1 associates with GluA2 subunits and is directly involved in their stabilisation at synaptic sites. Whilst no direct link has been established, ubiquitination decreases the levels of available GRIP1 in neurons as experiments looking at the decrease in GluA2 caused by GRIP1 decrease due to ubiquitination are blocked by the exposure of neurons to APV (Guo and Wang, 2007).

Whilst there are several mechanisms at play promoting AMPAR receptor down regulation in early postnatal stages synaptic unsilencing is a crucial mechanism in plasticity of early neural networks. Up to postnatal day 12, early LTP is driven almost entirely by the unsilencing of synapses in a PKA dependent manner (Abrahamsson et al., 2008).

1.3 Synaptogenesis

The formation of functional synaptic sites generally features a series of clearly defined stages. The stages encompass the apposition of nascent pre and postsynaptic sites leading up to the formation of synapses with all essential transmission machinery present (Waites et al., 2005). Peripheral synaptogenesis seems to differ slightly from central synaptogenesis in that much of the postsynaptic machinery is able to organise in the absence of a functional presynaptic partner (Yang et al., 2001). However much of our current understanding of central synaptogenesis suggests presynaptic specialisation occurs prior to that of postsynaptic formation and that the former may influence development of the latter (Friedman et al., 2000; Ziv, 2001). Current research has also revealed much about the cell derived factors which regulate these stages of synapse development. Expressed at various stages of CNS development and with a myriad of forms and properties, these proteins contribute to the complexity of neuronal circuits.

1.3.1 Axon extension and target recognition

The complexity with which neural networks in the CNS are formed begins with the initial target recognition between prospective connected neurons (Chen and Cheng, 2009; Salinas, 1999; Shen and Cowan, 2010). Generally this process is mediated by axonal outgrowth and regulated by signals derived by the postsynaptic cell (Waites et al., 2005). The specificity with which these axons form synaptic partners is a marvel considering the relative distance axons may have to travel. One such example of this specificity exists in the eye where retinal ganglion axons extend from the eye to the thalamus forming synapses only with thalamic cells (Shatz, 1996; Shatz, 1997).

Of essential importance to this process are the presence of diffusible axon guidance molecules (Terauchi and Umemori, 2011). The growing axons are directed toward their postsynaptic partner by signals derived from these targets (Waites et al., 2005). The result is an increasing concentration of chemo-attractant as the axon grows closer to its partner. Several types of guidance molecules (both attractant and repellent) exist operating in various brain regions. Netrins are an example of proteins both able to encourage and inhibit axonal growth (Kennedy et al., 1994). Commissural axons of the spinal cord are regulated by a gradient of netrin 1 and 2, whilst RGC axon growth is regulated by netrin 1 released by Optic nerve head (ONH) cells (Kennedy et al., 2006; Oster et al., 2004). Semaphorins are a second

group of secreted proteins which act to repel sensory, sympathetic and axons in the hippocampus (Chedotal et al., 1998). Several proteins initially identified as developmental or growth morphogens have also been identified as axon guidance proteins. Morphogens are substances involved in the regulation of cell and tissue fate acting in a concentration dependent manner (Turing, 1990). These include proteins such as Wnts, BMP, FGF, NGF and Hedgehog (Waites et al., 2005).

Close proximity between the pre and postsynaptic neurons initiates further specialisation and priming processes in first the presynaptic followed by the postsynaptic terminals. This stage is identified initially by a decrease in the outgrowth of the axon with a concomitant increase in volume of the axon terminal (Hall et al., 2000; Krylova et al., 2002). Concurrently accumulation of synapse specific proteins in the nascent synaptic site occurs (McAllister, 2007). It is important to note that whilst there may be no physical interaction between pre and post synaptic terminal at this stage signals derived from the postsynaptic terminal have been identified as acting in a retrograde manner to aid presynaptic development (Salinas, 2005; Tessier-Lavigne and Goodman, 1996). These signalling proteins include many of the same axon guidance family of proteins along with cell surface signalling proteins which come in to play as the surfaces come into contact. These factors include FGF's, Wnts, Cholesterol and TSP whilst cell surface signals include cadherins, CAMs, Narp, Neurexin/Neuroligin and Ephrin/Eph complexes (Waites et al., 2005).

1.3.2 Synapse maturation

The formation of a nascent synaptic site by apposition of pre and postsynaptic neurons initiates the maturation stage of synapse development. Via a complex array of signalling proteins and cascades the machinery for release presynaptically and postsynaptic neurotransmitter array is assembled. Presynaptically many of the proteins required at synaptic sites are transported together in the developing axon ready for delivery to designated sites (McAllister, 2007; Ziv and Garner, 2004). PTVs (piccolo transport vesicles) and STVs (synaptic vesicle protein transport vesicles) are two such cargo vesicles identified in young neurons. PTVs contain proteins such as piccolo, bassoon, Munc13, Munc18, syntaxin and Snap25 (Zhai et al., 2001). STVs colocalised strongly with markers for VAMP, SV2, Synapsin and amphiphysin (Ahmari et al., 2000). Many of these proteins rapidly accumulate in the early stages of synapse formation and these sites provide a reliable marker for future

functional sites (Gerrow et al., 2006). Presumably the early presence of scaffolding proteins generates a favourably 'set-down' point for subsequent synaptic vesicle delivery.

The maturation of the presynaptic apparatus is proceeded (approximately 30-45minutes later) by the development of the PSD, at glutamatergic synapses, beginning with scaffolding proteins such as SAP90/PSD95 (Friedman et al., 2000). Studies are divided as to the method of the method of PSD95 transport to nascent synaptic sites. Similar to presynaptic cargo vesicles, PSD95 transport has been observed in clusters (Prange and Murphy, 2001). Whilst several studies have observed a more gradual accumulation of the scaffolding protein at synaptic sites (Bresler et al., 2001; Mars et al., 2001). The presence of postsynaptic scaffolding proteins encourages receptor recruitment as evidenced by the short delay in receptor recruitment proceeding PSD-95 clustering (Friedman et al., 2000). PSD-Glutamate receptor insertion is tightly regulated at developing synapses, receptor present along dendrites or adjacent to synaptic sites may not be incorporated into synapses. Indeed various studies suggest receptor may be inserted directly into PSDs from adjacent (internal or surface) regions or shuffled into the PSD from either proximal or distal surface regions (Waites et al., 2005). These regulatory mechanisms seem to vary even between receptor subunits, where GluA2is more locally inserted than GluA1 (Passafaro et al., 2001). Interestingly other important components of the postsynaptic terminus seem to be recruited passively. CaMKII and the scaffolding proteins homer and shank are accumulated at the synapse by trapping of local cytosolic pools (Bresler et al., 2004; Okabe et al., 2001; Shen and Meyer, 1999).

Formation of inhibitory central synapses is less well understood. The majority of inhibitory synapses are formed directly onto the dendritic shaft suggesting maturation of a presynaptic terminal which then seeks out and promotes maturation of the postsynaptic terminal. Consistent with this, data from Wierenga and colleagues suggests GABAergic synapses form exclusively without the assistance of postsynaptic dendritic protrusions (Wierenga et al., 2008). In common with glutamatergic synapses this study also revealed a delay of approximately 60 minutes between the accumulation of presynaptic vGAT and Postsynaptic Gephyrin.

1.4 Synaptogenic Molecules

The rapid accumulation of both pre and post synaptic proteins are regulated by a range of secreted and cell surface molecules. Identification and study of this field of neuroscience has

expanded in the last decade revealing much about the mechanisms and roles these diverse groups of proteins have. Nominally these molecules can be grouped as either trans-synaptic or secreted molecules.

1.4.1 Trans-synaptic molecules

Neurexins and Neuroligins form heteromeric synaptic cleft spanning complexes which have varying effects on synapse formation. Postsynaptic neuroligins bind presynaptic neurexin to generate the fully signalling complex. Three genes each encode α-neurexin and β-neurexin however both spliced and unspliced gene products are present at synapses. Presynaptically neurexins interact with a range of scaffolding proteins such as CASK and actin binding proteins such as Band4.1 (Dean and Dresbach, 2006). Through these interactors neurexins are able to stabilise the developing presynaptic terminal and cytoskeleton. Differences in the splicing of both neurexins and neuroligins, generates differences in binding affinities of the pre and postsynaptic partners (Boucard et al., 2005). Of the five neuroligin genes found in humans 1, 2 and 3 are expressed in the brain (Dean and Dresbach, 2006). Neuroligins play a key role in directing the formation of excitatory or inhibitory synapses. Neuroligins 1 and 2 are enriched at excitatory and inhibitory synapses respectively (Graf et al., 2004; Song et al., 1999; Varoqueaux et al., 2004). Indeed overexpression studies by Chubykin and colleagues further elucidated the causative role of both neuroligins in synapse formation. Overexpression of Neuroligin 1 in hippocampal neurons increased both AMPA and NMDA mediated synaptic responses alongside an increase in overall synapse number, measured by synapsin and spine density (Chubykin et al., 2007). Neuroligin 2 overexpression had no such effect on excitatory synaptic response but showed a significant promoter effect on inhibitory synaptic response. Neuroligin 3 is found at both glutamatergic and GABAergic synapses in the hippocampus and is believed to play associate roles in synapse formation with Neuroligin 1 and 2 (Budreck and Scheiffele, 2007).

SynCAM are cell adhesion molecules found at many cell-cell junctions and highly enriched at synaptic sites. These proteins were initially identified as potential synaptogenic molecules due to their structural similarity to cadherin and neurexin-neuroligin complexes (Biederer et al., 2002). In particular the presence of several Ig domains coupled with a PDZ interacting domain identified this protein as a potential pro-synaptogenic molecule. Expression of full length SynCAM in neuronal cultures promoted functional synapse formation, in contrast with expression of a SynCAM containing a C-terminal deletion which inhibited synapse number

increase (Biederer et al., 2002). Interestingly SynCAM1 has been identified as a specific promoter of excitatory synapse number without having an effect on inhibitory synapses as defined by ultrastructural analysis (Robbins et al., 2010).

Eph Receptors form synapse spanning complexes with Ephrin molecules. Eph Receptors comprise the largest members of the receptor tyrosine kinase family. Both the Ephs and ephrins are expressed in A and B forms, which specify the presence of either a GPI moiety or transmembrane domain respectively. The Eph receptors are generally located postsynaptically whilst the ephrin molecules are found presynaptically, similar to the neuroligin complex there is specificity in the binding affinities of the various Ephs and ephrins. In general Eph A and B receptors have highest affinity for Ephrins of the same form. EphB2 overexpression in cortical neurons directly increases clustering of GluA2 and enhances spine formation (Kayser et al., 2006). Interestingly investigations into the binding of EphB with EphrinB show a direct interaction with NMDARs required for the effects on GluA2. Indeed activation of EphB enhances NMDAR mediated Ca2+ influx and gene expression (Takasu et al., 2002). However while in vivo studies of EphB2 null mice reveal minor defects in synaptic function they have failed to reveal defects in both synapse formation or spine morphology (Grunwald et al., 2001; Henderson et al., 2001). The discrepancies in the effects *in vivo* and *in vitro* are likely due to the array of Eph-Ephrin complexes generating a measure of compensatory mechanisms in KO experiments. In concurrence with this triple KO EphB1/B2/B3 mice form fewer synapses and do not form dendritic spines in the hippocampus (Henkemeyer et al., 2003). EphA4 signalling, also expressed at synaptic sites, inhibits spine formation. Exposure of cultured neurons to ephrin A1 or A3 (activators of EphA4 signalling) caused varying defects in spine density and size (Murai et al., 2003). This is mirrored by disorganisation of dendritic spines in EphA4 KO mice (Murai et al., 2003). The physiological relevance of this negative regulation may be to limit uncontrolled growth of spines believed to be important for later potentiation and memory paradigms (Lai and Ip, 2009). EphrinB reverse signalling is also important in synaptogenic regulation. In experiments by Kayser and colleagues heterologous 293T-neuronal co-cultures were generated. Overexpression of EphB in 293T cells provided a pseudo postsynaptic contact for axons, activating reverse EphrinB signalling. SV2 and FM4-64 sites were observed to increase on cultured neurons where EphrinB was activated (Kayser et al., 2006). In the developing xenopus optic-tectum, activation of EphrinB via infusion of

EphB2-Fc increases size and number of synaptobrevin and SNAP-25 clusters (Lim et al., 2008).

Wang and colleagues recently identified the Synaptic adhesion like molecule (SALM) family of transmembrane synaptic organising proteins (Wang et al., 2006). Currently 5 members of this family have been identified via human and mouse cDNA screens (Ko et al., 2006; Wang et al., 2006) SALM1 interacts with both NMDARs and overexpression increases NMDAR and PSD-95 clustering at synapses (Wang et al., 2006). SALM2 overexpression increased the number of excitatory synapses without any effect on the inhibitory synapses in hippocampal neurons (Ko et al., 2006). In the same study SALM2 was demonstrated to interact directly with PSD-95 whilst the synaptic protein organiser effects were reversed with knockdown.

1.4.2 Secreted molecules

Brain derived neurotrophic factor is emerging as regulator of synapse formation in the CNS. Signalling through synaptic TrkB receptors, have been identified as specific enhancers of excitatory synapse number (Vicario-Abejon et al., 1998). Consistent with this, studies using both BDNF gain of function and TrkB inactivation/activation models have demonstrated effects on both synaptic proteins and spine number/morphology (Luikart and Parada, 2006). Using organotypic hippocampal slice Tyler and Pozzo-Miller observed presynaptic enhancements after long term exposure to BDNF. BDNF exposure increased the number of docked vesicles at CA1 stratum radiatum synapses without affecting reserve pool size (Tyler and Pozzo-Miller, 2001). The same treatment enhanced both synapse number per neuron (observed via synaptobrevin) and spine density. The same study also used whole cell patch clamp technique to measure miniature excitatory currents (mEPSC). mEPSC frequency changes roughly represent presynaptic changes whilst postsynaptic changes are represented by amplitude changes (Fatt and Katz, 1952). Exposure of these synapses to BDNF saw an enhancement in mEPSC frequency with no change to amplitude size (Tyler and Pozzo-Miller, 2001). Electrophysiological studies have also observed BDNF induced enhancements in spontaneous synaptic firing rate and increases in both amplitude and frequency of EPSCs in hippocampal cultures (Levine et al., 1995). Interestingly the increases in EPSC frequency were occluded by use of postsynaptic TrkB inhibitor suggesting BDNF acts directly at both pre and postsynaptic sides of the synapse. BDNF also impacts synaptic function by directly influencing NMDAR activity. Exposure of hippocampal neurons to BDNF enhanced

NMDAR channel open probabilities whilst also causing an enhancement in plasma membrane associated expression of NMDAR (Caldeira et al., 2007; Levine et al., 1998). Concurring with BDNF *in vivo* gain of function studies TrkB signalling loss caused synaptic defects. Using a conditional TrkB KO mouse system Luikart and colleagues observed decreases in spine density alongside decreases in PSD-95 and synaptophysin stains in CA1 (Luikart et al., 2005). The severity of these defects increased proportionally with the length of TrkB signalling knockout and maintained from p20 into "adulthood". BDNF/TrkB signalling clearly plays a strong role in the formation and maintenance of synapses. Contrary to the old ideas of presynaptic enhancer molecules, BDNF is directly involved in regulation at both sides of the synapse.

The FGF family of proteins play a number of roles in the differentiation and proliferation of various cell types. These soluble proteins also function as synaptic organisers most commonly signalling through Fgf(R) receptors and heparan sulfate proteoglycans. Fgf2 was observed, by Dai and Peng when applied to spinal cord neurons, to increase both the number of synaptic vesicles and amount of synaptotagmin in axons (Dai and Peng, 1995). Several Fgfs were subsequently identified by Umemori and colleagues as potent presynaptic organiser in pontine and vestibular neurons. Exposure of pontine and vestibular cultures to recombinant Fgf22 promoted vesicle, SV2 and synapsin clustering (Umemori et al., 2004). Furthermore use of FGFR2bAP and inhibitor of endogenous Fgfs7, 10 and 22 resulted in defects in synapsin accumulation *in vitro* and mossy fibre varicosities (representing presynaptic sites) *in vivo*. With the use of postnatal FgfR2 KO mice, defects were observed in synapsin, bassoon and synaptophysin accumulation in the cerebellum of p14 animals (Umemori et al., 2004). Recent work has identified specificity in the synaptogenic properties of the various Fgfs. Terauchi and colleagues examined the expression of Fgfs22 and 7 and found these enriched in CA3 hippocampal layers. By measuring the accumulation of the excitatory marker vGlut and the inhibitory marker vGat in Fgf22 or Fgf7 KO animals defects were observed at excitatory and inhibitory synapses respectively (Terauchi et al., 2010). These defects appeared to be limited to the presynaptic terminals with no effect on postsynaptic gephyrin or PSD-95. mPSCs confirmed the imaging data by showing defects in excitatory or inhibitory mini frequency in Fgf22 or Fgf7 neurons respectively (Terauchi et al., 2010). Current data and work on Fgfs identify these target derived molecules to be essential for the presynaptic development post axonal outgrowth however much of the specific roles of each Fgf in synapse formation continues to be uncovered.

Semaphorins are another group of secreted axon guidance molecules which seem to be important at various stages in neural circuit formation (McAllister, 2007). Of particular interest the Class 3 and 4 semaphorins have been intensely studied and observed to have myriad effects on synapse maturation. Morita and colleagues demonstrated the ability of Sema3A to enhance clustering of PSD-95 and the number of PSD-95 associated spines on cortical neurons. The number of synapsin clusters associated with PSD-95 clusters also increased suggesting an enhancement of excitatory synapse number (Morita et al., 2006). Exposure of hippocampal neurons to Sema4D meanwhile causes a RhoA dependent increase in spine density (Lin et al., 2007). Sema4B has been observed acting both at excitatory and inhibitory sites. RNAi knockdown of Sema4B in neurons resulted in a decrease in synaptotagmin and GluA2 stained synapses alongside a decrease in both mEPSC frequency and amplitude (Paradis et al., 2007). Interestingly Sema4B and Sema4D knockdown inhibited GABAR/GAD67 colocalisation in hippocampal neurons suggesting both molecules have general synaptogenic properties (Paradis et al., 2007). Further research is required into the roles played by the various semaphorin classes at the various synapse types, in particular little is known of the role of class 3 semaphorins on GABAergic synapse formation. As such it is clear both class 4 and 3 semaphorin signalling is important in receptor and spine organisation in neurons.

Similar to many of the above examples of secreted proteins Wnts have been identified as important molecules in the regulation of synapse formation. Like Fgfs these proteins have long been studied for the variety of effects they have on embryo development, more recent research has begun to reveal roles for Wnts at various stages of neural circuit formation and maintenance. The experiments of this thesis primarily concern the abilities of Wnt proteins in brain development and so the next section will contain a detailed summary of our current understanding of these molecules.

1.5 Wnts

Wnts are a conserved group of highly glycosylated diffusible molecules. These proteins are found across both vertebrate and invertebrate and have a wide range of roles across the life span of an organism. The first Wnt protein discovered (Wnt1) was uncovered as a proto-oncogene which when over-expressed caused mammary tumours in mice (Nusse and Varmus, 1992).

1.5.1 Wnts: expression and secretion

The number of Wnt proteins expressed varies across vertebrate and invertebrate species, from 5 members in C.elegans to 19 in humans and rodents (Nusse and Varmus, 1992). The name Wnt is derived from a combination of the drosophila Wnt homologue wingless (Wg) and the mouse mammary gland tumour associated protein Int1 (Klaus and Birchmeier, 2008; Nusse et al., 1991). In 1982 Nusse and Varmus identified the Int1 gene as causative in the formation of mammary gland tumours (Nusse and Varmus, 1982). With the subsequent identification of the drosophila Int1 homologue Wg, which produced amongst other developmental defects a wingless phenotype the term Wnt was later used to describe genes and the downstream signalling pathways (Nusse et al., 1991). Interest into research of Wnts has been driven by their interesting combination of effects on both cancer proliferation and embryonic development. Due to this Wnt signalling have been identified and studied in carcinogenesis, body axis specification, neural tube development, cell polarity, stem cell proliferation and CNS development (Barker and Clevers, 2006; Budnik and Salinas, 2011; Cayuso and Marti, 2005; Katoh, 2007; Niehrs, 2010).

Wnt proteins are expressed throughout the body but most strongly enriched in the brain, lungs, kidneys and prostate(Kirikoshi et al., 2001). In the mammalian brain, with varying degrees of intensity various Wnt proteins are found in the cerebellum, cortex, hippocampus and olfactory bulb (rodents) of both young and adult animals (McCarthy, 2006). Indeed the expression of Wnt and Wnt signalling molecules postnatally and into adulthood provided early clues as to the role of Wnt in neural development and maintenance (Coyle-Rink et al., 2002).

The Wnt family of proteins are highly conserved secreted proteins post-translationally modified and approximately 40kDa in size (Port and Basler, 2010). The discovery that these proteins were highly hydrophobic, attaching to cell membranes and extracellular matrices,

was initially analogous to both sequence predictions and their function as secreted guidance molecules (Bradley and brown 1990). The discovery of several lipid moieties in association with most of the Wnt proteins explained the hydrophobic nature of the molecules. The first of these was a palmitate group attached to one of the many conserved cysteine residues on Wnt proteins. This was first discovered in Wnt3a and subsequently demonstrated in Wnt1 and Wnt5a (Galli et al., 2007; Kurayoshi et al., 2007; Willert et al., 2003)

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Whilst the lipid modifications to most Wnt species are responsible for their hydrophobicity these modifications are also essential for both Wnt secretion and activity. Mutation of Cys^{77} in Wnt3a and Cys^{104} Wnt5a, normally post-translationally palmitoylated, display no secretion defects but exhibit loss of Wnt signalling ability (Galli et al., 2007; Kurayoshi et al., 2007). Galli and colleagues identified porcupine mediated Wnt3a lipid modification as essential for signalling by analysing the downstream readout of Top/FopFlash renilla luciferase in 293T cells. TopFlash assays involve transfection of TCF (nuclear effector of Wnt signalling) reporter constructs. The experimenters knocked down porcupine expression and assessed the effect on TCF reporter activity, observing a decrease (Galli et al., 2007). Loss of palmitoylation decreases the ability of Wnt5a to inhibit Wnt3a induced Tcf-4 activity, to promote cell migration and bind to cell surface signalling receptors (Kurayoshi et al., 2007).

The multi-pass transmembrane protein Wntless (Wls) or Eveness interrupted (Evi) has recently been identified as an essential mediator of Wnt secretion. Banziger and colleagues investigated the effect of Wls in both human and C.elegans cell systems and found overexpression and knockdown of the protein inhibited release of Wnt species (Banziger et al., 2006). To date only WntD secretion is free from regulation by Wls (Ching et al., 2008). The subcellular localisation of Wls to the golgi and the subsequent accumulation of Wnt within the compartment during Wls knockdown, suggests Wls acts to facilitate Wnt exocytosis from the golgi network (Port et al., 2008).

Work from the lab of Vivian Budnik has recently demonstrated the release of synaptic vesicle containing both Evi and Wg suggesting a role for Evi/Wls in extracellular trafficking of Wnt species (Korkut et al., 2009). In confirmation of the study by Banziger and colleagues, Korkut et al. demonstrated a requirement for Evi in presynaptic Wg release and the resultant NMJ phenotype mimicked Wg loss. Imaging in this study revealed the presence of Evi both pre and postsynaptically and in transport across the cleft in vesicles with Wg (Korkut et al.,

2009). Interestingly postsynaptic Evi was observed to interact with dGRIP trafficking which plays a role in postsynaptic DFz2 signalling (Ataman et al., 2006). As yet the lack of hi-fidelity mammalian Wnt antibodies means these experiments are yet to be recapitulated in mammalian systems.

1.5.2 Wnt signalling pathways

Wnt proteins were initially established from studies in embryo development patterning. Injection of Wnt1 into developing xenopus embryo resulted in the formation of a bifurcated anterior and enlarged posterior tadpole (McMahon and Moon, 1989). From the mass effect on tadpole development and subsequent experiments Wnt proteins were established as important regulators of cell fate and cell behaviour.

The various members of the Wnt family were identified from the initial cloning of Wnt1. There are 19 Wnt proteins expressed in humans and mice, this number varies across both vertebrate and invertebrate systems. Interestingly from the earliest days of studies on cell behaviour it was clear the various Wnt species were able to elicit differing effects on cells. The first suggestion of functional differences were the differing expression patterns in cell layers found for Wnts 3a, 5a and 5b during gastrulation in mice (Takada et al., 1994). Further assays which looked at the transformational activity of the Wnt proteins on mouse mammary cells also recorded differences in the strength of their effect (Wong et al., 1994). As a result the Wnts tested were able to be grouped, by those which could transform and those which couldn't, Wnts 1, 3a and 7a in the former and Wnts 4, 5a and 6 into the latter (Wong et al., 1994).

Wnt signal transduction is key to the ways in which these extra cellular proteins are able to cause their vast range of effects on an equally large number of cell types. Wnts cause cellular effects by first binding to cell surface receptors, these then as typical receptors cause a downstream intracellular cascade. Wnts are known to bind receptors such as Ryk, ROR and Crypto however the most well understood relationship is between Wnts and frizzled receptors. Functional differences between Wnts are further complicated by the differential binding ability of specific Frizzleds for the ligand. The different complements of Wnts with frizzleds along with the recruitment of accessory receptors such as the LRPs results in activation of a variety of intracellular signalling pathways.

Wnt signalling by Frizzled receptor activates a multi module protein named Dishevelled (Dvl). Dvl a protein of about 750aa in size consists of three highly conserved domains. The amino terminal region is the Dix domain, which shares homology with the same domain of axin. The central domain is named the PDZ zone and the C-terminal domain is called the DEP Domain. Dvl associates with a wide range of intracellular proteins through these domains including Frodo, Pak1, Notch, GBP/Frat, β-arrestin, rhoA, MuSK, CK1&2 (Wharton, 2003). By having this wide array of binding partners Dvl is able to activate various signalling pathways.

The canonical Wnt signalling pathway directly results in the transcriptional up regulation of specific gene products. Canonical signalling acts to regulate the presence of the intracellular protein β-catenin. In the absence of Wnt, intracellular stores of β-catenin are phosphorylated, causing them to be recognised by TrCP and degraded by proteosomes following ubiquitination. Phosphorylation is mediated by the β-catenin degradation complex which is composed of the scaffolding proteins; Axin, Adenomatous Polyposis Coli (APC) and the responsible kinases; Glycogen Synthase Kinase-3β (GSK3β) and Casein Kinase Ia (CKIa) (Logan and Nusse, 2004). On binding of Wnt to frizzled receptor, LRP co-receptor is recruited to the complex and Dvl is activated within the cell. Dvl then acts to destabilise the β-catenin degradation complex. One way in which it has been proposed Dvl may affect the degradation complex is by the recruitment of axin from the intracellular compartment and the degradation complex to the membrane and receptors. Over expression studies in drosophila with Dvl have demonstrated increased axin recruitment to the membrane, whilst axin-GFP has been observed recruited to the plasma membrane in a Dvl dependent manner in drosophila (Cliffe et al., 2003). Dvl2, via its DIX domain is able to interact with Axin and directly influence its recruitment to the plasma membrane thereby disrupting the degradation complex.

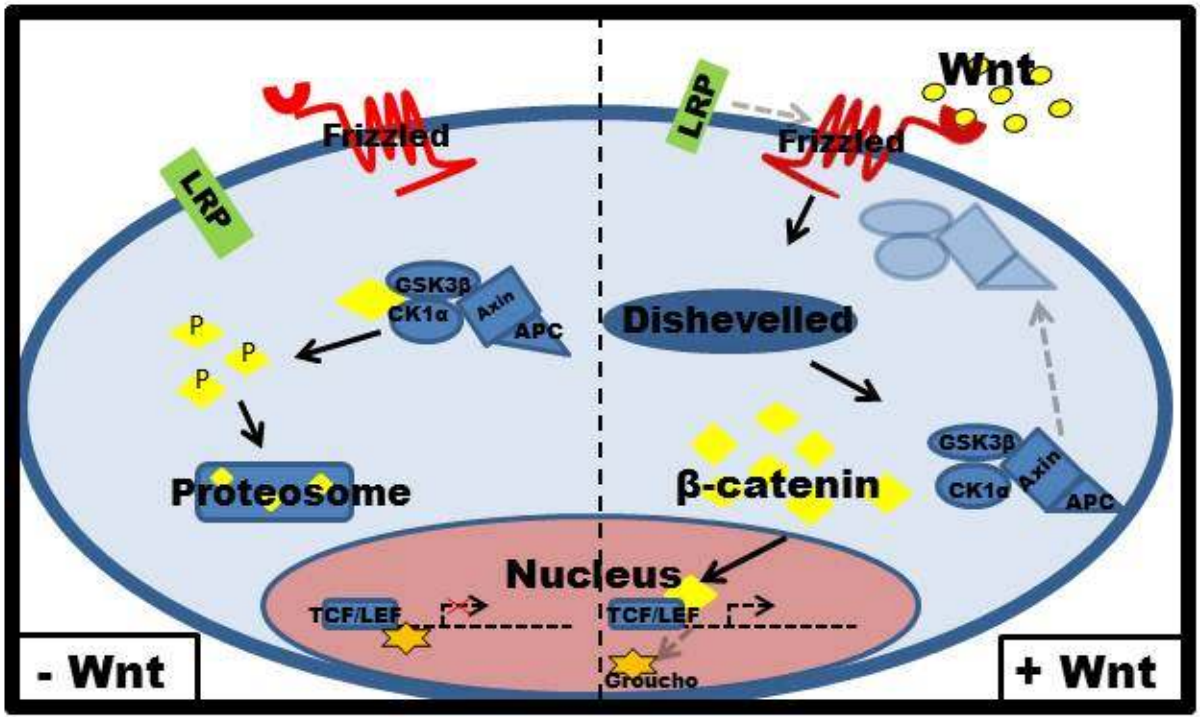

Figure 1.4 Canonical Wnt signalling. In the absence of Wnt (-Wnt), phosphorylated β-catenin is broken down by proteasomes. This is due to the phosphorylation of β-catenin by the destruction complex composed of CK1a, Axin, APC and GSK3β. In the presence of Wnt (+Wnt), frizzled receptor recruits the LRP co-receptor which through the activation of Dvl1 recruits the destruction complex to the surface receptor complex. B-catenin accumulates in the neuron and enters the nucleus displacing groucho from the TCF/LEF repressor complex. Transcription of Wnt responsive genes subsequently occurs.

Inhibition of the degradation complex results in an increase in the amount of intracellular β-catenin. β-catenin then translocates in high concentration to the nucleus where it interacts with the TCF repressor complex. In non-Wnt signalling conditions the repressor in association with a co-repressor groucho, prevents transcription (Cavallo et al., 1998). When stable β-catenin is able to enter the nucleus groucho is replaced with a histone acetylase (Cyclic AMP response element-binding protein) and becomes a transcriptional activator complex. These repressors control transcription of several genes many of which have been previously mentioned and some which act as feedback repressors/promoters of the signalling pathway.

Wnt also modifies cytoskeletal dynamics using members of the canonical pathway in a translation independent manner (Salinas, 2007). Here axin directly associates with microtubules and stabilizes these dynamic structures in opposition of the actions of MAP1B

(Ciani et al., 2004). Phosphorylated MAP1B negatively regulates microtubule stability (Hall et al., 2000). The presence of Dvl1 decreases the phosphorylation of MAP1B in likelihood via GSK3β inhibition (Ciani et al., 2004; Lucas et al., 1998). Decrease in the amount of MAP1B in association with microtubules in axons was also observed (Ciani et al., 2004). Endogenous Dvl tightly associated with microtubules and also enhances microtubule stability strengthening the pro microtubule effects of Wnt signalling here.

Wnt is also able to signal intracellularly through the actions of Dvl, independent of β-catenin. The first of these signalling pathways is described as the Planar cell polarity (PCP) pathway (Gordon and Nusse, 2006). The term PCP refers to the reorientation of cells during development e.g. during gastrulation (Veeman et al., 2003; Wallingford et al., 2002). In Dvl-Daam1-RhoA signalling Dvl binds to dishevelled associated activator of morphogenesis 1 (Daam1) reversing the auto-inhibited state of this protein (Liu et al., 2008). Together Dvl and Daam1 activate RhoA-GTPase which in turn activates the protein Rock resulting in the remodelling of cytoskeletal proteins (Habas et al., 2001).

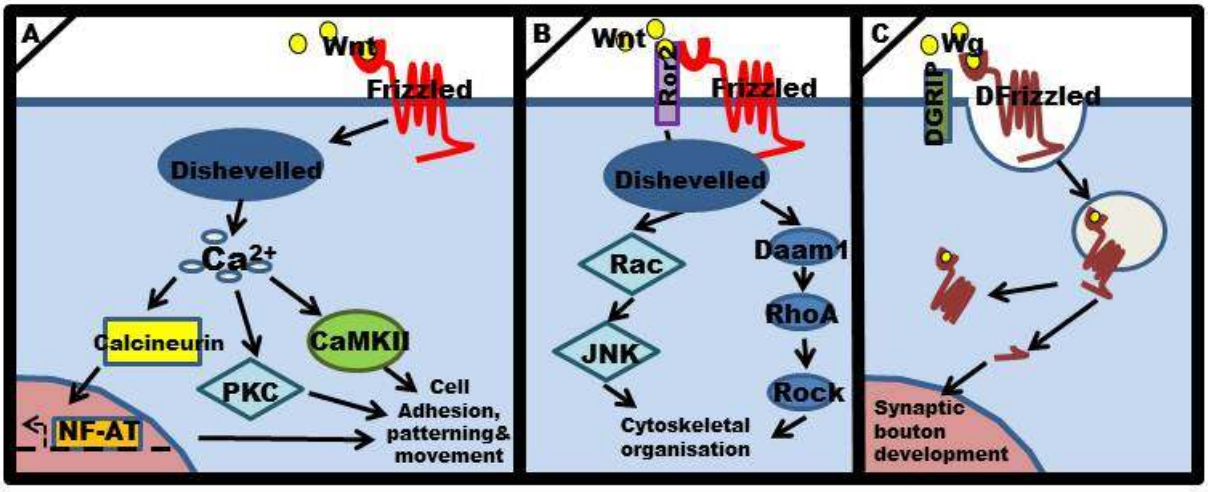

Figure 1.5 Non Canonical Wnt signalling pathways A) Wnt bound to Frizzled receptors activates intracellular Dvl to promote cellular calcium fluxes. The changes in cellular calcium concentration promote downstream signalling by CaMKII, PKC and calcineurin. These signalling pathways mediate cell adhesion, patterning and movement effects in neurons. B) Planar cell polarity signalling involves Wnt proteins signalling through ROR and Frizzled receptors to influence cytoskeletal organisation. Through these surface receptors dishevelled acts through Daam1, RhoA and Rock or a divergent signalling pathway involving Rac and

JNK. C) In drosophila Wnt is able to signal via a Dfrizzled nuclear import pathway. Here Wg (drosophila Wnt analogue) initiates the internalisation of the Wg-dFz complex. The frizzled N-terminal is cleaved intracellularly and enters the nucleus where it encourages synaptic bouton development.

Dvl can also activate a divergent non-canonical pathway activating the RhoGTPase, Rac. This in turn activates c-jun N-terminal kinase (JNK) resulting in modification to neuronal dendrite growth and cell polarity during xenopus gastrulation (Habas et al., 2001; Rosso et al., 2005).

Wnt frizzled interactions directly generate calcium fluxes resulting in a series of intracellular signalling cascades. These intracellular calcium concentration increases are sufficient to activate signalling via protein kinase C (PKC) and CaMKII (Semenov et al., 2007). Experiments overexpressing versions of Dvl lacking DIX domains caused intracellular increases in calcium flux, PKC and CaMKII activity suggesting an upstream position for Dvl in this pathway (Sheldahl et al., 2003). Experiments by Saneyoshi and colleagues identified a transcription factor (NF-AT) involved in Wnt/frizzled Ca2+ signalling. The activation of this transcription factor was observed in response to Wnt5a mediated calcium flux and is believed to be mediated by the calcium responsive protein calcineurin (Saneyoshi et al., 2002).

A more recently discovered Wnt signalling pathway has been described from experiments in the drosophila. Wg secretion initiates endocytosis of Drosophila frizzled 2 (DFz2) whereby the receptor is transported to the nucleus via transport vesicle (Mathew et al., 2005). Once at the nucleus the C-terminal portion is imported into the nucleus. Interestingly this process is mediated by drosophila GRIP (Ataman et al., 2006).

1.6 Wnts and Synapse formation

Through these signalling pathways Wnt and Wnt related species are able to regulate a great number of cellular processes. In the development of the nervous system many of these processes play essential roles. We have a good but incomplete understanding of the importance of Wnt signalling in processes guiding neurons from individual immature components to functional neural networks. The next section of my thesis aims to underline some of our understanding.

1.6.1 Dendritic and axonal development

Various Wnts have been demonstrated causing effects on neurons in terms of dendritic development. One of the early suggestions of these effects came from experiments where modulation of intra-cellular beta catenin resulted in enhanced dendritic arborisation (Yu and Malenka, 2003). Direct action of Wnt on dendritic arborisation was demonstrated in experiments using Wnt7b on hippocampal neurones (Rosso et al., 2005).Exposure of neurons to Wnt7b generated enhancements in total length and the number of branches in dendrites of hippocampal neurons blocked by secreted frizzled related protein (SFRP1) a Wnt7b sequestering protein. These effects were mimicked by Dvl1 overexpression in hippocampal neurons and mirrored in Dvl1 knockout mouse hippocampal cultures. This study also demonstrated activation of the canonical pathway did not stimulate dendritic growth suggesting that the work by Yu and Malenka involved a non-canonical signalling cascade enhancing beta catenin levels in the cell. Indeed the work by (Rosso et al., 2005) revealed a Dvl dependent pathway acting through RhoGTPases to regulate dendritic morphogenesis.

Looking at neurites in PC-12 and N1E-115 cells Kishida and colleagues identified Wnt3a regulated effects in neurite retraction. The group identified a neurite outgrowth inhibition mechanism signalling through the Rho-kinase pathway initated by Dvl1 overexpression or Wnt1 or Wnt3a exposure (Kishida et al., 2004). The contradiction in effects between this study and the previously mentioned study only confirm the complexity of Wnt signalling in terms of Wnt subtype and combination with various cell types.

More recent work has also demonstrated that cultured hippocampal neurons either transfected with Wnt-2 or treated with Wnt-2 conditioned media displayed both increased dendrite length and branching (Wayman et al., 2006). This study also demonstrates an activity dependent component to Wnt-2 mediated dendritic arborisation which will be discussed in further detail later in this chapter.

Wnt signalling in axonal growth and guidance has been in comparison to dendritic development been intensely studied. As previously discussed the development of the axonal compartment is particularly interesting because of the great distances and specificity with which these structures grow/translocate. Wnt proteins fit within the broad number of proteins involved in axon guidance and development. The initial clue for Wnt regulated axon growth was observed in drosophila studies using DWnt5. Overexpression disrupted the commissural

axon tracts in drosophila (Fradkin et al., 1995). Later studies identified the DWnt5 protein as a repulsive cue (Yoshikawa et al., 2003).

Subsequent studies began to reveal various Wnt proteins as axonal guidance (growth/retardant) cues along anterior-posterior axis of various species. Amongst these Wnt1, Wnt4, Wnt5a, Wnt7b, Frizzled3 and Ryk receptor have been studied (Dickson, 2005; Imondi and Thomas, 2003; Keeble et al., 2006; Liu et al., 2005; Lyuksyutova et al., 2003). Unsurprisingly with the myriad of proteins present and involved in axonal regulation there is interplay between various species. One such example is the relationship between Wnt3 and ephrin B1-EphB signalling. In the tectum this ephrin B1-EphB signalling generates attractive cues for incoming axons whilst Wnt3 signalling in this region is repulsive helping to direct the precise wiring of the Retinal ganglion cells synapses, reviewed in (Salinas and Zou, 2008).

1.6.2 Wnts as synaptic organisers

Whilst it is clear that Wnts have fundamental roles to play in the early development of the nervous system, particularly during path finding, less is known about the processes which follow. Some of the previously mentioned secreted factors (BDNF, FGF) are known to have synaptogenic effects along with these molecules cellular adhesion molecules such as Syn-Cams, Cadherins and most notably Neurexins and Neuroligins (Cowan et al., 2001; Siddiqui and Craig, 2011). The latter two molecules have also been demonstrated with having the ability to confer excitatory/inhibitory specificity at the synapse (Cowan et al., 2001). Even before neurons find their synaptic partners some of the components which will eventually form synaptic machinery are transported up and down axonal processes. A fully functional synapse is an extremely complex structure containing a vast number of components. The assembly of this structure requires site specific recruitment of these proteins, not just pre- but also post-synaptically. In this situation the Wnts would be ideal organiser candidates in the regulation of synapse formation due to their properties as soluble secreted molecules, able to move between neurons. Indeed several studies have generated evidence for the effects of Wnts in these subsequent processes.

The first indicators came in experiments looking at Wnt axon remodeling in cerebellar granule cells. In order to quantify the synaptic organizing power of Wnt proteins the investigators assessed synaptic protein accumulation in neurites a technique which gives a measure of nascent synaptic site formation (Chin et al., 1995). On addition of Wnt7a the

cultured granule cells were observed to have a higher number of synapsin clusters than controls (Lucas and Salinas, 1997). Further experiments showed that the total synapsin expression in the neurons were up regulated by Wnt addition (Lucas and Salinas, 1997). Additional evidence appeared later in work by the same group this time looking at dissociated cultures from mutant Wnt7a-/- mice. This time at glomerular rosettes the synapsin stained areas appeared to be much smaller than compared to wild type mice (Hall et al., 2000). Wnt and its downstream effector Dvl's effects on both synaptic puncta accumulation and synapse function were further investigated in a later study. Here both Dvl knockout mutant mice and Wnt7a/Dvl double mutant knockout mice were analyzed. In mossy fibers isolated from Dvl1 mutant mice puncta for VAMP2, were smaller and lower in number than in wild type cells (Ahmad-Annuar et al., 2006). This effect was also mimicked in cells from the Wnt7a/Dvl double mutant (Ahmad-Annuar et al., 2006).

The work thus far in the field had looked at the organization of synaptic molecules this left questions as to whether the delay in synapse accumulation of some synaptic components functionally affected synapse function. Mini post synaptic currents (mPSCs) from the granule cells of Wnt7a and Dvl1 deficient mutant mice were analyzed. Here brain slices were whole cell patch recorded and compared with wild type mice. The frequency of spontaneous events were found to be lower in the mutant mice than in comparison to wild type suggesting a defect caused by the lack of Wnt signaling (Ahmad-Annuar et al., 2006). The lack of effect to the amplitude of the events combined with EM images displaying 'normal' active zones suggested that this was specifically a pre-synaptic effect which affected the release of neurotransmitter (Ahmad-Annuar et al., 2006; Bekkers and Stevens, 1995).

It has been demonstrated certain Wnts are potent pro-synaptogenic factors however less data has been generated as to any opposing properties of the proteins. Some of the first observations of the repulsive activity of Wnts in synapse formation came from work done in the lab of Yimin Zou. As previously discussed this work demonstrated that Wnt proteins (wnt1 and 5a) produced by transfected COS cells were able to repel corticospinal tract (CST) neuron outgrowth (Liu et al., 2005). This was followed on by looking at the Wnt receptor, Ryk which was specifically expressed in CST neurons. The work done by this group suggested Wnts acting as repulsive cues through the Ryk receptor were responsible for CST axon guidance in spinal cord. Whilst it had been previously demonstrated that Wnts may act

as repulsive cues in the guidance of axons no evidence had been presented as to the negative effects of Wnts at synapses.

Work by Klassen and Shen has recently demonstrated Wnt mediated anti-synaptogenesis in the C.elegans system. In C.elegans the DA9 motor neuron forms precisely patterned *en passant* synapses along the anterior of the organism. By implanting cells in the posterior of the organism expressing Lin-44, the Wnt analogue in C.elegans, the group found that synapses proximal were disrupted. The generation of these asynaptic regions were shown to be mediated by both Lin-44 and the C.elegans frizzled analogue Lin-17 through Dvl1 (Klassen and Shen, 2007).

Another recent study in drosophila has demonstrated more evidence of Wnt anti-synaptogenesis. In this study differing target cues were identified between two adjacent muscle blocks (M12 andM13) which are innervated two specific motor neuron types (MN12 and MN13). From these muscle blocks Wnt4 proteins were particularly enriched in the M13 muscle. Both loss of function and gain of function experiments, with Wnt4, were able to exchange synapse formation specificity in the M12 andM13 muscle blocks (Inaki et al., 2007). Interestingly no studies have been published yet looking at the anti-synaptogenesis of Wnts at central synapses in mammalian models. A concise record of the characteristics different Wnt species possess in their synapse formation/inhibition ability is yet to be generated. An important question which remains unanswered is what are the differences between Wnts which cause them to have these different properties? Whether Wnts expressed in the CNS have opposing effects in terms of synaptogenesis will again be crucial in the topographic brain map of specific synapse formation. It would be particularly interesting if the formation of synapses between neurons was determined by the combination of Wnts and Frizzled receptors expressed by each neuron.

1.6.3 Wnts at stable synapses

Until fairly recently the drive within Wnt research has been directed at the role these proteins play in the target direction of neurons and the formation of synaptic sites. Relatively little however is currently understood about the effects of Wnts proteins at already formed (and functional) synapses. The changes at molecular level of a synapse are crucial to the efficiency and function of a neuron. From increasing the ease of transmission to silencing a synapse these modifications can have profound effects on neural pathways. To look at synaptic

efficiency electrophysiology methods are often applied, as these are able to give a direct translation of neuronal activity as they occur. This allows a real time insight into how the strength of how synaptic transmission changes. Using these techniques potentially also allows dissection of where physical changes are being made within the synapse. Some recent studies have combined these techniques with imaging to provide greater insight into Wnt signaling.

The first suggestion that Wnts may play a role in synaptic regulation came from the work by Ahmad-Annuar and colleagues. Miniature Post synaptic currents (mPSCs) were recorded from the granule cells of Wnt7a and Dvl1 null mice. mPSCs are spontaneous events of synaptic neurotransmitter containing vesicles being released into the synaptic cleft and generating small currents. These currents are able to provide a measure of readily releasable vesicle release probability and size along with post synaptic receptor clustering changes. In the above mentioned study, in comparison with GCs from wild type mice, miniature excitatory post synaptic currents (mEPSCs) showed significant decrease in frequency. Significantly in this study no effect was observed on amplitude of the post synaptic response of these mEPSCs.

Further work within our lab has led to interesting discoveries concerning the nature of Wnt at the synapse. An antagonist of Wnt signaling Secreted Frizzled Related Protein (SFRP) was used to look at the effect of blocking endogenous Wnt effect. SFRPs are proteins sharing homology with the CRD domain of Frizzled receptors. As such the proteins are able to modulate Wnt signaling by binding free extra cellular Wnts and preventing their interaction with cell bound Frizzled receptors. Much like Wnts there are several members of the family each with a specific affinity for binding partners. Experiments within our lab where 21DIV hippocampal neurons were exposed to a cocktail of SFRPs1, 2 and 3 for 16hours resulted in a decrease in mEPSC frequency but not change to mEPSC amplitude (Boyle and Salinas 2010). In particular the fact that at the 21DIV stage synaptogenic effect is decreasing suggests that this effect of blocking Wnt caused direct action on synapse function and not the result of synapse formation defects. A second important detail to note is that whilst the average frequency of mEPSCs was significantly decreased the amplitude was not (Boyle and Salinas, 2010). Changes in mPSC frequency specifically suggest increased release of neurotransmitter vesicles which may be affected in a number of ways. Frequency may be increased by changes to vesicle release probability, the size of the readily releasable vesicle pool or the number of release sites at a synapse (Bekkers and Stevens, 1995).

Some of the most recent work done in the Wnt field has been on the relationship the Wnt release has with neuronal activity. Studies have begun to investigate stimulated neurons and how these then cause both the increased expression and secretion of Wnt proteins. As previously mentioned Wayman and colleagues investigated the effect of increased excitatory activity on neurons and observed an increase in dendritic complexity (Wayman et al., 2006). This led to investigations into the possibility of a Wnt protein being responsible for this dendritic activity. Previously Beta-catenin over-expression had been demonstrated, increasing dendritic arborization and this provided the initial suspicions for Wnt involvement (Yu and Malenka, 2003). Continued investigation by Wayman and colleagues revealed a CAMKK mediated pathway resulting in up-regulation of CREB responsive gene expression. Wnt2 was identified as a CREB responsive gene and Wnt2 RNA was identified at an increased level after treatment with KCl and forskolin (Wayman et al., 2006).

These results observed in rat organotypic slices and cultures, has been followed up by recent discoveries in drosophila. Initial experiments, involving K^+ stimulations similar to those patterned learning paradigms which induce LTP, showed changes in presynaptic protrusions similar to the phenotype of drosophila with defects in wingless (Wg) signaling (Ataman et al., 2008). These stimulation paradigms also showed the ability to potentiate miniature excitatory junction potential (mEJP) frequency. By generating a wingless deficient heterozygous mutant the group were able to show in the presence of the stimulation paradigm the protrusion number decreased but was rescued by expression of Wg in motor neurons (Ataman et al., 2008). The link between, neuronal activity and Wg secretion was investigated by looking at Wg immune-reactivity around the postsynaptic density of motor neurons. When k^+ stimulation was evoked Wag staining was significantly increased, this was suppressed by elimination of $Ca2^+$ (Ataman et al., 2008).These findings have led to interesting questions as to the function of Wnts at these neurons? For instance do these proteins act in a cell autonomous and/or in a paracrine manner? Our understanding of this may increase our knowledge of whether Wnts have specific well defined local effects on release or are general enhancers of connectivity. What role will the release of these proteins play in the regulation of central synapses and their formation?

1.7 Project aims

My research project aimed to investigate the role that Wnt7a and Dvl1 signaling plays in regulating synaptic transmission. I used immunofluorescence and electrophysiological methods to study Wnt7a and Dvl1 effects based around the following hypotheses:

- Hippocampal neuronal cultures exposed to Wnt7a will show an increased accumulation of excitatory synaptic proteins in neurites.
- Hippocampal neuronal cultures exposed to Wnt7a or overexpressing Dvl1 will increase excitatory synaptic proteins on dendritic spines.
- Hippocampal neuronal cultures exposed to Wnt7a or overexpressing Dvl1 will have an increased number of excitatory synapses.
- Hippocampal neuronal cultures exposed to Wnt7a will demonstrate enhanced synaptic transmission whilst loss of Wnt7a/Dvl1 signaling will result in decreased synaptic transmission.

Chapter 2

Materials and Methods

2.1 Hippocampal Cell Culture

Hippocampal cultures were prepared from E18 Sprague-Dawley rat embryos. Cultures were prepared as described by Kaech and Banker (Kaech and Banker, 2006). Pregnant females were anaesthetised by use of CO_2 and killed by cervical dislocation. The embryos were removed and the hippocampi were then dissected from both hemispheres 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 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, and changed over to serum-free N2 and B27 containing culture medium after 2 hours. All cultures were maintained at 37°C in 95% CO_2/5% O_2 and received partial replacement of the culture medium with fresh medium once per week. Cultures were plated at 50 cells/mm^2 (low density) for recording evoked synaptic currents, 200 cells/mm^2 (high density) for recording mPSCs and calcium phosphate transfection, and 100 cells/mm^2 (medium density) or 200 cells/mm^2 for all other imaging experiments, unless otherwise stated.

2.2 Hippocampal cell calcium phosphate transfection

Hippocampal cultures were transfected at 8 DIV with Actin-eGFP and DVl1-HA constructs using Calcium Phosphate buffer. Control neurons were transfected with Actin-eGFP and the PSC2 empty vector which forms the backbone of the experimental DVL1-HA construct. . For each P60 culture dish transfected (containing 10 coverslips), 400ul of $CaCl_2$-DNA-HBS mixture was used. This mixture was carefully added drop wise directly onto the culture dish containing 3ml of serum free transfection media. DNA was applied to neurons in 3:1experimental DNA: Actin-eGFP ratio ensuring the majority of eGFP positive neurons were double transfected (1.5μg Actin-eGFP + 4.5μg Dvl1-HA, or 1.5μg Actin-eGFP + 4.5μg PCS2+ for control) and incubated for 10 minutes. The medium was immediately removed

from the culture dish, the cells washed twice with warm PBS, replaced with fresh medium and retained at 37°C. 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 at 37^{o}C.

2.3 Immunofluorescence

Coverslips were fixed for 18 minutes in 4% paraformaldehyde at room temperature. Cells were permeablized with 0.02% Triton for 1 minute, 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 alexa-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). GluR1 and GluR2 primary antibodies were applied to live neuronal cultures. Coverslips were incubated for 10 minutes with the appropriate dilutions of antibody, washed with PBS (37^{o}C) and subsequently fixed with paraformaldehyde and stained with secondary antibody as described above.

2.4 Image acquisition and analysis of synaptic puncta

Images were captured on Leica TCS SP1 or SP2 confocal microscope. Typically when imaging 3D samples using confocal microscopy Z-stack resolution (approximately half that of X or Y resolution) must be taken into consideration. In order to address this issue the number of Z-stacks taken per sample was optimized to intervals of half the resolution of the confocal lens. In my study samples were imaged on Leica TCS SP1 or SP2 confocal microscopes through 63x, 1.32NA APO lens. The Z-resolution of this lens is equivalent to 315nm (Zucker, 2006). Each sample was optimized to take Z-stack images every 158nm, double the Z resolution, whilst the upper and lower limits of the sample were determined manually (≈3µm). In reality due to light scatter on various surfaces of the microscope the actual axial resolution is lower than this (anywhere between 400nm-800nm). Typically hippocampal synaptic pre and postsynaptic terminals are larger than this axial resolution, therefore this setup will allow resolution of puncta colocalised on these parts of the synapse (Harris et al., 1992) Each imaging experiment was performed at least 3 times on independent cultures. 8-12 images were taken per condition and analysed using Volocity (Improvision). Volocity 3D imaging analysis software created by Improvision is an integrated acquisition,

visualisation and analysis tool for confocal images. Using this tool, confocal stacks acquired using Leica microscopes and acquisition software were reconstituted into 3D blocks where fluorescent puncta could be analysed for colocalisation in both the x, y and Z planes. By thresholding fluorescence in the x, y, z planes, 3D structures such as neurites, dendritic spines and receptors at synapses can be identified. Therefore puncta and neuritis are referred to in volumetric units. Using this method, overlapping thresholded regions of different fluorescence (corresponding to neurites, receptors, etc.) were identified using varies standardised protocols within the velocity software.

Regions used for analysis were selected based purely on consistency between images of either Tuj1 or EGFP-actin channels. Objects of interest (puncta of synaptic proteins or Tuj-1 labelled neurites) were delineated using intensity thresholds. 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. Co-localisation of synaptic puncta was determined using custom-built protocols in Volocity.

2.5 Dendritic spine-puncta colocalisation analysis

Spine analysis was performed manually. 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 head width of the spines determined with a line tool, the volume was determined using a custom built Volocity thresholding protocol. Analysis of spine and puncta colocalisation was determined by manual selection of spines followed by Volocity colocalisation protocol. 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.

2.6 Mouse breeding and genotyping

All mutant mice were maintained on a C57BL/6 background. *Wnt7a-/-;Dvl1-/-* double mutant mice were obtained from crosses of heterozygous *Wnt7a+/-* and *Dvl1+/-* mutant mice. 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′.

2.7 Acute slice preparation

Slices were prepared from P12-15 mice for recording miniature postsynaptic currents and evoked postsynaptic currents. Preparations were conducted as described by Bischofberger and colleagues (Bischofberger et al., 2006). 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 or a Leica VT1000S 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.

2.8 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 bicuculine and 1mM Mg^{2+}, whereas mIPSCs were recorded at 0mV in the presence of 10µM CNQX and 50µM AP-5.

Evoked postsynaptic currents were elicited by using a bipolar concentric electrode (FHC) attached to a Grass S48 stimulator to depolarise axons close to the patched cell in cell culture or axons of the stratum lucidum at MF-CA1 synapses in hippocampal slices. For paired-pulse recordings, the stimulus intensity was altered from cell to cell to give the minimum reproducible response. Minimal stimulus recordings were made by increasing stimulus voltage until single 'low' amplitude responses were generated. The responses were noted and compared in comparison to the number of evoked stimulations. 10mM QX-314(intracellular voltage gated sodium channel blocker) 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 lucidum approximately 100-200µm from the whole-cell patched neuron in the CA3 layer. In hippocampal cultures 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. For single pulse stimulus protocols the stimulus amplitude was manually increased until a minimal EPSC was established over a number of stimuli and maintained. During Paired pulse stimuli protocols the stimulus amplitude was modified throughout the length of the recording to maintain EPSC fidelity. All currents were recorded using an Axopatch 200A amplifier, filtered at 1 kHz and digitised onto computer at 10 kHz using WinEDR software.

2.9 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 Bekkers and Stevens (Bekkers and Stevens, 1995). 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 details of the templates, thresholds and filters used for detection are given in Table 2.1 below:

	Tau Rise	**Tau Decay**	**Area**	**Peak**
mEPSC	5ms-1000ms	0ms-1ms 15ms-1000ms	0pA/ms-1000pA/ms	0pA-1000pA
mIPSC	10ms-1000ms	0ms-5ms 100ms-1000ms	-1000pA/ms-0pA/ms	-1000pA-0pA

Table 2.1WinEDR mPSC filter settings

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 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.

2.10 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 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.

2.11 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. A randomisation test was also used to complement the above tests where data was non-normally distributed. Statistical significance is denoted in all figures as follows: * = $P<0.05$.

2.12 Solutions

Hippocampal plating medium – 50 ml

Neurobasal medium (Gibco)	Add to 50ml
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)	Add to 50ml
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
(pH to 8.5 with NaOH)	
Sterile filtered water	add to 400 ml

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	Add to 50ml

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	Add to 500ml
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)
Distilled water	Add to 500ml

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	
Sterile filtered water	add to 400 ml

Channel Blockers – Final molarity

Bicuculine-Methiodide (Ascent)	10µM
AP5(Ascent)	50 µM
DNQX (Ascent)	10 µM
TTX (Ascent)	100nM
QX-314 (Ascent)	10mM

Hanks balanced salt solution (HBSS)

(No calcium, no magnesium) Ordered from Gibco

2.13 Antibodies

Primary antibodies

Antibody and supplier	Dilution
Chicken anti-Tuj-1 (Chemicon)	1:1000
Rabbit anti-GluR1 (Affinity Bioreagents)	1:20 (Live)
Guinea pig anti-vGlut1 (Chemicon)	1:5000
Mouse anti-GluR2 (Synaptic Systems)	1:200 (Live)
Guinea pig anti-Synapsin (Synaptic Systems)	1:1000
Rat anti-HA (Roché)	1:1000
Chicken anti-GFP (Upstate)	1:500
Mouse anti-NR1 (synaptic systems)	1:500

SECONDARY ANTIBODIES

Antibody and supplier	Conjugate	Dilution
Goat anti-chicken IgG (Molecular Probes)	Alexa-647	1:600
Donkey anti-chicken IgG (Jackson Immunoresearch)	DyLight-488	1:600
Goat anti-guinea pig IgG (Molecular Probes)	Alexa-488	1:600

Goat anti-guinea pig IgG (Molecular Probes)	Alexa-568	1:600
Donkey anti-mouse IgG (Molecular Probes)	Alexa-488	1:600
Goat anti-mouse IgG (Molecular Probes)	Alexa-568	1:600
Goat anti-mouse IgG (Molecular Probes)	Alexa-647	1:600
Donkey anti-rabbit IgG (Molecular Probes)	Alexa-594	1:600
Goat anti-rat IgG (Molecular Probes)	Alexa-647	1:600

Chapter 3

Wnt7a signalling promotes the formation of functional glutamatergic synapses in the Hippocampus.

3.1 Introduction

The formation of a functional neural network requires precise regulation of the formation of a variety of different synapse types. A myriad number of proteins found in the brain play specific roles in the formation and maintenance of synaptic sites. The trans-synaptic proteins complexes formed by neuroligin proteins are one of the most well studied examples of a synaptogenic factor. The presence of specific neuroligin variants are associated exclusively with specific synapse types and help to direct the formation of these synapses (Chubykin et al., 2007). Neuroligins 1 and 2 are enriched at excitatory and inhibitory synapses respectively (Graf et al., 2004; Song et al., 1999; Varoqueaux et al., 2004). Similarly BDNF secreted proteins have also been identified in the regulation of specific synapses. Luikart and colleagues (2005) identified defects in excitatory synapse formation using a conditional TrkB mutant (BDNF loss of function model) (Luikart et al., 2005). Wnt proteins play a similar role to BDNF in the early development of neurons in development and are also found expressed through to adulthood in the brain (for review see (Salinas and Zou, 2008). The role of Wnt proteins in the postnatal development of synaptic sites is currently poorly understood however some recent studies have begun to reveal more about the role these proteins play.

The first evidence for a role of Wnt7a in promoting central synapse formation was discovered in studies in the cerebellum. Here cerebellar granule cells, grown in tissue culture on Wnt7a expressing cells displayed enhancements in synapsin1 clustering (Lucas and Salinas, 1997). The role of Wnt7a in central synaptogenesis was further investigated with the analysis of knockout mice. Loss of Wnt7a function causes defects in the formation of the mossy fibre-granule cell synapse as visualised at ultrastructural and immunochemical levels. Specifically loss of function reduces complexity of the synapse and size of the PSD whilst also decreasing the size of clusters of synapsin1 clusters (Hall et al., 2000). Double knockout Wnt7a-/-, Dvl1-/- mice exhibit defects in the frequency of AMPA mediated mEPSCs, suggesting these structural defects lead to malfunction in synaptic transmission (Ahmad-Annuar et al., 2006). These studies demonstrate the crucial regulatory role Wnt7a signalling plays in the formation of the cerebellar mossy fibre granule cell synapse.

The effect of Wnt7a on synapse specification in the cerebellum led to the question of whether this Wnt plays a similar role in other important brain regions. *In situ* hybridization studies have shown Wnt7a mRNA is expressed in various regions of the mammalian brain, most strongly in the cerebellum, hippocampus and olfactory bulb at various stages of development (Lucas and Salinas, 1997; McCarthy, 2006). Loss of Wnt7a function studies, were some of the earliest to suggest that specific Wnts regulate synapse formation within the CNS.

Whilst there is much data on the presence of Wnt proteins in the brain, their functions are still poorly understood. Gogolla and colleagues, through *in vivo* and *ex vivo* approaches, have investigated the expression of Wnts in the hippocampus in relation to environmental enrichment. Antibody staining for Wnt7a and Wnt7b revealed that these proteins are elevated in the hippocampus of animals exposed to an enriched environment (Gogolla et al., 2009).. This increase in expression is specific only to the CA3 region and not seen using antibodies specific for Wnt5 or Wnt3 (Gogolla et al., 2009). Furthermore the increased Wnt7a/b expression was correlated with an increased synapse number within the CA3 region of the hippocampus as determined by staining for synaptic markers such as bassoon and by the remodelling of large mossy fibre terminals (Gogolla et al., 2009). These experiments demonstrate a role of Wnt signalling in the formation of synapses in the hippocampus.

The above study demonstrates a role for Wnt signalling in activity and experience mediated changes to the circuitry of hippocampal synapses. However the manner in which these changes occur at synaptic level has not been assessed in detail. In particular, changes to the localisation of molecules at the synapse and the types of synapse each Wnt proteins act at is still poorly understood. Several recent studies have revealed differences in the action of Wnt species at central synapses focusing on Wnt3a and Wnt5a (Avila et al., 2010; Cuitino et al., 2010; Davis et al., 2008).Davis and colleagues (2008) examined the effects of Wnt3a, Wnt7a, Wnt7b and Dkk1 on accumulation of vGlut1 puncta on 10 DIV hippocampal cultures (Davis et al., 2008). Their normalised data suggests that all three Wnt species have a positive effect on the accumulation of this excitatory marker whilst Dkk1 an inhibitor of canonical Wnt signalling decreases presynaptic excitatory puncta accumulation.

More recently Avila and colleagues demonstrated the ability of Wnt3a to enhance both the number of presynaptic release sites by analysis of FM1-43 and synapsin staining (Avila et al., 2010). By examining mEPSC recordings from neurons treated with Wnt3a the same study

also revealed increases in mEPSC frequency suggesting likely increases in synapse number and or presynaptic release efficiency (Avila et al., 2010).

Cuitino and colleagues (2010) investigated the ability of Wnt5a to regulate inhibitory GABA synapses in the hippocampus (Cuitino et al., 2010). Wnt5a increases the number of GABAA receptors on neurons cultured for 18DIVs. In combination with this inhibitory spontaneous and miniature currents were recorded and analysed (Cuitino et al., 2010). Wnt5a increases the amplitude of both spontaneous and miniature currents. These experiments demonstrate the diversity of Wnt proteins in terms of the type of synapse they cause effects on.

My research aimed to provide greater understanding on the role of Wnt7a in the hippocampus. In particular, I have focused my attention on the function of Wnt7a at Glutamatergic synapses. Previous experiments in the lab have demonstrated that Wnt7a increases the co-localisation of the excitatory pre and postsynaptic markers, vGlut-1 and PSD-95 whilst having no effect on the colocalisation of pre- and postsynaptic markers for inhibitory synapses, vGat and Gephyrin (Ciani et al., 2011). This preference of Wnt7a action for excitatory hippocampal synapses poses the question, is this selective effect also manifested at the level of the postsynaptic receptors and can this be seen in transmission at these synapses. Furthermore questions still remain as to the characteristics of the effects of Wnt7a temporally and spatially across the hippocampus. Several studies indicate the second week of postnatal development in rats and mice as the peak period for synapse development (Nimchinsky et al., 2002). The coordinated peak of Wnt expression in hippocampus also occurs during this time period identifying a potential regulatory role for Wnt proteins at the various developmental periods. In this chapter, I addressed these questions by applying Wnt7a to hippocampal cultures and assessing the accumulation of the excitatory presynaptic protein vGlut and the post synaptic proteins GluA1, GluA2 and GluN1.

Interestingly the size and number of these synaptic proteins puncta increased in relation to Wnt7a exposure of both 3 hours and ≈18 hours. Along with an increase in puncta number, the various postsynaptic markers also increased in co-localisation with the presynaptic marker vGlut1. In order to assess how these Wnt7a mediated effects related to synaptic function I looked at miniature excitatory post synaptic currents (mEPSCs) in both 14div hippocampal cultures treated with Wnt7a (gain of function) and p14 Wnt7a-/-. Dvl1-/-, acute slices (loss of function). Exposure to acute (3hour) Wnt7a treatment increases mEPSC frequency and amplitude with no effects on the frequency or amplitude of mIPSCs. In the Wnt7a-/-. Dvl1-/-

mutant, a clear defect in mEPSC frequency and amplitude is observed. Surprisingly however neurons exposed to long (≈18hours) treatments of Wnt7a showed no enhancement in frequency or amplitude. These data suggest Wnt7a plays a pro-synaptogenic role at excitatory synapses in the hippocampus. This is manifested as an ability to regulate surface expression of GluA1 and GluA2 subunits and the total expression of GluN1 subunits.

3.2 Results

3.2.1 Wnt7a promotes the formation of excitatory pre and postsynaptic sites

Aggregation of synapse specific molecules such as synaptotagmin, bassoon, and VAMP or the colocalisation of pre and post synaptic proteins have previously been used to assess the ability of neuron derived proteins to affect synaptogenesis. In order to investigate the effects of Wnt7a, hippocampal neurons, cultured for 12-14 days, were exposed to recombinant Wnt7a, and stained (live in the case of GluA1 and GluA2 or fixed and stained for vGlut and GluN1). The numbers of clusters of each synaptic protein were quantified in relation to the volume of neurite they were present on. Neurites were identified by immuno-labelling with an antibody to β-3 tubulin (Tuj-1). I found that a 3 hour exposure to Wnt7a caused a near doubling in the number of GluA1 puncta in the neurites of 14 DIV hippocampal neurons (Fig3.1a, d). vGlut clusters in these cultures were also found to increase in number opposed to dendrites (Fig3.2a, b). vGlut and GluA1 puncta which were found to come into contact with each other on a neurite (overlapping by a pixel) were considered to be 'colocalised' and represent a potential synaptic site. After assessing the colocalisation of vGlut and GluA1 puncta I found that the percentage of the total number of each cluster opposed to the other, increased from 46 ± 1.04% (vehicle) to 57 ± 1.00% (Wnt7a) (Fig3.2c).

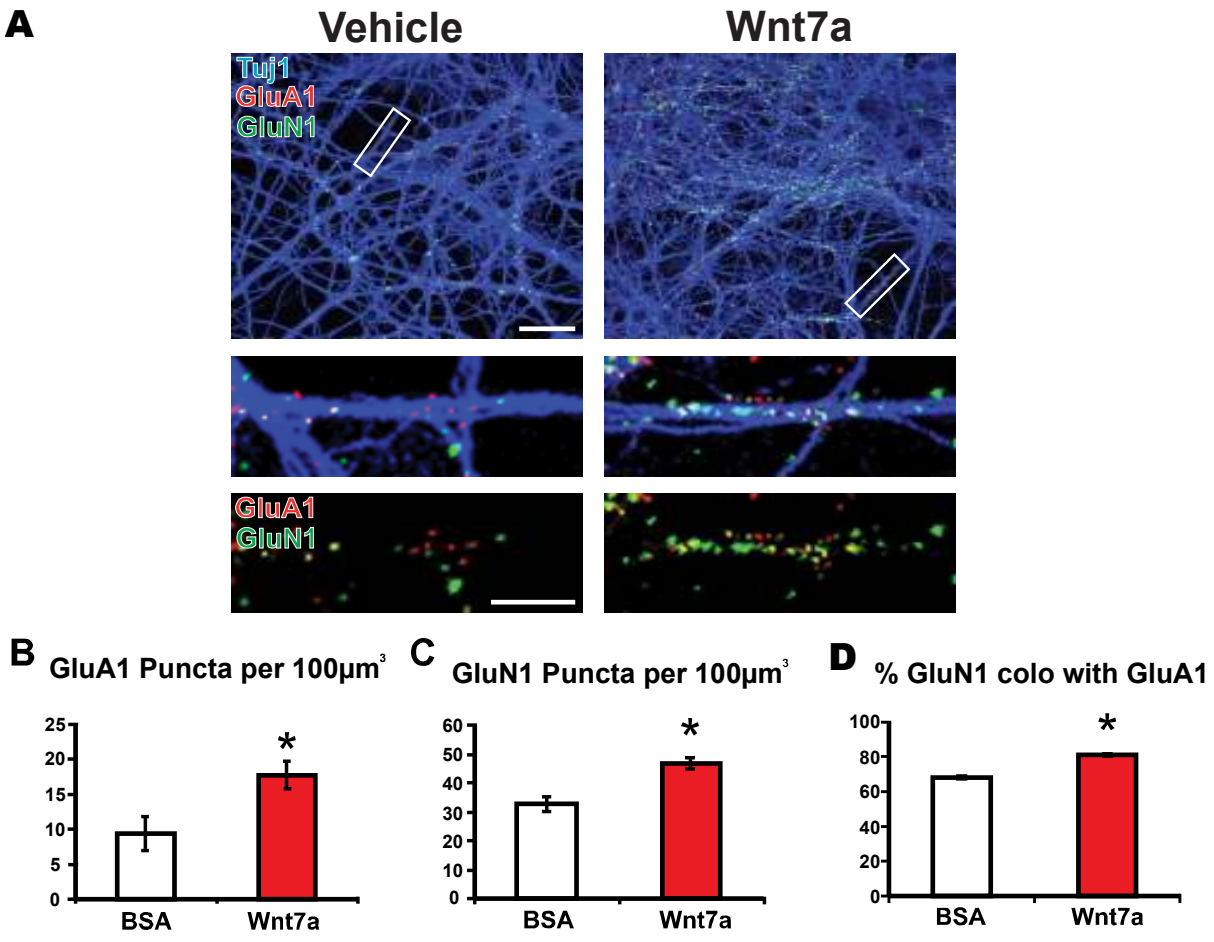

Figure 3.1 Wnt7a increases GluA1 and GluN1 puncta density and colocalisation. 14 DIV hippocampal cultures were treated with recombinant Wnt7a or BSA (vehicle) for 3 hours. (A) Images of treated neuronal cultures stained for Tuj1 (blue), GluA1 (Red) and GluN1 (Blue). The puncta number and colocalisation are increased by Wnt7a. White dashed boxes indicate the area shown in the enlarged panels. Scale bars in top panel = 30 µm and 10 µm in enlarged panels. (B) Shows quantification of increased GluA1 density. (C) Shows the quantification of GluN1 puncta increase between BSA and Wnt7a. (D) Quantification showing the increase in colocalisation between GluN1 and GluA1 puncta. * = $P<0.05$.

The increase in puncta size and colocalisation of vGlut and GluA1 puncta was mirrored by a similar sized increase in the number of GluA2 puncta in these cultures (Fig.3.3a, b). A 3 hour exposure of neurons to Wnt7a caused a 42% increase in GluN1 subunit

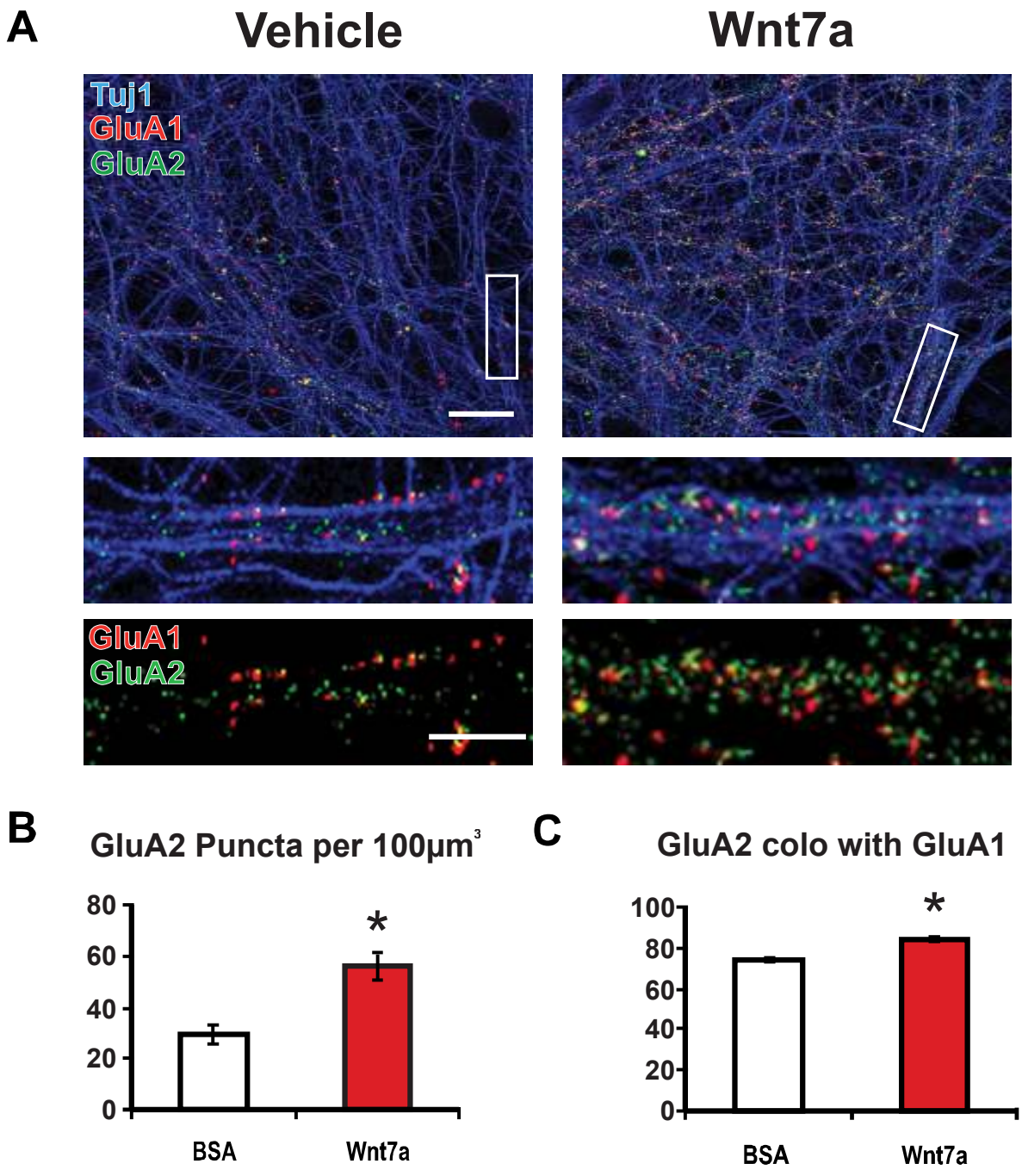

Figure 3.2 Wnt7a increases GluA2 puncta density and colocalisation of GluA2 with GluA1. 14 DIV hippocampal cultures were treated with recombinant Wnt7a or BSA (vehicle) for 3 hours. (A) Images of treated neuronal cultures stained for Tuj1 (green), GluA1 (Red) and GluA2 (Blue) the puncta number and colocalisation is increased by Wnt7a. White dashed boxes indicate the area shown in the enlarged panels. Scale bars in top panel = 30 µm and 10 µm in enlarged panels. (B) Quantification of increased GluA2 density. (C) Quantification of the increase in colocalisation between GluA2 and GluA1 puncta after Wnt7a treatment. * = $P<0.05$.

puncta number in hippocampal cultures (Fig3.1a, c). Interestingly in cultures stained with a combination of antibodies for GluA1 and GluN1 the colocalisation of these proteins increased from 68.2 ± 0.98% (vehicle) to 81 ± 1.12% (Wnt7a). Furthermore neurons exposed to Wnt7a showed an increase in colocalisation of GluA1 and GluA2 subunits; 73 ± 1.36% (vehicle) and 84 ± 1.64% (Wnt7a), and vGlut and GluA1; 45 ± 2.01% (vehicle) and 55 ± 2.93% (Wnt7a).

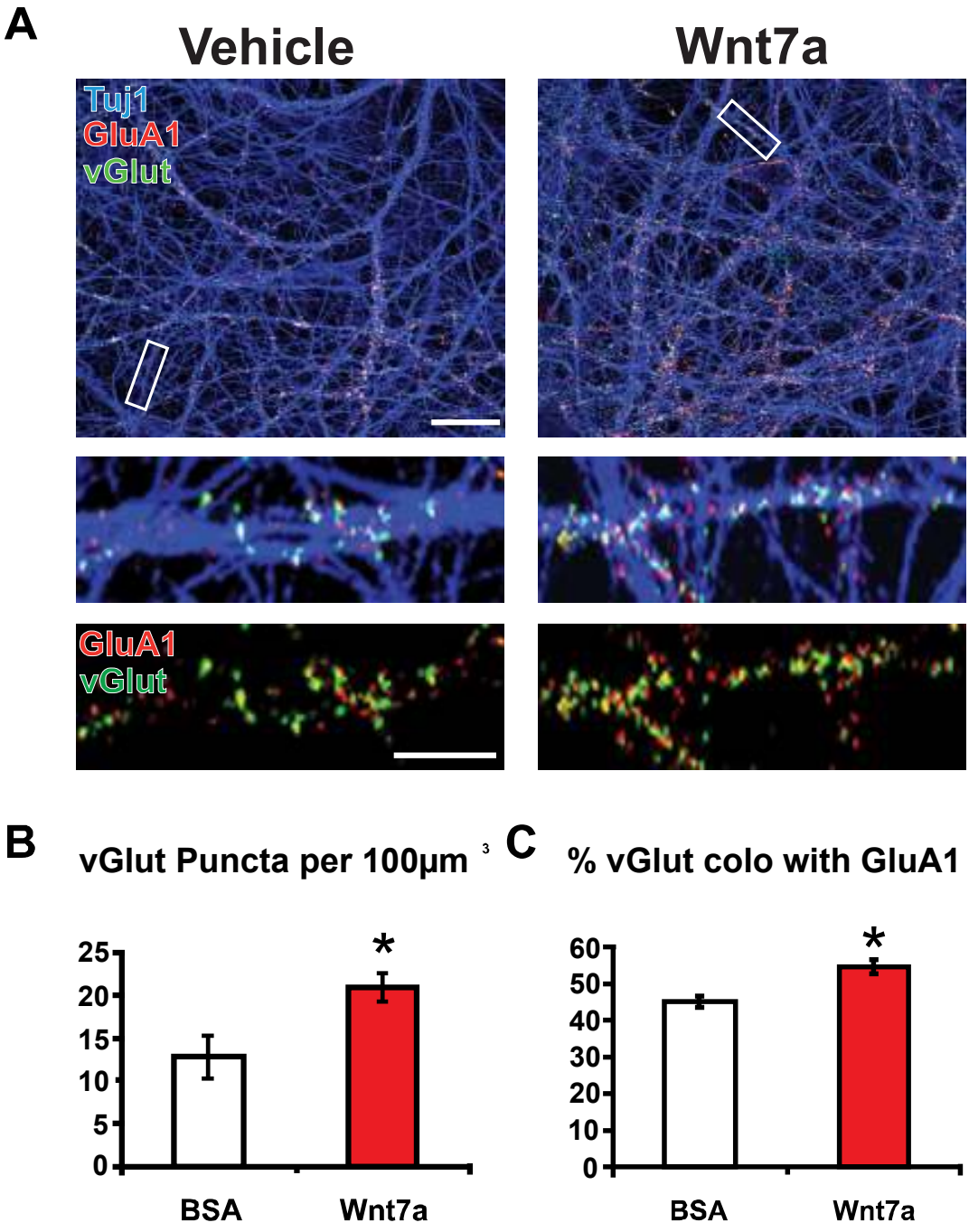

Figure 3.3 Wnt7a increases vGlut puncta density and colocalisation with GluA1. 14 DIV hippocampal cultures were treated with recombinant Wnt7a or BSA (vehicle) for 3 hours. (A) Images of treated neuronal cultures stained for Tuj1 (green), GluA1 (Red) and vGlut (Blue) the puncta number and colocalisation is increased by Wnt7a. White boxes indicate enlarged panels. Scale bars in top panel = 30 µm and 10 µm in enlarged panels. (B) Shows quantification of increased vGlut density. (C) Quantification showing the increase in colocalisation between vGlut and GluA1 puncta. * = P<0.05.

These data suggest that at this 14 DIV period, where synapse formation is at its peak, hippocampal neurons are susceptible to stimulation of synaptogenesis via Wnt7a signalling. In particular these data show that Wnt7a treatment results in both pre- and postsynaptic accumulation of synaptic machinery and that Wnt7a stimulates the coming together of the complementary parts of the synapse.

3.2.2 The synaptogenic effect of Wnt7a is related to neuronal maturity

Following the experiments described above I then investigated if there were Wnt7a effects in more mature 21 DIV cultures. The greater maturity of 21 DIV neurons and their connections provides a different environment in which to investigate the effects of Wnt7a. Whilst at 14 DIV there is high turnover of synapses with high net gain of new synapses in hippocampal cultures, at 21DIV the network is less dynamic in terms of new synapse formation(Nimchinsky et al., 2002). It was therefore interesting to ask if Wnt7a had a similar effect in stimulating synapse formation at this later stage as it does in less mature cultures. In order to do this hippocampal neurons were cultured for 3 weeks and then exposed to recombinant Wnt7a for a period of 3 hours followed by staining for pre- and postsynaptic markers. In stark contrast to the 14 DIV period, 3 hour exposure of the 21DIV cultures to Wnt7a did not cause an increase in the accumulation of the presynaptic protein vGlut1 (17.52 ± 2.61 (Vehicle) 22 ± 3.12 (Wnt7a) (Fig 3.4a,b).

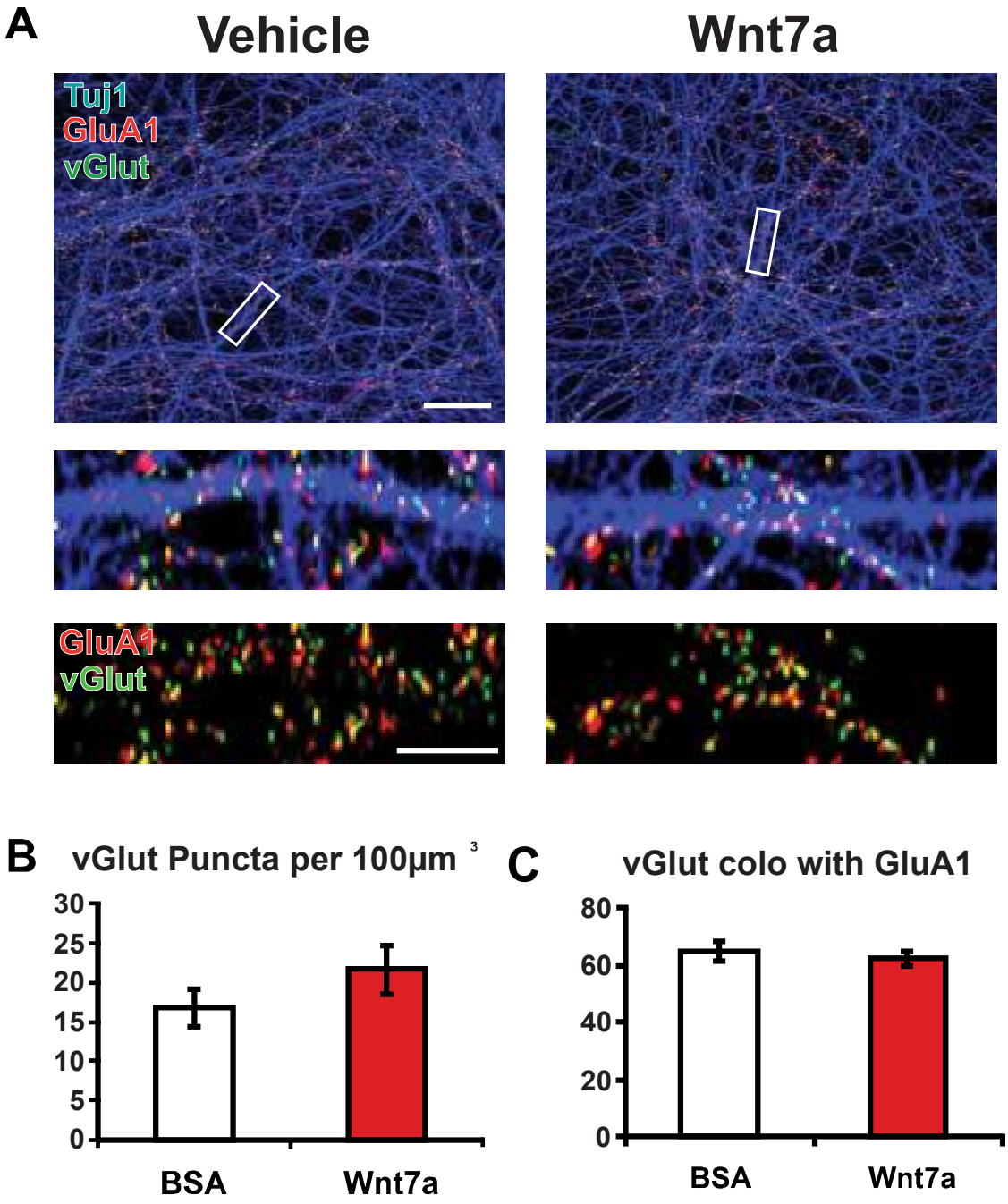

Figure 3.4 Wnt7a does not increase GluA1 and vGlut puncta density and colocalisation in mature cultures. 21 DIV hippocampal cultures were treated with recombinant Wnt7a or BSA (vehicle) for 3 hours. (A) Images of treated neuronal cultures stained for Tuj1 (green), GluA1 (Red) and vGlut (Blue) show that puncta number and colocalisation are not increased by Wnt7a. White dashed boxes indicate the area shown in the enlarged panels. Scale bars are: top panel = 30µm and 10µm in enlarged panels. (B) Shows the quantification of vGlut puncta between BSA and Wnt7a where no significant increase is observed. (C) Quantification shows no change in colocalisation between vGlut and GluA1 puncta. * = $P<0.05$.

Interestingly Wnt7a had no effect on the percentage of total vGlut puncta opposed to GluA1 puncta in these neurons (65 ± 4.23% (vehicle) 62 ± 3.21% (Wnt7a Fig3.4a, c). These data suggest that mature cultures lose the ability to not only regulate vGlut puncta accumulation in response to Wnt7a exposure but also lose the ability to localise vGlut puncta to sites which may be in apposition to postsynaptic receptors. I then looked at whether the localisation of GluA1 puncta was affected by Wnt7a exposure and whether GluA1 colocalisation with other synaptic markers was affected by Wnt7a in these more mature cultures.

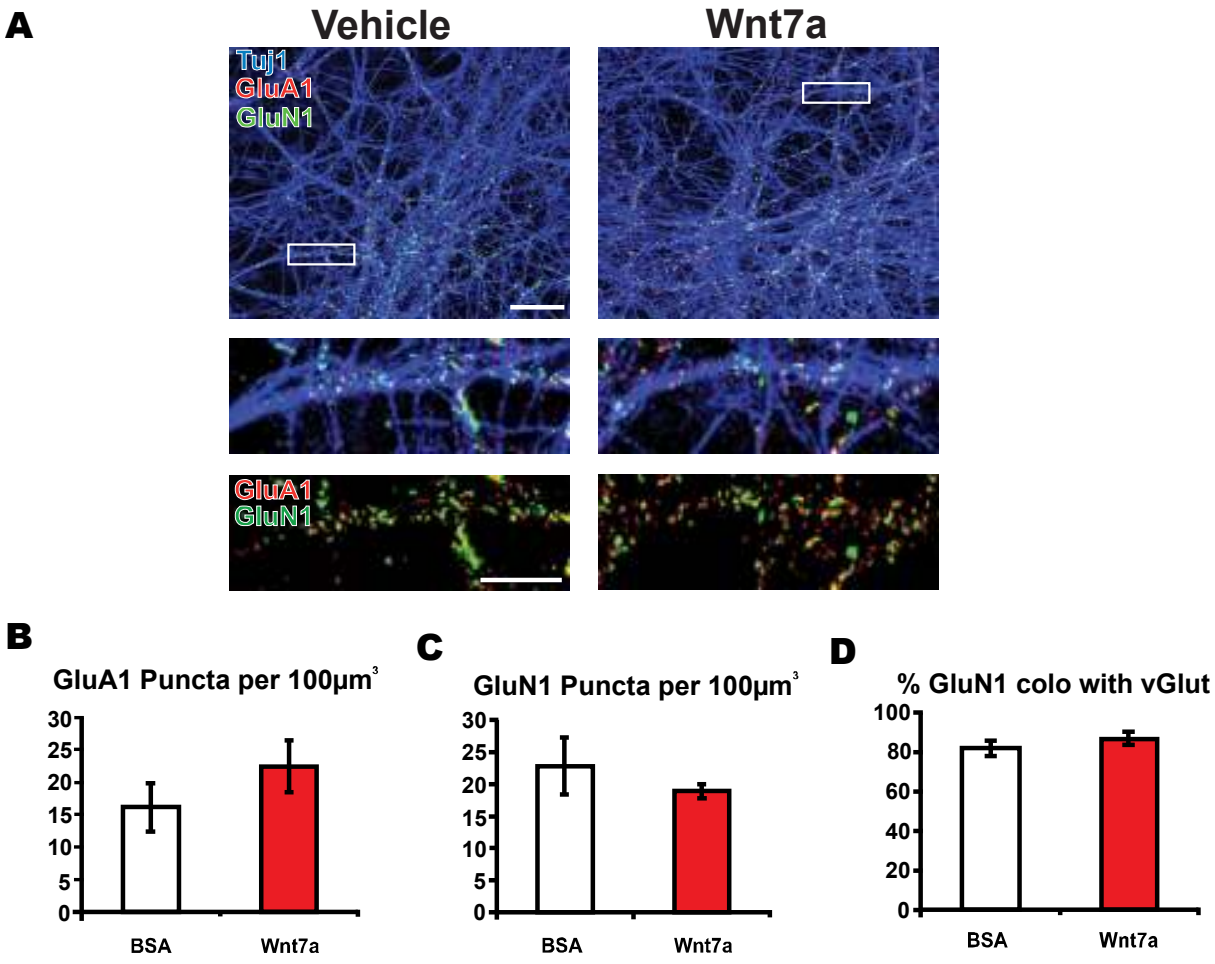

Figure 3.5 Wnt7a does not increases GluA1 or GluN1 puncta density or colocalisation at mature synapses. 21 DIV hippocampal cultures were treated with recombinant Wnt7a or BSA (vehicle) for 3 hours. (A) Images of treated neuronal cultures stained for Tuj1 (green), GluA1 (Red) and GluN1 (Blue) the puncta number and colocalisation were not increased by Wnt7a. White dashed boxes indicate the area shown in the enlarged panels. Scale bars: 30 µm top panel and 10 µm in enlarged panels. (B) Shows the quantification of vGlut puncta between BSA and Wnt7a where no significant increase is observed. (C) Quantification of GluN1 puncta between BSA and Wnt7a where no significant change was observed. (D) Quantification showing no change in colocalisation between vGlut and GluA1 puncta. * = $P<0.05$.

As observed for the presynaptic protein vGlut, neither GluA1 puncta per 100um^3 of dendrite; 39.71 ± 4.98 (vehicle), 35.21 ± 3.39 (Wnt7a) or GluA2 puncta; 16.13 ± 4.01 (vehicle), 22.41 ± 4.72 (Wnt7a) increased significantly in 21 DIV cultures after Wnt7a exposure for 3 hours

(Fig3.5a, b) (Fig3.6a, b). There were also no changes in the colocalisation of these two AMPA receptor subunits on dendrites; 61 ± 3.24% (vehicle), 68 ± 3.98% (Wnt7a) (fig3.6a,b). Finally, quantification of staining for the NMDAR GluN1 subunit showed there was no change in the number of GluN1 puncta along the dendrites or in their colocalisation with the GluA1 subunit (fig3.5a, c, d). These data suggest that Wnt7a does not affect the number of glutamatergic synapses in mature (21DIV) cultures.

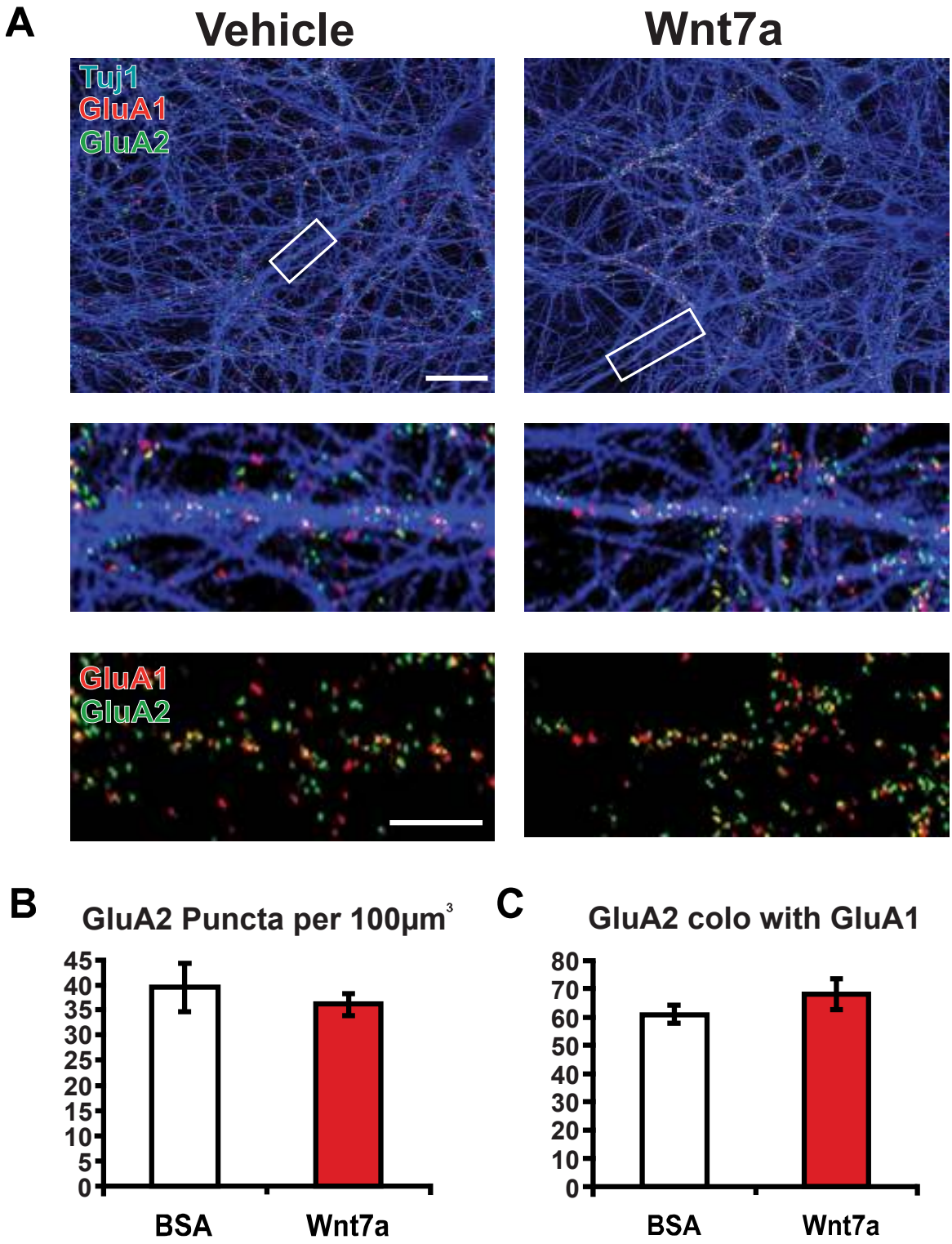

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Figure 3.6 Wnt7a does not increase GluA1 and GluA2 puncta density and colocalisation at mature synapses. 21 DIV hippocampal cultures were treated with recombinant Wnt7a or BSA (vehicle) for 3 hours. (A) Images of treated neuronal cultures stained for Tuj1 (green), GluA1 (Red) and GluA2 (Blue) show that puncta number and colocalisation are not changed by Wnt7a. White dashed boxes indicate the area of the image shown in the enlarged panels. Scale bars are 30 μm in the top panel and 10 μm in the enlarged panels. (B) Shows quantification of GluA2 puncta following BSA or Wnt7a treatment where no significant change was observed. (C) Quantification shows no change in colocalisation between GluA2 and GluA1 puncta. * = $P<0.05$.

3.2.3 Wnt7a signalling affects the formation of functional synapses in both mixed hippocampal cultures and at the MF-CA3 synapse.

The accumulation of both pre and post synaptic machinery observed using immunohistochemistry suggests only the potential of a synaptic site. The above data suggests that Wnt7a increases the colocalisation of synaptic machinery needed for synaptic transmission to occur. However the question of whether these putative synaptic sites are functional remained unanswered. In order to investigate this question I recorded mPSCs from 14 DIV neurons exposed either to Wnt7a or vehicle (BSA). This experiment allows effects on excitatory, glutamatergic synapses (AMPAR mediated) to be distinguished from effects of Wnt7a on inhibitory (GABAR mediated) synapses. Exposure of 14DIV neurons to Wnt7a for 3 hours caused a two-fold increase in the frequency of excitatory mEPSCs (fig3.7a, c). On average Wnt7a also caused a 50% increase in the amplitude of these mEPSCs (fig3.7a, d). This is in marked contrast to the lack of any effect on the frequency and amplitude of mIPSCs from inhibitory synapses. mIPSC frequency and amplitude did not change after exposure to Wnt7a (fig3.7b, c, d). An increase in mEPSC frequency may reflect both an increase in functional synapse number and/or maturation of the presynaptic release machinery whilst changes in amplitude are generally equated with changes in postsynaptic receptor efficiency as a result of changes in receptor localization or functional properties.

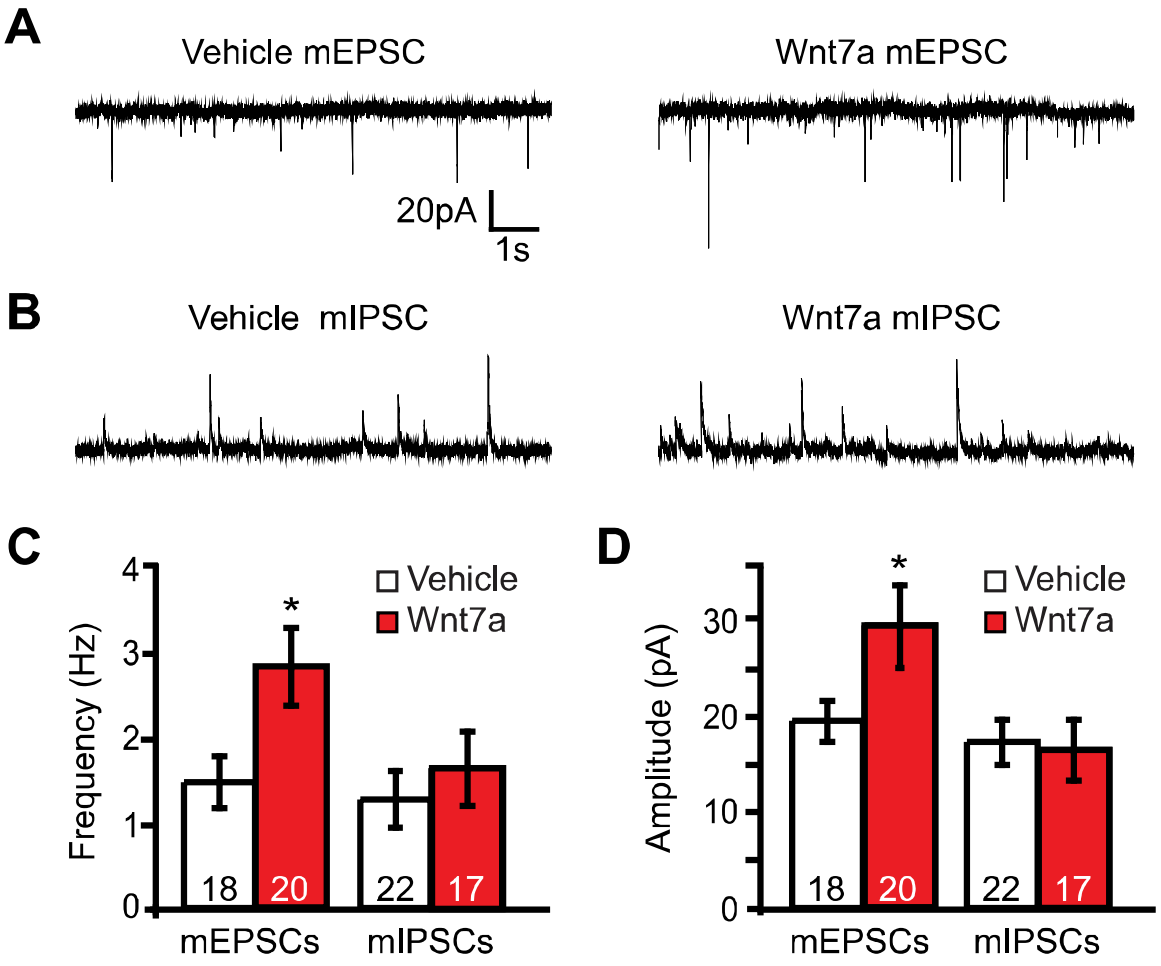

Figure 3.7 Wnt7a enhances excitatory AMPA receptor mediated mEPSCs with no effect on inhibitory mIPSCs. 14 DIV hippocampal cultures were treated with recombinant Wnt7a or BSA (vehicle) for 3 hours and miniature postsynaptic currents were recorded using the whole-cell patch-clamp technique. AMPA minis were isolated by perfusion of bath solution containing TTX, bicuculine, AP5 and Mg^{2+} whilst inhibitory currents were isolated using TTX, Mg^{2+}, AP5 and CNQX. (A) 10 second traces of mEPSCs from BSA or Wnt7a treated neurons illustrating enhancement of frequency and amplitude. (B) 10 second traces of mIPSCs from BSA or Wnt7a treated neurons illustrating no change to frequency or amplitude. (C) Quantification of the frequencies of both excitatory and inhibitory currents. (D) Quantification of the amplitude (-pA) of both excitatory and inhibitory currents (inset numbers give the number of cells recorded from per condition).

I then decided to investigate the effects of Wnt signalling in the intact hippocampus. Here I focused our investigation on the MF-CA3 synapse which has previously been shown to be highly enriched in Wnt7a expression (Gogolla et al., 2009). Using acute slices from p14 Wnt7a-/-, Dvl-/- knockout mice I assessed the effects of Wnt7a, Dvl1 loss of function on synaptic transmission. Here I saw a 27% decrease in the frequency of mEPSCs (fig3.8a, c).

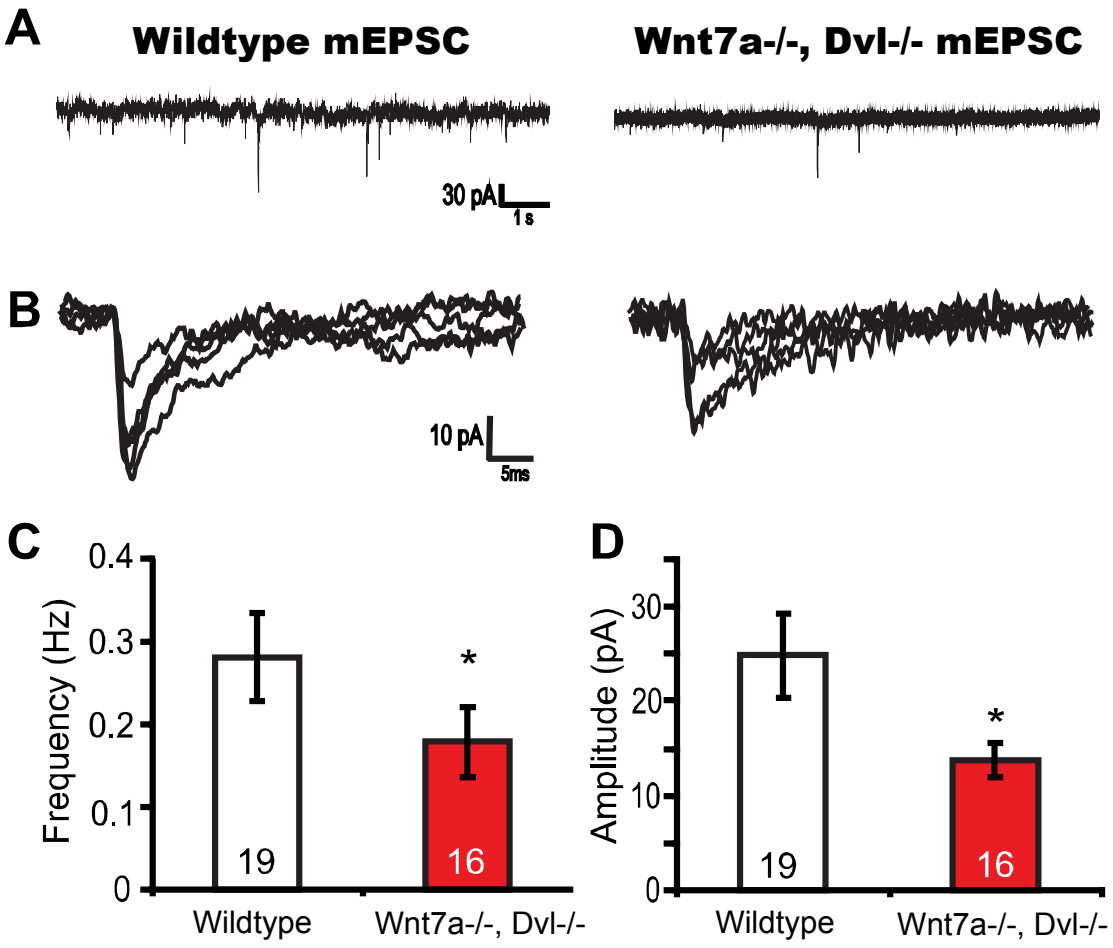

Figure 3.8 Loss of Wnt7a, Dvl1 signalling causes defects in AMPA receptor mediated mEPSC currents at MF-CA3 synapse. Acute hippocampal slices were prepared from p14 wild-type or double knockout (Wnt7a-/-, Dvl1-/-) mice. AMPA receptor mediated mEPSCs were isolated by perfusion of bath solution containing TTX, bicuculine, AP5 and Mg^{2+} whilst inhibitory currents were isolated using TTX, Mg^{2+}, AP5 and CNQX. (A) 10 second representative traces of mEPSCs recorded from wild-type or Wnt7a-/-, Dvl1-/- neurons. (B) Overlays of 5 consecutive minis recorded from wild-type or double knock-out animals illustrating the defect in mEPSC amplitude. (C) Quantification of the frequencies of mEPSCs from wild-type and double knockout neurons. (D) Quantification of the amplitude of

mEPSCs from wild-type and double knockout neurons (inset numbers give the number of cells recorded in each condition).

The effect of the likely loss of Wnt signalling in the double knock-out mice was consistent with the effect of application of Wnt7a we had seen cultures in that there was a 42% decrease in the mEPSC amplitude (fig3.8a, b, d). These data help to confirm the influence of Wnts and more specifically Wnt7a in the regulation of glutamatergic synapses in the hippocampus.

3.3 Discussion

3.3.1 Wnt7a regulates the formation of synapses both pre- and post-synaptically

The above data shows Wnt7a may play a role in both the pre and post-synaptic specialisation in hippocampal cultures. Pre-synaptically, this is displayed in an increase in the recruitment of the presynaptic specific marker vGlut1. Post-synaptically this effect is represented by an increase in the surface expression of GluA1 and GluA2 protein and total expression of GluN1 protein along dendrites. Corroborating with the above data, work by (Cerpa et al., 2008) has shown that Wnt7a regulates changes to the puncta of another presynaptic protein, synaptophysin in hippocampal neurons. In the above study the presynaptic organising ability of Wnt7a was compared to that of Wnt5a and the effects of Wnt7a as a postsynaptic organiser were also assessed. Here the investigators chose to study the post-synaptic scaffolding protein PSD-95 and did not see any changes in the recruitment of this protein in dendrites. These results may at first seem counter to the data presented in this chapter since generally post-synaptic modifications involving PSD-95 and glutamate receptors often observed to go hand in hand (El-Husseini et al., 2000). However the explanation for this discrepancy in results may be in the length of exposure to Wnt in these experiments. In the experiments conducted by Cerpa et al. hippocampal cultured neurons were exposed to Wnt7a for a total of 1 hour before the neurons were fixed and assessed for either pre or post synaptic effects. This is in contrast to the 3 hour exposure used in the experiments of this chapter.

Cerpa and colleagues (2008) found enhancements in synapsin recruitment in this time frame but no changes to PSD-95 whilst after 3 hours of Wnt7a exposure I detected changes both pre and post-synaptically in the staining for vGlut1 and GluA1, GluA2 and GluN1. The increased exposure to Wnt7a may have caused these effects by either allowing an increase in intracellular signalling or by allowing the extra time needed for post-synaptic effects to

develop. A second possibility may be that development of the post-synaptic specialisation is a slower process than pre-synaptic specialisation. Via time-lapse microscopy Friedman and colleagues (2000) have demonstrated these discrepancies by imaging functional sites of pre-synaptic release co-localised with various pre and post synaptic markers. Sites positively labelled with FM1-43 dye co-localised to a significant degree with sites positively labelled with bassoon, a pre-synaptic marker, within 45mins of the sites formation. This was in contrast to sites co-labelled for FM1-43 and SAP-90/PSD-96 (75-120mins) or FM1-43 and GluA1 or GluN1 (>45mins) (Friedman et al., 2000).

Beyond simply increasing the number of pre-synaptic vGlut1 and post-synaptic GluA1, GluA2 and GluN1 in neurites Wnt7a increases the localisation of these proteins to potential synaptic sites. This idea is borne out by the increase in the co-localisation of vGlut1 protein with GluA1, GluA1 protein with GluA2 protein and GluA1 protein with GluN1 protein.

The increases in pre-synaptic vGlut and post-synaptic surface GluA1 colocalisation demonstrate what is likely to be a Wnt7a induced increase in functional synaptic sites. The presence of GluA1 at these synaptic sites suggesting specifically that Wnt7a leads to an increase in the number of synapses which are not post-synaptically silenced. Such changes in the presence and localisation of GluA1 containing synapses following Wnt7a application have not previously been presented in published studies.

Within 3 hours, Wnt7a increased the number of surface GluA1 and GluA2 puncta which were colocalised. These data further strengthen the suggestion that Wnt7a causes an increase in the number of functional synapses in these cultures. The receptor subunit specific content of excitatory synapses during development is constantly modified as the neuron matures. AMPA receptor subunits are described as changing from predominantly GluA1/4 containing to GluA1/2, GluA2/3 (Hall and Ghosh, 2008). The increased presence not only of more surface GluA2 protein on neuronal dendrites but at the same sites (co-localisation) as GluA1 puncta suggests a large proportion of these new synaptic sites are also more mature.

Regulation of the surface expression of glutamate receptors by an exogenous cellular factor has previously been observed in the presence of TNF (Wallach et al., 1999). TNF uses the cell surface receptors TNF-R1 or TNF-R2 to transduce intracellular signals and these receptors are widely expressed in the hippocampus (Wallach et al., 1999). TNF-α caused an increase in surface expression of GluA1 subunits on hippocampal neurons in tissue culture but no change in surface GluA2 subunits (Stellwagen et al., 2005). Interestingly TNF-α

effects were seen within 15mins of treatment and the authors suggest, the receptors lacking GluA2 subunits would eventually be replaced by GluA2 containing receptors (Stellwagen et al., 2005).

In parallel with changes in surface GluA1 and GluA2 proteins, Wnt7a caused an increase in the localisation of GluN1 puncta in and on dendrites. This data in partnership with the increase in colocalisation of GluN1 and GluA1 puncta suggest a role for Wnt7a in regulating the maturation of potential synaptic sites. The presence of increased synaptic sites containing both GluA1 and GluN1 conveys the likelihood that these sites are functional sites as opposed to postsynaptically silent synapses (Kerchner and Nicoll, 2008).

During development, post-synaptically silent synapses are regulated in number within the hippocampus (Kerchner and Nicoll, 2008). The population of synapses lacking AMPA receptors steadily decreases in number from early postnatal to mature hippocampus. Alterations in the proportion of GluA1 (AMPAR) relative to NMDAR content of synapses have also been proposed to occur during LTP (Pickard et al., 2001). This form of potentiation at a population of synapses may be generated by the conversion of silent synapses to functional ones resulting in an increase in the post-synaptic response of a given neuron. These kinds of changes are similar to the effects observed in hippocampal cultures exposed to Wnt7a at 14DIV. These data suggest that Wnt7a promotes the maturation of synaptic sites by regulating the localisation of surface GluA1, GluA2 and total GluN1 protein located post-synaptically to potential pre-synaptic release sites.

3.3.2 Exposure of mature neurons to exogenous Wnt7a does not affect the localisation of pre- or post-synaptic proteins.

During development, neurons follow a defined temporal program as they mature and form synaptic connections (Garner et al., 2006). Neurons that are post cell specification, migrate to their correct location, undergo axonal guidance leading to synapse formation and later in the mature system these synapses are refined (Cowan et al., 2001). Wnt proteins are expressed in the hippocampus from embryonic stages and continuing into maturity (McCarthy, 2006). Wnt signalling has also been implicated in the regulation of many of the processes leading to a mature neural network (Salinas and Zou, 2008). Therefore it was important to ask whether the effects of Wnt7a on neurons at 14 DIV were present at more mature networks.

Interestingly the stages of network formation which have been observed *in vivo* are well conserved in hippocampal cultures (Garner et al., 2006; Grabrucker et al., 2009). Studying neuronal cultures at 21 DIV allowed analysis of the effects of Wnt7a on neurons largely with mature synapses. Interestingly, in these mature neurons Wnt7a exposure did not seem to have a significant effect on the localisation of either pre or postsynaptic proteins.

The lack of effect was consistent regardless of whether I looked at the surface expression of AMPA receptor subunits (GluA1, GluA2) or the total NMDA receptor subunit (GluN1). This lack of postsynaptic effect is interesting in that it is consistent with the results seen in the work of Cerpa and colleagues (2008). Indeed the neuronal cultures used in their experiments were taken from a range of time points *in vitro* (14-21DIV) (Cerpa et al., 2008).

One possible explanation for the lack of Wnt7a mediated effects in mature cultures may be that the Wnt7a signalling pathway is already saturated at this stage in culture (e.g. the concentration of Wnt7a in the culture has reached saturating level for the Frizzled receptors expressed by the neurons) or that some aspect of the signal transduction process has become rate-limiting. At 14 DIV Wnt7a increases the co-localisation of vGlut1 clusters with GluA1 puncta from a control value of 46% to 57%. At 21 DIV the control value is already at a higher value (65%) than the Wnt7a treated cultures at 14DIV. As a result the Wnt7a treated co-localisation value remains at a similar value (62%).

In terms of the formation of glutamatergic synapses these data fall in line with published data which suggests synapse formation is most dynamic in the 12-16 day period (*in vitro* or *in vivo*) (Friedman et al., 2000). At mature synapses the mechanisms that alter synaptic machinery may differ from that of nascent and immature synapses, perhaps requiring the presence of several Wnts and coordinated changes in synaptic activity. Indeed the complex interplay between neurotrophic factors and synaptic activity in altering synaptic strength at mature synapses has been well established (Hall and Ghosh, 2008; Li et al., 2007; Li and Keifer, 2009). Similar collaborative regulation of synapse maturation has been seen with neuronal activity and endogenous Wnts (Sahores et al., 2010).

Sahores and colleagues (2010) were able to demonstrate the requirement for exogenous Wnt species in activity dependent synapse formation. Using a soluble version of the Fz-5 CRD increases in synapse number (synapsin co-localisation with GluN1), generated by electrical stimulation paradigms were abolished (Sahores et al., 2010). Crucially Wnt7a is a direct

binding partner of Fz-5 suggesting in part these effects could be mediated via Wnt7a to Fz-5 signalling (Sahores et al., 2010).

3.3.3 Exposure of neuronal cultures to Wnt7a increases the amplitude and frequency of excitatory miniature synaptic currents without affecting inhibitory currents.

The above data illustrates the ability of Wnt7a to affect the formation of glutamatergic synapses by regulating the localisation of AMPA and NMDA receptors to nascent synaptic sites. Ultimately however these changes in localisation might have no effect on cell to cell synaptic signalling that occurs within these cultures. In order to further investigate the synaptogenic effects of Wnt7a on synaptic transmission, I measured mPSCs from neurons acutely exposed to Wnt7a at 14 DIV. mPSCs are post-synaptic currents generated by spontaneous action potential-independent release of neurotransmitter vesicles. The resulting post synaptic currents are therefore believed to be quantal events and provide a transmission based assessment of the state of synapse number and content (Bekkers and Stevens, 1995; Fatt and Katz, 1952).

Wnt7a exposure increased both the frequency and amplitude of excitatory post synaptic miniature currents without affecting either parameter in inhibitory post synaptic currents. Changes to frequency are believed to correlate with pre-synaptic changes whilst changes in amplitude are correlated with post-synaptic alterations (Fatt and Katz, 1952). This effect in mEPSCs strongly reinforces the immunofluorescence data showing increases in post synaptic AMPA receptors with presynaptic sites. The lack of changes to inhibitory currents also compliments immunofluorescence data showing no changes to inhibitory pre and post-synaptic proteins vGAT and Gephyrin, respectively (Ciani et al., 2011).

Inhibitory synaptic transmission in the hippocampus is mediated primarily by $GABA_A$ receptors. Cuitino and colleagues whilst investigating the effects of Wnt5a on $GABA_A$ receptors looked at their localisation in relation to Wnt7a exposure. Wnt7a in time-course experiments was unable to affect the surface localisation of receptor. This was in contrast to experiments using Wnt5a which enhanced the levels of surface $GABA_A$ receptor in the same time period (Cuitino et al., 2010).

Interestingly the above data from Cuitino et al. (2010) is just one of a growing number of studies that indicate different Wnt proteins regulate synapses with varying characteristics. Wnt3a has been shown to enhance the frequency of mEPSCs with no effect on current

amplitude which may suggest a pre-synaptic role for this Wnt in the hippocampus (Avila et al., 2010) and/or a role in establishment of the post-synaptic apparatus that does not include up-regulation of AMPAR number in the post-synaptic membrane. Wnt5a however appears to act in contrasting fashion to Wnt3a. Wnt5a gain of function in 10 DIV hippocampal cultures results in an enhancement in mEPSC amplitude with no effect on frequency (Varela-Nallar et al., 2010). Interestingly this study attempted to investigate NMDAR mediated mEPSCs and similarly detected an enhancement in current amplitude, although the NMDAR mediated mEPSCs described by Varela-Nallar et al (2010) do not have the expected time course of hippocampal NMDA currents (Lester et al., 1989) and it is not clear from this paper why the measured frequency of AMPAR mediated mEPSCs would be around 5-fold higher than that of the NMDAR mediated mEPSCs (Varela-Nallar et al., 2010 Figure 5 and Supplementary Figure S3), or why the amplitude of the AMPAR mediated currents in this study is much greater than observed in many previous studies of mEPSCs in hippocampal neurones in tissue culture.

Chapter 4

Wnt signalling regulates the localisation of glutamatergic receptors on dendritic spines by acting directly at the postsynaptic terminal.

4.1 Introduction

At central synapses, particularly in the hippocampus, cerebellum and cortex the majority of excitatory synapses are found on dendritic spines (Nimchinsky et al., 2002). Dendritic spines are present at central synapses in a variety of shapes and sizes. Changes in spine size and shape are often correlated with changes in synaptic strength. Glutamatergic AMPAR and NMDAR receptors are believed to mediate these changes by modifications in their localisation (Makino and Malinow, 2009; Petralia et al., 2009). However the extracellular signals which regulate localisation of glutamate receptors at spines are poorly understood.

The previous chapter's results indicate that Wnt7a plays a role in the regulation of excitatory synapse formation without effecting inhibitory synaptic transmission. Furthermore recent data from our lab indicates a role for Wnt7a acting directly at the postsynaptic specialisation.

We have recently demonstrated that Wnt7a plays an important role in the regulation of Spine morphogenesis in the hippocampus. Neurons cultured and exposed to Wnt7a showed an increase in both the size and density of dendritic spines (Ciani et al., 2011). This effect was partially mirrored in neurons which overexpressed Dvl1 specifically in the post-synaptic neuron. In these neurons spine size was significantly increased with no effect on the overall number of spines. Spine morphogenesis by Wnt7a-Dvl1 signalling in vivo was also assessed using knockout mice. These models displayed defects in size and number in both the CA1 and CA3 regions of the hippocampus (Ciani et al., 2011).

Spines are specialised structures on the dendrite which compartmentalise and concentrate machinery essential for efficient excitatory transmission. It is no surprise then that at the structural level changes in the morphology of dendritic spines have been correlated with changes in the content of synaptic proteins in these structures (Nusser et al., 1998). Inversely structural synaptic proteins such as actin filaments and PSD-95 (involved in the stabilisation of Glutamatergic synapses) are involved in the regulation of spine morphogenesis (El-Husseini et al., 2000; Fischer et al., 1998). The presence and activation of NMDARs play essential roles in the development of the excitatory neural landscape. Development of precise synaptic inputs is directly regulated by the presence of NMDA receptors in the

somatosensory cortex of rodents (Iwasato et al., 2000). Using cortical GluN1 KO mice Iwasato and colleagues observed the development of patterned topographic connections between the sensory periphery (whiskers) and the brain. Gross analysis of patterning in GluN1 KO somatosensory cortex revealed relatively normal whisker related patterning (Iwasato et al., 2000). Further analysis of the GluN1 KO mice revealed defects in barrels and barrel boundaries indicating a possible role in the consolidation of specific connections at these synapses for NMDARs. Furthermore NMDAR activation is important in the plasticity of spine morphology (Matsuzaki et al., 2004). Using two photon glutamate uncaging and the NMDAR antagonist AP5 matsuzaki and colleagues were able to observe the role of NMDAR in spine morphology changes. In the absence of AP5, dendritic spines are enlarged in response to uncaged glutamate. In the presence of AP5, spine morphology remained unchanged in response to glutamate (Matsuzaki et al., 2004).

With the above in mind, various interesting questions presented themselves. Knowing my studies indicate that Wnt7a affects the localisation of both AMPA and NMDA receptors in hippocampal neurons, is the trafficking of receptors spine specific? Furthermore how does the localisation of these glutamate receptors relate to changes in the size of spine morphology?

The cell exogenous nature of Wnt proteins provide an interesting subject of study at synaptic sites. The ability of these proteins to be expressed from either/or both pre and postsynaptic neuron whilst also taking action at either side of the synapse presents several questions about the specific role of Wnt proteins. As has been mentioned above and in previous chapters the various Wnt species play different roles in synapse formation and maintenance. These differing effects may be due to the presence of certain specific Wnt receptors exclusively pre or postsynaptically or the lack of specific intra-cellular signalling molecules required in the Wnt signalling pathway.

In the cerebellum Wnt7a seems to have an exclusive role in regulation of the presynaptic side of the synapse. Wnt7a produced by the post-synaptic cell acting in a retrograde manner enhances miniature synaptic current frequency but not amplitude (Ahmad-Annuar et al., 2006). There are however precedents for the action of Wnt signalling directly on post-synaptic compartments from work at peripheral nervous system synapses. In drosophila Wnt signalling via a post-synaptic DFz-2c nuclear import pathway regulates the correct formation of the NMJ (Mosca and Schwarz, 2010; Packard et al., 2002). Postsynaptic mutations in the

dfz2 resulted in the formation of ghost boutons and defects in the localisation of GluRs. Wnt11r directly signals to postsynaptic muscle at vertebrate NMJ (Jing et al., 2009). In the zebrafish Jing and colleagues demonstrated blockade of Dvl signalling in muscle prevented the formation of pre-patterned AChR clustering. Knockdown of endogenous Wnt11r via morpholinos also caused defects in AChR pre-patterning without affecting muscle development (Jing et al., 2009). Wnt3 in association with nerve derived agrin signalling regulates the mature clustering of AChR in the chick (Henriquez et al., 2008). Myotubes directly treated with Wnt3 show an increase in the prepatterning of AChR micro-clusters. Furthermore Wnt3 signalling in myotubes activated Rac1 activity whilst DN-Rac1 was shown to inhibit the clustering effect. Wnt postsynaptically plays a role in the regulation of hippocampal neuronal dendritogenesis (Rosso et al., 2005). Dvl1 gain and loss of function in neurons resulted in respective enhancement and defects in dendritic complexity.

The data in the previous chapter suggests hippocampus neurons exposed to Wnt7a exhibit enhancements on both sides of the synapse in the form of changes to pre and postsynaptic protein localisation. Whether these are direct effects of the Wnt7a signalling on pre and postsynaptic site are as yet unclear. These postsynaptic effects may be secondary effects mediated by the release of a secondary messenger from the presynaptic terminal. Alternatively an enhancement in presynaptic release caused by Wnt7a may act to enhance the trafficking of postsynaptic receptors. To investigate these hypotheses I used neuron specific overexpression of the intracellular Wnt signalling protein Dvl1. By activating Wnt signalling in the post-synaptic neuron I was able to investigate whether this would be able to mimic the effects of Wnt activation in pre and post synaptic neurons on receptor localisation.

4.2 Results

4.2.1 Exogenous Wnt7a increases accumulation of glutamate receptors and presynaptic inputs onto the dendritic spines of developing neurons.

To investigate how Wnt7a affects the accumulation of glutamatergic receptors on dendritic spines, hippocampal neurons were cultured for 8 days before being transfected with an eGFP-actin/pc12-empty vector mixed solution. The neurons were transfected using calcium phosphate and returned to culture either for an additional 6 days or 13 days at which point the cultures were appropriately exposed to Wnt7a, stained and fixed. The sparse transfection of neurons allowed individual cells and their dendritic spines to be identified by eGFP stain. In order to investigate changes in the number of synapses formed on dendritic spines the

cultures were stained with the presynaptic marker vGlut alongside the postsynaptic glutamate receptor markers for GluA1, GluA2 and GluN1.I began by investigating the effects of 3 hr. Wnt7a treatment on 14 DIV neurons.

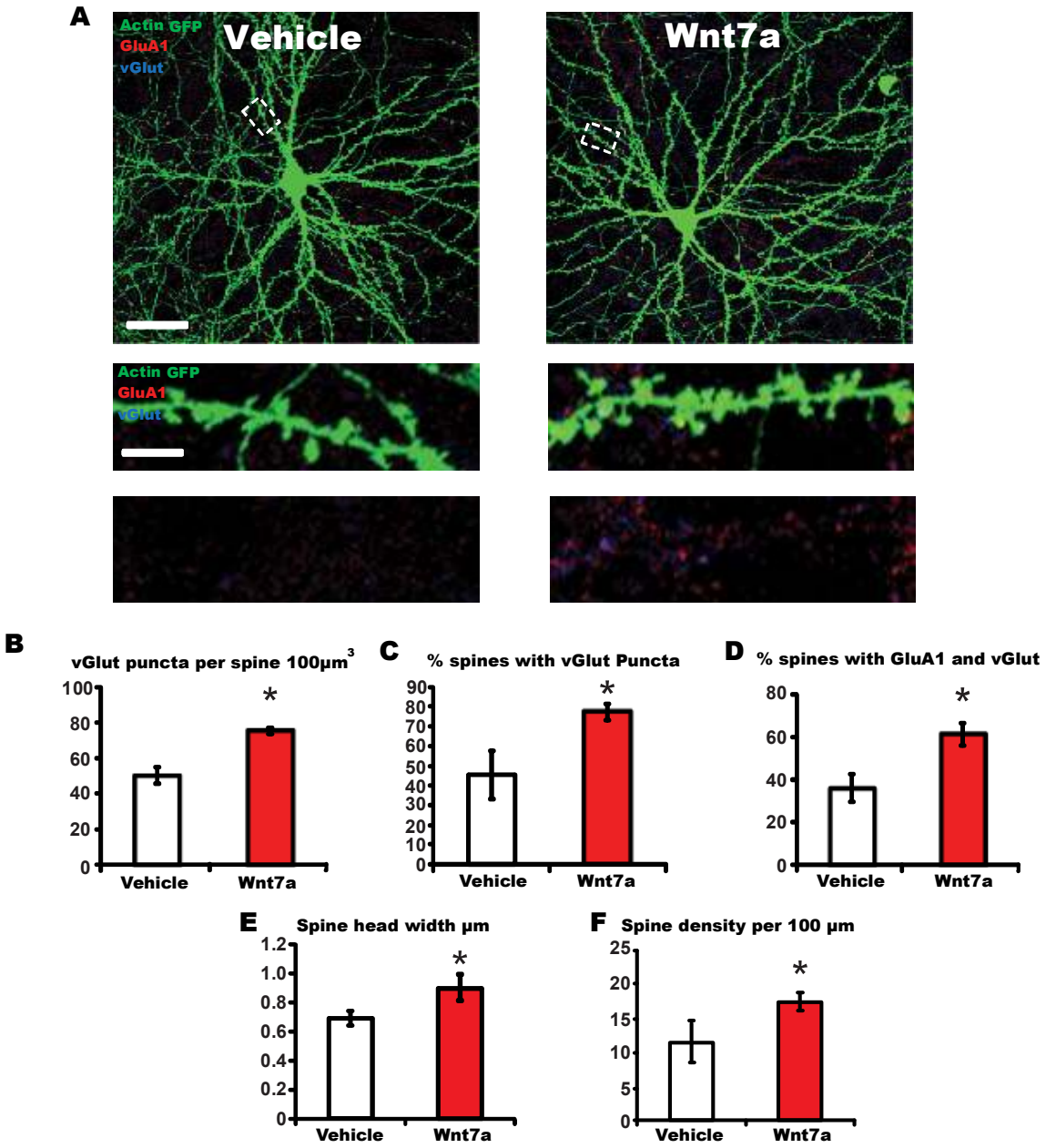

Figure 4.1 Wnt7a increases GluA1 and vGlut accumulation and localisation on dendritic spines of 14DIV neurons. Hippocampal neurons cultured for 14 days were treated with BSA (vehicle) or Wnt7a for 3hrs. Dendritic spines were analysed for GluA1 (red) and vGlut (blue) puncta. (A) Top panel displays images of treated neurons stained with Actin-gfp (green), GluA1 and vGlut (Scale bar =30um). Middle panels display high magnification

images of dashed box in low magnification images (Scale bar =5um). Lower panel displays GluA1 and vGlut puncta minus Spines and Dendrites. Wnt7a increases both the number and size of spines whilst increasing the number of GluA1 and vGlut associated. (B) Quantification of the number of vGlut puncta associated with spines per $100um^3$ of spine volume. (C) Quantification of the % of spines with vGlut puncta associated. (D) Quantification of % of total spines associated with both GluA1 and vGlut puncta. (E) Quantification of the width of spines. (F) Quantification of number of spines per 100μm length of dendrite * = $P<0.05$.

Wnt7a increases the number of vGlut puncta in contact with dendritic spines by 46% (fig. 3.1 A and B). 3 hr. Wnt7a exposure also increases the number of spines contacted with at least one vGlut puncta by 70% and similarly increases the number of spines positive for both vGlut and GluA1 puncta also by 70% (fig. 3.1 A, C and D). These increases demonstrate Wnt7a's ability to increase the organisation of presynaptic structures onto postsynaptic sites.

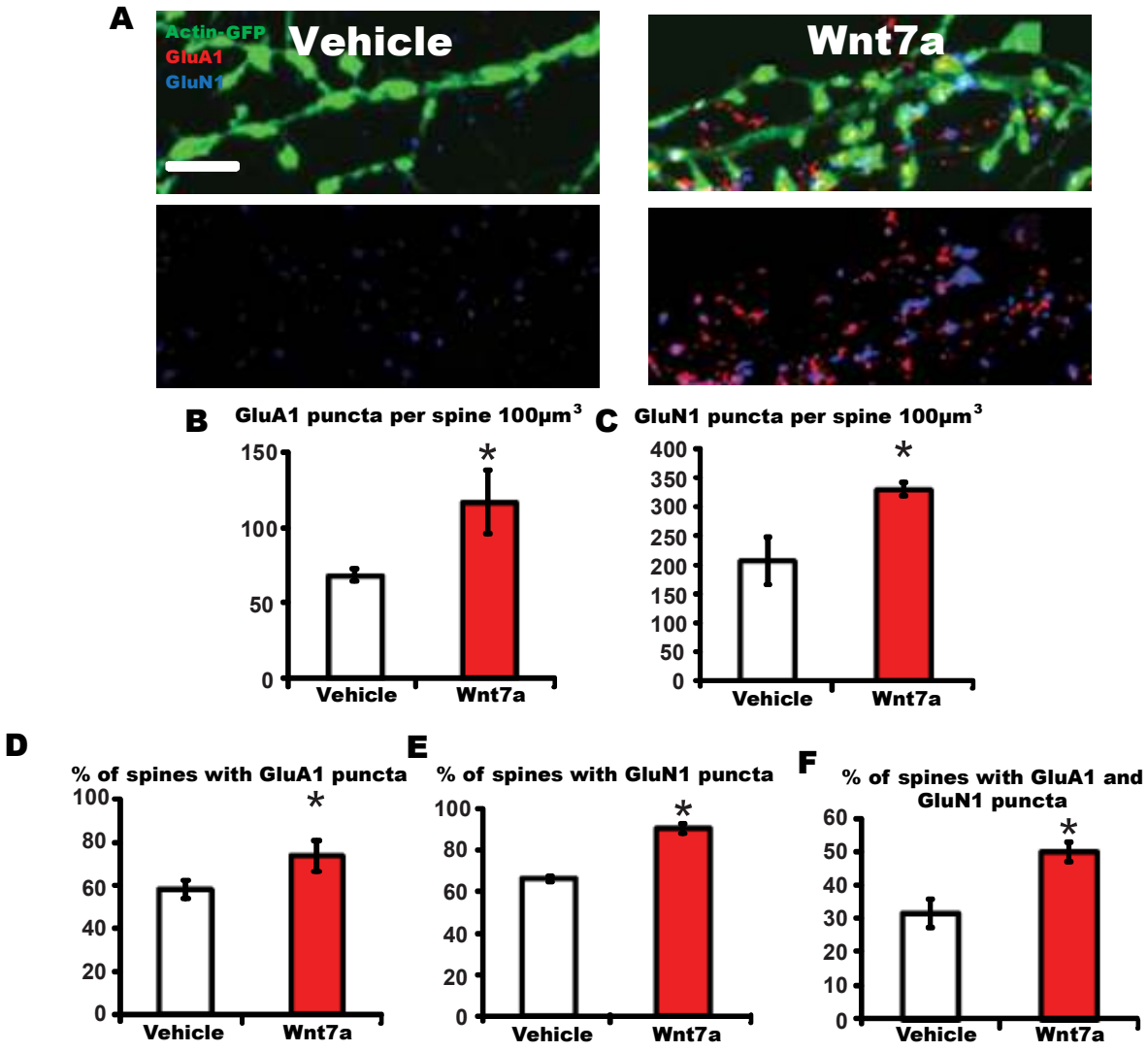

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Figure 4.2 Wnt7a increases GluA1 and GluN1 puncta localisation on spines and increases the number of spines associated with both proteins at 14 DIV. Hippocampal neurons cultured for 14 days were treated with BSA (vehicle) or Wnt7a for 3hrs. Dendritic spines were analysed for GluA1 (red) and GluN1 (blue) puncta. (A) High magnification images of isolated dendrites display the Wnt7a mediated increases in spine associated GluA1 and GluN1 puncta number (Scale bar= 5um). Top panel displays stain for Actin-gfp (green), GluA1 and GluN1 whilst lower panels display puncta images minus Actin-gfp. (B) Quantification of GluA1 puncta number per Spine volume displays the strong increase in GluA1 puncta associated with spines after Wnt7a treatment. (C) Quantification of GluN1 puncta number per spine volume displays the increase in puncta accumulation on spines per spine volume. (D) Quantification of the % of spines with GluA1 puncta associated reveals an increase with Wnt7a treatment. (E) Quantification of the percentage of total spines associated with GluN1 puncta revealed a 36% increase (F) Quantification of spines with both GluA1 and GluN1 puncta associated display a 56% increase. * = $P<0.05$.

GluA1 puncta associated with dendritic spines were also increased in number from 69 ± 5 pp100μm^3 to 115 ± 20pp100μm^3 (fig 4.2 A and B). Wnt7a also increases the number of GluN1 puncta associated with dendritic spines by 60% (fig. 4.2 A and C). The percentage of Spines which were associated with GluA1, GluN1 or both puncta in the same spine all increased by 26%, 36% and 56% respectively in response to Wnt7a exposure (fig. 4.2 A, D-F).

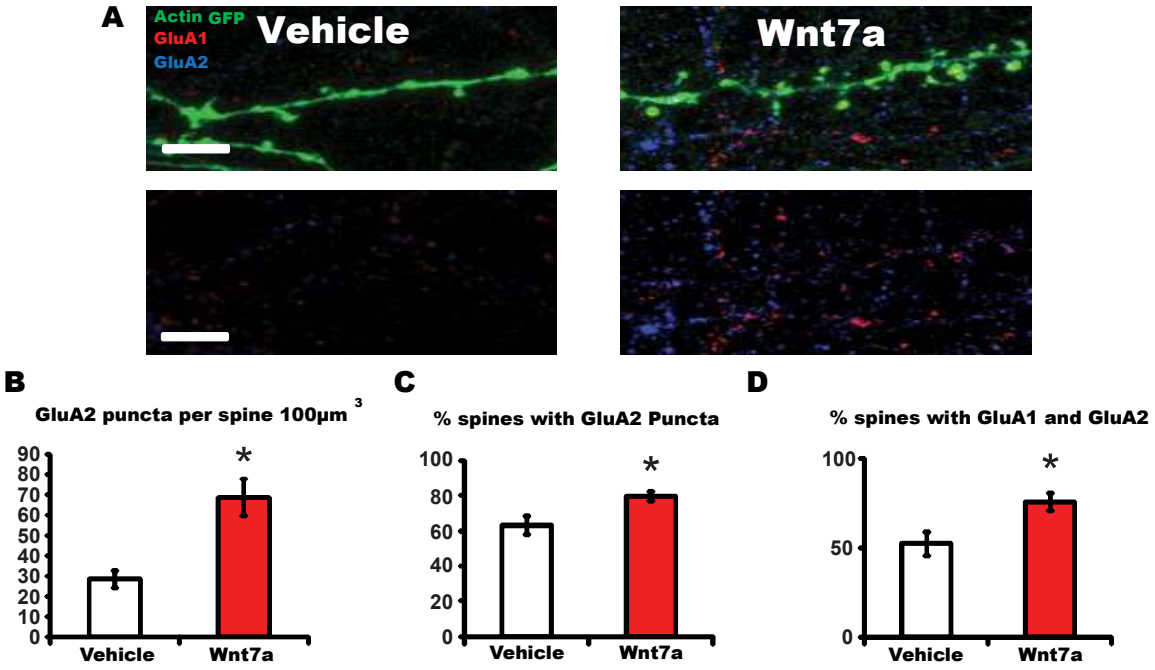

Figure 4.3 Wnt7a increases GluA1 and GluA2 puncta localisation on spines and increases the number of spines associated with both proteins at 14 DIV. Hippocampal neurons cultured for 14 days were treated with BSA (vehicle) or Wnt7a for 3hrs. Dendritic spines were analysed for GluA1 (red) and GluA2 (blue) puncta. (A) High magnification images of isolated dendrites display the Wnt7a mediated increases in spine associated GluA1 and GluN1 puncta number (Scale bar= 5um). Top panel displays stain for Actin-gfp (green), GluA1 and GluA2 whilst lower panels display puncta images minus Actin-gfp. (B) Quantification of GluA2 puncta number per spine volume displays the significant 138% increase in puncta accumulation on spines per spine volume. (C) Quantification of the percentage of total spines associated with GluA2 puncta revealed a 25% increase (D) Quantification of spines with both GluA1 and GluA2 puncta associated display a 45% increase. * = $P<0.05$.

Analysis of GluA2 stained cultures also revealed Wnt7a mediated enhancement in puncta accumulation on spines. GluA2 puncta associated with dendritic spines increased by 138% (fig. 4.3 A and B). The percentage of spines associated with GluA2 puncta or associated with GluA2 and GluA1 increased by 25% and 45% respectively (fig. 4.3 A, C and D). These results show that at 14DIV, Wnt7a not only increases the aggregation of glutamate receptors on dendritic spines but also increases the number of spines with GluA1, GluA2 and/or GluN1 in apposition with a presynaptic contact.

4.2.2 ***Exogenous Wnt7a does not affect accumulation of synaptic proteins onto the dendritic spines of mature neurons.***

The previous results demonstrate an ability of Wnt7a to encourage synaptic protein accumulation at dendritic spine sites on 14DIV neurons. I then decided to investigate whether Wnt7a was able to further regulate glutamate receptor organisation on the spines of more mature neurons. Neurons were maintained in culture for a further seven days until day 21 where they were treated with Wnt7a stained as appropriate and imaged. Contrary to the results seen at 14 DIV, Wnt7a exposure was unable to mediate changes in receptor accumulation.

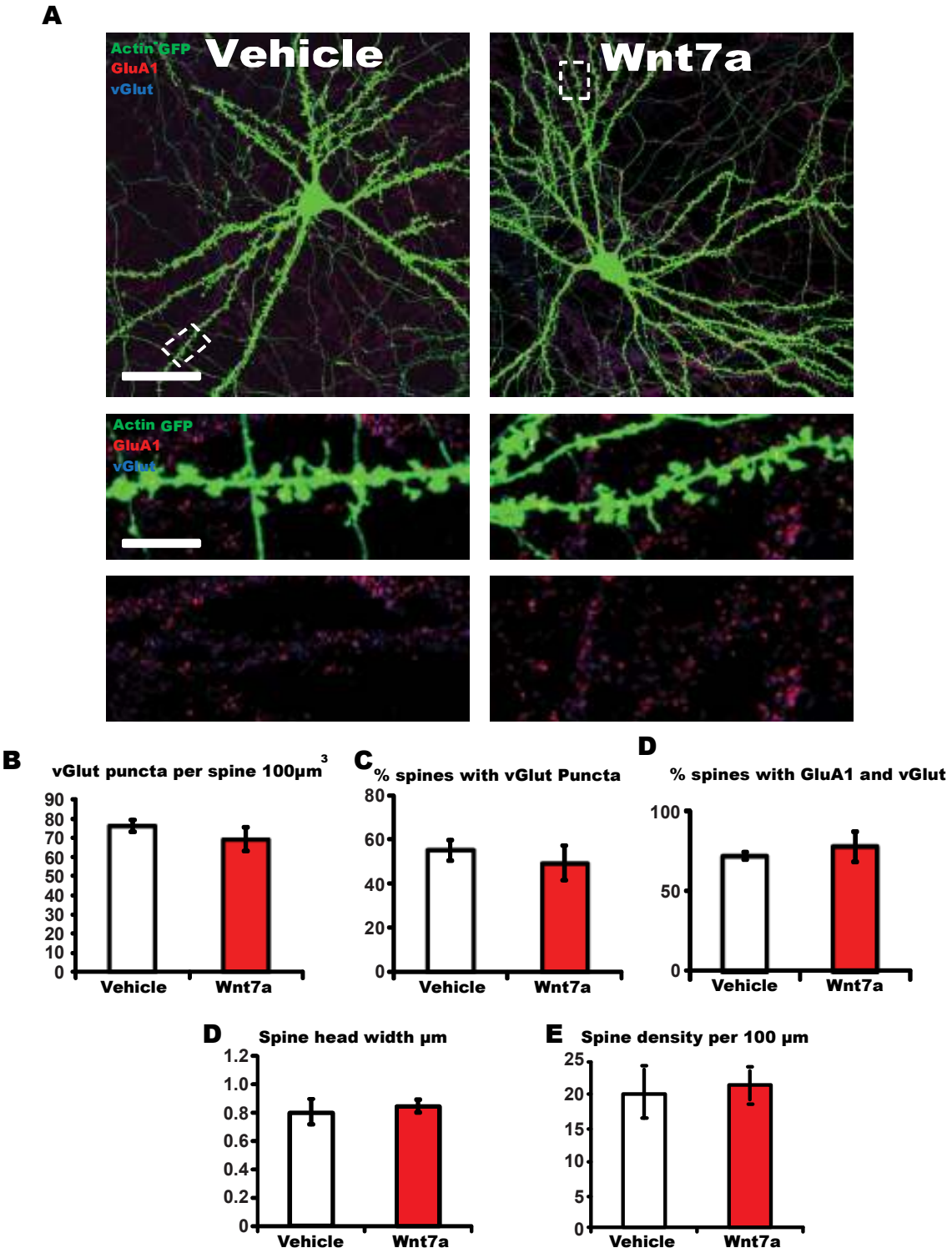

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Figure 4.4 Wnt7a does not affect GluA1 and vGlut accumulation and localisation on dendritic spines of 21DIV neurons. Hippocampal neurons cultured for 21 days were treated with BSA (vehicle) or Wnt7a for 3hrs. Dendritic spines were analysed for GluA1 (red) and vGlut (blue) puncta. (A) Top panel displays images of treated neurons stained with Actin-gfp (green), GluA1 and vGlut (Scale bar =50um). Middle panels display high magnification images of dashed box in low magnification images (Scale bar =5um). Lower panel displays GluA1 and vGlut puncta minus Spines and Dendrites (actin-gfp stain). Wnt7a does not increase the number or size of spines or the number of GluA1 and vGlut associated with these spines. (B) Quantification of the number of vGlut puncta associated with spines per $100um^3$ of spine volume shows no differences between vehicle and Wnt7a treated. (C) Quantification of the % of spines with vGlut puncta associated shows no difference between treatments. (D) Quantification of % of total spines associated with both GluA1 and vGlut puncta shows no significant difference between treatments. (E) Quantification of the width of spines shows no change in the size of spines heads with treatment. (F) Quantification of number of spines per 100μm length of dendrite demonstrates no change in the number of spines along the neurites * = $P<0.05$.

The number of vGlut puncta associated with dendritic spines did not significantly change (BSA: 77 ± 3pp$100\mu m^3$ Wnt7a: 69 ± 7 pp$100\mu m^{3)}$ (fig 4.4 A and B). The failure of Wnt7a to affect vGlut accumulation was mirrored in the percentage of spines associated with vGlut or vGlut and GluA1 (fig. 4.4 A, C and D). The failure of Wnt7a to increase the colocalisation of GluA1 and vGlut on dendritic spines indicates an inability of 3Hr Wnt treatment to increase synapse number at 21DIV.

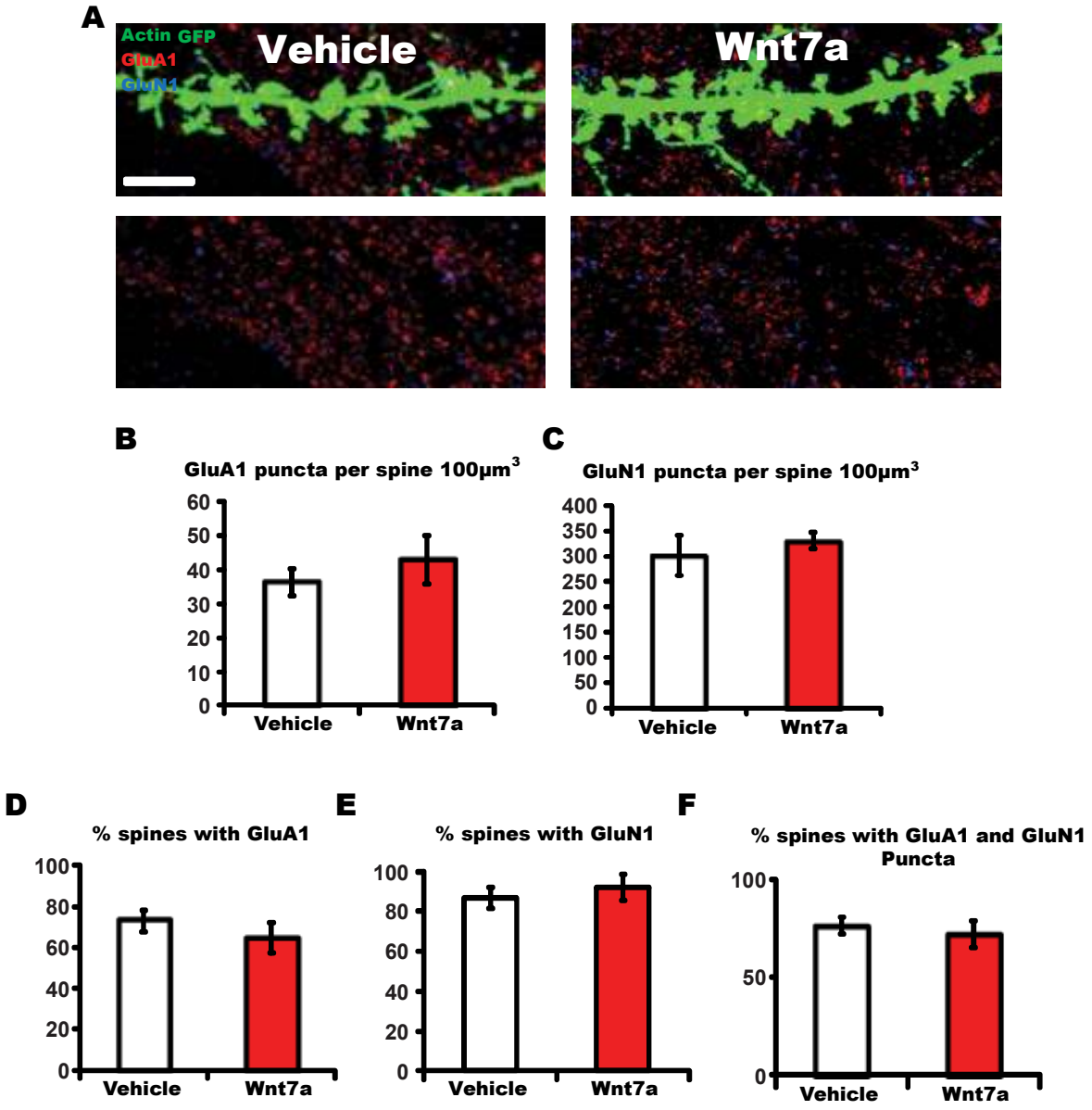

Figure 4.5 Wnt7a has no significant effect on GluA1 and GluN1 puncta localisation on spines and in 21 DIV cultured hippocampal neurons. Hippocampal neurons cultured for 21 days were treated with BSA (vehicle) or Wnt7a for 3hrs. Dendritic spines were analysed for GluA1 (red) and GluN1 (blue) puncta. (A) High magnification images of isolated dendrites display the Wnt7a mediated increases in spine associated GluA1 and GluN1 puncta number (Scale bar= 5um). Top panel displays stain for Actin-gfp (green), GluA1 and GluN1 whilst lower panels display puncta images minus Actin-gfp. (B) Quantification of GluA1 puncta number per Spine volume displays no significant change in GluA1 puncta associated with spines after Wnt7a treatment. (C) Quantification of GluN1 puncta number per spine volume reveals no change in puncta accumulation on spines per spine volume. (D) Quantification of the % of spines with GluA1 puncta associated reveals no significant change

between treatments. (E) Quantification of the percentage of total spines associated with GluN1 puncta revealed no difference (F) Quantification of spines with both GluA1 and GluN1 puncta associated display no difference between vehicle and Wnt7a treatments.

Indeed all 3 glutamate receptor markers; GluA1, GluA2 and GluN1 did not significantly increase in density on the dendritic spines of 21 DIV neurons in response to 3Hr Wnt7a exposure (fig. 4.5 A-C, fig. 4.6 A and B). In confirmation of Wnt7a's inability to mediate receptor accumulation the percentage of spines associated with the various glutamate receptors was also diminished. At 21 DIV 73 ± 5.1% of spines were associated with GluA1 puncta in control conditions becoming 64 ± 8.109% after 3hrs of Wnt7a treatment (fig. 4.5 A and D). These observations were mimicked in the number of spines associated with GluN1 (BSA: 87 ± 5.666%, Wnt7a: 92 ± 7.158%) or GluA1 and GluN1 (BSA: 76 ± 4.571%, Wnt7a: 72 ± 6.719%) (fig. 4.5A, E and F).

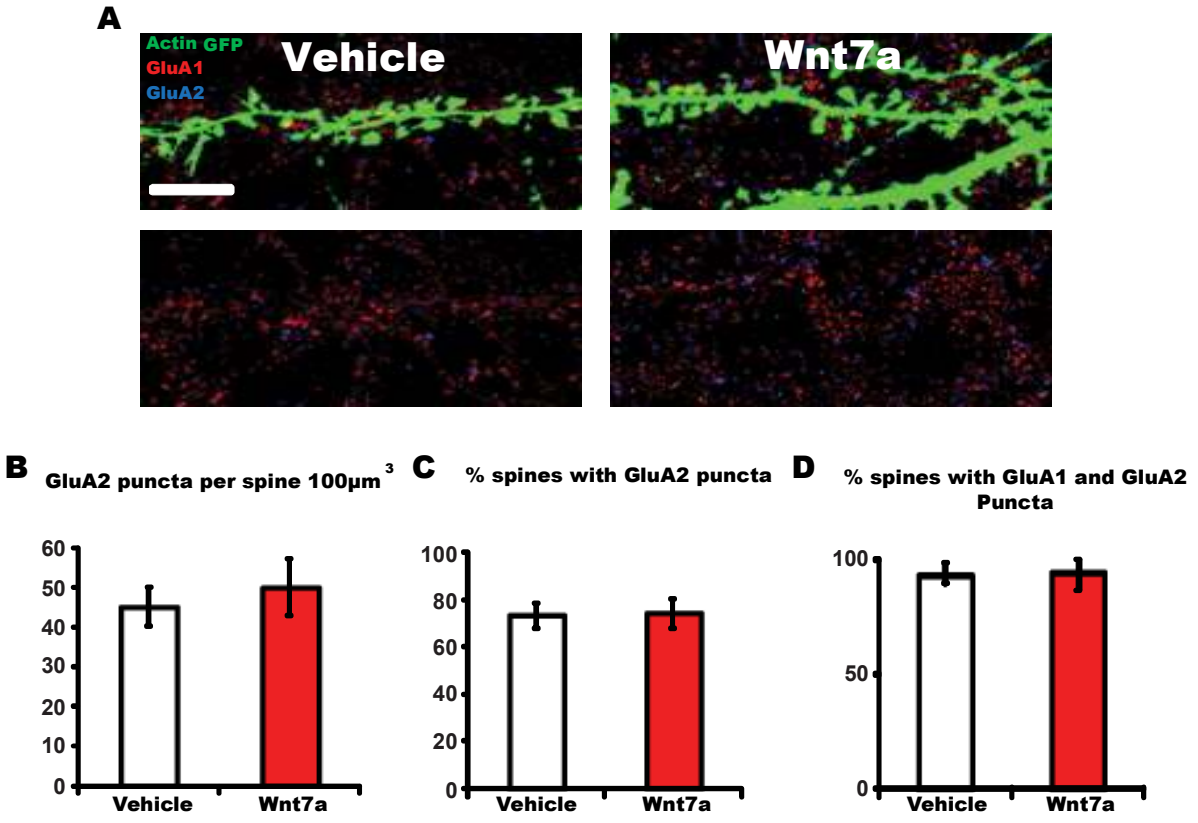

Figure 4.6 Wnt7a treatment does not increase GluA1 and/or GluA2 puncta localisation on spines or the number of spines associated with both puncta on 21 DIV neurons. Hippocampal neurons cultured for 21 days were treated with BSA (vehicle) or Wnt7a for 3hrs. Dendritic spines were analysed for GluA1 (red) and GluA2 (blue) puncta. (A) High magnification images of isolated dendrites display the Wnt7a mediated increases in spine associated GluA1 and GluA2 puncta number (Scale bar= 5um). Top panel displays stain for Actin-gfp (green), GluA1 and GluA2 whilst lower panels display puncta images minus Actin-gfp. (B) Quantification of GluA1 puncta number per Spine volume displays no significant change in GluA1 puncta associated with spines after Wnt7a treatment. (C) Quantification of GluA2 puncta number per spine volume reveals no change in puncta accumulation on spines per spine volume. (D) Quantification of the % of spines with GluA1 puncta associated reveals no significant change between treatments. (E) Quantification of the percentage of total spines associated with GluA2 puncta revealed no difference (F) Quantification of spines with both GluA1 and GluA2 puncta associated display no difference between vehicle and Wnt7a treatments.

These experiments suggest on the spines of 21 DIV neurons, 3hrs Wnt7a exposure does not affect the number of postsynaptically un-silenced synapses. GluA2 puncta number associated with spines remained constant between neurons treated with BSA (45 ± 5.227 pp100μm) and (Wnt7a: 50 ± 7.744) (fig. 4.6 A and B). The percentage of spines associated with GluA2 or GluA2 and GluA1 also remained constant between BSA and Wnt7a treatments (fig 4.6 A, C and D). These results show that on mature neurons Wnt7a is unable to regulate GluA2 receptor localisation on dendritic spines.

4.2.3 Postsynaptic Wnt activation increases accumulation of glutamate receptors and presynaptic inputs onto the dendritic spines of developing neurons.

Whilst the data above clearly indicate Wnt7a signalling regulates the localisation of synaptic proteins both pre and postsynaptically the location at which Wnt7a acts is still unclear. The ability of Wnt7a to act directly on axons to direct presynaptic development has been established (Ahmad-Annuar et al., 2006). The question remains whether the maturation of the postsynaptic compartment is the result of direct postsynaptic action by Wnt7a or secondary signalling as a consequence of presynaptic maturation.

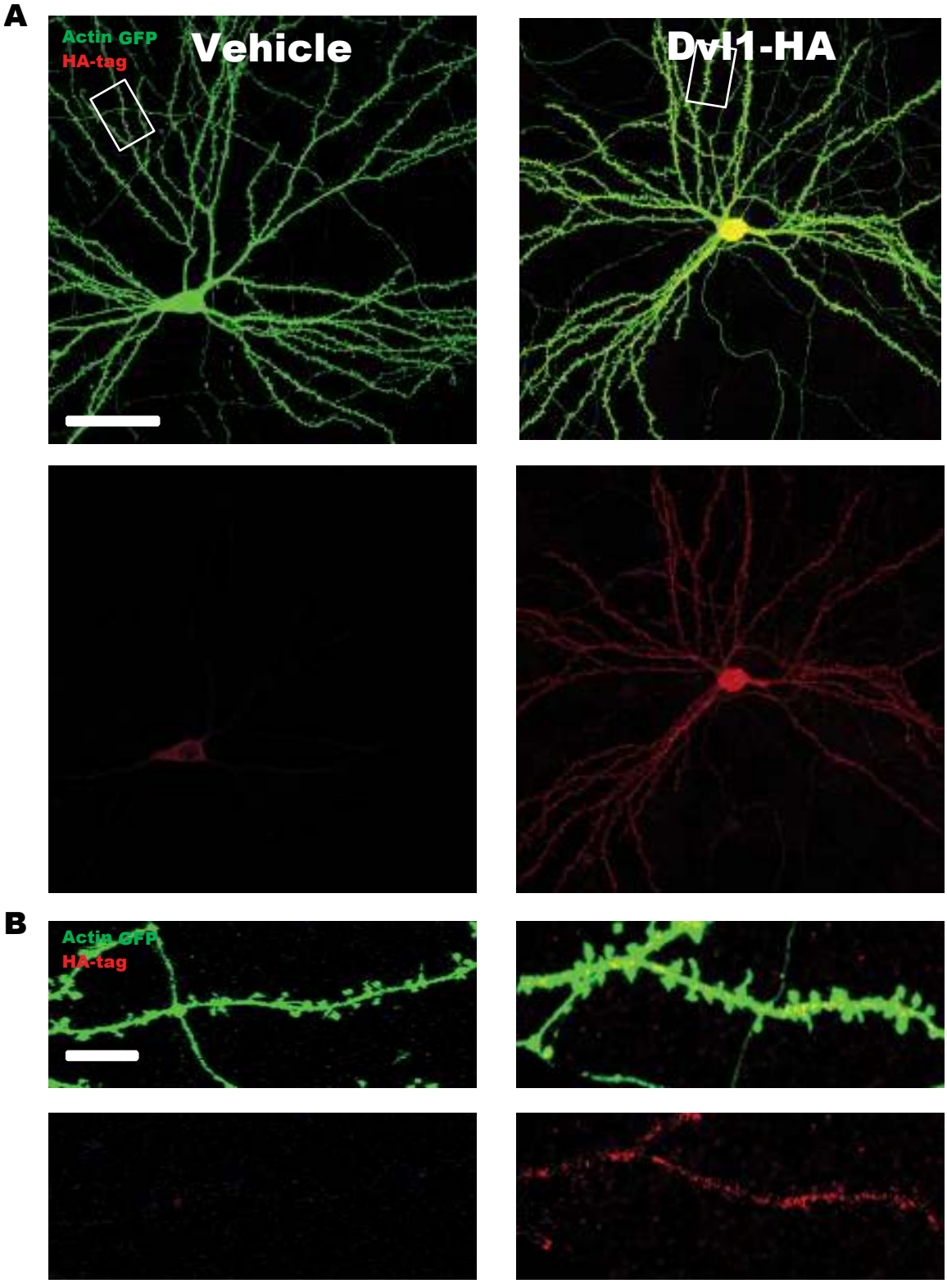

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Figure 4.7 Overexpression of Dvl1-HA in hippocampal neurons promotes increases in spine size. Hippocampal cultures were transfected with either a combination of Actin-GFP and Dvl1-HA expressing constructs or Actin-GFP and PCS2- empty vector constructs. (A) The top panels (left to right) show neurons transfected without or with Dvl1-HA respectively. Neurons were fixed and stained for actin-GFP (green) and HA-epitope (red) and spine morphology was observed. The HA-tag (Dvl1) was observed present throughout neurons in overexpressing neurons in contrast to the control transfected neurons where signal was only faintly present in the cell body (Scale bar=30μm). (B) Lower panels show magnified sections of dendrite illustrating the presence of HA signal (Dvl1) in dendrite and sparsely in dendritic spines (Scale bar=5μm).

To address this question I attempted to analyse neurons in which Wnt signalling was activated only in the postsynaptic neuron. To achieve this I co-transfected neurons with the eGFP-actin construct used to visualise spines and Dvl1-HA a downstream intracellular activator of Wnt signalling pathways (fig. 4.7). The neurons were transfected as in previous experiments at 8DIV and the constructs allowed to express up to 14 DIV to look at developing synapses or 21DIV to study more mature synapses.

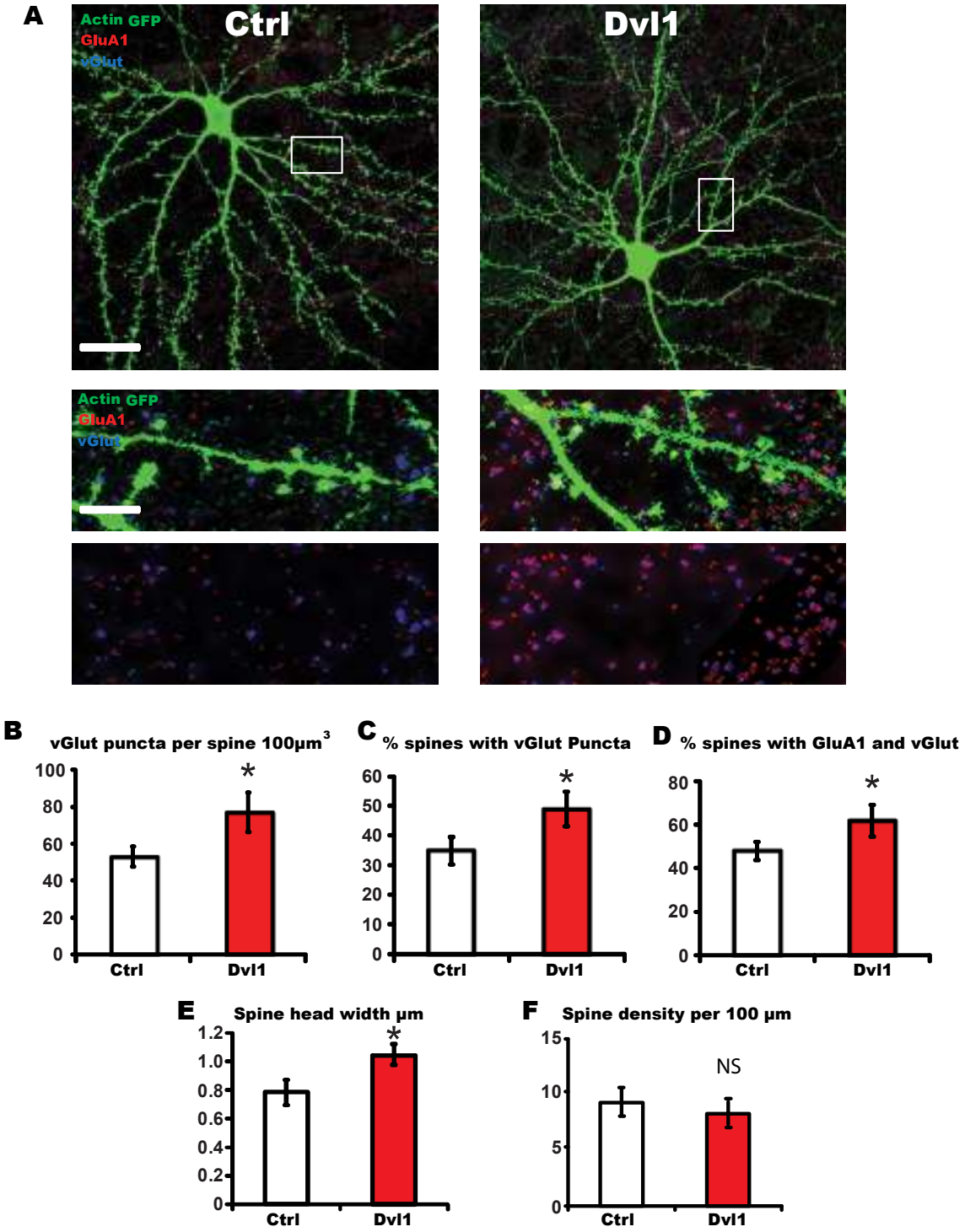

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Figure 4.8 Postsynaptic Wnt signalling activation increases GluA1 and vGlut accumulation and localisation on dendritic spines of 14DIV neurons. Cultured hippocampal neurons at 8DIV were co-transfected with either actin-gfp and control empty vector or actin-gfp and Dvl1-Ha constructs. Neurons were fixed and stained at 14DIV and dendritic spines were analysed for GluA1 (red) and vGlut (blue) puncta. (A) Top panels displays low magnification images of treated neurons stained with Actin-gfp (green), GluA1 and vGlut (Scale bar =30um). Middle panels display high magnification images of dashed box area in low magnification images (Scale bar =5um). Lower panel displays GluA1 and vGlut puncta minus Spines and Dendrites. Dvl overexpression increases both the number and size of spines whilst increasing the number of GluA1 and vGlut associated. (B) Quantification of the number of vGlut puncta associated with spines per $100um^3$ revealed a significant difference between treatments of 48%. (C) Quantification of the % of spines with vGlut puncta associated increased by 40%. (D) Quantification of % of total spines associated with both GluA1 and vGlut puncta was significantly increased by 32%. (E) Quantification of the width of spines shows a significant increase in spine size. (F) Quantification of number of spines per 100μm length of dendrite illustrates the lack of spine number change on neurons transfected with Dvl1-HA. * = $P<0.05$.

Post synaptic activation of Wnt signalling mediates a 23% increase in the number of GluA1 puncta on spines alongside a significant 107% increase in GluN1 puncta fig 4.8 A, B and C. These effects were complemented by a slight but significant increase in the percentage of spines associated with GluA1 puncta (6%), a strong increase in the percentage of spines associated with GluN1 puncta (81%) and a similar increase in the percentage of spines associated with both GluA1 and GluN1 puncta (42%) (fig. 4.8 A and D-F). This suggests postsynaptic activation alone is sufficient to increase both the localisation of AMPAR and NMDAR on spines concomitantly increasing the proportion of postsynaptically active synaptic sites.

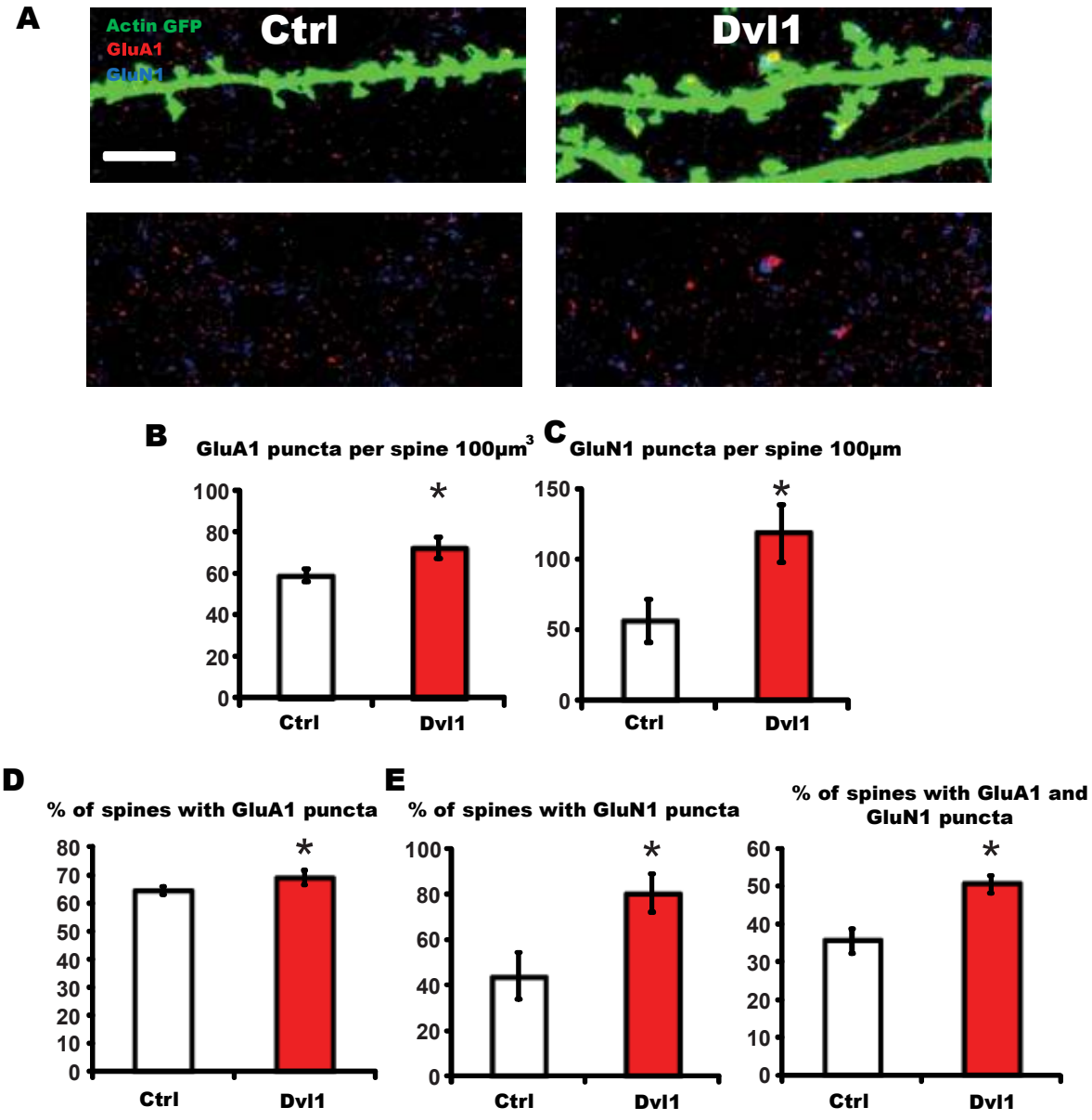

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Figure 4.9 Postsynaptic expression of Dvl1 increases GluA1 and GluN1 puncta localisation on spines and increases the number of spines associated with both proteins at 14 DIV. Cultured hippocampal neurons at 8DIV were co-transfected with either actin-gfp and control empty vector or actin-gfp and Dvl1-Ha constructs. Dendritic spines were analysed for GluA1 (red) and GluN1 (blue) puncta at 14DIV. (A) High magnification images of isolated dendrites display the Dvl1 mediated increases in spine associated GluA1 and GluN1 puncta number (Scale bar= 5um). Top panel displays stain for Actin-gfp (green), GluA1 and GluN1 whilst lower panels display puncta images minus Actin-gfp. (B) Quantification of GluA1 puncta number per Spine volume displays a 23% increase in GluA1 puncta associated with spines after Dvl1 overexpression. (C) Quantification of GluN1 puncta number per spine volume displays the strong 103% increase in puncta accumulation on spines per spine volume. (D) Quantification of the % of spines with GluA1 puncta associated reveals a small but significant 6% increase with Dvl1 overexpression. (E) Quantification of the percentage of total spines associated with GluN1 puncta revealed a 81% increase (F) Quantification of spines with both GluA1 and GluN1 puncta associated display a 42% increase. * = $P<0.05$.

Analysis of GluA2 localisation also revealed an increase in the density of puncta on spines from 62 ± 5.516 pp100μm^3 to 78 ± 3.122 pp100μm^3 (fig 4.8 A and B). This increase in GluA2 mobilisation resulted in an increase in the percentage of spines associated with GluA2 puncta up 39% from 31 ± 2.633% to 43 ± 6.959% (fig. fig 4.8 A and C). Interestingly the number of spines associated with both GluA1 and GluA2 also increases by 37%. Together these results indicate the ability of Dvl1 to mediate both maturation of synapses by increase of GluA2 into spines and to increase the total proportion of mature spines on a neuron.

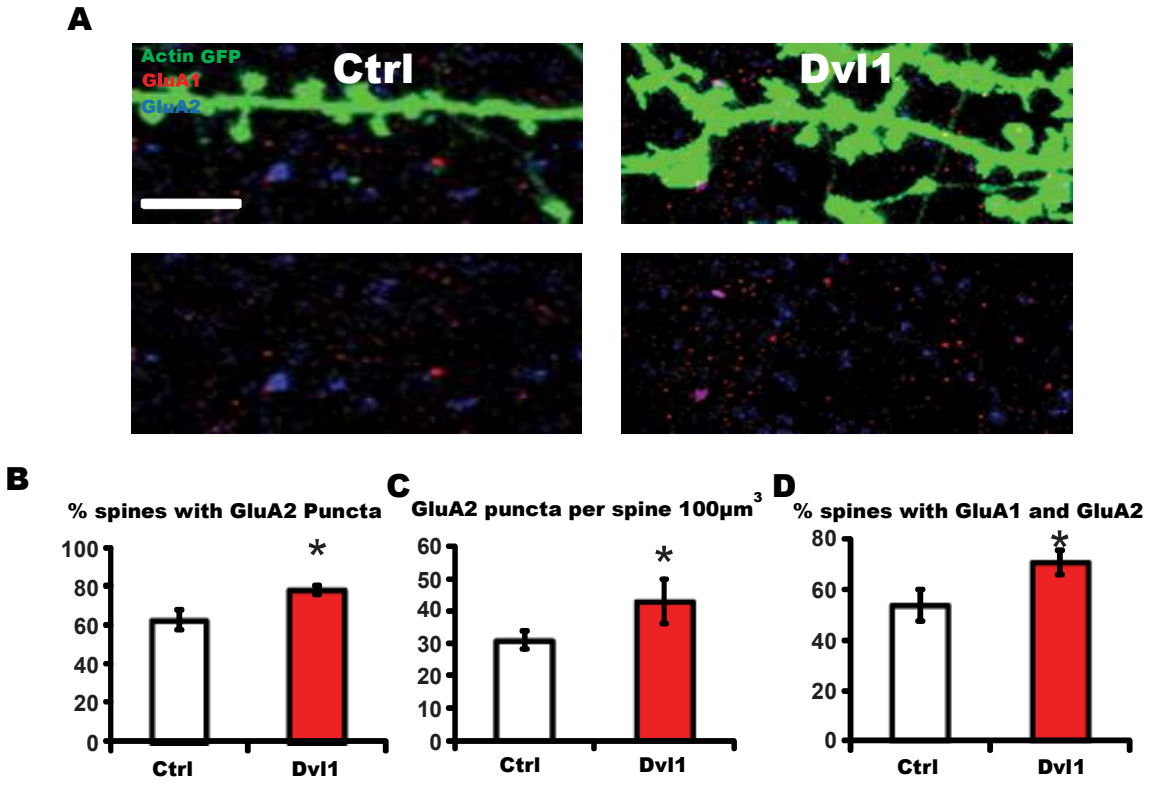

Figure 4.10 Dvl1 overexpression increases GluA1 and GluA2 puncta localisation on spines and increases the number of spines associated with both proteins at 14 DIV. Cultured hippocampal neurons at 8DIV were co-transfected with either actin-gfp and control empty vector or actin-gfp and Dvl1-Ha constructs. Dendritic spines were analysed for GluA1 (red) and GluA2 (blue) puncta at 14DIV. (A) High magnification images of isolated dendrites display the Dvl1 mediated increases in spine associated GluA1 and GluA2 puncta number (Scale bar= 5um). Top panel displays stain for Actin-gfp (green), GluA1 and GluA2 whilst lower panels display puncta images minus Actin-gfp. (B) Quantification of GluA2 puncta number per spine volume displays a 26% increase in puncta accumulation on spines per spine volume. (C) Quantification of the percentage of total spines associated with GluA2 puncta revealed a 39% increase (D) Quantification of spines with both GluA1 and GluA2 puncta associated display a 37% increase. * = $P<0.05$.

Neurons were also stained for the presynaptic marker vGlut to assess any changes postsynaptic Wnt activation has on the colocalisation of spine located glutamate receptors with presynaptic sites. Changes in these values likely represent changes to functional synaptic sites. Intriguingly the percentage of spines associated with vGlut puncta increases by 40%

(fig. 4.7A and C) whilst the percentage of spines associated with both GluA1 and vGlut increase by 32% from 47 ± 4.502% to 62 ± 6.799% (fig. 4.7A and D). These results suggest postsynaptic expression of Dvl1 activates a retrograde synaptic signalling pathway mediating enhanced presynaptic organisation onto the maturing spines.

4.2.4 Postsynaptic Wnt activation does not affect accumulation of synaptic proteins onto the dendritic spines of mature neurons.

The above data suggests activation of Wnt signalling postsynaptically acts specifically to increase synapse number and presence of subunits associated with maturity on dendritic spines. Although we are aware of these effects in developing, immature cultures the question over whether postsynaptic Dvl activation can further increase receptor localisation in more mature spine synapses remained. To investigate this I over-expressed Dvl1 in neurons at 8 DIV and allowed these neurons to remain in culture until DIV 21 where the neurons were fixed stained and the synaptic puncta analysed.

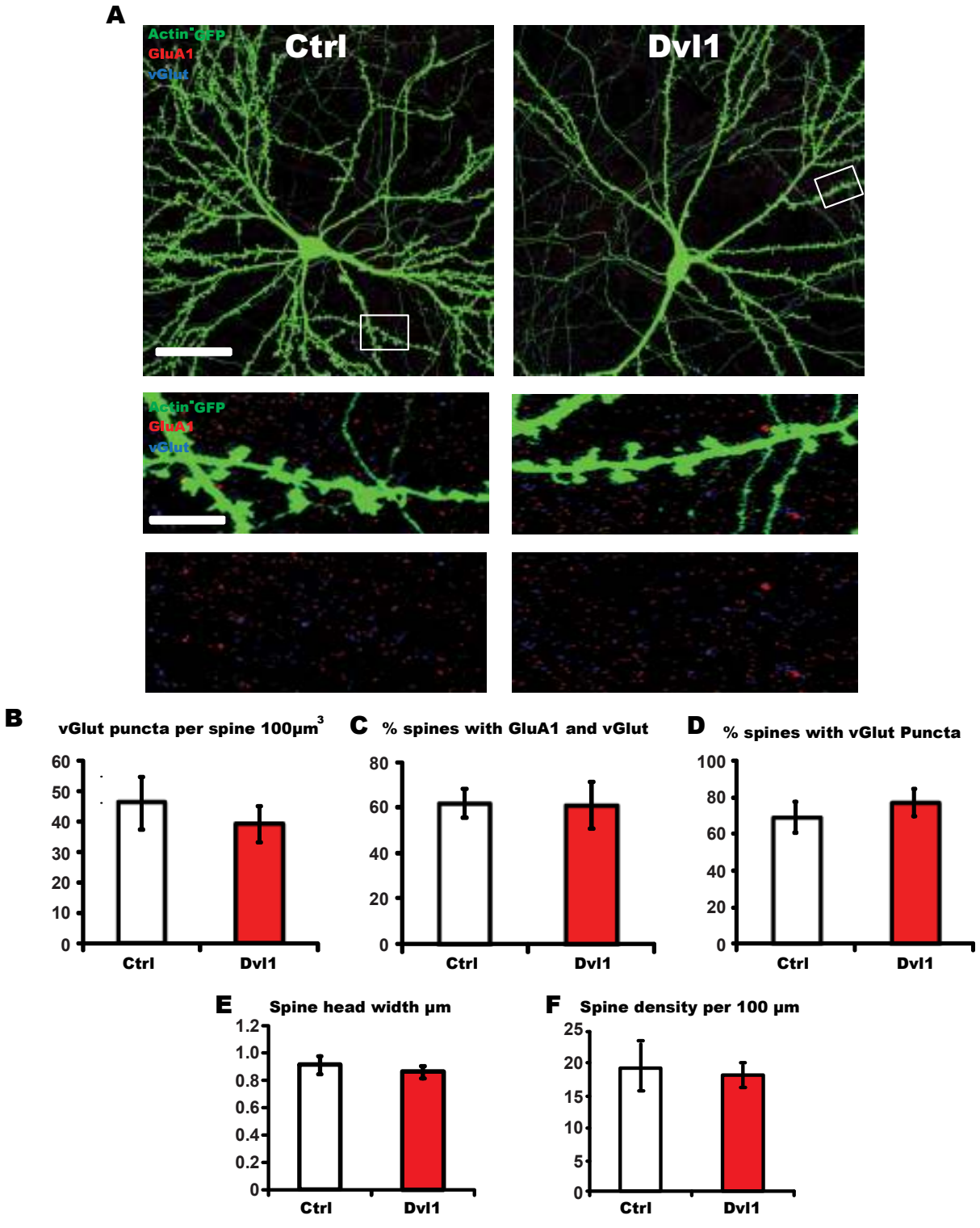

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Figure 4.11 Overexpression of Dvl1 does not affect GluA1 and/or vGlut localisation on dendritic spines of 21DIV neurons. Cultured hippocampal neurons at 8DIV were co-transfected with either actin-gfp and control empty vector or actin-gfp and Dvl1-Ha constructs. Neurons were fixed and stained at 14DIV and dendritic spines were analysed for GluA1 (red) and vGlut (blue) puncta. (A) Top panel displays images of treated neurons stained with Actin-gfp (green), GluA1 and vGlut (Scale bar =50um). Middle panels display high magnification images of dashed box in low magnification images (Scale bar =5um). Lower panel displays GluA1 and vGlut puncta minus Spines and Dendrites (actin-gfp stain). Dvl1 overexpression does not increase the number or size of spines or the number of GluA1 and vGlut associated with these spines. (B) Quantification of the number of vGlut puncta associated with spines per $100um^3$ of spine volume shows no differences between Control and Dvl1 transfected neurons. (C) Quantification of the % of spines with vGlut puncta associated shows no difference between treatments. (D) Quantification of % of total spines associated with both GluA1 and vGlut puncta shows no significant difference between treatments. (E) Quantification of the width of spines and (F) Quantification of number of spines per 100μm length of dendrite showed no change between ctrl and Dvl1 expressing neurons.

Mimicking Wnt7a at 21 DIV, Dvl1 overexpression does not affect synapse number or synaptic puncta accumulation. vGlut and GluA1 puncta associated with spines shows no significant increase in neurons overexpressing Dvl1 (fig. 4.10A and B, fig 4.11A and B). These effects are compounded by a lack of significant change in the percentage of spines associated with vGlut puncta (BSA: 61±7.132, Dvl1: 60±5.21%) or both vGlut and GluA1 puncta (BSA: 69±6.023%, Dvl1: 75±6.143%) (fig. 4.10A, C and D).

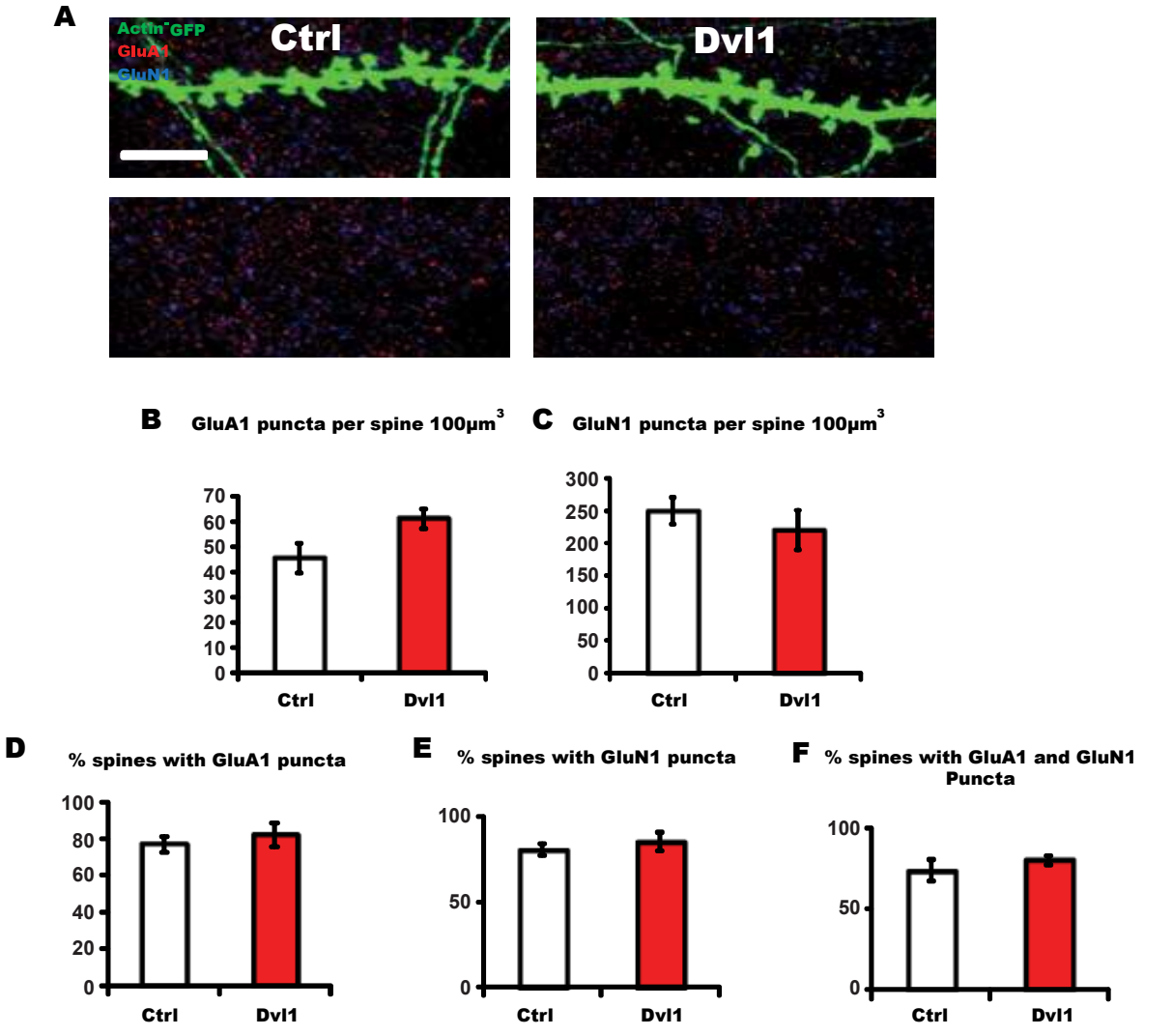

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Figure 4.12 Dvl1 overexpression has no significant effect on GluA1 and GluN1 puncta localisation on spines and in 21 DIV cultured hippocampal neurons. Cultured hippocampal neurons at 8DIV were co-transfected with either actin-gfp and control empty vector or actin-gfp and Dvl1-Ha constructs. Neurons were fixed and stained at 14DIV and dendritic spines were analysed for GluA1 (red) and GluN1 (blue) puncta. (A) High magnification images of isolated dendrites display the Dvl1 mediated increases in spine associated GluA1 and GluN1 puncta number (Scale bar= 5um). Top panel displays stain for Actin-gfp (green), GluA1 and GluN1 whilst lower panels display puncta images minus Actin-gfp. (B) Quantification of GluA1 puncta number per Spine volume displays no significant change in GluA1 puncta associated with spines after Dvl1 overexpression. (C) Quantification of GluN1 puncta number per spine volume reveals no change in puncta accumulation on spines per spine volume. (D) Quantification of the % of spines with GluA1 puncta associated reveals no significant change between treatments. (E) Quantification of the percentage of total spines associated with GluN1 puncta revealed no difference (F) Quantification of spines with both GluA1 and GluN1 puncta associated display no difference between vehicle and Dvl1 overexpression.

Neither GluN1 nor GluA2 receptor puncta were significantly altered in the spines of Dvl1 overexpressing neurons (fig. 4.11A and C, fig. 4.12A and B). This indicates postsynaptic activation of Wnt signalling is unable to alter the Ca^{2+} conductance of spines via GluA2 or GluN1 receptor localisation. Dvl1 overexpression similarly has no effect on the percentage of spines containing GluN1 puncta (fig. 4.11A and E), GluA2 puncta (fig. 4.12A and C), both GluN1 and GluA1 (fig. 4.11A and F) or GluA2 and GluA1 (fig. 4.12A and D).

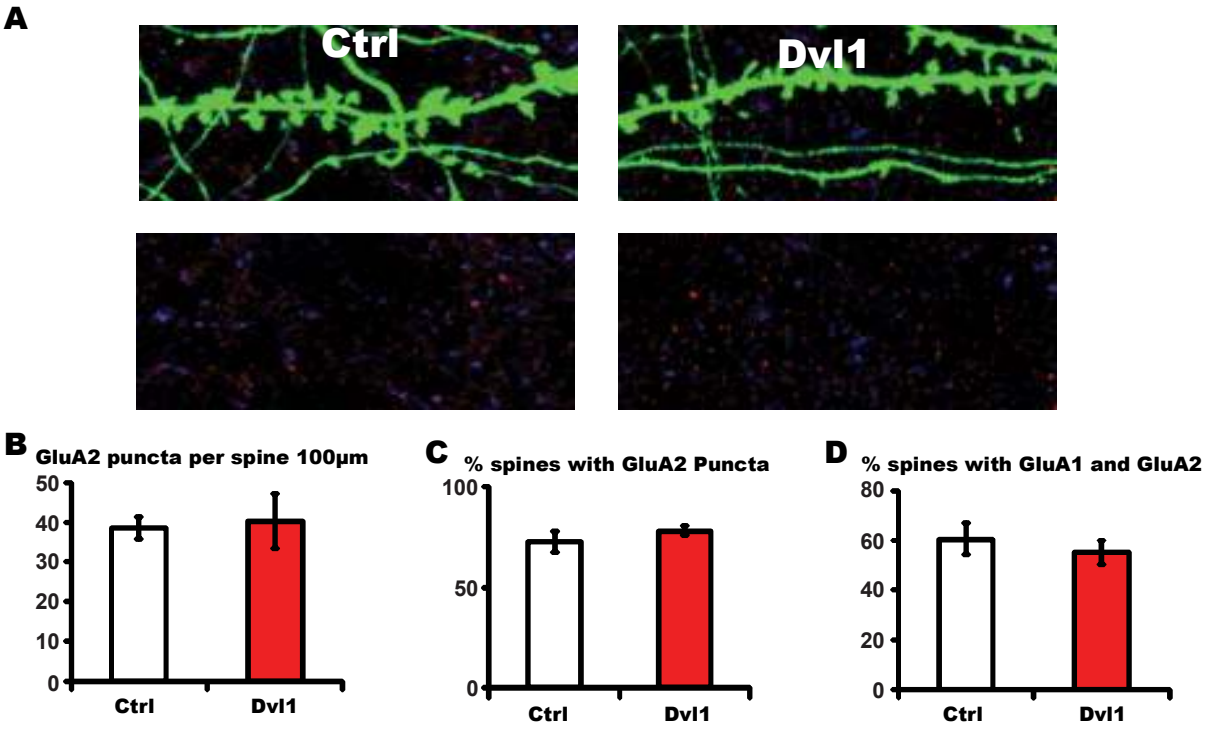

Figure 4.13 Dvl1 overexpression has no significant effect on GluA1 and GluA2 puncta localisation on spines and in 21 DIV cultured hippocampal neurons. Cultured hippocampal neurons at 8DIV were co-transfected with either actin-gfp and control empty vector or actin-gfp and Dvl1-Ha constructs. Neurons were fixed and stained at 14DIV and dendritic spines were analysed for GluA1 (red) and GluA2 (blue) puncta. (A) High magnification images of isolated dendrites display the Dvl1 mediated increases in spine associated GluA1 and GluA2 puncta number (Scale bar= 5um). Top panel displays stain for Actin-gfp (green), GluA1 and GluA2 whilst lower panels display puncta images minus Actin-gfp. (B) Quantification of GluA1 puncta number per Spine volume displays no significant change in GluA1 puncta associated with spines after Dvl1 overexpression. (C) Quantification of GluA2 puncta number per spine volume reveals no change in puncta accumulation on spines per spine volume. (D) Quantification of the % of spines with GluA1 puncta associated reveals no significant change between treatments. (E) Quantification of the percentage of total spines associated with GluA2 puncta revealed no difference (F) Quantification of spines with both GluA1 and GluA2 puncta associated display no difference between vehicle and Dvl1 overexpression.

4.3 Discussion

The above data shows that at 14 DIV Wnt7a plays a significant role in the regulation of glutamate receptor localisation on dendritic spines at synaptic sites. Furthermore the data presented above when taken with data presented by Ciani et al. (2011) indicates that whilst Wnt7a increases the number and size of dendritic spines, presynaptic input to these spines is also increased. This increase is further confirmed by both the changes to percentage of spines associated with either vGlut puncta alone or vGlut and GluA1 puncta. These significant increases indicate that presynaptic input increases to both the majority of spines but also more importantly to spines with GluA1, important for functional transmission.

4.3.1 The role of Wnt7a signalling on NMDAR to synaptic sites

Wnt7a exposure of neurons at 14 DIV induces a hereto novel ability to aggregate GluN1 subunits at the site of excitatory transmission on dendritic spines. Antibody specificity of the GluN1 subunit was such that in contrast to the GluA1 and GluA2 assays both intracellular and surface receptors were labelled. This detail might lead one to assume increases in GluN1 puncta may not be specific to synaptic sites but instead be part of intracellular pools. However the increase in colocalisation between GluN1 and GluA1 proteins suggest that the total increase in GluN1 on spines results in increased synaptic GluN1. The GluN1 subunit is an obligatory component of the functional signalling receptor and as such its increased presence is an important modification by Wnt7a. This increase in NMDAR density is likely to increase the ability of these neurons to potentiate/depotentiate in response to synaptic activity via intracellular calcium signalling.

Very little is known of soluble extracellular factors which regulate GluN1 trafficking into the synaptic and perisynaptic regions. However through interactions with PSD proteins, adhesion molecules seem to play similar roles to Wnt7a in synaptic NMDAR accumulation. SALM1overexpression in 14DIV neurons increases the number of surface NR2B subunits along dendrites (Wang et al., 2006). Similarly to Wnt7a proteins SALM proteins are believed to have a role specifically in excitatory synapse formation (Ko et al., 2006). NMDARs are believed to be maintained at the synapse by interactions with PSD proteins, knockdown of PSD-95 reveals moderate defects in NMDAR currents (Ehrlich et al., 2007; Petralia et al., 2009). Overexpression of PSD-95 is able to increase the surface expression of NMDAR in heterologous cell systems (Lin et al., 2006; Lin et al., 2004). Work from our lab has recently demonstrated the role of Wnt7a in regulating PSD-95 accumulation at synaptic sites (Ciani et

al., 2011). The increase of NMDAR as a result of Wnt7a activity may in part be due to this increase in its synaptic interacting partner.

4.3.2 Wnt7a signalling regulates increases in spine maturity through glutamate receptor complementation

Concurrently the increase in NR1 localisation at synaptic sites is complemented by increases in both GluA1 and GluA2 density. Increase in GluA1, the major contributory subunit in AMPAR transmission, on spines indicates that Wnt7a promotes the formation of new functional synaptic sites as opposed, AMPA silent synapses. This idea is strengthened by the increase in colocalisation % of GluA1 and GluN1 puncta. The increase in both GluA2 density and % of spines with GluA2 puncta or GluA2 and GluA1 puncta indicates Wnt7a mediated maturation of spines. GluA2 subunit containing receptors play important roles in basal synaptic transmission and CA1 LTP (Seidenman et al., 2003). GluA2 subunit containing receptors are also non permeable to Ca^{2+} influx providing a role in buffering neurons from excitotoxicity (Hollmann et al., 1991). Alongside increases in GluA2 accumulation recent data from our lab has indicated a functional role of Wnt7a signalling in increases in spine size (Ciani et al., 2011). Together these changes are classic indicators of excitatory synapse maturation (Chen et al., 2009; Dunaevsky et al., 1999; Hall and Ghosh, 2008). The GluA2 puncta density increase together with the increase in % of spines with GluA2 puncta suggests that the spines affected by wnt7a mediated puncta mobilisation (the newly formed subset or all spines) are more mature. Together with the increases in spines with both GluA1 and GluA2 the data suggests Wnt7a exposed neurons have increased numbers of functional mature excitatory spine synapses.

These effects of Wnt signalling most closely mimic the effect of Tumour necrosis factor alpha (TNFα) on GluA2 localisation. Experiments by Rainey-smith and colleagues (2010) demonstrate an increase in the surface expression of GluA2 protein in response to short term treatments of TNFα (Rainey-Smith et al., 2010). In these experiments Ca^{2+} influx was significantly decreased indicating a neuroprotective effect of the TNFα signalling molecule. Similarly vascular endothelial growth factor (VEGF) has a similar effect on the GluA2 expression in motor neurons (Bogaert et al., 2010). In these experiments GluA2 modifications were driven by increases in transcription.

The increased maturation of these synapses by changes in spine morphology and receptor localisation may explain why no significant modifications were observed at 21DIV. Both

recombinant Wnt7a and Dvl1 overexpression fail to enhance spine size and number or size respectively at 21 DIV (Ciani and Salinas, unpublished). These results suggest a number of possibilities; the excitatory spine capacity for receptor accumulation may have reached a plateau where no further increases are possible. This seems unlikely because for all of the markers assessed, 100% occupancy of spines was never reached. A second possibility may be that the level of Wnt activation (using Wnt7a bath application or overexpression) was insufficient to mobilise effects in what would be by 21 DIV a Wnt rich system. This idea is strengthened by a Wnt loss of function study in our lab using SFRPs in 21 DIV where loss of glutamatergic synaptic proteins is observed (Boyle and Salinas, 2010 unpublished).

4.3.3 Postsynaptic expression of Wnt signalling activates effects both post and presynaptically.

Whilst the role of Wnt proteins signalling to the presynaptic compartment are well established the role of Wnt at postsynaptic sites is less well defined. In this chapter I identified the effects of Wnt signalling postsynaptically by overexpression of Dvl1 in neurons and analysing the dendritic spines of these neurons. Previous studies by our lab revealed postsynaptic expression of Dvl1 mimicked increases in spine morphology but not spine density caused by recombinant Wnt7a. The changes to spine size were coordinated with increases in glutamate receptor localisation at 14 DIV but not at 21 DIV. Most interestingly this specific postsynaptic Wnt activation promotes an increase in presynaptic organisation onto these spines. The increases in vGlut puncta associated with spines indicate that presynaptic terminals may contain more release sites and/or more presynaptic sites may be synapsing onto each spine.

Studies have suggested Wnt7a plays a role in enhancements in presynaptic release however these studies have used bath applications of Wnt7a thereby allowing direct action of Wnt7a at presynaptic sites. The data in this study indicates that activation of Wnt signalling postsynaptically initiates retrograde signalling to enhance presynaptic function. Potentially this signal may be Wnt derived however with the lack of high fidelity Wnt specific antibodies this would be difficult to ascertain.

Non Wnt retrograde signals may also play a role in this form of regulation, one such example is neuronal nitric oxide synthase (nNOS). Poglia and colleagues (2010) identified an nNOS mediated trans-synaptic signalling pathway which enhanced the presynaptic specialisation.

Postsynaptic overexpression of PSD-95 caused an increase in the number of multiply innervated spines (MIS). This effect on MIS was diminished by blockade of nNOS function. Interestingly postsynaptic overexpression of Dvl1 increases PSD-95, whilst the overexpression of PSD-95 in the above study mimics the changes to spine morphology observed in Wnt activation (Ciani et al., 2011; Nikonenko et al., 2008).

Chapter 5

Wnt 7a regulates the number and strength of functional synaptic sites

5.1 Introduction

Recent advances in technology have improved our understanding of neuronal activity and synaptic transmission. Imaging tools have been used to visualise the gross functional changes in the brain caused by disorders such as schizophrenia, Alzheimer's and epilepsy (Duncan, 1997; Meyer-Lindenberg et al., 2008; Sperling et al., 2010). These studies have been invaluable in characterising the changes that occur in the normal brain and how the brain changes in disease conditions. This functional information in turn has been used to identify defects in protein expression which mimic these functional defects.

Functional synaptic communication within the brain is generally mediated by evoked transmission and it is here that most deleterious neurological defects take effect. In the example of epilepsy the functional phenotype is the occurrence of unregulated, prolonged and synchronized neuronal discharges propagating to surrounding structures (neural networks) (Vincent and Mulle, 2009). Whilst the ability to induce LTP at functional synapses is impaired in the brain of Alzheimer's sufferers (Gleichmann and Mattson, 2010). Interestingly the number and strength of functional synaptic contacts is developmentally determined by a number of processes. In early postnatal development, the vast majority of central synapses are postsynaptically silenced by regulation of the presence of specific GluA subunits (Gomperts et al., 1998; Kerchner and Nicoll, 2008). Presynaptically development and maturation of the presynaptic terminal increases the strength of synaptic transmission as development proceeds (Gasparini et al., 2000). The mechanisms that regulate processes such as synapse silencing, and presynaptic maturation by extracellular signalling molecules remain poorly understood.

As I have demonstrated in the previous two chapters, Wnt signalling plays a role in the localisation of excitatory receptors to synaptic sites. Functional studies at the synapse in relation to the regulation of Wnt signalling have recently become an area of great interest. Currently few studies have examined the functional effects of Wnt signalling in synaptic function. Measuring field postsynaptic currents, Chen and colleagues investigated the role of Wnt3a in hippocampal LTP. Using both loss and gain of Wnt signalling function in the form of Fz-8/Fc and Wnt3a respectively on hippocampal slices both impairment and enhancement

(whilst poor) of LTP were observed (Chen, 2006). Following this study, Beaumont and colleagues investigated the effects of Wnt signalling on transmission using small compounds that mimic canonical Wnt signalling (Beaumont et al., 2007). Using the compound WASP-1 (Wnt activating small molecule potentiator-1) on hippocampal cultures enhancements in both mEPSC frequency and amplitude were observed alongside increases in fEPSC slope and LTP pronouncement. These experiments indicate a role for Wnt signalling pathways in the functional transmission of synapses.

The effects of Wnt7a and Wnt5a on both field and local recordings at CA3-CA1 hippocampal synapses was analysed by Cerpa and colleagues (Cerpa et al., 2008). The authors observed a presynaptic enhancement in transmission with Wnt7a but not with Wnt5a from fEPSC recordings. Meanwhile Wnt7a also enhanced presynaptic function as determined by intracellular recording at the same CA3-CA1 synapse (Cerpa et al., 2008). The same group further investigated the effects of Wnt5a on synaptic function, analysing field recordings on hippocampal CA1 neurons, observing an increase in fEPSC normalised amplitude (Farias et al., 2009). Subsequently Wnt5a was observed to increase amplitude in both AMPA and NMDA derived mEPSC in hippocampal cultures (Varela-Nallar et al., 2010). These series of experiments suggests both Wnt7a and Wnt5a mediated transmission effects at the CA3-CA1 synapse in the hippocampus. Interestingly the effects of Wnt7a and Wnt5a seem to differ in specificity with Wnt5a proposed to act specifically at the postsynaptic side of the CA3-CA1 synapse. Both studies in this thesis and previous studies have demonstrated Wnt7a is able to affect synaptic function and receptor localisation presynaptically and postsynaptically (Ahmad-Annuar et al., 2006).

Whilst the localisation of synaptic receptors is a useful indicator of the mechanisms by which synapse modification occur, the relationship between receptor localisation and function is not always straightforward. Modulatory processes at the synapse may act to diminish the effect of increased synaptic receptor localisation by modification of presynaptic release (Pang et al., 2006). Therefore it was important to establish how the effects of receptor localisation by Wnt7a signalling impinge on the functional aspects of synaptic transmission at these synapses.

5.2 Results

5.2.1 Wnt7a gain of function enhances evoked postsynaptic current amplitude and decreases synaptic failure.

In order to access the effects of Wnt signalling on synaptic function 14DIV hippocampal cultures were treated with Wnt7a for 3hrs and patched. Concurrently a stimulating electrode was placed approximately 100-200μm to generate a post synaptic current from presynaptic axons. By modulating the stimulus intensity presynaptic axons were able to elicit minimal amplitude post synaptic currents alongside synaptic failures.

After 3hr exposure to Wnt7a hippocampal neurons exhibit an increase in the amplitude of synaptic currents by 53% (fig.5.1a and c). The increased amplitude is paired with a decrease in the number of stimulus events which fail to generate a significant current (fig5.1a and b). The percentage of these failures decreased from 29 ± 8.276% to 15±6.723% indicating a significant increase in the activity of Wnt7a exposed synapses. These results demonstrate the role of Wnt7a gain of function in increasing the generation of significant postsynaptic currents thereby enhancing synaptic efficiency.

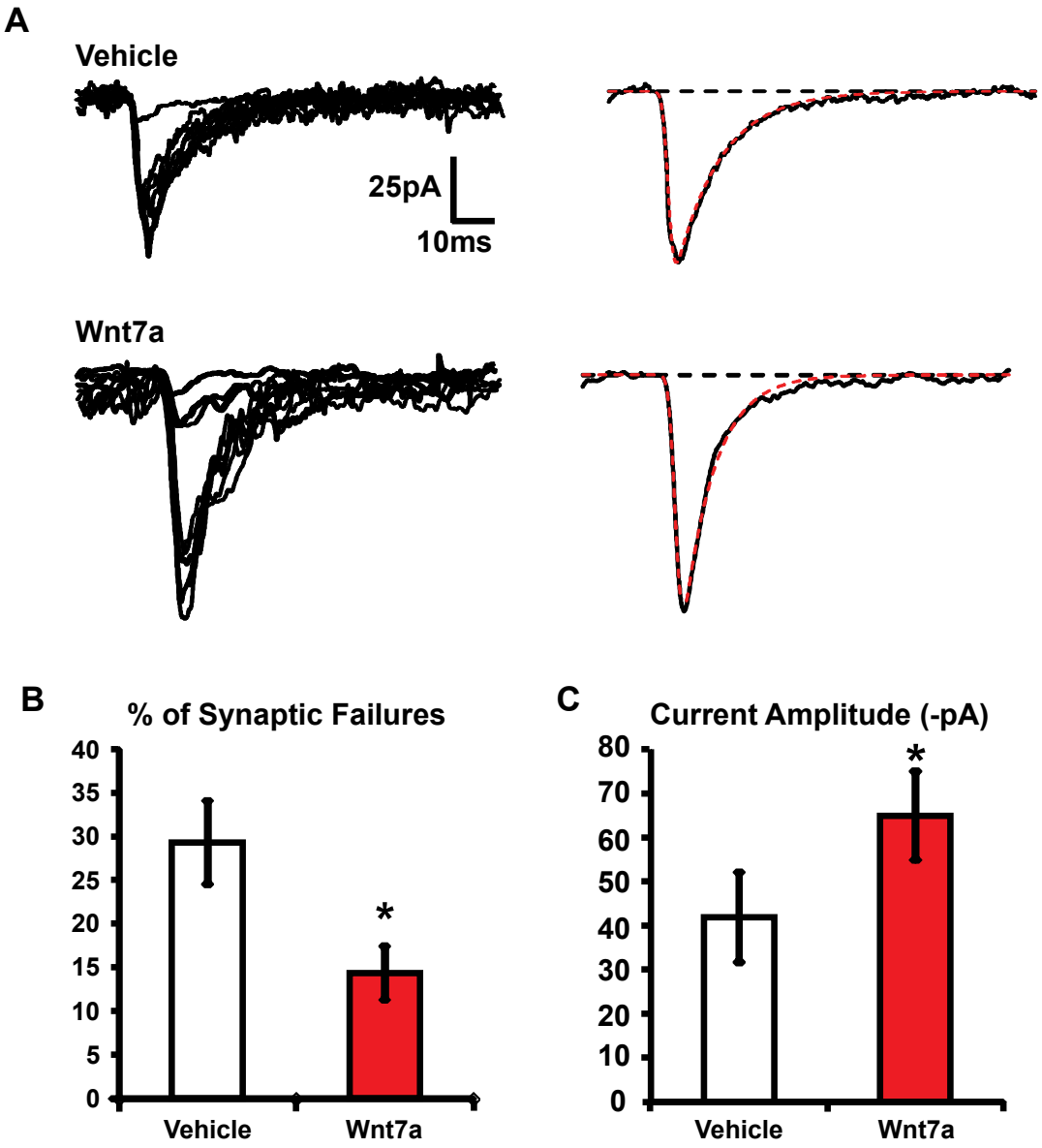

Figure 5.1 Wnt7a increases evoked postsynaptic currents and decreases synaptic failure rates. 14 DIV hippocampal cultures were treated with BSA (vehicle) or Wnt7a for 3 hours and postsynaptic currents were recorded by whole cell patch clamp. (A) Left panels show overlay of 10 consecutive stimulus runs with representative synaptic failures. Top panel shows currents from vehicle treated whilst lower panel displays currents from Wnt7a treated neuron. Right panels display average sweep from vehicle or Wnt7a treated cell. (B) Quantification of synaptic failure rates given as %failures of total stimulus events. (C) Quantification of averaged currents amplitude reveals an increase after Wnt7a treatment. N number of cells, BSA=12, Wnt7a=15 (* = P<0.05)

5.2.2 Wnt7a/Dvl1 loss of function at the MF-CA3 synapse decreases evoked postsynaptic current amplitude and increases the frequency of synaptic failures.

In order to look at both loss of function of Wnt signalling and to ascertain whether Wnt signalling plays a role *in vivo* I prepared acute brain slices from both wildtype and Wnt7a-/-, Dvl1-/- mutant mice. Prior data has suggested that the expression of Wnt7a and related signalling molecules may be strongest in the wildtype animal at the MF-CA3 synapse (Gogolla et al., 2009; Sahores et al., 2010) so I focused on this synapse. Postsynaptic currents were again elicited by the positioning of a stimulating electrode on the mossy fibre tracts of the stratum lucidum, activating presynaptic granule cell axons.

Postsynaptic currents generated in the double knockout animals are significantly smaller than those generated in wildtype animals. Indeed a 60% decrease in current amplitude was observed (fig5.2 a and c). The percentage of postsynaptic failures also increases by 22% in the double mutant when compared to the wildtype. Thus this is the converse effect to that obtained with Wnt gain of function studies. Here *in vivo* Wnt7-Dvl1 loss of function clearly results in defects in synaptic function. These results demonstrate a role for Wnt7a –Dvl1 signalling in enhancing the efficiency of currents generated in the postsynaptic component of the synapse.

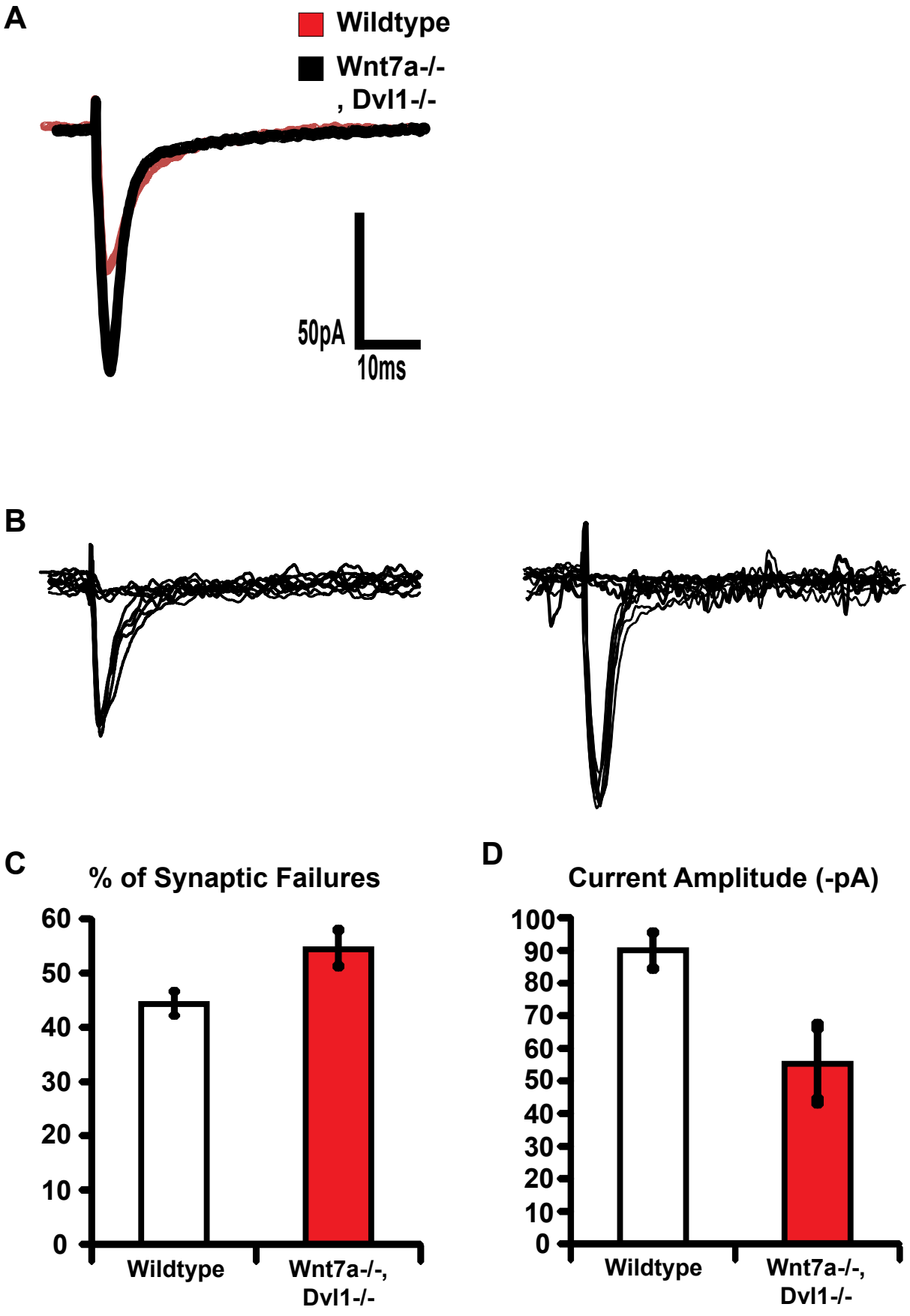

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Figure 5.2 Loss of Wnt7a-Dvl1 signalling decreases evoked postsynaptic currents and increases synaptic failure rates at the MF-CA3 synapse. Acute hippocampal slices were prepared from P14 wildtype and Wnt7a-/-, Dvl1-/- null mice and stimulus evoked currents were recorded by whole cell patch clamp. (A) Left panels show overlay of 10 consecutive stimulus runs with representative synaptic failures. Top panel shows currents from Wildtype slices whilst lower panel displays currents from double knockout slices. Right panels display average sweep from wildtype or double knockout cells. (B) Quantification of synaptic failure rates given as %failures of total stimulus events. (C) Quantification of averaged currents amplitude reveals an increase after Wnt7a treatment. N numbers refer to number of animals used, Wildtype=5, Wnt7a-/-, Dvl1-/-=7 (* = $P<0.05$)

5.2.3 Wnt7a gain of function decreases paired pulse ratios in hippocampal cultures.

My experiments looking at AMPAR mediated currents suggest that there is a strong relationship between the receptor acumulation described in the previous chapters and synaptic function. The changes in the amplitude of postsynaptic currents suggest the movement of AMPAR subunits contributes to a functional change at the synapse as opposed receptor mobilization into reserve pools around or near the synapse. The effects on failure rate suggest that Wnt signalling increases the activity at these synapses which may be the result of changes both pre or postsynaptically. The ability of Wnt7a to act as a presynaptic organiser has been demonstrated in various studies however the functional effects of Wnt7a at presynaptic compartments are poorly understood (recent reviews (Farias et al., 2010; Salinas and Zou, 2008).

In order to investigate the functional presynaptic effects of Wnt7a signalling, I assessed the paired pulse ratio (PPR) at the MF-CA3 synapse after Wnt7a exposure. PPR was determined by eliciting a pair of stimulus events separated by a short interval (50ms) and measuring the ratio of the first resultant current to the second. This form of short term potentiation or depression can be used to assess the presynaptic efficiency of a synapse. Where the PPR is modified positively (greater than 1) presynaptic release is likely to be inhibited whereas if

PPR is modified negatively (lower than 1) presynaptic release is likely to have been enhanced (Cerpa et al., 2008; Dobrunz and Stevens, 1997; Schulz et al., 1994).

In 14DIV hippocampal cultures, Wnt7a significantly decreases PPR from 1.03±0.053 in vehicle treated cultures to 0.84±0.012 (fig5.3 a and c). Confirming the previous single stimulus experiment (fig5.1), comparison of first current amplitude shows a 51% increase in amplitude between vehicle and Wnt7a treated neurons (fig5.3 a and b).

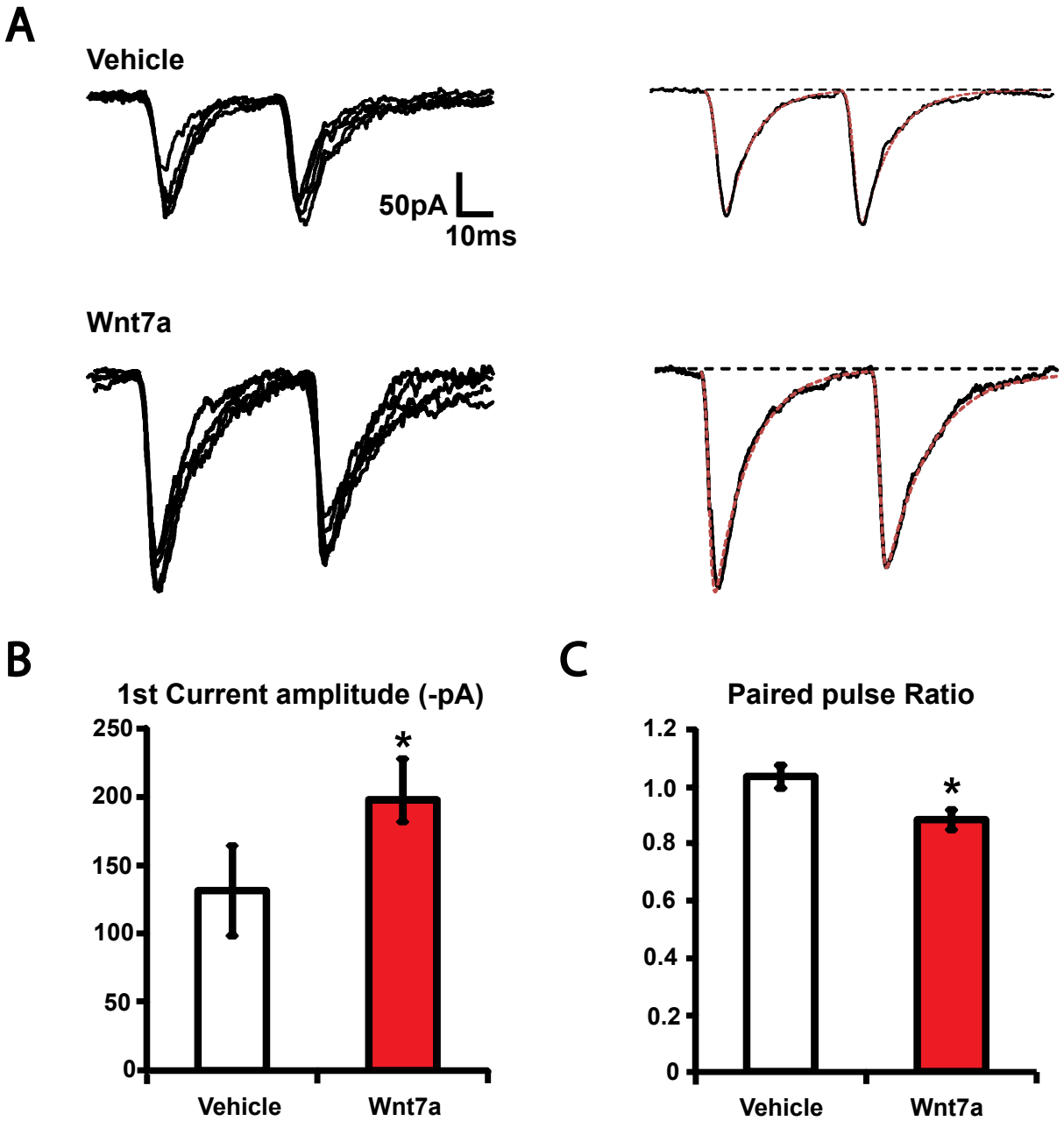

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Figure 5.3. Wnt7a signalling increases presynaptic efficacy and postsynaptic currents. 14 DIV hippocampal cultures were treated with BSA (vehicle) or Wnt7a for 3 hours and paired currents were recorded by whole cell patch clamp. Stimuli were separated by 50ms and currents were recorded and analysed (A) Left panels show overlay of 10 consecutive stimulus sweeps whilst top panel shows currents from vehicle treated whilst lower panel displays currents from Wnt7a treated neuron. Right panels display average sweep from vehicle or Wnt7a treated cell respectively. (B) Quantification of the first currents in the pair for each treatment. The figure clearly shows a significant increase in current amplitude in Wnt7a treated cells when compared to controls. (C) Quantification of current 2 amplitude/current 1 amplitude displayed as the PPR value Wnt7a decreases the amplitude of the second current in the pair, represented as a decrease in PPR value. N numbers of cells, BSA=12, Wnt7a=15 (* = $P<0.05$)

5.2.4 Wnt7a/Dvl1 loss of function at MF-CA3 synapses results in a decreased Paired pulse ratio.

Witnessing this effect of Wnt7a in cultured neurons, I then decided to test whether loss of function of Wnt signalling also affects PPR at the MF-CA3 synapse. The Wnt7a, Dvl1 double mutant exhibits an increase in PPR at the MF-CA3 synapse from 1.05±0.034 in the wildtype mice to 1.23±0.055 in the mutant mice. Loss of Wnt7a/Dvl1 function in the hippocampus also decreases the amplitude of the first currents by 28% (fig5.4 a and b). These results suggest that Wnt7a signalling plays a functional role in regulation of presynaptic strength in the hippocampus in addition to postsynaptic changes.

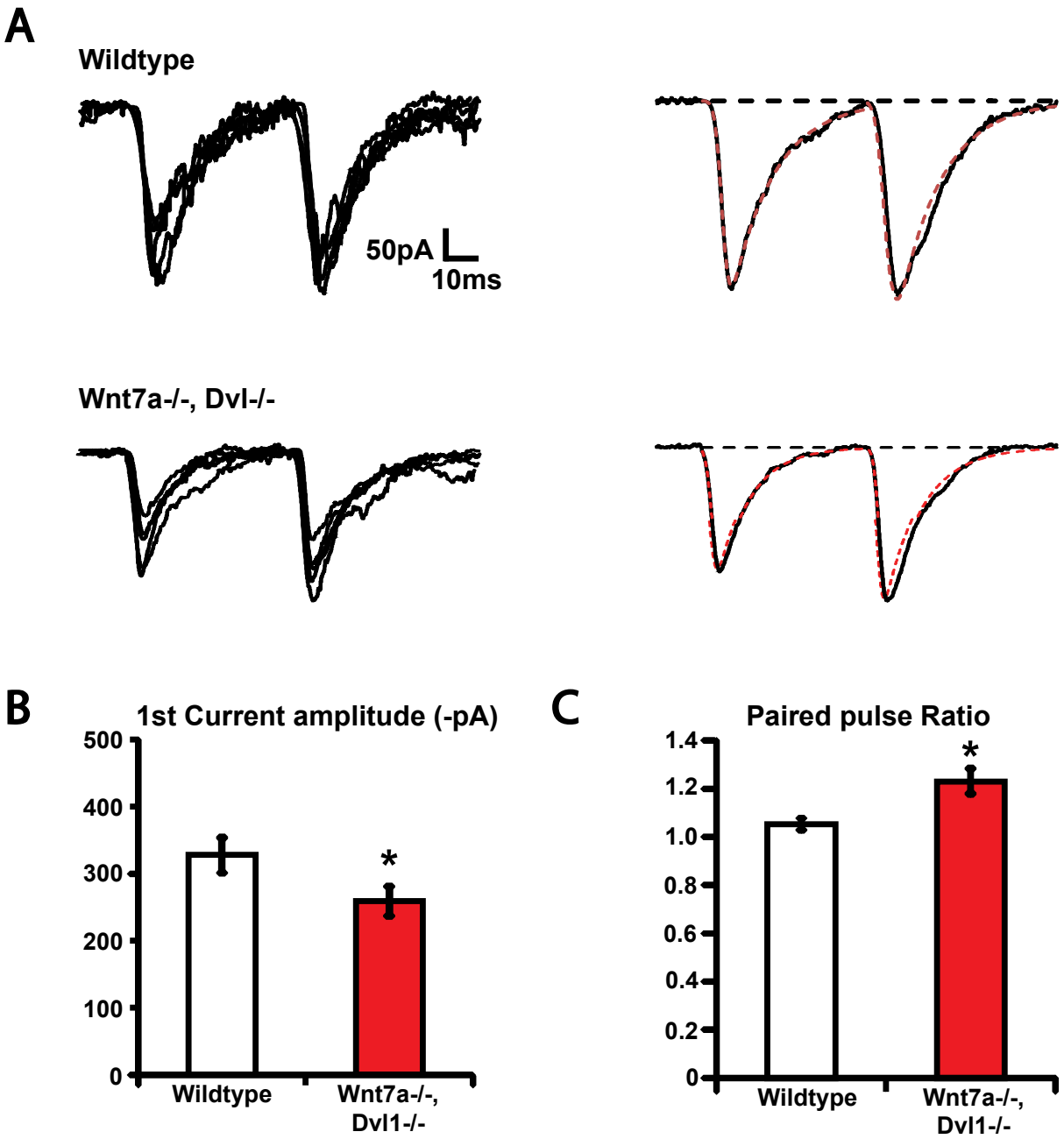

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Figure 5.4 Loss of Wnt7a-Dvl1 signalling decreases presynaptic efficacy and postsynaptic currents. Acute hippocampal slices were prepared from P14 wildtype and Wnt7a-/-, Dvl1-/- null mutant mice and paired currents were recorded by whole cell patch clamp. (A) Left panels show overlay of 10 consecutive stimulus runs. Top panel shows currents from wildtype slices whilst lower panel displays currents from double knockout slices. Right panels display average sweep from wildtype or double knockout cells. (B) Quantification of the first currents in the pair for each treatment. The figure shows a significant decrease in current amplitude in double knockout cells. (C) Quantification of current 2 amplitude/current 1 amplitude displayed as the PPR value loss of Wnt7a-Dvl1 signalling increases the amplitude of the second current in the pair, represented as an increase in PPR value. N numbers refers to number of animals used, Wildtype=4, Wnt7a-/-, Dvl1-/-=8 (* = $P<0.05$)

5.3 Discussion

The results presented in this chapter demonstrate the novel function of Wnt7a to enhance synaptic transmission at both the pre and postsynaptic terminals. Using minimal stimulation and 3 hour exposure to Wnt7a or slices from Wnt7a-Dvl1 null mice, I demonstrated that Wnt7a gain of function increases the number of successful presynaptically mediated postsynaptic events and increases the amplitude of these currents whilst in vivo loss of Wnt7a-Dvl signalling has the opposite effects. These results indicate that Wnt7a regulates both pre and postsynaptic sides of the synapse by increasing synaptic transmission. These results are consistent with findings presented in previous chapters showing that Wnt7a increases the levels of GluA1 and GluA2 subunits to synaptic sites and the co-localization of GluA1 and vGlut1. The increase in postsynaptic current amplitude specifically suggests new synapses Wnt7a promotes are also AMPAR functional sites.

5.3.1 Wnt signalling regulates AMPAR mediated function at hippocampal synapses.

Prior to my study the role of Wnt7a in functional synaptic transmission was poorly understood however studies with similarly synaptogenic molecules reveal similar effects to the Wnt7a mediated results demonstrated in this chapter. Neuroligin1 (NL-1) has been identified as an excitatory synaptogenic factor in hippocampal neurons (Chih et al., 2005; Levinson et al., 2005; Prange et al., 2004). Interestingly postsynaptic overexpression of NL-1

increases both AMPAR and NMDAR mediated currents via an NMDAR dependent mechanism (Chubykin et al., 2007). These results are particularly interesting in the light of results from our lab indicating an NMDAR dependent basis for Wnt7a mediated spine morphogenesis (Ciani and Salinas, unpublished).

The postsynaptic effects of loss of Wnt7a, Dvl1 signalling further confirms the importance of these molecules in development of the postsynaptic compartment. Interestingly the long-term deprivation of Wnt7a, Dvl1 signalling still generates normal basal synaptic transmission in the hippocampus (Boyle and Salinas, 2010). The loss of Wnt7a and Dvl1 however results in a defect of both the size and success of stimulus evoked currents. These results confirm the effects seen in the mEPSCs of the same animals suggesting loss of Wnt7a, Dvl1 signalling decreases synapse number and results in less functional strength at the MF-CA3 synapse. These defects also confirm the functional importance of Wnt7a signalling in the CA3 region of the hippocampus as demonstrated by Caroni and colleagues.

Interestingly Cerpa and colleagues' recent study suggests that Wnt5a but not Wnt7a mediates postsynaptic Wnt signalling (Cerpa et al., 2008). Their study implies that whilst Wnt5a alone has functional effects on NMDAR signalling neither Wnt5a nor Wnt7a signalling contribute to AMPAR transmission in the hippocampus. In contrast my studies clearly demonstrate a postsynaptic role for Wnt7a. The reason for the different results is unclear however, it is worth mentioning that Cerpa's experiments were performed by exposing cells to Wnt7a for a short period of time. This short term treatment may have been sufficient to initiate receptor mobilisation but not enough to register significant functional output. In contrast, my experiments were performed after 3 hours of Wnt7a exposure. Another important issue is the synapse studied. Wnt7a localises very strongly at the CA3 region of the hippocampus in comparison to the CA1 region (Gogolla et al., 2009). This data suggests that Wnt7a signalling plays a greater role at the MF-CA3 synapse region than at the CA3-MF synapse. Indeed by studies using Wnt7a; Dvl1 mutant mice demonstrate a clear defect at the CA3-MF synapses.

Considering the data presented in this chapter using both Wnt7a gain and loss of function we can conclude that these signalling proteins are crucial in the regulation of hippocampal synaptic strength. The localisation of Wnt7a signalling molecules indicate that this role is particularly significant at the MF-CA3 synapse where loss of Wnt7a signalling results in functional defects. In mixed CA3/CA1 hippocampal cultures however Wnt7a gain of function

over a 3hr period strongly enhances functional synaptic transmission pre and postsynaptically. The striking similarities between NL-1 and Wnt7a gain of function phenotypes further demonstrate the unique ability of these extracellular molecules to enhance postsynaptic function.

5.3.2 Wnt7a mediated enhancement in presynaptic function in the hippocampus.

Enhancement and defects in PPR in respective Wnt loss of function and gain of function experiments indicate Wnt7a mediated changes to the efficacy of presynaptic release at these synapses. This conclusion is further supported by both increase and decrease of spontaneous failures in response to loss or gain of Wnt7a signalling respectively.

An interesting similarity between my data and the data produced by Cerpa and colleagues (2008) mentioned in the last section was the identification of a functional role of Wnt7a signalling presynaptically in hippocampal synapses. Cerpa and colleagues observe a decrease in facilitation index after Wnt7a treatment in hippocampal CA1 neurons after 30min (Cerpa et al., 2008). Similarly, I showed that loss of function of Wnt7a signalling at the MF-CA3 synapse increases PPR. Taken together with the data presented in previous chapters showing both enhancements and decreases in mEPSC frequency in relation to Wnt7a gain and loss of function respectively these results suggest a role in presynaptic release efficiency.

BDNF shares many functional roles with the Wnt family of proteins and indeed plays a similar role in synaptic organisation and the function of presynaptic release in the hippocampus (Caldeira et al., 2007; Jovanovic et al., 2000). PPR is decreased in neurons CA1 neurons of the hippocampus when exposed to BDNF (Mohajerani et al., 2007). Similarly a decrease in synaptic failures was observed and an increase in the frequency of spontaneous (miniature) EPSCs. Interestingly it has been postulated that BDNF activity/release is related to presynaptic activity in a positive feedback loop to regulate presynaptic aspects of LTP. Enhanced neurotransmitter release as a result of LTP results in enhanced BDNF release which acts a secondary messenger at the synapse to further increase presynaptic release efficiency (Jia et al., 2010). The same relationship between Wnt signalling synaptic function and release have been established and investigated in various studies (Gogolla et al., 2009; Sahores et al., 2010). It is likely that that this form of Wnt7a mediated synaptic enhancement feedback loop may occur and have effects both pre and postsynaptically.

Chapter 6

Discussion

6.1 Summary of Results

During my PhD project I have investigated the role of Wnt signalling in various aspects of hippocampal synapse formation and function. I used a combination of cellular and electrophysiological techniques together with gain and loss of function approaches. My results demonstrate the crucial role of Wnt7a in the regulation of excitatory synapses in the hippocampus. Cellular changes induced by loss or gain of function of Wnt7a results in profound changes in various functional aspects of synaptic transmission. The key major findings are the demonstration that Wnt7a specifically promotes the formation of excitatory synapses and that Wnt7a increases synaptic strength. This is the first report that demonstrates Wnt signalling modulates the strength of synapses.

Wnt7a increases the formation of excitatory synapses, this is achieved by promoting the increased synaptic localisation of excitatory synaptic markers. Specifically, the presynaptic marker vGlut1 (responsible for the recycling of neurotransmitter and the surface expression of AMPAR receptor subunits) GluA1 and GluA2. These subunits comprise the major components of functional "AMPAR active" synapses on mature neurons (Bredt and Nicoll, 2003; Hall and Ghosh, 2008). The increase in AMPAR subunits is complemented by an increase in the total expression of GluN1 in dendritic spines. Furthermore, Wnt7a increases the association of excitatory presynaptic markers with postsynaptic markers, thus indicating the ability of Wnt7a to regulate the formation of excitatory synapses. Postsynaptic colocalisation assays also suggest greater association of the different glutamate receptor subunits (GluA1 with GluN1) with each other at these potential synaptic sites suggesting that these sites may be more postsynaptically active.

Consistent with a role for Wnt7a in excitatory synapse formation, Wnt7a enhances mEPSC frequency and amplitude without affecting inhibitory minis. mEPSCs recorded from CA3 neurons in acute slices prepared from *Wnt7a, Dvl1* mutant mice demonstrate that deficiency in Wnt7a;Dvl1 signalling results in defects in both frequency and amplitude of excitatory minis. Importantly, these results demonstrate the crucial role Wnt7a signalling plays in the development of the functional synaptic circuitry of the hippocampus. These results also demonstrate regulatory roles of Wnt7a both pre- and postsynaptically *in vitro* and *in vivo*. In contrast, this role of Wnt signalling is diminished on the synapses of mature neurons. Wnt

signalling gain of function experiments revealed no significant effect on the localisation of the synaptic markers; vGlut1, GluA1, GluA2 or GluN1.

In this thesis, I demonstrate a role for Wnt7a/Dvl1 signalling as a potent organiser of excitatory transmission. Wnt7a increases spines number and size whereas overexpression of Dvl1 specifically increases spine size. These morphological Wnt mediated changes are complemented by increases in the number of puncta for postsynaptic glutamatergic receptor subunits GluA1, GluA2 and GluN1 concomitantly with an increase in the number of the presynaptic marker vGlut1. These results demonstrate that Wnt7a increases excitatory innervation. As dendritic spines are the major site of excitatory synaptic transmission in the many regions of the brain these results indicate a major role for Wnt7a signalling in the regulation of excitatory transmission.

Finally, I demonstrate that Wnt7a signalling plays a role in AMPAR mediated synaptic transmission. In 14DIV hippocampal neurons, Wnt7a increases the amplitude of synaptic currents whilst decreasing the number of synaptic failures. Moreover, Wnt7a increases presynaptic release efficiency as determined by the PPR. These results from cultured neurons were strengthened by data obtained from recordings at the MF-CA3 synapse using acute slices from wild-type and *Wnt7a, Dvl1* knockout mice. These results confirm the role of Wnt7a by displaying defects in evoked current amplitude, increases in the number of synaptic failure and defects in presynaptic release efficiency as determined by PPR. The enhancements and defects in synaptic failure rates clearly demonstrate the role of wnt signalling in producing successful synaptic transmission. These results suggest modulation to levels of Wnt proteins at synapses may be a means to control synaptic transmission in an on/off mechanism as required by the neural circuitry. Combined with the changes to presynaptic release efficiency determined by PPR as a result of Wnt signalling it is clear Wnt7a signalling plays roles in pre and postsynaptic transmission.

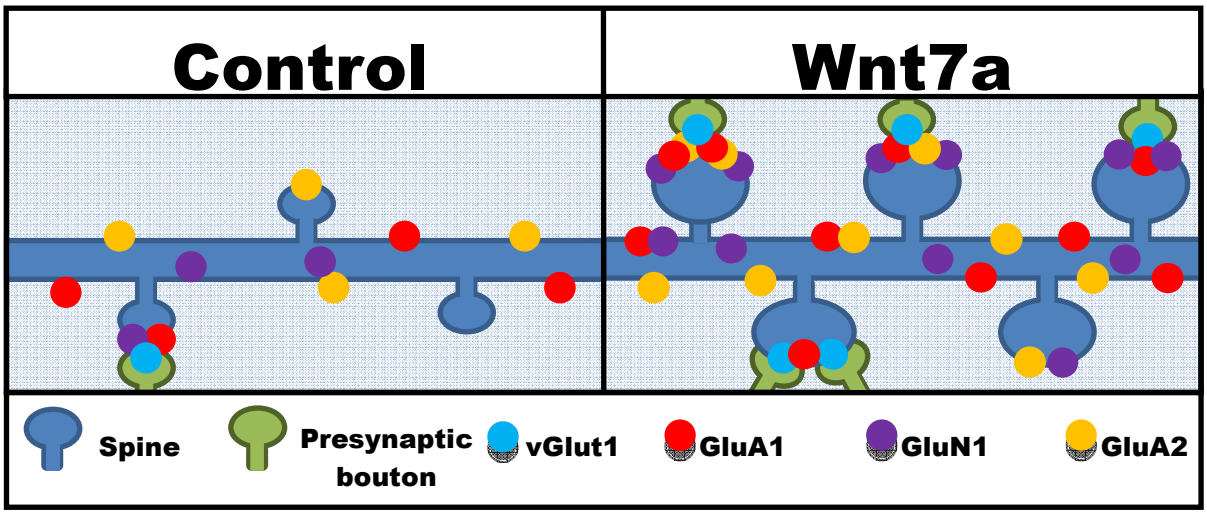

Figure 6.1 Wnt7a signalling increases excitatory synapse strength by enhancing synaptic protein localisation on spines. In control neurons vGlut1, GluN1, GluA1 and GluA2 are distributed throughout the dendrite whilst spine sizes vary from small/immature to larger/mature. In the presence of Wnt7a, the number and size of dendritic spines are increased. This is accompanied by an increase in the accumulation of postsynaptic glutamate receptors on spines. An increase in the apposition of presynaptic markers such as vGlut1 on dendritic spines also indicates an increase in the number of innervated spines.

6.2 The role of Wnt7a signalling in pre- and postsynaptic excitatory synapse formation.

The role of Wnts has been well established in the presynaptic regulation of neural circuit formation particularly preceding apposition of the pre and postsynaptic specialisations. Indeed, experiments performed by Ahmad-Annuar and colleagues (2006) demonstrated the role Wnt7a plays in the accumulation of presynaptic components such as bassoon and synapsin. Electrophysiological recordings of the mEPSCs in the cerebellum of *Wnt7a, Dvl1* knockout mice revealed a decrease in the frequency of currents but no effect on the amplitude of currents (Ahmad-Annuar et al., 2006). These results demonstrate the specific presynaptic role of Wnt7a signalling at the MF-Granule cell synapse.

The above role of Wnt7a in the cerebellum is interesting when compared to its role in hippocampus. At the MF-granule cells synapse, mEPSC recordings indicate a strong role for Wnt7a/Dvl1 signalling in the presynaptic side. This was also observed in hippocampal neurons. Changes in both failure rate and PPR in Wnt7a gain and loss of function experiments indicate a crucial role for these proteins at hippocampal synapses. However, the mechanisms by which Wnt regulates release efficiency are poorly understood. Alongside

changes to vGlut accumulation, changes in the accumulation of synaptophysin and synapsin at synaptic sites have been observed in response to Wnt signalling modification (Ahmad-Annuar et al., 2006; Cerpa et al., 2008). Postsynaptically, the effect of Wnt7a/Dvl1 loss is much less apparent, with the only significant defect being a decrease in PSD width as assessed by electron-microscopy in the cerebellum (Ahmad-Annuar et al., 2006). In contrast, loss of Wnt7a/Dvl1 signalling at the hippocampal MF-CA3 synapse generates defects in both the frequency and amplitude of mEPSCs. Further analysis of the ultrastructure of synapses at the MF-CA3 synapse in these double knockout animals reveals strong defects in PSD size (Ciani and Salinas, unpublished).

Understanding the differences in the roles of the various Wnt proteins in hippocampal synapse formation has garnered interest from a large number of groups in recent years. The data generated by the various labs is producing a clearer picture of the role each Wnt species plays in the hippocampus. My results demonstrate that Wnt7a signals to both the pre and postsynaptic sides to promote synapse formation. Specifically, analysis of neurons in which Dvl1 signalling is activated postsynaptically, reveal enhancements in both the postsynaptic increase of glutamate receptor subunits and the presynaptic gain of vGlut1 inputs alongside increases in dendritic spine volume (Ciani et al., 2011). This data is further complemented by experiments using similar postsynaptic activation of Dvl1 in postsynaptic neurons. The resultant mEPSCs display enhancements both in frequency and amplitude, perhaps indicating both pre and postsynaptic effects of postsynaptic activation of Wnt signalling (Ciani et al., 2011).

In comparison, recent publications describe the action of Wnt5a, which is also believed to act postsynaptically. Similar to the action of Wnt7a, Wnt5a increases spine density, and mEPSC amplitude (Varela-Nallar et al., 2010). Wnt5a mediated increases in NMDAR currents are demonstrated to be regulated by the activity of both PKC and JNK (Cerpa et al., 2011). Wnt5a also seems to be less specific in the synapses it interacts with, affecting both inhibitory and excitatory synapses. These results are interesting in light of data generated by our lab which demonstrate the role of CaMKII in Wnt7a/Dvl1 signalling at the postsynaptic side (Ciani et al., 2011). Whilst it is not unusual for two related signalling molecules to activate different signalling pathways it is none-the-less interesting that these pathways can be employed to induce different responses. These subtle variations in the effects different Wnts have at the synapse provide neuronal circuits with an array of system tweaking tools.

6.3 Wnt7a signalling strongly regulates synaptic transmission in the CA3 region of the hippocampus and the implications for memory and learning

InterestinglyWnt7a/Dvl1 signalling seems to contribute differentially to various sub-regions of the hippocampus. At the MF-CA3 synapse I observed defects in evoked synaptic amplitudes and PPR from Wnt7a;Dvl1 null mice. mEPSCs recorded at the CA3-CA1 synapse of Wnt7a/Dvl1 knockout mice revealed no significant defects in either frequency or amplitude (Boyle and Salinas, unpublished). Interestingly, the same study identifies defects in presynaptic release efficiency at this synapse suggesting that the role of Wnt7a/Dvl1 signalling at the MF-CA3 synapse may have knock on deleterious effects at downstream synapses in the hippocampus (Boyle and Salinas, 2010). Indeed experiments examining lesions in upstream regions of the hippocampal tri-synaptic circuit (dentate gyrus (DG), CA3 cell layer) generated results similar to complete hippocampal ablation in memory and learning tests (Emerich and Walsh, 1989; Handelmann and Olton, 1981; Sutherland et al., 1983; Walsh et al., 1986).

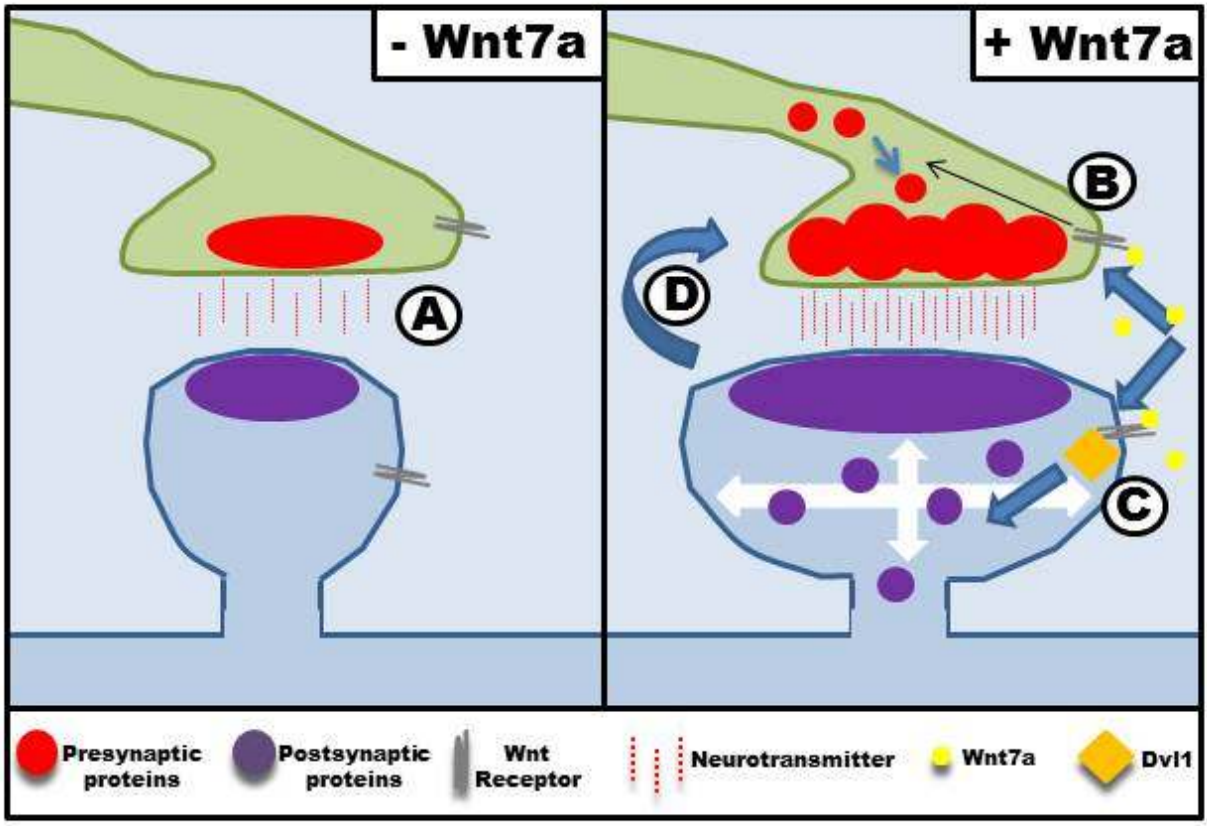

Figure 6.2 Wnt7a signals directly to both the pre and postsynaptic terminals. (A) In the absence of exogenous Wnt7a functional synaptic transmission is maintained. (B) In the presence of exogenous Wnt7a, Wnt binds directly to receptors on the presynaptic terminal. Here Wnt signalling enhances trafficking of presynaptic proteins to the site of release enhancing the strength of neurotransmitter release. (C) Wnt7a directly signals at postsynaptic sites and requires Dishevelled-1 to stimulate postsynaptic growth. Wnt7a signalling enhances both the localisation of postsynaptic receptor and scaffolding proteins whilst increasing spine head volume. (D) Changes to the postsynaptic spine promote retrograde signalling which may further enhance presynaptic input at these sites.

The hippocampus has long been identified as a primary brain area required for learning and memory. Our current understanding of the hippocampal circuitry suggests each region is responsible for different aspects of memory learning and consolidation and these differences are generated by addition inputs to the various sub-regions from the entorhinal cortex (Witter, 1993). Learning tasks requiring the rapid uptake of novel information have been demonstrated CA3 dependent (Lee and Kesner, 2003; Nakazawa et al., 2003). Some aspects of memory and learning appear to be uniquely mediated by the CA3 and its proximal, medial and distal sub-regions. Gilbert and Kesner (2003) identified defects in multiple trial tasks as a result of CA3 lesions not mimicked by lesions in the CA1 or DG regions (Gilbert and Kesner, 2003). The CA3 has also been implicated in the integration of arbitrary associative memory. This form of memory describes the association of two spatially separated memories such as the location of an object stored in the parietal cortex with the identity of an object stored in the temporal cortex (Kesner, 2007). Investigation using pharmacological block of activity of parietal and or temporal cortex afferents into the CA3 demonstrated significant defects in object-location trials of rats (Hunsaker et al., 2007), whilst CA3 lesion similarly impaired rats in specific object–location paired tests (Gilbert and Kesner, 2003).

The important role of CA3 synapses and neurons in the maintenance of learning and memory paradigms suggests impairment in their function may have serious effects in the behaviour of an organism. Both experiments using Wnt7a gain of function in mixed CA3/CA1 neuronal cultures and loss of Wnt7a/Dvl1 function at the MF-CA3 synapse indicate potential functional synaptic dysfunction in the hippocampus potentially resulting in defects to the normal behaviour of these animals. Interestingly lack of Wnt7a, Dvl1 signalling in null

animals birth results in normal development of the hippocampal architecture and neurons which are synaptically active. However the impairment rather than complete loss of activity generated by loss of Wnt7a/Dvl1 signalling suggests that the role Wnt7a plays is more subtle perhaps more involved in the modulation or up-regulation of synaptic strength and maturity in response to presynaptic activity from hippocampal inputs. These roles of Wnt7a fit with several studies implicating expression of Wnt7a and other Wnt species as a function of increased synaptic activity.

Two studies in particular relate specifically to Wnt7a expression in the hippocampus in relation to heightened synaptic activity/learning paradigms. Gogolla and colleagues demonstrated an increased expression of Wnt7a/7b, using immunocytochemistry specifically in the hippocampal CA3 (Gogolla et al., 2009). Whilst data from our lab (Sahores et.al 2010) demonstrates the enhanced release of species which bind the Fz-5 receptor (of which Wnt7a is member), in response to high frequency stimulation, promoting synapse formation (Sahores et al., 2010). My results from both gain and loss of Wnt7a function suggest that Wnt7a is able to mediate the synaptic demand for both greater presynaptic contacts and postsynaptic glutamate receptor proteins to transduce the enhanced presynaptic release activity.

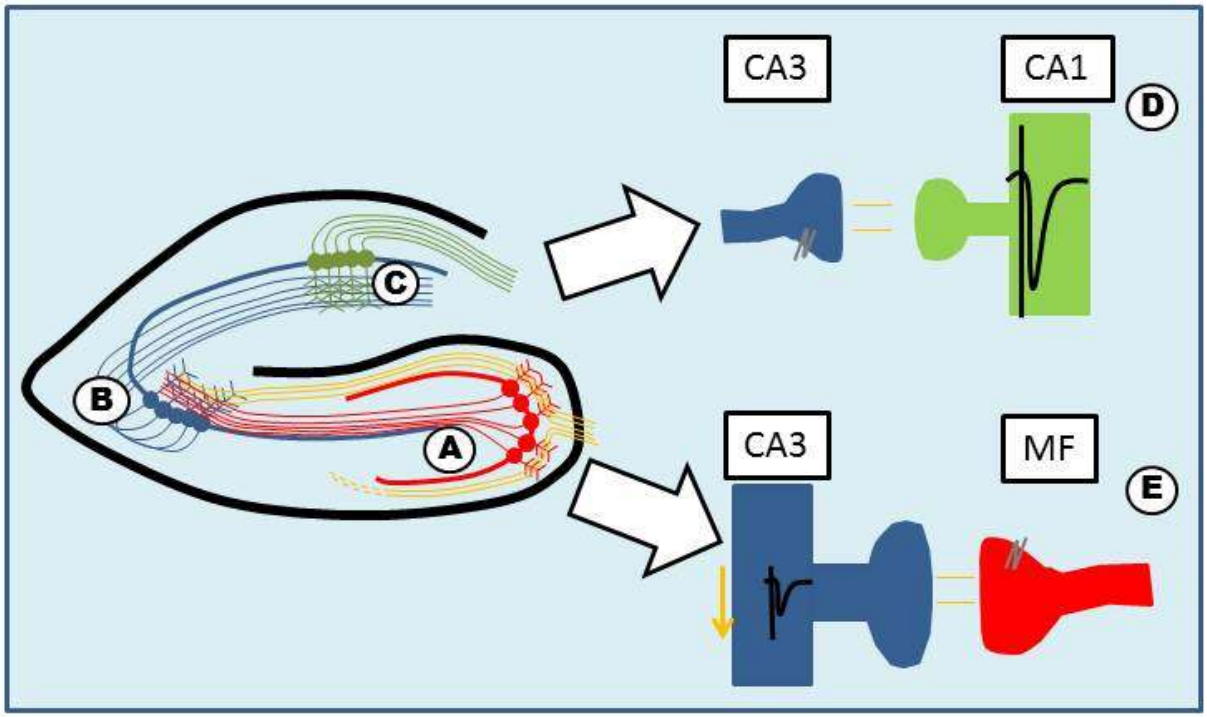

Figure 6.3 Wnt signalling within the Hippocampal circuitry regulates learning and memory. (A) Loss of Wnt7a/Dvl1 signalling at the mossy fibre axons negatively influences release of neurotransmitter at postsynaptic targets. The majority of these axons form large terminals at CA3 neurons but also synapse onto inhibitory interneurons. (B) Loss of Wnt7a/Dvl1 signalling decreases postsynaptic response in CA3 pyramidal neurons. (C) loss of Wnt7a/Dvl1 signalling generates defects in PPR and current amplitude at CA3-CA1 synapse further illustrating the importance of Wnt signalling in the hippocampus (Boyle and Salinas, unpublished). (D) Wnt receptors are present but Wnt7a and Dvl are no longer present at the CA3-CA1 synapse in the knockout mice. This loss of signalling here generates detectable presynaptic defects in PPR but not in EPSC amplitude. (E) At the MF-CA3 synapse loss of Wnt7a and Dvl1 negatively affect presynaptic release and postsynaptic amplitude.

It is interesting to speculate as to whether high levels of neuronal activity generating high levels of Wnt7a release are able to modulate hippocampal circuitry not just at the level of synapses but at the level of pathways by enhancing activity along one pathway to the extent that other pathways become less significant purely by the activity of Wnt7a alone. In this way modulation of hippocampal activity at the level of wiring may be mediated by Wnt7a or a battery of Wnt species.

6.4 The role of Wnt7a signalling in neurodegenerative and neurological disorders

Defects in the Wnt signalling pathway contribute to cancer, and effects on cell homeostasis and neuronal circuit formation and function. Recent studies have begun to reveal roles for these proteins in neurological disorders. The preferential role of Wnt7a/Dvl1 signalling in excitatory synaptic transmission (Ciani et al., 2011) and synapse formation may link the expression of these gene products to several neurological and neurodegenerative disorders.

The specific enhancement of excitatory synaptic activity over inhibitory synapses in the hippocampus is a phenotype often observed in epilepsy (Leite et al., 2005). Interestingly the epilepsy forming paradigm ‘kindling’ mimics several aspects of Wnt mediated excitatory synaptic enhancement. These changes include increases in synapse number in the basal hippocampus and LTP like increases in synaptic transmissions (Geinisman et al., 1990). Intriguingly, kindling eventually promotes the loss of postsynaptic neurons and the sprouting of new high frequency release mossy fibre terminals believed to play a role in the eventual epileptic events. Whilst the long term overexpression of Dvl1 in the hippocampal neurons

does not seem to cause such extreme defects it would be interesting to assess the cumulative effect of further Wnt activation on the basal activity of transmission. It is likely that a massive up-regulation of Wnt signalling (possibly involving several pro-synaptogenic Wnts for excitatory synapses) would be required to observe such epileptic activity.

Wnt signalling mediated regulation of both dendritic spines and spine associated receptors mimic phenotypes present in neurocognitive and neurodegenerative disorders such as Alzheimer's (AD), Parkinson disease and schizophrenia (D'Amelio et al., 2011; Garey et al., 1998; Zaja-Milatovic et al., 2005). Often these disorders are associated with loss or dismorphia of spine synapses, a phenotype similar to those observed in Wnt7a/Dvl1 loss of function (Ciani et al., 2011). Intriguingly up-regulated Wnt signalling is already believed to be a potential target in the future treatment of AD (Boonen et al., 2009).

In some models Familial Alzheimer's disease (FAD) has been linked to mutations in the presenilin gene family which in turn influence, Wnt signalling (Boonen et al., 2009). Presenilins promote the degradation of cytoplasmic β-catenin inhibiting canonical Wnt signalling (Kang et al., 2002). Analysis of the various FAD associated presenilin mutations (FAD-PSEN1) against various PSEN backgrounds generates a range of cell survival phenotypes which may contribute to AD pathogenesis (Boonen et al., 2009).

Tau proteins, upon hyper-phosphorylation, form the paired helical filaments which accumulate to produce the tangles so associated with AD pathology (Goedert et al., 2006). Tau hyper-phosphorylation represents a second model of AD pathogenesis with links to Wnt signalling. Hyper-phosphorylated Tau is unable to interact with microtubules affecting microtubule dynamics and promoting pathogenic tangle formation (Ballatore et al., 2007). GSK-3β, a key component of the canonical Wnt signalling pathway, is a major kinase that phosphorylates Tau and as such its activity plays a role in the regulation of AD pathogenesis (Lovestone et al., 1994).

Consistent with the view that dysfunction of Wnt signalling could contribute to AD, Dkk1 (Dickopf 1), an inhibitor of canonical Wnt signalling is linked to AD. Caricasole and colleagues, (2004) demonstrated beta amyloid induced expression of Dkk1 in cultured cortical neurons. Dkk1 expression was also observed in colocalisation with neurofibrillary tangles in the brains of AD patients (Caricasole et al., 2004). Data from our lab also reveals a strong anti-synaptogenic role of Dkk1 in the hippocampus resulting in synaptic disassembly

(Dickens and Salinas, unpublished). Indeed, increased levels of Dkk1 induced by Aß could contribute to decreased synapse density in the brains of AD sufferers (Scheff et al., 2006).

Excitingly a recent study by De Ferrari and colleagues (2007) has revealed the direct link of a Wnt signalling molecule to human late onset Alzheimer's disease. Mutations in the LRP6 co-receptor gene have been associated with Alzheimer's disease presenting a significant risk factor to carriers (De Ferrari et al., 2007). Interestingly Wnt7a signalling is believed to be mediated by a Fz-5/LRP6 receptor complex potentially indicating Wnt7a signalling to AD (Caricasole et al., 2003). Further studies are necessary to fully establish a role for Wnt signalling in AD.

Whilst the roles of Wnt signalling in AD and synapse formation are likely to require different signalling pathways this none the less represents an area in which greater research may help to produce therapies.

Discussing the work I have undertaken and the results I have observed during my PhD with friends outside of science I have often been asked of the significance my work may have in "the real world". I have always hoped that with our understanding of the role this gene product plays in both the enhancement of synapse number and function and the role it plays specifically at excitatory over inhibitory synapses that this may one day lead to therapies for disorders with complementary aberrant phenotypes. In particular the advent of effective gene therapies and continued research into the expression and function of Wnt proteins may allow treatments for diseases mentioned above such as epilepsy, Alzheimer's and Parkinson's. Furthermore, further research into the context specific release and function of Wnt proteins in response to activity and learning paradigms may far into the future help us develop a better picture of the molecular mechanisms and signalling molecules of memory and learning.

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