The role and regulation of Frizzled receptors in synapse formation

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I, Alessandro Bossio, declare that the work presented here 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 synapses is crucial for brain function. Secreted Wnt proteins signal through Frizzled and other receptors to regulate synaptogenesis. In particular, Wnt7a promotes synaptogenesis in the hippocampus. The receptor Fz5 mediates Wnt7a-induced presynaptic assembly, but the mechanisms underlying Fz5 regulation are not well understood. How Wnt7a signals at postsynaptic sites is also unknown. Fz7, another receptor binding Wnt7a, is hypothesised to have a role in this process.

To address these questions, I used biochemical and cell biology techniques combining *in vitro* and *in vivo* approaches. My findings demonstrate that Fz5 and Fz7 have distinct synaptic localisation. Fz5 is absent from dendritic spines - excitatory postsynaptic structures - and is not required for spine development. In contrast, Fz7 localises in spines and is required for Wnt7a-induced spine formation.

Our preliminary data suggested that Fz5 is palmitoylated, a post-translational lipid modification that affects protein distribution and function. I demonstrated that all Frizzled receptors can be palmitoylated. Using a palmitoylation-deficient Fz5 receptor, I showed that palmitoylation is required for Fz5 interaction with the scaffold protein Dishevelled, a key component of the Wnt signalosome, but has no impact on Fz5 degradation rate and lateral mobility at the plasma membrane. Palmitoylation-deficient Fz5 exhibits impaired axonal distribution, increased endocytosis and decreased surface levels. Expression of wild-type Fz5 in the hippocampus promotes presynaptic assembly, whereas palmitoylation-deficient Fz5 lacks synaptogenic activity. Palmitoylation is therefore a critical molecular mechanism that underpins Fz5 regulation and function *in vivo*.

These findings demonstrate that two distinct Frizzled receptors act pre- and postsynaptically to promote synaptogenesis, and reveal a previously uncharacterised lipid modification of Frizzled receptors, which is of critical functional importance. This work opens up new avenues to study the role of Frizzled palmitoylation in different biological contexts, from cell fate decisions to neuronal circuit formation and plasticity.

Impact Statement

During my PhD I studied the mechanisms that regulate Wnt signalling in the context of synapse formation in the brain. Wnts are secreted proteins that signal through Frizzled and other receptors to activate a variety of downstream signalling pathways. Wnt signalling plays a role in a wide range of physiological processes, from tissue patterning to stem cell biology and synaptogenesis. Aberrant Wnt signalling is associated with a number of pathological conditions, including several types of cancers and neurodegenerative disorders such as Alzheimer's and Parkinson's disease. Understanding how Wnt signalling is regulated is therefore crucial to dissect the mechanisms underlying this complex signalling cascade in health and disease.

The precise molecular mechanisms that regulate the main receptors of Wnt ligands, Frizzled receptors, remain poorly understood. In demonstrating that possibly all Frizzled receptors undergo palmitoylation, my work characterises a previously unidentified post-translational lipid modification of this class of receptors. Focussing on Fz5, one of the most studied Frizzled receptors expressed in mammals, I showed that palmitoylation is critical for the trafficking and functional activity of this Wnt receptor in promoting synaptogenesis in the developing hippocampus. These findings are novel and identify an important molecular mechanism that directly underpins Frizzled function *in vivo*.

In elucidating a novel mechanism of Frizzled regulation in the brain, my work lays the foundation to further study how Wnt signalling is regulated at the receptor level across a multitude of biological processes. As surface receptors are a classical drug target, investigating how palmitoylation can be modulated to tune Frizzled surface levels and activity has substantial therapeutic potential. My findings are therefore likely to have a significant impact on basic as well as clinical research.

We are planning to publish this study in a high impact scientific journal, and present this work at prestigious international meetings such as the Wnt Signalling Gordon Research Conference. My findings are of direct and

significant interest to the entire field of Wnt signalling research, which is a large community spread across the world and includes academic research groups as well as pharmaceutical companies with interests in this important signalling pathway. For instance, the Dementia Research Institute, which is the biggest initiative ever launched in UK to defeat dementia, has invested to study the role of Wnt signalling in synapse degeneration. In addition, on-going clinical trials in US are testing Wnt signalling inhibitors to cure different forms of cancer.

The impact of my findings can already be appreciated in the academic environment, as our laboratory has recently been awarded a grant to further study the impact of Fz5 palmitoylation in neuronal circuit assembly and function. Post doctoral fellows and students will benefit from taking part in this project. In the future, other research grants and fellowships could be proposed to study the role of palmitoylation in the regulation of other Frizzled receptors. Therefore, the work produced during my PhD has the required potential to influence future academic and private studies in the Wnt signalling research field.

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Abbreviations

AAV	(Adeno Associated Virus)
ABE	(acyl biotin exchange)
Ach	(achetylcholine)
AD	(Alzheimer's disease)
AZ	(active zone)
AMPAR	(α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate receptor)
APs	(action potentials)
APC	(Adenomatus polyposis coli)
APT	(Acyl-proteinthioesterase 1)
BBB	(blood brain barrier)
BDNF	(Brain derived neurotrophic factor)
β-TrCP	(Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase)
CaMKII	(Ca^{2+}/calmodulin-dependent protein kinase II)
CAMs	(Cell adhesion molecules)
CNS	(central nervous system)
CK1	(Casein kinase 1)
cKO	(conditional knock-out)
CRD	(Cys rich domain)
C-term	(carboxyl terminus)
Cys	(cysteine)
DG	(dentate gyrus)
DHHC	(Aspartate, Hystidine, Hystidine, Cystein)
Dkk	(Dickkopf)
Dishevelled	(Dvl)
DMSO	(dimethyl sulfoxide)
EAAT	(excitatory amino acid transporter)
EE	(enriched environment)
EphB	(erythropoietin-producing human hepatocellular receptors)
ER	(endoplasmic reticulum)
Erb	(Receptor tyrosine-protein kinase erbB-4)
ERAD	(endoplasmic reticulum-associated degradation)
ERK	(extracellular-signal-regulated kinase)
Evi	(Eveness interrupted in vertebrates)
FGF	(Fibroblast Growth Factor)

FRAP	(fluorescence recovery after photobleaching)
GABA	(γ-aminobutyric acid)
Gli	(glioma-associated oncogene homologue)
GPCRs	(G-protein Coupled receptors)
Gpr124	(probable G-protein coupled receptor 124)
GRIP1	(glutamate receptor interacting protein 1)
Gsk3β	(glycogen synthase kinase 3)
HFS/LFS	(high/low frequency stimulation)
ICV	(intracerebroventricular)
IP	(immunoprecipitation)
IP_3	(inositol triphosphate)
JNK	(Jun N-terminal kinase)
KD	(knock-down)
KI	(knock-in)
KO	(knock-out)
LEF/TCF	(lymphoid enhancer binding factor and transcription factor)
LRGs	(leucine-rich repeat-containing G-protein coupled receptor
LRP6	(low density lipoprotein related receptor 6)
LTP/LTD	(long-term potentiation/depression)
MAPK	(mitogen-activated protein kinase)
MBOAT	(membrane bound O-acyl transferase)
MuSK	(Muscle-specific kinase)
NCAM	(neural cell adeshion molecules)
NGF	(nerve growth factor)
Nlg	(Neuroligin)
NMDAR	(N-methyl-d-aspartate receptor)
NMJ	(neuromuscular junction)
Nrg	(Neuregulin)
Nrxn	(Neurexin)
NTs	(neurotrasnmitters)
N-term	(amino terminus)
O/N	(overnight)
PATs	(protein acyl transferases)
PCP	(planar cell polarity)
PLC	(Phospholipase C)
PKA/PKC	(protein kinase A, C)
PM	(plasma membrane)

Porc	(Porcupine)
PPT1	(Palmitoyl-protein thioesterase 1)
PSD	(postsynaptic densities)
Ptch	(Patched)
qPCR	(quantitative polymerase chain reaction)
RNF43	(Ring finger protein 43)
RT	(room temperature)
RUSH	(retention using selective hooks)
Ser	(serine)
SFRPs	(Secreted Frizzled related protiens)
Shh	(Sonic Hedgehog)
SM	(Sec1/Munc18)
Smo	(Smoothened)
SNARE	(soluble N-ethyl maleimide sensitive-factor attachment protein receptors)
SPARC	(secreted protein acidic enriched in cysteine)
SVs	(synaptic vesicles)
Syts	(Synaptotagmins)
SynGAP	(Ras-GTPase-activating protein)
SWIM	(secreted Wg-interacting molecule)
TNF	(Tumor Necrosis Factor)
TrkB	(Tropomysin receptor kinase B)
TSP	(Thrombospondin)
vGat	(vesicular GABA transporter)
VGCCs	(voltage gated Ca^{2+} channels)
vGlut	(vesicular Glutamate transporter)
WB	(western blot)
WIF-1	(Wnt inhibitory factor 1)
Wingless	(Wg)
Wls	(Wntless in *Drosophila*)
ZNRF3	(Zinc and ring finger 3)

CHAPTER 1: INTRODUCTION

1.1 General overview

What we do, from breathing and moving to more complex actions like performing an experiment or discussing Brexit, depends on the correct function of our brain. To perform these actions the brain relies on the transmission of information between nerve cells, or neurons. Neurons are organised in circuits formed by countless connections between cells. These connections, called synapses, are the hubs of communication between neurons and are crucial for the transmission of nerve impulses. Synapses are asymmetric connections between nerve cells characterised by the presence of highly specialised proteins that allow the transit of information from a pre- to a postsynaptic cell, thus enabling signal transmission along neuronal circuits. The correct development of synapses is crucial to sustain the function of our brain. In fact, aberrant synapse formation is associated with the onset of several neurological disorders. One of the key questions in developmental neurobiology is what are the molecular mechanisms that regulate the assembly and maturation of synapses?

The formation and maturation of synapses require the orchestrated recruitment and stabilisation of thousands of synaptic proteins at sites where new connections are formed. Synaptogenic factors are a broad group of molecules which regulate the formation of synapses. Remarkable progress has been made in understanding the role and regulation of synaptogenic factors during synapse formation, but in spite of such advancement we still do not fully understand how this complex phenomenon is regulated. In my PhD thesis I have studied the molecular mechanisms that regulate the trafficking and function of Frizzled receptors, which are the main receptors for Wnt ligands, a family of conserved secreted glycolipoproteins that act as potent synaptogenic factors in the central and peripheral nervous systems.

1.2 Structure and function of synapses

Synapses are small but highly dynamic structures specialised to transmit electro-chemical signalling between a presynaptic cell (a neuron) and a postsynaptic cell, which can be another neuron, a muscle fibre or a gland. Pre- and postsynaptic compartments exhibit a highly asymmetric and polarised architecture that is optimal for the transmission of electro-chemical signals from one cell to another. Glial cells, particularly astrocytes, are also important cellular components of the synapse, as they offer structural and functional support. The crucial role of astrocytes at synapses has given rise to the concept of tripartite synapse (Fig 1.1) (Araque et al. 1999; Perea et al. 2009).

1.2.1 The presynaptic side

The presynaptic terminal, or bouton, is where electrical stimuli in the form of action potentials (APs) arrive and are converted into chemical signals in the form of neurotransmitters (NTs). NTs are released to activate receptors on postsynaptic cells (Fig 1.1). Once an AP reaches a presynaptic terminal, it induces an influx of Ca^{2+} ions through voltage gated Ca^{2+} channels (VGCCs) (Takahashi and Momiyama 1993; Catterall 2011). This activates the Ca^{2+}-sensitive components of the NT release machinery and triggers the fusion of NTs-loaded synaptic vesicles (SVs) with the plasma membrane (PM), thus enabling the release of NTs in the synaptic cleft, a very small space (roughly 20 nm) that separates the pre- and postsynaptic side (Südhof 2012a; Midorikawa and Sakaba 2015) (Fig 1.1). The process of NT release occurs in less than 1ms and involves a vast number of proteins including all the components of the release machinery (Südhof 2013).

The release machinery is composed by a multitude of molecules; the best characterised are Synaptotagmins (Syts), members of the SNARE (soluble N-ethyl maleimide sensitive-factor attachment protein receptors) family and the SM (Sec1/Munc18) protein family (Rizo and Xu 2015). In response to elevated Ca^{2+} concentrations at presynaptic terminals, Syts interact with SNARE proteins enabling the exocytosis of SVs (Chapman 2008; Chapman

2018). SNARE proteins are the core components of the release machinery. This family includes over 60 members, of which SNAP25, Synaptobrevin1/2 and Syntaxin1 are the most studied. SNARE proteins do not only regulate SVs fusion but they are also involved in all types of vesicle exocytosis (Wu et al. 2014). The interaction between different SNAREs leads to the docking of vesicles to the PM. If sufficient Ca^{2+} enters the terminal, SVs fuse with the PM allowing the release of NTs (Chen and Scheller 2001; Duman and Forte 2003; Ungar and Hughson 2003; Han et al. 2017). SM proteins are fundamental regulators of exocytosis: they act as “clasps” to spatially and temporally coordinate the interaction between SNAREs (Dulubova et al. 2007; Südhof and Rothman 2009; Südhof and Rizo 2011).

At presynaptic terminals SVs loaded with NTs fuse with the PM at a specific area called the active zone (AZ) (Fig 1.1). The main function of the AZ is to favour the docking and fusion of SVs, to recruit VGCCs and to spatially coordinate the apposition of pre- and postsynaptic sides (Zhai and Bellen 2004; Südhof 2012b). The AZ is characterised by a complex cytoskeletal organisation, which is required to hold in place different components of the AZ (Cingolani and Goda 2008). Some of the key components of the AZ zones are Munc-13 (Protein Unc 13), RIM (regulating synaptic membrane exocytosis 1), Piccolo and Bassoon. Munc-13 binds directly to some SNAREs and is required for both spontaneous and evoked transmitter release (Südhof 2012b). RIM and RIM-BPs (RIM binding proteins) are key organizers of presynaptic terminals as they control SV docking and tether Ca^{2+} channels to the AZ. Piccolo and Bassoon have crucial functions at the AZ as they move SVs towards the PM, regulate the localisation of VGCCs and are involved in activity-induced remodelling of the actin cytoskeleton (Piccolo only) (Hallermann et al. 2010; Mukherjee et al. 2010; Gundelfinger et al. 2015).

NTs released into the synaptic cleft bind to postsynaptic receptors causing the influx of ions and affecting the electrical activity of postsynaptic cells (Snyder 2009; Smart and Paoletti 2012). At postsynaptic sites, NTs can induce depolarisation or hyperpolarisation, which are changes in the electrophysiological balance of neuronal cells caused by the influx of

positively or negatively charged ions. Depolarisation promotes neuronal excitation facilitating the propagation of electrical stimuli along postsynaptic cells, whereas hyperpolarisation inhibits this process.

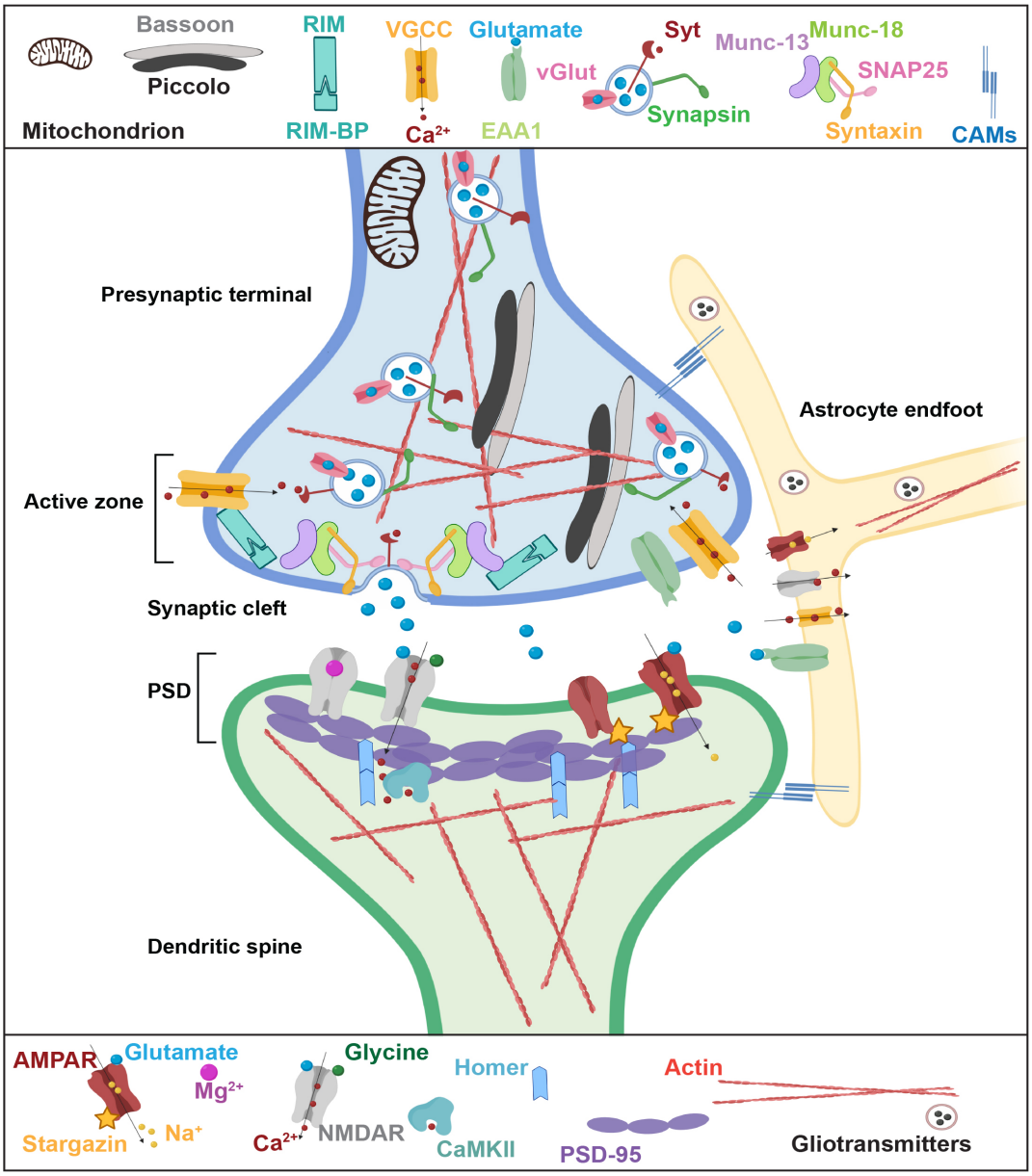

Fig 1.1: Schematic of pre- and postsynaptic structure
The presynaptic side is characterised by the presence of SVs loaded with NTs, components of the release machinery, VGCCs and the synaptic organizers Bassoon and Piccolo. The postsynaptic compartment is constituted by NT receptors, adhesion molecules, scaffold proteins and signalling molecules, which are concentrated in a protein-dense area called the PSD. Astrocytes are crucial components of central synapse as they provide structural support and engage in molecular signalling.

Hundreds of NTs exist, but the amino acid glutamate and GABA (γ-aminobutyric acid) are the most common excitatory and inhibitory NTs in the CNS (Meldrum 2000; Valenzuela et al. 2011; Snyder 2017). However, whether a NT induces excitation or inhibition depends on the electrophysiological environment of the receiving cells; for instance, early in development GABA induces depolarisation due to the high concentration of Cl^- ions found in young neurons (Ben-Ari 2002; Spitzer 2010). During development changes in the expression of the ion transporter NKCC1 (Na^+-K^+-$2Cl^-$ co-transporter 1) and KCC2 (K^+-Cl^- cotransporter 2) affect the electrical properties of neurons, causing a shift from excitation to inhibition upon activation of GABA receptors (Rivera et al. 1999; Tyzio et al. 2006; Leonzino et al. 2016). Dopamine, serotonin, acetylcholine (Ach) and glycine are also common NTs released at central and peripheral synapses (Valenzuela et al. 2011; Snyder 2017).

After NT release, SV recycling and NT re-uptake are absolutely required to replenish the SV pool and terminate signalling by clearing NTs from the synaptic cleft, a function that is largely dependent on astrocytes (Rizzoli 2014; Soykan et al. 2016). Two main classes of NT transporters exist: a) those localised on SVs, such as vGlut (vesicular Glutamate transporter) and vGAT (vesicular GABA transporter), which control the loading of NTs; b) those distributed at the PM, which terminate signalling by mediating the re-uptake of NTs from the synaptic cleft, like EAAT (excitatory amino acid transporter) and GAT (Danbolt 2001; Shigeri et al. 2004; Blakely and Edwards 2012; Scimemi 2014). Given the crucial role of NT transporters at synapses and the progress made in understanding their structure and function, these molecules have become one of the most important drug targets in the CNS (Gether et al. 2006; Iversen 2006).

In summary, presynaptic terminals are specialised structures to sense the arrival of electrical stimuli, induce the release of NTs and ensure the re-uptake of these molecules from the synaptic cleft to terminate signalling and undergo further cycles of transmission (Fig 1.1). Presynaptic terminals are made by hundreds of proteins that work together to ensure transmission of signals from a pre- to a postsynaptic cell. VGCCs, SVs loaded with NTs, the

release machinery and the AZ are the most important components of presynaptic terminals.

1.2.2 The postsynaptic side

The postsynaptic side is the structure of the synapse specialised for the reception of NTs. Binding of NTs to their postsynaptic receptors induces changes in the electrical properties of postsynaptic cells and results in the activation of downstream signalling (Fig 1.1) (Sheng and Kim 2011). For the purpose of this PhD thesis, I will focus mainly on the structure of excitatory postsynaptic sites and I will only briefly touch upon the organisation of inhibitory synapses.

Dendritic spines are the main postsynaptic structures for excitatory synapses. They were first described over a century ago by Ramon y Cajal in his astonishing drawings, following his observation of Golgi-stained brain tissues (Ramón y Cajal 1909). Since then, dendritic spines have been one of the most studied synaptic structures. Spines are small actin-rich (max width <1μm) protrusions extending from dendrites; they are extremely dynamic and diverse in their morphology (Häusser et al. 2000; Sorra and Harris 2000; Nimchinsky et al. 2002; Rochefort and Konnerth 2012). Dendritic spines are distributed along the entirety of dendritic branches at a density that varies greatly according to age and brain areas, ranging from 1-10 spines/10 μm (Huttenlocher 1990; Woolley et al. 1990; Benavides-Piccione et al. 2013; Jammalamadaka et al. 2013; Morales et al. 2014). In most cases dendritic spines are innervated by one presynaptic terminal, but multi-innervated spines also exist (Fiala et al. 1998; Giese et al. 2015). Spines, particularly mature ones, exhibit a defined head that comprises one or more postsynaptic densities (PSD), which are protein-dense areas formed by NT receptors, trans-synaptic proteins, scaffold proteins and signalling molecules (Walikonis et al. 2000; Boeckers 2006; Arellano et al. 2007; Kim and Sheng 2009). Based on their morphology dendritic spines are classified into three groups: a) mushroom spines, which exhibit a relatively big head that is connected to the dendritic branch by a very thin neck; b) thin spines, which have small

head and thin neck; and c) stubby spines, which do not present a clear separation between head and neck (Arellano et al. 2007; Berry and Nedivi 2017). There is a correlation between morphology, molecular composition and function of dendritic spines (Yuste and Bonhoeffer 2001; Arellano et al. 2007; Dent et al. 2011; Bosch and Hayashi 2012). Changes in dendritic spine size and morphology are some of the mechanisms underlying synaptic plasticity, which is the ability of synapses to dynamically adapt their structure and function in response to external stimuli (see section 1.3.3).

The PSD of dendritic spines is probably the most complex substructure at synapses. It is estimated that several hundreds of proteins are concentrated at the PSD (Walikonis et al. 2000; Collins et al. 2006), an astonishing number given the small size of this structure: roughly 200-800 nm long and 30-50 nm thick (Boeckers 2006). Among the molecules in the PSD, we find NTs receptors, ion channels, adhesion molecules, signalling enzymes and scaffold proteins. The most abundant proteins are CaMKII (Ca^{2+}/Calmodulin-dependent protein kinase II), SynGAP (Ras-GTPase-activating protein) and the scaffold protein PSD-95 (Kim and Sheng 2009). The PSD is localised at the tip of dendritic spine heads, where it exerts structural and functional roles. The PSD supports adhesion molecules and NT receptors and, through scaffold proteins, couples these membrane proteins to cytoplasmic signalling molecules (Fig 1.1) (Sheng and Kim 2011). The PSD itself is a very dynamic structure, and modifications of the molecular composition of the PSD are reflected in changes in spine structure and function (Hering and Sheng 2001).

Cell adhesion molecules (CAMs) of the PSD have critical synaptic functions as they bind presynaptic partners bridging the two sides of the synapse (Yamagata et al. 2003; Dalva et al. 2007). In addition, they mediate the interaction with other cell types like astrocytes (Togashi et al. 2009). CAMs also play a role in the recognition of synaptic targets, a process particularly important during the initial stages of synapse formation (Washbourne, Dityatev, et al. 2004). Moreover, by acting as trans-synaptic signalling molecules and binding intracellular scaffold proteins, they can modulate synapse structure and function activating downstream effectors (Dalva et al. 2007). Among the members of this family of proteins we find: Cadherin,

NCAM (Neural cell adhesion molecules) and SynCAM, which mainly form homophilic interactions across the synapse, and the presynaptic EphrinB and Neurexin with their respective postsynaptic partners EphB receptors and Neuroligin (Washbourne et al. 2004; Dalva et al. 2007; Missler et al. 2012). I will discuss these molecules in more details in the context of synapse formation (see section 1.3.2).

NTs receptors are other major components of the PSD. AMPAR and NDMAR (α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate and N-methyl-d-aspartate respectively) are the main glutamate receptors. They are formed by 4 subunits that assemble in many different combinations giving rise to diverse functional properties (Pickard et al. 2000; W. Lu et al. 2009; Traynelis et al. 2010; Gambrill and Barria 2011). AMPARs are opened by the binding of glutamate and mediate fast synaptic transmission by allowing the influx of Na^+ and Ca^{2+} cations (Prieto and Wollmuth 2010; Popescu 2012; Greger et al. 2017). NMDARs are activated by glutamate and glycine binding and by depolarization-mediated removal of Mg^{2+} cations which normally block NMDARs (Platt 2007; Sobolevsky 2015). NMDARs are mainly permeable to Ca^{2+} and Na^+ and mediate slow synaptic transmission (Burnashev 1998). Both AMPARs and NMDARs are key signalling molecules for synaptic plasticity (Hunt and Castillo 2012; Huganir and Nicoll 2013) (see section 1.3.3). NMDARs are stabilized at the PSD by direct binding with the major scaffold protein PSD-95 (Kornau et al. 1995; Husi et al. 2000; Sheng 2001), whereas AMPARs form a complex with PSD-95 through the interaction with other molecules such as Stargazing and GRIP1 (glutamate receptor interacting protein 1) (Fig 1.1) (Dong et al. 1999; Chen et al. 2000; Schnell et al. 2002; Bats et al. 2007). Thus, the PSD is fundamental for the structural and functional organisation of hundreds of postsynaptic proteins.

Inhibitory synapses are essential to regulate neuronal excitability and function of neuronal circuits. These types of synapses release GABA (in the brain) or Glycine (in the spinal cord), which act on postsynaptic receptor to induce hyperpolarisation and inhibit electrical activity in postsynaptic cells (Moss and Smart 2001). Inhibitory synapses are rarely found on spines (Chiu et al. 2013), they rather concentrate along the dendritic shaft, soma or at the

initial segment of axons. A recent paper proposed that specific temporally-restricted expression profiles characterise different populations of GABAergic neurons and establish the distribution of inhibitory synapses at different cellular locations (dendrites vs soma vs axons) (Favuzzi et al. 2019). GABA receptors (GABARs) are the main NT receptors at inhibitory synapses (Fig 1.2). $GABA_ARs$ are pentameric ionotropic receptors that assemble in many different combinations of α, β and γ subunits, and upon GABA binding they quickly inhibit electrical activity by allowing the influx of Cl^- (Olsen and Sieghart 2009; Miller and Aricescu 2014). $GABA_BRs$ are metabotropic receptors that assemble in heterodimers and act through a second messenger system to regulate slow and prolonged inhibitory neurotransmission (Chebib and Johnston 1999; Bettler et al. 2004). Gephyrin is a self-assembling scaffold protein and a key organizer molecule at inhibitory postsynaptic sites; its main function is to stabilize $GABA_ARs$ at synapses by facilitating the clustering of these receptors (Choii and Ko 2015) (Fig 1.2). Like PSD-95 and glutamate receptors at excitatory synapses, GABARs and Gephyrin are involved in mechanisms of synaptic plasticity at inhibitory synapses.

In summary, the postsynaptic compartment is a very specialized structure where binding of NTs to their highly concentrated receptors activate downstream cascades to propagate signalling. In contrast to presynaptic sites, excitatory and inhibitory postsynaptic compartments exhibit different structural organisation. Excitatory synapses are mainly formed on dendritic spines, which are highly specialised and dynamic structures extending from dendritic branches. Inhibitory synapses are mainly found at the soma and along the dendritic shaft. The PSD is the central component of postsynaptic sites; it incorporates hundreds of molecules including NT receptors, CAMs, scaffold proteins and signalling molecules to orchestrate signalling in response to NT release. In the following section I will introduce the molecular mechanisms that regulate the assembly of synapses.

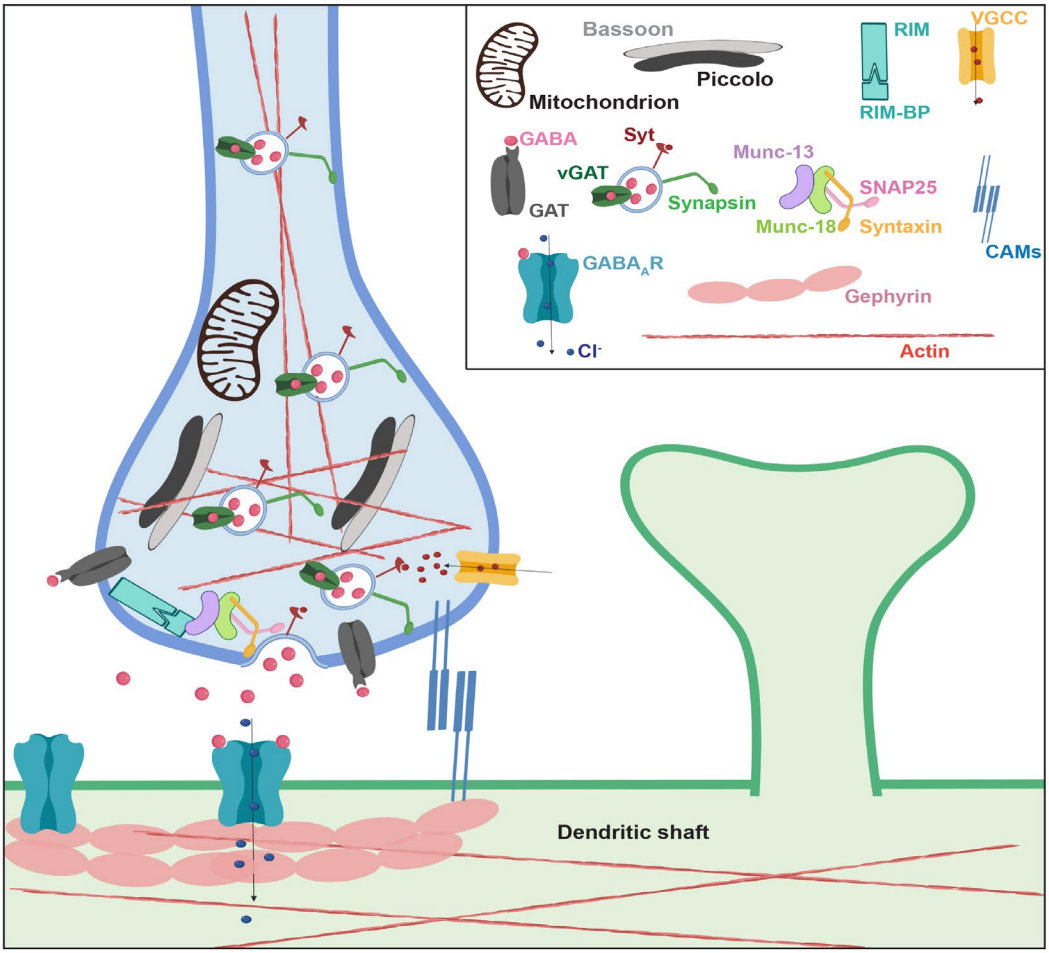

Fig 1.2: Schematic of an inhibitory synapse
Inhibitory synapses are usually found along the dendritic shaft or in proximity of the soma and rarely on dendritic spines. GABA is the main NT at inhibitory synapses and vGAT and GAT are vesicular and membrane transporters for this NT. GABA release activates the postsynaptic receptors $GABA_A$ allowing the influx of Cl^- ions in the postsynaptic compartment and inhibiting neuronal excitation. Gephyrin is the major component of the PSD at inhibitory synapses and is fundamental to cluster $GABA_A$Rs.

1.3 Synaptogenesis

The assembly of synapses is a key step for the formation of functional neuronal circuits, and is therefore essential for the proper function of the brain. The timing of synapse formation varies greatly between different brain areas (Huttenlocher and Dabholkar 1997; Dehorter et al. 2012). In rodents synaptogenesis peaks around the second week of life (P10-P15) (Semple et al. 2013), and it is followed by synaptic pruning, a period of selective elimination of unnecessary connections that results in roughly 50% reduction in synapse number by postnatal week 4-6 (Pressler and Auvin 2013). The rate of synapse formation during early postnatal life is astonishing, it has been estimated that synaptic density in the cortex on newborn rats increases from 200 million/mm^3 to 4 billion/mm^3 in just five weeks (DeFelipe et al. 1999). Highly regulated molecular mechanisms are in place to ensure such fast and precise expansion in synaptic connections. Moreover, synapses continue to form throughout the entire lifespan, in a balance with synapse elimination. Unlike synapse formation during postnatal development, synaptogenesis in adults is mainly experience-dependent and is one of the key mechanisms underlying synaptic plasticity (Zito and Svoboda 2002; Markham and Greenough 2004; Song et al. 2005; Holtmaat and Svoboda 2009), which is considered the cellular correlate of learning and memory.

1.3.1 Molecular mechanisms of synapse formation

The formation of new synapses requires the coordinated recruitment and stabilisation of thousands of proteins at both sides of the synapse; thus, synaptogenesis is an extremely complex process. The correct formation of synaptic connections is essential to sustain the cognitive performances of our brain; in fact, aberrant formation of synapses has been linked to onset of several neurodevelopmental disorders (Lepeta et al. 2016; Del Pino et al. 2018; Batool et al. 2019). Enormous progresses have been made in describing the sequence of events as well as the molecules involved in the formation of synapses; however, we still have much to learn about the mechanisms underlying this complex process. The steps towards the

formation of a new synapse can be chronologically ordered in this sequence: a) synthesis and clustering of synaptic proteins prior to axo-dendritic contacts; b) formation of axo-dendritic contacts; c) recruitment of synaptic proteins and synapse maturation (Fig 1.3) (McAllister 2007; Favuzzi and Rico 2018; Südhof 2018). In the next paragraphs I will introduce the main mechanisms that regulate these processes, with a specific focus on excitatory synapse formation.

Synthesis and trafficking of synaptic proteins prior to axo-dendritic contacts

The formation of axo-dendritic contacts is the first physical step into synapse development; however, mobile clusters of pre- and postsynaptic proteins already exist before these contacts are established (Ahmari et al. 2000; Zhai et al. 2001; Washbourne et al. 2002; Washbourne, Liu, et al. 2004) (Fig 1.3 A). The distribution and dynamics of these clusters seem to play an important role in driving the formation of axo-dendritic contacts; in fact, synapses are formed preferentially in correspondence of static or slow-moving pockets of synaptic proteins (Gerrow et al. 2006; Knott et al. 2006; Sabo et al. 2006). Further evidence supporting the importance of these clusters of synaptic proteins is the fact that synaptic transmission has been recorded upon seconds after the formation of axo-dendritic contacts, suggesting that components of the release machinery and certain postsynaptic receptors are already present at sites where synapses form (Munno and Syed 2003). In addition, spontaneous or induced glutamate release from presynaptic sites has been shown to shape dendritic filopodia and induce *de novo* formation of dendritic spines (Portera-Cailliau et al. 2003; Richards et al. 2005; Kwon and Sabatini 2011; Andreae and Burrone 2015; Andreae and Burrone 2018). However, the absolute requirement of NT release in synapse formation is still debated (see section 1.3.3). Thus, pre-existing clusters of synaptic proteins along neuronal processes constitute the first building blocks for the assembly of synapses, and neuronal activity seems to play a role in determining the sites of axo-dendritic contacts.

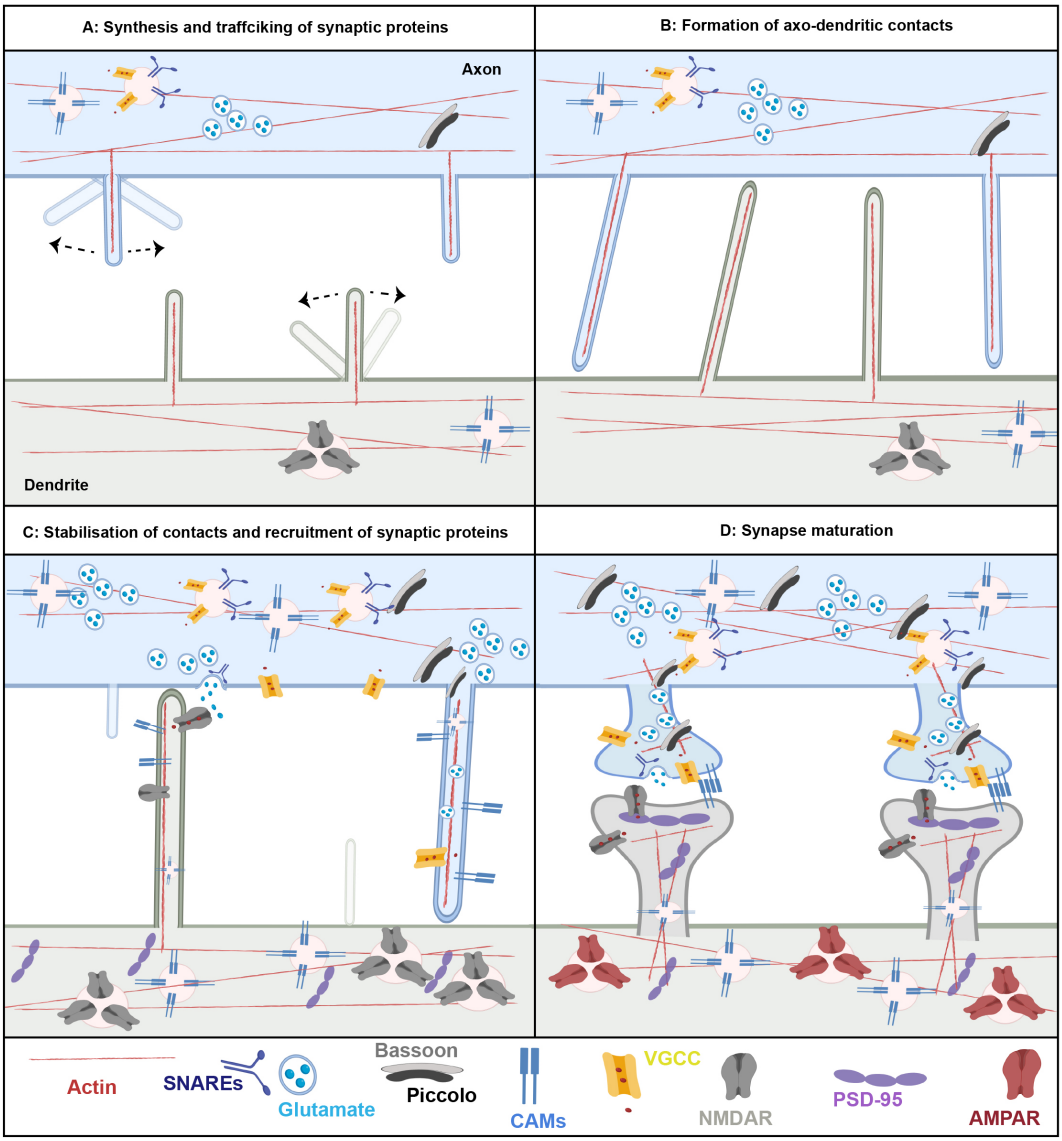

Fig 1.3: Stages of synaptogenesis
A: Some synaptic molecules are synthesised and trafficked along neuronal processes before axo-dendritic contacts are formed. **B:** Axons and dendrites extend highly motile filopodia to contact potential synaptic partners. **C:** Most contacts are not stabilised and rapidly retract, whereas others are maintained. Rapid recruitment of synaptic molecules is the first step in the process of synapse maturation. **D:** Axo-dendritic contacts are stabilised into mature synapses. The recruitment of AMPARs is one of the events determining the full maturation of synapses.

The formation of axo-dendritic contacts in synapse assembly

Axo-dendritic contacts are the first physical step in the formation of new synapses (Fig 1.3 B). Several types of connections are possible: axon-axon, dendrite-dendrite and axon-dendrite. I will only discuss axon-dendrite connections. Axons and dendrites are both able to grow actin-rich protrusions called filopodia, which interact with partner cells to establish initial

contacts (Ziv and Smith 1996; Fiala et al. 1998). These interactions occur very rapidly and the vast majority retract within seconds to minutes before maturing into functional synapses (Wong and Wong 2000; Bonhoeffer and Yuste 2002). It has been proposed that filopodia are stabilised in apposition of clusters of both pre- (Ruthazer et al. 2006; Sabo et al. 2006) and postsynaptic proteins (Niell et al. 2004; Gerrow et al. 2006; Knott et al. 2006). Several synaptogenic factors promote the growth of axonal and dendritic filopodia. For instance, NGF (nerve growth factor), BDNF (brain derived neurotrophic factor) and Wnt molecules promote the growth of filopodia (Menna et al. 2009; Schlessinger et al. 2009; Ketschek et al. 2011; Stamatakou et al. 2015). Dendritic filopodia are very numerous and dynamic during early development but decrease in density and motility in a directly proportional manner to synapse maturation (Jontes and Smith 2000; Portera-Cailliau et al. 2003). Neuronal activity rapidly increases the density and motility of dendritic filopodia (Maletic-Savatic et al. 1999; Portera-Cailliau et al. 2003). In addition, glutamate-independent local oscillations of Ca^{2+} at dendritic filopodia have been proposed to stabilise axo-dendritic contacts (Lohmann and Bonhoeffer 2008). Thus, axonal and dendritic filopodia are fundamental structures for the initial contacts between pre- and postsynaptic cells. These contacts can be stabilised into mature synapses in response to neuronal activity and certain signalling molecules (see below).

Synapse maturation

Synapse differentiation, or maturation, is the process of stabilisation of axo-dendritic contacts and recruitment of synaptic proteins to form a functional synapse (Fig 1.3 C-D). Although some pockets of synaptic proteins are pre-formed before synapse formation, the vast majority of synaptic proteins is coordinately transported to newly formed synapses after the formation of axo-dendritic contacts. Live-imaging studies have shown that, within 15-30 mins from the formation of axo-dendritic contacts, core presynaptic proteins including Piccolo, Bassoon and members of the release machinery are transported together to the newly formed synapses (Ahmari et al. 2000; Friedman et al. 2000; Zhai et al. 2001). CAMs play a fundamental role in stabilising the initial contacts between axon and dendrites by allowing the

recruitment of other synaptic components. Specific examples of the role of CAMs in synapse formation are discussed below (see section 1.3.2)

On the postsynaptic side, Neuroligin1 accumulates at synapses within a few minutes from the formation of axo-dendritic contacts, and recruits PSD-95 and NMDARs within 45 mins (Barrow et al. 2009). AMPARs are recruited after NMDARs, consistently with the fact that NMDAR transmission exceeds AMPAR transmission in early development (Hall and Ghosh 2008). The observation that newly formed synapses lack AMPARs gave rise to the concept of silent synapses, which have been proposed to acquire AMPARs in an activity-dependent manner (Isaac et al. 1995; Liao et al. 1995; Wu et al. 1996; Zhu et al. 2000). Activation of NMDARs trigger rapid and substantial incorporation of AMPARs at silent synapses, thus driving synapse maturation. However, these observations have been challenged by studies showing that NMDARs inhibit AMPAR insertion at developing synapses (Ju et al. 2004; Sutton et al. 2006; Hall et al. 2007; Hall and Ghosh 2008). It is now accepted that a molecular switch of the subunit composition of NMDARs is responsible for synaptic insertion of AMPARs. Until early postnatal life, NMDARs are formed primarily by NR1 and NR2B subunits, whereas at later stages NR2A outnumber NR2B subunits (Monyer et al. 1994; Sheng et al. 1994; Stephenson 2001; Liu et al. 2004; Elias et al. 2008). This switch allows AMPAR recruitment and synapse maturation (Massey et al. 2004; Kim et al. 2005; Hall et al. 2007; Gambrill and Barria 2011). Dendritic spine maturation occurs simultaneously to these events and is regulated by multiple factors including CAMs and secreted synaptogenic molecules (see section 1.3.2) (Sorra and Harris 2000; Tada and Sheng 2006; Hiester et al. 2013; Poon et al. 2013). The development of dendritic spines is characterised by a progressive reduction in the number of filopodia in favour of the formation of thin, stubby and mushroom spines (Cline 2001; Grutzendler et al. 2002; García-López et al. 2010).

In summary, synapse formation is a complex process that starts in late embryonic development, peaks in early postnatal life and continues throughout the entire life span. The assembly of excitatory synapses requires a series of events that include the synthesis of synaptic proteins, the

formation of physical contacts between a pre- and a postsynaptic cell and the recruitment of key synaptic molecules. Hundreds of proteins are presents at mature synapses; therefore, the orchestrated recruitment and interaction of these proteins requires a fine regulation by synaptic-organiser molecules (Siddiqui and Craig 2011). In the next section, I will introduce some of the key synaptogenic molecules that regulate synapse formation.

1.3.2 Synaptogenic molecules

Cell adhesion molecules (CAMs)

CAMs regulate the formation of physical contacts between axons and dendrites (Washbourne, Dityatev, et al. 2004; Togashi et al. 2009; Südhof 2018). The interaction between presynaptic Neurexin (Nrxn) and postsynaptic Neuroligin (Nlg) is one of the most extensively studied mechanisms of CAM-mediated synapse formation (Fig 1.4). In mammals three *Nrxn* genes exist (*Nrx1-2-3*) and each encodes different isoforms: α-, β- and the recently identified γ. *Nrxns* are further modified by splicing events generating hundreds of different isoforms (Yan et al. 2015). The synaptogenic role of Nrxn and Nlg was first described in cell line/neuron co-cultures, where the expression of Nlg in heterologous cells was sufficient to drive presynaptic differentiation in contacting neurons (Scheiffele et al. 2000; Dean et al. 2003; Graf et al. 2004). *In vivo* studies of *Nrxn* and *Nlg* KO (knock-out) mice have elucidated the roles of these molecules in regulating synapse maturation and synaptic transmission (Missler et al. 2003; Varoqueaux et al. 2006; Li et al. 2007; Banovic et al. 2010; Liang et al. 2015; L. Y. Chen et al. 2017). However, whether Nrxn and Nlg are required for the initial stages of synapse formation is still a matter of debate. In fact, triple *α-Nrx* (1-2-3) KO mice, which die postnatally, exhibit severe defects in Ca^{2+} channels localisation and function, but still form structurally normal synapses (Missler et al. 2003). Similarly, triple *Nlg* KO mice exhibit severe defects in synaptic transmission but unchanged synaptic density (Varoqueaux et al. 2006). Given the numerous Nrxn isoforms, two major challenges of KO studies are to establish the specificity of each Nrxns and to avoid compensatory effects. Recently, α and β pan-Nrxn cKO (conditional KO)

have been generated (Chen et al. 2017). Cre-mediated deletion of these genes at P0, to avoid lethality, showed complex and diverse phenotypes in different brain areas and cell types, supporting the hypothesis that Nrxn exert very distinct functions depending on the cellular context (L. Y. Chen et al. 2017). However, it should be noted that these mice still expressed the recently identified γ isoforms of Nrxn, complicating the interpretation of the data. A recent paper examined the synaptogenic role of γ-Nrxn in *C.elegans* and found that γ isoforms are required for synapse formation (Kurshan et al. 2018). Interestingly, it was proposed that γ-Nrxn is regulated by Wnt signalling, a major regulator of synapse formation (Kurshan et al. 2018). The authors showed that Wnt inhibits synaptogenesis in the posterior motor neurons of *C. elegans* by downregulating γ-Nrxn expression and by promoting endocytosis of Frizzled receptors (Kurshan et al. 2018). Thus, Nrxn and Nlg are crucial regulators of synapse maturation but their requirement for early stages of synapse assembly remains to be clarified.

Cadherins, NCAM (neural cell adhesion molecule) and SynCAM are expressed on both sides of the synapse and form mainly homophilic interactions across the synaptic cleft (Fig 1.4). Cadherins, as all the other CAMs at synapses, stabilise the physical interaction between pre-and postsynaptic sites (Arikkath and Reichardt 2008). In addition, through the interaction with Catenins, Cadherins act as signalling molecules to induce modifications of the actin cytoskeleton (Brusés 2006; Arikkath and Reichardt 2008; Friedman et al. 2015). Cadherins and Catenins, particularly N-cadherin and β-catenin, are involved in the assembly of SV clusters at early stages of synapse formation (Bamji et al. 2003), and are required for dendritic spine morphogenesis (Togashi et al. 2002).

NCAM is a single pass transmembrane protein highly expressed in the CNS and is recruited at newly formed synaptic sites within minutes from axo-dendritic contacts (Washbourne, Dityatev, et al. 2004). Experiments in dissociate hippocampal cultures have shown that NCAM promotes the assembly of synapses and modulates synaptic transmission through NMDARs; however, NCAM does not seem to be strictly required for synaptogenesis (Dityatev et al. 2000). Cadherins and NCAMs, are both

involved in the process of synaptic targeting; in fact, impairing Cadherin or NCAM signalling results in dispersal or mistargeting of synaptic sites (Brusés 2006; Arikkath and Reichardt 2008).

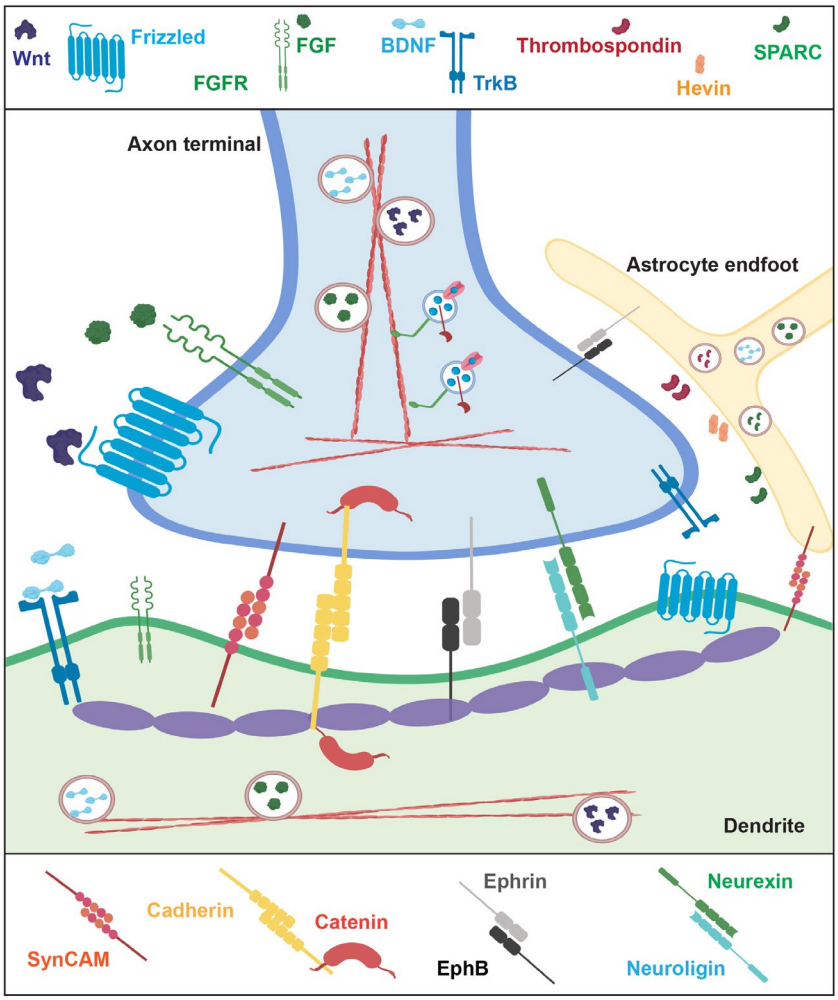

Fig 1.4: Synaptogenic factors
Synaptogenic factors are broadly divided in two main categories: trans-synaptic molecules, or CAMs, and secreted factors. CAMs bridge pre- and postsynaptic compartments ensuring structural support, alignment of these structures and signalling transduction across the synapse. Secreted factors are released at pre- and postsynaptic sites and act on their receptors to activate downstream signalling cascades that drive synapse assembly and maturation.

Alike Nrxn and Nlg, the role of SynCAM in synapse formation was first described co-culturing cell lines and dissociated neurons. These experiments have shown that expression of SynCAM in HEK293 cells is sufficient to drive presynaptic differentiation in contacting neurons (Biederer et al. 2002); a mechanism that can be promoted by both homophilic and heterophilic interactions of SynCAM (Fogel et al. 2007; Robbins et al. 2010). SynCAM

localises at growth cones where it shapes their morphology and stabilises axo-dendritic contacts (Stagi et al. 2010). In addition, *in vivo* gain and loss of function of SynCAM respectively increases and decreases excitatory synapse density, respectively, and it also affects synaptic plasticity in mature neurons (Robbins et al. 2010).

Presynaptic EphrinB and its postsynaptic tyrosine kinase receptor EphB are key modulators of synapse formation and function (Fig 1.4) (Hruska and Dalva 2012; Sloniowski and Ethell 2012). For instance, EphrinB-EphB signalling is required for filopodia motility (Kayser et al. 2008), and mice lacking different isoforms of EphB (EphB1-2-3) exhibit fewer presynaptic terminals, decreased content of NMDARs and defects in the formation of dendritic spines (Henkemeyer et al. 2003). In contrast, gain of function of EphB receptors increases the assembly of presynaptic sites and promotes the clustering of AMPA and NMDA receptors, the latter through direct interaction between EphB and NMDARs (Dalva et al. 2000). In summary, CAMs are key synaptogenic factors involved in different aspects of synaptogenesis, including stabilisation of axo-dendritic contacts, recruitment of synaptic proteins and maturation of functional synapses.

Secreted synaptogenic factors

Another class of synaptogenic factors comprises secreted molecules such as BDNF, FGF (Fibroblast Growth Factor), Neuregulin, Shh (Sonic Hedgehog) and Wnts (Fig 1.3). The fact that secreted molecules are expressed prior to the formation of axo-dendritic contacts suggests that these molecules may act upstream of CAMs in regulating synapse formation (Shen and Cowan 2010; Johnson-Venkatesh and Umemori 2010). I will focus on the aforementioned secreted factors, with the exception of Wnt molcules, whose role in synapse formation will be described in details in a separate section (see section 1.6.1).

BDNF is the best-characterised secreted synaptogenic factor. BDNF belongs to the neurotrophin family and is involved in several aspects of the CNS development, including stem cell proliferation, neuronal survival, axon-dendrite polarisation and guidance, synapse formation and synaptic plasticity

(Binder and Scharfman 2004; Park and Poo 2013). The action of BDNF at synapses was first described at neuromuscular connections where BNDF potentiates synaptic strength (Lohof et al. 1993; Wang et al. 1995; Stoop and Poo 1996). Similarly, BDNF was observed to modulate synaptic function in the CNS (Kang and Schuman 1995; Patterson et al. 1996; Levine et al. 1995), but the first evidence showing the synaptogenic activity of BDNF came in 1998, when this molecule was shown to induce the formation of excitatory and inhibitory synapses in cultured hippocampal neurons (Vicario-Abejón et al. 1998). Subsequently, a number of studies elucidated some of the mechanisms though which BDNF induces excitatory and inhibitory synapse formation, and described a role for this molecule in regulating synaptic plasticity and activity-mediated synaptogenesis (McAllister et al. 1999; Bramham and Messaoudi 2005; Leal et al. 2017). BDNF is secreted by neurons at pre- and postsynaptic sites, as well as by microglia and astrocytes (Lessmann and Brigadski 2009; Song et al. 2017). BDNF levels are elevated in response to increased neuronal activity (Hong et al. 2008; Sakata et al. 2013; Sleiman et al. 2016), and autocrine BDNF signalling within single spines modulates synaptic plasticity (Harward et al. 2016).

How does BDNF regulate synapse formation? BDNF binds, on both sides of the synapse, to TrkB receptors (Tropomyosin receptor kinase B), allowing local activation of downstream signalling pathways (Zhang and Poo 2002). During synaptogenesis clusters of TrkB receptors are present at axons and dendrites as well as in filopodia, indicating an ideal distribution to influence synapse formation (Gomes et al. 2006). *TrkB* KO mice exhibit defects in axonal branching, decreased density of SVs, reduced levels of SNARE proteins, and severe reduction in synapse number (Martínez et al. 1998). BDNF-TrkB signalling is involved in the formation of both excitatory and inhibitory synapses (Gottmann et al. 2009; Lessmann and Brigadski 2009). A variety of signalling molecules are activated downstream of TrkB. These include Ras GTPases, PI3K (phosphatidyl inositol-3 kinase), MAPK (mitogen-activated protein kinase), ERK (extracellular-signal-regulated kinase), CREB (cAMP response element-binding protein), PKA/PKC (protein kinase A, C) and CaMKII (Reichardt 2006; Kowiański et al. 2018). Thus,

acting through a multitude of downstream effectors BDNF-TrkB signalling affects different aspects of synapse formation, such as remodelling of the actin cytoskeleton, recruitment and organisation of key synaptic proteins, modulation of synaptic transmission and regulation of gene expression.

Neuregulin (Nrg) is another member of the neurotrophin family involved in synapse formation. The human genome contains four *NRG* genes, of which *NRG1* is the most extensively studied. In the CNS, Nrg1 signals through the tyrosine kinase receptors Erb. During embryonic development Nrg1-ErbB4 signalling regulates the migration and differentiation of inhibitory interneurons (Mei and Xiong 2008). In early postnatal life Nrg1-Erb signalling is required for the development of excitatory and inhibitory synapses (Rico and Marín 2011). ErbB4 receptors, which are expressed by inhibitory interneurons (Fazzari et al. 2010), promote the formation and function of inhibitory synapses on axons of pyramidal cells and on the dendrites of GABAergic neurons, where these receptors are expressed (Fazzari et al. 2010, Del Pino et al.2013). Nrg1-Erb signalling has also been proposed to regulate the development and maturation of dendritic spines (Barros et al. 2009). *Nestin-Cre*-mediated cKO of *ErbB2* and *ErbB4* diminishes dendritic spine number and size in pyramidal cells of the cortex and hippocampus (Barros et al. 2009). However, given that ErbB4 expression is largely restricted to inhibitory interneurons, ErbB4 loss in pyramidal cells does not affect excitatory synapses number and function, suggesting that the decreased spine density in *ErbB2-ErbB4* cKO mice is likely due to Nrg1-ErbB2 signalling or to effects that are not cell-autonomous (Fazzari et al. 2010). The crucial role of Nrg1 and Erb4 signalling in inhibitory synapse formation is reflected by the fact that mutations in these genes have been associated with the onset of schizophrenia (Stefansson et al. 2002; Mei and Xiong 2008; Walsh et al. 2008) a neurological disorders characterised by the disruption in the balance between excitatory and inhibitory connections (Jaaro-Peled et al. 2010).

Fibroblast growth factors (FGFs) are another important group of secreted synaptogenic molecules. FGFs are a family of 22 secreted growth factors that signal through 4 FGF receptors (FGFRs) and regulate a wide range of biological processes during development and adulthood (Ornitz and Itoh

2001). FGF signalling is a master regulator of cell proliferation, survival, migration and differentiation in several areas of the body including the CNS (Ornitz and Itoh 2015). Furthermore, FGFs have a synaptogenic role in the CNS and at the *Drosophila* NMJ (Umemori et al. 2004; Fox et al. 2007). FGF7 signals through FGFR1b and FGFR2b to promote inhibitory synapse formation in the hippocampus, whereas FGF22-FGFR1b signalling is required for the formation excitatory synapses (Terauchi et al. 2010; Dabrowski et al. 2015). Thus, beside their crucial role in early development, FGFs are fundamental regulators of synapse formation.

The Hedgehog signalling pathway is a major regulator of tissue morphogenesis in several organs including the brain (Varjosalo and Taipale 2008; Briscoe 2009; Garcia et al. 2018). Sonic Hedgehog (Shh) is the best characterised of three Hedgehog ligands expressed in mammals. In the canonical Shh signalling, the secreted glycolipoprotein Shh binds at the PM to Patched (Ptch) receptors, inactivating Ptch-mediated inhibition of the 7 transmembrane Smoothened (Smo) (Choudhry et al. 2014; Lee et al. 2016). Thus, in the presence of Shh, Smo activates downstream signalling. This results in the nuclear translocation of the transcription factors Gli (glioma-associated oncogene homologue), with consequent expression of Gli-target genes, which include *Ptch* and *Gli1*, several Wnt signalling components and many others (Choudhry et al. 2014; Lee et al. 2016). Like other morphogens, such as FGF, BMP (Bone morphogenic factor) and Wnts, Shh is highly expressed in the postnatal brain (Ahn and Joyner 2005; Palma et al. 2005), suggesting a role beyond tissue morphogenesis (Álvarez-Buylla and Ihrie 2014). Indeed, in the postnatal brain Shh regulates stem cell proliferation, axon guindance and was recently been shown to have a role in synapse formation (Belgacem et al. 2016; Garcia et al. 2018). Treatment of hippocampal neurons with Shh increases the number of presynaptic terminals (Mitchell et al. 2012), and cKO of *Shh* in a subset of layer V cortical neurons results in diminished formation of dendritic spines (Harwell et al. 2012). The non-canonical receptor Boc (brother of Cdo), which mediates Gli-independent signalling during axon guidance (Charron et al. 2003; Okada et al. 2006), is expressed in layers II-III of the cortex, which project to neurons

in layer V, where Shh is expressed (Harwell et al. 2012). Layer V neurons of *Boc* KO mice phenocopy the synaptic defect of *Shh* KO in layer II-III, indicating that this receptor is required to mediate Shh signalling at these specific synapses (Harwell et al. 2012). Thus, Shh, one of the master regulators of tissue morphogenesis, is also required postnatally to control synapse formation in the cortex.

Glial synaptogenic factors

Glial cells are other important regulators of synapse formation and function (Eroglu and Barres 2010; Allen 2013; Bosworth and Allen 2017). First, astrocytes, which are the most abundant glial cell type in the brain, are physical and functional constituents of synapses (Araque et al. 1999; Perea et al. 2009). Indeed, the maturation of glial cells occurs simultaneously to the formation of synapses, suggesting a role in this process (Eroglu and Barres 2010). Neuron-astrocyte co-cultures provided key information about the role of glia in synapse maturation. Pure neuronal populations of retinal ganglion cells develop fewer synapse than neurons cultured with astrocyte-conditioned medium (Meyer-Franke et al. 1995). Thrombospondin (TSP), an extracellular glycoprotein that mediates cell-cell and cell-matrix interactions (Fig 1.4), was identified as one of the factors present in the conditioned medium, and it was demonstrated that immunodepletion of TPS from the medium prevents the formation of synapses (Christopherson et al. 2005). In the rodent brain TPS1 and TPS2 are highly expressed during development and *Tps1* and *Tps2* null mice exhibit a marked reduction in the number of excitatory synapses (Christopherson et al. 2005). TPSs induce synaptogenesis by binding to the α-2 δ-1 subunit of neuronal Ca^{2+} channels (Eroglu et al. 2009). This subunit is also the target of the potent anti-epileptic drug Gabapentin, which inhibits synapse formation by competing with TPSs (Eroglu et al. 2009). Hevin and SPARC (Secreted Protein Acidic enRiched in Cysteine) are other proteins secreted by astrocytes that respectively promote and inhibit synapse formation (Fig 1.3) (Kucukdereli et al. 2011). Hevin stabilises synaptic connections by bridging α-Nrx1 and Nlg-1B (Singh et al. 2016), whereas SPARC specifically inhibits Hevin-mediated synaptogenesis (Kucukdereli et al. 2011). After synaptogenesis, astrocytes continue to

regulate synaptic function. At mature synapses astrocyte end-feet engulf synaptic terminals providing spatial limitations to the synaptic cleft, and consistently with the fact that they express NT transporters, astrocytes play a crucial role in NT re-uptake to terminate synaptic transmission (Araque et al. 1999; Perea et al. 2009). Moreover, astrocytes' end-feet contain NT receptors and are able to release gliotransmitters, such as Glutamate, GABA, ATP (Adenosine triphosphate) and TNF (Tumor Necrosis Factor), which act on pre- and postsynaptic sites affecting synaptic transmission. Therefore, these cells are capable of responding and regulating synaptic signals (Kimelberg 1995; Porter and McCarthy 1997; Araque et al. 2014).

In summary, a multitude of synaptogenic factors is involved in building a synapse and the effects of these molecules are diverse, ranging from promoting SVs clustering to modifying the actin cytoskeleton and regulating the distribution of NT receptors. CAMs bridge synaptic connections, provide structural support, and engage in signalling activation. In addition, secreted factors are key organizers of synaptic connections. Their receptors are localised at pre- and postsynaptic compartments to locally activate downstream signalling and regulate synapse assembly and maturation. Glial cells engulf synapses and provide spatial and functional support. Glial-derived factors are fundamental signalling molecules for synaptogenesis and synaptic function in development and adulthood. In the next section I will introduce some of the mechanisms of synaptic plasticity, including the process of activity-mediated synapse formation.

1.3.3 Synaptic plasticity and activity-mediated synapse formation

Beside the aforementioned synaptogenic factors, neuronal activity is another driving force of synapse formation. Neuronal activity is also required for synapse maturation and it regulates synthesis, trafficking and function of a plethora of synaptic molecules. Neuronal activity modulates synapse formation in two different manners: first, at very early stages of development, spontaneous release of NT promotes synaptogenesis (Andreae and Burrone 2018); second, experience-dependent patterns of neuronal activity induce

the formation of synapses in the developing and adult brain (Zito and Svoboda 2002; Holtmaat and Svoboda 2009; Fu and Zuo 2011).

During early development spontaneous NT release occurs along axons and at growth cones (Hume et al. 1983; Young and Poo 1983; Xie and Poo 1986; Gao and van den Pol 2000). Several pieces of evidence support a role for spontaneous release in the development of both pre- and postsynaptic sites. A series of Drosophila mutants, including null flies for the SNARE binding protein Complexin, exhibit enhanced spontaneous release that correlates with increased number of presynaptic boutons in motor neurons (Huntwork and Littleton 2007; Cho et al. 2015). Moreover, blockage of spontaneous release results in the development of smaller boutons (Choi et al. 2014). At the postsynaptic side, dendritic filopodia extend towards boutons that undergo spontaneous release, and pharmacological blockage of NMDARs at very early developmental stages, when only spontaneous release occurs, negatively affects dendritic arborisation and synapse formation (Andreae and Burrone 2015). In addition, spatially and temporally controlled release of glutamate or GABA can promote the formation of dendritic spines in the mouse developing cortex (Kwon and Sabatini 2011; Oh et al. 2016). However, contrasting pieces of evidence question the role of NT release in the early stages of synapse formation. In fact, dissociated hippocampal cultures and organotypic slices from *Munc-13* or *Munc-18* KO mice, which lack spontaneous and evoked transmission, still form structurally normal synapses (Varoqueaux et al. 2002; Sigler et al. 2017). However, analyses of embryonic brains (E16-E18), before the onset of neurodegeneration that causes the death of the animals, showed extensive reduction of synapse number, even if the remaining synapses are morphologically normal (Bouwman et al. 2004; Verhage et al. 2000). Thus, the precise role of NT release in synapse formation is still not fully understood. If synaptic transmission appears dispensable for the very early stages of synapse assembly, it is required for synapse maturation and maintenance.

Back in 1963 Wiesel and Hubel introduced the concept of experience-dependent plasticity by studying the effects of monocular deprivation during the development of the visual system in kittens. The authors found atrophy in

areas of the brain connected to the deprived eye, whereas those connected to the open eye were expanded (Wiesel and Hubel 1963). This suggested that experience-induced neuronal activity shapes the connectivity of the brain. It is now established that synaptic connections rapidly respond to external stimuli, or patterns of neuronal activity, by long-term structural and functional changes, which are thought to be the cellular and molecular bases of learning and memory formation (Ho et al. 2011; Stuchlik 2014; Batool et al. 2019). This process is generally referred to as synaptic plasticity.

In response to experience-induced patterns of neuronal activity, synapses can be formed or eliminated, and their molecular composition can be altered to modulate synaptic strength. For instance, whisker stimulation/deprivation shapes the development and structural organisation of the barrel cortex in rodents (Erzurumlu and Gaspar 2012). Specifically, the activation of sensory neurons by whisker stimulation enhances motility and turn-over rate of dendritic spines and filopodia, resulting in an overall increase of excitatory and inhibitory inputs (Lendvai et al. 2000; Knott et al. 2002; Trachtenberg et al. 2002). Consistently, whisker deprivation reduces the survival rate of newly formed spines and rapidly induces rearrangements of synaptic connectivity in the barrel cortex (Trachtenberg et al. 2002; Knott et al. 2006). Exposure to a socially and physically enriched environment (EE) is another extensively used paradigm to study experience-dependent neuronal development. Rodents kept in EE exhibit increased gliogenesis and neurogenesis, enhanced complexity of neuronal morphology, higher synaptic density and improved learning (van Praag et al. 2000; Nithianantharajah and Hannan 2006). Indeed, learning and memory formation are complex cognitive processes that require the coordination of synaptogenic factors and synaptic molecules to establish functional and long-lasting connections (Tronson and Taylor 2007; Xu et al. 2009; Johansen et al. 2011; Holtmaat and Caroni 2016). Thus, experience drives profound changes in the connectivity of the brain, but how are these processes regulated at the cellular and molecular level?

Neuronal activity acts on synapses in multiple ways. Activity controls cytoskeletal dynamics, regulates the trafficking, recruitment, and stabilisation

of synaptic proteins, as well as changes in gene expression (Hua and Smith 2004; Ho et al. 2011). The most extensively studied forms of synaptic plasticity are the NMDAR-dependent long-term potentiation and depression (LTP/LTD) of hippocampal synaptic connections. Activation of NMDARs, through specific patterns of presynaptic stimulation like high or low frequency stimulation (HFS/LFS), allows Ca^{2+} influx into the postsynaptic compartment with consequent activation of downstream effectors (Lüscher and Malenka 2012). For instance, HFS-mediated opening of NMDARs activates CaMKII and other signalling molecules, which in turn recruit more AMPARs at the surface of dendritic spines (Lisman et al. 2012; Herring and Nicoll 2016), thus increasing the size and strength of these structure (Matsuzaki et al. 2001; Hanley 2008). Long-term effects of LTP and LTD include the formation/disassembly of synaptic connections and the modulation of gene expression (Malenka and Bear 2004).

The synaptic expression and localisation of a number of synaptogenic factors, including BDNF and Wnt signalling components, is regulated by neuronal activity, pointing to a role for these molecules in synaptic plasticity (Sahores and Salinas 2011; Poon et al. 2013; Kowiański et al. 2018). For instance, neuronal activity regulates the transcription, local translation and secretion of BDNF, which in turn controls several aspects of synaptic potentiation, such as NT release (Jovanovic et al. 2000; Lu 2003; Yano et al. 2006), AMPAR trafficking (Narisawa-Saito et al. 2002; Caldeira et al. 2007), gene expression (Messaoudi et al. 2002) and dendritic spine morphology and density (Alonso et al. 2004; Rex et al. 2007). Interestingly, striking similarities are shared between BDNF and Wnt signalling in mechanisms of synaptic plasticity (McLeod and Salinas 2018). In the following sections, I will cover the role of Wnt signalling in synapse formation and plasticity (see section 1.6.2).

In summary, synapse formation does not only occur during early developmental phases but continues throughout the entire life. Neuronal activity is a fundamental regulator of synapse assembly and function. Synaptic transmission, either spontaneous or evoked, can induce the formation of new synaptic connections. Moreover, experience-induced neuronal activity from motor, sensorial or emotional inputs, shapes the

connectivity of our brain. Neuronal activity drives synapse formation by affecting the expression, localisation and function of many synaptic molecules and synaptogenic factors, including BNDF and Wnt signalling molecules. In the next section, I will introduce the components of the Wnt pathways and I will discuss the regulation and functions of these prominent cascades.

1.4 Wnt signalling

1.4.1 Overview

With the expression “Wnt signalling” we refer to a group of signalling pathways activated by secreted glyco-lipoproteins called Wnts. The discovery of Wnt molecules dates back to 1976, when it was observed that a mutation in a *Drosophila* gene caused loss of wing tissue, hence the name Wingless (Wg) (Sharma and Chopra 1976). Later in 1982 Roel Nusse and Harold Varmus discovered a new proto-oncogene named Int-1 (Nusse and Varmus 1982) with homology to *Wg* (Rijsewijk et al. 1987). The fusion of the names of these two genes gave birth to the nomenclature Wnt. In the following decades an incredible amount of work has elucidated the function of Wnt signalling in a number of key biological processes. Wnt signalling is crucial in early development, as it regulates body axis patterning, cell fate, cell proliferation and cell migration (Logan and Nusse 2004; Nusse 2012; Nusse and Clevers 2017). In adults, Wnt signalling is involved in tissue homeostasis by regulating maintenance and differentiation of stem cells in different organs including the skin, the intestines and the brain (Reya and Clevers 2005; Katoh and Katoh 2007; Clevers et al. 2014). Wnt signalling also plays a fundamental role in the nervous system: it regulates patterning of the nervous system, formation and function of the blood brain barrier, axon guidance, dendritic development, synaptogenesis, synaptic plasticity and maintenance (Liebner et al. 2008; Salinas 2012; Park and Shen 2012; Dickins and Salinas 2013). Given the variety of biological processes in which Wnt signalling is involved, it is not surprising that aberrant Wnt signalling is linked to several pathologies including cancer, autoimmune diseases and neurological disorders such as schizophrenia and Alzheimer’s disease (AD) (Nusse 2005; Johnson and Rajamannan 2006; Harvey and Marchetti 2014; Nusse and Clevers 2017).

1.4.2 Wnt signalling components

Wnt signalling is extremely complex: a multitude of extracellular, cytoplasmic and nuclear components, as well a plethora of membrane receptors and

modulators, interact in multiple ways through at least 5 different signalling cascades (Komiya and Habas 2008).

Wnt ligands

Wnt ligands are secreted glyco-lipoproteins and are the main activators of Wnt signalling. Wnts are highly conserved across all metazoans; 19 Wnts are expressed in mammals, and with 10 Frizzled receptors and many other Wnt signalling components they signal through complex molecular pathways (Komiya and Habas 2008; MacDonald et al. 2009). Considerable amount of work, especially in *Drosophila* and *C. elegans*, has described the key steps and modulators of Wnt biogenesis and secretion. In the ER (endoplasmic reticulum) Wnts are subjected to two major posttranslational modifications (PTMs) N-glycosylation and acylation. Glycosylation occurs at two asparagine residues on the N-term of Wnt ligands and has been proposed to regulate Wnt secretion and signalling (Komekado et al. 2007; Kurayoshi et al. 2007). However, the requirement of glycosylation for these functions has been debated for some Wnt ligands like Wnt1 (Mason et al. 1992).

Wnt ligands are also lipid modified. First, Wnts have been proposed to be palmitoylated on a cysteine (Cys) residue at the N-term (Cys77 in Wnt3a) (Willert et al. 2003). This modification was originally proposed to be necessary for Wnt signalling activation but dispensable for protein secretion (Willert et al. 2003). However, later reports questioned the role of this modification by showing the involvement of Cys77 in the formation of a intra-protein disulfite bond rather than in binding palmitate moieties (Janda et al. 2012; Janda and Garcia 2015). In the ER Wnt ligands are further modified by palmitoleoylation, which differs from palmitoylation (Fig 1.10 page 77), at a conserved serine (Ser) residue (Ser209 in Wnt3a) by Porcupine (Porc) multi-pass membrane O-acyl transferase (MBOAT) (Takada 2006). Wnt palmitoleoylation is considered absolutely required for Wnt secretion, as palmitoleoylation-deficient Wnt mutants are retained in the ER (Takada et al. 2006; Hausmann et al. 2007). In addition, palmitoleoylation regulates Wnt binding to Frizzled receptors (Janda et al. 2012; Janda and Garcia 2015; DeBruine et al. 2017; Nile et al. 2017). However, a very recent report just questioned this claim by showing that in *Xenopus laevis* several Wnts, in

which palmitoleoylation sites had been mutated, retained binding to Frizzled receptors and signalling capability (Speer et al. 2019). Thus, despite extensive studies the precise role of Wnt's PTMs remains to be fully elucidated.

The trafficking of Wnt ligands from the ER to the plasma membrane is regulated by another multi-pass membrane protein, Wls (Wntless in *Drosophila*) or Evi (Eveness interrupted in vertebrates) (Ching and Nusse 2006; Bartscherer and Boutros 2008; Das et al. 2012). Evi is distributed along the entire secretion pathway, interacts directly with Wnts and is required for the progression of these ligands along the secretion route (Bänziger et al. 2006; Bartscherer et al. 2006; Goodman et al. 2006). In Wls mutant flies Wg accumulates in the ER and these animals phenocopy Wg mutants (Bänziger et al. 2006; Bartscherer et al. 2006; Goodman et al. 2006). Therefore, this multi-pass transmembrane protein is an essential regulator of Wnt trafficking and function.

The retromer, a complex of molecules that shuttles proteins from endosomes to the Golgi apparatus and back to the PM, is a fundamental regulator of protein trafficking, including Wnt ligands (Seaman 2012). The retromer complex plays a crucial role in Wnt secretion by regulating the recycling of Evi from endosomes to the Golgi apparatus and enabling further cycles of Evi-mediated Wnt transport and secretion at the PM (Belenkaya et al. 2008; Port et al. 2008; Yang et al. 2008; Harterink et al. 2011). In addition, the ERAD (endoplasmic reticulum-associated degradation) system controls Evi degradation in response to Wnt activation, providing a mechanism of feedback loop inhibition (Glaeser et al. 2018). Thus, the secretion of Wnt ligands, and therefore their activity, is highly dependent on the correct function of these two protein-trafficking regulators.

However, caution should be taken when discussing Wnt secretion. In fact, the secretion of these molecules is a matter of vivid debate in the Wnt field. Is Wnt secretion required for signalling? It is commonly assumed that Wnt ligands must be secreted and diffuse in the extracellular space to signal. Several models for Wnt secretion and propagation have been proposed (Takada et al. 2017), including extracellular shuttling mediated by the Wnt-

binding proteins SWIM (secreted Wg-interacting molecule) (Mulligan et al. 2012); restricted diffusion along neighbouring cells through proteoglycans (Yan and Lin 2009); movements across filopodia-like protrusion, or cytonemes (Stanganello and Scholpp 2016); and release on extracellular vesicles like exosomes (Gross et al. 2012; Koles et al. 2012). Each model presents limitations and seems restricted to specific cellular-contexts. Recent work from the Vincent lab questioned the assumption that Wnt must diffuse in the extracellar space to signal. Flies in which Wg was replaced with a membrane-bound form of this protein, are viable, fertile, and exhibit only a minor delay in tissue morphogenesis, suggesting that Wnt diffusion is dispensable for signalling in these conditions (Alexandre et al. 2014). Thus, despite the huge progress made in understanding Wnt biogenesis, modification and secretion, there is still much to learn about their trafficking and signalling regulation at the PM and in the extracellular space.

Secreted Wnt inhibitors

SFRPs (secreted Frizzled related proteins) constitute the largest family of Wnt inhibitors (Cruciat and Niehrs 2013). In humans five *SFRPS* genes have been identified. These secreted molecules are characterised by the presence of a N-term CRD (Cys rich domain) domain that resembles the CRD region of Frizzled receptors. SFRPs inhibit Wnt signalling in two ways: a) by binding and sequestering Wnts in the extracellular space (Leyns et al. 1997; Lin et al. 1997; Wang et al. 1997); b) by interacting with the CRD domain of Frizzled receptors, thus competing with Wnts for binding (Bafico et al. 1999; Rodriguez et al. 2005). By chelating Wnts prior to Frizzled binding, SFRPs exert a global inhibition on Wnt signalling not directed to specific cascades (see section 1.4.3). However, by binding to Frizzled receptors, SFRPs have been shown to silence specific Wnt signalling pathways (Bovolenta et al. 2008). Interestingly, in addition to the classical inhibitory function of SFRPS, a role in Wnt signalling activation has also been proposed (Rodriguez et al. 2005). In fact, by interacting with both Frizzled and Wnt, SFRPs can favour the interaction between ligand and receptor; moreover, by bridging Frizzled-Frizzled interactions, SFRPs can also promote the formation of receptor dimers which are sufficient to initiate signalling even in the absence of Wnts

(Carron et al. 2003; Rodriguez et al. 2005). Moreover, SFRPs interact with molecule unrelated to Wnt signalling; therefore, independently of Wnt they are involved in biological processes such as cancer, axon guidance and BMP signalling (Bovolenta et al. 2008). Thus, the versatile mode of action of these molecules makes them an interesting target for Wnt signalling regulation.

Another group of Wnt antagonists is the Dkk (Dickkopf) protein family, which consists of four members of secreted glycoproteins. Dkk1, -2 and -4 exhibit high homology with each other and inhibit Wnt signalling by binding to the Wnt co-receptor LRP6 (Zorn 2001). Dkk binding to LRP6 blocks canonical Wnt signalling (see section 1.4.3) in two ways: a) Dkk1 competes with Wnt ligands for binding to LRP6; b) Dkk1 inhibits the assembly of the LRP6-Frizzled complex preventing the formation of the Wnt signalosome (Bafico et al. 2001; Niehrs 2006). Dkk1 and Dkk2 can also bind to other receptors, specifically Kremen1 and 2. Dkk1 binding to Kremen stabilises the formation of a complex with LRP6 and induces the endocytosis of this complex, thus decreasing surface levels of LRP6 and inhibiting canonical Wnt signalling (Mao et al. 2002). Dkk3 is the most divergent member of the family and its role in Wnt signalling is still poorly understood (Niehrs 2006). It has been proposed that Dkk3 binds to Kremen 1 and 2 but does not interact with LRP6 (Nakamura and Hackam 2010); therefore, it might not act as a Wnt antagonist.

Other secreted Wnt inhibitors are the de-acylase Notum, an enzyme that removes palmitoleic acid from Wnt ligands inhibiting their interaction with Frizzled receptors (Kakugawa et al. 2015), and WIF-1 (Wnt inhibitory factor 1), which similarly to SFRPs binds to Wnt ligands preventing their interaction with Frizzled receptors (Kawano and Kypta 2003). Therefore, Wnts can be modulated intracellularly and extracellularly by a variety of different proteins that affect the function of these ligands by adding/removing PTMs, by regulating Wnt trafficking, or by competing for binding with Wnt receptors.

Frizzled receptors

Frizzled are 7 transmembrane G-protein Coupled receptors (GPCRs) and are the main receptors for Wnt ligands. Like many of the Wnt signalling

components, Frizzled receptors were first identified in *Drosophila*, where Frizzled deficient flies revealed tissue polarity defects, such as disorganisation of wing hair, hence the name Frizzled (Gubb and García-Bellido 1982). However, only in 1996 it became clear that Frizzled are receptors for Wnt ligands (Bhanot et al. 1996). 10 Frizzled receptors are expressed in mammals, and they play critical roles in all Wnt signalling cascades. Frizzled receptors are essential for embryonic development, establishment of tissue and cell polarity, CNS formation and function, as well as regulation of cell proliferation in adults (Huang and Klein 2004; Wang et al. 2016; Zeng et al. 2018).

Frizzled receptors exhibit some typical features of GPCRs: they are formed by 7 transmembrane domains, they are glycosylated at the extracellular N-term region, they have a cytoplasmic C-term tail that can be modified by PTMs and interact with G protein subunits (Schulte and Bryja 2007). Frizzled can indeed signal as GPCRs by interacting with G protein subunits and by activating intracellular Ca^{2+} signalling typical of this class of receptors (Slusarski et al. 1997; Katanaev and Buestorf 2009; Koval and Katanaev 2011; Wright et al. 2018). However whether GPCRs-like activation of Frizzled is required in all Wnt cascades remains unknown (see section 1.4.3). Frizzled exhibits a fairly conserved N-term CRD (cysteine rich domain), which is required for Wnt binding (Fig 1.5) (Huang and Klein 2004; Wang et al. 2006). The CRD of these receptors contains a hydrophobic cavity that allows the interaction with the palmitoleate moiety of Wnt ligands (Janda et al. 2012; DeBruine et al. 2017; Nile et al. 2017). Recent works have shown that the lipid moiety of Wnt ligand bridges two or more CRD domains of Frizzled receptors (DeBruine et al. 2017; Nile et al. 2017), consistent with the notion that these receptors can form homo- and heterodimers at the cell surface (Dann et al. 2001; Carron et al. 2003). Dimerisation of Frizzled receptors is sufficient to initiate signalling (Dann et al. 2001; Carron et al. 2003), but whether Wnt binding induces Frizzled dimerisation has not been demonstrated yet. Despite the progresses made in understanding the interaction between Wnt and Frizzled at the cell surface, there is still much to learn about the molecular mechanisms that regulate this process.

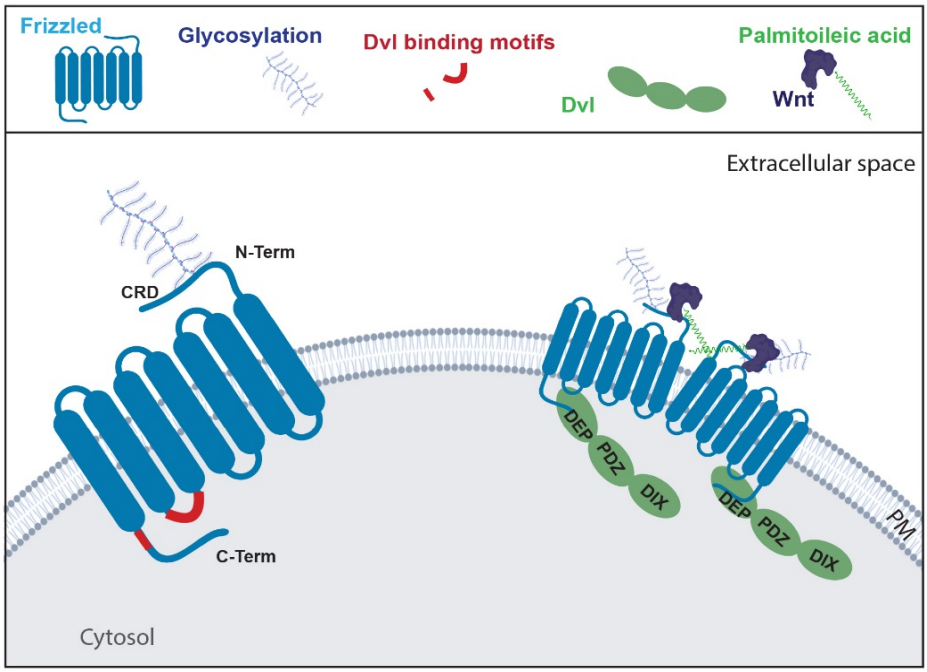

Fig 1.5: Frizzled receptors

Frizzled are the main receptors for Wnt ligands and are essential for Wnt signalling activation. The human genome contains 10 *FZD* genes encoding for proteins that exhibit a fairly similar glycosylated N-Term and highly variable C-Term tails, except for a conserved Dvl binding motif. The key scaffold protein Dvl interacts with Frizzled through the DEP domain. Wnt palmitoleoylation is required for Frizzled binding and induces dimerization of Frizzled receptors.

The C-term of Frizzled receptors varies greatly in length and amino acid composition, but exhibits a conserved Dishevelled (Dvl) binding motif in close proximity to the transmembrane domain (Wang et al. 2006). Dvl is a scaffold protein that is critically involved in all Wnt signalling cascades. Dvl is composed of three domains: DIX, PDZ and DEP. The DIX domain is required for Dvl polymerisation, which plays a role in signalling activation (see section 1.4.3), and is involved in Frizzled binding; the PDZ domain is required for Frizzled binding and ,as well as the DEP domain, for the interaction with other downstream components (Fig 1.5) (Gao and Chen 2010). In addition to the C-term, two motifs on the intracellular loops of Frizzled receptors (Fz5 precisely) are required for binding to Dvl1 (Tauriello et al. 2012). Frizzled activation by Wnt ligands induces membrane recruitment of Dvl, an event that is common and required for all Wnt signalling cascades (Gao and Chen 2010) (see section 1.4.3).

Like most GPCRs Frizzled receptors are modified by PTMs. First, Frizzled receptors are glycosylated on multiple sites at the N-term region and these modifications have been suggested to promote the maturation of Frizzled receptors and their trafficking to the PM (Yamamoto et al. 2005). The ER resident protein Shisa, inhibits Frizzled glycosylation, limiting their trafficking to the PM and down-regulating Wnt signalling (Fig 1.5) (Yamamoto et al. 2005). TMEM59 (transmembrane protein 59) is another protein that regulates Fz5 glycosylation and promotes its trafficking to and clustering at the PM (Gerlach et al. 2018). TMEM59 inhibits Fz5 glycosylation but, in contrast to Shisa, it promotes Frizzled-mediated signalling (Gerlach et al. 2018), thus questioning the function of Frizzled glycosylation. In fact, the authors showed that glycosylation-deficient mutant Fz5 receptors are normally trafficked to the PM and exhibit WT-like levels of signalling activation (Gerlach et al. 2018). Therefore, the physiological role of Frizzled glycosylation remains unclear and requires further investigations.

Frizzled receptors can also be phosphorylated. These receptors contain putative sites for PKA and PKC phosphorylation (Wang and Malbon 2004). Dvl-dependent phosphorylation of Fz3 has been suggested to down-regulate Wnt signalling (Yanfeng et al. 2006); whereas phosphorylation of Fz6 by the CK1 (Casein kinase 1) is required to maintain the surface levels of this receptor (Strakova et al. 2018). Thus, phosphorylation of Frizzled receptors can affect Wnt signalling but the precise role of this modification remains largely unexplored and deserves further attention. Independent observations demonstrated that ubiquitination/deubiquitination cycles control Frizzled internalisation and recycling at the PM (Hao et al. 2012; Koo et al. 2012; Madan et al. 2016). Frizzled receptors are multi-monoubiquitinated on lysine residues by the E3 ligases RNF43 (ring finger protein 43) and ZNRF3 (zinc and ring finger protein 3) (Hao et al. 2012; Koo et al. 2012). Ubiquitination of Frizzled induces the internalisation and lysosomal degradation of these receptors, reducing their surface levels and down-regulating Wnt signalling (Hao et al. 2012; Koo et al. 2012; Moffat et al. 2014). RNF43 and ZNRF3 are inhibited by the secreted molecules R-Spondins and their receptors LGRs (Leucine-rich repeat-containing G-protein coupled receptors), which are

potent activators of Wnt signalling (de Lau et al. 2014; Hao et al. 2016). R-Spondins bind to both RNF43/ZNRF3 and LGRs forming a trimeric complex (Hao et al. 2012). The binding of R-spondin causes ZNRF3 internalisation, inhibiting Frizzled ubiquitination and increasing the surface levels of these receptors (Hao et al. 2012). Consistently, loss of function mutations of RNF43 and ZNRF3 increase surface levels of Frizzled receptors resulting in aberrant activation of Wnt signalling, which is associated with cancer development (Hao et al. 2016; Katoh and Katoh 2017; Zeng et al. 2018). Thus, mounting evidence indicates that ubiquitination of Frizzled receptors plays a crucial role in Wnt signalling modulation.

The role of receptor endocytosis is a controversial topic in the Wnt signalling field (Gagliardi et al. 2008). Wnt-induced Clathrin and Caveolin-mediated endocytosis of Wnt receptors (Frizzled and LRP6) are required for signalling activation (Seto and Bellen 2006; Yamamoto et al. 2006; Gagliardi et al. 2014; Hagemann et al. 2014). However, endocytosis is also a mechanism to reduce surface levels of Wnt receptors and dampen Wnt signalling (Hao et al. 2012; Koo et al. 2012; Moffat et al. 2014; Madan et al. 2016; Agajanian et al. 2019). Therefore, whether endocytosis activates or inhibits Wnt signalling seems to depend on the cellular context, and the molecular mechanisms that regulate Frizzled membrane trafficking remain poorly undersood.

In summary, Frizzled are the main receptors for Wnt ligands and are essential for Wnt signalling activation. The function of these receptors is modulated by PTMs; in particular, glycosylation, phosphorylation and ubiquitination have been proposed to regulate the trafficking and signalling of these receptors. However, the molecular mechanisms that regulate Frizzled trafficking and their activity are still poorly understood and require further investigation. During my PhD, I have studied a novel mechanism of regulation of Frizzled trafficking and function.

The co-receptor LRP6 and other Wnt receptors

At the cell surface, Wnts bind to a variety of receptors beside the aforementioned Frizzled. LRP6 (Low-density lipoprotein receptor-related

protein 6) is a single pass transmembrane protein that acts as a co-receptor for Wnts in the canonical β-catenin pathway (see section 1.4.3). LRP6 has a long N-term extracellular domain which can bind multiple Wnt ligands, some of which simultaneously (Bourhis et al. 2010), and the Wnt inhibitors Dkk1 and Dkk2 (B T MacDonald and He 2012). Given the ability of binding Wnt ligands and Wnt antagonists, LRP6 is a fundamental regulator of Wnt signalling activation. In addition, the C-term of LRP6, which contains multiple phosphorylation sites, is essential for signalling transduction (Tamai et al. 2004; MacDonald et al. 2009). Wnt binding to LRP6 and Frizzled receptors induces the formation of a multimeric complex, which induces the PM recruitment of Dvl and other components of the Wnt cascade, including the scaffold protein Axin, the kinases CK1 and Gsk3β (glycogen synthase kinase 3 (see section 1.4.3) (Cong et al. 2004; He et al. 2004; Gao and Chen 2010). The role of LRP6 in Wnt signalling will be examined in more details in the context of the β-catenin pathway (see section 1.4.3). Beside Wnt signalling activation, LRP6 plays a fundamental role in lipid metabolism mediating cholesterol homeostasis (Go and Mani 2012).

Wnts can also bind to the tyrosine kinase receptors Ror and Ryk. Ror receptors contain a N-term Frizzled-like CRD domain that is required for Wnt binding, whereas Ryk receptors bind Wnt ligands through an extracellular domain similar to the Wnt inhibitor WIF (Green et al. 2014). Wnt binding to these receptors primes the formation of a complex between ligand, receptor (Frizzled) and co-receptor (Ror or Ryk), similarly to the interaction with LRP6. Signalling through Ror or Ryk has been shown to activate the planar cell polarity (PCP) pathway through the membrane recruitment of Dvl (see section 1.4.3) (Komiya and Habas 2008; Gao and Chen 2010; Minami et al. 2010). By signalling through this cascade, Ror and Ryk are fundamental regulators of tissue morphogenesis and play important functions in CNS development (Minami et al. 2010; Green et al. 2014). Moreover, the GPCR Gpr124 (Probable G-protein coupled receptor 124) and the metallo-protease inhibitor Reck (reversion inducing cysteine rich protein with kazal motifs) are important co-receptors for Wnt7a/Wnt7b and are essential for the development of the BBB (blood brain barrier) (Cho et al. 2017). Reck binds to

Gpr124, which in turn binds to Frizzled receptors facilitating the formation of a signalling complex (Cho et al. 2017). In addition, recent work has shown that Wnt7a/b can directly bind Reck, which then associates with other receptors including Frizzled and Gpr124 (Eubelen et al. 2018; Vallon et al. 2018). Thus, Wnt binding to a variety of different plasma membrane receptors and co-receptors results in a vast signalling diversification. In the next session, I will introduce the Wnt signalling cascades activated downstream of surface receptors.

1.4.3 Wnt signalling pathways

Canonical or β-catenin Wnt signalling

Canonical Wnt signalling is the most characterised Wnt signalling pathway. The key features of this cascade are the stabilisation of cytoplasmic β-catenin by inhibition of the β-catenin destruction complex, and the consequent activation of transcription factors that regulate the expression of Wnt target genes (Clevers 2006; Nusse and Clevers 2017).

In the canonical Wnt cascade, a series of signalling events determine whether β-catenin is degraded, down-regulating Wnt signalling, or whether it accumulates in the cell promoting signalling transduction. Gsk3β and CK1, together with the scaffold protein Axin, the cytoplasmic protein APC (Adenomatus polyposis coli), PP2A (protein phosphatase 2A) and the E3 ubiquitin ligase β-TrCP (Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase) are part of the β-catenin destruction complex. In the abscence of Wnts, the kinases of this complex phosphorylate β-catenin targeting it for ubiquitination by β-TrCP and consequent proteasomal degradation (Kimelman and Xu 2006; Stamos and Weis 2013).

The canonical pathway is initiated when Wnt binds to Frizzled and LRP6, an event that triggers the membrane recruitment of Dvl, promoting the formation of a signalling complex and the disassembly of the destruction complex (Komiya and Habas 2008; Nusse and Clevers 2017). Next, LRP6 is phosphorylated by Gsk3β and CK1, and Axin is recruited to the PM where it interacts with the phosphorylated C-term tail of LRP6 (Davidson et al. 2005;

Zeng et al. 2005). Upon ligand binding and phosphorylation of LRP6 the destruction complex is inhibited and cytoplasmic β-catenin is stabilised. Several models have proposed different sequences of events downstream of LRP6 activation that lead to β-catenin stabilisation: 1) in the initiation-amplification model is a feed-forward loop in which the formation of the LRP6-Frizzled-Dvl complex at the PM recruits Axin, which promotes GSK3β-mediated phosphorylation of LRP6, resulting in the recruitment of more Axin to the PM (MacDonald et al. 2008; Wolf et al. 2008). 2) In the signalosome model, DIX-domain-dependent polymerisation of Dvl promotes the clustering of multiple Frizzled-LRP6 complexes, which results in Axin recruitment and LRP6 phosphorylation by Gsk3β and CK1 (Schwarz-Romond, Fiedler, et al. 2007). Axin also contain a DIX domain, proposed to polymerise with Dvl (Schwarz-Romond, Metcalfe, et al. 2007). 3) The endosomal signalling model shares similarity with the signalosome model, but it proposes that assembly of the signalosome and signalling transduction occurs upon Caveolin-mediated internalisation of LRP6 and Frizzled receptors (Yamamoto et al. 2006; Bryan T MacDonald and He 2012b). However, canonical Wnt signalling is up-regulated in intestinal stem cells of *Caveolin*$^{-/-}$ mice, raising questions as to whether this model is restricted to specific cellular contexts (Li et al. 2005; Sotgia et al. 2005; Gagliardi et al. 2008; Bryan T MacDonald and He 2012b). 4) Another model suggests that Dvl activates PI4KIIα (phosphatidylinositol 4-kinase type II) and PIP5KI (phosphatidylinositol-4-phosphate 5-kinase type I) leading to the production of PIP_2 (phosphatidylinositol 4,5-bisphosphate). PIP_2, through unknown molecular mechanisms, promotes the clustering and phosphorylation of LRP6 by Gsk3β and CK1 (Pan et al. 2008). Thus, in spite of huge progress, the precise molecular mechanisms by which the canonical Wnt signalling is activated remain to be fully elucidated.

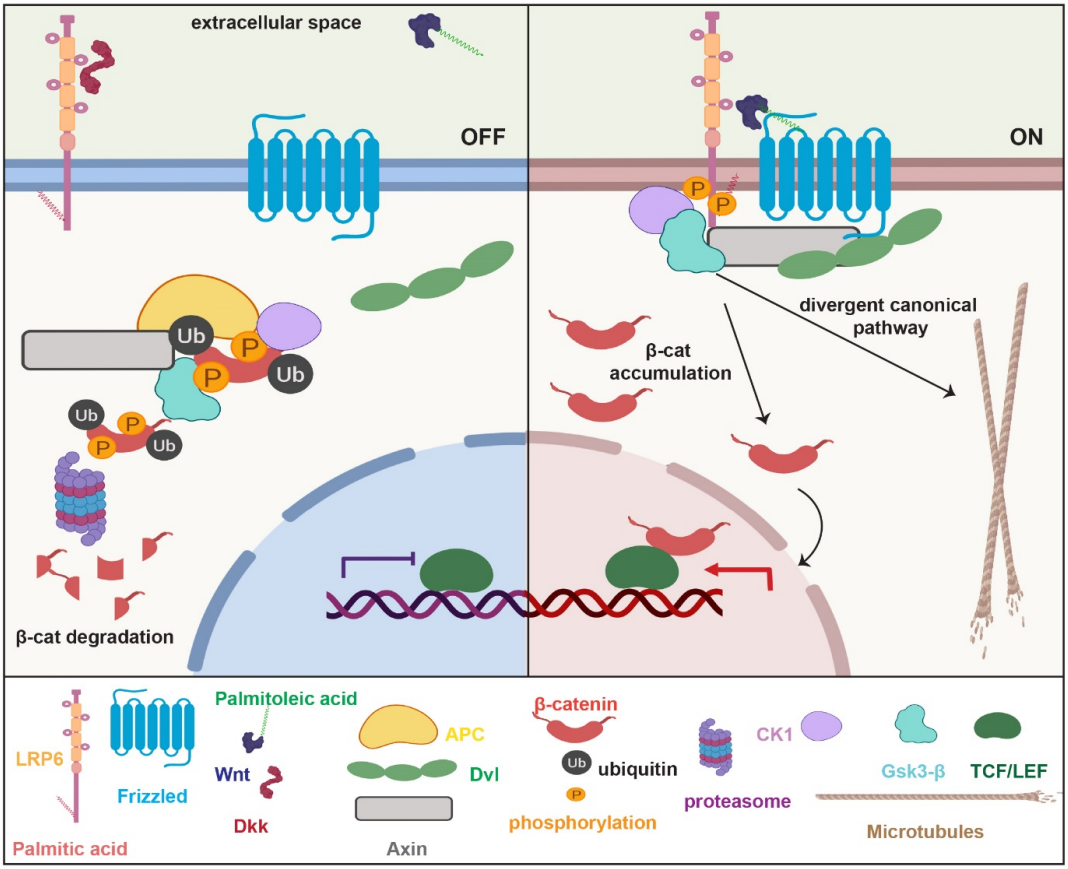

Fig 1.6: β-catenin and divergent canonical Wnt pathways

In the absence of Wnt ligands, or in the presence of Wnt inhibitors like Dkk1, the destruction complex formed by Gsk3-β, Axin, APC, CK1 and β-TrCP, phosphorylates and ubiquitinates β-catenin, targeting it to proteasomal degradation. Wnt binding to LRP6 and Frizzled induces PM recruitment of Dvl together with Axin, CK1 and Gsk3-β, leading to the accumulation of unphosphorylated β-catenin in the cytoplasm. Phosphorylation of LRP6 by CK1 and Gsk3-β activates dowstream signalling. Unphosphorylated β-catenin translocates to the nucleus, and drives the expression of Wnt target genes through TCF/LEF. Activation of a divergent canonical cascade downstream of Gsk3-β, independent of β-catenin and transcription, promotes microtubule remodelling.

Once β-catenin is stabilised in the cytoplasm, it translocates to the nucleus where it binds to the transcription factors LEF/TCF (lymphoid enhancer binding factor, and transcription factor) to drive the expression of Wnt target genes (Henderson and Fagotto 2002; Jamieson et al. 2012). Wnt target genes include regulators of cell proliferation, differentiation and migration as well as several Wnt signalling components such as Axin, LRP6, Dkk1, Notum and others, thus providing negative/positive feedback loops to regulate this signalling cascade (Gordon and Nusse 2006; Lien and Fuchs 2014; Ramakrishnan and Cadigan 2017).

The divergent canonical pathway

The divergent canonical pathway is activated through the initial steps of the canonical pathway but diverges at the level of Gsk3β and it does not involve regulation of gene transcription. This alternative cascade was initially proposed to regulate cell division in *C. elegans* blastomers (Schlesinger et al. 1999). Loss of function mutations of several Wnt-components (including Gsk3β), but not TCF or RNA polymerase mutants, exhibited defects in mitotic spindle orientation, indicating the requirement of Gsk3β but the dispensability of gene transcription (Schlesinger et al. 1999). Our laboratory has demonstrated that Axin and Dvl inhibit Gsk3β-mediated phosphorylation of microtubule-associated proteins, thereby stabilising microtubules and regulating microtubules organisation in developing axons (Ciani et al. 2004). Subsequent studies showed that this pathway is conserved at the *Drosophila* NMJ (Gögel et al. 2006). Studies in *Drosophila* and mammalian cells suggest that other canonical Wnt components such as APC, Axin and Dvl interact directly with the cytoskeleton and that Gsk3β phosphorylates microtubule-associated proteins as well as APC (Salinas 2007). Thus, classical members of the canonical Wnt cascade have key functions in modulating microtubule dynamics by acting downstream of Gsk3β, through a divergent signalling pathway that is independent of transcription.

The PCP pathway

The Planar Cell Polarity pathway is a β-catenin-independent Wnt cascade that is best characterised for its role in establishing cell and tissue polarity (Fig 1.6) (Gray et al. 2011; Devenport 2014). In the PCP pathway, Wnt ligands bind to Frizzled receptors independently of LRP6. The co-receptors Ror and Ryk have been shown to play a role in this cascade (Green et al. 2014), but whether they are always required for PCP signalling remains elusive. Like in the canonical pathway, Dvl is recruited to the PM, where it interacts with Daam1 (Dishevelled Associated Activator Of Morphogenesis 1) to activate two downstream pathways: the Rho-ROCK (Rho associated coiled-coil containing protein kinase) cascade, and the Rac-JNK (Jun N-

terminal kinase) pathway. These kinases phosphorylate a plethora of downstream targets; therefore, the precise molecular interactions downstream of ROCK and JNK depend on the specific cellular context (Amano et al. 2010; Zeke et al. 2016). The functional outcome of Rho-ROCK and Rac-JNK signalling is the remodelling of cytoskeletal structures (Schlessinger et al. 2009).

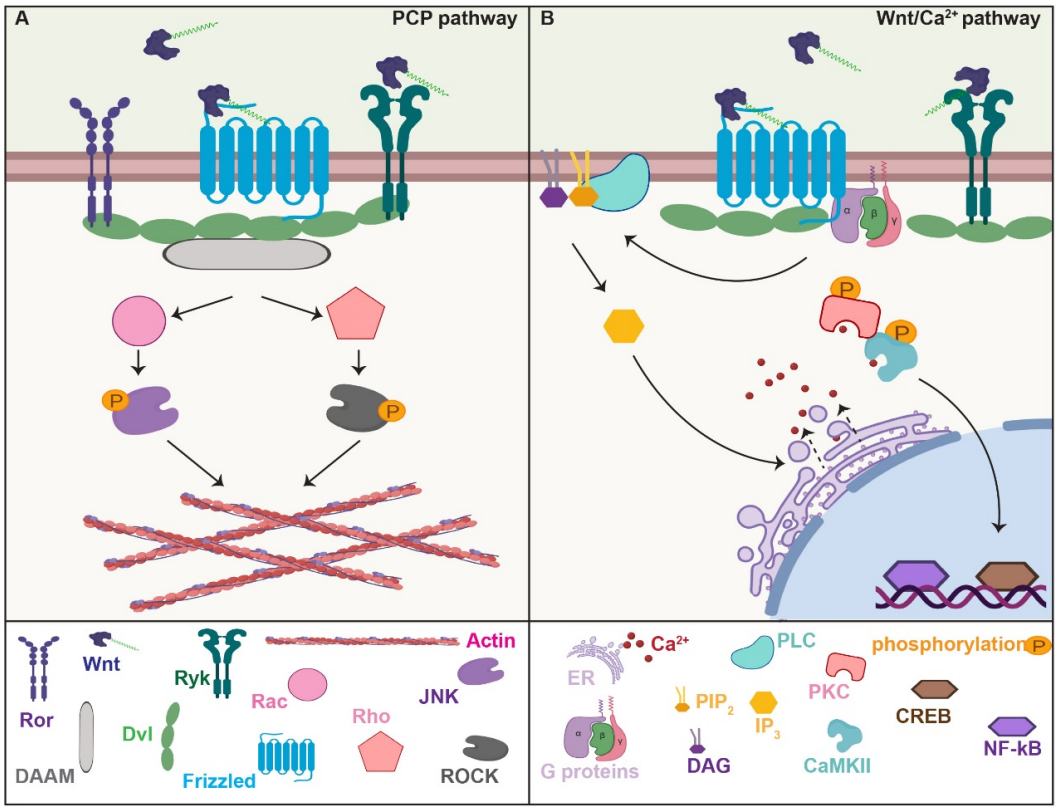

Fig 1.7: PCP and Wnt/Ca^{2+} pathways
A: In the PCP pathway, Wnt binding to Frizzled and the co-receptors Ryk and Ror activates the small GTPases Rho and Rac, which in turn activate ROCK and JNK driving remodelling of the actin cytoskeleton. **B:** In the Wnt/Ca^{2+} pathway, Wnt binding to Frizzled activates G proteins and PLC, which acts on PIP_2 and DAG to produce IP_3. IP_3 induces Ca^{2+} release from the ER activating the kinases PKC and CaMKII. Downstream signalling events include NFkB and CREB activation in the nucleus to drive gene transcription.

The Wnt/Ca^{2+} pathway

The Wnt Ca^{2+} pathway is another β-catenin-independent Wnt cascade (Fig 1.6). In this signalling pathway, Wnt ligands bind to Frizzled and in some circumstances to co-receptors, such as Knypek and Ror2, inducing recruitment of Dvl in a G protein dependent manner and activating Phospholipase C (PLC). PLC acts on PIP_2 (Phosphatidylinositol 4,5-

bisphosphate) increasing the levels of the second messengers IP_3 (Inositol trisphosphate) and DAG (diacylglycerol). IP_3 induces the rapid release of Ca^{2+} from the ER activating Ca^{2+}-dependent kinases such as PKC and CaMKII (Kühl et al. 2000; Sheldahl et al. 2003), which can signal to a number of downstream effectors including the transcription factors CREB (cAMP response element-binding protein) and NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells). Ca^{2+} signalling plays an important role during embryogenesis, CNS development and cancer (Komiya and Habas 2008; De 2011).

Frizzled nuclear import pathway

This Wnt pathway is distinguished from all the other Wnt cascades for the mechanism of Frizzled signalling (Fig 1.7). This cascade was described at the *Drosophila* NMJ to regulate synapse formation and function (Koles and Budnik 2012). Wg binding to DFz2, which is the homolog of human Fz5 and Fz8, causes receptor internalisation and cleavage (Mathew et al. 2005). DFz2 is cleaved at the C-term domain and this portion of protein trafficks to the nucleus in a GRIP1 (glutamate receptor interacting protein 1) dependent manner, whereas the rest of the DFz2 accumulates outside the nucleus (Ataman et al. 2006). Wg, DFz2 or GRIP1 null flies exhibit the same defects in synapse formation and function (Ataman 2006). The molecular mechanisms that control DFz2 nuclear function are unclear, but its nuclear import is followed by nuclear export of large complexes of transcripts for synaptic proteins (Speese et al. 2012). This mechanism of cleavage and nuclear import is unusual for Frizzled receptors but it is a quite common mode of action for other types of molecules such as tyrosine kinase receptors (Carpenter and Liao 2013; Chen and Hung 2015). For instance, Ryk is cleaved at the C-term and this portion of protein is transferred to the nucleus to regulate the differentiation of neuronal progenitor cells in the cortex (Lyu et al. 2008). Thus, Wnt-induced receptor cleavage and nuclear import seem to be additional Wnt signalling routes; however, further experiments are needed to address the role of this pathway in other biological contexts and in other organisms.

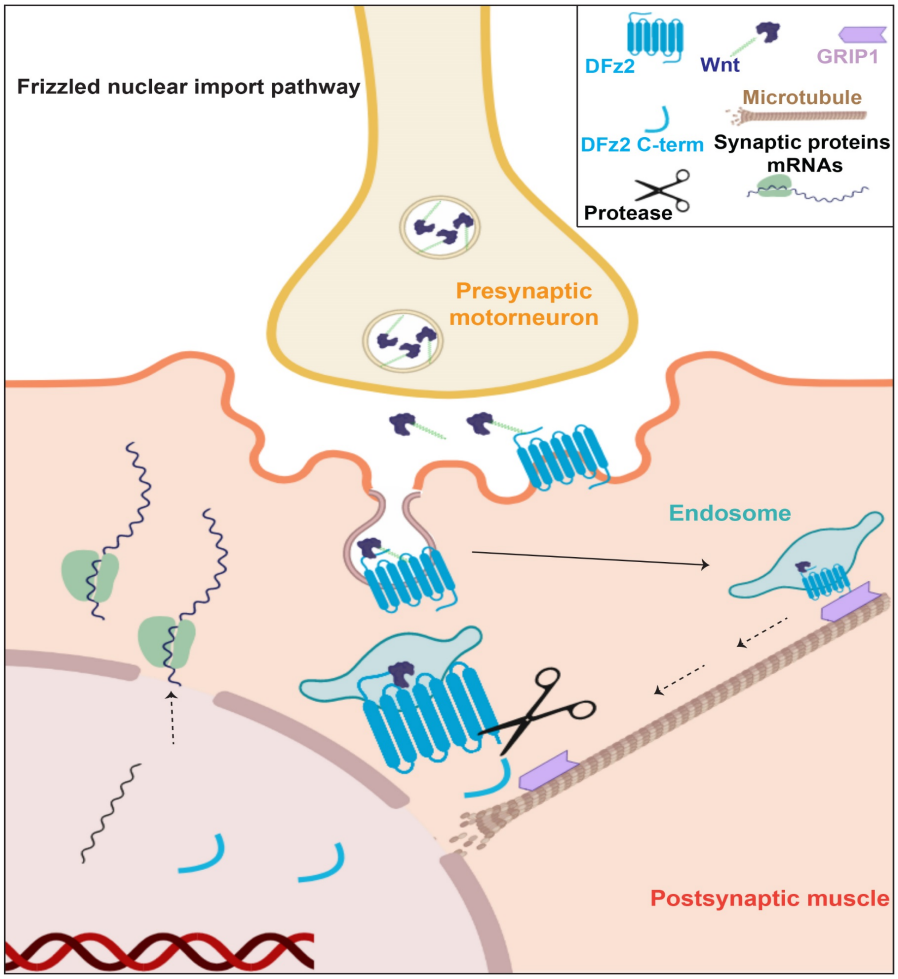

Fig 1.8 Frizzled nuclear import pathway

At the *Drosophila* NMJ Wnt is released by presynaptic motor neurons and binds to DFz2 on postsynaptic muscle cells. The binding induces internalisation of DFz2 and GRIP1-dependent trafficking along microtubules. DFz2 is cleaved at the C-term and, through yet unknown molecular mechanisms, this protein fragment is imported into the nucleus to promote the expression of genes encoding synaptic proteins.

1.5 Wnt signalling in neural development

Wnt signalling is involved in many key aspects of neural development and these functions are highly conserved across different species. From very early embryonic stages, Wnts promote the development of the neural tube, which is the precursor structure of the brain, and trigger site-directed migration of multipotent stem cells from the dorsal part of the neural tube, which is the neural crest (Ikeya et al. 1997; Dorsky et al. 1998; Pinson et al. 2000; Kiecker and Niehrs 2001; De Calisto et al. 2005; Bocchi et al. 2017). Once these multipotent stem cells have migrated and reached the appropriate location, Wnt signalling is required for their differentiation into mature neurons (Dickinson et al. 1994; Megason and McMahon 2002; Chesnutt et al. 2004; Hirabayashi et al. 2004). In differentiated neurons Wnts play a fundamental role in establishing neuronal polarity and promoting the growth of neuronal processes (Ciani and Salinas 2005; Salinas and Zou 2008; C.-W. He et al. 2018). Numerous pieces of evidence show that Wnt signalling plays a role in axon growth, guidance and branching in different model systems and in different areas of the CNS and PNS (Lucas and Salinas 1997; Krylova et al. 2002; Lyuksyutova et al. 2003; Yoshikawa et al. 2003; Rodriguez et al. 2005; Keeble et al. 2006; Hua et al. 2014). In addition, Wnts are also required for activity-mediated dendritic growth and branching (Yu and Malenka 2003; Rosso et al. 2005; Wayman et al. 2006; Kirszenblat et al. 2011; Lanoue et al. 2017; Ferrari et al. 2018).

In late embryonic development and early postnatal life, Wnt signalling plays a crucial role in synapse formation by modulating the assembly of pre- and postsynaptic sites (Budnik and Salinas 2011; Salinas 2012). Wnt signalling is also implicated in basal synaptic transmission as well as short- and long-term synaptic plasticity (Oliva et al. 2013a; McLeod and Salinas 2018). In the adult, Wnts control neurogenesis (Lie et al. 2005; Varela-Nallar and Inestrosa 2013) and mechanisms of synaptic maintenance (Inestrosa and Arenas 2010; Dickins and Salinas 2013; Purro et al. 2014; Buechler and Salinas 2018). Thus, Wnt signalling plays different roles at synapses, regulating their assembly during development to controlling their function and maintenance during adulthood.

Wnt signalling not only promotes the development and function of neuronal cells, but it is crucial for several other cell types in the brain. In particular, Wnt signalling is fundamental for the formation of the BBB (Liebner et al. 2008; Polakis 2008; Zhou and Nathans 2014; Cho et al. 2017), and regulates the maturation and function of oligodendrocytes, astrocytes and microglia (Halleskog et al. 2011; Yang et al. 2012; Tang 2014; Yao et al. 2016; Zheng et al. 2017; Soomro et al. 2018). In light of such a profound involvement at different levels of neural development, maturation, and function, it is not surprising that aberrant Wnt signalling is linked to several neurological disorders including brain patterning abnormalities, psychiatric conditions such as schizophrenia and bipolar disorders, and neurodegenerative pathologies like Alzheimer's (AD) and Parkinson's disease (Berwick and Harvey 2012; Panaccione et al. 2013; Inestrosa and Varela-Nallar 2014; Harvey and Marchetti 2014; Purro et al. 2014; Mulligan and Cheyette 2017; Hoseth et al. 2018).

1.6 Wnt signalling at the synapse

The role of Wnts at synapses was first described in the context of synaptogenesis, but studies in the last 20 years have also revealed a role for Wnt signalling in synaptic transmission, synaptic plasticity and synapse maintenance in adulthood. These key roles of Wnt signalling are directly translated into functional outcomes, such as the regulation of cognitive functions and complex behaviours. In the following sections, I will introduce evidence supporting the role of Wnt signalling in these processes.

1.6.1 Wnt signalling in synaptogenesis

Wnt molecules are potent synaptogenic factors able to induce pre and postsynaptic assembly. Our laboratory first described that exposure to Wnt7a increases the clustering of presynaptic proteins in cultured cerebellar granule cells (Lucas and Salinas 1997). These findings were soon corroborated by *in vivo* studies demonstrating that Wnt7a null mice exhibit defects in presynaptic organisation in the cerebellum, precisely at the mossy fibre-granule cell synapse (Hall et al. 2000). In primary hippocampal cultures, Wnt7a/Wnt7b promote the assembly of excitatory presynaptic terminals without affecting inhibitory synapse formation (Davis et al. 2008; Ciani et al. 2011). Expression of Fz5 in hippocampal neurons is sufficient to promote presynaptic assembly, whereas loss of function of this receptor reduces the number of presynaptic sites (Fig 1.9) (Sahores et al. 2010). Moreover, Fz5 receptors, which are present along axons, are required for Wnt7a-mediated presynaptic assembly (Fig 1.9) (Sahores et al. 2010). Furthermore, Wnt7a signalling, through removal of APC from the β-catenin destruction complex, has been suggested to promote clustering of nicotinic acetylcholine receptors at presynaptic site of hippocampal neurons (Farías et al. 2007).

Other Wnt ligands signal through different Wnt receptors to promote presynaptic assembly. For instance, the co-receptor LRP6 is a key regulator of synapse formation. *Lrp6* KO mice are embryonically lethal, but *in vivo* KD of LRP6 results in a reduction of excitatory pre- and postsynaptic terminals (Sharma et al. 2013). In addition, Wnt3a through LRP6 and Fz1 receptors

promotes excitatory presynaptic formation in hippocampal neurons (Varela-Nallar et al. 2009; Avila et al. 2010), and a role in presynaptic assembly for Wnt5a signalling through Ror1 and Ror2 has also been suggested (Paganoni et al. 2010). Furthermore, the scaffold protein Dvl1, which is required for signalling transduction, is localised at presynaptic sites and expression of this protein is sufficient to drive presynaptic assembly (Ahmad-Annuar et al. 2006). Most importantly, *Dvl1* null mice mimic cerebellar synaptic defects observed in *Wnt7a*$^{-/-}$ animals (Ahmad-Annuar et al. 2006), indicating that downstream signalling through Dvl1 is essential for presynaptic assembly. Thus, Wnt ligands signal through a number of PM receptors and downstream components to regulate the assembly of presynaptic terminals.

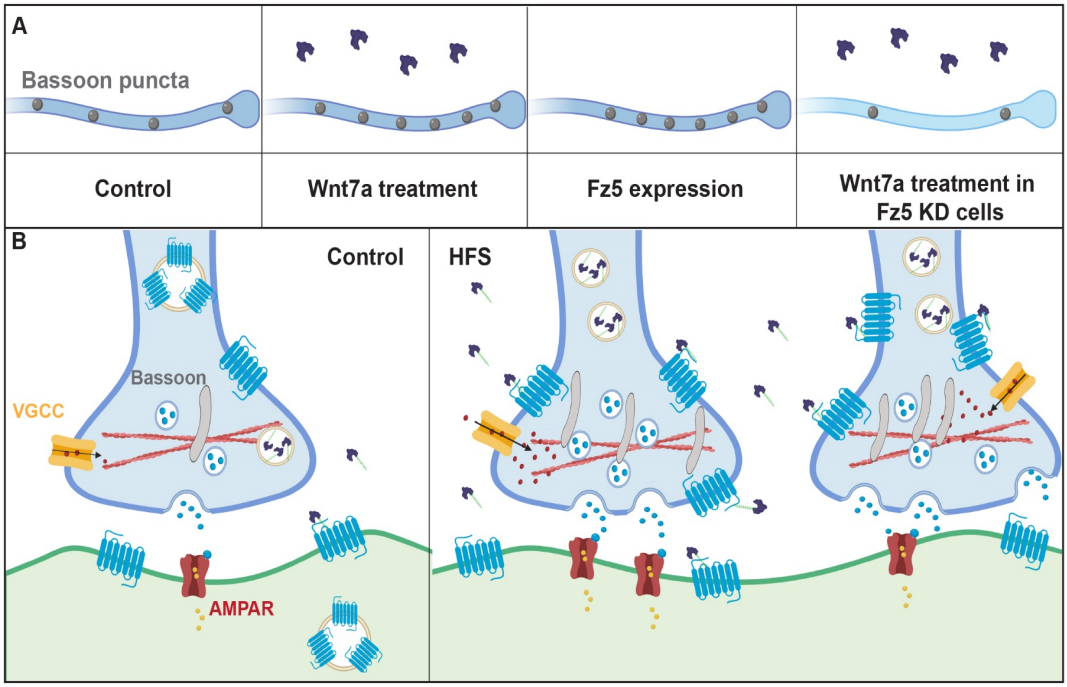

Fig 1.9. The role of Fz5 in presynaptic assembly

A: Wnt7a treatment induces presynaptic assembly in axons of dissociated hippocampal neurons (Sahores et al. 2010). Similarly, Fz5 expression mimics Wnt7a effects. Fz5 KD cells exhibit fewer presynaptic sites and are unable to respond to Wnt7a (Sahores et al. 2010). **B:** HFS of primary hippocampal neurons results in the formation of new synapses and promotes surface trafficking of Fz5 without changing the total levels of this receptor. Fz5 is required for HFS-induced presynaptic formation (not shown in this schematic) (Sahores et al. 2010).

Wnt signalling can also promote the formation of postsynaptic sites. For example, Wnt7a signals to the postsynaptic compartment to increase the number and size of dendritic spines, where excitatory synapses are mainly formed (Ciani et al. 2011). In contrast, Wnt7a does not affect the formation of inhibitory postsynaptic sites (Ciani et al. 2011) – a function that involves another Wnt ligand. Namely, Wnt5a ptomotes $GABA_AR$ clustering at inhibitory synapses (Cuitino et al. 2010). What is the downstream signalling involved in Wnt7a-mediated spine formation? The receptor that regulates Wnt7a signalling at dendritic spines site was unknown when I started my PhD. Data describing the role of two Wnt7a receptors (Fz5 and Fz7) at postsynaptic compartments will be presented in chapter 3 of this thesis. Downstream of Wnt receptors, Dvl1 is enriched at dendritic spines and its expression is sufficient to promote synapse growth in number and size (Ciani et al. 2011). Furthermore, Dvl1 is required for Wnt7a to induce postsynaptic development, and *Wnt7a*$^{-/-}$*;Dvl1*$^{-/-}$ double KO mice exhibit reduced spine number and size in pyramidal neurons of the hippocampus (Ciani et al. 2011). Wnt7a-Dvl1 signalling requires CaMKII activation to increase dendritic spine number and size (Ciani et al. 2011). Wnt7a-induced activation of CaMKII not only affects spine development but it also regulates the synaptic and surface recruitment of AMPARs (Ciani et al. 2011; McLeod et al. 2018). The activation of CaMKII, the delivery of AMPAR at the cell surface, and the increase in spine size occur very rapidly (within 10 mins of exposure to Wnt7a), suggesting that these are transcription independent effects (Ciani et al. 2011; McLeod et al. 2018). Thus, Wnt7a is a potent synaptogenic factor that signals bidirectionally to promote the assembly of pre- and postsynaptic sites.

Other Wnts and Wnt receptors have been proposed to regulate the development of excitatory postsynaptic sites but the data is controversial. In primary hippocampal neurons Wnt5a rapidly induces PSD-95 clustering through activation of JNK (Farías et al. 2009), and it has been suggested to increase dendritic spine number and size (Varela-Nallar et al. 2010). However, Wnt5a effects on spines were not observed in the previous report from the same group (Farías et al. 2009). Importantly, analyses of Wnt5a

cKO revealed no changes in the formation of dendritic spines (Chen et al. 2017), raising questions on the actual role of Wnt5a at spines. The co-receptor LRP6, which is required for presynaptic formation, is localised at dendritic spines and is necessary for PSD-95 clustering and spine formation, but it does not affect the assembly of inhibitory synapses (Sharma et al. 2013). Thus, Wnt signalling is a key regulator of postsynaptic development as it promotes the formation of dendritic spines and controls the localisation and function of several key postsynaptic proteins.

The study of the development of the NMJ has considerably enriched our understanding of the molecular mechanisms of Wnt-induced synapse formation. At the *Drosophila* NMJ, Wg is released by presynaptic motor neurons and is essential for the formation of proper synaptic boutons (Packard et al. 2002). *Wg*$^{-/-}$ flies exhibit abnormal presynaptic boutons, called "ghost boutons", which lack key presynaptic components such as mitochondria and the active zone (Packard et al. 2002; Ataman et al. 2006). At the NMJ, Wg is secreted through exosomes together with Wls and subsequently binds to the postsynaptic receptor DFz2 (Korkut et al. 2009). Wg binding to DFz2 induces the internalisation and cleavage of this receptor, resulting in the nuclear import of DFz2 C-term domain (Mathew et al. 2005; Ataman et al. 2006), which is then followed by nuclear export of large complexes of synaptic protein transcripts (Fig 1.8) (Speese et al. 2012).

In vertebrates, Wnt signalling is also required at the NMJ, where it acts through an interplay with the potent secreted synaptogenic factor Agrin and its receptor MuSK (Muscle-specific kinase) to regulate the assembly of acetylcholine receptors (AChRs) (Wu et al. 2010; Budnik and Salinas 2011). *Agrin*$^{-/-}$ and *Musk*$^{-/-}$ mice exhibit severe defects in NMJ development and AChRs clustering (DeChiara et al. 1996; Gautam et al. 1996). MuSK receptors, which bind to Dvl1 (Luo et al. 2002), contain a Frizzled-like CRD domain that allows their direct interaction with certain Wnt ligands (Jing et al. 2009). Wnt3, which is expressed by presynaptic motor neurons (Krylova et al. 2002), activates MuSK-Dvl1-Rac on muscle cells to induce the initial clustering of AChRs on the postsynaptic side; Agrin is then required to stabilise these AChRs clusters (Henriquez et al. 2008). Other molecular

interplays between Agrin and Wnt signalling occur at the level of APC and LRP4, a single pass receptor belonging to the same family of LRP5/6 receptors. APC interacts with AChRs receptors and is necessary for their clustering, a function that is conserved in central neurons (Temburni et al. 2004; Farías et al. 2007; Rosenberg et al. 2008). *Lrp4*$^{-/-}$ mice mimic the NMJ developmental defects of M*usk*$^{-/-}$ animals (Weatherbee et al. 2006); in fact, LRP4 is a co-receptor for Agrin and forms a complex with MuSK receptors that is necessary for the development of the NMJ (Kim et al. 2008; Zhang et al. 2008). Thus, Wnt signalling is required for the development of central and peripheral synapses in different model systems.

The findings presented until now support a role for Wnt signalling as positive regulator of synapse formation; however, Wnt signalling has also been described as inhibitory signalling for the development of the NMJ in *C. elegans* (Park and Shen 2012). In *C. elegans*, presynaptic sites of the DA9 motorneuron are not present in the tail of the worm, where a gradient of Lin-44 (*C. elegans* Wnt) is found (Klassen and Shen 2007). Lin-44 restricts Lin-17 (*C. elegans* Frizzled) expression to the tail of the worm, and loss of function of Lin-44, Lin-17 or Dsh1 (*C. elegans* Dvl) allows the ectopic formation of presynaptic sites in the posterior region of the worm, suggesting that active Wnt signalling is required to inhibit synapse formation (Klassen and Shen 2007). However, a recent report suggested that Lin-17 has actually a pro-synaptogenic function that is inhibited by Lin-44 through the induction of receptor endocytosis and down-regulation of γ-Neurexin, which was proposed to act in parallel to the Wnt signalling pathway through yet unspecified molecular mechanisms (Kurshan et al. 2018). The interplay between Frizzled receptors and Neurexins during synapse formation is a novel and exciting finding that deserves further investigation in vertebrate model systems.

In summary, Wnt signalling is a key regulator of central and peripheral synapses. Wnt ligands promote the formation of both excitatory and inhibitory synapses, organising the assembly of pre- and postsynaptic compartments. Several Wnt ligands, in particular Wnt7a, Wnt3a and Wnt5a, are potent synaptic regulators. However, the receptors and intracellular mechanisms

through which Wnt signalling drives synapse assembly remain to be fully elucidated.

1.6.2 Wnts in synaptic transmission, plasticity and maintenance

In addition to a crucial function in synapse formation, Wnts play a fundamental role in regulating synaptic transmission, synaptic plasticity and synaptic maintenance. Wnt signalling at the synapse controls the levels and dynamics of SVs, the localisation and functions of members of the NT release machinery, and the localisation of postsynaptic NT receptors (McLeod and Salinas 2018). Application of exogenous Wnts to hippocampal neurons increases the rate of SVs recycling, indicating an increment in NT release (Ahmad-Annuar et al. 2006; Cerpa et al. 2008; Varela-Nallar et al. 2009). Consistently, blockage of endogenous Wnts with SFRPs, or by genetic suppression of *Wnt7a* and *Dvl1,* impairs NT release (Ahmad-Annuar et al. 2006; Varela-Nallar et al. 2010; Cerpa et al. 2011; Ciani et al. 2011; Ciani et al. 2015). At presynaptic terminals, Dvl1 interacts with Syt1 (direct) and other SNAREs (indirect) to regulate NT release (Ciani et al. 2015). At postsynaptic sites, Wnt ligands increase postsynaptic currents by modulating the distribution and function of glutamate receptors (Ciani et al. 2011; Mcleod et al. 2018). Wnt7a increases AMPAR surface levels, enhancing AMPAR-mediated currents (McLeod et al. 2018), whereas Wnt5a signalling through Ror2 receptors strengthens NMDAR distribution and transmission and inhibits the activity of voltage gated K^+ channels (Cerpa et al. 2011; Cerpa et al. 2015; Parodi et al. 2015; McQuate et al. 2017). In addition, at inhibitory synapses Wnt5a controls $GABA_AR$ surface levels thereby increasing GABAergic transmission (Cuitino et al. 2010). Thus, Wnt ligands shape excitatory and inhibitory synaptic transmission by acting on both sides of the synapse.

An interesting aspect of Wnt signalling is the modulation of Wnt components by neuronal activity. Neuronal activity induces the expression and release of Wnt components, which are in turn required for activity-induced neuronal and synaptic plasticity (Budnik and Salinas 2011; Sahores and Salinas 2011; McLeod and Salinas 2018). For instance, neuronal activity promotes

dendritogenesis by inducing the transcription and release of Wnt ligands and by activating cytoplasmic β-catenin (independently of its nuclear function), CaMKII and CREB signalling (Yu and Malenka 2003; Wayman et al. 2006). Moreover, the surface levels of Fz5 are also regulated by neuronal activity. In cultured neurons, HFS (high frequency stimulation) increases, whereas LFS (low frequency stimulation) decreases, the levels of surface Fz5, and this receptor is required for activity-mediated synapse formation (Fig 1.9) (Sahores et al. 2010). Moreover, induction of LTP by electrical or chemical stimulation increases Wnt3a and Wnt7a/Wnt7b protein levels at synapses (Chen et al. 2006; McLeod et al. 2018). Wnt ligands facilitate LTP induction and blockage of Wnt signalling through SFRPs or Dkk1 inhibits the expression of LTP (Chen et al. 2006; Cerpa et al. 2011; Marzo et al. 2016; McLeod et al. 2018). In particular, Wnt5a increases NMDAR surface levels and increases NMDAR-mediated currents, facilitating LTP induction (Cerpa et al. 2011; Cerpa et al. 2015; McQuate et al. 2017). Wnt7a regulates the early signalling events that allow LTP expression by activating CaMKII and PKA, promoting phosphorylation and surface recruitment of AMPARs and growth of dendritic spines (McLeod et al. 2018). Furthermore, Wnt signalling is involved in activity-mediated synapse formation at the NMJ. Patterned stimulation of neuronal activity induces the formation of presynaptic ghost boutons in *Drosophila* motor neurons (Ataman et al. 2008). These events are respectively facilitated and abolished by the expression or suppression of Wg (Ataman et al. 2008). In addition, the expression of Wnt7a/Wnt7b ligands is increased in mice exposed to EE (enriched environment) – a behavioral paradigm that stimulates neuronal activity and increases synapse formation (van Praag et al. 2000; Nithianantharajah and Hannan 2006). Importantly, intracranial application of the Wnt inhibitors SFRPs suppresses EE-induced synapse formation in the hippocampus, strongly suggesting that Wnt signalling is required for experience-mediated synapse formation (Gogolla et al. 2009). Therefore, neuronal activity modulates the levels of Wnt components, which in turn are fundamental regulators of activity-induced synaptic plasticity.

The central functions played by Wnt signalling at synapses are further highlighted by the fact that positive or negative modulation of these cascades result in behavioural and cognitive changes. For instance, inhibition of Wnt signalling by Dkk1 infusion impairs fear memory consolidation in the amygdala (Maguschak and Ressler 2011). Similarly, hippocampal-mediated fear-conditioned learning is inhibited by local application of SFRPs or Dkk1, and enhanced by exposure to Wnt3a (Xu et al. 2015). In the striatum and hippocampus of adult mice, induced expression of Dkk1 triggers synapse disassembly, affects synaptic transmission, plasticity and memory without affecting cell viability (Galli et al. 2014; Marzo et al. 2016). Thus, Wnt signalling is a fundamental regulator of synaptic plasticity. By acting on cellular and molecular mechanisms of synaptic function and plasticity, Wnt signalling directly impacts on cognitive functions and complex behaviours.

In the adult brain, synapse formation and elimination are in a constant balance to maintain normal levels of synaptic density but at the same time allow changes in structural and functional plasticity of synapses during processes such as learning and memory. A disruption of this balance in favour of synapse elimination, for example, is presented at early stages of several neurodegenerative disorders (Selkoe 2002; Palop et al. 2006; Palop and Mucke 2010; Bellucci et al. 2016). Mounting evidence strongly suggests a link between deficient canonical Wnt signalling and AD (Inestrosa and Arenas 2010; Dickins and Salinas 2013; Purro et al. 2014). DKK1 levels are increased in post-mortem brain of AD patients and *Dkk1* expression is induced by exposure of hippocampal neurons to Amyloid β (Aβ) oligomers (Caricasole et al. 2004; Purro et al. 2012). Aβ is the main component of the characteristic extracellular plaques found AD patients' brains, and Aβ oligomers are the most toxic form of Aβ, initiating synaptotoxicity in the process of neurodegeneration (Benilova et al. 2012; Kayed and Lasagna-Reeves 2013). Dkk1 inhibition with neutralising antibodies prevents Aβ-mediated synapse disassembly in brain slices, strongly suggesting that Dkk1 acts downstream of Aβ (Purro et al. 2012). In addition, a genetic variant of the Wnt co-receptor LRP6 has been linked to late onset development of AD (De Ferrari et al. 2007). Moreover, conditional KO of *Lrp6* results in age-

dependent synapse loss and exacerbates neurodegeneration in a mouse model of AD (Liu et al. 2014). Furthermore, the microglia receptor TREM2 (Triggering Receptor Expressed On Myeloid Cells) has been identified as a risk factor in AD. TREM2 is required to stabilise β-catenin and promote survival of microglia cells (Zheng et al. 2017), which are key regulators of synapse function in health and disease (Hong et al. 2016; Salter and Stevens 2017). Thus, Wnt signalling play an important role in synaptic maintenance, and aberrant Wnt signalling is linked to synapse degeneration.

1.7 Receptor localisation and signalling, a role for post-translational modifications

Surface receptors are essential molecules for signalling in every cell of our organism. Their distribution at the PM determines the spatio-temporal dynamic of signalling activation. Therefore, studying the molecular mechanisms that regulate the trafficking and stabilisation of receptors at the PM is crucial to understand how signalling is modulated. Post-translational modifications (PTMs) are biochemical changes occurring on one or more amino acids after protein synthesis. PTMs are master regulator of protein trafficking and function and they target virtually every protein. The biochemical nature of these modifications varies greatly, ranging from the formation of intra-protein disulfide bonds to the covalent attachment of low or very high molecular weight groups. In spite of profound biochemical differences, all PTMs share one common feature: they increase the biochemical properties of proteins beyond those conferred by the amino acids that constitute these molecules. Dozens of PTMs exist but some, like phosphorylation and N-glycosylation, are far more common than others (Khoury et al. 2011). PTMs addition and removal are usually catalyzed by specific enzymes, which are localised essentially in all subcellular compartments, even in the extracellular space. The functions of PTMs are very diverse and reflect the great biochemical variety of these modifications.

Wnt signalling is a good example to illustrate the variety of functions of PTMs. Palmitoleoylation of Wnt ligands on Cys residues, which is catalyzed in the ER by Porc, regulates protein secretion and interaction with Frizzled receptors (Hausmann et al. 2007; Nile and Hannoush 2016). In the extracellular space the de-acylase enzyme Notum removes palmitoleate moiety from Wnt ligands thus inhibiting Wnt signalling activation (Kakugawa et al. 2015). β-catenin phosphorylation induces its ubiquitination and consequent degradation through the proteasome system (Stamos and Weis 2013). Palmitoylation controls protein folding and exit from the ER of the Wnt co-receptor LRP6; once at the PM, the turnover rate of LRP6 is controlled by an interplay between palmitoylation and ubiquitination (Abrami et al. 2008; Perrody et al. 2016). Frizzled receptors are glycosylated in the ER but the

physiological role of this modification remains to be elucidated. Fz3 phosphorylation inhibits Wnt signalling whereas phosphorylation of Fz6 has the opposite effects (Yanfeng et al. 2006; Strakova et al. 2018), and ubiquitination/deubiquitination cycles regulate Frizzled internalization and recycling at the PM (Hao et al. 2012; Koo et al. 2012; Madan et al. 2016). These are just a few PTMs of some components of the Wnt signalling pathway, but they illustrate the broad spectrum of functions controlled by PTMs and how these modifications directly impact cell signalling. In the following sections I will discuss in more details the functions of S-palmitoylation.

1.7.1 Lipid PTMs

Palmitoylation is the most common lipid PTM of proteins and is highly conserved across all eukaryotic organisms (Blanc et al. 2015). It consists of the covalent and reversible attachment of palmitic acid (16 C) on Cys residues. It is distinguished in S- and N-palmitoylation: S-palmitoylation (from now on palmitoylation) consists of the formation of a thioester bond between palmitic acid and Cys residues, whereas N-palmitoylation occurs via the formation of an amide bond (Fig 1.10) (Resh 2013). For instance, the morphogen Shh is N-palmitoylated by Haht (Hedgehog acyltransferase) a member of the MBOAT family (membrane bound O-acyl transferase) that differs from the enzymes that catalyse S-palmitoylation (see section 1.7.2) (Buglino and Resh 2012). Different from other lipid modifications such as miristoylation or prenylation, palmitoylation is reversible as it can be rapidly removed from protein substrates by specific enzymes, thus palmitoylation can dynamically regulate protein function (Nadolski and Linder 2007).

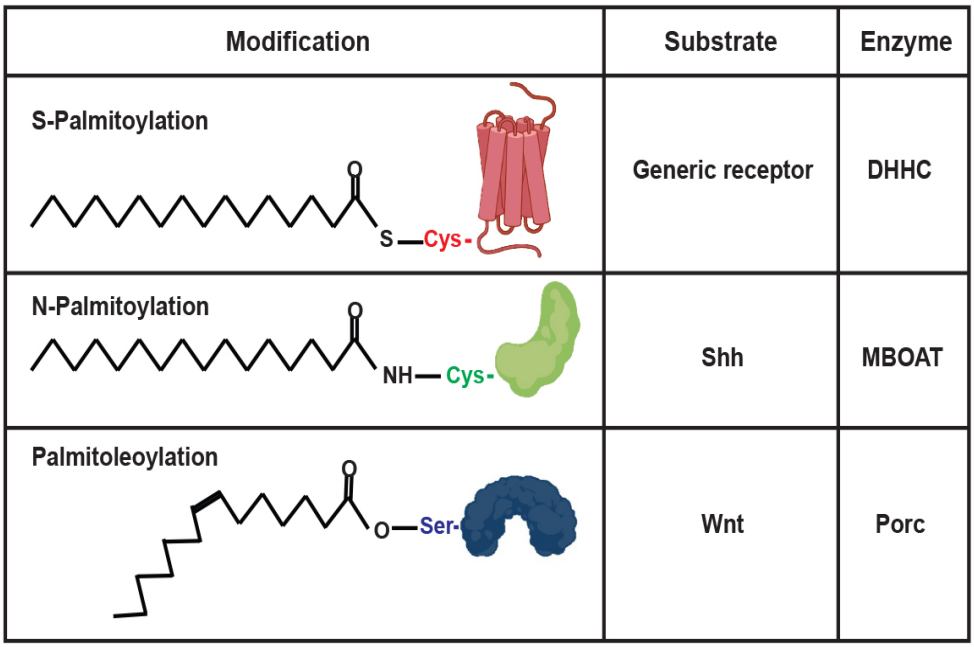

Fig 1.10: Different types of palmitoylation

S-palmitoylation is the most common lipid modification of proteins. Palmitic acid is attached to Cys residues via a thioester bond catalysed by DHHC enzymes. N-palmitoylation differs from S-palmitoylation as it binds Cys residues via an amide, catalaysed by members of the MBOAT family. Palmitoleoyaltion consists of the attachment of palmitoleic acid to Ser residues. This modification of Wnt ligands is catalysed by the enzyme Porcupine (Porc).

The overarching effect of palmitoylation is to make proteins more hydrophobic. If this is easily graspable as a strategy to anchor cytoplasmic protein to the PM, the function of palmitoylation for transmembrane proteins is less intuitive. However, palmitoylation affects protein function in multiple ways (Fig 1.11).

In the ER, palmitoylation together with ubiquitination can regulate protein folding and sorting, as for the Wnt co-receptor LRP6 (Abrami et al. 2008; Perrody et al. 2016), the anthrax toxic receptors TEM8 (tumor endothelial marker 8) and CMG2 (Capillary Morphogenesis Gene 2 Protein) (Abrami et al. 2006). Palmitoylation has recently been proposed to act as a major regulator of anterograde transport from the Golgi apparatus, where accumulation of palmitoylated proteins in certain areas of this organelle determines protein sorting, to the PM (Ernst et al. 2018). In addition, palmitoylation is a major regulator of protein trafficking and localisation. For instance, the small Ras GTPases H/N-Ras undergo

palmitoylation/depalmitoylation cycles that dynamically shuffle these proteins between the Golgi and the PM (Prior and Hancock 2001; Baekkeskov and Kanaani 2009). Similar observations have been made for transmembrane protein such as NMDARs and AMPARs, which are moved between the PM and endosomal compartments in a palmitoylation dependent manner (Baekkeskov and Kanaani 2009; Greaves et al. 2010; Salaun et al. 2010; Gladding and Raymond 2011; Sohn and Park 2019). Moreover, palmitoylation can target cytoplasmic as well as transmembrane proteins, particularly GPCRs, to specific membrane domains (Barnett-Norris et al. 2005; Goddard and Watts 2012). Indeed, palmitoylated proteins are often found in cholesterol-rich membrane fractions suggesting direct targeting to lipid rafts (Melkonian et al. 1999; Levental et al. 2010). In addition, protein-protein interactions are also regulated by palmitoylation. For instance, the clustering of PSD-95 or Gephyrin depends on their palmitoylation (El-Husseini et al. 2000; Dejanovic et al. 2014). By affecting several aspects of basic cell biology, palmitoylation has a profound impact on several physiological processes including cell metabolism, cell death, and formation of immune and neuronal synapses. Aberrant regulation of palmitoylation results in the development of pathological conditions such as infection diseases, cancer and neurological disorders (Resh 2012; Cho and Park 2016; Sobocińska et al. 2017; Chen et al. 2018).

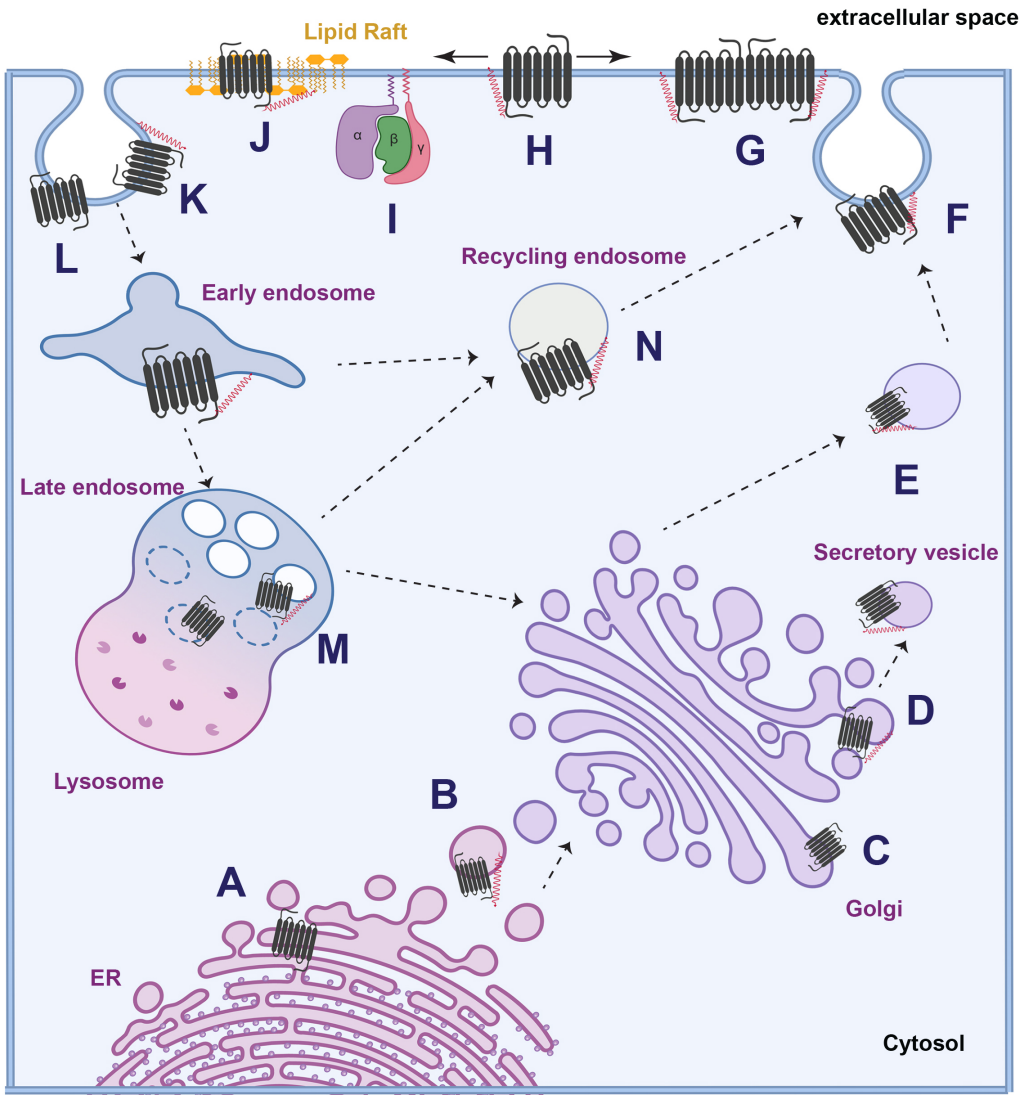

Fig 1.11: The effects of protein palmitoylation

Palmitoylation is a reversible lipid modification that affects protein trafficking and function is multiple ways. In the ER, palmitoylation can regulate protein folding (**A**) and trafficking to the Golgi (**B**). Certain proteins are retained in the Golgi until palmitate moieties are removed (**C**), whereas others require palmitoylation to exit the Golgi apparatus (**D**) and undergo sorting to specific subcellular compartments (**E**). In addition, palmitoylation can affect exocytosis at the PM (**F**), modulate protein-protein interaction (**G**), control lateral mobility of transmembrane proteins (**H**), induce membrane targeting of soluble proteins (**I**) and determine the localisation to specific membrane microdomains (**J**). Moreover, palmitoylation might favour or inhibit endocytosis (**K-L**), protein recycling (**N**) and degradation (**M**). Thus, palmitoylation is a master regulator of protein trafficking and function.

1.7.2 Palmitoylating and de-palmitoylating enzymes

Unlike other lipid PTMs, such as myristoylation and prenylation, palmitoylation does not require a clear consensus sequence other than the presence of a Cys to be catalysed. It has been suggested that the biochemical nature of neighbouring amino acids determine the occurrence of palmitoylation on Cys residues, but the precise molecular mechanisms that regulate enzyme-substrate recognition are still poorly understood (Yik and Weigel 2002; Tabaczar et al. 2017; Rana et al. 2019). For these reasons, the identification of the enzymes that catalyse protein palmitoylation has been particularly challenging. The enzymes responsible for S-palmitoyation were first identified in yeast as a group of PATs (protein acyl transferases) containing a DHHC (Aspartate, Hystidine, Hystine, Cysteine) conserved catalytic motif (Roth et al. 2002). 15 years ago, 23 DHHC genes were isolated from the mammalian genome and gain-of-function studies led to the identification of the enzymes that palmitoylate PSD-95 (Fukata et al. 2004). This work pioneered the study and the regulation of DHHC enzymes, particularly at synapses (see section 1.7.3).

DHHC enzymes have considerably different structures except for some common features: they all exhibit a conserved DHHC motif, have at least four transmembrane domains and the N-terminus and the C-terminus are exposed to the cytoplasmic region (Fig 1.12) (Gottlieb and Linder 2017). It has been proposed that DHHC enzymes undergo autopalmitoylation at the Cys residue within the DHHC motif and then transfer this palmitate group to protein substrates (Fig 1.12) (Jennings and Linder 2012; Gottlieb et al. 2015). However, only some DHHC enzymes exhibit this characteristic and further experiments are needed to determine whether other DHHCs share this catalytic mechanism. DHHCs exhibit tissue-specific expression and are present in different subcellular compartments (Ohno et al. 2006). Overexpression studies in HEK293 cells have shown that the majority of DHHCs localise between the ER and the Golgi apparatus and some are found on endosomes (Ohno et al. 2006). Although the molecular mechanisms that govern substrate recognition are still elusive, in the last 15

years numerous enzymes/substrate pairs have been identified (Tabaczar et al. 2017). Interestingly, several proteins like PSD95, SNAP25, AMPARs and others can be palmitoylated by several enzymes (Fukata et al. 2004; Hayashi et al. 2005; Greaves et al. 2010), increasing the challenge of understanding enzymes/substrate specificity.

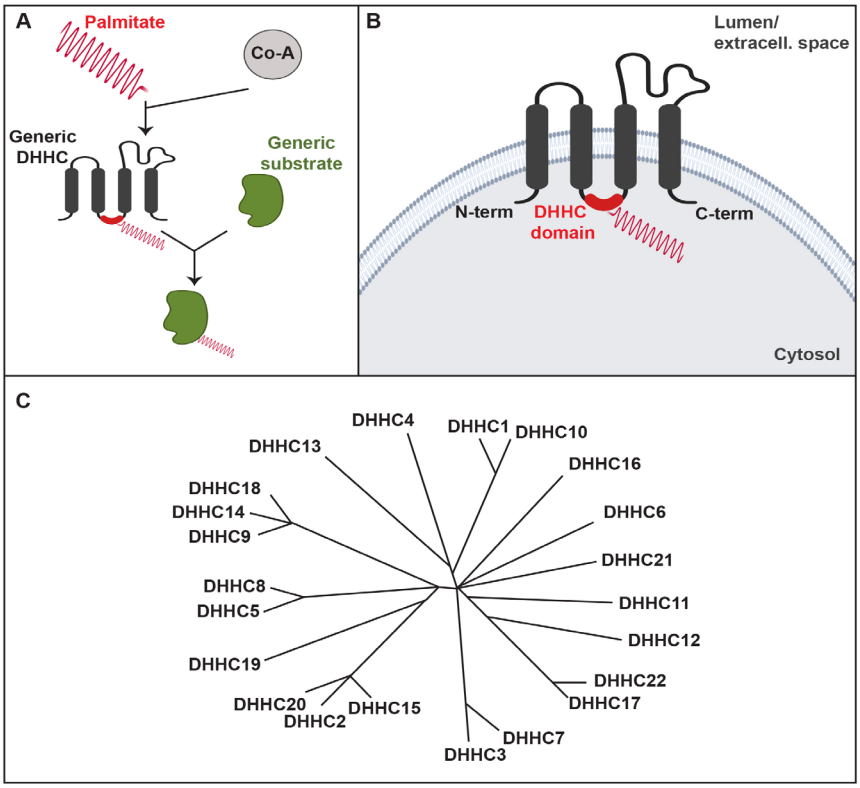

Fig 1.12: S-palmitoylation by DHHC protein acyl transferases

A: It has been proposed that several DHHCs undergo CoA-mediated autopalmitoylation of the Cys residue in the DHHC motif before transferring the palmitate moiety to a specific substrate. **B:** Most DHHCs have 4 transmembrane domains with cytoplasmic N- and C-term regions. The DHHC catalytic motif is conserved across all 23 DHHC genes. **C:** Phylogenetic tree of human DHHC genes (adapted from Ohno et al. 2006).

One of the key features of palmitoylation is its reversibility. The removal of palmitate is catalysed by a group of enzymes called protein acyl thioesterases. APT1 (acyl protein thioesterase) and PPT1 (palmitoyl protein thioesterase), and their homologs APT2 and PPT2, were first described to act as thioesterase on Ras GTPases and G proteins (Won et al. 2018). PPT1 is found exclusively on lysosomes and late endosomes, excluding its involvement in de-palmitoylation events occurring in the cytoplasm or at the

PM (Hernandez et al. 2013). PPT1 mutations are linked to the development of infantile forms of neuronal ceroid lipofuscinoses (NCL), aslo known as Batten disease (Zeidman et al. 2009; Kollmann et al. 2013), a severe neurodegenerative disorders characterised by the lysosomal accumulation of autofluorescent lipoparticles that leads eventually to cell death, particularly of neurons (Kyttälä et al. 2006; Kollmann et al. 2013). APT1 and 2 are ubiquitously expressed and are primarily cytoplasmic, but they are also associated with some internal membranes and with the PM (Vartak et al. 2014; Adachi et al. 2016; Won et al. 2016), most likely through the interaction with their own palmitate groups (Yang et al. 2010; Vartak et al. 2014). However, the distribution of APT1 and 2 has only been investigated by overexpression studies due to the lack of antibodies against endogenous APTs; thus, the precise localisation of endogenous APT1 and 2 remains to be established. Loss of function studies clearly showed that APTs KD affects palmitoylation of some substrates, like Huntingtin, but not others, like N-Ras and PSD-95, suggesting the presence of other thioesterases for cytosolic proteins (Lin and Conibear 2015). Indeed, ABHD proteins (α/β-hydrolase domain-containing protein) were recently screened for thioesterase activity and ABHD17s (isoforms A, B and C) were found to act as de-palmitoylating enzymes of N-Ras, PSD-95 and MAP6 (microtubule associated protein 6) (Lin and Conibear 2015; Yokoi et al. 2016; Tortosa et al. 2017). ABHD17 enzymes exhibit plasma membrane localisation (Yokoi et al. 2016), but the mechanisms that regulate their enzymatic function are still unknown. Thus, palmitoylation is a very dynamic PTM that controls multiple aspects of protein trafficking and functions, and the enzymes that catalyse or remove palmitoylation have started to be identified, significantly contributing to our understanding of the regulation of this PTM.

1.7.3 Palmitoylation at synapses

Hundreds of neuronal proteins are palmitoylated and this modification is crucial for different processes including axon and dendrite polarisation and also synapse growth and synaptic function (Fukata and Fukata 2010). Key synaptic proteins are palmitoylated, these include components of the release

machinery, scaffold proteins and NT receptors (Prescott et al. 2009; Thomas and Huganir 2013; Montersino and Thomas 2015; Matt et al. 2019). The list of synaptic proteins that are palmitoylated is long and ever growing. Here I will only touch upon some key examples.

Palmitoylation of SNAP23-25, Synaptotagmins, Syntaxin1 and VAMP2 is fundamental for their function at axonal terminals (Prescott et al. 2009). Palmitoylation of SNAP25, catalysed by DHHC3, DHHC7 and DHHC17, regulates stable membrane binding and ensures SNAP25 accumulation at exocytic sites (Koticha et al. 2002; Salaün et al. 2005). In addition, the majority of the 17 Synaptotagmin (Syt) isoforms contain at least one C-term Cys residue that is palmitoylated (Kang et al. 2004). Mutation of the Cys residue of SytI affects this protein in multiple ways: palmitoylation-mutant SytI has a more diffuse distribution and is less abundant at synapses, consistently with a role for palmitoylation in regulating SytI clustering at presynaptic sites (Kang et al. 2004). On postsynaptic sites, key scaffold proteins, as well as CAMs, signalling molecules and NT receptors are palmitoylated. For example, PSD-95 is dually palmitoylated at two Cys residues at the N-terminus, and this PTM is required for microtubule-dependent targeting of PSD-95 at synapses, and for PSD-95-mediated clustering of NT receptors (El-Husseini et al. 2000). Palmitoylation of PSD-95 is mediated by multiple DHHCs enzymes, such as DHHC2, DHHC3, DHHC7 and DHHC15 (Fukata et al. 2004). Furthermore, palmitoylation of certain kinases such as the cytoskeleton regulator LIMK1 (LIM domain kinase 1) and the non-receptor tyrosine kinase Fyn is required for their activation of NMDARs and their ability to signal at synapses (Montersino and Thomas 2015). Moreover, glutamate and GABA receptors are also palmitoylated. Dual palmitoylation of AMPARs and NMDARs has very distinct roles on their trafficking and function (Naumenko and Ponimaskin 2018). At the Golgi, palmitoylation is catalysed by DHHC3 and is required for retaining AMPARs and NMDARs on this organelle (Hayashi et al. 2005; Hayashi et al. 2009; Lin et al. 2009). At the PM, palmitoylation controls AMPAR and NMDAR internalisation in different manners: palmitoylation of AMPARs at the C-terminus facilitates activity-induced internalisation of these receptors by regulating its interaction

with 4.1N protein (Hayashi et al. 2005; Lin et al. 2009). Palmitoylation of NMDARs, also at the C-terminus, increases Fyn-mediated phosphorylation and stabilises this receptor at the PM by inhibiting constitutive internalisation (Hayashi et al. 2009). Thus, palmitoylation of many pre- and postsynaptic proteins is crucial for their correct localisation and function.

The reversibility of protein palmitoylation is fundamental at synapses, where activity-mediated regulation of protein function and localisation is one of the underlying mechanisms of synaptic plasticity. Several DHHC enzymes including DHHC2, DHHC5, DHHC8, DHHC9, and DHHC12 are regulated by neuronal activity (Noritake et al. 2009; Thomas et al. 2012; Brigidi et al. 2014; Dejanovic et al. 2014; Brigidi et al. 2015). For instance, induction of LTP increases DHHC5 catalytic activity and mobilises DHHC5 from dendritic spines to endosomes, where it palmitoylates δ-catenin (Brigidi et al. 2014; Brigidi et al. 2015). Palmitoylated δ-catenin is targeted to spines where it interacts with N-cadherin to modulate synaptic strength (Brigidi et al. 2014; Brigidi et al. 2015). Similarly, neuronal activity enhances DHHC8 catalytic activity, which increases GRIP1B palmitoylation stabilising AMPARs at the cell surface (Thomas et al. 2012). Moreover, long-term blockage of neuronal activity, which is associated with homeostatic plasticity (Turrigiano 2012), increases DHHC2-mediated palmitoylation of PSD-95, therefore enhancing its interaction with AMPARs and strengthening synaptic transmission (Noritake et al. 2009). Thus, DHHC enzymes affect the localisation and function of key synaptic proteins in an activity dependent manner, suggesting that they may directly regulate neural circuits and affect cognitive functions. Indeed, *Dhhc5* KO mice exhibit severe deficits in hippocampal-dependent behaviors (Li et al. 2010). Mice lacking *Dhhc17*, which palmitoylates PSD-95, AMPARs, SNAP25 and Huntingtin, have severe defects in synaptic transmission, synaptic plasticity and hippocampal-mediated memory (Milnerwood et al. 2013). Therefore, growing efforts have focused on studying the role of palmitoylating and depalmitoylating enzymes in physiological and pathological processes that affect synapses. For instance, aberrant activity of DHHCs or depalmitoylating enzymes is linked to disorders

characterised by synaptic failure, including AD, Huntington disease, schizophrenia and autism (Cho and Park 2016; Zaręba-Kozioł et al. 2018).

1.8 Regulation of Wnt7a-Frizzled signalling at synapses, a role for palmitoylation.

Wnt7a is a potent synaptogenic factor that promotes the assembly of excitatory synapses by signalling to both sides of the synapse. Wnt7a also regulates synaptic transmission and synaptic plasticity (Sahores et al. 2010; Ciani et al. 2011). Our laboratory identified Fz5 as the receptor for Wnt7a at presynaptic sites (Sahores et al. 2010). Moreover, Fz5 surface levels are regulated by neuronal activity (Sahores et al. 2010), suggesting that dynamic mechanisms are in place to regulate the trafficking of this receptor at the synapse.

Given the dynamic modulation of Fz5 surface levels in response to neuronal activity, our laboratory started to investigate the molecular mechanisms that could regulate this process. First, Dr Eleanna Stamatakou, a previous PhD in our laboratory, identified possible sites for PTMs in the C-term of Fz5. The C-terminus of Fz5 contains three Cys residues that could be targeted by S-palmitoylation (Fig 4.2). These three Cys are indeed predicted to be palmitoylated by the CCS-palm 4.0 software (Stamatakou and Salinas unpublished data). Using different techniques including the acyl-biotin exchange (ABE) assay (Wan et al. 2007), incorporation of radioactive palmitate, and site-directed mutagenesis of the three Cys to Ser (Fz5 3CS), Dr Stamatakou confirmed that Fz5 is indeed palmitoylated (Fig 1.13) (Stamatakou and Salinas unpublished results). Moreover, Fz5 palmitoylation is regulated by activity as the levels of this modification are increased in cultured neurons stimulated by HFS and in the hippocampus of mice exposed to EE (Fig 1.13) (Stamatakou and Salinas unpublished results). Importantly, preliminary studies using the 3CS Fz5 mutant showed that palmitoylation is required for Fz5 synaptogenic activity (Fig 1.13). To our knowledge, this is the first demonstration that Frizzled receptors are palmitoylated. These are novel and exciting findings for the Wnt field, as they uncover a previously uncharacterised PTM of Frizzled receptors, unravelling a new mechanism for Wnt signalling regulation. In my PhD project, I extended this investigation by examining whether other Frizzled receptors

are palmitoylated and what are the molecular mechanisms through which palmitoylation regulates Fz5.

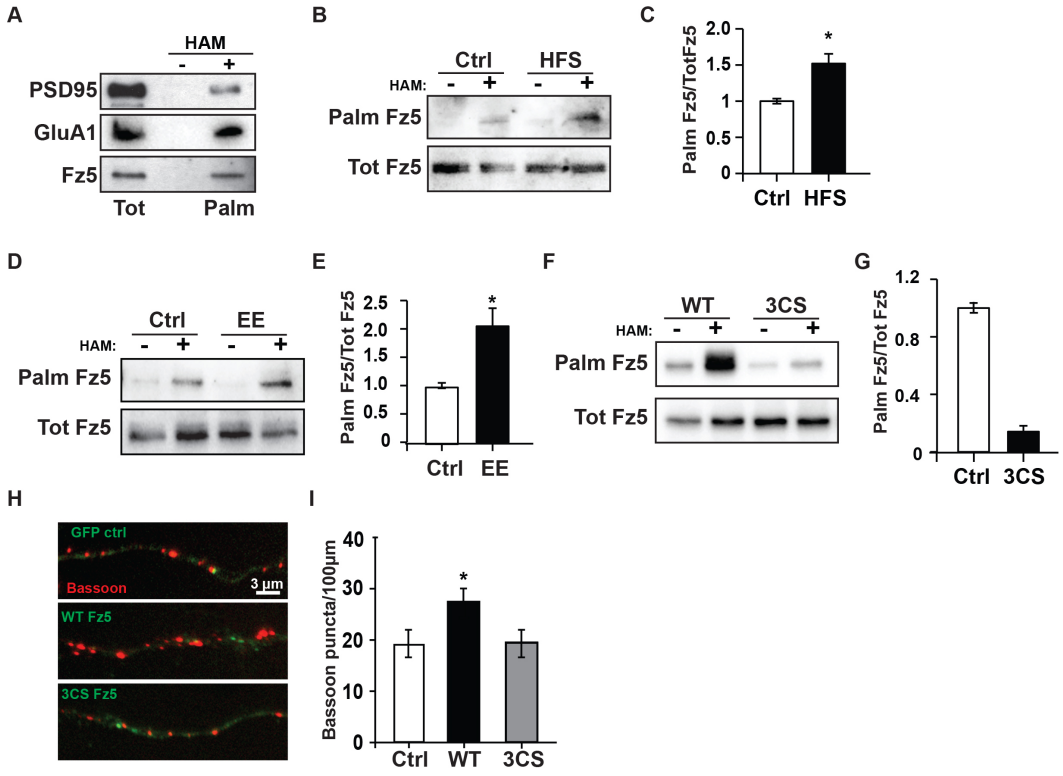

Fig 1.13. Fz5 is palmitoylated and this modification is important for its synaptogenic activity
A: ABE assay from mouse brains shows that Fz5 is palmitoylated *in vivo*. PSD-95 and GluA1 were used as positive controls. HAM (hydroxylamine) was used to isolate palmitoylated proteins (see section 2.15 material and methods). **B-E:** Preliminary data show that Fz5 palmitoylation is increased upon HFS of primary hippocampal cells and in the hippocampus of mice exposed to EE when compared to controls. **F-G:** Site-directed mutagenesis of these Cys to Ser abolished palmitoylation of Fz5 indicating that these residues are targeted for palmitoylation. **H-I:** Palmitoylation is required for Fz5 function, as palmitoylation-deficient Fz5 receptors (3CS Fz5) fail to promote presynaptic assembly in axons of dissociated hippocampal neurons.

1.9 Thesis aims

Wnt signalling plays a critical role at the synapse by regulating synapse assembly during development and by modulating synaptic function and maintenance in the adult brain. In particular, Wnt7a is a potent synaptogenic factor that promotes the assembly of pre- and postsynaptic sites, but the molecular mechanism underlying these processes remain elusive. Specifically, although Fz5 is a receptor required for Wnt7a-induced presynaptic assembly, the receptor mediating Wnt7a-dependent was unknown. Furthermore, palmitoylation, a previously uncharacterised PTM of Frizzled receptors, seems to be required for Fz5 function at synapses. However, whether other Frizzled receptors are palmitoylated, and the molecular mechanisms through which palmitoylation controls Fz5 function, remain completely unknown. Finally, although the role of Fz5 in synapse formation has been shown *in vitro* (Sahores et al. 2010), its role in synapse formation and the role of Fz5 palmitoylation *in vivo* are unknown. During my PhD, I used a combination of biochemical, molecular and cell biology techniques to study the function of two Wnt7a receptors (Fz5 and Fz7) at the synapse. In particular, I focused my attention on the molecular mechanism by which palmitoylation affects Fz5 trafficking and function. The experiments presented in the next chapters were aimed to address the following specific questions:

1) Which is the receptor for Wnt7a that is required for postsynaptic development?
2) Are other Frizzled receptors palmitoylated?
3) Which are the enzymes that palmitoylate Fz5?
4) What are the molecular mechanisms by which palmitoylation controls Fz5?
5) What is the role of Fz5 and its palmitoylation for synapse formation *in vivo*?

CHAPTER 2: MATERIAL AND METHODS

2.1 DNA constructs, cloning and viruses

WT Fz5-HA was a kind gift from professor Xi He (Harvard University, US). Dvl1-HA and WT Fz5-SEP constructs were previously generated in house, following standard restriction-enzymes-based cloning techniques. The plasmids for the expression of all Frizzled receptors (mouse) with C-term 1D4 tag were purchased from Addgene (references: Fz1 42263; Fz2 42264; Fz3 42265; Fz4 42266; Fz5 42267; Fz6 42268; Fz7 42269; Fz8 42270; Fz9 42271; Fz10 42272). 3CS and double Cys mutant Fz5 plasmids were generated by Dr Eleanna Stamatakou and Dr Laura-Nadine Schuhmacher respectively. QuikChangeLightning Site-Directed Mutagenesis kit (Stratagene) was used to clone 3CS Fz5, whereas the Phusion PCR kit was used to clone Double Cys mutant Fz5 according to manufacturer's instructions. The following primers were used to generate these constructs:

Table 2.1: Primers used for Fz5 cloning

Constructs	Forward and reverse primers
3CS Fz5	Fw 5′-CACCAGCCGCAGCAGCAGCCGCCCGCG-3′
	Rv 5′-CGCGGGCGGCTGCTGCTGCGGCTGGTG-3′
C537-538S	Fw 5'-GTTTCACCAGCCGCAGCAGCTGCCG-3'
	Rv 5'-CGGCTGGTGAAACGCCGCCACGACT-3'
C537-539S	Fw 5'-TGGCGGCGTTTCACCAGCCGCAGCTG-3'
	Rv 5'-GTGAAACGCCGCCACGACTCCACCGT-3'
C538-539S	Fw 5'-CACCAGCCGCTGCAGCAGCCGCCCGC-3'
	Rv 5'-TGCAGCGGCTGGTGAAACGCCGCCACGA-3'

All DHHCs plasmids were a kind gift from Prof Akio Kihara and Dr Yusuke Ohno from the University of Hokkaido, Japan. These plasmids contain human DHHCs coding sequences tagged with Myc or Flag at the N-term or C-term, depending on the effect of the tag on each protein. The terminology of DHHC enzymes needs clarification, as the names commonly used by research groups do not always correspond to the nomenclature reported on gene databases:

Table 2.2: Nomenclature of DHHC enzymes

Commonly used nomenclature		**Nomenclature on gene databases**		**Accession numbers**	
Human	**Mouse**	**Human**	**Mouse**	**Human**	**Mouse**
DHHC1	*Dhhc1*	*ZDHHC1*	*Zdhhc1*	NM_013304	BC026570
DHHC2	*Dhhc2*	*ZDHHC2*	*Zdhhc2*	BC050272	NM_178395
DHHC3	*Dhhc3*	*ZDHHC3*	*Zdhhc3*	NM_016598	NM_026917
DHHC4	*Dhhc4*	*ZDHHC4*	*Zdhhc4*	NM_018106	NM_028379
DHHC5	*Dhhc5*	*ZDHHC5*	*Zdhhc5*	NM_015457	NM_144887
DHHC6	*Dhhc6*	*ZDHHC6*	*Zdhhc6*	BC007213	NM_025883
DHHC7	*Dhhc7*	*ZDHHC7*	*Zdhhc7*	NM_017740	NM_133967
DHHC8	*Dhhc8*	*ZDHHC8*	*Zdhhc8*	NM_013373	AY668947
DHHC9	*Dhhc9*	*ZDHHC9*	*Zdhhc9*	BC012826	AK032233
DHHC10*	***Dhhc10***	***ZDHHC11***	***Zdhhc11***	NM_024786	AY668948
DHHC11	***Dhhc11***	***ZDHHC23***	***Zdhhc23***	NM_173570	AY668949
DHHC12	*Dhhc12*	*ZDHHC12*	*Zdhhc12*	NM_032799	BC021432
DHHC13	***Dhhc13***	***ZDHHC24***	***Zdhhc24***	NM_207340	BC071194
DHHC14	*Dhhc14*	*ZDHHC14*	*Zdhhc14*	NM_024630	BC059814
DHHC15	*Dhhc15*	*ZDHHC15*	*Zdhhc15*	BC103980	NM_175358
DHHC16	*Dhhc16*	*ZDHHC16*	*Zdhhc16*	NM_032327	XM_129300
DHHC17	*Dhhc17*	*ZDHHC17*	*Zdhhc17*	NM_015336	NM_172554
DHHC18	*Dhhc18*	*ZDHHC18*	*Zdhhc18*	NM_032283	AY668950
DHHC19	*Dhhc19*	*ZDHHC19*	*Zdhhc19*	NM_144637	BC049761
DHHC20	*Dhhc20*	*ZDHHC20*	*Zdhhc20*	NM_153251	AY668951
DHHC21	*Dhhc21*	*ZDHHC21*	*Zdhhc21*	NM_178566	NM_026647
DHHC22	***Dhhc22***	***ZDHHC13***	***Zdhhc13***	AB024495	NM_028031
- **	***Dhhc23***	**-**	***Zdhhc25***	**-**	BC049767
DHHC24	***Dhhc24***	***ZDHHC22***	***Zdhhc22***	NM_174976	NM_001089 43

* Mismatches between common nomenclature and genes' names on databases are reported in bold; ** The *DHHC23* gene does not exist in the human genome.

shRNA plasmids (Scr, Fz5, Fz7, DHHC3 and DHHC7) were purchased from Vectorbuilder. The backbone is a standard plasmid compatible with packaging into AAV for *in vivo* studies. shRNA target sequences were expressed under the U6 shRNA-specific promoter, whereas mCherry, which was used as a marker to identify transfected cells and evaluate their morphology, was expressed under the CMV promoter.

Table 2.3 : shRNA target sequences used for loss-of-function studies

shRNA plasmids	Target sequences and vector ID on vectorbuilder.com	Species
Scrambled	GGCGTTACGTCCTAACATGCG VB180530-1036dvh	-
Fz5 shRNA #1	GAACTCGCTACGAGGCTTTGT VB170405-1058suz	rat
Fz5 shRNA #3	GCACAGTCGTCTTCCTCTTAG VB170405-1059dar	rat
Fz5 shRNA #4	CTGTAGCGACCTTCCTCATTG VB170405-1060mvy	rat
Fz7 shRNA #1	GCTAACGGCCTCATGTACTTT VB170130-1035jtg	rat
Fz7 shRNA #2	GCTATCCAGAGCGACCCATTA VB170130-1036eez	rat
Fz7 shRNA #3	GGTGGGTCATTCTTTCCCTCA VB170130-1037euk	rat
DHHC3 shRNA #1	CCCAAAGGAAATGCCACTAAA VB180924-1027yhy	human
DHHC3 shRNA #2	GTATAGCATCATCAACGGAAT VB180924-1028dkz	human
DHHC7 shRNA #1	GAAGGGATGAAGTCCGTCTTT VB180925-1279gjq	human
DHHC7 shRNA #2	GATAACTGTAATCCTGTTGAT VB180925-1285yhg	human

The Fz7 rescue construct was designed to express a shRNA-resistant form of mouse Fz7 together with Fz7 shRNA (Vector ID on Vectobuilder.com: VB161028-1050thj). Therefore, each transfected cell expressed Fz7 cDNA and Fz7-shRNA simultaneously. GFP ctrl, WT Fz5, 3CS Fz5 were also purchased from Vectobuilder (Vectorbuilder IDs: VB150925-10026; VB170901-1016acm; VB170901-1018czc). In these plasmids, WT and 3CS Fz5 sequences are expressed downstream of EGFP and the P2A linker, which self-cleaves after translation (Fig 6.1 chapter 6) (Kim et al. 2011). Thus, cells expressing these plasmids are GFP and Fz5 positive. Ultra-purified high

titer AAV9 viruses (>10^{12} viral particles/mL) for *in vivo* experiments were purchased by Vectorbuilder.

2.2 qPCR analyses

Quantitative PCR (qPCR) analyses were performed in collaboration with Dr Ernest Palomer Vila. Hippocampal tissue and cultured cells were processed to evaluate the efficacy of Fz5, Fz7 and DHHC3-7 KD (knock-down). One hippocampus per animal, or one 60 mm dish of 90% confluent cell lines, were homogenised using 400 µl of Trizol (Ambion/RNA Life Technologies). RNA was extracted using Direct-zol RNA kit (Zymo Research) according to manufacturer's instructions. Reverse transcription was performed using the RevertAid-H-Minus First Strand cDNA kit from 1000 ng of RNA. cDNA was then used for pPCR analyses, which were performed according to manufacturer's instructions using GoTaq qPCR Master Mix kit and a BioRad pPCR thermocycler. Primers (Sigma-Aldrich) were used at 0.5 µM concentration. Several house-keeping genes were used for qPCR quantification using the comparative Ct method.

Table 2.4: primers used for qPCR analyses

Gene	Species	Forward and reverse primers
Fzd5	Rat	Fw 5'- TCTGTTATGTGGGCAACCAA -3' Rv 5'- CCAAGACAAAGCCTCGTAGC -3'
Fzd7	Rat	Fw 5'- GCAGTGGCTGAAAAGACTCC - 3' Rv 5'- CAGTTAGCATCGTCCTGCAA -3'
ZDHHC3	Human	Fw 5'- TACAGTTGAAGCCTGGGCAG -3' Rv 5'- TCTTCCGAATGCACCGCTTA -3'
ZDHHC7	Human	Fw 5'- GCTGTATTAAACCCGAGCGC -3' Rv 5'- CACAATTGTTCACCCACGGG -3'
Gapdh	Rat	Fw 5'- ATGGCCTTCCGTGTTCCTAC-3 Rv 5'-CATACTTGGCAGGTTTCTCCA-3'
GUSB	Human	Fw 5'- GGTTGGAGAGCTCATTTGGA -3' Rv 5'- CTCTCGTCGGTGACTGTTCA -3'
PGK1	Human	Fw 5'- CAGTTTGGAGCTCCTGGAAG -3' Rv 5'- CACAGGAACTAAAAGGCAGGA -3'

2.3 Animal use and intracerebroventricular (ICV) injections of AAV9

Experiments carried out on rats and mice were performed as stated under personal and project licences granted by the UK Home Office in compliance of the Animals (Scientific Procedures) Act 1986. C57BL/6 wild type mice were used for ICV injections. P0-P1 new-born pups were injected in the lateral ventricles with adeno-associated virus serotype 9 (AAV9) with minimal variations to what previously described (J.-Y. Kim et al. 2014).

Before collecting the pups for injections the mother was moved to a different cage and kept isolated for the entire length of the procedure (10-30 mins depending on the number of pups to be injected). Manipulation of neonatal mice can result in rejection and cannibalism by the mother. To cover up external smells that could induce pups rejection, I thoroughly rubbed my hands with beddings from the original cage before touching the animals. Next, pups were gently collected and placed in tissue pockets on a pre-warmed pad to avoid hypothermia. One pup at the time was processed for injection: the head was held flat with two fingers and a Hamilton syringe with a 32G needle was mounted on a stereotaxic frame to freely press the plunger without risking of displacing the needle and used to dispense the virus. Injection sites were located halfway between the Lambda and Bregma sutures, approximately 1 mm laterally of the sagittal midline (see Fig 6.1) (J.-Y. Kim et al. 2014). The needle was inserted to a depth of 2.5 µm from the surface of the mouse's head. Up to 2.5 µL of virus were injected and the needle slowly retracted to repeat the procedure on the other hemisphere. For identification purposes, front and/or back paws were poked using a small needle covered in tattoo paste. Before returning to the cage, the pups were gently rubbed with beddings to cover external smells that could potentially induce rejection by the mother. Finally, the mother was re-introduced to the cage. Pups were collected 14-15 days post-injection.

2.4 Brain dissection, fixation and freezing

Animals were sacrificed according to Schedule 1 protocol (culled with an overdose of CO_2 and decapitated), brains were rapidly dissected and either

snap frozen in liquid nitrogen for WB (western blot) and qPCR (quantitative polymerase chain reaction) analyses or fixed with 4% PFA/PBS O/N at 4 ˚C (Table 2.13). 24 hrs later, fixed brains were rinsed twice with PBS and placed for 2-3 days at 4 ˚C in a 30% sucrose/PBS solution for cryopreservation. The brains were then frozen in dry-ice-cold isopentane (2-methylbutane, Sigma) and stored at -80 ˚C until cryosectioning.

2.5 Cryosectioning and Immunofluorescence analyses of brain slices

Frozen brains were cut using a Leica CM1850 cryostat. The brains were introduced in the cryostat, equilibrated at -20 ˚C for 15-20 min and then embedded in OCT (VWR Chemicals). 30 µm thick sections were cut sagittally, collected in cryo-freezing solution (2.14) and placed at -20 ˚C for long term storage. Sections were rinsed from cryo-freezing solution with PBS (3x10 mins washes) before being immersed in blocking solution (10% donkey serum, 0.5 % Triton X-100 in PBS) with gentle shaking for 1-2 hrs at RT. Primary antibodies (Table 2.5) were diluted in blocking solution and incubated O/N at 4 ˚C with vigorous shaking. The following day the slices were washed 3 times for 10 min with PBS and incubated in the dark for 3-4 hrs with secondary antibodies diluted 1:600 in blocking solution. After 3x10 mins washes with PBS, slices were mounted on glass microscope slides using Fluoromount-G (Southern Biotech) and let dry in the dark for at least 48 hrs before being imaged.

2.6 Cell cultures, transfection methods and pharmacological treatments

Primary rat hippocampal cultures

Primary hippocampal cultures were prepared from E18 Sprague-Dawley rat embryos as previously described (Dotti et al. 1988). After removing the brain from the skull, the tissue was kept in ice cold Hank's balanced salt solution (Gibco) for the duration of the hippocampal dissection, which was performed using a Leica dissecting microscope. After completion of the dissection, the hippocampi were placed at 37 ˚C for 18 mins in Hank's solution supplemented with 0.5% trypsin (Invitrogen) for chemical digestion. Next, the

hippocampi were rinsed three times with Hank's solution and transferred to DMEM plating medium (Table 2.8) for mechanical dissociation, which was carried out with three glass Pasteur pipettes of different size. Cells were counted using a haemocytometer and plated at required density: 250 cells/mm^2 for high-density cultures, or 125 cells/mm^2 for low-density cultures. Cells were plated onto 12mm wide glass coverslips pre-treated with nitric acid and coated O/N (over-night) with 1 mg/mL poly-D-lysine in boric buffer (Sigma). After two hrs at 37 ˚C with 95% O_2 and 5% CO_2, DMEM plating media was replaced with Neurobasal maintenance medium supplemented with nutrition factors (Gibco, Table 2.9). Cells were kept in culture for up to 2 weeks and fresh medium was added when necessary to compensate for reduction in volume due to evaporation.

Hippocampal neurons were transfected using the Ca^{2+} phosphate method (Dudek et al. 2001). Depending on the aim of the experiment, transfection was performed at DIV (days *in vitro*) 5-9. Briefly, 20 mins before transfection Neurobasal maintenance medium of a P60 dish was replaced with 3 mL of transfection medium (Table 2.10). For each 60mm dish, 5 µg of plasmid DNA, 10 µL of 2.5 M $CaCl_2$ and H_2O were mixed to reach a final volume of 100 µL, which was then added drop-wise to 100 µL of 2x HEPES-buffer saline solution (Table 2.11) and incubated in the dark for 20 mins at RT. Next, the mix was added to the cells for 5-10 mins at 37 ˚C. The cells were then washed 3 times with pre-equilibrated transfection medium and returned to the incubator with 4 mL of original maintenance medium supplemented with Pen/Strep.

HEK293, NRK and NB2A cultures

HEK293 (human embryonic kidney 293) and NRK (normal rat kidney) were cultured in DMEM medium (Gibco) containing Glutamax, D-glucose (4.5 g/L), pyruvate, 10 % FBS (Gibco) and 1 % v/v Pen/Strep (Gibco). Cells were kept in a humidifier incubator at 37 ˚C with 95% O_2 and 5% CO_2. Cells were transfected using Lipofectamine or AMAXA nucleofection methods.

Lipofectamine transfection

Lipofectamine transfection was performed according to manufacturer's instructions (Thermo Fisher). Briefly, 3.5-5x10^5 cell were plated in one well of 6-well plate with 2 mL of DMEM maintenance medium. The following day the cells were transfected with up to 2.5 µg of plasmid DNA. For each well, 0.2-2.5 µg of DNA and 5 µL of reagent 3000 were added to 125 µL of OPTIMEM medium (Gibco). In parallel, 5 µL of Lipofectamine were added to 125 µL of OPTIMEM, immediately vortexed and added to the tube containing DNA and reagent 3000. This mix was incubated at RT for 10-15 mins and then added to the cells in a drop-wise manner. Cells were harvested 24-48 hrs post transfection.

AMAXA nucleofection

NRK cells were transfected by AMAXA nucleofection (Lonza) following manufacturer's instruction. Briefly, after trypsinisation cells were pelleted and resuspended in 100 µL (per condition) of WT-EM transfection solution (Table 2.12). 1x10^6 cells/per condition were mixed gently with up to 5 µg of plasmid DNA. The cells were immediately transferred to glass cuvettes and nucleofected using the appropriate program in a Lonza nucleofector device (NRK cells were nucleofected using the X-01 program). 10 mins after nucleofection, 500 µL of pre-equilibrate DMEM medium were added to each cuvette, the cells were collected using a thin Pasteur pipette and plated at the desired density.

Pharmacological treatments

To examine Fz5 degradation rate NRK cells were treated with the protein translation blocker Cycloheximide (Cell Signalling Technology, 50 µg/ml) or with DMSO (dimethyl sulfoxide, control vehicle) for different time points (0 mins - 2 hrs). To inhibit palmitoylation and examine Fz5 surface levels, HEK293 cells were treated with 2BP (2-Bromohexadecanoic acid, Sigma) at a final concentration of 100 µM or with DMSO.

2.7 Antibody feeding experiments

NRK cells expressing WT or 3CS Fz5 were placed on an ice block in the cold room for 15-20 mins to stop membrane trafficking. Primary HA antibodies were diluted 1:500 in ice cold DMEM maintenance media. The mix was added to the cells, still on ice, for 30 mins to allow antibody binding to the extracellular HA tag of surface Fz5 receptors. The cells were then washed 3 times with ice cold PBS and a subset, used as control (t=0), was immediately fixed with ice cold PFA. Another subset of cells was placed in pre-equilibrated warm DMEM and returned to the incubator for 15 mins to allow for receptor endocytosis. These cells were then fixed with warm 4% PFA in PBS and processed for immunofluorescence analyses (see section 2.9).

2.8 Fluorescent Recovery After Photobleaching (FRAP)

For FRAP experiments NRK cells were imaged using a Leica TCS SP8 inverted confocal microscope with a 60x objective (NA 1.35). The external chamber of the microscope was equilibrated at 37 ˚C. NRK cells expressed WT or 3CS Fz5 tagged at the N-term with SEP (super ecliptic pHluorin), a GFP variant that does not fluoresce at acid pH, normally found in intracellular vesicles, but emits fluorescence at neutral pH, normally found extracellularly (Miesenböck et al. 1998). Therefore, the fluorescence signal detected came from Fz5 receptors at the cell surface. To reduce background fluorescence and stabilise the pH, NRK cells were cultured with DMEM without phenol red and supplemented with HEPES buffer (Gibco). A baseline of 15 single stack images (256x256 pixels = 82x82 µm; 0.32 µm voxel) was taken every 0.25 sec prior to bleaching. Samples were flashed 10 times with 100% laser power every 0.25 sec to bleach the fluorescence signal in three small areas of the field of view: a) outside the cell (blank); b) at the edge of the cell; c) in the middle of the cell. The *“blank”* area at the edge of the cell was used for quantification of fluorescence recovery, which was measured by imaging at the following intervals and frequencies: 1 image / 0.25 sec for the first 12.5 sec; 1 image / sec for the next 30 sec; 1 image / 5 sec for the next 60 sec (total = 102,5 sec).

2.9 Immunofluorescence analyses of cultured cells

Hippocampal and cell line cultures were stained using a standard immunofluorescence protocol. Briefly, cells were rinsed with warm PBS and fixed in 4% PFA/PBS for 20 mins at RT. When fixing hippocampal neurons, 4% PFA was supplemented with 4% sucrose (Table 2.13). After fixation, cells were rinsed 3 times with PBS and either stored at 4 ˚C or processed for immunostaining. Cells were permeabilised with 0.05 % Triton X-100 in PBS for 5 min, washed once with PBS, and placed for at least 45 mins at RT in blocking buffer (5% BSA/PBS) in a humidified chamber. Primary antibodies were diluted in 1% BSA/PBS and added onto the cells O/N at 4 ˚C in a humidified chamber. The following day, after 3 washes with PBS, secondary antibodies were diluted in 1% BSA/PBS and added onto the cells for 1 hr in the dark at RT in a humidified chamber. Cells were then washed 3 more times with PBS and mounted on glass microscope slides with a drop of FluorSave (Calbiochem) mounting medium. Dry samples were placed at 4 ˚C or at -20 ˚C for short or long term storage.

2.10 Confocal imaging

Hippocampal neurons and cell lines were imaged on an Olympus FV1000 inverted confocal microscope. An oil-immersion 60x (NA 1.35) objective was used to acquire images at 207 nm/pixel. 6-12 z-step of 0.25 µm were imaged to analyse dendritic spines, synaptic puncta, endocytosed receptors and other trafficking markers. Brain slices were imaged on a Leica TCS SP8 inverted confocal microscope using an oil-immersion 60x (NA 1.35) objective to acquire images at 207 nm/pixel. 8 z-steps of 0.25 µm or 15-25 z-steps of 1 µm were acquired to image synaptic puncta or entire cells/brain areas respectively. For experiments on hippocampal neurons or cell lines 7-15 images from at least 3 independent biological samples were acquired. For brain slices, a minimum of 6 images from 3 slices was acquired for each animal.

2.11 Image analyses

Volocity software (PerkinElmer) was used to design protocols for quantification of all confocal images. When possible, analyses were carried out blind to the experimental condition/genotype. The number and size of dendritic spines of hippocampal neurons were measured manually. Briefly, 3-4 secondary dendrites were cropped from each cell and used for quantification. Morphological features commonly accepted in the field (Arellano et al. 2007; Basu et al. 2018) were used to identify stubby, mushroom and thin spines and exclude filopodia-like structures. The number of spines was normalised to the length of dendrites, which was calculated manually using a measuring tools of the Volocity software. Using the same tool, the maximum width of the head of each spine, here called spine size, was calculated. The average value of all the spines of each cell was used for quantification.

Similar criteria were used to identify synaptic puncta and vesicles of endocytosed receptors. To identify objects of interests, thresholds were designed according to two main features: fluorescence intensity and minimum/maximum size of the object. The software automatically measured number and volume of the identified objects. When analysing presynaptic assembly in isolated axons, the number of synaptic puncta was normalised to the length of the axon. To analyse images of brain slices, three different squares were cropped from each image to avoid areas of non-puncta staining, such as cell bodies or blood vessels, which would skew the number of puncta when normalising for total volume. For antibody-feeding experiments, the number of puncta of endocytosed receptors was normalised per cell. Only cells with similar size were imaged.

2.12 Preparation of protein samples and western blotting (WB)

Cultured cells were first rinsed with ice cold PBS and then lysed in appropriate amount of lysis buffer (Table 2.15) supplemented with PMSF (Sigma-Aldrich, final concentration 1 mM), 1x phosphatase and protease inhibitors (Roche). Unless stated otherwise, lysates were transferred to

Eppendorf tubes and incubated for 10 mins on ice before max speed centrifugation (10 mins at 13000 rpm at 4 ˚C). The pellet was discarded and the supernatant collected for protein quantification, which was performed using the BCA kit following manufacturer's instructions (Thermo Scientific). After adjusting protein concentrations to equal amounts, 5x loading buffer containing DTT (Table 2.27) was added to a final dilution of 1x, and the samples were incubated at 65 ˚C for 10 mins to denature protein structures. 5 to 30 µg of protein were loaded on SDS PAGE gels following standard Western Blot (WB) guide lines. Nitrocellulose or PVDF membranes were blocked for at least 1 hr at RT in 5% milk or BSA/TBS-t (Table 2.26) and then probed O/N at 4˚C with primary antibodies (Table 2.5). The following day, after 3x10 mins washes with TBS-t, the membranes were incubated for 1hr at RT with fluorescent or HRP-conjugated secondary antibodies and then washed again 3x10-30 mins before detection of fluorescence or chemiluminescence signals. When HRP-conjugated secondaries were used, ECL substrate (Amersham) was added to the membranes according to manufacturer's instructions to visualise chemiluminescence signals. A ChemiDoc (Bio-Rad) and ImageLab software (Bio-Rad) were used to imaging WB membranes. ImageJ software was used for quantification of signal intensity.

2.13 Surface biotinylation

Surface biotinylation experiments were performed as previously described (Sahores et al. 2010). One 60mm dish of HEK293 cells at 80-90% confluency was used for each condition. Cells were first placed on an ice block for 10 mins to stop membrane trafficking. All the following incubations and centrifugations were performed at 4 ˚C unless stated otherwise. Cells were washed twice with PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and then treated for 30 mins with 0.25 mg/mL of membrane-impermeable biotin/PBS (EZ-Link Sulfo-NHS-Biotin, Thermo Fisher Scientific) to biotinylate surface proteins. 10 mM glycine in PBS was used for 3x5 min washes to neutralise excess biotin and prevent biotinylation of intracellular proteins at the moment of lysis. Each dish was lysed with 500 µL of RIPA buffer (Table

2.16) supplemented with protease and phosphatase inhibitors. The samples were then passed 3 times through a 25G needle to maximise lysis efficacy and shear DNA and large portions of membranes. 10 mins after centrifugation at 13000 rpm, supernatants were collected and pellets discarded. Protein concentration was determined by BCA assay as described before (section 2.12). 10-50 µL were kept as total lysate (input), whereas the rest of the lysate (200-400 µg of protein) was added to 50 µL of streptavidin agarose beads (Pierce Streptavidin Agarose, Thermo Fisher Scientific) pre-equilibrated by three washes with RIPA buffer. Samples were placed on a wheel for end-over-end rotation for 2 hrs. Next, they were washed 3-4 times with RIPA buffer containing protease and phosphatase inhibitors. Each wash consisted of spinning down the samples at 1000 rpm for 1 min to precipitate streptavidin beads, discarding the supernatant, and adding 500 µL of fresh RIPA buffer to the beads. After the final wash, residual RIPA buffer was removed using a 30G needle, whose gauge is too narrow to aspirate beads. 50-60 µL of 2.5x loading buffer were added to elute biotinylated proteins from beads (IP or surface fraction). IP and input samples were then incubated at 65 ˚C for 10 mins to complete protein denaturation.

2.14 Co-IP (co-immunoprecipitation) experiments

Co-IP experiments were performed in HEK293 cells using GFP-trap beads (Chromotek). Importantly, these GFP beads recognise numerous variants of GFP proteins (Chromotek), including SEP, a pH-sensitive derivate of EGFP (Miesenböck et al. 1998). WT and/or 3CS Fz5-SEP were transfected in HEK293 in order to use GFP-trap and examine the interaction with Dvl1-HA and Fz5-HA. 24h hrs after transfection, one 60 mm dish of 80-90% confluent HEK293 cells was rinsed with PBS and lysed in 500 µL Triton Lysis Buffer (Table 2.22) supplemented with protease and phosphatase inhibitors. The samples were then incubated at 4 ˚C for 20 mins on an end-over-end rotating wheel and then spun at 13000 rpm for 10 mins. Protein quantification was determined, concentrations adjusted, and 20-50 µL of total lysate kept as input. The rest (200-400 µg of proteins) was added to 15-20 µL of GFP-trap beads pre-equilibrated by 3 washes with lysis buffer. The specimens were

incubated at 4 ˚C for 2-3 hrs on an end-over-end rotating wheel before being washed and processed for SDS PAGE analysis (same procedure as for surface biotinylation samples, section 2.13). Proteins co-immunoprecipitated together with WT or 3CS Fz5 were detected by WB, probing the IP fraction for proteins of interest (Dvl-HA or Fz5-HA). Relative levels of the co-immunoprecipitated proteins were calculated as a ratio over the total protein in the lysate.

2.15 Acyl-Biotin exchange (ABE) assay

ABE assay was performed from brain, hippocampal cultures and HEK293 lysates to identify palmitoylated proteins. The procedure was carried out with minimal variations to what previously reported (Wan et al. 2007). This protocol is based on the chemical exchange of palmitate groups with biotin, followed by standard streptavidin-mediated immunoprecipitation of biotinylated proteins. The protocol is divided in three consecutive days, followed by standard WB analyses on SDS PAGE gels.

Chloroform-methanol (CM) protein precipitation

This technique was used to wash protein samples from different components in the various buffers used throughout the protocol. All the steps of CM precipitation were performed at RT. Briefly, 1.2 mL of lysate were transferred to 15 mL plastic Falcons and 4.8 mL of methanol, 1.5 mL of chloroform and 3.6 mL of water were added. The mix was immediately vortexed and spun in a J2-MI Beckman centrifuge with a JS-7.5 swinging bucket rotor at 4000 rpm for 20 mins. After this centrifugation three phases are formed: top aqueous phase (water and methanol), a solid pancake-like interphase (proteins) and a bottom phase (chloroform). The top aqueous phase was carefully aspirated without disturbing the protein interphase. 3.6 mL of methanol were gently added to the tube and the solution slowly inverted 2-3 times to mix chloroform and methanol. The samples were centrifuged at 4000 rpm for 5 mins to pellet the solid protein interphase. The CM mix was discarded and the tubes air-dried for 2-3 min to facilitate the resuspension of the pellet. This is a critical step: left over traces of CM from insufficient drying, as well as

over-drying the samples, result in a slow and incomplete resuspension of the pellets. Pellets were resuspened in 300 µL of 4SB buffer (Table 2.19) unless stated otherwise. After adding 4SB, samples were placed in a water bath at 37 ˚C and frequently tapped and vortexed to resuspend pellets. This step can take from 10- up to 30 mins depending on the amount of protein present in the lysate. After complete resuspension, unless stated otherwise, 900 µL of ABE lysis buffer with 0.2% triton were added and the procedure was repeated.

Day1: blockage of free Cys residues

Flash-frozen brain tissue was homogenised with a pestel in 1 mL of ABE lysis buffer (Table 2.17) supplemented with 10 mM NEM (*N*-Ethylmaleimide) (Sigma), 1 mM PMSF and 1x protease inhibitors cocktail (PI, Table 2.18). When working with cells, two 60mm dishes of hippocampal neurons or HEK293 cells were washed twice with ice cold PBS and then lysed in 1 mL (500 µL/dish) of ABE lysis buffer with the same supplements mentioned above. In this protocol NEM is used to block all the free Cys residues, thus avoiding biotinylation in the following steps. Poor efficacy of NEM blockage can result in background signals in negative control samples (-HAM, see day 2). After lysis, cells were passed 3-5 times through a 25G needle and 85 µL of Triton X-100 were added (final concentration 1.7%). Lysates were then passed 3 more times through a 25G needle to ensure complete homogenisation. Samples were placed at 4 ˚C for 1hr on an end-over-end rotating wheel (10 rpm) before centrifugation at low speed (0.3 rcf at 5 mins at 4 ˚C) to remove big particulates. Next, lysates were washed by chloroform-methanol (CM) precipitation (see above) and resuspended in 300 µL of 4SB buffer supplemented with 10mM NEM. 900 µL of ABE lysis buffer containing 10 mM NEM, 1mM PMSF, 1x PI and 0.2% triton were added and samples were incubated O/N at 4 ˚C on a rotating wheel (10 rpm).

Day 2: NEM wash out - acyl-biotin exchange

In the second day of the protocol NEM is washed out, palmitate groups removed by treatment with Hydroxilamine (HAM) and newly exposed Cys residues biotinylated. Excessive NEM was washed by 3 sequential CM

precipitations. The protocol can be stopped after each CM precipitation and lysates can be stored at -20 ˚C in 4SB buffer. At the end on the 3rd CM the pellet was resuspended in 600 µL of 4SB and each sample was divided into two tubes. Half of the samples were treated with 900 µL of +HAM buffer (Table 2.20) and half of the samples were treated with 900 µL of -HAM buffer (Table 2.20). The samples were incubated in the dark at RT for 1hr on a rotating wheel at 10 rpm. +HAM buffer contained 0.7 M HAM (Sigma), which breaks the thioester bonds between Cys residues and palmitate groups. -HAM buffer contained water; therefore, thioester bonds between Cys residues and palmitate groups were unaffected. During this incubation with HAM buffers, free Cys residues, which were originally palmitoylated, were biotinylated with HPDP-biotin that was added to both + and - HAM buffers. HAM was then washed out by one CM precipitation and protein pellets resuspended in 300 µL 4SB. To ensure complete biotinylation of free Cys residues, 900 µL of low HPDP-biotin buffer (Table 2.21) were added and the samples incubated in the dark at RT for 1hr on a rotating wheel at 10 rpm. To remove excessive HPDP-biotin before streptavidin pull-down (day 3), the samples were subjected to three consecutive CM precipitations. The protocol was stopped by freezing the samples after the 1st or 2nd CM precipitation.

Day 3: immunoprecipitation of biotinylated (=palmitoyated) proteins

Samples were thawed at RT and the remaining CM precipitation (1 or 2, depending on the stopping point at the end of day 2) were carried out. After the last CM, pellets were resuspended in 50 µL of 2SB buffer to dilute SDS concentration and ensure efficient streptavidin pull-down. SDS was further diluted to 0.1% by adding 950 µL of ABE lysis buffer supplemented with 1 mM PMSF and 1x PI. The samples were incubated in the dark at RT for 30 mins on a wheel rotating at max speed (40 rpm) to ensure complete dissolution of protein pellet and washing from residual SDS. Next, lysates were spun at max speed for 1 min in a bench-top centrifuge at RT to remove large particulates before the pull-down. Protein concentration was determined with BCA kit and adjusted for each sample. 100 µL of total lysate were kept as input and the rest of the lysates were added to 80 µL of streptavidin agarose beads pre-equilibrated with ABE lysis buffer containing

PMSF and PI. The samples were incubated for 2-3 hrs in the dark, at RT, on a wheel (10 rpm). Next, they were washed 3-4 times with ABE lysis buffer and, as previously described for surface biotinylation and Co-IP experiments, left over solution was aspirated with thin needles. Proteins were eluted from beads by adding 50-60 µL of 2.5x loading buffer and by heating samples for 10 min at 65 ˚C. The specimens were then used for standard WB procedures.

2.16 Statistical analyses

Statistical analyses were carried out using GraphPad Prism. First, normality was tested using either D'agostino-Pearson, Saphiro-Wilk or Kolmogorov-Smirnov tests. For all the experiments each animal/culture was considered as independent experimental unit, with the exception of the analyses of Fz5 and Fz7 KD in hippocampal neurons (chapter 3) and FRAP live-imaging experiments, in which each cell was treated as an independent experimental unit. One sample t-test and two-tailed unpaired Student's t-test were used, where appropriate, to calculate statistical significance between two conditions. Mann-Whitney test was used when comparing two data-sets not normally distributed. One-way and Two-way ANOVA were performed on data sets composed of 3 or more conditions. One Way ANOVA followed by Tukey's post hoc test was used on normally distributed samples whereas Kruskall-Wallis and Dunn's post hoc test were run on not-normally distributed data sets.

Table 2.5: List of primary antibodies

Antibody	species	Supplier and cat number	Dilution/application
HA	rat	Roche, 11867423001	1:1000 (IF and WB)
Homer1	rabbit	Synaptic Systems,16003	1:1000 (IF)
GFAP	chicken	Abcam, ab5541	1:500 (IF)
Iba-1	rabbit	Synaptic Systems, 234003	1:500 (IF)
MAP2	chicken	Abcam, ab5392	1:2000 (IF)
EEA1	rabbit	Cell Signalling, 3288	1:100-200 (IF)
Rab11	rabbit	Cell Signalling, 5589	1:100-200 (IF)
Rab7	rabbit	Cell Signalling, 9367	1:100-200 (IF)
GFP	chicken	Millipore, 06-896	1:500 (IF)
GFP	rabbit	Invitrogen, A6455	1:500 (IF brain slices); 1:2000 (IF cultured cells);1:5000 (WB)
mCherry	rabbit	Abcam, ab167453	1:500 (IF)
β-actin	rabbit	Cell Signalling, 4970S	1:5000 (WB)
β-tubulin	mouse	Sigma, T9026	1:10000 (WB)
GAPDH	rabbit	Abcam, ab181602	1:5000 (WB)
Vinculin	mouse	Sigma, v4505	1:1000-2000 (WB)

List of solutions

10x phosphate buffer saline (PBS) 1L

Compounds and final concentration	From stock
1.37 M NaCl	80 g
27 mM KCl	2 g
20 mM KH_2HPO_4	2.4 g
100 mM Na_2HPO_4	14.4 g
dH_2O	Up to 1L
pH to 7.4 with HCl and NaOH	

Borate buffer (400 mL)

Compounds and final concentration	**From stock**
50 mM boric acid	1.24 g
25 mM borax	2 g
dH_2O	Up to 400 mL
pH to 8.5 and filter. Store at 4 ˚C	

Hippocampal cultures plating medium (50 mL)

Compounds and final concentration	**From stock**
Horse serum (Gibco)	5 mL
Penicillin / Streptomycin (Gibco)	200 µL
DMEM, GlutaMAX, D-Gluocse (4.5 g/L), pyruvate	45 mL

Hippocampal cultures maintenance medium (50 mL)

Compounds and final concentration	**From stock**
B27 (Gibco)	1 mL
N2 (Gibco)	500 µL
1 mM Glutamine (Gibco)	250 µL
Glucose (Sigma)	500 µL
Neurobasal medium (Gibco)	up to 50 mL

Neuronal transfection medium (50 mL)

Compounds and final concentration	**From stock**
Glucose (Sigma)	666 µL
Neurobasal medium (Gibco)	up to 50 mL

2x HEPES-buffered saline (HeBBS) (200 mL)

Compounds and final concentration	**From stock**
274 mM NaCl	3.2 g
10 mM KCl	142 mg
1.4 mM Na_2HPO_4	40 mg
15 mM D-glucose	540 mg
42 mM HEPES	2 g

mQH_2O	up to 200 mL
pH to 7.06 - 7.14 with NaOH and filter	

WT-EM nucleofection solution for cell lines (200mL)

Compounds and final concentration	**From stock**
15 mM NaH_2PO_4	3 mL (from 1M solution)
35 mM Na_2HPO_4	7 mL (from 1M solution)
5 mM KCl	1 mL (from 1M solution)
10 mM $MgCl_2$	2 mL (from 1M solution)
11 mM Glucose	2.2 mL (from 1M solution)
100 mM NaCl	4 mL (from 5M solution)
20 mM HEPES	4 ml (from 1M solution)
mQ H_2O	176.8 mL
pH to 7.2 with NaOH	

4% PFA (50 mL)

Compounds and final concentration	**From stock**
4 % paraformaldehyde	2 g
4 % sucrose	2 mg
0.4 mM NaOH	200 µL (from 0.1M stock)
PBS	5 mL of PBS 10x
Check pH (7-7.5)	

Cryo-freezing solution (800 mL)

Compounds and final concentration	**From stock**
11 mM NaH_2PO_4 - H_2O	1.25 g
30 mM Na_2HPO_4 - $2H_2O$	4.36 g
30 % ethylene glycol	240 mL
30 % glycerol	240 mL
dH_2O	up to 800 mL

Standard WB lysis buffer (50 mL)

Compounds and final concentration	**From stock**

150 mM NaCl	1.5 mL (From 5M solution)
1.0% NP-40 or Triton X-100	500 µL (From 100%solution)
50 mM Tris pH 8.0	2.5 mL (from 1M solution)

Supplement with protease and phosphatase inhibitors

RIPA lysis buffer (50 mL)

Compounds and final concentration	**From stock**
10 mM Tris (pH 8)	0.5 mL (from 1M solution)
100 mM NaCl	1 mL (from 5 M solution)
1 mM EDTA (pH 8)	100 µL (from 0.5 M solution)
1 % NP-40	0.5 mL (from 100% solution)
0.1 % SDS	0.5 mL (from 10 % solution)
0.5 % deoxycholate	0.25 g
dH_2O	up to 50 mL

Supplement with protease and phosphatase inhibitors

ABE assay, lysis Buffer (50 mL)

Compounds and final concentration	**From stock**
150 mM NaCl	1.53 mL (from 5 M solution)
50 mM Tris pH 7.4	2.5 mL (from 1M solution)
5 mM EDTA (pH 8)	500 µL (from 0.5 M solution)

pH to 7.4 and keep at 4 ˚C

When required supplement with protease and phosphatase inhibitors and Triton X-100 to final concentration of 0.2%. When required supplement with NEM (Pierce, 23030). Final concentration 10 mM. **1M stock has to be prepared fresh the day of the experiment** (125 g NEM in 1 mL of 100% ethanol).

ABE assay, 100x protease inhibitors cocktail (1 mL)

Compounds and final concentration	**From stock**
25 µg/mL Pepstatin (Sigma)	250 µL (from 1 mg/mL stock dissolved in methanol and kept at -20 ˚C)

25 µg/mL Leupeptin (Sigma)	25 µL (from 10 mg/mL stock dissolved in mQ H_2O and kept at -20 ˚C)
25 µg/mL Antipain (Sigma)	25 µL (from 10 mg/mL stock dissolved in DMSO and kept at -20 ˚C)
25 µg/mL Chymostatin (Sigma)	25 µL (from 10 mg/mL stock dissolved in ethanol and kept at -20 ˚C)
Ethanol	675 µL

ABE assay, 2% and 4% SDS buffer (20 mL)

Compounds and final concentration	**From stock**
2% or 4% SDS	4 or 8 mL (from 10% solution)
50 mM Tris pH 7.4	1 mL (from 1M solution)
5 mM EDTA	0.2 ml (from 0.5M solution)
dH_2O	10.8 or 14.8 mL

Keep at RT until finished. When required supplement with NEM (**NEM has to be added fresh every time**)

ABE assay, +/- HAM buffers (5 mL)

Compounds and final concentration	**From stock**
0.7 M Hydroxylamine (Sigma)	3.5 mL (from 1M solution). The day of the experiment dissolve 1.4 gr in 15 mL dH2O. pH to 7.4 with roughly 1.5 mL of 10M NaOH and bring to 20 mL final volume.

1 mM HPDP–biotin (Pierce, 21341)	1.25 ml from 4 mM HPDP–biotin. HPDP- biotin is dissolved at 50 mM in DMSO and stored at -20 ˚C. **The day of the experiment** dilute the 50 mM stock to 4mM in N,N-dimethyl formamide (Sigma)
0.2% Triton X-100	50 µL (from 20% solution)
1x protease inhibitors	50 µL (from 100x solution)
1 mM PMSF	50 µL (from 100mM solution)
dH_2O	50 µL

-HAM solution has the same components, but HAM is substituted by 250 µL of 1M Tris pH 7.4 and 3.25 mL dH2O.

ABE assay, Low HPDP–biotin buffer (10 mL)

Compounds and final concentration	**From stock**
0.2 mM HPDP–biotin	0.5 mL ml from 4 mM HPDP–biotin.
150 mM NaCl	300 µL (from 5M solution)
50 mM Tris pH 7.4	500 µL (from 1M solution)
5 mM EDTA	100 µL (from 0.5M solution)
0.2% Triton X-100	100 µL (from 20% solution)
1x protease inhibitors	100 µL (from 100x solution)
1 mM PMSF	100 µL (from 100mM solution)
dH_2O	8.2 mL

Co-IP lysis buffer (50mL)

Compounds and final concentration	**From stock**
50 mM Tris pH 7.5 or 8	2.5 mL (from 1M solution)
150 mM NaCl	1.5 mL (from 5M solution)
1 mM EDTA	100 µL (from 0.5M solution)
0.5% Triton X-100	1 ml (from 25% solution)
dH_2O	up to 50 mL

Supplement with protease and phosphate inhibitors

10x Tris-glycine buffer (1 L)

Compounds and final concentration	**From stock**
250 mM Tris	30 g (of Trizma base)
1.9 M glycine	144 g
dH_2O	up to 1 L

1x Running buffer

Compounds and final concentration	**From stock**
0.1 % SDS	10 mL (from 10 % solution)
Running buffer to 1x	100 mL (from 10x solution)
dH_2O	up to 1 L

1x Transfer buffer

Compounds and final concentration	**From stock**
20 % ethanol	200 mL
Running buffer to 1x	100 mL (from 10x solution)
dH_2O	up to 1 L

10x TBS buffer (1 L)

Compounds and final concentration	**From stock**
1.37 M NaCl	80 g
26.8 mM KCl	2 g
250 mM Tris	30 g (of Trizma base)
dH_2O	up to 1 L

1x TBS-t buffer (10 L)

Compounds and final concentration	**From stock**
1x TBS buffer	1 L from 10x solution
0.1 % Tween 20	10 mL
dH_2O	Up to 10 L

5x Laemali buffer with DTT (20 mL)

Compounds and final concentration	**From stock**
250mM Tris (pH 6.8)	0.6 g (of Trizma base)
500 mM DTT	5 mL (from 2M solution)
4 % SDS	0.8 g
1 % bromophenol blue	0.2 g
50 % glycerol	10 mL
dH2O	up to 20 mL

CHAPTER 3: THE ROLE OF Fz5 AND Fz7 IN PRE- AND POSTSYNAPTIC DEVELOPMENT

3.1 Introduction

Wnt molecules are key organizers of embryonic development (MacDonald et al. 2009; Nusse and Clevers 2017; Wiese et al. 2018). In addition, numerous research demonstrates that Wnt signalling plays a pivotal role in controlling several aspects of CNS development and function, such as BBB formation, dendritogenesis, axon guidance, synapse formation, synaptic plasticity and maintenance (Liebner et al. 2008; Budnik and Salinas 2011; Salinas 2012; McLeod and Salinas 2018; Oliva et al. 2013b).

The synaptogenic activity of Wnt molecules was first described over 20 years ago. Our laboratory demonstrated that exogenous Wnt7a induces axonal remodelling and the assembly of presynaptic sites in cerebellar granule cells (Lucas and Salinas 1997). Importantly, the effects of Wnt7a were confirmed *in vivo* at granule cell/mossy fibre synapses using a *Wnt7a*$^{-/-}$ mouse model (Hall et al. 2000). Since then, mounting evidence has unravelled the role of Wnt7a in orchestrating the formation and function of both sides of the synapse. On the presynaptic side, Wnt7a promotes the assembly of key presynaptic structures (Lucas and Salinas 1997; Hall et al. 2000; Davis et al. 2008; Sahores et al. 2010) and regulates synaptic transmission by controlling vesicle recycling and neurotransmitter release (Ahmad-Annuar et al. 2006; Cerpa et al. 2008; Ciani et al. 2015). On the postsynaptic side, Wnt7a is required for the formation and growth of dendritic spines (Ciani et al. 2011), which are the postsynaptic structures for the vast majority of excitatory inputs. Moreover, on the postsynaptic side Wnt7a regulates the trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), strengthening synaptic plasticity (McLeod et al. 2018). Therefore, Wnt7a promotes synapse assembly by acting on both sides of the synapse. However, a key question still remains: how do Wnts drive synaptogenesis?

The action of different Wnts on their receptors, and the activation of downstream cascades, determines the function of these molecules. At the cell surface Wnt ligands can bind to a number of different receptors, including the canonical co-receptor LRP6, the non-canonical co-receptors Ror and Ryk and the main Wnt receptors Frizzled (van Amerongen 2012). The role of Frizzled receptors in Wnt-mediated synaptogenesis is poorly understood. Work from our lab has shown that the synaptogenic factor Wnt7a can bind to Fz5 and Fz7, which are both found in synaptosome fractions (Sahores et al. 2010; McLeod et al. 2018). Fz5 is enriched at presynaptic sites and overexpression of this receptor in hippocampal dissociated neurons is sufficient to induce presynaptic differentiation, mimicking the effect of Wnt7a (Sahores et al. 2010). Importantly, loss of function studies demonstrated that Fz5 is required for Wnt7a-mediated presynaptic assembly in cultured neurons (Sahores et al. 2010). However, how Wnt7a signalling is mediated at postsynaptic sites remained unknown. In this chapter, I will present gain and loss of function studies performed on dissociated hippocampal neurons where I compared the role of Fz5 and Fz7 in the development of both sides of the synapse. I have addressed the following specific questions:

1) Are Fz5 and Fz7 similarly localised at dendritic spines?
2) Does Fz5, a presynaptic receptor for Wnt7a, have a role in postsynaptic development?
3) Is Fz7 required for postsynaptic development?
4) Does Wnt7a-Fz7 signalling drive dendritic spine formation?
5) Is Fz7 also required for presynaptic differentiation?

3.2 Results

3.2.1 Fz5 and Fz7 localisation at dendritic spines

Wnt7a signals on axons to induce presynaptic assembly (Lucas and Salinas 1997; Hall et al. 2000; Davis et al. 2008), a function mediated by Fz5 receptors (Fig 1.9) (Sahores et al. 2010). On the postsynaptic side, Wnt7a ligands regulate dendritic spine formation and morphology (Ciani et al. 2011). However, the receptor required for Wnt7a to induce postsynaptic development was unknown. A binding assay screen revealed that Wnt7a can also bind Fz7, which is expressed in the brain and found in synaptosome fractions (McLeod et al. 2018). In a work recently published (McLeod et al. 2018), we compared the localisation and function of Fz5 and Fz7 to identify the receptor required for Wnt7a to induce postsynaptic development.

Using immunofluorescence and confocal microscopy, I first examined the localisation of these two Wnt receptors in dissociated hippocampal neurons. We have previously used antibodies against endogenous Fz5 to examine its distribution along axons and dendrites, but not dendritic spines (Sahores et al. 2010). Unfortunately, these antibodies are no longer available; therefore, the distribution of Fz5 was analysed by expressing Fz5-HA in cultured neurons. EGFP-actin was co-expressed with Fz5-HA to visualise the morphology of the cells in particular dendritic spines, where actin is enriched (Fig 3.1). The cells were examined at 12-14 DIV (days *in vitro*), within the peak of synaptogenesis (12-16 DIV), unless otherwise stated. As previously observed (Sahores et al. 2010), I found that Fz5 receptors were present in the soma and along axons and dendrites. Although Fz5 was clearly present along dendrites, it was almost never observed within dendritic spines (Fig 3.2 A), suggesting that this receptor might not be involved in mechanisms of spine development and function. In contrast, primary antibodies against endogenous Fz7 revealed the presence of this receptor in dendritic spines (Fig 3.3 A). Although these data were obtained using different approaches (overexpression vs endogenous), these findings indicate that these two Wnt receptors exhibit different localisation: Fz5 is not localised at dendritic spines

whereas Fz7 is. These observations suggest that Fz5 and Fz7 might have different functions at dendritic spines.

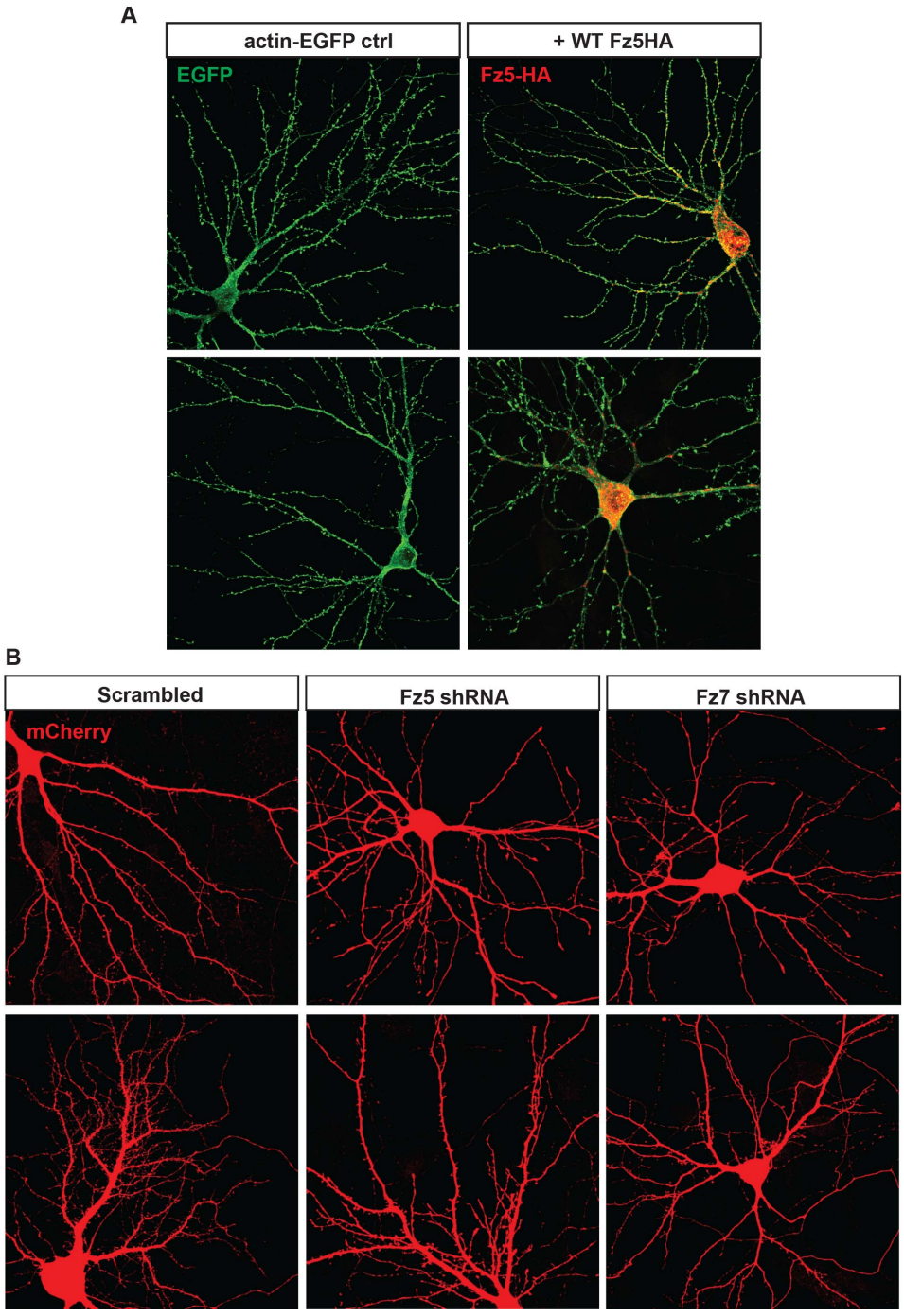

Fig 3.1: Gallery of neurons selected for *in vitro* studies
Confocal images showing examples of primary hippocampal cells transfected with different expression plasmids. Cells were transfected at 7-9 DIV by calcium phosphate method and fixed at 12-14 DIV for immunofluorescence analyses. Exclusively pyramidal cells were selected. Pyramidal cells were identified by the typical triangular shape of the cell body and by the dendritic arborisation. EGFP-Actin or mCherry were co-expressed to allow for the visualisation of dendritic branches and spines. 3-4 secondary branches per cell were selected to measure dendritic spine number and size.

3.2.2 Fz5 expression is neither sufficient nor required for dendritic spine formation

To examine whether Fz5 regulates postsynaptic development, I performed gain and loss of function experiments. First, hippocampal neurons were transfected with either EGFP-actin alone (control) or together with Fz5-HA (Fig 3.1 and 3.2). Pyramidal neurons, identified by the shape of the cell body and dendritic arbour, were selected for the analysis of spine density and size (Fig 3.1 and 3.2). Expression of Fz5 did not change spine number or size, indicating that this receptor does not promote postsynaptic development of these structures (Fig 3.2).

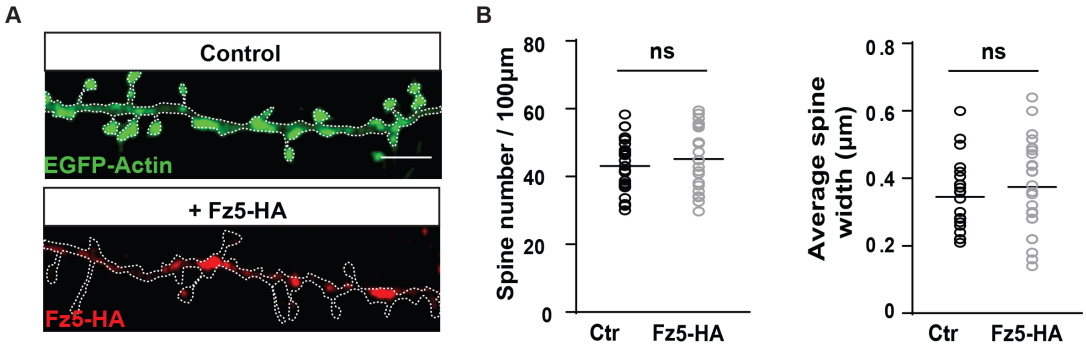

Fig 3.2: Fz5 gain-of function does not affect dendritic spines
A: Confocal images of 13-14 DIV cells expressing EGFP-Actin control (green, top panel) or EGFP-Actin and Fz5-HA (red). The white dotted lines delineate the perimeter of EGFP-Actin staining. Note that Fz5-HA is present along the dendritic shaft but almost completely absent from spine heads. Scale bar: 5 µm. **B:** Quantification of dendritic spine number (left) and width (right). Fz5 gain of function does not affect spine number and size. (Data presented as mean with raw values for each cell analysed; n = 28-32 cells per condition from 3 independent cultures; Student's t-test).

Next, loss-of-function experiments were performed to test the requirement of Fz5 receptor for synapse formation. Three shRNAs targeting different regions of rat Fz5 were tested in NRK (Normal Rat Kidney cells). The cells were AMAXA nucleofected with either Scrambled (Scr) or Fz5-specific shRNAs and harvested 48 hrs later for qPCR analyses (these qPCR experiments were performed in collaboration with Dr Ernest Palomer Vila). We found that shRNA 1, 2 and 3 significantly reduced Fz5 mRNA levels compared to Scr ctrl (Fig 3.3 A). The percentage of reduction roughly reflected the transfection rate observed (not shown), indicating that the

overall levels of knockdown depended on the level of transfection efficiency. Since shRNA 1 induced the strongest decrease in Fz5 mRNA levels, this construct was used for loss of function experiments. Primary hippocampal neurons were transfected with Scr or Fz5-shRNA 1 at DIV 7-9 and fixed for immunostaining analyses at DIV 12-14. For these experiments, mCherry was co-expressed to identify transfected cells and visualise dendritic arborisation and spines (Fig 3.1). I found that Fz5 KD did not affect dendritic spine number or size (Fig 3.3 B-C) compared to control neurons expressing Scr ShRNA. Together, these gain and loss of function experiments strongly suggest that Fz5, which is required for presynaptic assembly (Sahores et al. 2010), does not affect the formation and morphology of dendritic spines.

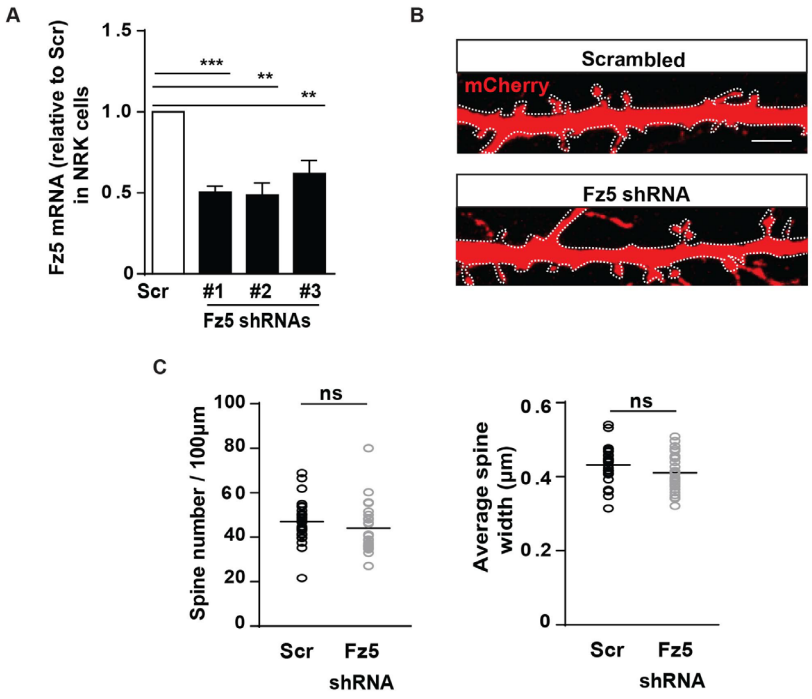

Fig 3.3: Fz5 loss-of-function does not affect dendritic spines
A: qPCR analyses of Fz5 mRNA levels in NRK (normal rat kidney) cells transfected with Scrambled or three different Fz5-shRNA clones. The graph shows the fold change relative to scrambled shRNA control. Fz5 shRNA clone 1 was selected for functional studies in hippocampal neurons. (Data presented as mean ± SEM; n = 4 repeats from 4 independent cultures; Student's t-test **P < 0.01, ***P < 0.001). **B:** Confocal images showing dendritic branches of hippocampal neurons expressing Scrambled or Fz5-shRNA 1. The white dotted line delineates the perimeter of the mCherry staining (red). Scale bar = 2.5 µm. **C:** Quantification of dendritic spine number and width. KD of Fz5 does not affect spine density or size. (Data presented as mean with raw values for each cell analysed; n = 27 cells per condition from 3 independent cultures; Student's t-test).

3.2.3 Fz7 is required for postsynaptic development and Wnt7a-mediated spine formation

The role of Fz7 in postsynaptic development was tested by loss of function experiments in dissociated hippocampal neurons. First, KD (knock-down) of Fz7 was tested in NRK cells as described for Fz5. NRK cells transfected with Fz7-shRNA 1, 2 and 3 showed a significant reduction of Fz7 mRNA levels compared to Scr ctrl (Fig 3.4 B). I then tested the impact of the KD in primary neurons. Scr and shRNA 3 were transfected into primary hippocampal neurons at 7-9 DIV and the dendritic phenotype was examined at 12-14 DIV when spines are formed. I found that KD of Fz7 resulted in a significant decrease in the number of dendritic spines when compared to neurons expressing Scr shRNA (Fig 3.4 C-D). However, spine size was unchanged between Scr and KD cells (Fig 3.4 E-F). To rule out off-target effects, I performed rescue experiments by co-expressing Fz7-ShRNA together with shRNA-resistant Fz7 cDNA (rescue). Expression of the shRNA-resistant Fz7 fully rescued the number of dendritic spines to Scr levels (Fig 3.4 C-D), demonstrating that the spine defect was not due to off target effects.

Next, I tested whether Fz7 is required for Wnt7a to signal on the postsynaptic side. For this, neurons (12-14 DIV) were treated for 3 hrs with recombinant Wnt7a and the effects on spines were analysed. As expected, spine size and number were significantly increased in Scr expressing cells exposed to Wnt7a compared to controls (BSA treated) (Fig 3.4 E-F) (Ciani et al. 2011). However, the increase in spine size and number was completely abolished in Fz7 KD cells, suggesting that these cells were unable to respond to Wnt7a (Fig 3.4 E-F). Altogether, these data indicate that Fz7 is necessary for the development of dendritic spines and is required for Wnt7a-mediated spine growth.

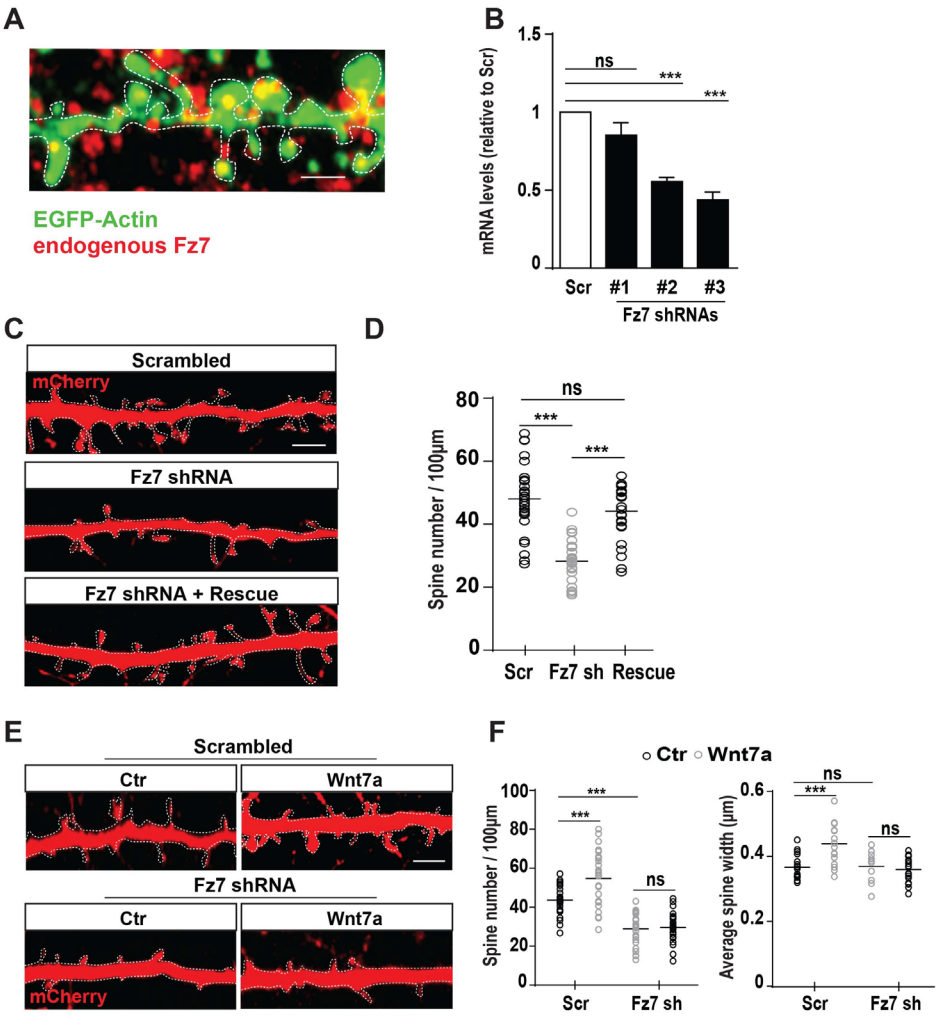

Fig 3.4: Fz7 is required for spine formation and Wnt7a-induced structural plasticity

A: Confocal image of a dendrite of a hippocampal cell expressing EGFP-Actin (green). Endogenous Fz7 (red) can be observed along the dendritic shaft and inside spine heads. **B:** qPCR analyses of Fz7 mRNA levels in NRK cells expressing Scr or three different Fz7-shRNA clones. Fz7 shRNA clone 3 was selected for functional studies in hippocampal neurons. (Data presented as mean ± SEM; n = 4 repeats from 4 independent cultures; **P < 0.01, ***P < 0.001; Student's t-test). **C:** Dendritic branches of hippocampal neurons 12-14 DIV expressing Scrambled, Fz7-shRNA 3 or Fz7 shRNA 3 together with a Fz7 rescue construct. Scale bar = 2.5 µm. **D:** KD of Fz7 reduces spine density, which is fully rescued upon expression of a Fz7 rescue construct (data presented as mean with raw values for each cell analysed; n = 27 cells per condition from 3 independent cultures; ***P < 0.001, One-way ANOVA with multiple comparisons). **E:** Dendritic branches of hippocampal neurons 12-14 DIV expressing Scrambled or Fz7-shRNA, exposed to BSA (control) or Wnt7a for 3 hrs. **F:** Fz7 KD prevents the increase in spine number and size induced by Wnt7a. Note that Fz7 KD does not affect spine size in basal conditions. (Data presented as mean with raw values for each cell analysed; n = 27 cells per condition from 3 independent cultures; ***P < 0.001 One-way ANOVA with multiple comparisons).

To extend the comparison between Fz5 and Fz7 receptors, the requirement of Fz7 for presynaptic assembly was also tested. Low-density cultures were transfected with Scr and shRNA Fz7 constructs at 5-6 DIV when the formation of presynaptic boutons begins and fixed at 10 DIV for immunofluorescence analyses (Fig 3.5). Isolated axons were selected, and the number of Bassoon puncta, a marker that labels both excitatory and inhibitory presynaptic sites, was counted. I found that Fz7 KD did not affect the number of presynaptic sites (Fig 3.5), indicating that Fz7, differentially from Fz5, is not required for presynaptic assembly. These results, together with previous work from our laboratory (Sahores et al. 2010; Ciani et al. 2011), demonstrate that Wnt7a signals through Fz5 and Fz7 receptors to regulate pre and postsynaptic assembly at excitatory synapses.

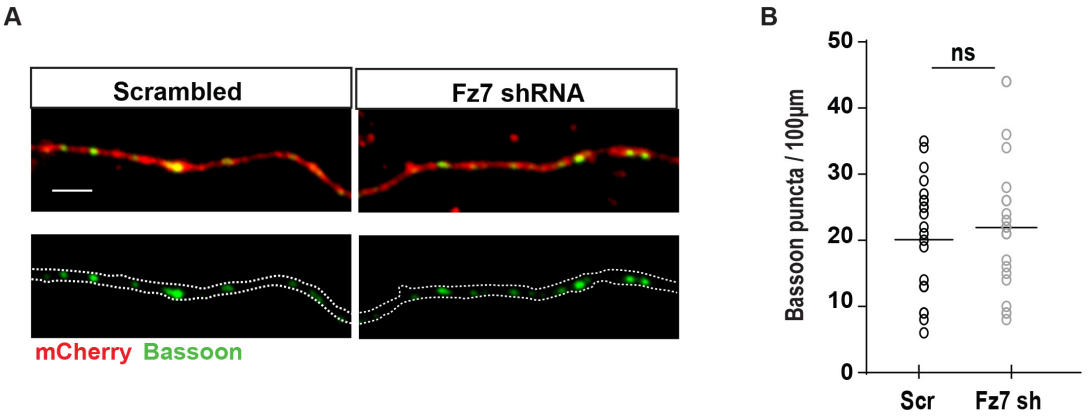

Fig 3.5: Fz7 is not required for presynaptic assembly
A: Confocal images showing isolated axons from 10 DIV cells expressing Scrambled or Fz7 shRNA. The presynaptic marker Bassoon is labelled in green, and the white dotted lines delineate the perimeter of mCherry staining. Scale bar: 5µm. **B:** Quantification of Bassoon density along 100µm of axon. Fz7 KD does not reduce the number of Bassoon puncta along axons. (Data presented as mean with raw values for each cell analysed; n = 21 cells per condition from 3 independent cultures; Student's t-test).

3.3 Discussion

Some of the Wnts (4 of the 19 Wnts) expressed in mammals have been shown to play a crucial role in synapse formation (Salinas 2012). Particularly, the Wnt7a ligand promotes the formation of both pre and postsynaptic sides at excitatory synapses (Hall et al. 2000; Sahores et al. 2010; Ciani et al. 2011). Wnt7a signals through Fz5 on axons to promote presynaptic assembly (Sahores et al. 2010). However, the postsynaptic receptor for Wnt7a was unknown until now. Here, I have characterised the localisation of two Wnt7a receptors, Fz5 and Fz7, at dendritic spines and I have examined their function at both sides of the synapse.

3.3.1 The distribution of Fz5 and Fz7 at dendritic spines

Biochemical analyses reported that both Fz5 and Fz7 are present at synapses (Sahores et al. 2010; McLeod et al. 2018). Using immunofluorescence analyses, I found that these receptors exhibit distinct localisation at dendritic spines, where Fz7, but not Fz5, is detected (Fig 3.2 A; Fig 3.4 A). It should be noted that the distribution of Fz5 was studied using overexpression techniques due to the lack of suitable primary antibodies. Although this approach can sometimes create artefacts in protein localisation, gain and loss of function experiments presented here confirmed that Fz5 is not involved in postsynaptic development. In the general discussion (see chapter 7), I will consider hypotheses on what molecular mechanisms may establish the different localisation and function of Fz5 and Fz7.

3.3.2 Fz5 is not required for dendritic spine development

Our laboratory demonstrated that Fz5 is required and is sufficient to drive presynaptic assembly in cultured neurons (Sahores et al. 2010). Moreover, Fz5 is required for Wnt7a- and activity-induced formation of presynaptic boutons (Sahores et al. 2010). However, the role of this receptor in the development of dendritic spines had not been explored before. Here, I showed that neither gain nor loss of function of Fz5 affected spine size and number. Altogether these data demonstrate that Fz5, whose function at the

presynaptic side was previously reported, is not required for spine development.

One question still remains, what is the function of Fz5 in dendrites? Given the distribution of this receptor along the dendritic shaft, we could hypothesise a role for Fz5 in the formation of inhibitory postsynaptic sites, which are mostly found on the shaft rather than on dendritic spines (Moss and Smart 2001). In chapter 6 I will present data showing the role of Fz5 expression in excitatory synapse formation *in vivo*. We are currently using the same samples to examine inhibitory synapse formation. Very preliminary data suggest that Fz5 is not involved in the formation of inhibitory synapses in the developing hippocampus; consistently, other preliminary data from cultured neurons suggest that Fz5 puncta in the shaft do not seem to colocalise with inhibitory postsynaptic structures (Bossio and Salinas unpublished results). Therefore, the function of Fz5 receptors in dendrites remains to be elucidated.

3.3.3 The role of Fz7 at pre- and postsynaptic sites

The data presented here strongly suggest that Fz7 is not required for presynaptic assembly. Whether Fz7 plays a role in axons still needs to be addressed. Beside presynaptic assembly, Wnt signalling plays an important role in regulating axon growth cone dynamics, SVs recycling and neurotransmitter release (Ciani et al. 2004; Purro et al. 2008; Stamatakou et al. 2015; Ciani et al. 2015). Thus, although Fz7 does not affect presynaptic assembly its role in these Wnt-mediated presynaptic functions should be investigated.

Data from our lab showed that the synaptogenic factor Wnt7a binds to Fz7 (McLeod et al. 2018), which localises at dendritic spines (Fig 3.4 A). I found that in basal conditions loss of Fz7 down-regulates the density of dendritic spines but not their size. In addition, loss of function of Fz7 prevented Wnt7a-induced increase in spine number and size, strongly suggesting that Fz7 mediates Wnt7a effects at dendritic spines. An interesting question arises from these data. If Fz7 does not regulate spine size, how does it block Wnt7a-mediated enlargement of these structures?

The size of dendritic spine is determined by the area of the postsynaptic density, the amount of postsynaptic receptors and the cytoskeleton within the spine head and neck (Arellano et al. 2007). Molecular mechanisms regulating these factors are in place to continuously maintain the size of dendritic spines. My data indicate that in basal conditions KD of Fz7 does not affect dendritic spines, suggesting that this receptor is dispensable for steady state maintenance of spines size. However, it is important to examine these data in the context of structural plasticity of dendritic spines. Spines rapidly increase or decrease their size in response to external stimuli, such as experience-dependent or induced patterns of neuronal activity (Hering and Sheng 2001; Rochefort and Konnerth 2012; Bosch and Hayashi 2012). How does neuronal activity control spine size? It is established that neuronal activity promotes the expression and secretion of several synaptogenic molecules, including BDNF and Wnts, which play a role in shaping spine morphology (Thoenen 1991; Harward et al. 2016; McLeod and Salinas 2018). Our lab has recently demonstrated that during the very early stages of LTP induction (within 10 mins) Wnt7a levels are increased at synapse (McLeod et al. 2018). In addition, we showed that Fz7 receptors are required for LTP-induced spine enlargement and, consistently, we found that Fz7 is required for the induction of LTP. In light of these findings, the fact that Wnt7a does not increase spine size in Fz7 KD cells indicates that this receptor is required for structural plasticity of dendritic spines.

The work presented in this chapter enriches our understanding of the role of Wnt receptors in synapse formation. Several Wnt receptors, including LRP6, Ryk, Ror and Frizzled have been shown to be required for pre and/or postsynaptic assembly (Mathew et al. 2005; Varela-Nallar et al. 2009; Paganoni et al. 2010; Sahores et al. 2010; Sharma et al. 2013). Wnt7a is the best-characterised synaptogenic Wnt ligand; however, the receptor required to mediate its function at dendritic spines was unknown until now. My data, alongside previous work from our lab (Sahores et al. 2010), describe distinct localisation and function of Fz5 and Fz7 at both sides of the synapse, shedding light on the molecular mechanisms of Wnt7a-mediated synapse formation.

CHAPTER 4: PALMITOYLATION OF FRIZZLED RECEPTORS

4.1 Introduction

The cell-surface recruitment and localisation of receptors is crucial for proper signalling in every cell. This is often achieved by regulating protein trafficking and function through post-translational modifications (PTMs). The biochemistry of these modifications can be very diverse, ranging from the attachment of a single phosphate group to large carbohydrate or lipid chains. Thus, PTMs have a direct impact on the structure, localisation and function of proteins.

Extensive efforts have been made to understand how key synaptic proteins are trafficked to synapses, and how their function is regulated in response to different stimuli (Lau and Zukin 2007; Anggono and Huganir 2012; Bassani et al. 2013; Choquet and Triller 2013; Diering and Huganir 2018). However, much less is known about the synaptic recruitment and regulation of synaptogenic factors and their receptors, particularly Frizzled receptors. The surface levels of the synaptogenic receptor Fz5 are up and downregulated by HFS and LFS (high/low frequency stimulation) respectively, and Fz5 is required for Wnt7a- and activity-mediated synapse formation in hippocampal neurons (Sahores et al. 2010). In light of these results, we started to investigate potential molecular mechanisms that could control Fz5 trafficking in a dynamic manner.

We decided to examine for specific features at the C-terminal domain of the receptor, which is crucial for signalling (Tauriello et al. 2012) and could determine Fz5 trafficking and retention at the PM and at synapses. Observations made by a former member of our lab, Dr E. Stamatakou, revealed the presence of three cysteine (Cys) residues at the C-term of Fz5; these Cys could be sites for PTMs such as palmitoylation (Stamatakou and Salinas unpublished). Indeed, by mutagenesis of the three Cys to Ser, which cannot be palmitoylayed, and radiolabelling, Dr. Stamatakou confirmed that Fz5 is palmitoylated (Fig 1.13). In addition, preliminary observations suggest that palmitoylation is required for Fz5 synaptogenic activity in cultured neurons (Fig 1.13) (Stamatakou and Salinas, unpublished). These results

were exciting because they revealed for the first time that Frizzled receptors can be palmitoylated and that this modification might be crucial for their function. Therefore, the study of this modification is novel and of extreme interest for the Wnt signalling field.

We focused on palmitoylation because it is one of the most common lipid modifications of synaptic proteins and it regulates their trafficking and function at synapses (Fukata and Fukata 2010; Globa and Bamji 2017). S-palmitoylation, distinguished from N-palmitoylation (Fig 1.10), consists of the reversible attachment of a long saturated chain of palmitic acid on Cys residues, and is the most common lipid modification of proteins (Resh 2016; Linder and Deschenes 2007). As well as increasing protein hydrophobicity, palmitoylation can alter protein conformation, as in the case of the Wnt co-receptor LRP6 (Abrami et al. 2008), arrest or allow the progression of proteins along the trafficking route, and target molecules to specific membrane domains (Charollais and Van Der Goot 2009; Aicart-Ramos et al. 2011). In addition, protein-protein interactions and the interplay with other PTMs can be affected by palmitoylation (Salaun et al. 2010; Blaskovic et al. 2013).

S-palmitoylation is catalysed by a specific family of protein acyl transferases (PATs) named DHHC enzymes (Fukata et al. 2006; Guan and Fierke 2011). DHHC enzymes are specifically localised withtin the cell (ER, Golgi, endosomes, plasma membrane) and between tissues (Ohno et al. 2006). Many of these enzymes are expressed in the brain and their substrates have only just started to be identified (Fukata and Fukata 2010). The identification of DHHC-substrates has been instrumental in elucidating the molecular mechanisms that control protein trafficking and function at the synapse (Fukata and Fukata 2010; Globa and Bamji 2017).

Although our studies demonstrate that Fz5 is palmitoylated (Fig 1.13), we did not know whether other Frizzled receptors are also palmitoylated and whether all the Cys at the C-term of Fz5 can be palmitoylated. Furthermore, I have asked which are the enzymes that palmitoylate Fz5, and performed gain and loss of function studies on members of the DHHC family of protein

acyl transferases. The novelty of the data presented in this and the next chapter opens new doors to unravel the mechanisms of regulation of Wnt signalling at the level of Frizzled receptors. Understanding how Wnt receptors are regulated will help us to comprehend and, where necessary, manipulate Frizzled function in different biological processes in which Wnt signalling is involved. In this chapter I will present data that address these specific questions:

1. Which Fz receptors are palmitoylated?
2. Which Cys residues are palmitoylated in Fz5?
3. Which DHHC enzymes can palmitoylate Fz5?
4. Which DHHC enzymes are required for Fz5 palmitoylation?

4.2 Results

4.2.1 Some Frizzled receptors are palmitoylated

Data from our laboratory indicate that Fz5 is palmitoylated *in vivo* and *in vitro* (Fig 1.13, Stamatakou and Salinas unpublished results). However, whether other Frizzled receptors are palmitoylated had not been investigated before. To test this, I first used the CSS-Palm 4.0 software (Ren et al. 2008) to predict putative sites of palmitoylation in the entire protein sequence of all Frizzled receptors. Each of the 10 Frizzled receptors (human sequences) was predicted to be palmitoylated on at least one Cys residue (Fig 4.1). These Cys residues are spread across different protein domains including the N-term, transmembrane (TM) regions and the C-term. Although the C-terms of Fz3, Fz4, Fz5, Fz6, Fz8, Fz9 and Fz10 contain at least one Cys (Fig 4.2), only those of Fz5 and its homolog Fz8 were predicted to be palmitoylated (Fig 4.1). Taken together, these computational analyses suggest that all Frizzled receptors might be palmitoylated on several Cys residues. However, given the heterogeneous distribution of these Cys residues (e.g N-term vs TM vs C-term), we could speculate that palmitoylation, if present, might have very distinct functions for different Frizzled receptors.

Frizzled receptors were screened for palmitoylation using the ABE biochemical assay (Wan et al. 2007). All the receptors were expressed in HEK293 cells. Palmitoylation was clearly detectable for Fz3, Fz4, Fz5, Fz6, and Fz9 (Fig 4.3 A). Fz1, Fz7 and Fz8 exhibited lower levels of palmitoylation and only very weak signals were observed for Fz2 and Fz10 after long exposure of the WB membrane (Fig 4.3 A). These results indicate that several Frizzled receptors are palmitoylated, most likely on the Cys residues identified with CSS-Palm-4.0. However, to rule out false positives, especially for those Frizzled that only exhibited very weak palmitoylation levels, it would be important to confirm these results by different techniques such as mass spectrometry, incorporation of radioactive palmitate and mutagenesis of putative palmitoylation sites, as we have done for Fz5 (Stamatakou and Salinas, unpublished results). Taken together, these bioinformatic and

biochemical analyses strongly suggest that Frizzled receptors are palmitoylated, a novel and important finding for the Wnt signalling field.

	Residue	Domain	Peptide	Score	Cut-off
Fz1	330	1st TM	IGIWSVLCCASTLFT	5.469	3.419
Fz2	255	1st TM	ILTWSVLCCASTFFT	5.717	3.419
	256	1st TM	LTWSVLCCASTFFTV	4.887	4.222
Fz3	36	N-term	EPITLRMCQDLPYNT	8.98	4.222
Fz4	261	2nd TM	PIIFLSMCYNIYSIA	4.893	4.222
Fz5	537	C-term	WRRFTSRCCCRPRRG	7.601	3.419
	538	C-term	RRFTSRCCCRPRRGH	9.306	3.419
	539	C-term	RFTSRCCCRPRRGHK	5.719	4.222
Fz6	10	Sign peptide	MFTFLLTCIFLPLLR	37.698	4.222
	32	N-term	EPITVPRCMKMAYNM	4.832	4.222
	211	1st TM	TVSIFCLCATLFTFL	4.352	4.222
Fz7	264	1st TM	VGVWSVLCCASTLFT	7.902	3.419
Fz8	620	C-term	WRSLCTRCCWASKGA	5.671	3.419
Fz9	47	N-term	QAVEIPMCRGIGYNL	5.245	4.222
	100	N-term	CSLYAPMCTDQVSTP	6.574	4.222
	273	2nd TM	PIIFLSMCYNVYSLA	6.867	4.222
Fz10	18	Sign peptide	VLQVMGSCAAISSMD	30.68	4.222
	42	N-term	QPIEIPMCKDIGYNM	5.038	4.222
	95	N-term	CSLYAPMCTEQVSTP	4.969	4.222
	110	N-term	IPACRVMCEQARLKC	4.527	4.222
	269	2nd TM	PIIFLSMCYCVYSVG	5.352	4.222

Fig 4.1: Prediction of palmitoylation of Frizzled receptors
Summary of palmitoylation predictions run using CSS-Palm 4.0. Cys residues predicted to be palmitoylated are highlighted in red. The position number (2nd column) and the protein domain (3rd column) are given for each Cys residue predicted to be palmitoylated. Prediction scores (5th column) were calculated using an automatic high-threshold cut-off (6th column). Cys residues within the signalling peptide of Frizzled receptors are shown in light grey, as these residues are cleaved in the ER and will not be part of mature proteins.

	C-term protein sequence
Fz1	SGKTLNSWRKFYTRLTNSKQGETTV
Fz2	SGKTLHSWRKFYTRLTNSRHGETTV
Fz3	GSKKTCFEWASFFHGRRKKEIVNESRQVLQEPDFAQSLLRDPNTPIIRK SRGTSTQGTSTHASSTQLAMVDDQRSKAGSIHSKVSSYHGSLHRSRD GRYTPCSYRGMEERLPHGSMSRLTDHSRHSSSHRLNEQSRHSSIRDL SNNPMTHITHGTSMNRVIEEDGTSA
Fz4	KTLHTWQKCSNRLVNSGKVKREKRGNGWVKPGKGSETVV
Fz5	WSGKTVESWRRFTSRCCCRPRRGHKSGGAMAAGDYPEASAALTGRT GPPGPAATYHKQVSLSHV
Fz6	GSKKTCTEWAGFFKRNRKRDPISESRRVLQESCEFFLKHNSKVKHKKK HYKPSSHKLKVISKSMGTSTGATANHGTSAVAITSHDYLGQETLTEIQTS PETSMREVKADGASTPRLREQDCGEPASPAASISRLSGEQVDGKGQA GSVSESARSEGRISPKSDITDTGLAQSNNLQVPSSSEPSSLKGSTSLLV HPVSGVRKEQGGGCHSDT
Fz7	SGKTLQSWRRFYHRLSHSSKGETAV
Fz8	SGKTLESWRSLCTRCCWASKGAAVGGGAGATAAGGGGGPGGGGGGG GPGGGGGPGGGGGSLYSDVSTGLTWRSGTASSVSYPKQMPLSQV
Fz9	SSKTFQTWQSLCYRKIAAGRARAKACRAPGSYGRGTHCHYKAPTVVL HMTKTDPSLENPTHL
Fz10	TSKTLQSWQQVCSRRLKKKSRRKPASVITSGGIYKKAQHPQKTHHGKY EIPAQSPTCV

Fig 4.2: Alignment of the C-term of Frizzled receptors
C-terms of human Frizzled receptors. Cys residues are highlighted in red. Note that the C-term domains exhibit considerably variable length and amino acid composition. All Frizzled receptors except Fz1, Fz2 and Fz7 contain Cys residues at the C-term, but only those in Fz5 and Fz8 are predicted to be palmitoylated (see Fig 4.1)

4.2.2 Fz5 is palmitoylated on each of the 3 Cys residues at the C-term

Previous findings from our lab have shown that mutations of the three Cys residues to Ser, which cannot be palmitoylated, abolish Fz5 palmitoylation (Fig 1.13). To address which of the three Cys residues is palmitoylated, we generated Fz5 mutants in which two of the Cys were mutated to Ser and only one residue was left unchanged (Note: the cloning of these double mutants was performed by Dr Laura-Nadine Schuhmacher, a postdoc in the lab). I will refer to these mutants with the following annotations: C537-538S, C538-539S and C537-539S. Palmitoylation was reduced by roughly 50% in each of these mutants, and essentially abolished when all three Cys were mutated to

Ser (3CS Fz5) (Fig 4.3 B-C). These data reproduce previous observations from lab and show that the three Cys at the C-term are required for Fz5 palmitoylation. In addition, these findings demonstrate that each of these Cys residues can be palmitoylated.

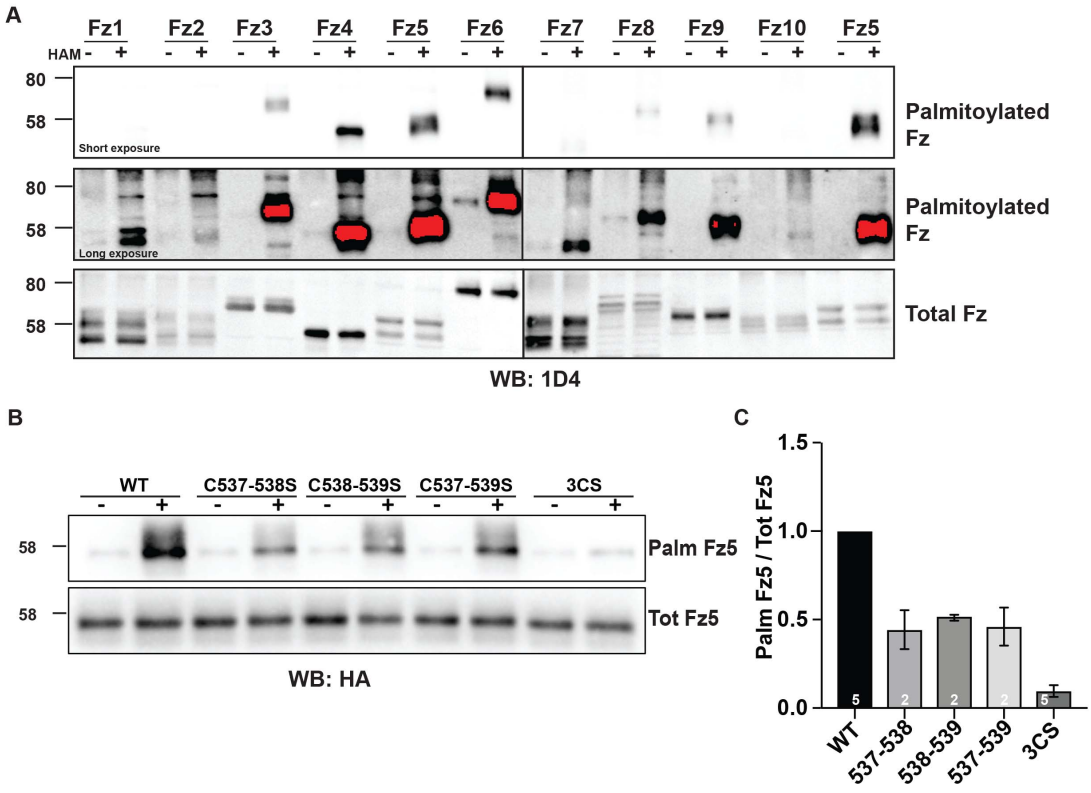

Fig 4.3: Frizzled receptors palmitoylation - Fz5 palmitoylation at the C-term
A: ABE assays from HEK293 cells expressing Frizzled receptors tagged with 1D4. HAM (hydroxylamine) is the chemical agent used to remove palmitate groups from Cys residues. The presence of + and – indicate samples treated with HAM or vehicle (negative controls). The top panel shows the streptavidin pull-down fraction of palmitoylated Frizzled receptors exposed for a short time to reveal high-intensity signals for Fz3, Fz4, Fz5, Fz6 and Fz9. The middle panel shows the same membrane exposed for longer to reveal low-intensity signals from Fz1, Fz2, Fz7, Fz8, and Fz10. The bottom panel shows the input from total lysates. Fz5 was loaded on each membrane as internal control (n = 3). **B:** ABE assays showing palmitoylation of WT and double Cys mutant Fz5 receptors. **C:** Quantification of Fz5 palmitoylation expressed as a ratio of palmitoylated to total Fz5 and normalised to WT Fz5. Palmitoylation is reduced by roughly 50% in each Fz5 double mutant, and is decreased almost to background levels when all three Cys are mutated (3CS Fz5). Data presented as mean ± SEM; n = 2-5 independent experiments.

4.2.3 Investigating which DHHC enzymes palmitoylate Fz5

To understand how Fz5 palmitoylation is regulated, it is crucial to identify the enzymes responsible for its palmitoylation. Therefore, I have performed gain and loss of function experiments to address this question. First, cDNAs of all 23 DHHC enzymes (a kind gift from Professor Akio Kihara and Dr Yusuke Ohno), were expressed together with Fz5-HA in HEK293 cells, and palmitoylation levels of Fz5 were examined by ABE assay (see section 2.15 chapter 2). Fz5 palmitoylation was increased over 2-folds by six enzymes: DHHC3, DHHC6, DHHC7, DHHC9, DHHC14 and DHHC15. In particular, expression of DHHC3 and DHHC7, which belong to the same subfamily (Fig 1.12), increased Fz5 palmitoylation over 6 folds. In contrast, palmitoylation of Fz5 was reduced by more than 30% in the presence of DHHC12 (-32%), DHHC13 (-48%) and DHHC24 (-41%) (Fig 4.4), suggesting that the presence of these enzymes activates some signalling pathways that inhibit Fz5 palmitoylation.

To address which enzymes are required for Fz5 palmitoylation, I performed loss of function experiments on the two most promising candidates: DHHC3 and DHHC7. Two shRNAs for each enzyme were validated in HEK293 cells, as shown by the decrease of DHHC3 and DHHC7 mRNA levels 48h after transfection of each shRNA clone (Fig 4.5 A-B). These shRNAs were then used to KD endogenous DHHC3 or DHHC7 while expressing Fz5-HA. To avoid potential compensatory effects, I decided to also KD both enzymes simultaneously. However, neither single nor double KD of DHHC3 and DHHC7 reduced Fz5-HA palmitoylation significantly (Fig 4.5 C-D). These data suggest that either these enzymes are not required for Fz5 palmitoylation or that the presence of other DHHCs might compensate for the loss of DHHC3 and DHHC7. Further experiments, which are discussed below, are needed to clarify the discrepancy between gain and loss of function analyses.

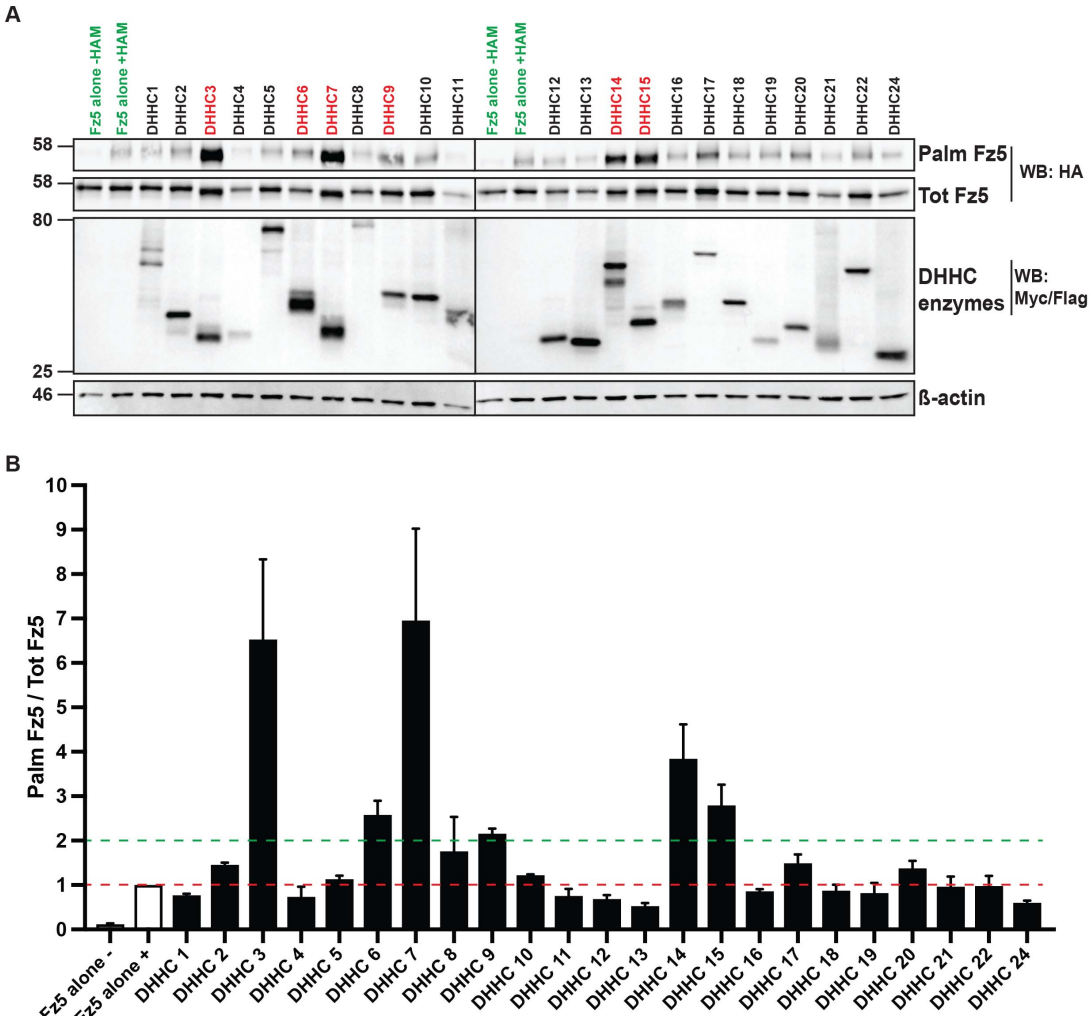

Fig 4.4: Gain-of-function screen for the DHHC enzymes that palmitoylate Fz5
A: ABE assays showing palmitoylation of Fz5 by different DHHC enzymes. All 23 DHHCs, tagged with either Myc or Flag, were co-expressed with Fz5-HA in HEK293 cells. "*Fz5-HA alone* + *or* – HAM" were loaded on each gel and used as internal controls. **B:** Quantification of Fz5 palmitoylation in the presence of DHHC enzymes was normalised to the level of *Fz5 alone* + (red dotted line). Expression of DHHC3, DHHC6, DHHC7, DHHC9, DHHC14, DHHC15 increased Fz5 palmitoylation over 2 fold (green dotted line). Data presented as mean ± SEM; n = 2 independent experiments.

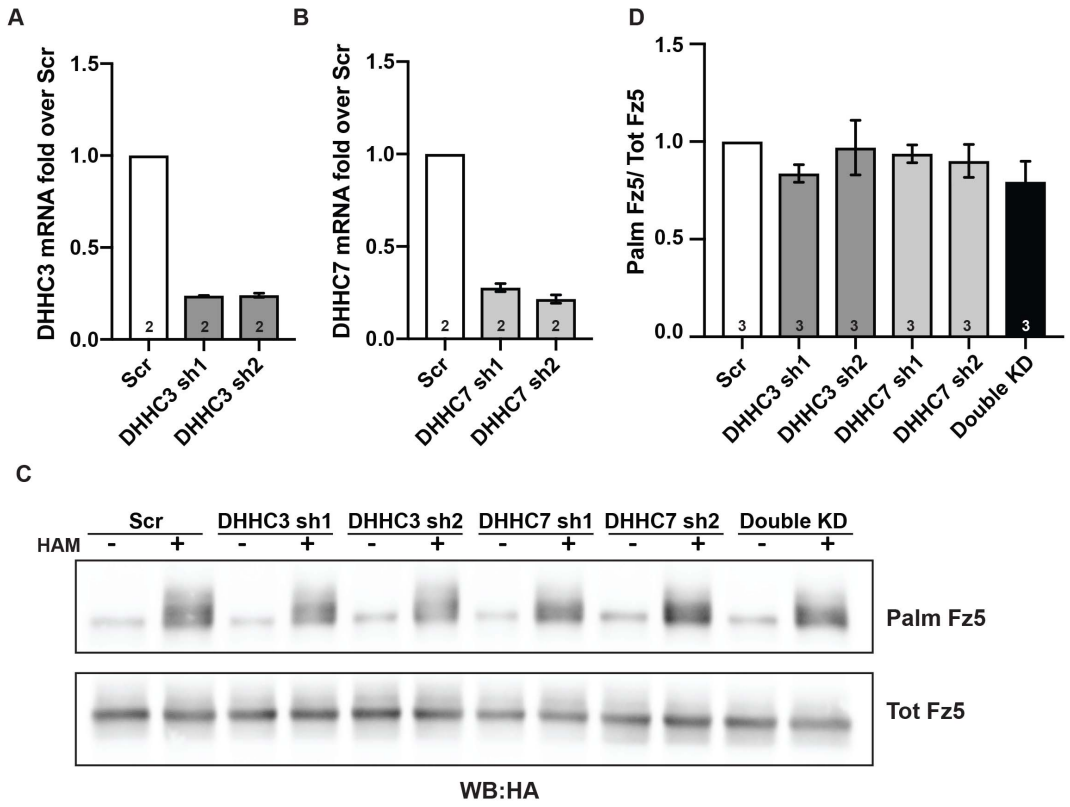

Fig 4.5 The effect of DHHC3 and DHHC7 loss-of-function on Fz5 palmitoylation
A-B: qPCR showing DHHC3 and DHHC7 mRNA levels upon shRNA-mediated KD. Each pair of shRNA clones resulted in roughly 80% decrease of DHHC3 or DHHC7 mRNA respectively. Data presented as mean ± SEM; n = 2 independent experiments. **C:** Representative WB from ABE assays showing palmitoylation of Fz5-HA upon DHHC3 and DHHC7 KD. **D:** Quantification of Fz5-HA palmitoylation. Values were normalised to Scr shRNA. Single or double KD of DHHC3 and DHHC7 did not significantly change in the levels of Fz5 palmitoylation. Data presented as mean ± SEM; n = 3 independent experiments; One sample t-test.

4.3 Discussion

Wnt signalling through Frizzled receptors is central in many biological processes. Studying how Frizzled receptors are regulated at the molecular level is crucial to understand the function of these molecules. Here I have shown that potentially all Frizzled receptors are palmitoylated. I have also determined the sites where Fz5 is palmitoylated and I have identified six enzymes that can catalyse this PTM. The results presented in this chapter uncover a novel PTM of Frizzled receptors and open new avenues to study the role of palmitoylation on these family of proteins.

4.3.1 Frizzled receptors are palmitoylated – Fz5 palmitoylation at the C-term

Previous observations from our lab showed that Fz5 is palmitoylated (Fig 1.13), but whether other Frizzled receptors are subjected to the same modification was unknown. By analysing the structure of Frizzled receptors I found that each of these proteins contain at least one Cys residue predicted to be palmitoylated. The Cys residues are located across diverse domains in different Frizzled receptors. My biochemistry experiments strongly suggest that Fz3, Fz4, Fz5, Fz6 and Fz9 are palmitoylated, whereas further analyses are required to address palmitoylation of Fz1, Fz2, Fz7, Fz8 and Fz10. Although the biological role of Fz5 palmitoylation will be addressed in the next chapter, some general considerations can be made for the whole Frizzled family.

The fact that Cys residues are located across different protein domains (N-term vs TM vs C-term) implies that palmitoylation may have different roles on Frizzled receptors, and it also suggests that this modification might be regulated by different mechanisms. For instance, palmitoylation of Frizzled receptors may occur in different subcellular compartments and might be catalysed by different DHHC enzymes. In addition, bioinformatic analyses revealed that Cys residues are present at the C-term of most Frizzled receptors (Fz3, Fz4, Fz5, Fz6, Fz8, Fz9, Fz10), but only those of Fz5 and its homologous, Fz8, were predicted to be palmitoylated. Thus, it is possible that

palmitoylation at the C-term of Frizzled receptors might have evolved to regulate specific function of Fz5 and its homolog Fz8.

Preliminary data from our lab showed that the 3 Cys residues at the C-term of Fz5 are required for palmitoylation, but whether palmitoylation can occur on each of these Cys was not addressed before. Here I found that each of the three Cys can be palmitoylated. This observation raises the question of whether palmitoylation on different residues has diverse biological effects. Although it is unknown whether palmitoylation on adjacent Cys can regulate distinct biological processes, several examples exist for proteins in which Cys residues are not adjacent. For instance AMPARs and NMDARs are palmitoylated on distinct TM or C-term Cys residues, which respectively control the retention of these receptors at the Golgi or regulate their internalisation from the plasma membrane (Hayashi et al. 2005; Hayashi et al. 2009). In my PhD I have investigated the role of palmitoylation using the triple Cys Fz5 mutant (Fz5 3CS, see chapter 5 and 6); therefore, the role of palmitoylation on each specific Cys residues remains unknown. Altogether, these results uncover a novel PTM of Frizzled receptors and open a whole new areas of research in the Wnt signalling field.

4.3.2 Palmitoylation of Fz5 by members of the DHHC family

Identifying enzyme-substrate pairs is fundamental to understand how palmitoylation is regulated, but it is still a major challenge in the field of protein palmitoylation. To identify the enzymes that palmitoylate Fz5, I performed gain- and loss-of-function experiments of members of the DHHC family of protein acyl transferases (PAT) (Fukata et al. 2006; Ohno et al. 2006). The gain-of-function screen presented here shows that DHHC3 and DHHC7, but also DHHC6, 9, 14, and 15, can palmitoylate Fz5 in HEK293 cells. Particular attention was given to DHHC3 and DHHC7, which increased Fz5 palmitoylation around 6 to 7fold. These enzymes, which belong to the same DHHC subfamily (Fig 1.12), are expressed in the CNS and many other tissues and localise at the Golgi apparatus (Ohno et al. 2006). Many substrates, including several pre- and postsynaptic proteins, are

palmitoylated by both of these enzymes (Globa and Bamji 2017; Matt et al. 2019), suggesting either redundancy or a coordinated mechanism that requires both enzymes. However, the requirement of DHHC3 and DHHC7 was not confirmed by shRNA mediated KD of these enzymes. The most likely explanation is that other DHHC enzymes compensate for the loss of DHHC3 and DHHC7.

How can we avoid compensatory effects? The first question that needs to be addressed is whether palmitoylation by six different DHHC6 might be an artefact induced by overexpression of these enzymes. This same question has recently been addressed in the context of palmitoylation of γ subunits of $GABA_A$Rs (Kilpatrick et al. 2016). In HEK293 cells both DHHC3 and DHHC7 palmitoylate $GABA_A$Rs (Fang et al. 2006); however, only *Dhhc3*$^{-/-}$ mice, and not *Dhhc7*$^{-/-}$ animals, exhibit defects in $GABA_A$Rs trafficking and function (Kilpatrick et al. 2016). The authors elegantly demonstrated that this phenotype is due to a subtle difference in localisation of these enzymes at the Golgi apparatus: DHHC3 and DHHC7 localise at the *cis*- and *trans*-Golgi respectively (Kilpatrick et al. 2016). In HEK293 cells, overexpression causes aberrant localisation of DHHC7 to the *cis*-Golgi, enabling palmitoylation of $GABA_A$Rs (Kilpatrick et al. 2016). Thus, palmitoylation of $GABA_A$Rs by DHHC7 seems to be an artefact of protein overexpression (Kilpatrick et al. 2016). Therefore, examining Fz5 palmitoylation in a more physiological context, such as using *DHHCs* KO mouse models, could unravel the role of these enzymes for Fz5 palmitoylation *in vivo*.

Another approach could be to extend the analysis of the effects of DHHC KD to Fz5-specific functions. Indeed, several proteins that are palmitoylated on more than one Cys residue exhibit residue-specific function of palmitoylation. For instance, NMDARs are palmitoylated at the C-term on two distinct clusters of Cys residues (Hayashi et al. 2009). Palmitoylation of a first cluster of Cys mediates the retention of NDMARs in the Golgi, whereas palmitoylation of a second group of Cys is important for the membrane stability of the receptor (Hayashi et al. 2009). Although we do not know whether palmitoylation of the three Cys residues at the C-term of Fz5 results in residues-specific effects, it would be important to examine the effects of

DHHCs KD on Fz5-specific function. In fact, while overall palmitoylation levels might be compensated by other DHHCs, specific functional outcomes might be impaired by the loss of one or more enzymes. In this respect, it would be interesting to examine the impact of DHHCs KD on Fz5-induced synapse formation. In collaboration with other members of the lab we are currently working on this hypothesis.

Despite the inconclusive data on the requirement of DHHC enzymes, what can we learn from the gain-of-function screen? DHHC3, DHHC6, DHHC7 DHHC9, DHH14 and DHHC15 increase Fz5 palmitoylation. In HEK293 cells these enzymes localise between the ER and the Golgi (Ohno et al. 2006). Therefore, it seems reasonable to assume that palmitoylation of Fz5 occurs before the receptor reaches the cell surface, and palmitoylation could regulate the trafficking of this receptor to different subcellular locations. Knowing where palmitoylation is attached to Fz5 could help us to dissect the role of this modification.

Taken together my data strongly suggest that several if not all Frizzled receptors are palmitoylated. I also demonstrated that each of the Cys residues at the C-term of Fz5 is palmitoylated, and that at least 6 different DHHC enzymes can palmitoylate Fz5 in cell lines. Further experiments are needed to address the requirement of these enzymes for Fz5 palmitoylation. Thus, my findings uncover a previously uncharacterised PTM of Frizzled receptors. Studying the molecular mechanisms and biological functions of Frizzled palmitoylation will unravel novel mechanisms of regulation of Wnt signalling.

CHAPTER 5: THE IMPACT OF PALMITOYLATION ON Fz5 SIGNALLING AND TRAFFICKING

5.1 Introduction

Frizzled are the main receptors for Wnt ligands and are essential for signalling. However, surprisingly little is known about the molecular mechanisms that control the function and distribution of these receptors. In light of the crucial roles that Frizzled receptors play in a variety of biological processes, including cancer, stem cell biology and neuroscience, it is fundamental to study how their function is regulated. In the previous chapter, I have introduced palmitoylation, a previously uncharacterised modification of Frizzled receptors, with special attention to Fz5, a receptor for the synaptogenic factor Wnt7a. Preliminary data from our lab show that palmitoylation-deficient Fz5 (3CS Fz5) is unable to promote synapse formation *in vitro* (Fig 1.13), suggesting that palmitoylation is crucial for Fz5 function. Here, I have investigated the role of this modification further, and used biochemical and cell biology techniques to elucidate how palmitoylation affects Fz5.

Palmitoylation can affect proteins in a number of different ways. First, palmitoylation can positively or negatively control protein turnover (Percherancier et al. 2001; Roberts et al. 2016). Second, palmitoylation can regulate the exit of proteins from the ER and Golgi, determining progression along the trafficking route and sorting to specific subcellular compartments (Greaves and Chamberlain 2007; Salaun et al. 2010; Ernst et al. 2018). This is particularly important in neurons, where selective trafficking to axon or dendrites is crucial for cell polarity and for synapse formation and function (Fukata and Fukata 2010; Yokoi et al. 2012; Montersino and Thomas 2015; Holland and Thomas 2017; Yogev and Shen 2017). Third, the attachment of lipid chains onto proteins can affect their lateral mobility across the PM, thereby affecting signalling (Delint-Ramirez et al. 2011; S. Kim et al. 2014). Fourth, palmitoylation is a major regulator of exocytosis, endocytosis and recycling of membrane proteins, thus controlling their surface levels and determining signalling strength and duration (Resh 2006; Nadolski and

Linder 2007; Sugita 2008; Doherty and McMahon 2009; Grant and Donaldson 2009). Fifth, palmitoylation can control protein-protein interactions such as ligand-receptor binding, oligomerisation, and the interaction with scaffold proteins (Simons and Toomre 2000; Resh 2006; Charollais and Van Der Goot 2009). By affecting one or more of these cell biology mechanisms, palmitoylation has direct consequences on several aspects of protein function.

The fact that the palmitoylation deficient mutant 3CS Fz5 is unable to promote synapse formation suggests that this post-translational modification might regulate several of the cell biology processes listed above. Therefore, I have performed a series of experiments, in cell lines and primary hippocampal neurons, to establish the role of Fz5 palmitoylation in each of these processes. The data presented here shed new light into the role of this previously uncharacterised PTM of Frizzled receptors, hence enriching our understanding of the regulation of these molecules. The following questions will be addressed:

1. Does palmitoylation affect Fz5 protein turnover?
2. Does palmitoylation regulate Fz5 interaction with key components of the Wnt signalosome?
3. Does palmitoylation regulate Fz5 localisation in hippocampal neurons?
4. Does palmitoylation control Fz5 membrane trafficking?

5.2 Results

5.2.1 Fz5 turnover is independent of protein palmitoylation

Palmitoylation controls the turnover rate of many proteins including the A1 adenosine receptor (Gao et al. 1999) and the chemokine and HIV receptor CCR5 (Percherancier et al. 2001). In some cases, like for anthrax toxin receptors and the Wnt co-receptor LRP6, it does so by preventing protein ubiquitination (Abrami et al. 2006; Abrami et al. 2008). To test whether Fz5 turnover is regulated by palmitoylation, I blocked protein translation in NRK (normal rat kidney) cells and monitored the degradation of WT and 3CS Fz5 over time by WB (western blot). Both WT and 3CS Fz5 exhibited a half-life of roughly 60 mins, strongly suggesting that palmitoylation does not regulate the turnover rate of Fz5 (Fig 5.1). My data are consistent with previous reports on the half-life of other Frizzled receptors (Mukai et al. 2010; Hao et al. 2012). In addition, the relatively rapid turnover rate of Fz5 receptors is comparable to the half-life of Smoothened (Smo) (Milenkovic et al. 2009), a seven transmembrane protein which belongs to the Frizzled family of GPCRs. Smo is a key component of the Hedgehog signalling, and has an half-life of roughly 60-120 mins (Milenkovic et al. 2009). In contrast, other GPCRs like adenosine, adrenergic, cannabinoid and glutamate metabotropic receptors exhibit considerably longer half-life (10-48 hrs) (McIntosh et al. 1998; Drake et al. 2006; Hazell et al. 2012), suggesting regulation by different molecular mechanisms than the Frizzled/Smo family. Thus, these experiments indicate that palmitoylation does not affect Fz5 turnover.

5.2.2 Palmitoylation is required for Fz5 interaction with Dvl1 but dispensable for Fz5/Fz5 interaction

Palmitoylation deficient Fz5 lacks synaptogenic activity (Fig 1.13), suggesting deficiency in Wnt signalling. The binding of Wnt ligands to their receptors triggers the formation of a signalosome comprising ligand/receptor/co-receptors and intracelullar molecules including the scaffold protein Dishevelled (Dvl), which is essential for all Wnt signalling cascades (Gao and

Chen 2010; Nusse and Clevers 2017). Frizzled receptors form dimers at the cell surface through the N-term CRD domain and this is is sufficient to activate Wnt signalling (Dann et al. 2001; Carron et al. 2003). In addition, Wnt ligands interact with Frizzled receptors by spanning their CRD domain with the lipid moiety (DeBruine et al. 2017; Nile et al. 2017), perhaps stabilising this interaction. However, the role of the C-term tail in Frizzled/Frizzled binding remains unknown; therefore, I decided to examine whether this interaction is regulated by palmitoylation.

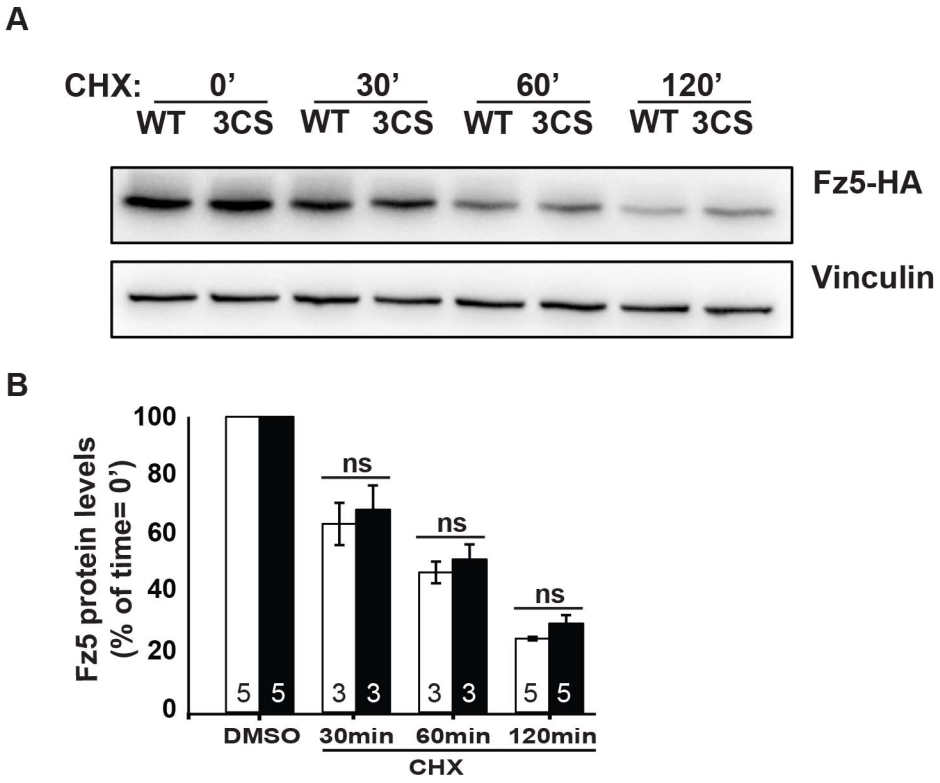

Fig 5.1: Palmitoylation does not regulate Fz5 turnover
A: WB analyses showing WT and 3CS Fz5 degradation rate in NRK cells treated with DMSO (control vehicle) or Cycloheximide (CHX, 50 µg/ml) to block protein translation. **B:** Quantification of Fz5 protein levels upon treatment with CHX revealed no difference between WT and 3CS. Values were normalised to the loading control Vinculin, which is not degraded over 120 mins. Data presented as mean ± SEM; n=3 independent experiments; two-way ANOVA.

The interaction between Fz5/Fz5 was evaluated by co-immunoprecipitation (Co-IP) experiments. Fz5-HA and Fz5-SEP (WT Fz5-HA / WT Fz5-SEP or 3CS Fz5-HA / 3CS Fz5-SEP) were co-expressed in HEK293 cells. SEP (Superecliptic pHluorin) is a pH sensitive GFP variant (Miesenböck et al.

1998), but in this case it was simply used as a GFP tag to perform GFP-trap mediated pull-down (Chromotek). Co-IP experiments were performed by pulling down Fz5-SEP and probing for HA tagged Fz5. The interaction between Fz5/Fz5 was unchanged between WT and 3CS receptors (Fig 5.2 A-B). Thus, these data suggest that palmitoylation is not required for Fz5/Fz5 interaction.

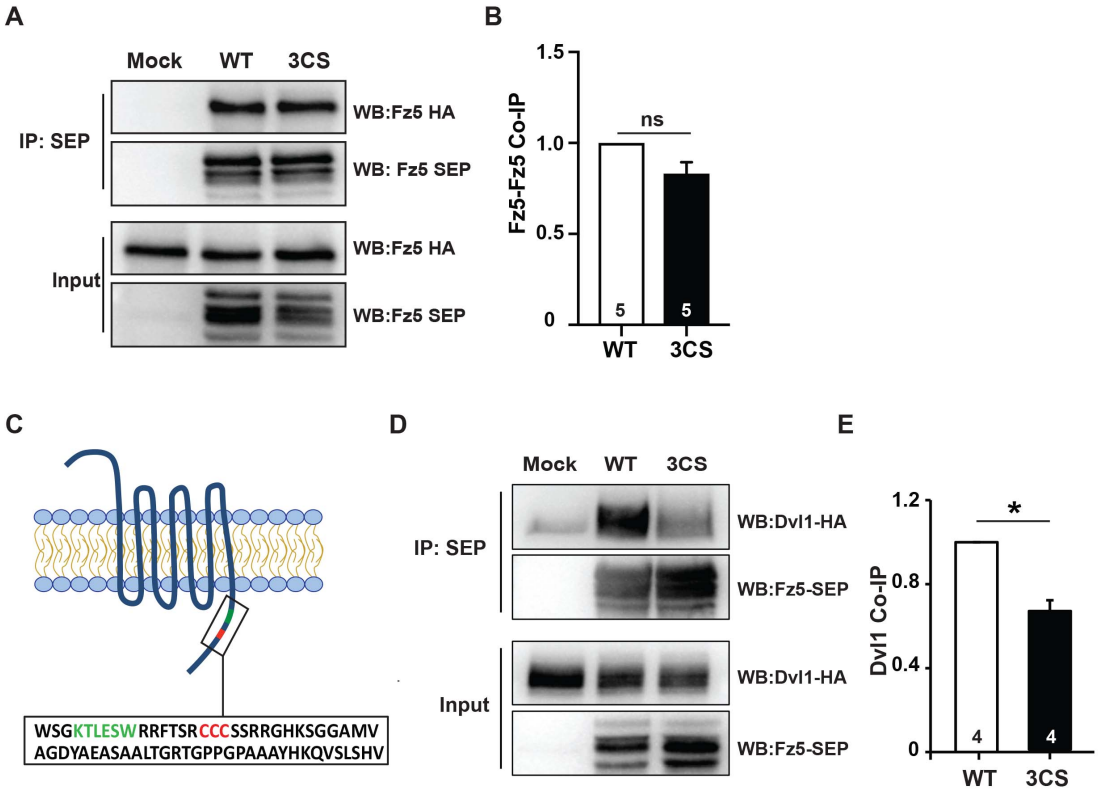

Fig 5.2: Palmitoylation is dispensable for Fz5/Fz5 interaction but required for binding to Dvl1
A: WB showing Fz5/Fz5 interaction from Co-IP analyses. WT or 3CS Fz5-SEP were expressed in HEK293 together with WT or 3CS Fz5-HA. GFP-trap beads were used to pull down Fz5-SEP. Mock condition is the negative control in which cells express Fz5-HA but not Fz5-SEP. **B:** Quantifiation of Fz5/Fz5 interaction normalised to WT Fz5. Data presented as mean ± SEM; n=5 independent experiments; One sample t-test. **C:** The schematic shows the C-term of Fz5, where the Dvl1 binding domain is highlighted in green and the three palmitoylated Cys are labelled in red. **D:** WB showing Fz5/Dvl1 interaction from Co-IP analyses. **E:** Quantification of Fz5-Dvl1 interaction normalised to WT Fz5. The binding to Dvl1 is significantly reduced in the presence of 3CS Fz5. Data presented as mean ± SEM; n=4 independent experiments; One sample t-test; *= p value <0.05.

Another fundamental event for the formation of the Wnt signalosome and signalling transduction is the interaction between Frizzled and Dvl. At the C-term Fz5 is palmitoylated on three Cys residues (Fig 4.3 B-C), which are located in close proximity (6 amino acids away) to one of the Dvl1 binding motifs (Tauriello et al. 2012). Given such proximity, it is possible that palmitoylation could regulate binding to Dvl1. Therefore, I tested this hypothesis by expressing WT or 3CS Fz5-SEP and Dvl1-HA in HEK293 cells and measuring their interaction as previously described for Fz5-SEP/Fz5-HA. The interaction with Dvl1 was reduced by roughly 40% in the presence of 3CS Fz5 compared to WT receptors (Fig 5.2 D-E), indicating that palmitoylation is involved in Fz5 binding to Dvl1. Taken together, these data suggest that palmitoylation is dispensable for Fz5/Fz5 interaction but is important for Dvl1 binding.

5.2.3 Palmitoylation regulates Fz5 distribution along axons but not dendrites and soma of hippocampal neurons

Palmitoylation is a major regulator of protein trafficking and sorting. This function is particularly important in highly polarised cells like neurons; in fact, several neuronal proteins exhibit palmitoylation-dependent sorting (Fukata and Fukata 2010). For instance, palmitoylation is required to target GAP-43 to growth cones (Skene and Virág 1989; Liu et al. 1991), to direct the growth factor DLK1 to axonal trafficking vesicles (Holland et al. 2016) and to sort Synaptotagmin 1 to presynaptic compartments (Kang et al. 2004). Preliminary data indicate that 3CS Fz5 fails to induce synapse formation (Fig 1.13), suggesting that Fz5 function in axons is compromised. Therefore, I hypothesised that WT and 3CS Fz5 might exhibit different localisation patterns, which could explain the lack of synaptogenic activity of palmitoylation deficient Fz5-receptors.

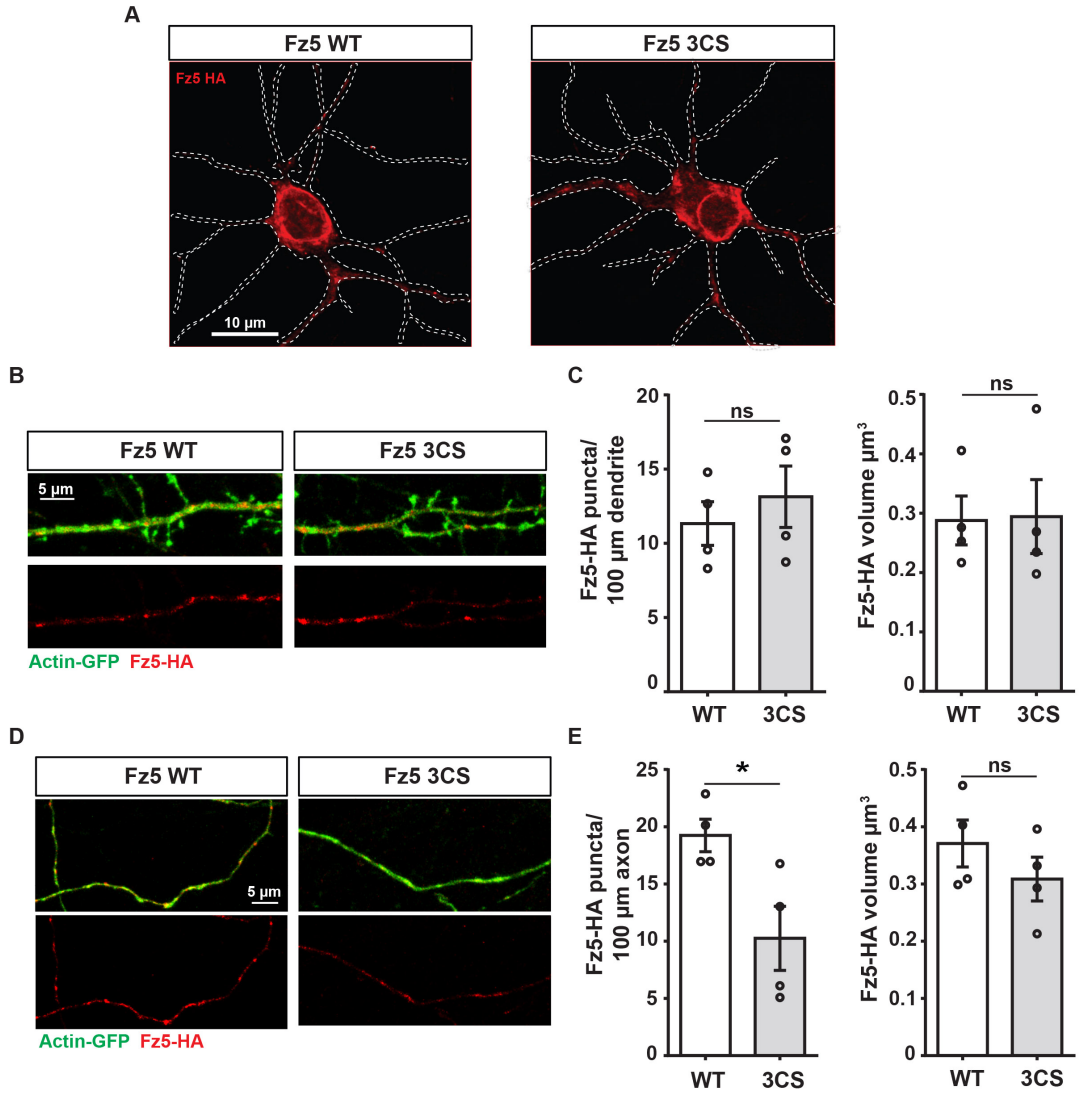

Fig 5.3: Palmitoylation controls Fz5 distribution in axons but not dendrites or soma

A: Confocal images of dissociated hippocampal neurons DIV 9-11 expressing WT or 3CS Fz5-HA (red). Fz5-HA receptors are present in the soma, particularly around the perinuclear region. The white dotted lines correspond to the perimeter of actin-EGFP staining (not shown) and mark the morphology of the cells. **B** and **D:** Confocal images of dendritic branches (B) and axons (D) of neurons expressing actin-EGFP (green) and WT or 3CS Fz5 (red). As previously reported (Fig 3.2 A) WT Fz5 is not present in dendritic spines. Similarly 3CS Fz5 is also confined to the dendritic shaft. **C** and **E**: Quantification of Fz5 puncta number and volume along dendrites (C) and axons (E). Data expressed as mean ± SEM with average of raw values for each experiment; n=4 independent experiments, 8-12 images per condition were measured and averaged for each experiment; Student's t-test, *=p value <0.05.

To address this hypothesis, primary hippocampal neurons were transfected at 5-7 days in vitro (DIV) with WT or 3CS Fz5-HA plasmids, and the distribution of Fz5 receptors was examined by confocal microscopy at 9-11 DIV. WT and 3CS Fz5 receptors were present in the cell body and exhibited

a punctate distribution along axons and dendrites of primary hippocampal neurons. At the cell body, WT and 3CS receptors were concentrated in the perinuclear area, most likely the ER, and appeared undistinguishable (Fig 5.4 A). Similarly, the number and volume of Fz5 puncta along dendrites was not different between WT and 3CS receptors (Fig 5.4 B-C). 3CS Fz5, alike what I have previously shown for WT Fz5 (Fig 3.2 A), was almost completely absent from dendritic spines. These data indicate that palmitoylation does not regulate Fz5 localisation along the dendritic shaft and spines. In contrast, the number of Fz5 puncta along axons was significantly reduced for mutant receptors compared to WT Fz5 (Fig 5.4 D-E), whereas the volume of the puncta was unchanged. These data suggest that Fz5 localisation in hippocampal neurons is in part dependent on palmitoylation, specifically along axons, where Fz5 has been shown to regulate presynaptic assembly (Sahores et al. 2010). These observations are consistent with the fact that palmitoylation-deficient Fz5 lacks synaptogenic activity (Fig 1.13).

5.2.4 The role of palmitoylation in Fz5 stability at the PM

A balance between exocytosis, endocytosis and recycling regulates the levels of transmembrane proteins at the cell surface (Sugita 2008; Doherty and McMahon 2009; Grant and Donaldson 2009). The internalisation of a vast number of proteins is regulated either positively or negatively by palmitoylation (Goddard and Watts 2012; Naumenko and Ponimaskin 2018). For instance, constitutive internalisation is increased for palmitoylation-mutant NMDARs (Hayashi et al. 2009) and Transferrin receptors (Alvarez et al. 1990), whereas palmitoylation is required for the endocytosis of a number of other receptors, including AMPARs, dopamine D3 receptor, PAR2 (Protease activated receptor 2) and many others (Hayashi et al. 2005; Lin et al. 2009; Adams et al. 2011; Zhang et al. 2016). Therefore, I have performed antibody-feeding experiments to address whether palmitoylation affects the internalisation of Fz5 receptors.

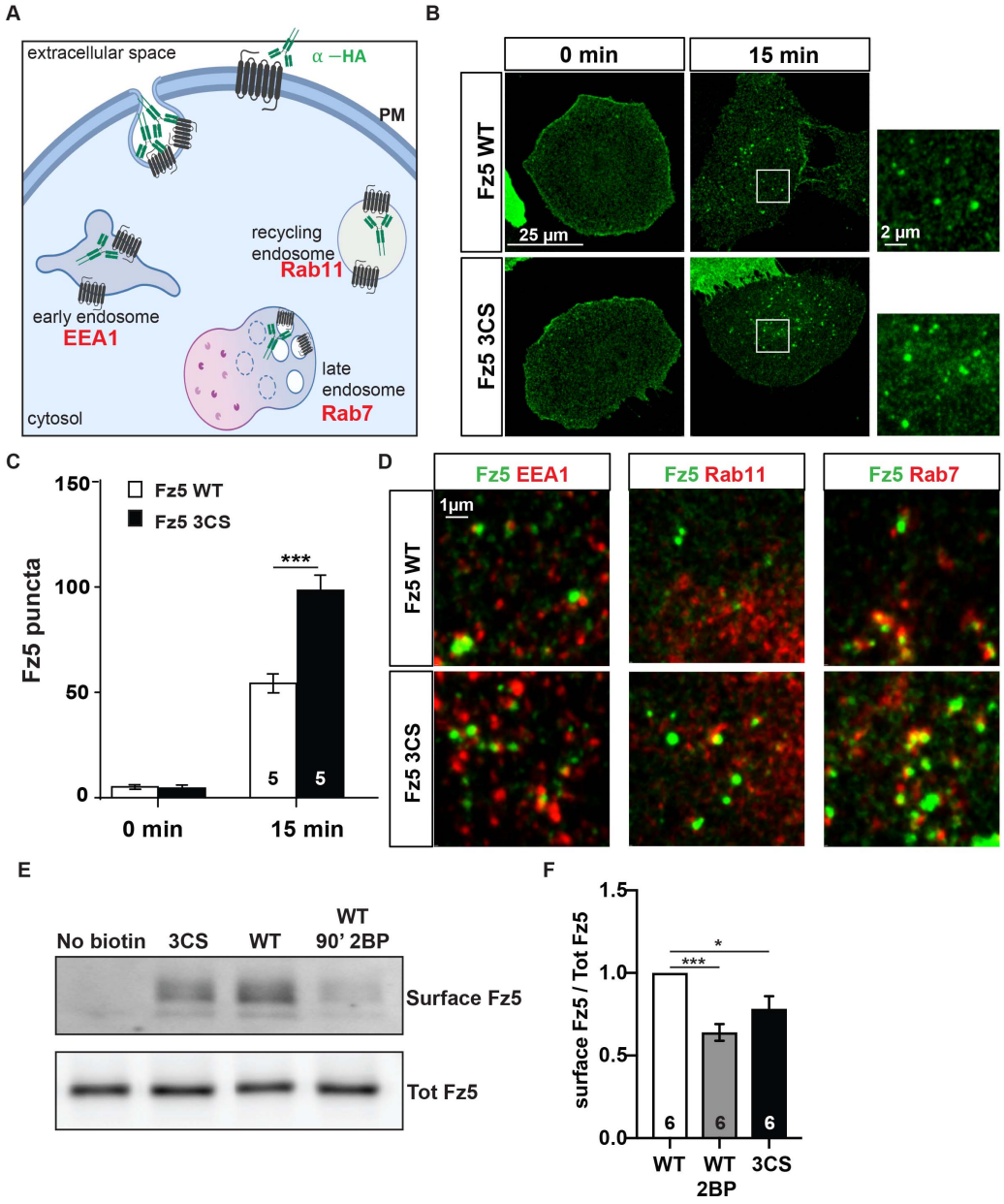

Fig 5.4: Palmitoylation regulates Fz5 membrane stability
A: The schematic illustrates the principle of antibody feeding experiments. Cells were placed on ice to block trafficking and label surface Fz5 receptors with HA antibodies. Cells were then returned to 37 ˚C to allow for internalisation of surface proteins. **B:** Confocal images of NRK cells expressing WT or 3CS Fz5-HA. Insets on the right show clear formation of endocytic clustes. **C:** Quantification of Fz5 endocytic puncta. At t=15 mins (after being retunerd to 37 ˚C) 3CS Fz5 puncta were double than WT Fz5. Data presented as mean ± SEM; n=5 independent experiments, 10-12 cells per condition were measured and averaged for each experiment; Student's t-test, ***=p value < 0.0005. **D:** Confocal images of Fz5 puncta (green) colocalising with endosomal markers (red, EAA1, Rab11, Rab7). **E:** Surface biotinylation analyses of HEK293 cells expressing WT or 3CS Fz5-HA. Cells expressing WT Fz5 were treated for 90 mins with 2BP (100 µM), to inhibit protein palmitoylation. **F:** Quantification of surface levels of Fz5 normalised to total protein levels and to WT Fz5 treated with vehicle ctrl. Data presented as mean ± SEM; n=6 independent experiments; one-way ANOVA, */***=p value < 0,05 and 0.0005.

NRK cells expressing either WT or 3CS Fz5-HA were placed on ice to block trafficking, and then incubated with primary antibodies against the HA tag at the N-term extracellular region of Fz5 receptors. Next, cells were either fixed (time=0) or returned at 37˚C to allow receptor internalisation (Fig 5.4 A). The formation of typical endocytic clusters of Fz5-HA was analysed by confocal microscopy. 15 mins after returning the cells to 37˚C, WT and 3CS Fz5 showed remarkable differences in the levels of internalisation (Fig 5.5 A-B). The number of endocytic clusters was double for 3CS Fz5 compared to WT Fz5 (Fig 5.5 B). To confirm that these puncta were indeed clusters of internalised receptors, I examined their colocalisation with common markers of early, late and recycling endosomes (EEA1, Rab7 and Rab11 respectively). I found that Fz5 clusters co-localised with each one of these markers (Fig 5.5 C). Altogether these data strongly suggest that internalisation of palmitoylation-deficient Fz5 is accelerated, suggesting that palmitoylation inhibits constitutive endocytosis of this receptor.

Given that 3CS is internalised faster than WT Fz5, I examined whether the surface levels of the mutant receptor were reduced. Surface biotinylation experiments in HEK239 cells showed a small (20%) but statistically significant reduction of surface 3CS Fz5 compared to WT receptors (Fig 5.5), consistently with an increase in the rate of steady-state internalisation. Importantly, this result was confirmed by pharmacological treatment of WT Fz5 with 2-BP (Fig 5.5), a drug that blocks palmitoylation by preventing the addition of new palmitate groups to Cys residues (Davda et al. 2013). Overall these data strongly suggest that genetic or pharmacological inhibition of palmitoylation decrease the surface levels of Fz5.

Another important aspect of receptor stability at the PM is their lateral mobility, a phenomenon that can be regulated by palmitoylation. For instance, palmitoylation and phosphorylation regulate the synaptic recruitment by lateral diffusion of extrasynaptic AMPARs, a process that is fundamental for induction of synaptic plasticity (Hayashi et al. 2005; Lin et al. 2009; Makino and Malinow 2009). In addition, palmitoylation-deficient mutants of large conductance Ca^{2+} channels move faster across the PM (S. Kim et al. 2014). Except for one study where several Wnts have been shown to increase

lateral mobility of Fz6 (Kilander et al. 2014), the lateral mobility of Frizzled receptors has not been investigated. Therefore, I asked whether lateral diffusion of Fz5 might be affected by palmitoylation.

To address this question, I performed FRAP (Fluorescence Recovery After Photobleaching) experiments on NRK cells expressing WT or 3CS Fz5 . These receptors were tagged at the N-term with SEP, a pH sensitive GFP variant that does not fluoresce at acidic pH, normally found in intracellular vesicles, but is excited at neutral pH, normally found in the extracellular space (Miesenböck et al. 1998). The use of SEP tagged-receptors, combined with FRAP, allowed us to monitor the lateral movement of Fz5 at the cell surface (Georgiou et al. 2002; Ashby et al. 2004; Hildick et al. 2012). Three small regions per field of view were bleached: one located outside the cell to be used as blank, one around the perinuclear area and one at the edge of the cell. The blank region and the one at the edge of the cell were used for quantification of fluorescence recovery. After bleaching, the recovery of fluorescence intensity of SEP tagged proteins is achieved by lateral diffusion of nearby receptors and/or insertion of new ones. Given the small size of the bleached area and the short time of the recording, it is extremely unlikely that exocytosis occurred in the regions of interest. Therefore, it is reasonable to assume that the recovery of fluorescence intensity is due to lateral diffusion of nearby receptors (Martin et al. 2009; Hildick et al. 2012). I found that the recovery of fluorescence intensity was unchanged between WT and 3CS Fz5 (Fig 5.6 A-B). Moreover, I extrapolated the pools of mobile and immobile receptors from the maximal percentage of fluorescence recovery. The reason why the recovery of fluorescence signal does not reach 100% is because a portion of receptors are immobile at the PM and therefore they are not replaced by lateral diffusion of nearby receptors (Zheng et al. 2011; Hildick et al. 2012). I found that 80% of receptors appeared to be mobile and 20% immobile for both WT and 3CS Fz5 (Fig 5.6 C), suggesting that lateral mobility of Fz5 does not depend on palmitoylation. Altogether the data presented in this chapter strongly suggest that palmitoylation affects Dvl1 interaction, Fz5 distribution along axons, Fz5 internalisation and surface levels of this receptor. In contrast, Fz5 turnover rate, Fz5/Fz5 interaction, Fz5

localisation in the soma and dendrites of hippocampal neurons, as well as the lateral mobility of the receptor, seem to be independent of its palmitoylation.

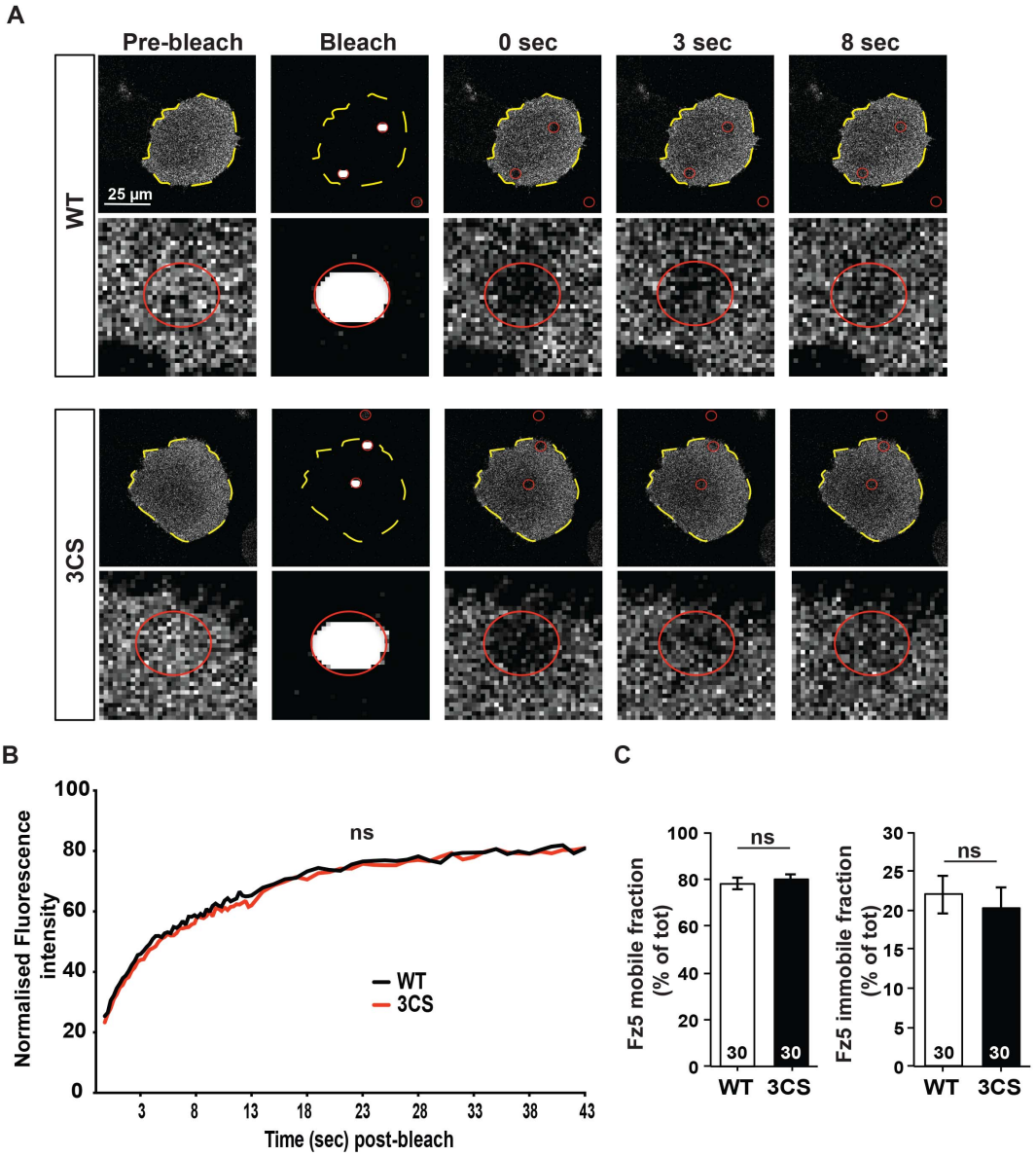

Fig 5.5: Palmitoylation does not affect Fz5 lateral mobility
A: Confocal images of NRK cells expressing WT or 3CS Fz5-SEP (white) imaged at different time points after photobleaching. Three regions were bleached (red circles): one outside the cell (used as blank); one around the perinuclear area and one at the edge of the cell (used for quantification). The yellow dotted lines delineate the perimeter of the cell. **B:** Quantification of fluorescence intensity in the region of interest normalised to t=0 sec after bleaching. Data presented as mean; n=30 cells per condition; two-way ANOVA. **C:** quantification of mobile and immobile fractions of Fz5 receptors; data presented as mean ± SEM; n=30 cells per condition; Student's t-test.

5.3 Discussion

Findings from our lab suggest that palmitoylation is required for Fz5 to induce presynaptic assembly *in vitro* (Fig 1.13) (Stamatakou and Salinas, unpublished results), implying that this modification is required for Fz5 function. Here I have asked which aspects of Fz5 function are regulated by palmitoylation. Using biochemical and cell biology approaches I found that palmitoylation is required for Fz5-Dvl1 interaction, for the stability of the receptor at the PM and for its distribution along axons, where Fz5 is required for presynaptic assembly (Sahores et al. 2010). In contrast, Fz5 palmitoylation seems to be dispensable for Fz5/Fz5 interaction, Fz5 turnover rate, its lateral mobility at the PM and for the distribution of this receptor in the soma or along dendritic processes. These findings represent the first characterisation of the molecular mechanisms through which palmitoylation affects a Frizzled receptor.

5.3.1 Palmitoylation regulates Fz5 localisation in hippocampal neurons

The precise localisation of protein at specific subcellular compartments is fundamental in every cell, but particularly in neurons because of their high degree of polarisation. Palmitoylation can regulate the sorting and clustering of proteins into neuronal process and at synapses. For instance, Paralemmin and GAP-43 require the attachment of palmitate moieties to be directed to dendritic spines and axonal growth cones (El-Husseini et al. 2001; Greaves and Chamberlain 2007). Moreover, PSD-95 and Gephyrin exhibit palmitoylation dependent-clustering in dendrites (El-Husseini et al. 2000; Dejanovic et al. 2014). Given that palmitoylation-mutant Fz5 receptors lack synaptogenic activity, I hypothesised that the localisation of Fz5 in axonal processes might be impaired. The data presented here indicate that Fz5 is less abundant in axons of dissociated hippocampal neurons, as observed by the decreased number of axonal puncta compared to WT Fz5. In contrast, the localisation at the cell body and along dendrites was unchanged between WT and 3CS Fz5, suggesting the existence of axon-specific mechanisms that regulate Fz5 sorting and/or clustering.

Two non-mutually exclusive mechanisms could underlie the impaired distribution of mutant Fz5 in axons. First, Fz5 sorting into axons might be compromised. At present we do not have evidence supporting a role for palmitoylation in Fz5 sorting, but we are planning to address this question combining live-imaging of GFP-tagged Fz5 with the RUSH (retention using selective hooks) technology, which allows synchronised protein trafficking from the ER to the PM (Boncompain et al. 2012). Thus, comparing WT and 3CS trafficking into axons could elucidate the role of palmitoylation in this process. A second possibility is that Fz5 clustering in axons might be dependent on palmitoylation. Given that Fz5/Fz5 interaction is unchanged between WT and 3CS receptors we could speculate that palmitoylation is dispensable for Fz5 clustering. However, my data also indicate that the interaction with the scaffold protein Dvl1 is dependent on palmitoylation; suggesting that Dvl-mediated clustering might be impaired in 3CS Fz5 receptors. Interestingly, Dvl1 is involved in several aspects of presynaptic function including synapse formation, vesicle recycling and neurotransmitter release (Ahmad-Annuar et al. 2006; Ciani et al. 2015). Therefore, it would be important to address whether Fz5/Dvl1 interaction is necessary for Fz5 clustering and function along neuronal processes and at presynaptic sites.

5.3.2 The role of palmitoylation in Fz5 membrane trafficking

Given the prominent role of palmitoylation in regulating receptor endocytosis, I examined its impact on Fz5 internalisation. The data presented here strongly suggest that palmitoylation decreases the endocytosis rate of Fz5 stabilising it at the cell surface, but does not affect the lateral mobility of this receptor at the PM. Together with the observation that palmitoylation-mutant Fz5 exhibits impaired interaction with Dvl1 (Fig 5.2) and lacks synaptogenic activity (Fig 1.13), these data strongly suggest that, in this context, endocytosis of Fz5 receptors has a negative impact on signalling activation.

The role of receptor endocytosis in signalling activation is a matter of debate in the Wnt field. On one hand, endocytosis of Frizzled and the co-receptors LRP5-6 has been shown to be required for β-catenin stabilisation in different

model systems (Yamamoto et al. 2006; Gagliardi et al. 2014; Hagemann et al. 2014; Seto and Bellen 2006). On the other hand, the opposite has also been proposed: clathrin-mediated endocytosis of LRP6 provides a negative feedback on Wnt signalling (Agajanian et al. 2019), and ubiquitination-induced internalisation of Frizzled receptors is a mechanism to down-regulate signalling (Hao et al. 2012; Koo et al. 2012; de Lau et al. 2014; Moffat et al. 2014; Zebisch and Jones 2015; Madan et al. 2016). Thus, the role endocytosis for signalling activation/down-regulation seems to be dependet on the cellular context and the molecular mechanisms are not fully understood.

Given the role of ubiquitination in Frizzled endocytosis (Hao et al. 2016), it would be interesting to investigate whether this modification is co-regulated together with palmitoylation to control Fz5 surface levels, as it has been shown for the Wnt-co-receptor LRP6 (a separate function from the role of palmitoylation/ubiquitination in protein folding) (Abrami et al. 2008). In addition, it would be important to address the impact of palmitoylation in ligand-induced endocytosis of Frizzled receptors, which has been shown to be required for signalling activation (Yamamoto et al. 2006). These questions represent some of the next challenges to unravel the role of palmitoylation in Frizzled endocytosis, and will be discussed in more details in the general discussion (see chapter 7). Overall my data describe a role for palmitoylation in regulating Fz5 membrane levels and endocytosis. Since aberrant Wnt signalling, which is often a consequence of deregulation of Wnt receptors, is linked to the development of several pathological conditions including cancer and neurodegenerative disorders (Johnson and Rajamannan 2006; Anastas and Moon 2013; Wang et al. 2016), these data shed light on a novel mechanism of regulation of Fz5, opening new avenues to examine the trafficking and function of Frizzled receptors in health and disease.

CHAPTER 6: THE ROLE OF Fz5 AND ITS PALMITOYLATION IN THE FORMATION OF SYNAPSES *IN VIVO*

6.1 Introduction

In the previous chapters, I have presented findings that characterise an unidentified PTM of Frizzled receptors, and I have addressed the molecular mechanisms by which this modification affects Fz5 trafficking. The data presented until now were obtained using *in vitro* model systems, specifically dissociated hippocampal cultures and cell lines. Here, I will focus on the role of Fz5 and its palmitoylation in the assembly of synapses *in vivo*, namely in the developing hippocampus of new-born mice.

Almost 20 years ago, the study of the postnatal brain of *Wnt7a*$^{-/-}$ null mice revealed that Wnts are synaptogenic factors (Hall et al. 2000). Since then, the roles for Wnt signalling in synapse formation and function have been reported. *Wnt7a*$^{-/-}$*-Dvl1*$^{-/-}$ double KO mice exhibit a severe synaptic phenotype in the cerebellum (Ahmad-Annuar et al. 2006) and impaired spine morphogenesis and synaptic transmission deficits in the hippocampus (Ciani et al. 2011; Ciani et al. 2015). Furthermore, complex cognitive functions and locomotion tasks, which require correct synapse formation and function, depend on Wnt signalling (Maguschak and Ressler 2008; Maguschak and Ressler 2011; Gogolla et al. 2009; C.-M. Chen et al. 2017; Xu et al. 2015; Tabatadze et al. 2012; Galli et al. 2014; Marzo et al. 2016). Therefore, Wnt signalling plays a crucial role for synapse development and function *in vivo*.

A role for Fz5, a receptor for the synaptogenic ligand Wnt7a, in presynaptic assembly has been demonstrated *in vitro* using dissociated hippocampal cultures (Fig 1.9) (Sahores et al. 2010), but the role of this receptor in the CNS remains largely uncharacterised, especially in the context of synapse formation. Moreover, the *in vivo* role of palmitoylation of Fz5 is unknown. In the postnatal brain, Fz5 is expressed in the thalamus (Shimogori et al. 2004; Liu et al. 2008), where it regulates neuronal survival (Liu et al. 2008). Furthermore, the expression of Fz5 increases during synaptogenesis in the hippocampus (Davis et al. 2008; Sahores et al. 2010), where Fz5 localises at

synapses (Sahores et al. 2010). Importantly, Wnt7a is also expressed in the hippocampus (Shimogori et al. 2004; Davis et al. 2008; Gogolla et al. 2009; Ciani et al. 2011), where it has been shown to regulate synapse formation and plasticity (Gogolla et al. 2009; Ciani et al. 2011; McLeod et al. 2018). Thus, I asked whether Fz5 promotes synapse formation in the hippocampus and whether palmitoylation is required for this process *in vivo*.

To address the role of this receptor and its palmitoylation in the formation of synapses *in vivo,* I have performed intracerebroventricular (ICV) injections of Adeno Associated Virus (AAV) to deliver WT and 3CS Fz5 cDNA in the developing brain of new-born mice. This approach has been used before to study central synapses (Lu et al. 2013; C. X. He et al. 2018; Passini and Wolfe 2001; Kim et al. 2013; Ge et al. 2018). Upon expression of WT or 3CS Fz5, I evaluated synaptic density by immunofluorescence and confocal microscopy, as routinely performed in our laboratory (McLeod et al. 2017). The results presented in the following section are the first *in vivo* investigation of the role of Fz5 and its palmitoylation in the developing hippocampus. Thus, they contribute to a better understanding of the role of this receptor at synapses, and provide novel and valuable knowledge on the function of a previously unidentified PTM of Fz5 receptors. Here I will address the following specific questions:

1) Does WT Fz5 promote synapse formation in the developing hippocampus?
2) Is palmitoylation required for Fz5-induced synapse formation in the developing hippocampus?

6.2 Results

6.2.1 Effects of ICV injections on hippocampal anatomy and proliferation of glial cells

To express Fz5 in the brain of new-born mice, AAV9 viruses carrying WT or 3CS Fz5 cDNA were injected in the lateral ventricles of each hemisphere at P0-P1 and animals sacrificed at P14, within the peak of synaptogenesis (Semple et al. 2013). To identify infected cells Fz5 was expressed together with EGFP. Fz5 and EGFP were translated from the same transcript but immediately separated by autocleavage of the P2A linker (Fig 6.1 A-C) (Kim et al. 2011). Importantly, cellular localisation was undistinguishable between Fz5 expressed from a standard PCS2+ vector or from the construct packaged into AAV9 (Fig 6.1 A-C).

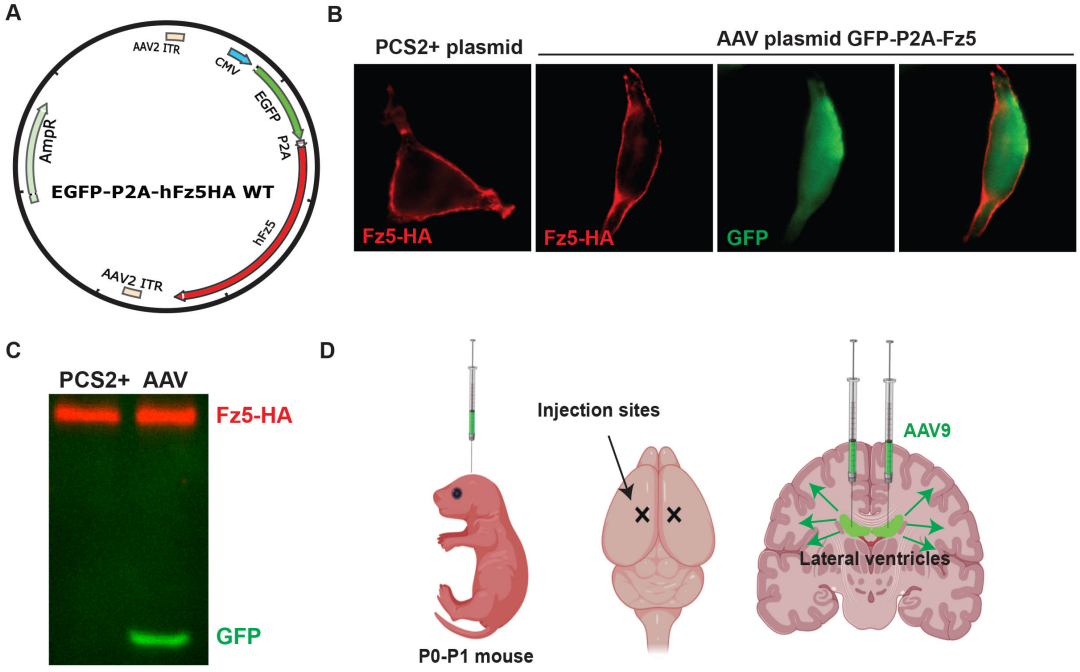

Fig 6.1: ICV injections in new born mice
A: Map of AAV plasmid used to express WT or 3CS Fz5 in the brain of new-born mice. The expression is driven by the CMV promoter. **B:** The P2A linker between GFP (green) and Fz5-HA does not affect Fz5 localisation. As shown in these epifluorescence images of HEK293 cells, Fz5 (red) is still localised at the PM in an identical manner to Fz5 receptors expressed using a standard PCS2+ plasmid. **C:** WB from HEK293 lysate showing complete P2A cleavage. GFP and Fz5 run at the expected molecular weight. **D:** 2.5 µL of AAV9 viruses were injected in the lateral ventricle of each hemisphere of P0-P1 new-born mice. The injection site was located in between the Lambda and Bregma sutures, approximately 1mm laterally of the midline suture.

Several reports have demonstrated that the serotype of viral capsids and injection timing determine the number and type of cells that get infected. Specifically, ICV injections of AAV8 or AAV9 within the first 24 hrs of life results in maximal infection rate and neuronal specificity compared to other AAV serotypes like AAV1 or AAV2 (Chakrabarty et al. 2013; Gholizadeh et al. 2013). Therefore, 2.5 µL of AAV9 (>10^{12} viral particle/mL) were injected in each hemisphere of C57BL/6 mice at P0, with minimal modifications from what has been previously reported (Fig 6.1 A) (see section 2.3 of material and methods) (J.-Y. Kim et al. 2014).

The injection of AAV9 (GFP ctrl; WT Fz5; 3CS FZ5) resulted in widespread infection of several areas of the brain including the hippocampus, cortex and olfactory bulb (Fig 6.2). The axons projecting to the stratum lacunosum moleculare (SLM), which make synapses onto dendrites of CA1 cells (Fig 6.2 B), consistently exhibited very high infection rate (Fig 6.2 A). These axons originate in part from cells in the CA3 region of the hippocampus and partially from cells in the upper layers of the entorhinal cortex (EC), which were both highly infected by AAV9 (Fig 6.2 A). Therefore, regions of interest in the SLM were selected for quantification of synapse density (Fig 6.2 C). As expected, neurons were the most infected cell type (Fig 6.2 A bottom left panel). However, several astrocytes were also infected (Fig 6.2 A bottom left panel). Before examining the impact on synapses, I ruled out whether ICV injections caused abnormalities in hippocampal anatomy or aberrant activation and proliferation of glial cells. DAPI staining did not reveal obvious differences in gross hippocampal anatomy between animals injected with PBS or AAV (GFP ctrl, WT Fz5, 3CS Fz5) (Fig 6.3 A). Similarly, the number and morphology of astrocytes (GFAP positive) and microglia (Iba1 positive) did not reveal any obvious differences (Fig 6.3 B). In addition, no obvious abnormalities were observed in the size of injected animals (data not shown). Taken together, these qualitative analyses strongly suggest that ICV injections of AAV do not affect the overall development of the hippocampus, consistent with previous work where the same approach has been used (Broekman et al. 2006; Chakrabarty et al. 2013; Gholizadeh et al. 2013;

McLean et al. 2014). Therefore, I proceeded to analyse the effects that genetic manipulation of Fz5 had on synapse formation.

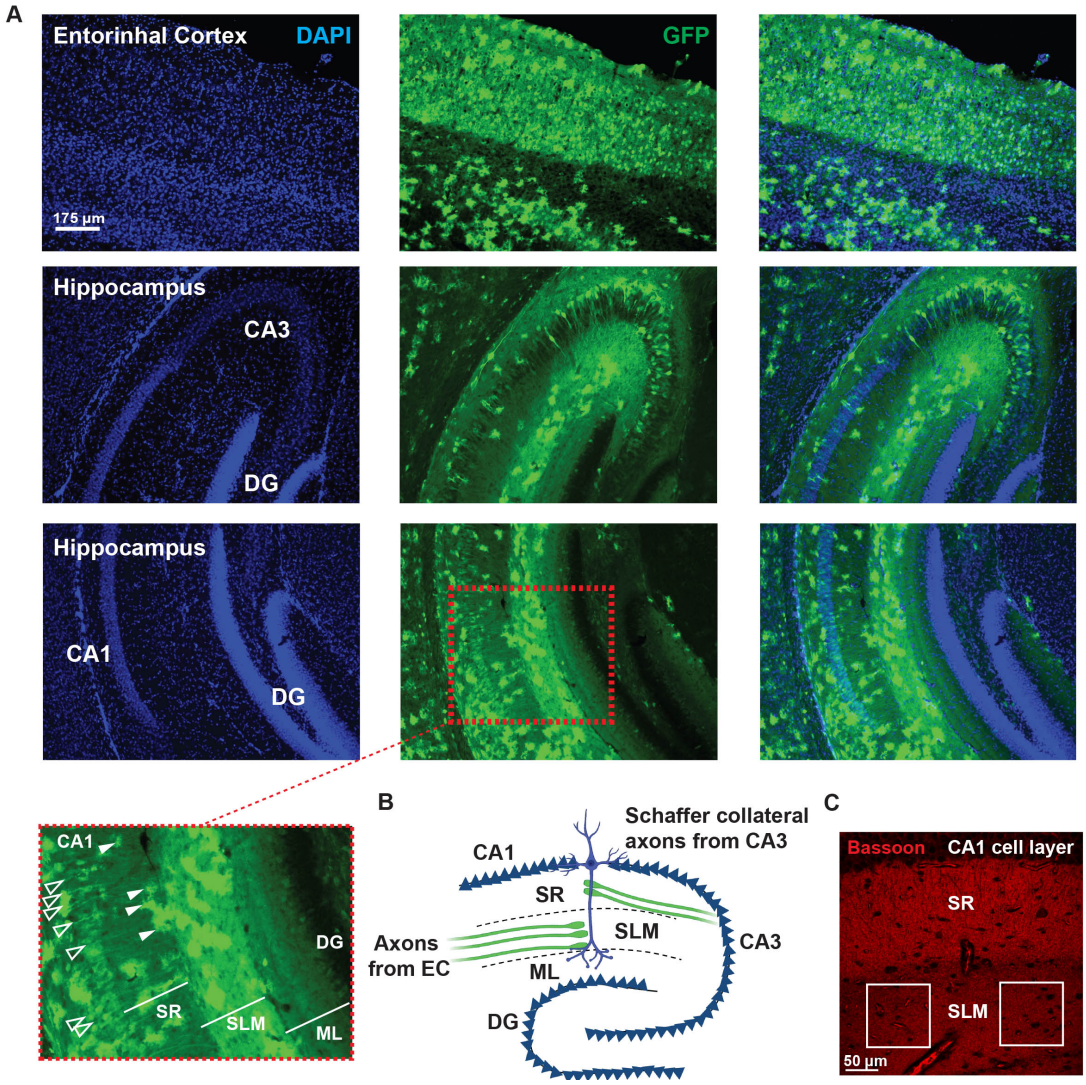

Fig 6.2: Spread of AAV9 viruses
A: Confocal images showing infection of different brain areas. Cell nuclei are labelled in blue (DAPI) and infected cells in green (GFP). The upper layers of the EC, which project axons to the SLM, were highly infected by AAV9. Consistently, the SLM exhibited the highest GFP staining intensity compared to other layers of the hippocampus (SR and ML). The bottom left panel (zoom in) shows that AAV9 infected mostly neuronal cells (empty arrowhead) but also several astrocytes (white-filled arrowhead). **B:** The schematic illustrates hippocampal connectivity. Distal CA1 dendrites receive input from the EC in the SLM. **C:** Confocal image of the hippocampus of a P14 mouse. The different layers of the mouse hippocampus are easily identifiable by variations in Bassoon staining intensity. Synapse density was evaluated in regions of interest (white boxes) within the SLM. SML=stratum lacunosum moleculare; ML= molecular layer; DG=dentate gyrus.

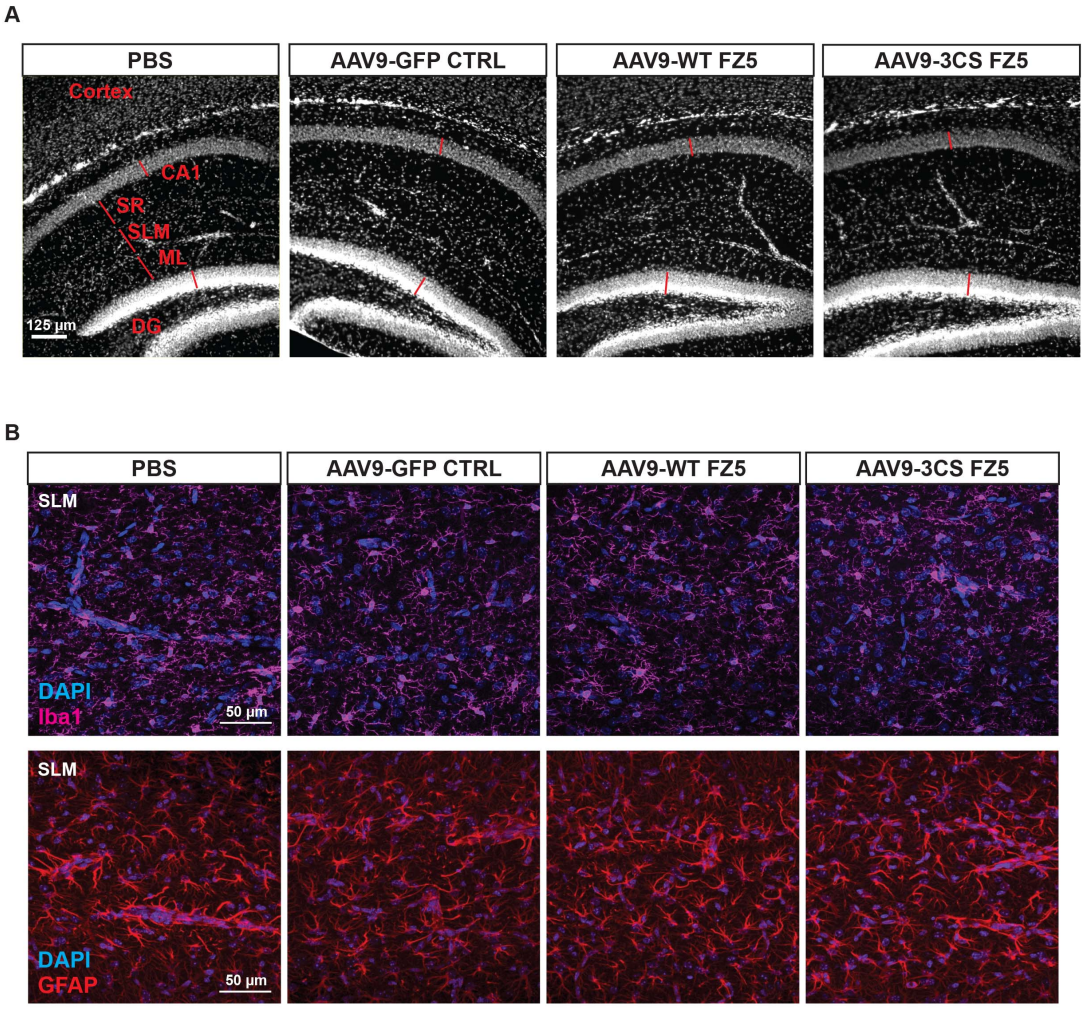

Fig 6.3: ICV injections did not affect hippocampal morphology or proliferation of glial cells
A: Confocal images showing CA1 and DG regions of the hippocampus of 14 days old mice. Cell nuclei were stained with DAPI (white). No obvious differences in the gross morphology of the hippocampus were observed in mice injected with PBS or with any AAV9 used. The thickness of CA1 and DG cell layers, (red lines) as well as the size of SR-SLM-ML, were unchanged, suggesting that injections of AAV9 did not cause cell death or major defects in hippocampal development. **B:** Confocal images of SLM regions of the hippocampus in mice injected with PBS or different AAV9 viruses. No obvious differences in the number of astrocytes (GFAP positive cells, red) or microglia (Iba1 positive cells, magenta) were observed between PBS or AAV9-injected animals, suggesting that AAV9 did not affect proliferation of glial cells.

6.2.2 Fz5 promotes synaptogenesis *in vivo* in a palmitoylation-dependent manner

To address the in vivo role of Fz5 palmitoylation in presynaptic assembly, P0-P1 mice were injected with AAV9 GFP (ctrl), and WT or 3CS Fz5. Synaptic density was evaluated by colocalisation of the endogenous

presynaptic marker Bassoon and the postsynaptic marker Homer1. Expression of WT Fz5 induced a statistically significant increase in the number of Bassoon puncta in the SLM compared to GFP ctrl animals (Fig 6.4). In contrast, the number and volume of Homer1 puncta were unchanged in animals expressing WT Fz5 compared to GFP ctrl. Importantly, these results are consistent with our obervations in cultured hippocampal neurons (Fig 3.2). Expression of Fz5 3CS mutant completely failed to induce presynaptic assembly, and, albeit not statistically significant, the levels of the postsynaptic marker Homer1 exhibited a marked trend towards a decrease (Fig 6.4 B). The volume of Homer1 was unchanged between mice expressing GFP ctrl and WT or 3CS Fz5 (Fig 6.4 B). The overall number of excitatory synapses, measured by the colocalisation between Bassoon and Homer1, was not statistically different between GFP ctrl and WT Fz5, nor between GFP ctrl and 3CS Fz5, most likely because of the high variability observed for Homer1 staining (Fig 6.4 B). However, a statistically significant difference in synapse density was observed when comparing WT and 3CS Fz5 (Fig 6.4 B), indicating that palmitoylation is required for the correct function of this receptor.

In summary, the data presented here strongly suggest that WT Fz5 boosts presynaptic assembly *in vivo* and that Fz5 palmitoylation is required for this function. My data also suggest that expression of palmitoylation-deficient Fz5 might have a negative impact on postsynaptic development. However, this observation requires further investigation. Altogether, these findings represent the first *in vivo* investigation of the role of WT and 3CS Fz5 in synapse assembly in the developing hippocampus.

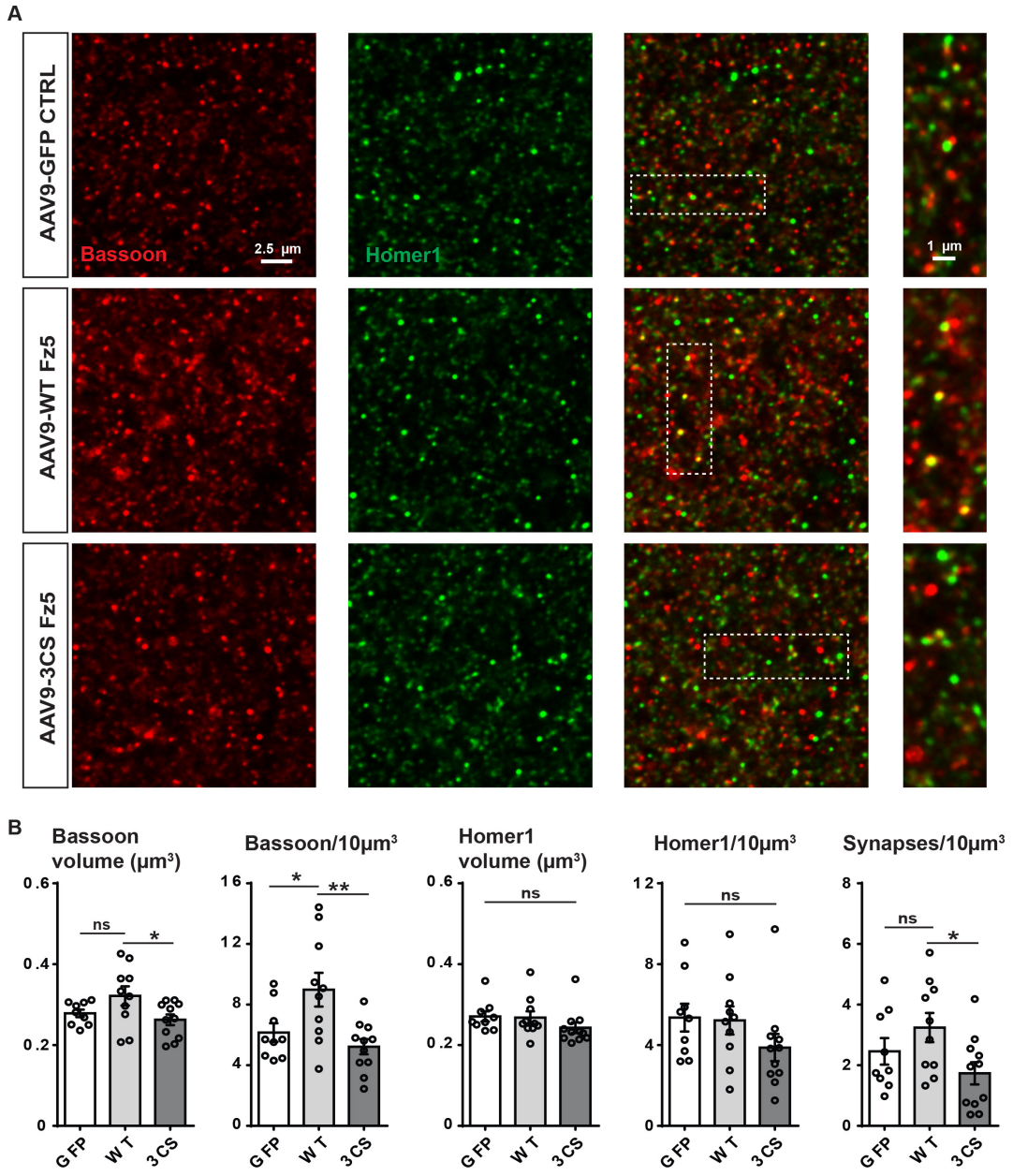

Fig 6.4: Expression of WT Fz5, but not the mutant 3CS Fz5, promotes presynaptic assembly in the developing hippocampus

A: Confocal images of the SLM of mice injected with AAV9 GFP ctrl, WT Fz5 and 3CS Fz5. Bassoon-positive presynaptic sites are shown in red and Homer1 positive-excitatory postsynaptic sites in green. Scale bar: 2.5µm (big panels) and 1µm (small panels). **B:** Quantification of the number and volume of pre- and postsynaptic puncta. Expression of WT Fz5 induced a significant increase in the number and volume of Bassoon puncta whereas no significant differences were observed for Homer1 number and volume. Synapse number was statistically different in WT Fz5 compared to 3CS Fz5 but unchanged compared GFP ctrl. (Data presented as mean with raw values for each animal analysed; n = 9 GFP, 10 WT Fz5, 11 3CS Fz5 animals; * p-value < 0.05, <0.005; One-way ANOVA with multiple comparisons).

6.3 Discussion

In this last chapter, I demonstrated a role for Fz5 in the formation of synapses *in vivo*. Moreover, I have asked whether palmitoylation is required for Fz5 function *in vivo*. My findings strongly suggest that expression of WT Fz5 increases presynaptic development without affecting excitatory postsynaptic differentiation. In contrast, palmitoylation-deficient Fz5 fails to induce presynaptic assembly and might have a negative impact of the development of excitatory postsynaptic structures. Importantly, the findings are consistent with our previous results obtained *in vitro* (Fig 1.13 and Fig 3.2). These results indicate that palmitoylation is essential for Fz5 function, and represent the first *in vivo* study addressing the importance of this modification for Frizzled receptors signalling. Therefore, this study paves the way for future investigations into the impact of palmitoylation on Frizzled function in different cellular processes.

6.3.1 Fz5 expression induces presynaptic assembly *in vivo*

Several members of the Frizzled family are expressed in the CNS (Wang et al. 2016), and have been implicated in the formation of synapses using *in vitro* model systems (Varela-Nallar et al. 2009; Sahores et al. 2010; Ramírez et al. 2016; McLeod et al. 2018). Although KO models exist for all Frizzled receptors (Wang et al. 2016), the role of these proteins at the synapse *in vivo* remains largely unknown, with the exception of studies of Frizzled receptors in other organisms such as *Drosophila* and *C.elegans*. DFz2 receptors are required for NMJ formation in *Drosophila* (Mathew et al. 2005) and Win-17 (*C.elegans* Frizzled) inhibits synapse formation in posterior motor neurons of *C.elegans* (Klassen and Shen 2007). Therefore, the *in vivo* role of Frizzled receptors in synaptogenesis in rodents remained uncharacterised until now.

My studies indicate that expression of Fz5 is sufficient to boost presynaptic assembly in the developing hippocampus, where its ligand, Wnt7a, is expressed (Shimogori et al. 2004; Davis et al. 2008; Gogolla et al. 2009; Ciani et al. 2011; McLeod et al. 2018). Expression of WT Fz5 not only increases the number of puncta for the presynaptic marker Bassoon compared to 3CS Fz5, but it also increases the volume of these structures,

suggesting a possible increase in synaptic strength. Further functional investigations using electrophysiology could address whether Fz5 has an impact in presynaptic function. In contrast, expression of WT Fz5 did not affect the number and size of Homer1-positive postsynaptic compartments, consistent with data presented in chapter 3 (Fig 3.2), where I have shown that gain and loss of function of Fz5 do not affect dendritic spine size and number in hippocampal cultures. In spite of a significant increase in presynaptic terminals, the number of excitatory synapses is not statistically changed between ctrl GFP and WT Fz5 expressing mice. A possible explanation for this result lies in the variability of Homer1 staining, which could have inevitably affected the number of Bassoon puncta colocalising with Homer1. Using different post-synaptic markers, such as PSD-95, and increasing the size of the data set might reveal differences in the total number of synapses between mice expressing GFP ctrl and WT Fz5. Another possible explanation is that the increase in Bassoon puncta, which include excitatory and inhibitory presynaptic terminals, might reflect an increase in inhibitory synapses. However, as I mentioned in section 3.3.2, very preliminary data (not shown) suggest that expression of WT Fz5 in the hippocampus does not affect inhibitory synapse formation, consistently with the fact that Wnt7a, a ligand for Fz5 receptors, does not affect inhibitory synapse formation in cultured neurons (Ciani et al. 2011). In summary, the data presented here represent novel and important findings that increase our understanding of the function Frizzled receptors have at synapses.

6.3.2 Palmitoylation is required for Fz5-induced synapse formation *in vivo*

The experiments presented here indicate that Fz5 requires palmitoylation to promote the assembly of presynaptic sites *in vivo*, consistently with previous observation from cultured neurons (Fig 1.13). In addition, my data revealed a trend towards a decrease in postsynaptic development (Homer1 count) upon expression of 3CS Fz5 compared to ctrl or WT Fz5. However, further experiments are needed to reach statistical significance. If Fz5 is not required for postsynaptic development (Fig 3.2), how does 3CS Fz5 affect

the postsynaptic compartment? One possibility is that 3CS Fz5 impairs the function of presynaptic terminals and therefore, indirectly, it affects the formation or stability of postsynaptic sites. Consistently with the fact that 3CS Fz5 does not increase presynaptic assembly and might have negative effect on postsynaptic development, I detected a significant difference in the numbers of excitatory synapses between mice expressing WT and 3CS Fz5. Therefore, these data indicate that palmitoylation is required for Fz5-mediated excitatory synapse assembly in the developing hippocampus. Given the novelty of Frizzled palmitoylation, our studies represent the first experimental pieces of evidence addressing the importance of this lipid modification for Frizzled function in any model systems. Together with the results presented in chapter 5, these data shed light into novel mechanisms by which Frizzled receptors could be regulated in a number of biological processes where Wnt signalling is involved, from cell fate decisions to the formation and function of neuronal circuits.

CHAPTER 7: DISCUSSION AND FUTURE DIRECTIONS

7.1 Summary of project aims and findings

During my PhD, I studied the molecular mechanisms by which Wnt signalling regulates synapse formation. In particular, I focused my attention on the role and regulation of Frizzled receptors, the main receptors for Wnt ligands. The aim of this PhD project was to study two aspects of Frizzled function in synaptogenesis: first, I investigated the molecular mechanisms by which the synpatogenic factor Wnt7a promotes both pre- and postsynaptic development through Fz5 and Fz7 receptors; second, I have characterised the role of palmitoylation, a novel post-translational modification (PMT) of Frizzled receptors, in regulating Fz5 trafficking and function in the context of synapse formation.

To address how Wnt7a affects pre- and postsynaptic development, I performed gain and loss of function experiments for two Wnt7a receptors, Fz5 and Fz7, and compared their localisation and function at both sides of the synapse. Fz5, which is required for Wnt7a-mediate presynaptic assembly (Sahores et al. 2010), does not localise to dendritic spines. I have shown that gain and loss of function of this receptor do not affect dendritic spine development in primary hippocampal neurons (McLeod et al. 2018). In contrast, I found that Fz7 localises at dendritic spines and is required for Wnt7a-induced spine formation and growth (McLeod et al. 2018). In addition, I examined the role of Fz5 in the developing hippocampus of new-born mice. Consistent with *in vitro* experiments, my data show that expression of Fz5 promotes presynaptic assembly without affecting postsynaptic development in the hippocampus. Importantly, I found that palmitoylation, a previously uncharacterised PTM of Frizzled receptors, is required for Fz5 function *in vivo*, as palmitoylation-deficient Fz5 (3CS Fz5) receptors fail to promote presynaptic assembly in the developing hippocampus. I also investigated the molecular mechanisms underlying palmitoylation-dependent regulation of Fz5. First, I established that each of the Cys residues in the C-term of Fz5 can be palmitoylated. Second, I examined which enzymes palmitoylate Fz5 and found that DHHC3 and DHHC7 strongly increase Fz5 palmitoylation

when expressed in HEK293 cells. However, loss of function of these two enzymes did not seem to affect palmitoylation levels of Fz5, likely because of compensatory effects by other enzymes. In fact, DHHC6, DHHC9, DHHC14 and DHHC15 were also found to increase Fz5 palmitoylation albeit less than DHHC3 and DHHC7. Third, I examined whether palmitoylation affects Fz5 stability and found no differences between WT and 3CS Fz5 degradation rate. Next, I investigated whether palmitoylation affects key interactions required for the formation of the Wnt signalosome and found that this modification does not affect Fz5-Fz5 interaction, but it is important for the binding to the scaffold protein Dvl1. In light of the crucial role played by palmitoylation in regulating protein trafficking, I examined whether 3CS Fz5 exhibits trafficking defects compared to WT receptors. Consistently with deficient presynaptic function, 3CS Fz5 is less abundant than WT Fz5 along the axons of primary hippocampal neurons. Furthermore, palmitoylation is required for Fz5 trafficking as my data strongly suggest that this modification inhibits constitutive internalization of Fz5 and is required to maintain the surface levels of this receptor; however, FRAP experiments suggest that palmitoylation does not affect Fz5 lateral mobility at the PM. Finally, I found that other Frizzled receptors, potentially the entire Frizzled receptor family, are palmitoylated - a finding that opens up new avenues to study how these fundamental receptors are regulated.

My findings contribute to our understanding of the molecular mechanisms underlying the role of Frizzled receptors in synapse formation. In particular, my work sheds new light on the role and regulation of the Frizzled receptors required for Wnt7a-induced pre- and postsynaptic development and demonstrates for the first time that palmitoylation of Frizzled proteins is a critical molecular mechanism underpinning localisation and function of these receptors. These findings represent a novel and important discovery for the Wnt signalling field, as they uncover a previously uncharacterised mechanism of regulation of Frizzled receptors, which are crucial in all Wnt signalling cascades.

7.2 Fz5 and Fz7 exhibit different synaptic distribution and mediate pre- and postsynaptic development respectively

Wnt7a regulates excitatory synapse formation by promoting the assembly of pre- and postsynaptic sites (Hall et al. 2000; Sahores et al. 2010; Ciani et al. 2011). Our lab identified Fz5 as the receptor for Wnt7a on the presynaptic side (Sahores et al. 2010), but the postsynaptic receptor was unknown when I started my PhD. Therefore, I examined which receptor is required for Wnt7a signalling at dendritic spines. I found that Fz5 and Fz7 receptors exhibit strikingly different localisation in dendrites: Fz5 distribution is limited to the dendritic shaft whereas Fz7 is also found in dendritic spines (Fig 7.1). Fz5 is required for Wnt7a-mediated presynaptic assembly (Sahores et al. 2010), but I found that this receptor does not affect dendritic spine development. In contrast, Fz7 is fundamental in mediating Wnt7a-induced development of dendritic spines without affecting presynaptic assembly (Fig. 7.1) (McLeod et al. 2018). My *in vivo* studies fully corroborate these *in vitro* observations, as Fz5 expression in the developing hippocampus promotes presynaptic assembly without affecting postsynaptic differentiation. Taken together, these findings strongly suggest that Fz5 and Fz7 have distinct roles on the opposite sides of the synapse.

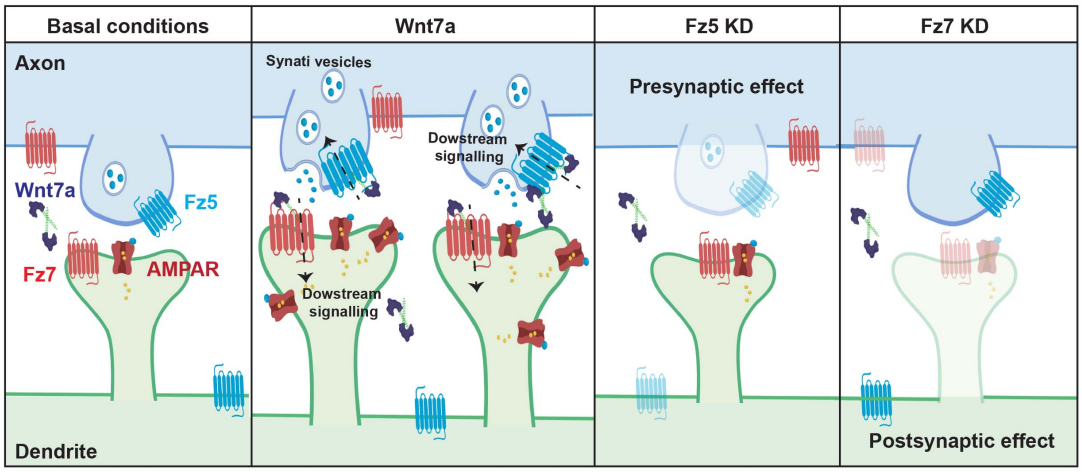

Fig 7.1: Fz5 and Fz7 regulate pre- and postsynaptic development respectively

Wnt7a promotes pre- and postsynaptic development (Hall et al. 2000; Ciani et al. 2011). In dissociated hippocampal neurons Fz5 (light blue) is required for presynaptic assembly and regulates Wnt7a (dark blue) effects on this side of the synapse (Sahores et al. 2010). My data suggest that Fz5 is not involved in dendritic spine development, whereas loss of Fz7 (red) results in decreased spine number and blocks Wnt7a-induced growth of dendritic spines without affecting presynaptic

assembly. Wnt7a-Fz7 signalling is also important for synaptic plasticity and AMPARs (dark red) trafficking (McLeod et al. 2018).

What are the mechanisms that control the localisation of Fz5 and Fz7 at different synaptic compartments? What are the signalling events downstream of these receptors that lead to synapse formation? One potential explanation lies in the different structures of these two receptors, in particular at the C-terminal domain. While both receptors can bind Wnt7a through their N-term domain, their C-term domains are strikingly different. Fz7 has a short tail (24 amino acids), whereas Fz5 has a longer one (63 amino acids), which can be multiply palmitoylated. These structural differences suggest that the interactome of Fz7 might be very different from Fz5. Therefore, it is concievable that the C-term of Frizzled receptors, perhaps through palmitoylation or other PTMs, determines Frizzled localisation, interactome and function. Could such differences in the C-term influence signalling downstream of Frizzled receptors? These questions could be addressed by generating Frizzled receptors chimeras and analysing how their function is affected. Using this approach, it has been demonstrated that the specificity of Wnt9-Fz9 signalling during stem cell development in *Zebrafish* is determined by just two intracellular domains of Fz9, which cannot be substituted with domains of other Frizzled receptors (Grainger et al. 2018). To address whether Frizzled receptor C-term domains determine synaptic localisation and function, future experiments should examine whether (1) Fz5 receptors with the Fz7 C-term domain localise at dendritic spines and promote spine development; (2) whether Fz7 with the Fz5 C-term promotes presynaptic gain-of-function, and whether this is palmitoylation-dependent.

Many other molecular mechanisms, which are not mutually exclusive, could be underpinning the distinct functions of Fz5 and Fz7. Previous findings indicate that - except for the involvement of Dvl1, which is required for both pre- and postsynaptic development (Ahmad-Annuar et al. 2006; Ciani et al. 2011) - Wnt7a induces different downstream signalling cascades at pre- and postsynaptic sites. At presynaptic sites of cultured hippocampal neurons, pharmacological inhibition of Gsk3β blocks the effects of Wnt7a; however,

Wnt7a-induced presynaptic assembly does not require transcription, suggesting that the divergent canonical pathway is involved in Wnt7a-mediated presynaptic assembly (E. Dickens PhD thesis, unpublished data). Whether Fz5 and its palmitoylation are involved in the activation of the divergent canonical pathway remains to be determined. At dendritic spines, Wnt7a-Fz7 signalling activates the Ca^{2+} cascade by activating CaMKII and PKA, which in turn phosphorylate AMPARs resulting in changes in their trafficking (McLeod et al. 2018). Thus, the divergent canonical pathway seems to be involved in Wnt7a-induced presynaptic formation, whereas Wnt7a-Fz7 signalling activates the Ca^{2+} signalling cascade at dendritic spines (McLeod et al. 2018). The next step will be to understand how the distinct signalling pathways are activated downstream of Fz5 and Fz7 at synapses.

7.3 Frizzled receptors are palmitoylated

My finding demonstrate that each of the three Cys residues at the C-term of Fz5 can be palmitoylated and that other Frizzled receptors are also lipid-modified. Palmitoylation was observed for Fz3, Fz4, Fz5, Fz6 and Fz9, and at lower levels also for Fz1, Fz2, Fz7, Fz8 and Fz10. The discovery that Frizzled receptors are palmitoylated is novel and suggests that there is a previously unrecognised molecular mechanism by which these receptors could be regulated.

These novel findings raise a number of important questions about the role of palmitoylation for each Frizzled receptors and how this impacts on Wnt signalling. Although I focused on the role of Fz5 palmitoylation, some cautious extrapolations can be made for the other members of the Frizzled family. All Frizzled receptors are predicted to be palmitoylated on at least one Cys residue located in different protein domains (N-term, transmembrane, C-term) (Fig 7.2). Except for Fz1, Fz2 and Fz7, which belong to the same subfamily, all Frizzled receptors contain Cys residues at the C-term. However, only Fz5 and its homolog Fz8 are predicted to be palmitoylated on the C-term (Fig 7.2). Given the different locations of Cys residues in Frizzled receptors, it is reasonable to assume that palmitoylation will have different effects on these receptors.

Fig 7.2: Frizzled receptors are palmitoylated

All Frizzled receptors are predicted to be palmitoylated on at least one Cys site (C). These residues are distributed on diverse protein domains (N-term, TM, and C-term) in different Frizzled receptors. In this schematic the receptors are grouped according to Frizzled subfamilies. It appears that memebrs of different subfamilies exhibit some patterns in distribution of Cys residues.

What can the distribution of these Cys residues tell us about palmitoylation of Frizzled receptors? Frizzled receptors belonging to the same subfamily share similarities in the distribution of Cys sites across different protein domains (Fig 7.2). For instance, Fz5 and its homolg Fz8 are the only ones predicted to

be palmitoylated on the C-term. The Fz1, Fz2, Fz7 subfamily is predicted to be palmitoylated on the 1st TM domain. The Fz4, Fz9, Fz10 subfamily might be palmitoylated on the 2nd TM domain and at the N-term. Similarly, Fz3 and Fz6 could also be palmitoylated in the extracellular region. Therefore, it seems that the localisation of these residues might correlate with the evolution of Frizzled receptors. This interesting observation should be taken into account when planning to address the function of palmitoylation for each Frizzled receptor, as similar molecular mechanisms might be shared by members of the same subfamily. However, caution should be taken regarding the prediction of Frizzled palmitoylation at the N-term, which was suggested by the CSS 4.0 software for Fz3, Fz6, Fz9 and Fz10 (Fig 4.1). First, extremely little is known about palmitoylation on extracellular domains of membrane proteins. In contrast, palmitoylation of Cys within TM or cytoplasmic domains has been observed in a number of TM proteins and has been widely shown to regulate multiple cell biology functions (Greaves and Chamberlain 2007; Linder and Deschenes 2007; Salaun et al. 2010; Blaskovic et al. 2013; Chamberlain and Shipston 2015). Second, the CRD domain of Frizzled receptors contains 10 conserved Cys residues, and in Fz3 and Fz8 these Cys all engage in disulfide bonds (Dann et al. 2001), suggesting that they might not be available sites for palmitoylation. It will be important to address whether this is true for all Frizzled receptors, especially those predicted to be palmitoylated at the N-term. However, if palmitoylation does occur at the N-term, its impact on ligand binding should be examined, as this interaction occurs between the CRD domain of Frizzled receptors and the lipid moiety on Wnt ligands (Janda et al. 2012; DeBruine et al. 2017; Nile et al. 2017; Nile and Hannoush 2018). Therefore, the position of Cys residues within Frizzled receptors needs to be taken into account when further studying the role of this modification for the function of these receptors.

7.4 Palmitoylation is essential for Fz5 function

My findings demonstrate that palmitoylation is required *in vivo* for Fz5-induced synapse formation. Consistent with preliminary observations in hippocampal cultures (Fig 1.13), palmitoylation-deficient Fz5 receptors do not promote the assembly of presynaptic sites in the developing hippocampus (Fig 7.3). In addition, expression of mutant Fz5 receptors (3CS Fz5) might have a negative impact on postsynaptic differentiation (Fig 7.3), although further experiments are required to support this conclusion. These novel and exciting findings open up new avenues to the study of Frizzled receptor regulation and Wnt signallig-mediated cellular functions.

How does palmitoylation regulate Fz5 function? I performed a series of experiments to address this question, focusing specifically on the role of palmitoylation for key interactions of Fz5 with the Wnt signalosome components and on the impact of this modification on Fz5 trafficking (see section 7.6). The correct assembly of the Wnt signalosome is crucial for activation of the pathway. A key event in this process is the recruitment of the scaffold protein Dvl to the PM, where Dvl interacts with the C-term domain of Frizzled receptors (Gao and Chen 2010). Frizzled dimerisation is another key event for Wnt signalling activation (Carron et al. 2003). My findings suggest that palmitoylation does not affect Fz5/Fz5 interaction, which is unsuprising given that Fz-Fz interactions occur via the extracellular CRD domain of these receptors (DeBruine et al. 2017; Nile et al. 2017). However, palmitoylation is important for Fz5 binding to Dvl1. The interaction between Dvl1 and Fz occurs between the DEP domain of Dvl1 and a discontinuous Dvl-binding motif over the C-term and the 3rd intracellular loop of Fz5 (Tauriello et al. 2012). At the C-term of Fz5, the Dvl1-binding motif is located in close proximity (6 amino acids apart) to the three Cys residues that are palmitoylated, suggesting that palmitoylation could affect Dvl1 interaction with the C-term of Fz5. However, it should be noted that this Dvl-binding domain is conserved across all Frizzled receptors (Wang et al. 2006), which, except for Fz8 (the Fz5 homolog), either do not contain Cys residues at the C-term or have Cys sites that are not predicted to be palmitoylated. Thus, if palmitoylation affects the interaction with Dvl1 through this motif, it is likely to

be a Fz5/Fz8-specific feature. Structural analyses will elucidate how palmitoylation modulates Dvl1 interaction at the C-term of Fz5 receptors. Furthermore, it should be examined whether Fz5 interaction with other components of the Wnt signalosome, such as the Wnt co-receptor LRP6, is palmitoylation-sensitive, and how different Wnt signalling cascades are affected by the removal of palmitate groups from Fz5 receptors. I am currently investigating whether removal of palmitate groups from Fz5 affects a specific Wnt cascade.

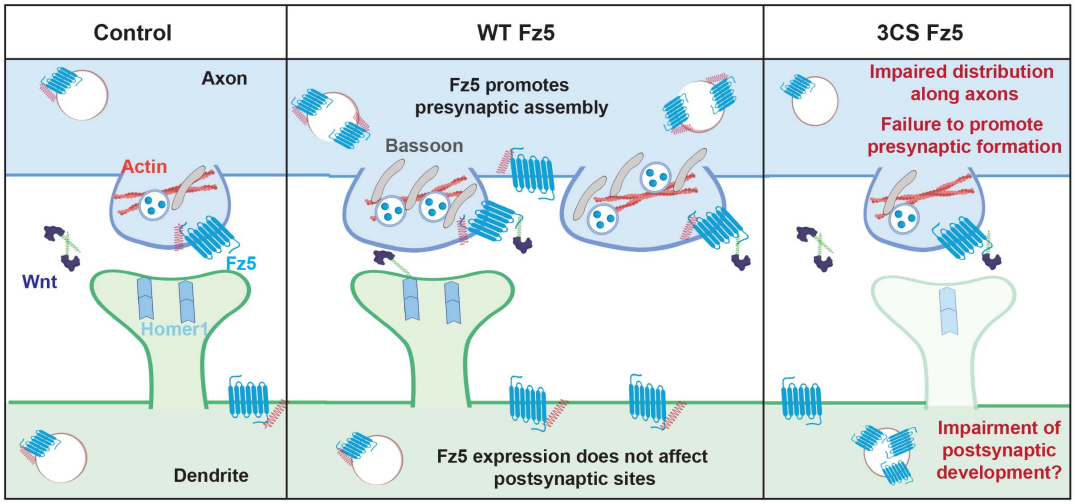

Fig 7.3: Fz5 expression promotes presynaptic assembly *in vivo* in a palmitoylation-dependent manner

Fz5 expression was driven in the developing hippocampus of new-born mice. Expression of WT Fz5 increases the number and volume of presynaptic sites without affecting postsynaptic compartments. In contrast, expression of palmitoylation deficient Fz5 fails to promote presynaptic assembly and might have a detrimental effect on the development of postsynaptic sites.

Interestingly, unpublished data from our lab show that Fz5 palmitoylation is regulated by neuronal activity (Fig 1.13) (Stamatakou and Salinas unpublished results), which is well established as one of the key mechanisms driving synapse formation and plasticity (Colón‐Ramos 2009; B. Lu et al. 2009; Choquet and Triller 2013; Andreae and Burrone 2014). As Wnt signalling components are regulated by neuronal activity and are crucial for activity-mediated synapse formation and function (Budnik and Salinas 2011; Sahores and Salinas 2011; McLeod and Salinas 2018), this suggests that

Fz5 palmitoylation could be a key molecular step in translating neuronal activity into enhanced synapse formation and function.

It is of particular interest to understand how neuronal activity affects Wnt7a/Wnt7b and Fz5 expression (at the protein level) and function. In the hippocampus, Wnt7a/Wnt7b protein levels are elevated upon induction of HFS (high frequqncy stimulation) (McLeod et al. 2018), and are increased in mice exposed to an EE (enriched environment) (Gogolla et al. 2009), a paradigm used to study activity-mediated synapse formation and remodelling (van Praag et al. 2000; Nithianantharajah and Hannan 2006). Fz5, whose surface levels are up- and down-regulated in response to HFS and LFS respectively (Sahores et al. 2010), is required for HFS-induced synapse formation in primary hippocampal neurons (Sahores et al. 2010). Unpublished data from our lab show that Fz5 palmitoylation is elevated upon HFS in primary neurons and by EE in the hippocampus of young mice (Fig 1.13). Therefore, it is intriguing to speculate that Fz5 and its palmitoylation are required for synapse formation downstream of activity-induced Wnt7a/Wnt7b. It is possible that Fz5 palmitoylation is necessary for Wnt7a/Wnt7b to induce synapse formation in mice exposed to EE. Furthermore, given that Fz5 palmitoylation is modulated by neuronal activity, the regulation of palmitoylating and de-palmitoylating enzymes in this context should be addressed. Several DHHC enzymes are positively regulated by neuronal activity, and their function is required for mechanisms of synaptic plasticity (Noritake et al. 2009; Thomas et al. 2012; Brigidi et al. 2014; Dejanovic et al. 2014; Brigidi et al. 2015). Studying the molecular mechanisms underlying activity-induced Fz5 palmitoylation and how this affects synapse formation will provide further insight into the complex interactions and dependencies that govern the process of synaptogenesis.

7.5 Palmitoylation regulates Fz5 trafficking and membrane stability

Palmitoylation is a master regulator of protein trafficking and function (Linder and Deschenes 2007). While palmitoyaltion does not affect Fz5 turnover and does not regulate the mobility of this receptor across the PM, palmitoylation is required to maintain Fz5 surface levels by inhibiting constitutive internalisation of this receptor (Fig 7.4). Moreover, palmitoylation-deficient Fz5 receptors are less abundant along axons of hippocampal neurons (Fig 5.3), suggesting defects in polarised sorting. These findings strongly suggest that palmitoylation is an important regulator of Fz5 trafficking.

Palmitoylation plays a fundamental role in regulating protein sorting and localisation (Greaves and Chamberlain 2007), especially in highly polarised cells like neurons (Fukata and Fukata 2010; Globa and Bamji 2017). The fact that palmitoylation-deficient Fz5 receptors fail to induce synapse formation suggested that the localisation of 3CS Fz5 receptors could be affected in hippocampal neurons. Indeed, although palmitoylation does not affect Fz5 distribution at the soma and along dendrites, it is required for Fz5 localisation along axons (Fig 5.3). Several neuronal proteins exhibit palmitoylation-dependent sorting into axons or dendrites (El-Husseini et al. 2001; Fukata and Fukata 2010; Globa and Bamji 2017). For instance, palmitoylation of PSD-95 regulates both trafficking into dendrites as well as its clustering at synapses (Craven et al. 1999; El-Husseini et al. 2000). Similarly, DLK (Dual leucine-zipper kinase), which is an essential kinase for axon-soma retrograde signalling after nerve injury, is targeted to axonal trafficking vesicles in a palmitoylation-dependent manner, and this modification is required for the assembly of DLK signalling complexes (Holland et al. 2016). Thus, palmitoylation can control both polarised sorting and protein clustering in neurons.

In section 5.3 I have discussed how palmitoylation might regulate Fz5 localisation in axons through these two non-mutually exclusive mechanisms. Recent findings suggest that palmitoylation at the Golgi is an essential signal for anterograde transport to the PM (Ernst et al. 2018). Given that I did not detect obvious accumulation of 3CS Fz5 at the ER or Golgi apparatus (Fig 5.3), major changes along the exocytic route are not expected. Nevertheless,

palmitoylation might be involved in polarised sorting into axons, thus explaining the decreased distribution of Fz5 3CS along these processes.To answer this question, we are planning to perform time-course and time-lapse experiments to compare the trafficking of WT and 3CS into axons and to presynaptic sites. To perform these analyses, we will take advantage of the RUSH (retention using selective hooks) system technology, which allows synchronised trafficking of proteins of interest from a specific organelle to other subcellular compartments (Boncompain et al. 2012; Boncompain and Perez 2014). This experiment will answer two specific questions: does palmitoylation regulate the delivery of Fz5 to the PM? Is this modification involved in Fz5 polarised trafficking to axons and presynaptic terminals? These are fundamental questions to fully elucidate the role of palmitoylation in Fz5 trafficking.

In addition to sorting and exocytosis, palmitoylation is a major regulator of protein stability at the PM, suggesting that surface levels of Fz5 could be affected by this modification. Indeed, palmitoylation inhibits Fz5 constitutive internalisation and is required to maintain Fz5 surface levels, but does not affect the lateral mobility of this receptor (Fig 5.4 and Fig 5.5). Another PTM, ubiquitination, has previously been shown to regulate Fz5 surface levels (Koo et al. 2012; Hao et al. 2012), raising the question whether palmitoylation and ubiquitination are co-regulated to control the levels of Fz5 at the PM. The transmembrane E3 ubiquitin ligases RNF43 and ZNRF3 interact directly with Frizzled receptors and ubiquitinate them, triggering their internalisation and, in some cases, lysosomal degradation (Hao et al. 2012; Koo et al. 2012; Bryan T MacDonald and He 2012a; de Lau et al. 2014). In endosomes, Frizzled receptors can be de-ubiquitinated by USP enzymes and recycled to the PM, or they can be targeted to degradation as a mechanism to down-regulate Wnt signalling (Mukai et al. 2010; Madan et al. 2016). My findings demonstrate that internalisation of 3CS Fz5 is increased compared to WT receptors, suggesting that ubiquitination might be increased in the absence of palmitate groups. What could be the molecular mechanisms of this co-regulation? One possibility is that palmitoylation may reduce the interaction of Fz5 with RNF43 and/or ZNRF3, perhaps by restricting the distribution of

Fz5 at PM domains where these ligases are not present. Given the prominent role of palmitoylation and ubiquitination in modulating membrane trafficking of Frizzled receptors, I am currently planning further experiments to address this important aspect of Frizzled receptor regulation.

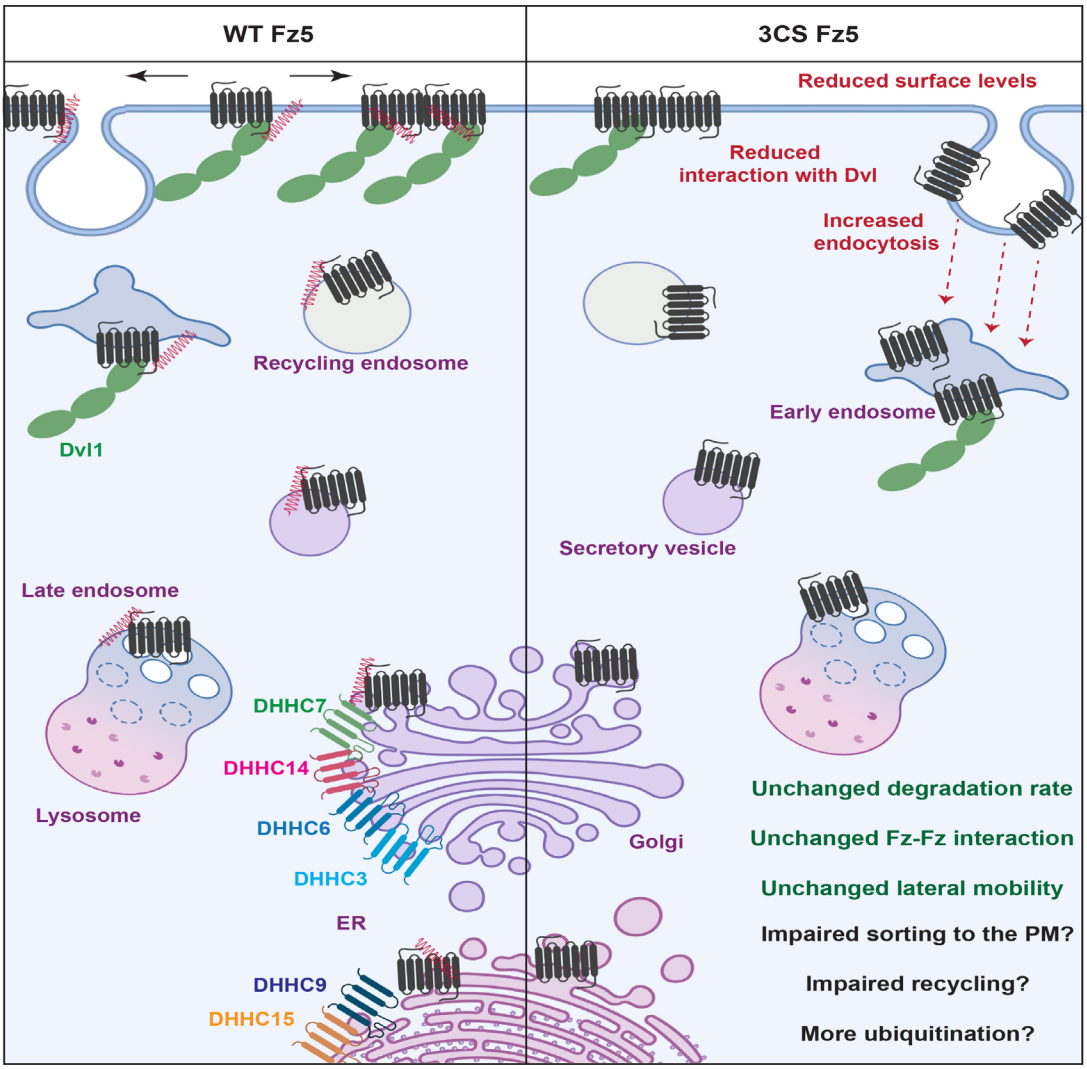

Fig 7.4: The role of palmitoylation in Fz5 trafficking

Palmitoylation is dispensable for certain aspects of Fz5 trafficking such as turnover rate, Fz5-Fz5 interaction and lateral mobility at the PM. In contrast, palmitoylation by one or more DHHC enzymes (DHHC3, DHHC6, DHHC7, DHHC9, DHHC14 and DHHC15) inhibits Fz5 internalisation, is required to maintain this receptor at the cell surface and modulates the interaction with the key scaffold protein Dvl1. Other aspects of Fz5 trafficking, such as sorting and recycling to the PM as well as ubiquitin-dependent internalisation, remain to be investigated.

Aside from its major role in protein endocytosis, palmitoylation is fundamental for the PM recycling of a number of surface receptors (McCormick et al. 2008; Charollais and Van Der Goot 2009; Naumenko and Ponimaskin 2018), suggesting that palmitoylation could also determine Fz5 recycling to the PM after endocytosis. The fact that protein turnover is unchanged, but surface levels of 3CS Fz5 are reduced, suggests that palmitoylation-deficient receptors accumulate on some endosomal compartments. My qualitative experiments show colocalisation of 3CS Fz5 with common endosome markers (EEA1, Rab7 and Rab11), but the recycling rate of WT vs 3CS Fz5 remains to be examined. A functional recycling assay, ideally coupled with the use of super-resolution microscopy, could reveal whether 3CS Fz5 fails to recycle to the PM.

Another interesting question is whether palmitoylation affects ligand-induced endocytosis of Fz5. Although the role of Wnt receptors internalisation in downstream signalling is still debated, ligand-induced endocytosis has been proposed as a model of Wnt signalling activation. Both Clathrin and Caveolin control the endocytosis of the Wnt signalosome (Gagliardi et al. 2008; Brunt and Scholpp 2018; Yamamoto et al. 2006; Blitzer and Nusse 2006). Wnt3a induces the internalisation of Fz5 through Clathrin in the absence of LRP6, whereas the Caveolin route is preferred when LRP6 is present (Yamamoto et al. 2006). As my antibody-feeding experiments (Fig 5.4) were performed without treating cells with exogenous Wnt ligands, they serve mainly as a read-out of constitutive internalisation rather than ligand-induced endocytosis, although cell lines do express some endogenous Wnts. Are WT and 3CS Fz5 equally internalised together with functional signalosomes upon Wnt binding? The reduced interaction between 3CS Fz5 and Dvl1 (Fig 5.2) suggests that the internalisation of the mutant receptor together with the Wnt signalosome might be impaired. TIRF (total internal reflection fluorescence) imaging coupled with super-resolution microscopy (Guo et al. 2018) will further elucidate the role of Fz5 palmitoylation in ligand-induced endocytosis.

The study of Frizzled lateral mobility is an understudied topic in the Frizzled receptor field. Given that palmitoylation-deficient Fz5 exhibits impaired membrane stability and reduced interaction with the scaffold protein Dvl1, the

lateral mobility at the PM could also be regulated by this modification. However, FRAP experiments in NRK cells suprisisingly revealed no differences in the lateral mobility of WT and 3CS Fz5, suggesting that palmitoylation is not involved in this process. However, further experiments should test how the binding of Wnt ligands affects the movements of WT and 3CS Fz5 across the PM. It has been proposed that several, but not all, Wnt ligands significantly accelerate Fz6 mobility at the PM (Kilander et al. 2014). Does palmitoylation regulate this phenomenon for Fz5 receptors? This question is particularly interesting in the context of synapses, where Wnt7a is rapidly increased upon induction of neuronal activity (McLeod et al. 2018). Furthermore, lateral mobility of surface receptors has profound effects on synapse strength and function (Groc et al. 2004; Lau and Zukin 2007; Delgado and Selvin 2018; Park 2018). For instance, changes in the lateral mobility of NMDARs and AMPARs, which are modulated by several factors including Wnt ligands (McQuate et al. 2017; McLeod et al. 2018), affect synaptic transmission and plasticity (Groc et al. 2004; Lau and Zukin 2007; Heine et al. 2008). However, nothing is known about the membrane dynamics of Frizzled receptors at the synapse. While my findings from cell line experiments suggest that palmitoylation is not involved in this process, it would be important to study further how the lateral mobility of Frizzled receptors is regulated to modulate synapse formation and function.

7.6 Multiple DHHC enzymes can palmitoylate Fz5

Identifying the enzymes that catalyse Fz5 palmitoylation and de-palmitoylation is crucial to understand the molecular mechanisms that regulate Fz5 function, and I conducted gain and loss of function experiments aimed at identifying these enzymes. Similarly to what has been reported for other palmitoylated substrates like PSD-95, Gα subunit, GRIP1B, δ-catenin, SNAP25 and others (Fukata et al. 2004; Hayashi et al. 2005; Hayashi et al. 2009; Thomas et al. 2012; Brigidi et al. 2014; Tsutsumi et al. 2009), Fz5 can be palmitoylated by multiple enzymes. Expression of the Golgi-resident DHHC3 or DHHC7 increased Fz5 palmitoylation by roughly 6-7 folds (Fig 4.4), but double KD of these two enzymes did not affect Fz5 palmitoylation. Other enzymes, including DHHC6, DHHC9, DHHC14 and DHHC15 (localised between ER and Golgi) are also able to palmitoylate Fz5 and could therefore compensate for the loss of DHHC3 and DHHC7 (Fig 7.4). Therefore, the requirement of specific DHHC enzymes for Fz5 palmitoylation remains to be fully elucidated.

How can it be determined which DHHC enzymes are essential for Fz5 palmitoylation? Understanding the recognition of Cys sites by specific palmitoylating enzymes is a major challenge in the field of protein palmitoylation. Some studies have proposed mechanisms of recognition based on the amino acid microenvironment around palmitoylation sites, and based on the molecular structure of DHHC enzymes (Resh 2006; Huang et al. 2009; Ohno et al. 2012). However, these observations are either very general or restricted to a small group of substrates. In the absence of computational tools for the prediction of enzyme/substrate pairs, researchers resort to gain and loss of function screens to tackle this question.

In the case of Fz5, potential compensatory effects by 4 different DHHCs may occur when down-regulating DHHC3 and DHHC7 expplastuicression. As discussed in section 4.3, the ability of 6 DHHCs to palmitoylate Fz5 might be the result of protein overexpression in cell lines. Therefore, a first crucial step is to understand whether each of these enzymes can palmitoylate Fz5 in a more physiological context. Besides biochemical analyses of palmitoylation levels, the functional outcomes of DHHCs KD should be examined, as this

strategy could reveal the requirement of certain enzymes in Fz5-specific functions, such as for the assembly of presynaptic sites. Another strategy to examine DHHC activity in a more specific manner is to examine whether each of these enzymes can palmitoylate all three Cys residues, or whether they exhibit residue-specific activity. If the latter is true, palmitoylation by different DHHCs couls have different effects on Fz5. This molecular mechanism has been demonstrated for certain synaptic proteins. For instance, PSD-95 is dually palmitoylated at the N-term by DHHC3, which mediates constitutive palmitoylation in the Golgi (El-Husseini et al. 2000; Fukata et al. 2004), but in dendrites, DHHC2 catalyses activity-induced palmitoylation of PSD-95, which is required for mechanisms of synaptic plasticity (Noritake et al. 2009; Fukata et al. 2013; Jeyifous et al. 2016). Using the double Cys mutants of Fz5 (see chapter 4), it is possible to examine whether DHHC enzymes can palmitoylate all Cys residues or whether their activity is restricted to a specific site. It can also be investigated whether palmitoylation of each Cys is involved in a specific function, such as constitutive versus activity-mediated palmitoylation. However, it is unknown whether palmitoylation events on adjacent Cys sites have diverse effects on proteins, and whether these modifications can be catalysed by different DHHC enzymes.

Another important aspect of protein palmitoylation is the study of the de-palmitoylation process. Different from other lipid modifications, palmitoylation is reversible, and the de-attachment of palmitate chains is catalysed by acyl protein thioesterases (Yokoi et al. 2016; Hornemann 2015; Lin and Conibear 2015; Won et al. 2018). Mutations of de-palmitoylating enzymes have been linked to several disorders, including AD and Batten disease (Kollmann et al. 2013; Cho and Park 2016). Examining the regulation of Frizzled de-palmitoylation will be of interest for future investigations. More comprehensive characterisations of palmitoylating and de-palmitoylationg enzymes will contribute enormously to our understanding of how Frizzled receptors are trafficked and regulated, and will shed light on potential targets that modulate Frizzled function. Given the prominent role that Wnt receptors play in several biological processes, including neuroscience, stem cell

biology and cancer, studying the role and regulation of Frizzled palmitoylating and de-palmitoylating enzymes is of considerable importance to basic as well as clinical research.

7.7 Conclusions

In spite of the prominent role of Wnt signalling in many and diverse biological processes, surprisingly little is known about the mechanisms that regulate the main receptors for Wnt ligands, Frizzleds. In my PhD project, I have studied the role and regulation of Frizzled receptors in synapse formation, with a particular focus on a previously uncharacterised PTM of Fz5. My results indicate that Fz7 and Fz5, two receptors for the synaptogenic ligand Wnt7a, have distinct roles in pre- and postsynaptic development, respectively. My findings also show for the first time that palmitoylation is required for the synaptogenic function of Fz5 *in vivo*. This previously uncharacterised PTM is essential for Fz5 membrane stability and for the interaction with the key scaffold protein Dvl1. Aside from Fz5, others and potentially all Frizzled receptors are palmitoylated. Together, my findings elucidate the molecular mechanisms that regulate Fz5 trafficking and function, opening up new avenues to study the mechanisms that regulate Frizzled receptors in health and disease.

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Bibliography

Abrami, L., Kunz, B., Iacovache, I. and van der Goot, F.G. 2008. Palmitoylation and ubiquitination regulate exit of the Wnt signalling protein LRP6 from the endoplasmic reticulum. *Proceedings of the National Academy of Sciences of the United States of America* 105(14), pp. 5384–5389.

Abrami, L., Leppla, S.H. and van der Goot, F.G. 2006. Receptor palmitoylation and ubiquitination regulate anthrax toxin endocytosis. *The Journal of Cell Biology* 172(2), pp. 309–320.

Adachi, N., Hess, D.T., McLaughlin, P. and Stamler, J.S. 2016. S-Palmitoylation of a Novel Site in the β2-Adrenergic Receptor Associated with a Novel Intracellular Itinerary. *The Journal of Biological Chemistry* 291(38), pp. 20232–20246.

Adams, M.N., Christensen, M.E., He, Y., Waterhouse, N.J. and Hooper, J.D. 2011. The role of palmitoylation in signalling, cellular trafficking and plasma membrane localisation of protease-activated receptor-2. *Plos One* 6(11), p. e28018.

Agajanian, M.J., Walker, M.P., Axtman, A.D., Ruela-de-Sousa, R.R., Serafin, D.S., Rabinowitz, A.D., Graham, D.M., Ryan, M.B., Tamir, T., Nakamichi, Y., Gammons, M.V., Bennett, J.M., Couñago, R.M., Drewry, D.H., Elkins, J.M., Gileadi, C., Gileadi, O., Godoi, P.H., Kapadia, N., Müller, S., Santiago, A.S., Sorrell, F.J., Wells, C.I., Fedorov, O., Willson, T.M., Zuercher, W.J. and Major, M.B. 2019. WNT Activates the AAK1 Kinase to Promote Clathrin-Mediated Endocytosis of LRP6 and Establish a Negative Feedback Loop. *Cell reports* 26(1), pp. 79–93.e8.

Ahmad-Annuar, A., Ciani, L., Simeonidis, I., Herreros, J., Fredj, N.B., Rosso, S.B., Hall, A., Brickley, S. and Salinas, P.C. 2006. Signalling across the synapse: a role for Wnt and Dishevelled in presynaptic assembly and neurotransmitter release. *The Journal of Cell Biology* 174(1), pp. 127–139.

Ahmari, S.E., Buchanan, J. and Smith, S.J. 2000. Assembly of presynaptic active zones from cytoplasmic transport packets. *Nature Neuroscience* 3(5), pp. 445–451.

Ahn, S. and Joyner, A.L. 2005. In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. *Nature* 437(7060), pp. 894–897.

Aicart-Ramos, C., Valero, R.A. and Rodriguez-Crespo, I. 2011. Protein palmitoylation and subcellular trafficking. *Biochimica et Biophysica Acta* 1808(12), pp. 2981–2994.

Alexandre, C., Baena-Lopez, A. and Vincent, J.-P. 2014. Patterning and growth control by membrane-tethered Wingless. *Nature* 505(7482), pp. 180–185.

Allen, N.J. 2013. Role of glia in developmental synapse formation. *Current Opinion in Neurobiology* 23(6), pp. 1027–1033.

Alonso, M., Medina, J.H. and Pozzo-Miller, L. 2004. ERK1/2 activation is necessary for BDNF to increase dendritic spine density in hippocampal CA1 pyramidal neurons. *Learning & Memory* 11(2), pp. 172–178.

Alvarez, E., Gironès, N. and Davis, R.J. 1990. Inhibition of the receptor-mediated endocytosis of diferric transferrin is associated with the covalent modification of the transferrin receptor with palmitic acid. *The Journal of Biological Chemistry* 265(27), pp. 16644–16655.

Álvarez-Buylla, A. and Ihrie, R.A. 2014. Sonic hedgehog signalling in the postnatal brain. *Seminars in Cell & Developmental Biology* 33, pp. 105–111.
Amano, M., Nakayama, M. and Kaibuchi, K. 2010. Rho-kinase/ROCK: A key regulator of the cytoskeleton and cell polarity. *Cytoskeleton* 67(9), pp. 545–554.
Van Amerongen, R. 2012. Alternative Wnt pathways and receptors. *Cold Spring Harbor Perspectives in Biology* 4(10).
Anastas, J.N. and Moon, R.T. 2013. WNT signalling pathways as therapeutic targets in cancer. *Nature Reviews. Cancer* 13(1), pp. 11–26.
Andreae, L.C. and Burrone, J. 2015. Spontaneous Neurotransmitter Release Shapes Dendritic Arbors via Long-Range Activation of NMDA Receptors. *Cell reports* 10(6), pp. 873–882.
Andreae, L.C. and Burrone, J. 2014. The role of neuronal activity and transmitter release on synapse formation. *Current Opinion in Neurobiology* 27, pp. 47–52.
Andreae, L.C. and Burrone, J. 2018. The role of spontaneous neurotransmission in synapse and circuit development. *Journal of Neuroscience Research* 96(3), pp. 354–359.
Anggono, V. and Huganir, R.L. 2012. Regulation of AMPA receptor trafficking and synaptic plasticity. *Current Opinion in Neurobiology* 22(3), pp. 461–469.
Araque, A., Carmignoto, G., Haydon, P.G., Oliet, S.H.R., Robitaille, R. and Volterra, A. 2014. Gliotransmitters travel in time and space. *Neuron* 81(4), pp. 728–739.
Araque, A., Parpura, V., Sanzgiri, R.P. and Haydon, P.G. 1999. Tripartite synapses: glia, the unacknowledged partner. *Trends in Neurosciences* 22(5), pp. 208–215.
Arellano, J.I., Benavides-Piccione, R., Defelipe, J. and Yuste, R. 2007. Ultrastructure of dendritic spines: correlation between synaptic and spine morphologies. *Frontiers in Neuroscience* 1(1), pp. 131–143.
Arikkath, J. and Reichardt, L.F. 2008. Cadherins and catenins at synapses: roles in synaptogenesis and synaptic plasticity. *Trends in Neurosciences* 31(9), pp. 487–494.
Ashby, M.C., Ibaraki, K. and Henley, J.M. 2004. It's green outside: tracking cell surface proteins with pH-sensitive GFP. *Trends in Neurosciences* 27(5), pp. 257–261.
Ataman, B., Ashley, J., Gorczyca, D., Gorczyca, M., Mathew, D., Wichmann, C., Sigrist, S.J. and Budnik, V. 2006. Nuclear trafficking of Drosophila Frizzled-2 during synapse development requires the PDZ protein dGRIP. *Proceedings of the National Academy of Sciences of the United States of America* 103(20), pp. 7841–7846.
Ataman, B., Ashley, J., Gorczyca, M., Ramachandran, P., Fouquet, W., Sigrist, S.J. and Budnik, V. 2008. Rapid activity-dependent modifications in synaptic structure and function require bidirectional Wnt signalling. *Neuron* 57(5), pp. 705–718.
Avila, M.E., Sepúlveda, F.J., Burgos, C.F., Moraga-Cid, G., Parodi, J., Moon, R.T., Aguayo, L.G., Opazo, C. and De Ferrari, G.V. 2010. Canonical Wnt3a modulates intracellular calcium and enhances excitatory neurotransmission in hippocampal neurons. *The Journal of Biological Chemistry* 285(24), pp. 18939–18947.

Azzolin, L., Panciera, T., Soligo, S., Enzo, E., Bicciato, S., Dupont, S., Bresolin, S., Frasson, C., Basso, G., Guzzardo, V., Fassina, A., Cordenonsi, M. and Piccolo, S. 2014. YAP/TAZ incorporation in the β-catenin destruction complex orchestrates the Wnt response. *Cell* 158(1), pp. 157–170.
Baekkeskov, S. and Kanaani, J. 2009. Palmitoylation cycles and regulation of protein function (Review). *Molecular membrane biology* 26(1), pp. 42–54.
Bafico, A., Gazit, A., Pramila, T., Finch, P.W., Yaniv, A. and Aaronson, S.A. 1999. Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signalling. *The Journal of Biological Chemistry* 274(23), pp. 16180–16187.
Bafico, A., Liu, G., Yaniv, A., Gazit, A. and Aaronson, S.A. 2001. Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nature Cell Biology* 3(7), pp. 683–686.
Bamji, S.X., Shimazu, K., Kimes, N., Huelsken, J., Birchmeier, W., Lu, B. and Reichardt, L.F. 2003. Role of beta-catenin in synaptic vesicle localisation and presynaptic assembly. *Neuron* 40(4), pp. 719–731.
Banovic, D., Khorramshahi, O., Owald, D., Wichmann, C., Riedt, T., Fouquet, W., Tian, R., Sigrist, S.J. and Aberle, H. 2010. Drosophila neuroligin 1 promotes growth and postsynaptic differentiation at glutamatergic neuromuscular junctions. *Neuron* 66(5), pp. 724–738.
Bänziger, C., Soldini, D., Schütt, C., Zipperlen, P., Hausmann, G. and Basler, K. 2006. Wntless, a conserved membrane protein dedicated to the secretion of Wnt proteins from signalling cells. *Cell* 125(3), pp. 509–522.
Barnett-Norris, J., Lynch, D. and Reggio, P.H. 2005. Lipids, lipid rafts and caveolae: their importance for GPCR signalling and their centrality to the endocannabinoid system. *Life Sciences* 77(14), pp. 1625–1639.
Barros, C.S., Calabrese, B., Chamero, P., Roberts, A.J., Korzus, E., Lloyd, K., Stowers, L., Mayford, M., Halpain, S. and Müller, U. 2009. Impaired maturation of dendritic spines without disorganisation of cortical cell layers in mice lacking NRG1/ErbB signalling in the central nervous system. *Proceedings of the National Academy of Sciences of the United States of America* 106(11), pp. 4507–4512.
Barrow, S.L., Constable, J.R., Clark, E., El-Sabeawy, F., McAllister, A.K. and Washbourne, P. 2009. Neuroligin1: a cell adhesion molecule that recruits PSD-95 and NMDA receptors by distinct mechanisms during synaptogenesis. *Neural Development* 4, p. 17.
Bartscherer, K. and Boutros, M. 2008. Regulation of Wnt protein secretion and its role in gradient formation. *EMBO Reports* 9(10), pp. 977–982.
Bartscherer, K., Pelte, N., Ingelfinger, D. and Boutros, M. 2006. Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. *Cell* 125(3), pp. 523–533.
Bassani, S., Folci, A., Zapata, J. and Passafaro, M. 2013. AMPAR trafficking in synapse maturation and plasticity. *Cellular and Molecular Life Sciences* 70(23), pp. 4411–4430.
Basu, S., Saha, P.K., Roszkowska, M., Magnowska, M., Baczynska, E., Das, N., Plewczynski, D. and Wlodarczyk, J. 2018. Quantitative 3-D morphometric analysis of individual dendritic spines. *Scientific reports* 8(1), p. 3545.
Batool, S., Raza, H., Zaidi, J., Riaz, S., Hasan, S. and Syed, N.I. 2019. Synapse formation: from cellular and molecular mechanisms to

neurodevelopmental and neurodegenerative disorders. *Journal of Neurophysiology* 121(4), pp. 1381–1397.
Bats, C., Groc, L. and Choquet, D. 2007. The interaction between Stargazin and PSD-95 regulates AMPA receptor surface trafficking. *Neuron* 53(5), pp. 719–734.
Belenkaya, T.Y., Wu, Y., Tang, X., Zhou, B., Cheng, L., Sharma, Y.V., Yan, D., Selva, E.M. and Lin, X. 2008. The retromer complex influences Wnt secretion by recycling wntless from endosomes to the trans-Golgi network. *Developmental Cell* 14(1), pp. 120–131.
Belgacem, Y.H., Hamilton, A.M., Shim, S., Spencer, K.A. and Borodinsky, L.N. 2016. The many hats of sonic hedgehog signalling in nervous system development and disease. *Journal of developmental biology* 4(4).
Bellucci, A., Mercuri, N.B., Venneri, A., Faustini, G., Longhena, F., Pizzi, M., Missale, C. and Spano, P. 2016. Review: Parkinson's disease: from synaptic loss to connectome dysfunction. *Neuropathology and Applied Neurobiology* 42(1), pp. 77–94.
Ben-Ari, Y. 2002. Excitatory actions of gaba during development: the nature of the nurture. *Nature Reviews. Neuroscience* 3(9), pp. 728–739.
Benavides-Piccione, R., Fernaud-Espinosa, I., Robles, V., Yuste, R. and DeFelipe, J. 2013. Age-based comparison of human dendritic spine structure using complete three-dimensional reconstructions. *Cerebral Cortex* 23(8), pp. 1798–1810.
Benilova, I., Karran, E. and De Strooper, B. 2012. The toxic Aβ oligomer and Alzheimer's disease: an emperor in need of clothes. *Nature Neuroscience* 15(3), pp. 349–357.
Berry, K.P. and Nedivi, E. 2017. Spine dynamics: are they all the same? *Neuron* 96(1), pp. 43–55.
Berwick, D.C. and Harvey, K. 2012. The importance of Wnt signalling for neurodegeneration in Parkinson's disease. *Biochemical Society Transactions* 40(5), pp. 1123–1128.
Bettler, B., Kaupmann, K., Mosbacher, J. and Gassmann, M. 2004. Molecular structure and physiological functions of GABA(B) receptors. *Physiological Reviews* 84(3), pp. 835–867.
Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J. and Nusse, R. 1996. A new member of the frizzled family from Drosophila functions as a Wingless receptor. *Nature* 382(6588), pp. 225–230.
Biederer, T., Sara, Y., Mozhayeva, M., Atasoy, D., Liu, X., Kavalali, E.T. and Südhof, T.C. 2002. SynCAM, a synaptic adhesion molecule that drives synapse assembly. *Science* 297(5586), pp. 1525–1531.
Binder, D.K. and Scharfman, H.E. 2004. Brain-derived neurotrophic factor. *Growth Factors* 22(3), pp. 123–131.
Blakely, R.D. and Edwards, R.H. 2012. Vesicular and plasma membrane transporters for neurotransmitters. *Cold Spring Harbor Perspectives in Biology* 4(2).
Blanc, M., David, F., Abrami, L., Migliozzi, D., Armand, F., Bürgi, J. and van der Goot, F.G. 2015. SwissPalm: Protein Palmitoylation database. [version 1; peer review: 3 approved]. *F1000Research* 4, p. 261.

Blaskovic, S., Blanc, M. and van der Goot, F.G. 2013. What does S-palmitoylation do to membrane proteins? *The FEBS Journal* 280(12), pp. 2766–2774.
Blitzer, J.T. and Nusse, R. 2006. A critical role for endocytosis in Wnt signalling. *BMC Cell Biology* 7, p. 28.
Bocchi, R., Egervari, K., Carol-Perdiguer, L., Viale, B., Quairiaux, C., De Roo, M., Boitard, M., Oskouie, S., Salmon, P. and Kiss, J.Z. 2017. Perturbed Wnt signalling leads to neuronal migration delay, altered interhemispheric connections and impaired social behavior. *Nature Communications* 8(1), p. 1158.
Boeckers, T.M. 2006. The postsynaptic density. *Cell and Tissue Research* 326(2), pp. 409–422.
Boncompain, G., Divoux, S., Gareil, N., de Forges, H., Lescure, A., Latreche, L., Mercanti, V., Jollivet, F., Raposo, G. and Perez, F. 2012. Synchronization of secretory protein traffic in populations of cells. *Nature Methods* 9(5), pp. 493–498.
Boncompain, G. and Perez, F. 2014. Synchronization of secretory cargos trafficking in populations of cells. *Methods in Molecular Biology* 1174, pp. 211–223.
Bonhoeffer, T. and Yuste, R. 2002. Spine motility. Phenomenology, mechanisms, and function. *Neuron* 35(6), pp. 1019–1027.
Bosch, M. and Hayashi, Y. 2012. Structural plasticity of dendritic spines. *Current Opinion in Neurobiology* 22(3), pp. 383–388.
Bosworth, A.P. and Allen, N.J. 2017. The diverse actions of astrocytes during synaptic development. *Current Opinion in Neurobiology* 47, pp. 38–43.
Bourhis, E., Tam, C., Franke, Y., Bazan, J.F., Ernst, J., Hwang, J., Costa, M., Cochran, A.G. and Hannoush, R.N. 2010. Reconstitution of a frizzled8.Wnt3a.LRP6 signalling complex reveals multiple Wnt and Dkk1 binding sites on LRP6. *The Journal of Biological Chemistry* 285(12), pp. 9172–9179.
Bouwman, J., Maia, A.S., Camoletto, P.G., Posthuma, G., Roubos, E.W., Oorschot, V.M.J., Klumperman, J. and Verhage, M. 2004. Quantification of synapse formation and maintenance in vivo in the absence of synaptic release. *Neuroscience* 126(1), pp. 115–126.
Bovolenta, P., Esteve, P., Ruiz, J.M., Cisneros, E. and Lopez-Rios, J. 2008. Beyond Wnt inhibition: new functions of secreted Frizzled-related proteins in development and disease. *Journal of Cell Science* 121(Pt 6), pp. 737–746.
Bramham, C.R. and Messaoudi, E. 2005. BDNF function in adult synaptic plasticity: the synaptic consolidation hypothesis. *Progress in Neurobiology* 76(2), pp. 99–125.
Brigidi, G.S., Santyr, B., Shimell, J., Jovellar, B. and Bamji, S.X. 2015. Activity-regulated trafficking of the palmitoyl-acyl transferase DHHC5. *Nature Communications* 6, p. 8200.
Brigidi, G.S., Sun, Y., Beccano-Kelly, D., Pitman, K., Mobasser, M., Borgland, S.L., Milnerwood, A.J. and Bamji, S.X. 2014. Palmitoylation of δ-catenin by DHHC5 mediates activity-induced synapse plasticity. *Nature Neuroscience* 17(4), pp. 522–532.
Briscoe, J. 2009. Making a grade: Sonic Hedgehog signalling and the control of neural cell fate. *The EMBO Journal* 28(5), pp. 457–465.

Broekman, M.L.D., Comer, L.A., Hyman, B.T. and Sena-Esteves, M. 2006. Adeno-associated virus vectors serotyped with AAV8 capsid are more efficient than AAV-1 or -2 serotypes for widespread gene delivery to the neonatal mouse brain. *Neuroscience* 138(2), pp. 501–510.
Brunt, L. and Scholpp, S. 2018. The function of endocytosis in Wnt signalling. *Cellular and Molecular Life Sciences* 75(5), pp. 785–795.
Brusés, J.L. 2006. N-cadherin signalling in synapse formation and neuronal physiology. *Molecular Neurobiology* 33(3), pp. 237–252.
Budnik, V. and Salinas, P.C. 2011. Wnt signalling during synaptic development and plasticity. *Current Opinion in Neurobiology* 21(1), pp. 151–159.
Buechler, J. and Salinas, P.C. 2018. Deficient wnt signalling and synaptic vulnerability in alzheimer's disease: emerging roles for the LRP6 receptor. *Frontiers in synaptic neuroscience* 10, p. 38.
Buglino, J.A. and Resh, M.D. 2012. Palmitoylation of Hedgehog proteins. *Vitamins and Hormones* 88, pp. 229–252.
Burnashev, N. 1998. Calcium permeability of ligand-gated channels. *Cell Calcium* 24(5-6), pp. 325–332.
Caldeira, M.V., Melo, C.V., Pereira, D.B., Carvalho, R., Correia, S.S., Backos, D.S., Carvalho, A.L., Esteban, J.A. and Duarte, C.B. 2007. Brain-derived neurotrophic factor regulates the expression and synaptic delivery of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor subunits in hippocampal neurons. *The Journal of Biological Chemistry* 282(17), pp. 12619–12628.
De Calisto, J., Araya, C., Marchant, L., Riaz, C.F. and Mayor, R. 2005. Essential role of non-canonical Wnt signalling in neural crest migration. *Development* 132(11), pp. 2587–2597.
Caricasole, A., Copani, A., Caraci, F., Aronica, E., Rozemuller, A.J., Caruso, A., Storto, M., Gaviraghi, G., Terstappen, G.C. and Nicoletti, F. 2004. Induction of Dickkopf-1, a negative modulator of the Wnt pathway, is associated with neuronal degeneration in Alzheimer's brain. *The Journal of Neuroscience* 24(26), pp. 6021–6027.
Carpenter, G. and Liao, H.-J. 2013. Receptor tyrosine kinases in the nucleus. *Cold Spring Harbor Perspectives in Biology* 5(10), p. a008979.
Carron, C., Pascal, A., Djiane, A., Boucaut, J.-C., Shi, D.-L. and Umbhauer, M. 2003. Frizzled receptor dimerization is sufficient to activate the Wnt/beta-catenin pathway. *Journal of Cell Science* 116(Pt 12), pp. 2541–2550.
Catterall, W.A. 2011. Voltage-gated calcium channels. *Cold Spring Harbor Perspectives in Biology* 3(8), p. a003947.
Cerpa, W., Gambrill, A., Inestrosa, N.C. and Barria, A. 2011. Regulation of NMDA-receptor synaptic transmission by Wnt signalling. *The Journal of Neuroscience* 31(26), pp. 9466–9471.
Cerpa, W., Godoy, J.A., Alfaro, I., Farías, G.G., Metcalfe, M.J., Fuentealba, R., Bonansco, C. and Inestrosa, N.C. 2008. Wnt-7a modulates the synaptic vesicle cycle and synaptic transmission in hippocampal neurons. *The Journal of Biological Chemistry* 283(9), pp. 5918–5927.
Cerpa, W., Latorre-Esteves, E. and Barria, A. 2015. RoR2 functions as a noncanonical Wnt receptor that regulates NMDAR-mediated synaptic transmission. *Proceedings of the National Academy of Sciences of the United States of America* 112(15), pp. 4797–4802.

Chakrabarty, P., Rosario, A., Cruz, P., Siemienski, Z., Ceballos-Diaz, C., Crosby, K., Jansen, K., Borchelt, D.R., Kim, J.-Y., Jankowsky, J.L., Golde, T.E. and Levites, Y. 2013. Capsid serotype and timing of injection determines AAV transduction in the neonatal mice brain. *Plos One* 8(6), p. e67680.
Chamberlain, L.H. and Shipston, M.J. 2015. The physiology of protein S-acylation. *Physiological Reviews* 95(2), pp. 341–376.
Chapman, E.R. 2018. A ca2+ sensor for exocytosis. *Trends in Neurosciences* 41(6), pp. 327–330.
Chapman, E.R. 2008. How does synaptotagmin trigger neurotransmitter release? *Annual Review of Biochemistry* 77, pp. 615–641.
Charollais, J. and Van Der Goot, F.G. 2009. Palmitoylation of membrane proteins (Review). *Molecular membrane biology* 26(1), pp. 55–66.
Charron, F., Stein, E., Jeong, J., McMahon, A.P. and Tessier-Lavigne, M. 2003. The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell* 113(1), pp. 11–23.
Chebib, M. and Johnston, G.A. 1999. The "ABC" of GABA receptors: a brief review. *Clinical and Experimental Pharmacology & Physiology* 26(11), pp. 937–940.
Chen, B., Sun, Y., Niu, J., Jarugumilli, G.K. and Wu, X. 2018. Protein lipidation in cell signalling and diseases: function, regulation, and therapeutic opportunities. *Cell chemical biology* 25(7), pp. 817–831.
Chen, C.-M., Orefice, L.L., Chiu, S.-L., LeGates, T.A., Hattar, S., Huganir, R.L., Zhao, H., Xu, B. and Kuruvilla, R. 2017. Wnt5a is essential for hippocampal dendritic maintenance and spatial learning and memory in adult mice. *Proceedings of the National Academy of Sciences of the United States of America* 114(4), pp. E619–E628.
Chen, J., Park, C.S. and Tang, S.-J. 2006. Activity-dependent synaptic Wnt release regulates hippocampal long term potentiation. *The Journal of Biological Chemistry* 281(17), pp. 11910–11916.
Chen, L., Chetkovich, D.M., Petralia, R.S., Sweeney, N.T., Kawasaki, Y., Wenthold, R.J., Bredt, D.S. and Nicoll, R.A. 2000. Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. *Nature* 408(6815), pp. 936–943.
Chen, L.Y., Jiang, M., Zhang, B., Gokce, O. and Südhof, T.C. 2017. Conditional deletion of all neurexins defines diversity of essential synaptic organizer functions for neurexins. *Neuron* 94(3), pp. 611–625.e4.
Chen, M.-K. and Hung, M.-C. 2015. Proteolytic cleavage, trafficking, and functions of nuclear receptor tyrosine kinases. *The FEBS Journal* 282(19), pp. 3693–3721.
Chen, Y.A. and Scheller, R.H. 2001. SNARE-mediated membrane fusion. *Nature Reviews. Molecular Cell Biology* 2(2), pp. 98–106.
Chesnutt, C., Burrus, L.W., Brown, A.M.C. and Niswander, L. 2004. Coordinate regulation of neural tube patterning and proliferation by TGFbeta and WNT activity. *Developmental Biology* 274(2), pp. 334–347.
Ching, W. and Nusse, R. 2006. A dedicated Wnt secretion factor. *Cell* 125(3), pp. 432–433.
Chiu, C.Q., Lur, G., Morse, T.M., Carnevale, N.T., Ellis-Davies, G.C.R. and Higley, M.J. 2013. Compartmentalization of GABAergic inhibition by dendritic spines. *Science* 340(6133), pp. 759–762.

Cho, C., Smallwood, P.M. and Nathans, J. 2017. Reck and Gpr124 Are Essential Receptor Cofactors for Wnt7a/Wnt7b-Specific Signalling in Mammalian CNS Angiogenesis and Blood-Brain Barrier Regulation. *Neuron* 95(5), pp. 1056–1073.e5.
Cho, E. and Park, M. 2016. Palmitoylation in Alzheimer's disease and other neurodegenerative diseases. *Pharmacological Research* 111, pp. 133–151.
Cho, R.W., Buhl, L.K., Volfson, D., Tran, A., Li, F., Akbergenova, Y. and Littleton, J.T. 2015. Phosphorylation of Complexin by PKA Regulates Activity-Dependent Spontaneous Neurotransmitter Release and Structural Synaptic Plasticity. *Neuron* 88(4), pp. 749–761.
Choi, B.J., Imlach, W.L., Jiao, W., Wolfram, V., Wu, Y., Grbic, M., Cela, C., Baines, R.A., Nitabach, M.N. and McCabe, B.D. 2014. Miniature neurotransmission regulates Drosophila synaptic structural maturation. *Neuron* 82(3), pp. 618–634.
Choii, G. and Ko, J. 2015. Gephyrin: a central GABAergic synapse organizer. *Experimental & Molecular Medicine* 47, p. e158.
Choquet, D. and Triller, A. 2013. The dynamic synapse. *Neuron* 80(3), pp. 691–703.
Choudhry, Z., Rikani, A.A., Choudhry, A.M., Tariq, S., Zakaria, F., Asghar, M.W., Sarfraz, M.K., Haider, K., Shafiq, A.A. and Mobassarah, N.J. 2014. Sonic hedgehog signalling pathway: a complex network. *Annals of neurosciences* 21(1), pp. 28–31.
Christopherson, K.S., Ullian, E.M., Stokes, C.C.A., Mullowney, C.E., Hell, J.W., Agah, A., Lawler, J., Mosher, D.F., Bornstein, P. and Barres, B.A. 2005. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. *Cell* 120(3), pp. 421–433.
Ciani, L., Boyle, K.A., Dickins, E., Sahores, M., Anane, D., Lopes, D.M., Gibb, A.J. and Salinas, P.C. 2011. Wnt7a signalling promotes dendritic spine growth and synaptic strength through Ca^{2+}/Calmodulin-dependent protein kinase II. *Proceedings of the National Academy of Sciences of the United States of America* 108(26), pp. 10732–10737.
Ciani, L., Krylova, O., Smalley, M.J., Dale, T.C. and Salinas, P.C. 2004. A divergent canonical WNT-signalling pathway regulates microtubule dynamics: dishevelled signals locally to stabilize microtubules. *The Journal of Cell Biology* 164(2), pp. 243–253.
Ciani, L., Marzo, A., Boyle, K., Stamatakou, E., Lopes, D.M., Anane, D., McLeod, F., Rosso, S.B., Gibb, A. and Salinas, P.C. 2015. Wnt signalling tunes neurotransmitter release by directly targeting Synaptotagmin-1. *Nature Communications* 6, p. 8302.
Ciani, L. and Salinas, P.C. 2005. WNTs in the vertebrate nervous system: from patterning to neuronal connectivity. *Nature Reviews. Neuroscience* 6(5), pp. 351–362.
Cingolani, L.A. and Goda, Y. 2008. Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. *Nature Reviews. Neuroscience* 9(5), pp. 344–356.
Clevers, H. 2006. Wnt/beta-catenin signalling in development and disease. *Cell* 127(3), pp. 469–480.
Clevers, H., Loh, K.M. and Nusse, R. 2014. Stem cell signalling. An integral program for tissue renewal and regeneration: Wnt signalling and stem cell control. *Science* 346(6205), p. 1248012.

Cline, H.T. 2001. Dendritic arbor development and synaptogenesis. *Current Opinion in Neurobiology* 11(1), pp. 118–126.
Collins, M.O., Husi, H., Yu, L., Brandon, J.M., Anderson, C.N.G., Blackstock, W.P., Choudhary, J.S. and Grant, S.G.N. 2006. Molecular characterization and comparison of the components and multiprotein complexes in the postsynaptic proteome. *Journal of Neurochemistry* 97 Suppl 1, pp. 16–23.
Colón‐Ramos, D.A. 2009. Chapter 2 Synapse Formation in Developing Neural Circuits. In: *Development of Neural Circuitry*. Current Topics in Developmental Biology. Elsevier, pp. 53‐79.
Cong, F., Schweizer, L. and Varmus, H. 2004. Wnt signals across the plasma membrane to activate the beta-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP. *Developmen*t 131(20), pp. 5103–5115.
Craven, S.E., El-Husseini, A.E. and Bredt, D.S. 1999. Synaptic targeting of the postsynaptic density protein PSD-95 mediated by lipid and protein motifs. *Neuron* 22(3), pp. 497–509.
Cruciat, C.-M. and Niehrs, C. 2013. Secreted and transmembrane wnt inhibitors and activators. *Cold Spring Harbor Perspectives in Biology* 5(3), p. a015081.
Cuitino, L., Godoy, J.A., Farías, G.G., Couve, A., Bonansco, C., Fuenzalida, M. and Inestrosa, N.C. 2010. Wnt-5a modulates recycling of functional GABAA receptors on hippocampal neurons. *The Journal of Neuroscience* 30(25), pp. 8411–8420.
Dabrowski, A., Terauchi, A., Strong, C. and Umemori, H. 2015. Distinct sets of FGF receptors sculpt excitatory and inhibitory synaptogenesis. *Development* 142(10), pp. 1818–1830.
Dalva, M.B., McClelland, A.C. and Kayser, M.S. 2007. Cell adhesion molecules: signalling functions at the synapse. *Nature Reviews. Neuroscience* 8(3), pp. 206–220.
Dalva, M.B., Takasu, M.A., Lin, M.Z., Shamah, S.M., Hu, L., Gale, N.W. and Greenberg, M.E. 2000. EphB receptors interact with NMDA receptors and regulate excitatory synapse formation. *Cell* 103(6), pp. 945–956.
Danbolt, N.C. 2001. Glutamate uptake. *Progress in Neurobiology* 65(1), pp. 1–105.
Dann, C.E., Hsieh, J.C., Rattner, A., Sharma, D., Nathans, J. and Leahy, D.J. 2001. Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. *Nature* 412(6842), pp. 86–90.
Das, S., Yu, S., Sakamori, R., Stypulkowski, E. and Gao, N. 2012. Wntless in Wnt secretion: molecular, cellular and genetic aspects. *Frontiers in biology* 7(6), pp. 587–593.
Davda, D., El Azzouny, M.A., Tom, C.T.M.B., Hernandez, J.L., Majmudar, J.D., Kennedy, R.T. and Martin, B.R. 2013. Profiling targets of the irreversible palmitoylation inhibitor 2-bromopalmitate. *ACS Chemical Biology* 8(9), pp. 1912–1917.
Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., Glinka, A. and Niehrs, C. 2005. Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* 438(7069), pp. 867–872.
Davis, E.K., Zou, Y. and Ghosh, A. 2008. Wnts acting through canonical and noncanonical signalling pathways exert opposite effects on hippocampal synapse formation. *Neural Development* 3, p. 32.

De, A. 2011. Wnt/Ca2+ signalling pathway: a brief overview. *Acta biochimica et biophysica Sinica* 43(10), pp. 745–756.
Dean, C., Scholl, F.G., Choih, J., DeMaria, S., Berger, J., Isacoff, E. and Scheiffele, P. 2003. Neurexin mediates the assembly of presynaptic terminals. *Nature Neuroscience* 6(7), pp. 708–716.
DeBruine, Z.J., Ke, J., Harikumar, K.G., Gu, X., Borowsky, P., Williams, B.O., Xu, W., Miller, L.J., Xu, H.E. and Melcher, K. 2017. Wnt5a promotes Frizzled-4 signalosome assembly by stabilizing cysteine-rich domain dimerization. *Genes & Development* 31(9), pp. 916–926.
DeChiara, T.M., Bowen, D.C., Valenzuela, D.M., Simmons, M.V., Poueymirou, W.T., Thomas, S., Kinetz, E., Compton, D.L., Rojas, E., Park, J.S., Smith, C., DiStefano, P.S., Glass, D.J., Burden, S.J. and Yancopoulos, G.D. 1996. The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell* 85(4), pp. 501–512.
DeFelipe, J., Marco, P., Busturia, I. and Merchán-Pérez, A. 1999. Estimation of the number of synapses in the cerebral cortex: methodological considerations. *Cerebral Cortex* 9(7), pp. 722–732.
Dehorter, N., Vinay, L., Hammond, C. and Ben-Ari, Y. 2012. Timing of developmental sequences in different brain structures: physiological and pathological implications. *The European Journal of Neuroscience* 35(12), pp. 1846–1856.
Dejanovic, B., Semtner, M., Ebert, S., Lamkemeyer, T., Neuser, F., Lüscher, B., Meier, J.C. and Schwarz, G. 2014. Palmitoylation of gephyrin controls receptor clustering and plasticity of GABAergic synapses. *PLoS Biology* 12(7), p. e1001908.
Delgado, J.Y. and Selvin, P.R. 2018. A revised view on the role of surface AMPAR mobility in tuning synaptic transmission: limitations, tools, and alternative views. *Frontiers in synaptic neuroscience* 10, p. 21.
Delint-Ramirez, I., Willoughby, D., Hammond, G.R.V., Ayling, L.J. and Cooper, D.M.F. 2011. Palmitoylation targets AKAP79 protein to lipid rafts and promotes its regulation of calcium-sensitive adenylyl cyclase type 8. *The Journal of Biological Chemistry* 286(38), pp. 32962–32975.
Dent, E.W., Merriam, E.B. and Hu, X. 2011. The dynamic cytoskeleton: backbone of dendritic spine plasticity. *Current Opinion in Neurobiology* 21(1), pp. 175–181.
Devenport, D. 2014. The cell biology of planar cell polarity. *The Journal of Cell Biology* 207(2), pp. 171–179.
Dickins, E.M. and Salinas, P.C. 2013. Wnts in action: from synapse formation to synaptic maintenance. *Frontiers in Cellular Neuroscience* 7, p. 162.
Dickinson, M.E., Krumlauf, R. and McMahon, A.P. 1994. Evidence for a mitogenic effect of Wnt-1 in the developing mammalian central nervous system. *Development* 120(6), pp. 1453–1471.
Diering, G.H. and Huganir, R.L. 2018. The AMPA receptor code of synaptic plasticity. *Neuron* 100(2), pp. 314–329.
Dityatev, A., Dityateva, G. and Schachner, M. 2000. Synaptic strength as a function of post- versus presynaptic expression of the neural cell adhesion molecule NCAM. *Neuron* 26(1), pp. 207–217.
Doherty, G.J. and McMahon, H.T. 2009. Mechanisms of endocytosis. *Annual Review of Biochemistry* 78, pp. 857–902.

Dong, H., Zhang, P., Song, I., Petralia, R.S., Liao, D. and Huganir, R.L. 1999. Characterization of the glutamate receptor-interacting proteins GRIP1 and GRIP2. *The Journal of Neuroscience* 19(16), pp. 6930–6941.
Dorsky, R.I., Moon, R.T. and Raible, D.W. 1998. Control of neural crest cell fate by the Wnt signalling pathway. *Nature* 396(6709), pp. 370–373.
Dotti, C.G., Sullivan, C.A. and Banker, G.A. 1988. The establishment of polarity by hippocampal neurons in culture. *The Journal of Neuroscience* 8(4), pp. 1454–1468.
Drake, M.T., Shenoy, S.K. and Lefkowitz, R.J. 2006. Trafficking of G protein-coupled receptors. *Circulation Research* 99(6), pp. 570–582.
Dudek, H., Ghosh, A. and Greenberg, M.E. 2001. Calcium phosphate transfection of DNA into neurons in primary culture. *Current Protocols in Neuroscience* Chapter 3, p. Unit 3.11.
Dulubova, I., Khvotchev, M., Liu, S., Huryeva, I., Südhof, T.C. and Rizo, J. 2007. Munc18-1 binds directly to the neuronal SNARE complex. *Proceedings of the National Academy of Sciences of the United States of America* 104(8), pp. 2697–2702.
Duman, J.G. and Forte, J.G. 2003. What is the role of SNARE proteins in membrane fusion? *American Journal of Physiology. Cell Physiology* 285(2), pp. C237–49.
El-Husseini, A. el-D., Craven, S.E., Brock, S.C. and Bredt, D.S. 2001. Polarized targeting of peripheral membrane proteins in neurons. *The Journal of Biological Chemistry* 276(48), pp. 44984–44992.
El-Husseini, A.E., Craven, S.E., Chetkovich, D.M., Firestein, B.L., Schnell, E., Aoki, C. and Bredt, D.S. 2000. Dual palmitoylation of PSD-95 mediates its vesiculotubular sorting, postsynaptic targeting, and ion channel clustering. *The Journal of Cell Biology* 148(1), pp. 159–172.
Elias, G.M., Elias, L.A.B., Apostolides, P.F., Kriegstein, A.R. and Nicoll, R.A. 2008. Differential trafficking of AMPA and NMDA receptors by SAP102 and PSD-95 underlies synapse development. *Proceedings of the National Academy of Sciences of the United States of America* 105(52), pp. 20953–20958.
Ernst, A.M., Syed, S.A., Zaki, O., Bottanelli, F., Zheng, H., Hacke, M., Xi, Z., Rivera-Molina, F., Graham, M., Rebane, A.A., Björkholm, P., Baddeley, D., Toomre, D., Pincet, F. and Rothman, J.E. 2018. S-Palmitoylation Sorts Membrane Cargo for Anterograde Transport in the Golgi. *Developmental Cell* 47(4), pp. 479–493.e7.
Eroglu, C., Allen, N.J., Susman, M.W., O'Rourke, N.A., Park, C.Y., Ozkan, E., Chakraborty, C., Mulinyawe, S.B., Annis, D.S., Huberman, A.D., Green, E.M., Lawler, J., Dolmetsch, R., Garcia, K.C., Smith, S.J., Luo, Z.D., Rosenthal, A., Mosher, D.F. and Barres, B.A. 2009. Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. *Cell* 139(2), pp. 380–392.
Eroglu, C. and Barres, B.A. 2010. Regulation of synaptic connectivity by glia. *Nature* 468(7321), pp. 223–231.
Erzurumlu, R.S. and Gaspar, P. 2012. Development and critical period plasticity of the barrel cortex. *The European Journal of Neuroscience* 35(10), pp. 1540–1553.
Eubelen, M., Bostaille, N., Cabochette, P., Gauquier, A., Tebabi, P., Dumitru, A.C., Koehler, M., Gut, P., Alsteens, D., Stainier, D.Y.R., Garcia-Pino, A. and

Vanhollebeke, B. 2018. A molecular mechanism for Wnt ligand-specific signalling. *Science* 361(6403).
Fang, C., Deng, L., Keller, C.A., Fukata, M., Fukata, Y., Chen, G. and Lüscher, B. 2006. GODZ-mediated palmitoylation of GABA(A) receptors is required for normal assembly and function of GABAergic inhibitory synapses. *The Journal of Neuroscience* 26(49), pp. 12758–12768.
Farías, G.G., Alfaro, I.E., Cerpa, W., Grabowski, C.P., Godoy, J.A., Bonansco, C. and Inestrosa, N.C. 2009. Wnt-5a/JNK signalling promotes the clustering of PSD-95 in hippocampal neurons. *The Journal of Biological Chemistry* 284(23), pp. 15857–15866.
Farías, G.G., Vallés, A.S., Colombres, M., Godoy, J.A., Toledo, E.M., Lukas, R.J., Barrantes, F.J. and Inestrosa, N.C. 2007. Wnt-7a induces presynaptic colocalisation of alpha 7-nicotinic acetylcholine receptors and adenomatous polyposis coli in hippocampal neurons. *The Journal of Neuroscience* 27(20), pp. 5313–5325.
Favuzzi, E., Deogracias, R., Marques-Smith, A., Maeso, P., Jezequel, J., Exposito-Alonso, D., Balia, M., Kroon, T., Hinojosa, A.J., F Maraver, E. and Rico, B. 2019. Distinct molecular programs regulate synapse specificity in cortical inhibitory circuits. *Science* 363(6425), pp. 413–417.
Favuzzi, E. and Rico, B. 2018. Molecular diversity underlying cortical excitatory and inhibitory synapse development. *Current Opinion in Neurobiology* 53, pp. 8–15.
Fazzari, P., Paternain, A.V., Valiente, M., Pla, R., Luján, R., Lloyd, K., Lerma, J., Marín, O. and Rico, B. 2010. Control of cortical GABA circuitry development by Nrg1 and ErbB4 signalling. *Nature* 464(7293), pp. 1376–1380.
De Ferrari, G.V., Papassotiropoulos, A., Biechele, T., Wavrant De-Vrieze, F., Avila, M.E., Major, M.B., Myers, A., Sáez, K., Henríquez, J.P., Zhao, A., Wollmer, M.A., Nitsch, R.M., Hock, C., Morris, C.M., Hardy, J. and Moon, R.T. 2007. Common genetic variation within the low-density lipoprotein receptor-related protein 6 and late-onset Alzheimer's disease. *Proceedings of the National Academy of Sciences of the United States of America* 104(22), pp. 9434–9439.
Ferrari, M.E., Bernis, M.E., McLeod, F., Podpolny, M., Coullery, R.P., Casadei, I.M., Salinas, P.C. and Rosso, S.B. 2018. Wnt7b signalling through Frizzled-7 receptor promotes dendrite development by coactivating CaMKII and JNK. *Journal of Cell Science* 131(13).
Fiala, J.C., Feinberg, M., Popov, V. and Harris, K.M. 1998. Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. *The Journal of Neuroscience* 18(21), pp. 8900–8911.
Fox, M.A., Sanes, J.R., Borza, D.-B., Eswarakumar, V.P., Fässler, R., Hudson, B.G., John, S.W.M., Ninomiya, Y., Pedchenko, V., Pfaff, S.L., Rheault, M.N., Sado, Y., Segal, Y., Werle, M.J. and Umemori, H. 2007. Distinct target-derived signals organize formation, maturation, and maintenance of motor nerve terminals. *Cell* 129(1), pp. 179–193.
Friedman, H.V., Bresler, T., Garner, C.C. and Ziv, N.E. 2000. Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron* 27(1), pp. 57–69.

Friedman, L.G., Benson, D.L. and Huntley, G.W. 2015. Cadherin-based transsynaptic networks in establishing and modifying neural connectivity. *Current Topics in Developmental Biology* 112, pp. 415–465.
Fu, M. and Zuo, Y. 2011. Experience-dependent structural plasticity in the cortex. *Trends in Neurosciences* 34(4), pp. 177–187.
Fukata, M., Fukata, Y., Adesnik, H., Nicoll, R.A. and Bredt, D.S. 2004. Identification of PSD-95 palmitoylating enzymes. *Neuron* 44(6), pp. 987–996.
Fukata, Y., Bredt, D.S. and Fukata, M. 2006. Protein palmitoylation by DHHC protein family. In: Kittler, J. T. and Moss, S. J. eds. *The dynamic synapse: molecular methods in ionotropic receptor biology*. Frontiers in Neuroscience. Boca Raton (FL): CRC Press/Taylor & Francis.
Fukata, Y., Dimitrov, A., Boncompain, G., Vielemeyer, O., Perez, F. and Fukata, M. 2013. Local palmitoylation cycles define activity-regulated postsynaptic subdomains. *The Journal of Cell Biology* 202(1), pp. 145–161.
Fukata, Y. and Fukata, M. 2010. Protein palmitoylation in neuronal development and synaptic plasticity. *Nature Reviews. Neuroscience* 11(3), pp. 161–175.
Gagliardi, M., Hernandez, A., McGough, I.J. and Vincent, J.-P. 2014. Inhibitors of endocytosis prevent Wnt/Wingless signalling by reducing the level of basal β-catenin/Armadillo. *Journal of Cell Science* 127(Pt 22), pp. 4918–4926.
Gagliardi, M., Piddini, E. and Vincent, J.-P. 2008. Endocytosis: a positive or a negative influence on Wnt signalling? *Traffic* 9(1), pp. 1–9.
Galli, S., Lopes, D.M., Ammari, R., Kopra, J., Millar, S.E., Gibb, A. and Salinas, P.C. 2014. Deficient Wnt signalling triggers striatal synaptic degeneration and impaired motor behaviour in adult mice. *Nature Communications* 5, p. 4992.
Gambrill, A.C. and Barria, A. 2011. NMDA receptor subunit composition controls synaptogenesis and synapse stabilization. *Proceedings of the National Academy of Sciences of the United States of America* 108(14), pp. 5855–5860.
Gao, C. and Chen, Y.-G. 2010. Dishevelled: The hub of Wnt signalling. *Cellular Signalling* 22(5), pp. 717–727.
Gao, X.B. and van den Pol, A.N. 2000. GABA release from mouse axonal growth cones. *The Journal of Physiology* 523 Pt 3, pp. 629–637.
Gao, Z., Ni, Y., Szabo, G. and Linden, J. 1999. Palmitoylation of the recombinant human A1 adenosine receptor: enhanced proteolysis of palmitoylation-deficient mutant receptors. *The Biochemical Journal* 342 (Pt 2), pp. 387–395.
Garcia, A.D.R., Han, Y.-G., Triplett, J.W., Farmer, W.T., Harwell, C.C. and Ihrie, R.A. 2018. The elegance of sonic hedgehog: emerging novel functions for a classic morphogen. *The Journal of Neuroscience* 38(44), pp. 9338–9345.
García-López, P., García-Marín, V. and Freire, M. 2010. Dendritic spines and development: towards a unifying model of spinogenesis--a present day review of Cajal's histological slides and drawings. *Neural plasticity* 2010, p. 769207.
Gautam, M., Noakes, P.G., Moscoso, L., Rupp, F., Scheller, R.H., Merlie, J.P. and Sanes, J.R. 1996. Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell* 85(4), pp. 525–535.

Ge, Y., Kang, Y., Cassidy, R.M., Moon, K.-M., Lewis, R., Wong, R.O.L., Foster, L.J. and Craig, A.M. 2018. Clptm1 limits forward trafficking of GABAA receptors to scale inhibitory synaptic strength. *Neuron* 97(3), pp. 596–610.e8.
Georgiou, G., Bahra, S.S., Mackie, A.R., Wolfe, C.A., O'Shea, P., Ladha, S., Fernandez, N. and Cherry, R.J. 2002. Measurement of the lateral diffusion of human MHC class I molecules on HeLa cells by fluorescence recovery after photobleaching using a phycoerythrin probe. *Biophysical Journal* 82(4), pp. 1828–1834.
Gerlach, J.P., Jordens, I., Tauriello, D.V.F., van' ' 't Land-Kuper, I., Bugter, J.M., Noordstra, I., van der Kooij, J., Low, T.Y., Pimentel-Muiños, F.X., Xanthakis, D., Fenderico, N., Rabouille, C., Heck, A.J.R., Egan, D.A. and Maurice, M.M. 2018. TMEM59 potentiates Wnt signalling by promoting signalosome formation. *Proceedings of the National Academy of Sciences of the United States of America* 115(17), pp. E3996–E4005.
Gerrow, K., Romorini, S., Nabi, S.M., Colicos, M.A., Sala, C. and El-Husseini, A. 2006. A preformed complex of postsynaptic proteins is involved in excitatory synapse development. *Neuron* 49(4), pp. 547–562.
Gholizadeh, S., Tharmalingam, S., Macaldaz, M.E. and Hampson, D.R. 2013. Transduction of the central nervous system after intracerebroventricular injection of adeno-associated viral vectors in neonatal and juvenile mice. *Human gene therapy methods* 24(4), pp. 205–213.
Giese, K.P., Aziz, W., Kraev, I. and Stewart, M.G. 2015. Generation of multi-innervated dendritic spines as a novel mechanism of long-term memory formation. *Neurobiology of Learning and Memory* 124, pp. 48–51.
Gladding, C.M. and Raymond, L.A. 2011. Mechanisms underlying NMDA receptor synaptic/extrasynaptic distribution and function. *Molecular and Cellular Neurosciences* 48(4), pp. 308–320.
Glaeser, K., Urban, M., Fenech, E., Voloshanenko, O., Kranz, D., Lari, F., Christianson, J.C. and Boutros, M. 2018. ERAD-dependent control of the Wnt secretory factor Evi. *The EMBO Journal* 37(4).
Globa, A.K. and Bamji, S.X. 2017. Protein palmitoylation in the development and plasticity of neuronal connections. *Current Opinion in Neurobiology* 45, pp. 210–220.
Go, G.-W. and Mani, A. 2012. Low-density lipoprotein receptor (LDLR) family orchestrates cholesterol homeostasis. *The Yale journal of biology and medicine* 85(1), pp. 19–28.
Goddard, A.D. and Watts, A. 2012. Regulation of G protein-coupled receptors by palmitoylation and cholesterol. *BMC Biology* 10, p. 27.
Gögel, S., Wakefield, S., Tear, G., Klämbt, C. and Gordon-Weeks, P.R. 2006. The Drosophila microtubule associated protein Futsch is phosphorylated by Shaggy/Zeste-white 3 at an homologous GSK3beta phosphorylation site in MAP1B. *Molecular and Cellular Neurosciences* 33(2), pp. 188–199.
Gogolla, N., Galimberti, I., Deguchi, Y. and Caroni, P. 2009. Wnt signalling mediates experience-related regulation of synapse numbers and mossy fiber connectivities in the adult hippocampus. *Neuron* 62(4), pp. 510–525.
Gomes, R.A., Hampton, C., El-Sabeawy, F., Sabo, S.L. and McAllister, A.K. 2006. The dynamic distribution of TrkB receptors before, during, and after synapse formation between cortical neurons. *The Journal of Neuroscience* 26(44), pp. 11487–11500.

Goodman, R.M., Thombre, S., Firtina, Z., Gray, D., Betts, D., Roebuck, J., Spana, E.P. and Selva, E.M. 2006. Sprinter: a novel transmembrane protein required for Wg secretion and signalling. *Development* 133(24), pp. 4901–4911.
Gordon, M.D. and Nusse, R. 2006. Wnt signalling: multiple pathways, multiple receptors, and multiple transcription factors. *The Journal of Biological Chemistry* 281(32), pp. 22429–22433.
Gottlieb, C.D. and Linder, M.E. 2017. Structure and function of DHHC protein S-acyltransferases. *Biochemical Society Transactions* 45(4), pp. 923–928.
Gottlieb, C.D., Zhang, S. and Linder, M.E. 2015. The Cysteine-rich Domain of the DHHC3 Palmitoyltransferase Is Palmitoylated and Contains Tightly Bound Zinc. *The Journal of Biological Chemistry* 290(49), pp. 29259–29269.
Gottmann, K., Mittmann, T. and Lessmann, V. 2009. BDNF signalling in the formation, maturation and plasticity of glutamatergic and GABAergic synapses. *Experimental Brain Research* 199(3-4), pp. 203–234.
Graf, E.R., Zhang, X., Jin, S.-X., Linhoff, M.W. and Craig, A.M. 2004. Neurexins induce differentiation of GABA and glutamate postsynaptic specializations via neuroligins. *Cell* 119(7), pp. 1013–1026.
Grainger, S., Nguyen, N., Richter, J., Setayesh, J., Lonquich, B., Oon, C.H., Wozniak, J.M., Barahona, R., Kamei, C.N., Houston, J., Carrillo-Terrazas, M., Drummond, I.A., Gonzalez, D., Willert, K. and Traver, D. 2018. EGFR confers exquisite specificity of Wnt9a-Fzd9b signalling in hematopoietic stem cell development. *BioRxiv*.
Grant, B.D. and Donaldson, J.G. 2009. Pathways and mechanisms of endocytic recycling. *Nature Reviews. Molecular Cell Biology* 10(9), pp. 597–608.
Gray, R.S., Roszko, I. and Solnica-Krezel, L. 2011. Planar cell polarity: coordinating morphogenetic cell behaviors with embryonic polarity. *Developmental Cell* 21(1), pp. 120–133.
Greaves, J. and Chamberlain, L.H. 2007. Palmitoylation-dependent protein sorting. *The Journal of Cell Biology* 176(3), pp. 249–254.
Greaves, J., Gorleku, O.A., Salaun, C. and Chamberlain, L.H. 2010. Palmitoylation of the SNAP25 protein family: specificity and regulation by DHHC palmitoyl transferases. *The Journal of Biological Chemistry* 285(32), pp. 24629–24638.
Green, J., Nusse, R. and van Amerongen, R. 2014. The role of Ryk and Ror receptor tyrosine kinases in Wnt signal transduction. *Cold Spring Harbor Perspectives in Biology* 6(2).
Greger, I.H., Watson, J.F. and Cull-Candy, S.G. 2017. Structural and Functional Architecture of AMPA-Type Glutamate Receptors and Their Auxiliary Proteins. *Neuron* 94(4), pp. 713–730.
Groc, L., Heine, M., Cognet, L., Brickley, K., Stephenson, F.A., Lounis, B. and Choquet, D. 2004. Differential activity-dependent regulation of the lateral mobilities of AMPA and NMDA receptors. *Nature Neuroscience* 7(7), pp. 695–696.
Gross, J.C., Chaudhary, V., Bartscherer, K. and Boutros, M. 2012. Active Wnt proteins are secreted on exosomes. *Nature Cell Biology* 14(10), pp. 1036–1045.
Grutzendler, J., Kasthuri, N. and Gan, W.-B. 2002. Long-term dendritic spine stability in the adult cortex. *Nature* 420(6917), pp. 812–816.

Guan, X. and Fierke, C.A. 2011. Understanding protein palmitoylation: biological significance and enzymology. *Science China. Chemistry* 54(12), pp. 1888–1897.
Gubb, D. and García-Bellido, A. 1982. A genetic analysis of the determination of cuticular polarity during development in Drosophila melanogaster. *Journal of embryology and experimental morphology* 68, pp. 37–57.
Gundelfinger, E.D., Reissner, C. and Garner, C.C. 2015. Role of Bassoon and Piccolo in Assembly and Molecular Organisation of the Active Zone. *Frontiers in synaptic neuroscience* 7, p. 19.
Guo, M., Chandris, P., Giannini, J.P., Trexler, A.J., Fischer, R., Chen, J., Vishwasrao, H.D., Rey-Suarez, I., Wu, Y., Wu, X., Waterman, C.M., Patterson, G.H., Upadhyaya, A., Taraska, J.W. and Shroff, H. 2018. Single-shot super-resolution total internal reflection fluorescence microscopy. *Nature Methods* 15(6), pp. 425–428.
Hagemann, A.I.H., Kurz, J., Kauffeld, S., Chen, Q., Reeves, P.M., Weber, S., Schindler, S., Davidson, G., Kirchhausen, T. and Scholpp, S. 2014. In vivo analysis of formation and endocytosis of the Wnt/β-Catenin signalling complex in zebrafish embryos. *Journal of Cell Science* 127(24), p. 5331.
Hall, A.C., Lucas, F.R. and Salinas, P.C. 2000. Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signalling. *Cell* 100(5), pp. 525–535.
Hall, B.J. and Ghosh, A. 2008. Regulation of AMPA receptor recruitment at developing synapses. *Trends in Neurosciences* 31(2), pp. 82–89.
Hall, B.J., Ripley, B. and Ghosh, A. 2007. NR2B signalling regulates the development of synaptic AMPA receptor current. *The Journal of Neuroscience* 27(49), pp. 13446–13456.
Hallermann, S., Fejtova, A., Schmidt, H., Weyhersmüller, A., Silver, R.A., Gundelfinger, E.D. and Eilers, J. 2010. Bassoon speeds vesicle reloading at a central excitatory synapse. *Neuron* 68(4), pp. 710–723.
Halleskog, C., Mulder, J., Dahlström, J., Mackie, K., Hortobágyi, T., Tanila, H., Kumar Puli, L., Färber, K., Harkany, T. and Schulte, G. 2011. WNT signalling in activated microglia is proinflammatory. *Glia* 59(1), pp. 119–131.
Han, J., Pluhackova, K. and Böckmann, R.A. 2017. The multifaceted role of SNARE proteins in membrane fusion. *Frontiers in physiology* 8, p. 5.
Hanley, J.G. 2008. AMPA receptor trafficking pathways and links to dendritic spine morphogenesis. *Cell Adhesion & Migration* 2(4), pp. 276–282.
Hao, H.-X., Jiang, X. and Cong, F. 2016. Control of Wnt Receptor Turnover by R-spondin-ZNRF3/RNF43 Signalling Module and Its Dysregulation in Cancer. *Cancers* 8(6).
Hao, H.-X., Xie, Y., Zhang, Y., Charlat, O., Oster, E., Avello, M., Lei, H., Mickanin, C., Liu, D., Ruffner, H., Mao, X., Ma, Q., Zamponi, R., Bouwmeester, T., Finan, P.M., Kirschner, M.W., Porter, J.A., Serluca, F.C. and Cong, F. 2012. ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Natur*e 485(7397), pp. 195–200.
Harterink, M., Port, F., Lorenowicz, M.J., McGough, I.J., Silhankova, M., Betist, M.C., van Weering, J.R.T., van Heesbeen, R.G.H.P., Middelkoop, T.C., Basler, K., Cullen, P.J. and Korswagen, H.C. 2011. A SNX3-dependent retromer pathway mediates retrograde transport of the Wnt sorting receptor

Wntless and is required for Wnt secretion. *Nature Cell Biology* 13(8), pp. 914–923.
Harvey, K. and Marchetti, B. 2014. Regulating Wnt signalling: a strategy to prevent neurodegeneration and induce regeneration. *Journal of Molecular Cell Biology* 6(1), pp. 1–2.
Harward, S.C., Hedrick, N.G., Hall, C.E., Parra-Bueno, P., Milner, T.A., Pan, E., Laviv, T., Hempstead, B.L., Yasuda, R. and McNamara, J.O. 2016. Autocrine BDNF-TrkB signalling within a single dendritic spine. *Nature* 538(7623), pp. 99–103.
Harwell, C.C., Parker, P.R.L., Gee, S.M., Okada, A., McConnell, S.K., Kreitzer, A.C. and Kriegstein, A.R. 2012. Sonic hedgehog expression in corticofugal projection neurons directs cortical microcircuit formation. *Neuron* 73(6), pp. 1116–1126.
Hausmann, G., Bänziger, C. and Basler, K. 2007. Helping Wingless take flight: how WNT proteins are secreted. *Nature Reviews. Molecular Cell Biology* 8(4), pp. 331–336.
Häusser, M., Spruston, N. and Stuart, G.J. 2000. Diversity and dynamics of dendritic signalling. *Science* 290(5492), pp. 739–744.
Hayashi, T., Rumbaugh, G. and Huganir, R.L. 2005. Differential regulation of AMPA receptor subunit trafficking by palmitoylation of two distinct sites. *Neuron* 47(5), pp. 709–723.
Hayashi, T., Thomas, G.M. and Huganir, R.L. 2009. Dual palmitoylation of NR2 subunits regulates NMDA receptor trafficking. *Neuron* 64(2), pp. 213–226.
Hazell, G.G.J., Hindmarch, C.C., Pope, G.R., Roper, J.A., Lightman, S.L., Murphy, D., O'Carroll, A.-M. and Lolait, S.J. 2012. G protein-coupled receptors in the hypothalamic paraventricular and supraoptic nuclei--serpentine gateways to neuroendocrine homeostasis. *Frontiers in Neuroendocrinology* 33(1), pp. 45–66.
He, C.-W., Liao, C.-P. and Pan, C.-L. 2018. Wnt signalling in the development of axon, dendrites and synapses. *Open biology* 8(10).
He, C.X., Arroyo, E.D., Cantu, D.A., Goel, A. and Portera-Cailliau, C. 2018. A versatile method for viral transfection of calcium indicators in the neonatal mouse brain. *Frontiers in Neural Circuits* 12, p. 56.
He, X., Semenov, M., Tamai, K. and Zeng, X. 2004. LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signalling: arrows point the way. *Development* 131(8), pp. 1663–1677.
Heine, M., Groc, L., Frischknecht, R., Béïque, J.-C., Lounis, B., Rumbaugh, G., Huganir, R.L., Cognet, L. and Choquet, D. 2008. Surface mobility of postsynaptic AMPARs tunes synaptic transmission. *Science* 320(5873), pp. 201–205.
Henderson, B.R. and Fagotto, F. 2002. The ins and outs of APC and beta-catenin nuclear transport. *EMBO Reports* 3(9), pp. 834–839.
Henkemeyer, M., Itkis, O.S., Ngo, M., Hickmott, P.W. and Ethell, I.M. 2003. Multiple EphB receptor tyrosine kinases shape dendritic spines in the hippocampus. *The Journal of Cell Biology* 163(6), pp. 1313–1326.
Henriquez, J.P., Webb, A., Bence, M., Bildsoe, H., Sahores, M., Hughes, S.M. and Salinas, P.C. 2008. Wnt signalling promotes AChR aggregation at the neuromuscular synapse in collaboration with agrin. *Proceedings of the*

National Academy of Sciences of the United States of America 105(48), pp. 18812–18817.
Hering, H. and Sheng, M. 2001. Dendritic spines: structure, dynamics and regulation. *Nature Reviews. Neuroscience* 2(12), pp. 880–888.
Hernández, A.R., Klein, A.M. and Kirschner, M.W. 2012. Kinetic responses of β-catenin specify the sites of Wnt control. S*cience* 338(6112), pp. 1337–1340.
Hernandez, J.L., Majmudar, J.D. and Martin, B.R. 2013. Profiling and inhibiting reversible palmitoylation. C*urrent Opinion in Chemical Biology* 17(1), pp. 20–26.
Herring, B.E. and Nicoll, R.A. 2016. Long-Term Potentiation: From CaMKII to AMPA Receptor Trafficking. A*nnual Review of Physiology* 78, pp. 351–365.
Hiester, B.G., Galati, D.F., Salinas, P.C. and Jones, K.R. 2013. Neurotrophin and Wnt signalling cooperatively regulate dendritic spine formation. M*olecular and Cellular Neurosciences* 56, pp. 115–127.
Hildick, K.L., González-González, I.M., Jaskolski, F. and Henley, J.M. 2012. Lateral diffusion and exocytosis of membrane proteins in cultured neurons assessed using fluorescence recovery and fluorescence-loss photobleaching. *Journal of Visualized Experiments* (60).
Hirabayashi, Y., Itoh, Y., Tabata, H., Nakajima, K., Akiyama, T., Masuyama, N. and Gotoh, Y. 2004. The Wnt/beta-catenin pathway directs neuronal differentiation of cortical neural precursor cells. *Development* 131(12), pp. 2791–2801.
Ho, V.M., Lee, J.-A. and Martin, K.C. 2011. The cell biology of synaptic plasticity. *Science* 334(6056), pp. 623–628.
Holland, S.M., Collura, K.M., Ketschek, A., Noma, K., Ferguson, T.A., Jin, Y., Gallo, G. and Thomas, G.M. 2016. Palmitoylation controls DLK localisation, interactions and activity to ensure effective axonal injury signalling. *Proceedings of the National Academy of Sciences of the United States of America* 113(3), pp. 763–768.
Holland, S.M. and Thomas, G.M. 2017. Roles of palmitoylation in axon growth, degeneration and regeneration. *Journal of Neuroscience Research* 95(8), pp. 1528–1539.
Holtmaat, A. and Caroni, P. 2016. Functional and structural underpinnings of neuronal assembly formation in learning. *Nature Neuroscience* 19(12), pp. 1553–1562.
Holtmaat, A. and Svoboda, K. 2009. Experience-dependent structural synaptic plasticity in the mammalian brain. *Nature Reviews. Neuroscience* 10(9), pp. 647–658.
Hong, E.J., McCord, A.E. and Greenberg, M.E. 2008. A biological function for the neuronal activity-dependent component of Bdnf transcription in the development of cortical inhibition. *Neuron* 60(4), pp. 610–624.
Hong, S., Dissing-Olesen, L. and Stevens, B. 2016. New insights on the role of microglia in synaptic pruning in health and disease. *Current Opinion in Neurobiology* 36, pp. 128–134.
Hornemann, T. 2015. Palmitoylation and depalmitoylation defects. *Journal of Inherited Metabolic Disease* 38(1), pp. 179–186.
Hoseth, E.Z., Krull, F., Dieset, I., Mørch, R.H., Hope, S., Gardsjord, E.S., Steen, N.E., Melle, I., Brattbakk, H.-R., Steen, V.M., Aukrust, P., Djurovic, S., Andreassen, O.A. and Ueland, T. 2018. Exploring the Wnt signalling pathway in schizophrenia and bipolar disorder. *Translational psychiatry* 8(1), p. 55.

Hruska, M. and Dalva, M.B. 2012. Ephrin regulation of synapse formation, function and plasticity. *Molecular and Cellular Neurosciences* 50(1), pp. 35–44.
Hua, J.Y. and Smith, S.J. 2004. Neural activity and the dynamics of central nervous system development. *Nature Neuroscience* 7(4), pp. 327–332.
Hua, Z.L., Jeon, S., Caterina, M.J. and Nathans, J. 2014. Frizzled3 is required for the development of multiple axon tracts in the mouse central nervous system. *Proceedings of the National Academy of Sciences of the United States of Americ*a 111(29), pp. E3005–14.
Huang, H.-C. and Klein, P.S. 2004. The Frizzled family: receptors for multiple signal transduction pathways. *Genome Biology* 5(7), p. 234.
Huang, K., Sanders, S., Singaraja, R., Orban, P., Cijsouw, T., Arstikaitis, P., Yanai, A., Hayden, M.R. and El-Husseini, A. 2009. Neuronal palmitoyl acyl transferases exhibit distinct substrate specificity. *The FASEB Journal* 23(8), pp. 2605–2615.
Huganir, R.L. and Nicoll, R.A. 2013. AMPARs and synaptic plasticity: the last 25 years. *Neuron* 80(3), pp. 704–717.
Hume, R.I., Role, L.W. and Fischbach, G.D. 1983. Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membranes. *Nature* 305(5935), pp. 632–634.
Hunt, D.L. and Castillo, P.E. 2012. Synaptic plasticity of NMDA receptors: mechanisms and functional implications. *Current Opinion in Neurobiology* 22(3), pp. 496–508.
Huntwork, S. and Littleton, J.T. 2007. A complexin fusion clamp regulates spontaneous neurotransmitter release and synaptic growth. *Nature Neuroscience* 10(10), pp. 1235–1237.
Husi, H., Ward, M.A., Choudhary, J.S., Blackstock, W.P. and Grant, S.G. 2000. Proteomic analysis of NMDA receptor-adhesion protein signalling complexes. *Nature Neuroscience* 3(7), pp. 661–669.
Huttenlocher, P.R. 1990. Morphometric study of human cerebral cortex development. *Neuropsychologia* 28(6), pp. 517–527.
Huttenlocher, P.R. and Dabholkar, A.S. 1997. Regional differences in synaptogenesis in human cerebral cortex. *The Journal of Comparative Neurology* 387(2), pp. 167–178.
Ikeya, M., Lee, S.M., Johnson, J.E., McMahon, A.P. and Takada, S. 1997. Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature* 389(6654), pp. 966–970.
Inestrosa, N.C. and Arenas, E. 2010. Emerging roles of Wnts in the adult nervous system. *Nature Reviews. Neuroscience* 11(2), pp. 77–86.
Inestrosa, N.C. and Varela-Nallar, L. 2014. Wnt signalling in the nervous system and in Alzheimer's disease. *Journal of Molecular Cell Biology* 6(1), pp. 64–74.
Isaac, J.T., Nicoll, R.A. and Malenka, R.C. 1995. Evidence for silent synapses: implications for the expression of LTP. *Neuron* 15(2), pp. 427–434.
Jaaro-Peled, H., Ayhan, Y., Pletnikov, M.V. and Sawa, A. 2010. Review of pathological hallmarks of schizophrenia: comparison of genetic models with patients and nongenetic models. *Schizophrenia Bulletin* 36(2), pp. 301–313.
Jamieson, C., Sharma, M. and Henderson, B.R. 2012. Wnt signalling from membrane to nucleus: β-catenin caught in a loop. *The International Journal of Biochemistry & Cell Biology* 44(6), pp. 847–850.

Jammalamadaka, A., Banerjee, S., Manjunath, B.S. and Kosik, K.S. 2013. Statistical analysis of dendritic spine distributions in rat hippocampal cultures. *BMC Bioinformatics* 14, p. 287.
Janda, C.Y. and Garcia, K.C. 2015. Wnt acylation and its functional implication in Wnt signalling regulation. *Biochemical Society Transactions* 43(2), pp. 211–216.
Janda, C.Y., Waghray, D., Levin, A.M., Thomas, C. and Garcia, K.C. 2012. Structural basis of Wnt recognition by Frizzled. *Science* 337(6090), pp. 59–64.
Jennings, B.C. and Linder, M.E. 2012. DHHC protein S-acyltransferases use similar ping-pong kinetic mechanisms but display different acyl-CoA specificities. *The Journal of Biological Chemistry* 287(10), pp. 7236–7245.
Jeyifous, O., Lin, E.I., Chen, X., Antinone, S.E., Mastro, R., Drisdel, R., Reese, T.S. and Green, W.N. 2016. Palmitoylation regulates glutamate receptor distributions in postsynaptic densities through control of PSD95 conformation and orientation. *Proceedings of the National Academy of Sciences of the United States of America* 113(52), pp. E8482–E8491.
Jing, L., Lefebvre, J.L., Gordon, L.R. and Granato, M. 2009. Wnt signals organize synaptic prepattern and axon guidance through the zebrafish unplugged/MuSK receptor. *Neuron* 61(5), pp. 721–733.
Johansen, J.P., Cain, C.K., Ostroff, L.E. and LeDoux, J.E. 2011. Molecular mechanisms of fear learning and memory. *Cell* 147(3), pp. 509–524.
Johnson, M.L. and Rajamannan, N. 2006. Diseases of Wnt signalling. *Reviews in Endocrine & Metabolic Disorders* 7(1-2), pp. 41–49.
Jontes, J.D. and Smith, S.J. 2000. Filopodia, spines, and the generation of synaptic diversity. *Neuron* 27(1), pp. 11–14.
Jovanovic, J.N., Czernik, A.J., Fienberg, A.A., Greengard, P. and Sihra, T.S. 2000. Synapsins as mediators of BDNF-enhanced neurotransmitter release. *Nature Neuroscience* 3(4), pp. 323–329.
Ju, W., Morishita, W., Tsui, J., Gaietta, G., Deerinck, T.J., Adams, S.R., Garner, C.C., Tsien, R.Y., Ellisman, M.H. and Malenka, R.C. 2004. Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. *Nature Neuroscience* 7(3), pp. 244–253.
Kakugawa, S., Langton, P.F., Zebisch, M., Howell, S., Chang, T.-H., Liu, Y., Feizi, T., Bineva, G., O'Reilly, N., Snijders, A.P., Jones, E.Y. and Vincent, J.-P. 2015. Notum deacylates Wnt proteins to suppress signalling activity. *Nature* 519(7542), pp. 187–192.
Kang, H. and Schuman, E.M. 1995. Long-lasting neurotrophin-induced enhancement of synaptic transmission in the adult hippocampus. *Science* 267(5204), pp. 1658–1662.
Kang, R., Swayze, R., Lise, M.F., Gerrow, K., Mullard, A., Honer, W.G. and El-Husseini, A. 2004. Presynaptic trafficking of synaptotagmin I is regulated by protein palmitoylation. *The Journal of Biological Chemistry* 279(48), pp. 50524–50536.
Katanaev, V.L. and Buestorf, S. 2009. The G protein-coupled receptor identity of the frizzled proteins. *Cell Communication and Signalling* 7(Suppl 1), p. A19.
Katoh, Masuko and Katoh, Masaru 2017. Molecular genetics and targeted therapy of WNT-related human diseases (Review). *International Journal of Molecular Medicine* 40(3), pp. 587–606.

Katoh, Masuko and Katoh, Masaru 2007. WNT signalling pathway and stem cell signalling network. *Clinical Cancer Research* 13(14), pp. 4042–4045.
Kawano, Y. and Kypta, R. 2003. Secreted antagonists of the Wnt signalling pathway. *Journal of Cell Science* 116(Pt 13), pp. 2627–2634.
Kayed, R. and Lasagna-Reeves, C.A. 2013. Molecular mechanisms of amyloid oligomers toxicity. *Journal of Alzheimer's Disease* 33 Suppl 1, pp. S67–78.
Kayser, M.S., Nolt, M.J. and Dalva, M.B. 2008. EphB receptors couple dendritic filopodia motility to synapse formation. *Neuron* 59(1), pp. 56–69.
Keeble, T.R., Halford, M.M., Seaman, C., Kee, N., Macheda, M., Anderson, R.B., Stacker, S.A. and Cooper, H.M. 2006. The Wnt receptor Ryk is required for Wnt5a-mediated axon guidance on the contralateral side of the corpus callosum. *The Journal of Neuroscience* 26(21), pp. 5840–5848.
Khoury, G.A., Baliban, R.C. and Floudas, C.A. 2011. Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. *Scientific reports* 1.
Kiecker, C. and Niehrs, C. 2001. A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in Xenopus. *Development* 128(21), pp. 4189–4201.
Kilander, M.B.C., Dahlström, J. and Schulte, G. 2014. Assessment of Frizzled 6 membrane mobility by FRAP supports G protein coupling and reveals WNT-Frizzled selectivity. *Cellular Signalling* 26(9), pp. 1943–1949.
Kilpatrick, C.L., Murakami, S., Feng, M., Wu, X., Lal, R., Chen, G., Du, K. and Luscher, B. 2016. Dissociation of Golgi-associated DHHC-type Zinc Finger Protein (GODZ)- and Sertoli Cell Gene with a Zinc Finger Domain-β (SERZ-β)-mediated Palmitoylation by Loss of Function Analyses in Knock-out Mice. *The Journal of Biological Chemistry* 291(53), pp. 27371–27386.
Kim, E. and Sheng, M. 2009. The postsynaptic density. *Current Biology* 19(17), pp. R723–4.
Kim, J.H., Lee, S.-R., Li, L.-H., Park, H.-J., Park, J.-H., Lee, K.Y., Kim, M.-K., Shin, B.A. and Choi, S.-Y. 2011. High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice. *Plos One* 6(4), p. e18556.
Kim, J.-Y., Ash, R.T., Ceballos-Diaz, C., Levites, Y., Golde, T.E., Smirnakis, S.M. and Jankowsky, J.L. 2013. Viral transduction of the neonatal brain delivers controllable genetic mosaicism for visualising and manipulating neuronal circuits in vivo. *The European Journal of Neuroscience* 37(8), pp. 1203–1220.
Kim, J.-Y., Grunke, S.D., Levites, Y., Golde, T.E. and Jankowsky, J.L. 2014. Intracerebroventricular viral injection of the neonatal mouse brain for persistent and widespread neuronal transduction. *Journal of Visualized Experiments* (91), p. 51863.
Kim, M.J., Dunah, A.W., Wang, Y.T. and Sheng, M. 2005. Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signalling and AMPA receptor trafficking. *Neuron* 46(5), pp. 745–760.
Kim, N., Stiegler, A.L., Cameron, T.O., Hallock, P.T., Gomez, A.M., Huang, J.H., Hubbard, S.R., Dustin, M.L. and Burden, S.J. 2008. Lrp4 is a receptor for Agrin and forms a complex with MuSK. *Cell* 135(2), pp. 334–342.

Kim, S., Kim, H. and Um, J.W. 2018. Synapse development organized by neuronal activity-regulated immediate-early genes. *Experimental & Molecular Medicine* 50(4), p. 11.
Kim, S., Lee, B.-C., Lee, A.-R., Won, S. and Park, C.-S. 2014. Effects of palmitoylation on the diffusional movement of BKCa channels in live cells. *FEBS Letters* 588(5), pp. 713–719.
Kimelberg, H.K. 1995. Receptors on astrocytes--what possible functions? *Neurochemistry International* 26(1), pp. 27–40.
Kimelman, D. and Xu, W. 2006. beta-catenin destruction complex: insights and questions from a structural perspective. *Oncogene* 25(57), pp. 7482–7491.
Kirszenblat, L., Pattabiraman, D. and Hilliard, M.A. 2011. LIN-44/Wnt directs dendrite outgrowth through LIN-17/Frizzled in C. elegans Neurons. *PLoS Biology* 9(9), p. e1001157.
Klassen, M.P. and Shen, K. 2007. Wnt signalling positions neuromuscular connectivity by inhibiting synapse formation in C. elegans. *Cell* 130(4), pp. 704–716.
Knott, G.W., Holtmaat, A., Wilbrecht, L., Welker, E. and Svoboda, K. 2006. Spine growth precedes synapse formation in the adult neocortex in vivo. *Nature Neuroscience* 9(9), pp. 1117–1124.
Knott, G.W., Quairiaux, C., Genoud, C. and Welker, E. 2002. Formation of dendritic spines with GABAergic synapses induced by whisker stimulation in adult mice. *Neuron* 34(2), pp. 265–273.
Koles, K. and Budnik, V. 2012. Wnt signalling in neuromuscular junction development. *Cold Spring Harbor Perspectives in Biology* 4(6).
Koles, K., Nunnari, J., Korkut, C., Barria, R., Brewer, C., Li, Y., Leszyk, J., Zhang, B. and Budnik, V. 2012. Mechanism of evenness interrupted (Evi)-exosome release at synaptic boutons. *The Journal of Biological Chemistry* 287(20), pp. 16820–16834.
Kollmann, K., Uusi-Rauva, K., Scifo, E., Tyynelä, J., Jalanko, A. and Braulke, T. 2013. Cell biology and function of neuronal ceroid lipofuscinosis-related proteins. *Biochimica et Biophysica Acta* 1832(11), pp. 1866–1881.
Komekado, H., Yamamoto, H., Chiba, T. and Kikuchi, A. 2007. Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. *Genes To Cells* 12(4), pp. 521–534.
Komiya, Y. and Habas, R. 2008. Wnt signal transduction pathways. *Organogenesis* 4(2), pp. 68–75.
Koo, B.-K., Spit, M., Jordens, I., Low, T.Y., Stange, D.E., van de Wetering, M., van Es, J.H., Mohammed, S., Heck, A.J.R., Maurice, M.M. and Clevers, H. 2012. Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. *Nature* 488(7413), pp. 665–669.
Korkut, C., Ataman, B., Ramachandran, P., Ashley, J., Barria, R., Gherbesi, N. and Budnik, V. 2009. Trans-synaptic transmission of vesicular Wnt signals through Evi/Wntless. *Cell* 139(2), pp. 393–404.
Kornau, H.C., Schenker, L.T., Kennedy, M.B. and Seeburg, P.H. 1995. Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science* 269(5231), pp. 1737–1740.
Koticha, D.K., McCarthy, E.E. and Baldini, G. 2002. Plasma membrane targeting of SNAP-25 increases its local concentration and is necessary for

SNARE complex formation and regulated exocytosis. *Journal of Cell Science* 115(Pt 16), pp. 3341–3351.
Koval, A. and Katanaev, V.L. 2011. Wnt3a stimulation elicits G-protein-coupled receptor properties of mammalian Frizzled proteins. *The Biochemical Journal* 433(3), pp. 435–440.
Kowiański, P., Lietzau, G., Czuba, E., Waśkow, M., Steliga, A. and Moryś, J. 2018. BDNF: A Key Factor with Multipotent Impact on Brain Signalling and Synaptic Plasticity. *Cellular and Molecular Neurobiology* 38(3), pp. 579–593.
Krylova, O., Herreros, J., Cleverley, K.E., Ehler, E., Henriquez, J.P., Hughes, S.M. and Salinas, P.C. 2002. WNT-3, expressed by motoneurons, regulates terminal arborization of neurotrophin-3-responsive spinal sensory neurons. *Neuron* 35(6), pp. 1043–1056.
Kucukdereli, H., Allen, N.J., Lee, A.T., Feng, A., Ozlu, M.I., Conatser, L.M., Chakraborty, C., Workman, G., Weaver, M., Sage, E.H., Barres, B.A. and Eroglu, C. 2011. Control of excitatory CNS synaptogenesis by astrocyte-secreted proteins Hevin and SPARC. *Proceedings of the National Academy of Sciences of the United States of America* 108(32), pp. E440–9.
Kühl, M., Sheldahl, L.C., Malbon, C.C. and Moon, R.T. 2000. Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus. *The Journal of Biological Chemistry* 275(17), pp. 12701–12711.
Kurayoshi, M., Yamamoto, H., Izumi, S. and Kikuchi, A. 2007. Post-translational palmitoylation and glycosylation of Wnt-5a are necessary for its signalling. *The Biochemical Journal* 402(3), pp. 515–523.
Kurshan, P.T., Merrill, S.A., Dong, Y., Ding, C., Hammarlund, M., Bai, J., Jorgensen, E.M. and Shen, K. 2018. γ-Neurexin and Frizzled Mediate Parallel Synapse Assembly Pathways Antagonized by Receptor Endocytosis. *Neuron* 100(1).
Kwon, H.-B. and Sabatini, B.L. 2011. Glutamate induces de novo growth of functional spines in developing cortex. *Nature* 474(7349), pp. 100–104.
Kyttälä, A., Lahtinen, U., Braulke, T. and Hofmann, S.L. 2006. Functional biology of the neuronal ceroid lipofuscinoses (NCL) proteins. *Biochimica et Biophysica Acta* 1762(10), pp. 920–933.
Lanoue, V., Langford, M., White, A., Sempert, K., Fogg, L. and Cooper, H.M. 2017. The Wnt receptor Ryk is a negative regulator of mammalian dendrite morphogenesis. *Scientific reports* 7(1), p. 5965.
Lau, C.G. and Zukin, R.S. 2007. NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. *Nature Reviews. Neuroscience* 8(6), pp. 413–426.
De Lau, W., Peng, W.C., Gros, P. and Clevers, H. 2014. The R-spondin/Lgr5/Rnf43 module: regulator of Wnt signal strength. *Genes & Development* 28(4), pp. 305–316.
Leal, G., Bramham, C.R. and Duarte, C.B. 2017. BDNF and hippocampal synaptic plasticity. *Vitamins and Hormones* 104, pp. 153–195.
Lee, R.T.H., Zhao, Z. and Ingham, P.W. 2016. Hedgehog signalling. *Development* 143(3), pp. 367–372.
Lendvai, B., Stern, E.A., Chen, B. and Svoboda, K. 2000. Experience-dependent plasticity of dendritic spines in the developing rat barrel cortex in vivo. *Nature* 404(6780), pp. 876–881.

Leonzino, M., Busnelli, M., Antonucci, F., Verderio, C., Mazzanti, M. and Chini, B. 2016. The Timing of the Excitatory-to-Inhibitory GABA Switch Is Regulated by the Oxytocin Receptor via KCC2. *Cell reports* 15(1), pp. 96–103.
Lepeta, K., Lourenco, M.V., Schweitzer, B.C., Martino Adami, P.V., Banerjee, P., Catuara-Solarz, S., de La Fuente Revenga, M., Guillem, A.M., Haidar, M., Ijomone, O.M., Nadorp, B., Qi, L., Perera, N.D., Refsgaard, L.K., Reid, K.M., Sabbar, M., Sahoo, A., Schaefer, N., Sheean, R.K., Suska, A., Verma, R., Vicidomini, C., Wright, D., Zhang, X.-D. and Seidenbecher, C. 2016. Synaptopathies: synaptic dysfunction in neurological disorders - A review from students to students. *Journal of Neurochemistry* 138(6), pp. 785–805.
Lessmann, V. and Brigadski, T. 2009. Mechanisms, locations, and kinetics of synaptic BDNF secretion: an update. *Neuroscience Research* 65(1), pp. 11–22.
Levental, I., Lingwood, D., Grzybek, M., Coskun, U. and Simons, K. 2010. Palmitoylation regulates raft affinity for the majority of integral raft proteins. *Proceedings of the National Academy of Sciences of the United States of America* 107(51), pp. 22050–22054.
Levine, E.S., Dreyfus, C.F., Black, I.B. and Plummer, M.R. 1995. Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. *Proceedings of the National Academy of Sciences of the United States of America* 92(17), pp. 8074–8077.
Leyns, L., Bouwmeester, T., Kim, S.H., Piccolo, S. and De Robertis, E.M. 1997. Frzb-1 is a secreted antagonist of Wnt signalling expressed in the Spemann organizer. *Cell* 88(6), pp. 747–756.
Li, J., Ashley, J., Budnik, V. and Bhat, M.A. 2007. Crucial role of Drosophila neurexin in proper active zone apposition to postsynaptic densities, synaptic growth, and synaptic transmission. *Neuron* 55(5), pp. 741–755.
Li, J., Hassan, G.S., Williams, T.M., Minetti, C., Pestell, R.G., Tanowitz, H.B., Frank, P.G., Sotgia, F. and Lisanti, M.P. 2005. Loss of caveolin-1 causes the hyper-proliferation of intestinal crypt stem cells, with increased sensitivity to whole body gamma-radiation. *Cell Cycle* 4(12), pp. 1817–1825.
Li, V.S.W., Ng, S.S., Boersema, P.J., Low, T.Y., Karthaus, W.R., Gerlach, J.P., Mohammed, S., Heck, A.J.R., Maurice, M.M., Mahmoudi, T. and Clevers, H. 2012. Wnt signalling through inhibition of β-catenin degradation in an intact Axin1 complex. *Cell* 149(6), pp. 1245–1256.
Li, Y., Hu, J., Höfer, K., Wong, A.M.S., Cooper, J.D., Birnbaum, S.G., Hammer, R.E. and Hofmann, S.L. 2010. DHHC5 interacts with PDZ domain 3 of post-synaptic density-95 (PSD-95) protein and plays a role in learning and memory. *The Journal of Biological Chemistry* 285(17), pp. 13022–13031.
Liang, J., Xu, W., Hsu, Y.T., Yee, A.X., Chen, L. and Südhof, T.C. 2015. Conditional neuroligin-2 knockout in adult medial prefrontal cortex links chronic changes in synaptic inhibition to cognitive impairments. *Molecular Psychiatry* 20(7), pp. 850–859.
Liao, D., Hessler, N.A. and Malinow, R. 1995. Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature* 375(6530), pp. 400–404.
Lie, D.-C., Colamarino, S.A., Song, H.-J., Désiré, L., Mira, H., Consiglio, A., Lein, E.S., Jessberger, S., Lansford, H., Dearie, A.R. and Gage, F.H. 2005.

Wnt signalling regulates adult hippocampal neurogenesis. *Nature* 437(7063), pp. 1370–1375.
Liebner, S., Corada, M., Bangsow, T., Babbage, J., Taddei, A., Czupalla, C.J., Reis, M., Felici, A., Wolburg, H., Fruttiger, M., Taketo, M.M., von Melchner, H., Plate, K.H., Gerhardt, H. and Dejana, E. 2008. Wnt/beta-catenin signalling controls development of the blood-brain barrier. *The Journal of Cell Biology* 183(3), pp. 409–417.
Lien, W.-H. and Fuchs, E. 2014. Wnt some lose some: transcriptional governance of stem cells by Wnt/β-catenin signalling. *Genes & Development* 28(14), pp. 1517–1532.
Lin, D.-T., Makino, Y., Sharma, K., Hayashi, T., Neve, R., Takamiya, K. and Huganir, R.L. 2009. Regulation of AMPA receptor extrasynaptic insertion by 4.1N, phosphorylation and palmitoylation. *Nature Neuroscience* 12(7), pp. 879–887.
Lin, D.T.S. and Conibear, E. 2015. ABHD17 proteins are novel protein depalmitoylases that regulate N-Ras palmitate turnover and subcellular localisation. *eLife* 4, p. e11306.
Lin, K., Wang, S., Julius, M.A., Kitajewski, J., Moos, M. and Luyten, F.P. 1997. The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signalling. *Proceedings of the National Academy of Sciences of the United States of America* 94(21), pp. 11196–11200.
Linder, M.E. and Deschenes, R.J. 2007. Palmitoylation: policing protein stability and traffic. *Nature Reviews. Molecular Cell Biology* 8(1), pp. 74–84.
Lisman, J., Yasuda, R. and Raghavachari, S. 2012. Mechanisms of CaMKII action in long-term potentiation. *Nature Reviews. Neuroscience* 13(3), pp. 169–182.
Liu, C., Wang, Y., Smallwood, P.M. and Nathans, J. 2008. An essential role for Frizzled5 in neuronal survival in the parafascicular nucleus of the thalamus. *The Journal of Neuroscience* 28(22), pp. 5641–5653.
Liu, C.-C., Tsai, C.-W., Deak, F., Rogers, J., Penuliar, M., Sung, Y.M., Maher, J.N., Fu, Y., Li, X., Xu, H., Estus, S., Hoe, H.-S., Fryer, J.D., Kanekiyo, T. and Bu, G. 2014. Deficiency in LRP6-mediated Wnt signalling contributes to synaptic abnormalities and amyloid pathology in Alzheimer's disease. *Neuron* 84(1), pp. 63–77.
Liu, X.-B., Murray, K.D. and Jones, E.G. 2004. Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. *The Journal of Neuroscience* 24(40), pp. 8885–8895.
Liu, Y.C., Chapman, E.R. and Storm, D.R. 1991. Targeting of neuromodulin (GAP-43) fusion proteins to growth cones in cultured rat embryonic neurons. *Neuron* 6(3), pp. 411–420.
Logan, C.Y. and Nusse, R. 2004. The Wnt signalling pathway in development and disease. *Annual Review of Cell and Developmental Biology* 20, pp. 781–810.
Lohmann, C. and Bonhoeffer, T. 2008. A role for local calcium signalling in rapid synaptic partner selection by dendritic filopodia. *Neuron* 59(2), pp. 253–260.
Lohof, A.M., Ip, N.Y. and Poo, M.M. 1993. Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. *Nature* 363(6427), pp. 350–353.

Lu, B. 2003. BDNF and activity-dependent synaptic modulation. *Learning & Memory* 10(2), pp. 86–98.
Lu, B., Wang, K.H. and Nose, A. 2009. Molecular mechanisms underlying neural circuit formation. *Current Opinion in Neurobiology* 19(2), pp. 162–167.
Lu, W., Bushong, E.A., Shih, T.P., Ellisman, M.H. and Nicoll, R.A. 2013. The cell-autonomous role of excitatory synaptic transmission in the regulation of neuronal structure and function. *Neuron* 78(3), pp. 433–439.
Lu, W., Shi, Y., Jackson, A.C., Bjorgan, K., During, M.J., Sprengel, R., Seeburg, P.H. and Nicoll, R.A. 2009. Subunit composition of synaptic AMPA receptors revealed by a single-cell genetic approach. *Neuron* 62(2), pp. 254–268.
Lucas, F.R. and Salinas, P.C. 1997. WNT-7a induces axonal remodeling and increases synapsin I levels in cerebellar neurons. *Developmental Biology* 192(1), pp. 31–44.
Luo, Z.G., Wang, Q., Zhou, J.Z., Wang, J., Luo, Z., Liu, M., He, X., Wynshaw-Boris, A., Xiong, W.C., Lu, B. and Mei, L. 2002. Regulation of AChR clustering by Dishevelled interacting with MuSK and PAK1. *Neuron* 35(3), pp. 489–505.
Lüscher, C. and Malenka, R.C. 2012. NMDA receptor-dependent long-term potentiation and long-term depression (LTP/LTD). *Cold Spring Harbor Perspectives in Biology* 4(6).
Lyu, J., Yamamoto, V. and Lu, W. 2008. Cleavage of the Wnt receptor Ryk regulates neuronal differentiation during cortical neurogenesis. *Developmental Cell* 15(5), pp. 773–780.
Lyuksyutova, A.I., Lu, C.-C., Milanesio, N., King, L.A., Guo, N., Wang, Y., Nathans, J., Tessier-Lavigne, M. and Zou, Y. 2003. Anterior-posterior guidance of commissural axons by Wnt-frizzled signalling. *Science* 302(5652), pp. 1984–1988.
MacDonald, Bryan T and He, X. 2012a. A finger on the pulse of Wnt receptor signalling. *Cell Research* 22(10), pp. 1410–1412.
MacDonald, B T and He, X. 2012. Frizzled and LRP5/6 receptors for Wnt/beta-catenin signalling. *Cold Spring Harb Perspect Biol* 4(12).
MacDonald, Bryan T and He, X. 2012b. Frizzled and LRP5/6 receptors for Wnt/β-catenin signalling. *Cold Spring Harbor Perspectives in Biology* 4(12).
MacDonald, B.T., Tamai, K. and He, X. 2009. Wnt/beta-catenin signalling: components, mechanisms, and diseases. *Developmental Cell* 17(1), pp. 9–26.
MacDonald, B.T., Yokota, C., Tamai, K., Zeng, X. and He, X. 2008. Wnt signal amplification via activity, cooperativity, and regulation of multiple intracellular PPPSP motifs in the Wnt co-receptor LRP6. *The Journal of Biological Chemistry* 283(23), pp. 16115–16123.
Madan, B., Walker, M.P., Young, R., Quick, L., Orgel, K.A., Ryan, M., Gupta, P., Henrich, I.C., Ferrer, M., Marine, S., Roberts, B.S., Arthur, W.T., Berndt, J.D., Oliveira, A.M., Moon, R.T., Virshup, D.M., Chou, M.M. and Major, M.B. 2016. USP6 oncogene promotes Wnt signalling by deubiquitylating Frizzleds. *Proceedings of the National Academy of Sciences of the United States of America* 113(21), pp. E2945–54.
Maguschak, K.A. and Ressler, K.J. 2008. Beta-catenin is required for memory consolidation. *Nature Neuroscience* 11(11), pp. 1319–1326.

Maguschak, K.A. and Ressler, K.J. 2011. Wnt signalling in amygdala-dependent learning and memory. *The Journal of Neuroscience* 31(37), pp. 13057–13067.
Makino, H. and Malinow, R. 2009. AMPA receptor incorporation into synapses during LTP: the role of lateral movement and exocytosis. *Neuron* 64(3), pp. 381–390.
Malenka, R.C. and Bear, M.F. 2004. LTP and LTD: an embarrassment of riches. *Neuron* 44(1), pp. 5–21.
Maletic-Savatic, M., Malinow, R. and Svoboda, K. 1999. Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. *Science* 283(5409), pp. 1923–1927.
Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mechler, B.M., Delius, H., Hoppe, D., Stannek, P., Walter, C., Glinka, A. and Niehrs, C. 2002. Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. *Nature* 417(6889), pp. 664–667.
Markham, J.A. and Greenough, W.T. 2004. Experience-driven brain plasticity: beyond the synapse. *Neuron Glia Biology* 1(4), pp. 351–363.
Martin, S., Henley, J.M., Holman, D., Zhou, M., Wiegert, O., van Spronsen, M., Joëls, M., Hoogenraad, C.C. and Krugers, H.J. 2009. Corticosterone alters AMPAR mobility and facilitates bidirectional synaptic plasticity. *Plos One* 4(3), p. e4714.
Martínez, A., Alcántara, S., Borrell, V., Del Río, J.A., Blasi, J., Otal, R., Campos, N., Boronat, A., Barbacid, M., Silos-Santiago, I. and Soriano, E. 1998. TrkB and TrkC signalling are required for maturation and synaptogenesis of hippocampal connections. *The Journal of Neuroscience* 18(18), pp. 7336–7350.
Marzo, A., Galli, S., Lopes, D., McLeod, F., Podpolny, M., Segovia-Roldan, M., Ciani, L., Purro, S., Cacucci, F., Gibb, A. and Salinas, P.C. 2016. Reversal of synapse degeneration by restoring wnt signalling in the adult hippocampus. *Current Biology* 26(19), pp. 2551–2561.
Mason, J.O., Kitajewski, J. and Varmus, H.E. 1992. Mutational analysis of mouse Wnt-1 identifies two temperature-sensitive alleles and attributes of Wnt-1 protein essential for transformation of a mammary cell line. *Molecular Biology of the Cell* 3(5), pp. 521–533.
Massey, P.V., Johnson, B.E., Moult, P.R., Auberson, Y.P., Brown, M.W., Molnar, E., Collingridge, G.L. and Bashir, Z.I. 2004. Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. *The Journal of Neuroscience* 24(36), pp. 7821–7828.
Mathew, D., Ataman, B., Chen, J., Zhang, Y., Cumberledge, S. and Budnik, V. 2005. Wingless signalling at synapses is through cleavage and nuclear import of receptor DFrizzled2. *Science* 310(5752), pp. 1344–1347.
Matsuzaki, M., Ellis-Davies, G.C., Nemoto, T., Miyashita, Y., Iino, M. and Kasai, H. 2001. Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. *Nature Neuroscience* 4(11), pp. 1086–1092.
Matt, L., Kim, K., Chowdhury, D. and Hell, J.W. 2019. Role of palmitoylation of postsynaptic proteins in promoting synaptic plasticity. *Frontiers in Molecular Neuroscience* 12, p. 8.

McAllister, A.K. 2007. Dynamic aspects of CNS synapse formation. *Annual Review of Neuroscience* 30, pp. 425–450.
McAllister, A.K., Katz, L.C. and Lo, D.C. 1999. Neurotrophins and synaptic plasticity. *Annual Review of Neuroscience* 22, pp. 295–318.
McCormick, P.J., Dumaresq-Doiron, K., Pluviose, A.-S., Pichette, V., Tosato, G. and Lefrancois, S. 2008. Palmitoylation controls recycling in lysosomal sorting and trafficking. *Traffic* 9(11), pp. 1984–1997.
McIntosh, H.H., Song, C. and Howlett, A.C. 1998. CB1 cannabinoid receptor: cellular regulation and distribution in N18TG2 neuroblastoma cells. *Brain research. Molecular brain research* 53(1-2), pp. 163–173.
McLean, J.R., Smith, G.A., Rocha, E.M., Hayes, M.A., Beagan, J.A., Hallett, P.J. and Isacson, O. 2014. Widespread neuron-specific transgene expression in brain and spinal cord following synapsin promoter-driven AAV9 neonatal intracerebroventricular injection. *Neuroscience Letters* 576, pp. 73–78.
McLeod, F., Bossio, A., Marzo, A., Ciani, L., Sibilla, S., Hannan, S., Wilson, G.A., Palomer, E., Smart, T.G., Gibb, A. and Salinas, P.C. 2018. Wnt Signalling Mediates LTP-Dependent Spine Plasticity and AMPAR Localisation through Frizzled-7 Receptors. *Cell reports* 23(4), pp. 1060–1071.
McLeod, F., Marzo, A., Podpolny, M., Galli, S. and Salinas, P. 2017. Evaluation of synapse density in hippocampal rodent brain slices. *Journal of Visualized Experiments* (128).
McLeod, F. and Salinas, P.C. 2018. Wnt proteins as modulators of synaptic plasticity. *Current Opinion in Neurobiology* 53, pp. 90–95.
McQuate, A., Latorre-Esteves, E. and Barria, A. 2017. A wnt/calcium signalling cascade regulates neuronal excitability and trafficking of nmdars. *Cell reports* 21(1), pp. 60–69.
Megason, S.G. and McMahon, A.P. 2002. A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* 129(9), pp. 2087–2098.
Mei, L. and Xiong, W.-C. 2008. Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. *Nature Reviews. Neuroscience* 9(6), pp. 437–452.
Meldrum, B.S. 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. *The Journal of Nutrition* 130(4S Suppl), p. 1007S–15S.
Melkonian, K.A., Ostermeyer, A.G., Chen, J.Z., Roth, M.G. and Brown, D.A. 1999. Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. Many raft proteins are acylated, while few are prenylated. *The Journal of Biological Chemistry* 274(6), pp. 3910–3917.
Messaoudi, E., Ying, S.-W., Kanhema, T., Croll, S.D. and Bramham, C.R. 2002. Brain-derived neurotrophic factor triggers transcription-dependent, late phase long-term potentiation in vivo. *The Journal of Neuroscience* 22(17), pp. 7453–7461.
Meyer-Franke, A., Kaplan, M.R., Pfrieger, F.W. and Barres, B.A. 1995. Characterization of the signalling interactions that promote the survival and growth of developing retinal ganglion cells in culture. *Neuron* 15(4), pp. 805–819.
Midorikawa, M. and Sakaba, T. 2015. Imaging exocytosis of single synaptic vesicles at a fast CNS presynaptic terminal. *Neuron* 88(3), pp. 492–498.

Miesenböck, G., De Angelis, D.A. and Rothman, J.E. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* 394(6689), pp. 192–195.
Milenkovic, L., Scott, M.P. and Rohatgi, R. 2009. Lateral transport of Smoothened from the plasma membrane to the membrane of the cilium. *The Journal of Cell Biology* 187(3), pp. 365–374.
Miller, P.S. and Aricescu, A.R. 2014. Crystal structure of a human GABAA receptor. *Nature* 512(7514), pp. 270–275.
Milnerwood, A.J., Parsons, M.P., Young, F.B., Singaraja, R.R., Franciosi, S., Volta, M., Bergeron, S., Hayden, M.R. and Raymond, L.A. 2013. Memory and synaptic deficits in Hip14/DHHC17 knockout mice. *Proceedings of the National Academy of Sciences of the United States of America* 110(50), pp. 20296–20301.
Minami, Y., Oishi, I., Endo, M. and Nishita, M. 2010. Ror-family receptor tyrosine kinases in noncanonical Wnt signalling: their implications in developmental morphogenesis and human diseases. *Developmental Dynamics* 239(1), pp. 1–15.
Missler, M., Zhang, W., Rohlmann, A., Kattenstroth, G., Hammer, R.E., Gottmann, K. and Südhof, T.C. 2003. Alpha-neurexins couple Ca2+ channels to synaptic vesicle exocytosis. *Nature* 423(6943), pp. 939–948.
Mitchell, N., Petralia, R.S., Currier, D.G., Wang, Y.-X., Kim, A., Mattson, M.P. and Yao, P.J. 2012. Sonic hedgehog regulates presynaptic terminal size, ultrastructure and function in hippocampal neurons. *Journal of Cell Science* 125(Pt 18), pp. 4207–4213.
Moffat, L.L., Robinson, R.E., Bakoulis, A. and Clark, S.G. 2014. The conserved transmembrane RING finger protein PLR-1 downregulates Wnt signalling by reducing Frizzled, Ror and Ryk cell-surface levels in C. elegans. *Development* 141(3), pp. 617–628.
Montersino, A. and Thomas, G.M. 2015. Slippery signalling: Palmitoylation-dependent control of neuronal kinase localisation and activity. *Molecular membrane biology* 32(5-8), pp. 179–188.
Monyer, H., Burnashev, N., Laurie, D.J., Sakmann, B. and Seeburg, P.H. 1994. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12(3), pp. 529–540.
Morales, J., Benavides-Piccione, R., Dar, M., Fernaud, I., Rodríguez, A., Anton-Sanchez, L., Bielza, C., Larrañaga, P., DeFelipe, J. and Yuste, R. 2014. Random positions of dendritic spines in human cerebral cortex. *The Journal of Neuroscience* 34(30), pp. 10078–10084.
Moss, S.J. and Smart, T.G. 2001. Constructing inhibitory synapses. *Nature Reviews. Neuroscience* 2(4), pp. 240–250.
Mukai, A., Yamamoto-Hino, M., Awano, W., Watanabe, W., Komada, M. and Goto, S. 2010. Balanced ubiquitylation and deubiquitylation of Frizzled regulate cellular responsiveness to Wg/Wnt. *The EMBO Journal* 29(13), pp. 2114–2125.
Mukherjee, K., Yang, X., Gerber, S.H., Kwon, H.-B., Ho, A., Castillo, P.E., Liu, X. and Südhof, T.C. 2010. Piccolo and bassoon maintain synaptic vesicle clustering without directly participating in vesicle exocytosis. *Proceedings of the National Academy of Sciences of the United States of America* 107(14), pp. 6504–6509.

Mulligan, K.A. and Cheyette, B.N.R. 2017. Neurodevelopmental perspectives on wnt signalling in psychiatry. *Molecular neuropsychiatry* 2(4), pp. 219–246.
Mulligan, K.A., Fuerer, C., Ching, W., Fish, M., Willert, K. and Nusse, R. 2012. Secreted Wingless-interacting molecule (Swim) promotes long-range signalling by maintaining Wingless solubility. *Proceedings of the National Academy of Sciences of the United States of America* 109(2), pp. 370–377.
Munno, D.W. and Syed, N.I. 2003. Synaptogenesis in the CNS: an odyssey from wiring together to firing together. *The Journal of Physiology* 552(Pt 1), pp. 1–11.
Nadolski, M.J. and Linder, M.E. 2007. Protein lipidation. *The FEBS Journal* 274(20), pp. 5202–5210.
Nakamura, R.E.I. and Hackam, A.S. 2010. Analysis of Dickkopf3 interactions with Wnt signalling receptors. *Growth Factors* 28(4), pp. 232–242.
Narisawa-Saito, M., Iwakura, Y., Kawamura, M., Araki, K., Kozaki, S., Takei, N. and Nawa, H. 2002. Brain-derived neurotrophic factor regulates surface expression of alpha-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid receptors by enhancing the N-ethylmaleimide-sensitive factor/GluR2 interaction in developing neocortical neurons. *The Journal of Biological Chemistry* 277(43), pp. 40901–40910.
Naumenko, V.S. and Ponimaskin, E. 2018. Palmitoylation as a functional regulator of neurotransmitter receptors. *Neural plasticity* 2018, p. 5701348.
Niehrs, C. 2006. Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene* 25(57), pp. 7469–7481.
Niell, C.M., Meyer, M.P. and Smith, S.J. 2004. In vivo imaging of synapse formation on a growing dendritic arbor. *Nature Neuroscience* 7(3), pp. 254–260.
Nile, A.H. and Hannoush, R.N. 2018. Fatty acid recognition in the Frizzled receptor family. *The Journal of Biological Chemistry*.
Nile, A.H. and Hannoush, R.N. 2016. Fatty acylation of Wnt proteins. *Nature Chemical Biology* 12(2), pp. 60–69.
Nile, A.H., Mukund, S., Stanger, K., Wang, W. and Hannoush, R.N. 2017. Unsaturated fatty acyl recognition by Frizzled receptors mediates dimerization upon Wnt ligand binding. *Proceedings of the National Academy of Sciences of the United States of America* 114(16), pp. 4147–4152.
Nimchinsky, E.A., Sabatini, B.L. and Svoboda, K. 2002. Structure and function of dendritic spines. *Annual Review of Physiology* 64, pp. 313–353.
Nithianantharajah, J. and Hannan, A.J. 2006. Enriched environments, experience-dependent plasticity and disorders of the nervous system. *Nature Reviews. Neuroscience* 7(9), pp. 697–709.
Noritake, J., Fukata, Y., Iwanaga, T., Hosomi, N., Tsutsumi, R., Matsuda, N., Tani, H., Iwanari, H., Mochizuki, Y., Kodama, T., Matsuura, Y., Bredt, D.S., Hamakubo, T. and Fukata, M. 2009. Mobile DHHC palmitoylating enzyme mediates activity-sensitive synaptic targeting of PSD-95. *The Journal of Cell Biology* 186(1), pp. 147–160.
Nusse, R. 2005. Wnt signalling in disease and in development. *Cell Research* 15(1), pp. 28–32.
Nusse, R. 2012. Wnt signalling. *Cold Spring Harbor Perspectives in Biology* 4(5).
Nusse, R. and Clevers, H. 2017. Wnt/β-Catenin Signalling, Disease, and Emerging Therapeutic Modalities. *Cell* 169(6), pp. 985–999.

Nusse, R. and Varmus, H.E. 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 31(1), pp. 99–109.
Oh, W.C., Lutzu, S., Castillo, P.E. and Kwon, H.-B. 2016. De novo synaptogenesis induced by GABA in the developing mouse cortex. *Science* 353(6303), pp. 1037–1040.
Ohno, Y., Kashio, A., Ogata, R., Ishitomi, A., Yamazaki, Y. and Kihara, A. 2012. Analysis of substrate specificity of human DHHC protein acyltransferases using a yeast expression system. *Molecular Biology of the Cell* 23(23), pp. 4543–4551.
Ohno, Y., Kihara, A., Sano, T. and Igarashi, Y. 2006. Intracellular localisation and tissue-specific distribution of human and yeast DHHC cysteine-rich domain-containing proteins. *Biochimica et Biophysica Acta* 1761(4), pp. 474–483.
Okada, A., Charron, F., Morin, S., Shin, D.S., Wong, K., Fabre, P.J., Tessier-Lavigne, M. and McConnell, S.K. 2006. Boc is a receptor for sonic hedgehog in the guidance of commissural axons. *Nature* 444(7117), pp. 369–373.
Oliva, C.A., Vargas, J.Y. and Inestrosa, N.C. 2013a. Wnt signalling: role in LTP, neural networks and memory. *Ageing Research Reviews* 12(3), pp. 786–800.
Oliva, C.A., Vargas, J.Y. and Inestrosa, N.C. 2013b. Wnts in adult brain: from synaptic plasticity to cognitive deficiencies. *Frontiers in Cellular Neuroscience* 7, p. 224.
Olsen, R.W. and Sieghart, W. 2009. GABA A receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology* 56(1), pp. 141–148.
Ornitz, D.M. and Itoh, N. 2001. Fibroblast growth factors. *Genome Biology* 2(3), p. REVIEWS3005.
Ornitz, D.M. and Itoh, N. 2015. The Fibroblast Growth Factor signalling pathway. *Wiley interdisciplinary reviews. Developmental biology* 4(3), pp. 215–266.
Packard, M., Koo, E.S., Gorczyca, M., Sharpe, J., Cumberledge, S. and Budnik, V. 2002. The Drosophila Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. *Cell* 111(3), pp. 319–330.
Paganoni, S., Bernstein, J. and Ferreira, A. 2010. Ror1-Ror2 complexes modulate synapse formation in hippocampal neurons. *Neuroscience* 165(4), pp. 1261–1274.
Palma, V., Lim, D.A., Dahmane, N., Sánchez, P., Brionne, T.C., Herzberg, C.D., Gitton, Y., Carleton, A., Alvarez-Buylla, A. and Ruiz i Altaba, A. 2005. Sonic hedgehog controls stem cell behavior in the postnatal and adult brain. *Development* 132(2), pp. 335–344.
Palop, J.J., Chin, J. and Mucke, L. 2006. A network dysfunction perspective on neurodegenerative diseases. *Nature* 443(7113), pp. 768–773.
Palop, J.J. and Mucke, L. 2010. Amyloid-beta-induced neuronal dysfunction in Alzheimer's disease: from synapses toward neural networks. *Nature Neuroscience* 13(7), pp. 812–818.
Pan, W., Choi, S.-C., Wang, H., Qin, Y., Volpicelli-Daley, L., Swan, L., Lucast, L., Khoo, C., Zhang, X., Li, L., Abrams, C.S., Sokol, S.Y. and Wu, D. 2008. Wnt3a-mediated formation of phosphatidylinositol 4,5-bisphosphate regulates LRP6 phosphorylation. *Science* 321(5894), pp. 1350–1353.

Panaccione, I., Napoletano, F., Forte, A.M., Kotzalidis, G.D., Del Casale, A., Rapinesi, C., Brugnoli, C., Serata, D., Caccia, F., Cuomo, I., Ambrosi, E., Simonetti, A., Savoja, V., De Chiara, L., Danese, E., Manfredi, G., Janiri, D., Motolese, M., Nicoletti, F., Girardi, P. and Sani, G. 2013. Neurodevelopment in schizophrenia: the role of the wnt pathways. *Current neuropharmacology* 11(5), pp. 535–558.
Park, H. and Poo, M. 2013. Neurotrophin regulation of neural circuit development and function. *Nature Reviews. Neuroscience* 14(1), pp. 7–23.
Park, M. 2018. AMPA receptor trafficking for postsynaptic potentiation. *Frontiers in Cellular Neuroscience* 12, p. 361.
Park, M. and Shen, K. 2012. WNTs in synapse formation and neuronal circuitry. *The EMBO Journal* 31(12), pp. 2697–2704.
Parodi, J., Montecinos-Oliva, C., Varas, R., Alfaro, I.E., Serrano, F.G., Varas-Godoy, M., Muñoz, F.J., Cerpa, W., Godoy, J.A. and Inestrosa, N.C. 2015. Wnt5a inhibits K(+) currents in hippocampal synapses through nitric oxide production. *Molecular and Cellular Neurosciences* 68, pp. 314–322.
Passini, M.A. and Wolfe, J.H. 2001. Widespread gene delivery and structure-specific patterns of expression in the brain after intraventricular injections of neonatal mice with an adeno-associated virus vector. *Journal of Virology* 75(24), pp. 12382–12392.
Patterson, S.L., Abel, T., Deuel, T.A., Martin, K.C., Rose, J.C. and Kandel, E.R. 1996. Recombinant BDNF rescues deficits in basal synaptic transmission and hippocampal LTP in BDNF knockout mice. *Neuron* 16(6), pp. 1137–1145.
Percherancier, Y., Planchenault, T., Valenzuela-Fernandez, A., Virelizier, J.L., Arenzana-Seisdedos, F. and Bachelerie, F. 2001. Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. *The Journal of Biological Chemistry* 276(34), pp. 31936–31944.
Perea, G., Navarrete, M. and Araque, A. 2009. Tripartite synapses: astrocytes process and control synaptic information. *Trends in Neurosciences* 32(8), pp. 421–431.
Perrody, E., Abrami, L., Feldman, M., Kunz, B., Urbé, S. and van der Goot, F.G. 2016. Ubiquitin-dependent folding of the Wnt signalling coreceptor LRP6. *eLife* 5.
Pickard, L., Noël, J., Henley, J.M., Collingridge, G.L. and Molnar, E. 2000. Developmental changes in synaptic AMPA and NMDA receptor distribution and AMPA receptor subunit composition in living hippocampal neurons. *The Journal of Neuroscience* 20(21), pp. 7922–7931.
Del Pino, I., Rico, B. and Marín, O. 2018. Neural circuit dysfunction in mouse models of neurodevelopmental disorders. *Current Opinion in Neurobiology* 48, pp. 174–182.
Pinson, K.I., Brennan, J., Monkley, S., Avery, B.J. and Skarnes, W.C. 2000. An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 407(6803), pp. 535–538.
Platt, S.R. 2007. The role of glutamate in central nervous system health and disease--a review. *Veterinary Journal* 173(2), pp. 278–286.
Polakis, P. 2008. Formation of the blood-brain barrier: Wnt signalling seals the deal. *The Journal of Cell Biology* 183(3), pp. 371–373.
Poon, V.Y., Choi, S. and Park, M. 2013. Growth factors in synaptic function. *Frontiers in synaptic neuroscience* 5, p. 6.

Popescu, G.K. 2012. Modes of glutamate receptor gating. *The Journal of Physiology* 590(1), pp. 73–91.
Port, F., Kuster, M., Herr, P., Furger, E., Bänziger, C., Hausmann, G. and Basler, K. 2008. Wingless secretion promotes and requires retromer-dependent cycling of Wntless. *Nature Cell Biology* 10(2), pp. 178–185.
Porter, J.T. and McCarthy, K.D. 1997. Astrocytic neurotransmitter receptors in situ and in vivo. *Progress in Neurobiology* 51(4), pp. 439–455.
Portera-Cailliau, C., Pan, D.T. and Yuste, R. 2003. Activity-regulated dynamic behavior of early dendritic protrusions: evidence for different types of dendritic filopodia. *The Journal of Neuroscience* 23(18), pp. 7129–7142.
Van Praag, H., Kempermann, G. and Gage, F.H. 2000. Neural consequences of environmental enrichment. *Nature Reviews. Neuroscience* 1(3), pp. 191–198.
Prescott, G.R., Gorleku, O.A., Greaves, J. and Chamberlain, L.H. 2009. Palmitoylation of the synaptic vesicle fusion machinery. *Journal of Neurochemistry* 110(4), pp. 1135–1149.
Pressler, R. and Auvin, S. 2013. Comparison of Brain Maturation among Species: An Example in Translational Research Suggesting the Possible Use of Bumetanide in Newborn. *Frontiers in neurology* 4, p. 36.
Prieto, M.L. and Wollmuth, L.P. 2010. Gating modes in AMPA receptors. *The Journal of Neuroscience* 30(12), pp. 4449–4459.
Prior, I.A. and Hancock, J.F. 2001. Compartmentalization of Ras proteins. *Journal of Cell Science* 114(Pt 9), pp. 1603–1608.
Purro, S.A., Ciani, L., Hoyos-Flight, M., Stamatakou, E., Siomou, E. and Salinas, P.C. 2008. Wnt regulates axon behavior through changes in microtubule growth directionality: a new role for adenomatous polyposis coli. *The Journal of Neuroscience* 28(34), pp. 8644–8654.
Purro, S.A., Dickins, E.M. and Salinas, P.C. 2012. The secreted Wnt antagonist Dickkopf-1 is required for amyloid β-mediated synaptic loss. *The Journal of Neuroscience* 32(10), pp. 3492–3498.
Purro, S.A., Galli, S. and Salinas, P.C. 2014. Dysfunction of Wnt signalling and synaptic disassembly in neurodegenerative diseases. *Journal of Molecular Cell Biology* 6(1), pp. 75–80.
Ramakrishnan, A.-B. and Cadigan, K.M. 2017. Wnt target genes and where to find them. [version 1; peer review: 3 approved]. *F1000Research* 6, p. 746.
Ramírez, V.T., Ramos-Fernández, E., Henríquez, J.P., Lorenzo, A. and Inestrosa, N.C. 2016. Wnt-5a/Frizzled9 Receptor Signalling through the Gαo-Gβγ Complex Regulates Dendritic Spine Formation. *The Journal of Biological Chemistry* 291(36), pp. 19092–19107.
Ramón y Cajal, S. 1909. *Histologie du système nerveux de l'homme & des vertébrés.* Paris :: Maloine,.
Rana, M.S., Lee, C.-J. and Banerjee, A. 2019. The molecular mechanism of DHHC protein acyltransferases. *Biochemical Society Transactions* 47(1), pp. 157–167.
Reichardt, L.F. 2006. Neurotrophin-regulated signalling pathways. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 361(1473), pp. 1545–1564.
Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y. and Yao, X. 2008. CSS-Palm 2.0: an updated software for palmitoylation sites prediction. *Protein Engineering, Design & Selection* 21(11), pp. 639–644.

Resh, M.D. 2013. Covalent lipid modifications of proteins. *Current Biology* 23(10), pp. R431–5.
Resh, M.D. 2016. Fatty acylation of proteins: The long and the short of it. *Progress in lipid research* 63, pp. 120–131.
Resh, M.D. 2006. Palmitoylation of ligands, receptors, and intracellular signalling molecules. *Science's STKE: Signal Transduction Knowledge Environment* 2006(359), p. re14.
Resh, M.D. 2012. Targeting protein lipidation in disease. *Trends in Molecular Medicine* 18(4), pp. 206–214.
Rex, C.S., Lin, C.-Y., Kramár, E.A., Chen, L.Y., Gall, C.M. and Lynch, G. 2007. Brain-derived neurotrophic factor promotes long-term potentiation-related cytoskeletal changes in adult hippocampus. *The Journal of Neuroscience* 27(11), pp. 3017–3029.
Reya, T. and Clevers, H. 2005. Wnt signalling in stem cells and cancer. *Nature* 434(7035), pp. 843–850.
Richards, D.A., Mateos, J.M., Hugel, S., de Paola, V., Caroni, P., Gähwiler, B.H. and McKinney, R.A. 2005. Glutamate induces the rapid formation of spine head protrusions in hippocampal slice cultures. *Proceedings of the National Academy of Sciences of the United States of America* 102(17), pp. 6166–6171.
Rico, B. and Marín, O. 2011. Neuregulin signalling, cortical circuitry development and schizophrenia. *Current Opinion in Genetics & Development* 21(3), pp. 262–270.
Rijsewijk, F., Schuermann, M., Wagenaar, E., Parren, P., Weigel, D. and Nusse, R. 1987. The Drosophila homolog of the mouse mammary oncogene int-1 is identical to the segment polarity gene wingless. *Cell* 50(4), pp. 649–657.
Rivera, C., Voipio, J., Payne, J.A., Ruusuvuori, E., Lahtinen, H., Lamsa, K., Pirvola, U., Saarma, M. and Kaila, K. 1999. The K+/Cl- co-transporter KCC2 renders GABA hyperpolarizing during neuronal maturation. *Nature* 397(6716), pp. 251–255.
Rizo, J. and Xu, J. 2015. The synaptic vesicle release machinery. *Annual review of biophysics* 44, pp. 339–367.
Rizzoli, S.O. 2014. Synaptic vesicle recycling: steps and principles. *The EMBO Journal* 33(8), pp. 788–822.
Robbins, E.M., Krupp, A.J., Perez de Arce, K., Ghosh, A.K., Fogel, A.I., Boucard, A., Südhof, T.C., Stein, V. and Biederer, T. 2010. SynCAM 1 adhesion dynamically regulates synapse number and impacts plasticity and learning. *Neuron* 68(5), pp. 894–906.
Roberts, B.J., Svoboda, R.A., Overmiller, A.M., Lewis, J.D., Kowalczyk, A.P., Mahoney, M.G., Johnson, K.R. and Wahl, J.K. 2016. Palmitoylation of desmoglein 2 is a regulator of assembly dynamics and protein turnover. *The Journal of Biological Chemistry* 291(48), pp. 24857–24865.
Rochefort, N.L. and Konnerth, A. 2012. Dendritic spines: from structure to in vivo function. *EMBO Reports* 13(8), pp. 699–708.
Rodriguez, J., Esteve, P., Weinl, C., Ruiz, J.M., Fermin, Y., Trousse, F., Dwivedy, A., Holt, C. and Bovolenta, P. 2005. SFRP1 regulates the growth of retinal ganglion cell axons through the Fz2 receptor. *Nature Neuroscience* 8(10), pp. 1301–1309.

Rosenberg, M.M., Yang, F., Giovanni, M., Mohn, J.L., Temburni, M.K. and Jacob, M.H. 2008. Adenomatous polyposis coli plays a key role, in vivo, in coordinating assembly of the neuronal nicotinic postsynaptic complex. *Molecular and Cellular Neurosciences* 38(2), pp. 138–152.
Rosso, S.B., Sussman, D., Wynshaw-Boris, A. and Salinas, P.C. 2005. Wnt signalling through Dishevelled, Rac and JNK regulates dendritic development. *Nature Neuroscience* 8(1), pp. 34–42.
Roth, A.F., Feng, Y., Chen, L. and Davis, N.G. 2002. The yeast DHHC cysteine-rich domain protein Akr1p is a palmitoyl transferase. *The Journal of Cell Biology* 159(1), pp. 23–28.
Ruthazer, E.S., Li, J. and Cline, H.T. 2006. Stabilization of axon branch dynamics by synaptic maturation. *The Journal of Neuroscience* 26(13), pp. 3594–3603.
Sabo, S.L., Gomes, R.A. and McAllister, A.K. 2006. Formation of presynaptic terminals at predefined sites along axons. *The Journal of Neuroscience* 26(42), pp. 10813–10825.
Sahores, M., Gibb, A. and Salinas, P.C. 2010. Frizzled-5, a receptor for the synaptic organizer Wnt7a, regulates activity-mediated synaptogenesis. *Development* 137(13), pp. 2215–2225.
Sahores, M. and Salinas, P.C. 2011. Activity-mediated synapse formation a role for Wnt-Fz signalling. *Current Topics in Developmental Biology* 97, pp. 119–136.
Sakata, K., Martinowich, K., Woo, N.H., Schloesser, R.J., Jimenez, D.V., Ji, Y., Shen, L. and Lu, B. 2013. Role of activity-dependent BDNF expression in hippocampal-prefrontal cortical regulation of behavioral perseverance. *Proceedings of the National Academy of Sciences of the United States of America* 110(37), pp. 15103–15108.
Salaün, C., Gould, G.W. and Chamberlain, L.H. 2005. The SNARE proteins SNAP-25 and SNAP-23 display different affinities for lipid rafts in PC12 cells. Regulation by distinct cysteine-rich domains. *The Journal of Biological Chemistry* 280(2), pp. 1236–1240.
Salaun, C., Greaves, J. and Chamberlain, L.H. 2010. The intracellular dynamic of protein palmitoylation. *The Journal of Cell Biology* 191(7), pp. 1229–1238.
Salinas, P.C. 2007. Modulation of the microtubule cytoskeleton: a role for a divergent canonical Wnt pathway. *Trends in Cell Biology* 17(7), pp. 333–342.
Salinas, P.C. 2012. Wnt signalling in the vertebrate central nervous system: from axon guidance to synaptic function. *Cold Spring Harbor Perspectives in Biology* 4(2).
Salinas, P.C. and Zou, Y. 2008. Wnt signalling in neural circuit assembly. *Annual Review of Neuroscience* 31, pp. 339–358.
Salter, M.W. and Stevens, B. 2017. Microglia emerge as central players in brain disease. *Nature Medicine* 23(9), pp. 1018–1027.
Scheiffele, P., Fan, J., Choih, J., Fetter, R. and Serafini, T. 2000. Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 101(6), pp. 657–669.
Schlesinger, A., Shelton, C.A., Maloof, J.N., Meneghini, M. and Bowerman, B. 1999. Wnt pathway components orient a mitotic spindle in the early Caenorhabditis elegans embryo without requiring gene transcription in the responding cell. *Genes & Development* 13(15), pp. 2028–2038.

Schlessinger, K., Hall, A. and Tolwinski, N. 2009. Wnt signalling pathways meet Rho GTPases. *Genes & Development* 23(3), pp. 265–277.
Schnell, E., Sizemore, M., Karimzadegan, S., Chen, L., Bredt, D.S. and Nicoll, R.A. 2002. Direct interactions between PSD-95 and stargazin control synaptic AMPA receptor number. *Proceedings of the National Academy of Sciences of the United States of America* 99(21), pp. 13902–13907.
Schulte, G. and Bryja, V. 2007. The Frizzled family of unconventional G-protein-coupled receptors. *Trends in Pharmacological Sciences* 28(10), pp. 518–525.
Schwarz-Romond, T., Fiedler, M., Shibata, N., Butler, P.J.G., Kikuchi, A., Higuchi, Y. and Bienz, M. 2007. The DIX domain of Dishevelled confers Wnt signalling by dynamic polymerization. *Nature Structural & Molecular Biology* 14(6), pp. 484–492.
Schwarz-Romond, T., Metcalfe, C. and Bienz, M. 2007. Dynamic recruitment of axin by Dishevelled protein assemblies. *Journal of Cell Science* 120(Pt 14), pp. 2402–2412.
Scimemi, A. 2014. Structure, function, and plasticity of GABA transporters. *Frontiers in Cellular Neuroscience* 8, p. 161.
Seaman, M.N.J. 2012. The retromer complex - endosomal protein recycling and beyond. *Journal of Cell Science* 125(Pt 20), pp. 4693–4702.
Selkoe, D.J. 2002. Alzheimer's disease is a synaptic failure. *Science* 298(5594), pp. 789–791.
Semple, B.D., Blomgren, K., Gimlin, K., Ferriero, D.M. and Noble-Haeusslein, L.J. 2013. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. *Progress in Neurobiology* 106-107, pp. 1–16.
Seto, E.S. and Bellen, H.J. 2006. Internalization is required for proper Wingless signalling in Drosophila melanogaster. *The Journal of Cell Biology* 173(1), pp. 95–106.
Sharma, K., Choi, S.-Y., Zhang, Y., Nieland, T.J.F., Long, S., Li, M. and Huganir, R.L. 2013. High-throughput genetic screen for synaptogenic factors: identification of LRP6 as critical for excitatory synapse development. *Cell reports* 5(5), pp. 1330–1341.
Sharma, R.P. and Chopra, V.L. 1976. Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. *Developmental Biology* 48(2), pp. 461–465.
Sheldahl, L.C., Slusarski, D.C., Pandur, P., Miller, J.R., Kühl, M. and Moon, R.T. 2003. Dishevelled activates Ca2+ flux, PKC, and CamKII in vertebrate embryos. *The Journal of Cell Biology* 161(4), pp. 769–777.
Sheng, M. 2001. The postsynaptic NMDA-receptor--PSD-95 signalling complex in excitatory synapses of the brain. *Journal of Cell Science* 114(Pt 7), p. 1251.
Sheng, M., Cummings, J., Roldan, L.A., Jan, Y.N. and Jan, L.Y. 1994. Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature* 368(6467), pp. 144–147.
Sheng, M. and Kim, E. 2011. The postsynaptic organisation of synapses. *Cold Spring Harbor Perspectives in Biology* 3(12).
Shigeri, Y., Seal, R.P. and Shimamoto, K. 2004. Molecular pharmacology of glutamate transporters, EAATs and VGLUTs. *Brain Research. Brain Research Reviews* 45(3), pp. 250–265.

Shimogori, T., VanSant, J., Paik, E. and Grove, E.A. 2004. Members of the Wnt, Fz, and Frp gene families expressed in postnatal mouse cerebral cortex. *The Journal of Comparative Neurology* 473(4), pp. 496–510.
Siddiqui, T.J. and Craig, A.M. 2011. Synaptic organizing complexes. *Current Opinion in Neurobiology* 21(1), pp. 132–143.
Sigler, A., Oh, W.C., Imig, C., Altas, B., Kawabe, H., Cooper, B.H., Kwon, H.-B., Rhee, J.-S. and Brose, N. 2017. Formation and maintenance of functional spines in the absence of presynaptic glutamate release. *Neuron* 94(2), pp. 304–311.e4.
Simons, K. and Toomre, D. 2000. Lipid rafts and signal transduction. *Nature Reviews. Molecular Cell Biology* 1(1), pp. 31–39.
Singh, S.K., Stogsdill, J.A., Pulimood, N.S., Dingsdale, H., Kim, Y.H., Pilaz, L.-J., Kim, I.H., Manhaes, A.C., Rodrigues, W.S., Pamukcu, A., Enustun, E., Ertuz, Z., Scheiffele, P., Soderling, S.H., Silver, D.L., Ji, R.-R., Medina, A.E. and Eroglu, C. 2016. Astrocytes assemble thalamocortical synapses by bridging nrx1α and NL1 via hevin. *Cell* 164(1-2), pp. 183–196.
Skene, J.H. and Virág, I. 1989. Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. *The Journal of Cell Biology* 108(2), pp. 613–624.
Sleiman, S.F., Henry, J., Al-Haddad, R., El Hayek, L., Abou Haidar, E., Stringer, T., Ulja, D., Karuppagounder, S.S., Holson, E.B., Ratan, R.R., Ninan, I. and Chao, M.V. 2016. Exercise promotes the expression of brain derived neurotrophic factor (BDNF) through the action of the ketone body β-hydroxybutyrate. *eLife* 5.
Sloniowski, S. and Ethell, I.M. 2012. Looking forward to EphB signalling in synapses. *Seminars in Cell & Developmental Biology* 23(1), pp. 75–82.
Slusarski, D.C., Corces, V.G. and Moon, R.T. 1997. Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. *Nature* 390(6658), pp. 410–413.
Smart, T.G. and Paoletti, P. 2012. Synaptic neurotransmitter-gated receptors. *Cold Spring Harbor Perspectives in Biology* 4(3).
Snyder, S.H. 2017. A life of neurotransmitters. *Annual Review of Pharmacology and Toxicology* 57, pp. 1–11.
Snyder, S.H. 2009. Neurotransmitters, receptors, and second messengers galore in 40 years. *The Journal of Neuroscience* 29(41), pp. 12717–12721.
Sobocińska, J., Roszczenko-Jasińska, P., Ciesielska, A. and Kwiatkowska, K. 2017. Protein palmitoylation and its role in bacterial and viral infections. *Frontiers in immunology* 8, p. 2003.
Sobolevsky, A.I. 2015. Structure and gating of tetrameric glutamate receptors. *The Journal of Physiology* 593(1), pp. 29–38.
Sohn, H. and Park, M. 2019. Palmitoylation-mediated synaptic regulation of AMPA receptor trafficking and function. *Archives of Pharmacal Research*.
Song, H., Kempermann, G., Overstreet Wadiche, L., Zhao, C., Schinder, A.F. and Bischofberger, J. 2005. New neurons in the adult mammalian brain: synaptogenesis and functional integration. *The Journal of Neuroscience* 25(45), pp. 10366–10368.
Song, M., Martinowich, K. and Lee, F.S. 2017. BDNF at the synapse: why location matters. *Molecular Psychiatry* 22(10), pp. 1370–1375.

Soomro, S.H., Jie, J. and Fu, H. 2018. Oligodendrocytes development and wnt signalling pathway. *International Journal of Human Anatomy* 1(3), pp. 17–35.
Sorra, K.E. and Harris, K.M. 2000. Overview on the structure, composition, function, development, and plasticity of hippocampal dendritic spines. *Hippocampus* 10(5), pp. 501–511.
Sotgia, F., Williams, T.M., Cohen, A.W., Minetti, C., Pestell, R.G. and Lisanti, M.P. 2005. Caveolin-1-deficient mice have an increased mammary stem cell population with upregulation of Wnt/beta-catenin signalling. *Cell Cycle* 4(12), pp. 1808–1816.
Soykan, T., Maritzen, T. and Haucke, V. 2016. Modes and mechanisms of synaptic vesicle recycling. *Current Opinion in Neurobiology* 39, pp. 17–23.
Speer, K.F., Sommer, A., Tajer, B., Mullins, M.C., Klein, P.S. and Lemmon, M.A. 2019. Non-acylated Wnts Can Promote Signalling. *Cell reports* 26(4), pp. 875–883.e5.
Speese, S.D., Ashley, J., Jokhi, V., Nunnari, J., Barria, R., Li, Y., Ataman, B., Koon, A., Chang, Y.-T., Li, Q., Moore, M.J. and Budnik, V. 2012. Nuclear envelope budding enables large ribonucleoprotein particle export during synaptic Wnt signalling. *Cell* 149(4), pp. 832–846.
Spitzer, N.C. 2010. How GABA generates depolarization. *The Journal of Physiology* 588(Pt 5), pp. 757–758.
Stagi, M., Fogel, A.I. and Biederer, T. 2010. SynCAM 1 participates in axo-dendritic contact assembly and shapes neuronal growth cones. *Proceedings of the National Academy of Sciences of the United States of America* 107(16), pp. 7568–7573.
Stamatakou, E., Hoyos-Flight, M. and Salinas, P.C. 2015. Wnt Signalling Promotes Actin Dynamics during Axon Remodelling through the Actin-Binding Protein Eps8. *Plos One* 10(8), p. e0134976.
Stamos, J.L. and Weis, W.I. 2013. The β-catenin destruction complex. *Cold Spring Harbor Perspectives in Biology* 5(1), p. a007898.
Stanganello, E. and Scholpp, S. 2016. Role of cytonemes in Wnt transport. *Journal of Cell Science* 129(4), pp. 665–672.
Stefansson, H., Sigurdsson, E., Steinthorsdottir, V., Bjornsdottir, S., Sigmundsson, T., Ghosh, S., Brynjolfsson, J., Gunnarsdottir, S., Ivarsson, O., Chou, T.T., Hjaltason, O., Birgisdottir, B., Jonsson, H., Gudnadottir, V.G., Gudmundsdottir, E., Bjornsson, A., Ingvarsson, B., Ingason, A., Sigfusson, S., Hardardottir, H., Harvey, R.P., Lai, D., Zhou, M., Brunner, D., Mutel, V., Gonzalo, A., Lemke, G., Sainz, J., Johannesson, G., Andresson, T., Gudbjartsson, D., Manolescu, A., Frigge, M.L., Gurney, M.E., Kong, A., Gulcher, J.R., Petursson, H. and Stefansson, K. 2002. Neuregulin 1 and susceptibility to schizophrenia. *American Journal of Human Genetics* 71(4), pp. 877–892.
Stephenson, F. 2001. Subunit characterization of NMDA receptors. *Current Drug Targets* 2(3), pp. 233–239.
Stoop, R. and Poo, M.M. 1996. Synaptic modulation by neurotrophic factors: differential and synergistic effects of brain-derived neurotrophic factor and ciliary neurotrophic factor. *The Journal of Neuroscience* 16(10), pp. 3256–3264.
Strakova, K., Kowalski-Jahn, M., Gybel, T., Valnohova, J., Dhople, V.M., Harnos, J., Bernatik, O., Ganji, R.S., Zdrahal, Z., Mulder, J., Lindskog, C.,

Bryja, V. and Schulte, G. 2018. Dishevelled enables casein kinase 1-mediated phosphorylation of Frizzled 6 required for cell membrane localisation. *The Journal of Biological Chemistry* 293(48), pp. 18477–18493.
Südhof, T.C. 2012a. Calcium control of neurotransmitter release. *Cold Spring Harbor Perspectives in Biology* 4(1), p. a011353.
Südhof, T.C. 2013. Neurotransmitter release: the last millisecond in the life of a synaptic vesicle. *Neuron* 80(3), pp. 675–690.
Südhof, T.C. 2012b. The presynaptic active zone. *Neuron* 75(1), pp. 11–25.
Südhof, T.C. 2018. Towards an understanding of synapse formation. *Neuron* 100(2), pp. 276–293.
Südhof, T.C. and Rizo, J. 2011. Synaptic vesicle exocytosis. *Cold Spring Harbor Perspectives in Biology* 3(12).
Südhof, T.C. and Rothman, J.E. 2009. Membrane fusion: grappling with SNARE and SM proteins. *Science* 323(5913), pp. 474–477.
Sugita, S. 2008. Mechanisms of exocytosis. *Acta Physiologica* 192(2), pp. 185–193.
Sutton, M.A., Ito, H.T., Cressy, P., Kempf, C., Woo, J.C. and Schuman, E.M. 2006. Miniature neurotransmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. *Cell* 125(4), pp. 785–799.
Tabaczar, S., Czogalla, A., Podkalicka, J., Biernatowska, A. and Sikorski, A.F. 2017. Protein palmitoylation: Palmitoyltransferases and their specificity. *Experimental Biology and Medicine*, p. 1535370217707732.
Tabatadze, N., Tomas, C., McGonigal, R., Lin, B., Schook, A. and Routtenberg, A. 2012. Wnt transmembrane signalling and long-term spatial memory. *Hippocampus* 22(6), pp. 1228–1241.
Tada, T. and Sheng, M. 2006. Molecular mechanisms of dendritic spine morphogenesis. *Current Opinion in Neurobiology* 16(1), pp. 95–101.
Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T. and Takada, S. 2006. Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. *Developmental Cell* 11(6), pp. 791–801.
Takada, S., Fujimori, S., Shinozuka, T., Takada, R. and Mii, Y. 2017. Differences in the secretion and transport of Wnt proteins. *Journal of Biochemistry* 161(1), pp. 1–7.
Takahashi, T. and Momiyama, A. 1993. Different types of calcium channels mediate central synaptic transmission. *Nature* 366(6451), pp. 156–158.
Tamai, K., Zeng, X., Liu, C., Zhang, X., Harada, Y., Chang, Z. and He, X. 2004. A mechanism for Wnt coreceptor activation. *Molecular Cell* 13(1), pp. 149–156.
Tang, S.-J. 2014. Synaptic activity-regulated Wnt signalling in synaptic plasticity, glial function and chronic pain. *CNS & Neurological Disorders Drug Targets* 13(5), pp. 737–744.
Tauriello, D.V.F., Jordens, I., Kirchner, K., Slootstra, J.W., Kruitwagen, T., Bouwman, B.A.M., Noutsou, M., Rüdiger, S.G.D., Schwamborn, K., Schambony, A. and Maurice, M.M. 2012. Wnt/β-catenin signalling requires interaction of the Dishevelled DEP domain and C terminus with a discontinuous motif in Frizzled. *Proceedings of the National Academy of Sciences of the United States of America* 109(14), pp. E812–20.
Temburni, M.K., Rosenberg, M.M., Pathak, N., McConnell, R. and Jacob, M.H. 2004. Neuronal nicotinic synapse assembly requires the adenomatous

polyposis coli tumor suppressor protein. *The Journal of Neuroscience* 24(30), pp. 6776–6784.
Terauchi, A., Johnson-Venkatesh, E.M., Toth, A.B., Javed, D., Sutton, M.A. and Umemori, H. 2010. Distinct FGFs promote differentiation of excitatory and inhibitory synapses. *Nature* 465(7299), pp. 783–787.
Thomas, G.M., Hayashi, T., Chiu, S.-L., Chen, C.-M. and Huganir, R.L. 2012. Palmitoylation by DHHC5/8 targets GRIP1 to dendritic endosomes to regulate AMPA-R trafficking. *Neuron* 73(3), pp. 482–496.
Thomas, G.M. and Huganir, R.L. 2013. Palmitoylation-dependent regulation of glutamate receptors and their PDZ domain-containing partners. *Biochemical Society Transactions* 41(1), pp. 72–78.
Togashi, H., Abe, K., Mizoguchi, A., Takaoka, K., Chisaka, O. and Takeichi, M. 2002. Cadherin regulates dendritic spine morphogenesis. *Neuron* 35(1), pp. 77–89.
Togashi, H., Sakisaka, T. and Takai, Y. 2009. Cell adhesion molecules in the central nervous system. *Cell Adhesion & Migration* 3(1), pp. 29–35.
Tortosa, E., Adolfs, Y., Fukata, M., Pasterkamp, R.J., Kapitein, L.C. and Hoogenraad, C.C. 2017. Dynamic Palmitoylation Targets MAP6 to the Axon to Promote Microtubule Stabilization during Neuronal Polarization. *Neuron* 94(4), pp. 809–825.e7.
Trachtenberg, J.T., Chen, B.E., Knott, G.W., Feng, G., Sanes, J.R., Welker, E. and Svoboda, K. 2002. Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* 420(6917), pp. 788–794.
Traynelis, S.F., Wollmuth, L.P., McBain, C.J., Menniti, F.S., Vance, K.M., Ogden, K.K., Hansen, K.B., Yuan, H., Myers, S.J. and Dingledine, R. 2010. Glutamate receptor ion channels: structure, regulation, and function. *Pharmacological Reviews* 62(3), pp. 405–496.
Tronson, N.C. and Taylor, J.R. 2007. Molecular mechanisms of memory reconsolidation. *Nature Reviews. Neuroscience* 8(4), pp. 262–275.
Tsutsumi, R., Fukata, Y., Noritake, J., Iwanaga, T., Perez, F. and Fukata, M. 2009. Identification of G protein alpha subunit-palmitoylating enzyme. *Molecular and Cellular Biology* 29(2), pp. 435–447.
Turrigiano, G. 2012. Homeostatic synaptic plasticity: local and global mechanisms for stabilizing neuronal function. *Cold Spring Harbor Perspectives in Biology* 4(1), p. a005736.
Tyzio, R., Cossart, R., Khalilov, I., Minlebaev, M., Hübner, C.A., Represa, A., Ben-Ari, Y. and Khazipov, R. 2006. Maternal oxytocin triggers a transient inhibitory switch in GABA signalling in the fetal brain during delivery. *Science* 314(5806), pp. 1788–1792.
Umemori, H., Linhoff, M.W., Ornitz, D.M. and Sanes, J.R. 2004. FGF22 and its close relatives are presynaptic organizing molecules in the mammalian brain. *Cell* 118(2), pp. 257–270.
Ungar, D. and Hughson, F.M. 2003. SNARE protein structure and function. *Annual Review of Cell and Developmental Biology* 19, pp. 493–517.
Valenzuela, C.F., Puglia, M.P. and Zucca, S. 2011. Focus on: neurotransmitter systems. *Alcohol research & health : the journal of the National Institute on Alcohol Abuse and Alcoholism* 34(1), pp. 106–120.
Vallon, M., Yuki, K., Nguyen, T.D., Chang, J., Yuan, J., Siepe, D., Miao, Y., Essler, M., Noda, M., Garcia, K.C. and Kuo, C.J. 2018. A RECK-WNT7

Receptor-Ligand Interaction Enables Isoform-Specific Regulation of Wnt Bioavailability. *Cell reports* 25(2), pp. 339–349.e9.
Varela-Nallar, L., Alfaro, I.E., Serrano, F.G., Parodi, J. and Inestrosa, N.C. 2010. Wingless-type family member 5A (Wnt-5a) stimulates synaptic differentiation and function of glutamatergic synapses. *Proceedings of the National Academy of Sciences of the United States of America* 107(49), pp. 21164–21169.
Varela-Nallar, L., Grabowski, C.P., Alfaro, I.E., Alvarez, A.R. and Inestrosa, N.C. 2009. Role of the Wnt receptor Frizzled-1 in presynaptic differentiation and function. *Neural Development* 4, p. 41.
Varela-Nallar, L. and Inestrosa, N.C. 2013. Wnt signalling in the regulation of adult hippocampal neurogenesis. *Frontiers in Cellular Neuroscience* 7, p. 100.
Varjosalo, M. and Taipale, J. 2008. Hedgehog: functions and mechanisms. *Genes & Development* 22(18), pp. 2454–2472.
Varoqueaux, F., Aramuni, G., Rawson, R.L., Mohrmann, R., Missler, M., Gottmann, K., Zhang, W., Südhof, T.C. and Brose, N. 2006. Neuroligins determine synapse maturation and function. *Neuron* 51(6), pp. 741–754.
Varoqueaux, F., Sigler, A., Rhee, J.-S., Brose, N., Enk, C., Reim, K. and Rosenmund, C. 2002. Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. *Proceedings of the National Academy of Sciences of the United States of America* 99(13), pp. 9037–9042.
Vartak, N., Papke, B., Grecco, H.E., Rossmannek, L., Waldmann, H., Hedberg, C. and Bastiaens, P.I.H. 2014. The autodepalmitoylating activity of APT maintains the spatial organisation of palmitoylated membrane proteins. *Biophysical Journal* 106(1), pp. 93–105.
Verhage, M., Maia, A.S., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., van den Berg, T.K., Missler, M., Geuze, H.J. and Südhof, T.C. 2000. Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* 287(5454), pp. 864–869.
Vicario-Abejón, C., Collin, C., McKay, R.D. and Segal, M. 1998. Neurotrophins induce formation of functional excitatory and inhibitory synapses between cultured hippocampal neurons. *The Journal of Neuroscience* 18(18), pp. 7256–7271.
Walikonis, R.S., Jensen, O.N., Mann, M., Provance, D.W., Mercer, J.A. and Kennedy, M.B. 2000. Identification of proteins in the postsynaptic density fraction by mass spectrometry. *The Journal of Neuroscience* 20(11), pp. 4069–4080.
Walsh, T., McClellan, J.M., McCarthy, S.E., Addington, A.M., Pierce, S.B., Cooper, G.M., Nord, A.S., Kusenda, M., Malhotra, D., Bhandari, A., Stray, S.M., Rippey, C.F., Roccanova, P., Makarov, V., Lakshmi, B., Findling, R.L., Sikich, L., Stromberg, T., Merriman, B., Gogtay, N., Butler, P., Eckstrand, K., Noory, L., Gochman, P., Long, R., Chen, Z., Davis, S., Baker, C., Eichler, E.E., Meltzer, P.S., Nelson, S.F., Singleton, A.B., Lee, M.K., Rapoport, J.L., King, M.-C. and Sebat, J. 2008. Rare structural variants disrupt multiple genes in neurodevelopmental pathways in schizophrenia. *Science* 320(5875), pp. 539–543.
Wan, J., Roth, A.F., Bailey, A.O. and Davis, N.G. 2007. Palmitoylated proteins: purification and identification. *Nature Protocols* 2(7), pp. 1573–1584.

Wang, H., Liu, T. and Malbon, C.C. 2006. Structure-function analysis of Frizzleds. *Cellular Signalling* 18(7), pp. 934–941.
Wang, H.Y. and Malbon, C.C. 2004. Wnt-frizzled signalling to G-protein-coupled effectors. *Cellular and Molecular Life Sciences* 61(1), pp. 69–75.
Wang, S., Krinks, M., Lin, K., Luyten, F.P. and Moos, M. 1997. Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. *Cell* 88(6), pp. 757–766.
Wang, T., Xie, K. and Lu, B. 1995. Neurotrophins promote maturation of developing neuromuscular synapses. *The Journal of Neuroscience* 15(7 Pt 1), pp. 4796–4805.
Wang, Y., Chang, H., Rattner, A. and Nathans, J. 2016. Frizzled receptors in development and disease. *Current Topics in Developmental Biology* 117, pp. 113–139.
Washbourne, P., Bennett, J.E. and McAllister, A.K. 2002. Rapid recruitment of NMDA receptor transport packets to nascent synapses. *Nature Neuroscience* 5(8), pp. 751–759.
Washbourne, P., Dityatev, A., Scheiffele, P., Biederer, T., Weiner, J.A., Christopherson, K.S. and El-Husseini, A. 2004. Cell adhesion molecules in synapse formation. *The Journal of Neuroscience* 24(42), pp. 9244–9249.
Washbourne, P., Liu, X.-B., Jones, E.G. and McAllister, A.K. 2004. Cycling of NMDA receptors during trafficking in neurons before synapse formation. *The Journal of Neuroscience* 24(38), pp. 8253–8264.
Wayman, G.A., Impey, S., Marks, D., Saneyoshi, T., Grant, W.F., Derkach, V. and Soderling, T.R. 2006. Activity-dependent dendritic arborization mediated by CaM-kinase I activation and enhanced CREB-dependent transcription of Wnt-2. *Neuron* 50(6), pp. 897–909.
Weatherbee, S.D., Anderson, K.V. and Niswander, L.A. 2006. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. *Development* 133(24), pp. 4993–5000.
Wen, L., Lu, Y.-S., Zhu, X.-H., Li, X.-M., Woo, R.-S., Chen, Y.-J., Yin, D.-M., Lai, C., Terry, A.V., Vazdarjanova, A., Xiong, W.-C. and Mei, L. 2010. Neuregulin 1 regulates pyramidal neuron activity via ErbB4 in parvalbumin-positive interneurons. *Proceedings of the National Academy of Sciences of the United States of America* 107(3), pp. 1211–1216.
Wiese, K.E., Nusse, R. and van Amerongen, R. 2018. Wnt signalling: conquering complexity. *Development* 145(12).
Wiesel, T.N. and Hubel, D.H. 1963. SINGLE-CELL RESPONSES IN STRIATE CORTEX OF KITTENS DEPRIVED OF VISION IN ONE EYE. *Journal of Neurophysiology* 26, pp. 1003–1017.
Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R. and Nusse, R. 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 423(6938), pp. 448–452.
Wolf, J., Palmby, T.R., Gavard, J., Williams, B.O. and Gutkind, J.S. 2008. Multiple PPPS/TP motifs act in a combinatorial fashion to transduce Wnt signalling through LRP6. *FEBS Letters* 582(2), pp. 255–261.
Won, S.J., Cheung See Kit, M. and Martin, B.R. 2018. Protein depalmitoylases. *Critical Reviews in Biochemistry and Molecular Biology* 53(1), pp. 83–98.
Won, S.J., Davda, D., Labby, K.J., Hwang, S.Y., Pricer, R., Majmudar, J.D., Armacost, K.A., Rodriguez, L.A., Rodriguez, C.L., Chong, F.S., Torossian,

K.A., Palakurthi, J., Hur, E.S., Meagher, J.L., Brooks, C.L., Stuckey, J.A. and Martin, B.R. 2016. Molecular Mechanism for Isoform-Selective Inhibition of Acyl Protein Thioesterases 1 and 2 (APT1 and APT2). *ACS Chemical Biology* 11(12), pp. 3374–3382.
Wong, W.T. and Wong, R.O. 2000. Rapid dendritic movements during synapse formation and rearrangement. *Current Opinion in Neurobiology* 10(1), pp. 118–124.
Woolley, C.S., Gould, E., Frankfurt, M. and McEwen, B.S. 1990. Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. *The Journal of Neuroscience* 10(12), pp. 4035–4039.
Wright, S.C., Cañizal, M.C.A., Benkel, T., Simon, K., Le Gouill, C., Matricon, P., Namkung, Y., Lukasheva, V., König, G.M., Laporte, S.A., Carlsson, J., Kostenis, E., Bouvier, M., Schulte, G. and Hoffmann, C. 2018. FZD5 is a Gαq-coupled receptor that exhibits the functional hallmarks of prototypical GPCRs. *Science Signalling* 11(559).
Wu, G., Malinow, R. and Cline, H.T. 1996. Maturation of a central glutamatergic synapse. *Science* 274(5289), pp. 972–976.
Wu, H., Xiong, W.C. and Mei, L. 2010. To build a synapse: signalling pathways in neuromuscular junction assembly. *Development* 137(7), pp. 1017–1033.
Wu, L.-G., Hamid, E., Shin, W. and Chiang, H.-C. 2014. Exocytosis and endocytosis: modes, functions, and coupling mechanisms. *Annual Review of Physiology* 76(1), pp. 301–331.
Xie, Z.P. and Poo, M.M. 1986. Initial events in the formation of neuromuscular synapse: rapid induction of acetylcholine release from embryonic neuron. *Proceedings of the National Academy of Sciences of the United States of America* 83(18), pp. 7069–7073.
Xu, N., Zhou, W.-J., Wang, Y., Huang, S.-H., Li, X. and Chen, Z.-Y. 2015. Hippocampal wnt3a is necessary and sufficient for contextual fear memory acquisition and consolidation. *Cerebral Cortex* 25(11), pp. 4062–4075.
Xu, T., Yu, X., Perlik, A.J., Tobin, W.F., Zweig, J.A., Tennant, K., Jones, T. and Zuo, Y. 2009. Rapid formation and selective stabilization of synapses for enduring motor memories. *Nature* 462(7275), pp. 915–919.
Yamagata, M., Sanes, J.R. and Weiner, J.A. 2003. Synaptic adhesion molecules. *Current Opinion in Cell Biology* 15(5), pp. 621–632.
Yamamoto, A., Nagano, T., Takehara, S., Hibi, M. and Aizawa, S. 2005. Shisa promotes head formation through the inhibition of receptor protein maturation for the caudalizing factors, Wnt and FGF. *Cell* 120(2), pp. 223–235.
Yamamoto, H., Komekado, H. and Kikuchi, A. 2006. Caveolin is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of beta-catenin. *Developmental Cell* 11(2), pp. 213–223.
Yan, D. and Lin, X. 2009. Shaping morphogen gradients by proteoglycans. *Cold Spring Harbor Perspectives in Biology* 1(3), p. a002493.
Yan, Q., Weyn-Vanhentenryck, S.M., Wu, J., Sloan, S.A., Zhang, Y., Chen, K., Wu, J.Q., Barres, B.A. and Zhang, C. 2015. Systematic discovery of regulated and conserved alternative exons in the mammalian brain reveals NMD modulating chromatin regulators. *Proceedings of the National Academy of Sciences of the United States of America* 112(11), pp. 3445–3450.

Yanfeng, W.A., Tan, C., Fagan, R.J. and Klein, P.S. 2006. Phosphorylation of frizzled-3. *The Journal of Biological Chemistry* 281(17), pp. 11603–11609.
Yang, C., Iyer, R.R., Yu, A.C.H., Yong, R.L., Park, D.M., Weil, R.J., Ikejiri, B., Brady, R.O., Lonser, R.R. and Zhuang, Z. 2012. β-Catenin signalling initiates the activation of astrocytes and its dysregulation contributes to the pathogenesis of astrocytomas. *Proceedings of the National Academy of Sciences of the United States of America* 109(18), pp. 6963–6968.
Yang, P.-T., Lorenowicz, M.J., Silhankova, M., Coudreuse, D.Y.M., Betist, M.C. and Korswagen, H.C. 2008. Wnt signalling requires retromer-dependent recycling of MIG-14/Wntless in Wnt-producing cells. *Developmental Cell* 14(1), pp. 140–147.
Yang, W., Di Vizio, D., Kirchner, M., Steen, H. and Freeman, M.R. 2010. Proteome scale characterization of human S-acylated proteins in lipid raft-enriched and non-raft membranes. *Molecular & Cellular Proteomics* 9(1), pp. 54–70.
Yano, H., Ninan, I., Zhang, H., Milner, T.A., Arancio, O. and Chao, M.V. 2006. BDNF-mediated neurotransmission relies upon a myosin VI motor complex. *Nature Neuroscience* 9(8), pp. 1009–1018.
Yao, K., Qiu, S., Tian, L., Snider, W.D., Flannery, J.G., Schaffer, D.V. and Chen, B. 2016. Wnt Regulates Proliferation and Neurogenic Potential of Müller Glial Cells via a Lin28/let-7 miRNA-Dependent Pathway in Adult Mammalian Retinas. *Cell reports* 17(1), pp. 165–178.
Yik, J.H.N. and Weigel, P.H. 2002. The position of cysteine relative to the transmembrane domain is critical for palmitoylation of H1, the major subunit of the human asialoglycoprotein receptor. *The Journal of Biological Chemistry* 277(49), pp. 47305–47312.
Yogev, S. and Shen, K. 2017. Establishing Neuronal Polarity with Environmental and Intrinsic Mechanisms. *Neuron* 96(3), pp. 638–650.
Yokoi, N., Fukata, M. and Fukata, Y. 2012. Synaptic plasticity regulated by protein-protein interactions and posttranslational modifications. *International review of cell and molecular biology* 297, pp. 1–43.
Yokoi, N., Fukata, Y., Sekiya, A., Murakami, T., Kobayashi, K. and Fukata, M. 2016. Identification of PSD-95 Depalmitoylating Enzymes. *The Journal of Neuroscience* 36(24), pp. 6431–6444.
Yoshikawa, S., McKinnon, R.D., Kokel, M. and Thomas, J.B. 2003. Wnt-mediated axon guidance via the Drosophila Derailed receptor. *Nature* 422(6932), pp. 583–588.
Young, S.H. and Poo, M.M. 1983. Spontaneous release of transmitter from growth cones of embryonic neurones. *Nature* 305(5935), pp. 634–637.
Yu, X. and Malenka, R.C. 2003. Beta-catenin is critical for dendritic morphogenesis. *Nature Neuroscience* 6(11), pp. 1169–1177.
Yuste, R. and Bonhoeffer, T. 2001. Morphological changes in dendritic spines associated with long-term synaptic plasticity. *Annual Review of Neuroscience* 24, pp. 1071–1089.
Zaręba-Kozioł, M., Figiel, I., Bartkowiak-Kaczmarek, A. and Włodarczyk, J. 2018. Insights Into Protein S-Palmitoylation in Synaptic Plasticity and Neurological Disorders: Potential and Limitations of Methods for Detection and Analysis. *Frontiers in Molecular Neuroscience* 11, p. 175.
Zebisch, M. and Jones, E.Y. 2015. ZNRF3/RNF43--A direct linkage of extracellular recognition and E3 ligase activity to modulate cell surface

signalling. *Progress in Biophysics and Molecular Biology* 118(3), pp. 112–118.
Zeidman, R., Jackson, C.S. and Magee, A.I. 2009. Protein acyl thioesterases (Review). *Molecular membrane biology* 26(1), pp. 32–41.
Zeke, A., Misheva, M., Reményi, A. and Bogoyevitch, M.A. 2016. JNK Signalling: Regulation and Functions Based on Complex Protein-Protein Partnerships. *Microbiology and Molecular Biology Reviews* 80(3), pp. 793–835.
Zeng, C.-M., Chen, Z. and Fu, L. 2018. Frizzled receptors as potential therapeutic targets in human cancers. *International Journal of Molecular Sciences* 19(5).
Zeng, X., Tamai, K., Doble, B., Li, S., Huang, H., Habas, R., Okamura, H., Woodgett, J. and He, X. 2005. A dual-kinase mechanism for Wnt co-receptor phosphorylation and activation. *Nature* 438(7069), pp. 873–877.
Zhai, R.G. and Bellen, H.J. 2004. The architecture of the active zone in the presynaptic nerve terminal. *Physiology* 19, pp. 262–270.
Zhai, R.G., Vardinon-Friedman, H., Cases-Langhoff, C., Becker, B., Gundelfinger, E.D., Ziv, N.E. and Garner, C.C. 2001. Assembling the presynaptic active zone: a characterization of an active one precursor vesicle. *Neuron* 29(1), pp. 131–143.
Zhang, B., Luo, S., Wang, Q., Suzuki, T., Xiong, W.C. and Mei, L. 2008. LRP4 serves as a coreceptor of agrin. *Neuron* 60(2), pp. 285–297.
Zhang, X. hui and Poo, M. 2002. Localized synaptic potentiation by BDNF requires local protein synthesis in the developing axon. *Neuron* 36(4), pp. 675–688.
Zhang, Xiaowei, Le, H.T., Zhang, Xiaohan, Zheng, M., Choi, B.-G. and Kim, K.-M. 2016. Palmitoylation on the carboxyl terminus tail is required for the selective regulation of dopamine D2 versus D3 receptors. *Biochimica et Biophysica Acta* 1858(9), pp. 2152–2162.
Zheng, C.-Y., Petralia, R.S., Wang, Y.-X. and Kachar, B. 2011. Fluorescence recovery after photobleaching (FRAP) of fluorescence tagged proteins in dendritic spines of cultured hippocampal neurons. *Journal of Visualized Experiments* (50).
Zheng, H., Jia, L., Liu, C.-C., Rong, Z., Zhong, L., Yang, L., Chen, X.-F., Fryer, J.D., Wang, X., Zhang, Y.-W., Xu, H. and Bu, G. 2017. TREM2 Promotes Microglial Survival by Activating Wnt/β-Catenin Pathway. *The Journal of Neuroscience* 37(7), pp. 1772–1784.
Zhou, Y. and Nathans, J. 2014. Gpr124 controls CNS angiogenesis and blood-brain barrier integrity by promoting ligand-specific canonical wnt signalling. *Developmental Cell* 31(2), pp. 248–256.
Zhu, J.J., Esteban, J.A., Hayashi, Y. and Malinow, R. 2000. Postnatal synaptic potentiation: delivery of GluR4-containing AMPA receptors by spontaneous activity. *Nature Neuroscience* 3(11), pp. 1098–1106.
Zito, K. and Svoboda, K. 2002. Activity-dependent synaptogenesis in the adult Mammalian cortex. *Neuron* 35(6), pp. 1015–1017.
Ziv, N.E. and Smith, S.J. 1996. Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron* 17(1), pp. 91–102.
Zorn, A.M. 2001. Wnt signalling: antagonistic Dickkopfs. *Current Biology* 11(15), pp. R592–5.