The role of Wnt signalling in synapse maintenance in the hippocampus

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Declaration

I, Douglas M Lopes, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Wnt signalling is well known for its role during development and formation of central synapses. Interestingly, many studies have demonstrated that Wnts and their receptors are continuously expressed after development in the adult brain, suggesting that Wnt signalling might play a role in synaptic maintenance during adulthood. Our lab has demonstrated that Dickkopf-1 (Dkk1), an endogenous Wnt antagonist, can rapidly induce the disassembly of synapses in hippocampal cultures (Purro et al., 2012). In addition, recent studies demonstrated that Dkk1 expression is increased in the brain of Alzheimer's disease (AD) patients, as well as in some transgenic mouse models of AD, which coincides with synaptic loss and impaired memory. These results suggest that Wnt signalling may be involved in the maintenance of synapses in the adult brain.

To investigate the role of Wnt signalling in the adult central nervous system, I used an inducible transgenic mouse line that expresses Dkk1 in the hippocampus under the control of tetracycline. I found that induction of Dkk1 expression in the hippocampus of adult mice leads to profound synaptic changes. Dkk1 expression induces the disassembly of excitatory synapses in the hippocampus, without affecting inhibitory synapses or compromising cell viability. Morphological analyses of dendritic spines showed a decrease in number and size of spines in the hippocampus CA1 area. Supplementing these findings, behavioural analysis showed that adult Dkk1 transgenic mice exhibit impaired spatial and long term memory. Furthermore, in this study I also give insight to the mechanism of action of Dkk1, demonstrating that Dkk1-mediated synaptic disassembly is dependent on protein degradation. In summary, my studies demonstrate that Wnt signalling is crucial for synaptic maintenance in the adult hippocampus and for normal hippocampal-dependent memory.

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List of abbreviations

AchR	Acetylcholine receptor
AD	Alzheimer Disease
APC	Adenomatous polyposis complex coli
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
APP	Amyloid precursor protein
Aβ	Amyloid-beta (or Beta-amyloid)
aCSF	Artificial cerebral spine fluid
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CAM	Cell adhesion molecule
CaMKII	Calcium/calmodulin dependent protein kinase II
CF	Climbing fibres
CK1	Casein kinase-1
CNS	Central nervous system
CRD	Cysteins-rich domain
CSF	Cerebral spine fluid
D1R	Dopamine receptor subtype 1
D2R	Dopamine receptor subtype 2
DG	Dentate gyrus
Dkk	Dickopff
Dvl	Dishevelled
EC	Entorhinal cortex
Eph	Ephrin
EPM	Elevated plus-maze
FGF	Fibroblast-growth factor
Fz	Frizzled receptor
GABA	γ - aminobutyric acid
GC	Granule cells
GFP	green fluorescent protein
Gsk-3β	Glycogen synthase kinase-3

JNK	c-Jun N-terminal kinases
KA-R	Kainate receptors
Krm	Kremen receptor
LDLR	Low-density lipoprotein receptors
LiCl	Lithium chloride
LRP5/6	Lipoprotein receptor related-protein class 5 and 6
LRRK2	Leucine-rich repeated kinase 2
LTD	Long-term depression
LTP	Long-term potentiation
MF	Mossy fibres
MWM	Morris water-maze
NFTA	Nuclear factor associated with T cells
NFTA	Neurofribilliary tangles
NGF	Nerve growth factor
NL	Neuroligin
NMDAR	N-methyl-D-aspartate receptors
NMJ	Neuro muscular junction
NOR	Novel Object Recognition
NRX	Neurexin
NT	Neurotrophin
OF	Open-field
PC	Purkinje cells
PCP	Planar cell polarity
PD	Parkinson's disease
PFA	Paraformaldehyde
PI	Propidium Iodide
PKA	Protein kinase A
PS1	Presenilin-1
PS2	Presenilin-2
PSD	Postsynaptic density
PSD95	Postsynaptic density protein 95
ROCK	Rho-associated coiled-coil containing protein kinase

RT-PCR	Reverse transcription polymerase chain reaction
rtTA	Tetracycline reverse transcriptional activator
SFRP	Secreted frizzled-related protein
SPRC	Synapse-associated polyribosome complexes
SynCam	Synaptic cell adhesion molecule
TetO	tetracycline-responsive promoter
Trk	Tyrosine kinase receptors
UPS	Ubiquitin proteasome system
VAMP	Vesicle associated membrane proteins
VGAT	Vesicular γ-aminobutyric transporter
vGlut	Vesicular glutamate transporter
wg	wingless
WIF1	Wnt-inhibitory factor-1
Wls	Wntless gene

Chapter 1

1. Introduction

1.1. The hippocampus

The current view, that the hippocampus plays a crucial role in memory, cognition and emotion evolved from the study of many scientists. The publication of the detailed structure of the hippocampus by Camillo Golgi, illustrating the unique organisation of the hippocampus, generated interest, debate and curiosity to the function of this large structure plays (Andersen, 2007). The first and most influential neuroanatomical hypothesis came to light in the end of the 1930s by Papez, who described the hippocampus structure as part of a circuit that is involved in emotions (Papez, 1937). Following Papez' theory, the major study that established the function of hippocampus was performed by Scoville and Milner (Scoville & Milner, 1957), when studying a patient with a damaged brain. This breakthrough study put the hippocampus as a crucial structure for episodic memory reporting the case of the HM patient, who had part of their hippocampus lesioned in an attempt to relieve seizures. Unfortunately, this operation had tremendous unforeseen consequences that led to the patient's inability to form new episodic memories (Andersen, 2007; Corkin, 2002; Scoville & Milner, 1957). Since then, many studies using patients and animal models confirmed that subjects with hippocampus lesions suffer with retrograde amnesia (S. J. Martin et al., 2005; Neves et al., 2008; Spiers et al., 2001; Vargha-Khadem et al., 1997) and further confirm the importance of the hippocampus in memory formation.

The use of experimental animals helped extensively to the understanding of different types of memories and what brain structures are involved in each one of these distinct memories processes. A strong wave of studies led by distinctive scientists in the 70s, looking into the hippocampus function, using systems ranging from rodents to primates, led to the conclusion that the hippocampus and related structures are involved in recognition, contextual retrieval and associative memory

(Squire, 2004; Squire et al., 2004). Together these studies demonstrate the importance of the hippocampus in declarative memory and the ability of this structure to detect and encode an event that happened in a specific time and place. Furthermore, another breakthrough study shed light on a unique feature of the hippocampus, the capability to form spatial maps.

The work from O'Keefe and Dostrovsky (J. O'Keefe & Dostrovsky, 1971) recording the activity of single cells from the hippocampus in an awake animal demonstrate that the pattern of firing of certain neurons are correlated to the location of the animal in the environment (J. O'Keefe & Dostrovsky, 1971). This was the first evidence that the hippocampus is involved in the formation of a cognitive environmental map. This pioneering study demonstrated that the hippocampus holds the information necessary to function as a map (J. O'Keefe & Dostrovsky, 1971) and this information is largely dependent of special neurons called place cells. These neurons were reported to be "silent" as the animal moved around its environment, until it entered in a small patch of the environment, also called place field, when some particular cells becomes active and started to fire (Andersen, 2007; J. O'Keefe & Dostrovsky, 1971). Furthermore, these hippocampal pyramidal cells can fire different patterning bursts, giving and processing information about the environment the animal is located (J. O'Keefe & Dostrovsky, 1971; John O'Keefe & Nadel, 1978). The existence of place cells demonstrated the role of the hippocampus in spatial adaptation and awareness, in addition to already described function in learning and memory.

1.2. The Hippocampal Anatomy

Anatomically, the hippocampus is located at the medial temporal lobe, along with associated structures (entorhinal, perirhinal, and parahippocampal cortices), together forming the medial temporal lobe memory system (Squire & Zola-Morgan, 1991) (Fig 1.1). The structures of the medial temporal lobe play a pivotal role in encoding, consolidation and retrieval of episodic memories (Brun et al., 2002). The hippocampus is a waning moon-shaped structure, subdivided in three regions: the

dentate gyrus (DG), the hippocampus proper (consisted of CA1, CA2 and CA3 - Fig 1.1) and the subiculum (van Strien et al., 2009). Each of these sub regions is formed of anatomically distinct layers giving unique characteristics for each region.

The deepest layer is composed of mixture of interneurons and afferent and efferent fibres are found; in the DG this layer is called hilus, whereas in the CA area it is called stratum oriens. Above the latter there is a layer composed of principal cells and interneurons. In the cortex this layer is called cell layer, in the DG it is called granule layer, and in the CA are referred as pyramidal cell layer (or stratum pyramidale) (Fig 1.2). The most superficial layer is the molecular layer (also called stratum moleculare), sharing the same name in the DG and subiculum. In the CA area however, this layer is subdivided, from the deepest to the most superficial, stratum lucidum, stratum radiatum and the stratum lacunosum moleculare (Fig 1.2). Importantly, due to anatomical differences, the stratum lucidum layer is not present in the CA2 and CA1 areas (van Strien et al., 2009). The hippocampus and its sub layers are ultimately the recipient of projections from cortical structures, which are located hierarchy earlier in the processing of information (Squire et al., 2004). The interconnectivity of these distinct hippocampal and cortical regions is formed by various different pathways, giving rise to a functional circuit (Fig. 1.2), described below.

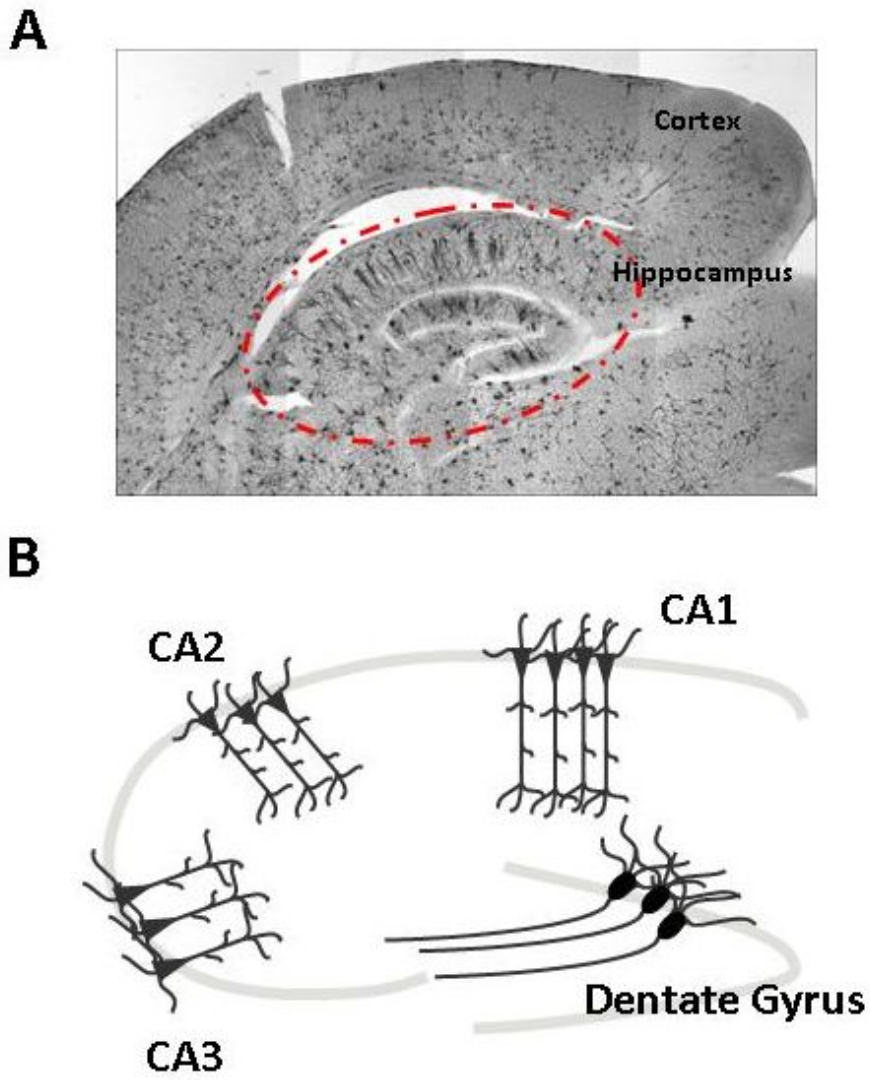

Figure 1.1 - The Hippocampus. A: Golgi staining of a sagittal section of the mouse forebrain. The hippocampus is located at the centre of the picture (within the dashed red line), below the cortical area. **B:** Schematic representation of the hippocampus proper, its organised subregions - CA1, CA2, CA3 and Dentate gyrus (DG) – and the principal neurons of each region.

1.2.1. The perforant pathway (EC-DG)

The projection from the entorhinal cortex (EC) to the hippocampus is called the perforant pathway (Fig 1.2). Traditionally this pathway is mainly characterised by the projection from the EC layer II to the DG. This projection is considered one of the main inputs into the hippocampus pathway (Amaral et al., 1990; Canto et al., 2008;

Koganezawa et al., 2008). Importantly, layers III, V and VI also contribute to this projection, but in a smaller scale (van Strien et al., 2009). The EC cells projects onto apical dendrites of the principal neurons, as well as to interneurons. Interestingly, the input from the EC is sparsely dispersed among the DG granule cells, and it is believed that each of these cells can only carry a fraction of the information coming from the EC, which gives the DG the ability to distinguish, disambiguate or overlap small differences in inputs creating a firing map (Leutgeb et al., 2007). This feature generates a sparse firing rate in granule cells, providing clues to the main function of this circuit, called pattern separation (Leutgeb et al., 2007). Pattern separation is defined as the key step during the processing of encoding of episodic memories that amplify and distinguish between new representations and representations already existent in the network (Leutgeb et al., 2007). Although the mechanism of pattern separation remains elusive (Leutgeb & Moser, 2007), recent studies in behaving animals demonstrate that this process is located within the granule cells of the DG (Rolls & Kesner, 2006).

1.2.2. The temporoammonic pathway (EC-CA1)

The EC has another direct set of projections into the hippocampus proper by making synapses directly to CA1 neurons (Fig 1.2), a distinct perforated pathway to the one described above. This direct pathway, also called temporoammonic pathway, comes from the EC layer III and targets the distal part of the apical dendrite (stratum lacunosum moleculare) of the CA1 region (Kajiwara et al., 2008). Importantly, this perforant input coming from the EC also targets interneurons that anatomically are located in the border of the stratum radiatum- lacunosum moleculare layers (Kajiwara et al., 2008).

Interestingly, a circuit loop between EC $\leftrightarrow$ CA1 has also been described (Lavenex et al., 2007). Such connection-loop was proposed after the observation of cycles of electrophysiological reverberation at the EC, once this region was stimulated. Indeed, a study using retrograde dying to trace the connections of the neurons between the EC and CA1 regions, demonstrated that entorhinal inputs to the CA1 are feedback to

the same EC column (Tamamaki & Nojyo, 1995). Furthermore, this study, together with electrophysiological recordings, led to the proposal that the reverberant activity play in important role for the formation of memories (Tamamaki & Nojyo, 1995). Although the functional role of the temporoammonic pathway in information processing and storage in the hippocampal network is yet not clear, a recent study demonstrated a distinct feature of this pathway.

An elegant study, recording plasticity in an awake animal, demonstrated that induction of long term plasticity at these synapses have different properties (lower induction but more robust response) in comparison to other synapses within the hippocampus circuit, including the mossy fiber-CA3 and Schaffer collateral-CA1 synapses (Aksoy-Aksel & Manahan-Vaughan, 2013). More importantly, the response of induced long term depression, an electrophysiological paradigm that leads to structural and molecular synaptic changes and plasticity in the brain (Massey & Bashir, 2007), in this same pathway is strongly modulated and controlled by the input coming from a distinct area of the hippocampus, the CA3 region (Aksoy-Aksel & Manahan-Vaughan, 2013) (CA3-CA1 input, discussed later). Together these studies show the interconnectivity of the different pathways within the hippocampus circuitry, which suggests that disruption of one specific pathway, may affect the function of the other.

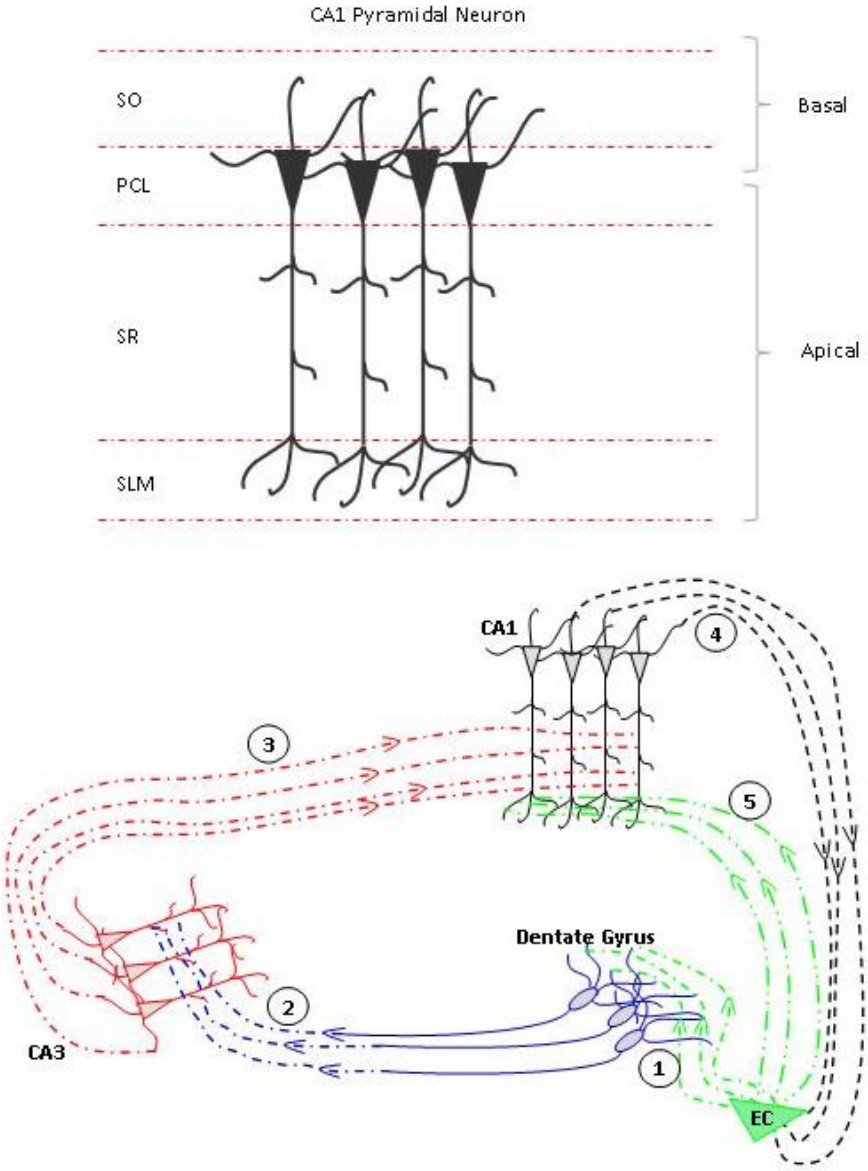

Figure 1.2 - The hippocampus anatomy. Top: Illustration of a CA1 pyramidal neuron, with their subdivided dendritic tree. From the basal to the apical dendritic tree: SO, stratum oriens; PCL, pyramidal cell layer; SLM, stratum lacunosum-moleculare and SR, stratum radiatum. **Bottom:** Pattern of connections of the hippocampal regions. **(1)** The projection from the entorhinal cortex (EC) to the hippocampus is called the perforant pathway. Traditionally this pathway is mainly characterised by the projection from the EC layer II to the DG. **(2)**The mossy fibre pathway is a direct projection from granule cells of the DG hilus to the CA3 area of the hippocampus **(3)**The Schaffer Collateral pathway refers to the CA3 pyramidal cells projection to the CA1 area of the hippocampus, which is one of the most studied pathways. **(4)** The temporoammonic pathway, comes from the EC layer III and targets directly the distal part of the CA1 apical dendrite. **(5)** The CA1 area synapses back to the EC, forming a circuit loop.

1.2.3. DG – CA3 pathway (mossy fibres)

The target of the granule cells projection from the DG hilus is the CA3 area of the hippocampus (Fig 1.2). This pathway was named after the "mossy appearance" of the projections, which are unmyelinated axons with varicosities and thorny excrescences covering the proximal dendrites of the mossy cells (Andersen, 2007; R.G. Morris, 2001). An interesting feature of this pathway is that a very large number of mossy cells innervate CA3 pyramidal cells with very little convergence – with an estimated innervation of 50 granule cells per pyramidal cell - and these innervations are formed by specialised presynaptic giant mossy fibre boutons (Amaral & Dent, 1981; Henze et al., 2002; R.G. Morris, 2001). Importantly many studies looking into the hippocampus connectivity show the existence of a back projection pathway, where CA3 pyramidal cells project back to the hilus and molecular layer of the DG (van Strien et al., 2009). Although it is not clear the nature (inhibitory of excitatory) and the function of these back projections, but given presence of interneurons in this subarea of the hippocampus together with the fact that granule cells express both excitatory and inhibitory neurotransmitters (Frotscher et al., 2006), it can be speculated that these projections may tune in neurotransmitter release on to CA3 pyramidal cells and avoid its hyper-excitability estate (Frotscher et al., 2006).

Inputs from the DG to the CA3 are implicated in memory and spatial representation in different manners. Studies in live animals carrying a lesion in the CA3 area have demonstrated that this pathway is necessary to store spatial memory, but not to recall them (Lassalle et al., 2000; Rolls, 2013). Furthermore, there is substantial evidence demonstrating the importance of the CA3 for the completion of associative/spatial memory tasks (Rolls, 2013). The so-called pattern completion, proposes that excitatory receptors in the CA3 region are crucial to "fill the blanks" if cues in the environment are removed in a reference memory task (Nakazawa et al., 2002; Rolls, 2013). Clear illustration of this CA3 function is demonstrated by experiments in which animals had this area lesioned or genetically manipulated (Kesner, 2013). For instance, NMDA receptors knockout mice in the CA3 area are unable to perform the Morris water maze task, if some of the familiar cues are

removed from the environment after training (Nakazawa et al., 2002). Additional studies further demonstrating the CA3 pattern completion function in different spatial memory tasks, such as the cheese board maze, has also been demonstrated (Kesner, 2013). In addition to spatial representation, it has also been demonstrated the involvement of the DG-CA3 in pathological states such epilepsy, as it has been demonstrated that the DG becomes hyperexcitable and spontaneously epileptic in the context of brain injury and neuronal or interneuron loss (Lothman et al., 1992; Sloviter et al., 2012).

1.2.4. The Schaffer Collateral pathway, SC (CA3 – CA1)

The extensive axon collateral system of the CA3 pyramidal cells and the detailed anatomical projection to the CA1 area of the hippocampus (Fig 1.2), then called "collateral fibre system", was first described by the Hungarian neurologist Karoly Schaffer (1982), hence the name of the pathway (Szirmai et al., 2012). Different studies demonstrate the interesting topology of the CA3-CA1 connection: the distal part of the CA3 pyramidal cells project to the most proximal dendrites of the CA1, and the proximal CA3 projects to the most proximal dendrites of the CA1 (van Strien et al., 2009). Further in the projection topography, the mid-portion of the CA3 connects to dendrites located between proximal and distal part of CA1 cells (van Strien et al., 2009). Notably, the CA2 pyramidal neurons do not receive CA3 mossy fibre projections. It is estimated that each single CA1 pyramidal neuron receives approximately 30,000 excitatory inputs, being most from this input (over 60%) located at the stratum radiatum layer (Megias et al., 2001). Importantly, this vast innervations coming from CA3 neurons is believed to be in a patchy distribution along the population of CA1 pyramidal cells, where some neurons receive very dense innervations, whereas others have a rather sparse number of inputs (X. G. Li et al., 1994).

The SC is nowadays one of the most studied pathways, from electrophysiology to cell biology. The pattern of firing of the CA3 area and its impact on CA1 cells allowed scientists to propose a mathematical model that describes this pathway and analyse

numerically how the information is processed and represented in the neuronal network (Schultz & Rolls, 1999). Using a combination of mathematical techniques based on theoretical models, electrophysiology, together with heavy statistics analysis, the authors were able to give precise information about many variables, including neuronal firing rates and firing pattern, during the process of storage and retrieval of information (Schultz & Rolls, 1999). Further to the already known involvement of the hippocampus network in memory, extra attention is given to the SC for two other main reasons: the CA3 is the point of convergence of information coming from different cortical areas and the CA1 area is known to be particularly affected in brain damages caused by ischemia and epilepsy (Schultz & Rolls, 1999).

1.3. The synapse

Ever since the basic concepts underlying nervous system function in the end 18th Century, neurosciences has thrived. The interest of Santiago Ramon y Cajal (1852-1934) in understanding the structure of the central nervous system (CNS) led him to observations and concepts key for understanding how the nervous system develops and functions. Ramon y Cajal's theory published in 1888 postulated the concept of "neuron independence" in which he describes neurons as cells with ramifications and dendrites that are in close contact with tiny axon branches with a nearby neuron. Furthermore, Ramon y Cajal proposed that nervous impulses are propagated through contact, by a kind of electrical impulse (Lopez-Munoz et al., 2006; Ramón y Cajal, 1923). It was based on Ramon y Cajal's neuron doctrine, that Sir Charles Sherrington (1857 – 1952) provided a concept of the integrative nervous system and neuron communication: the synapse (Sherrington, 1906).

The synapse is a perennial concept still study nowadays. Sherrington's synapse concept came from the Greek word "clasp". His book postulates that through synapses, the information coming from a unique neuron is reflected in an integrative manner within the nervous system. In addition, Sherrington defined the unidirectional property of synaptic transmission, from the pre to the postsynaptic

neuron. Furthermore, his theory also proposes that stimuli work in a reciprocal and auto-regulative manner, implementing the notion of excitatory and inhibitory synapses (Andersen, 2007; Levine, 2007; Sherrington, 1906). A further level of understanding of the synapses came five decades later with the advance of imaging and microscopy techniques (Fig 1.3). Observations of structures in the shape of vesicles from electron micrograph images raised the interest of many scientists (Wells, 2005). Studies by Palay & Palade (Palay & Palade, 1955) and Robert & Bennet (De Robertis, 1955) confirm the early predictions of Ramon y Cajal and Sherrigton that neurons communicate via pre- and postsynaptic structures and that there is no cytoplasmic continuity with each other. Furthermore, the works of De Robertis & Bennet (1955) made the connection between the structures they observed and the "granular fractions" that had been previously described by other authors - which they observed to be vesicles carrying neurotransmitters (Wells, 2005). This booming of information in the last century generated long last concepts and theories, such as synapses, synaptic vesicles and neurotransmitter release, which helped the scientific community to understand the way neurons work.

Decades of research allowed much progress in understanding of synapses and also their function. Now it is well established that synapses are complex structures with an asymmetric organisation and with specialised function. The main components are the presynaptic site containing synaptic vesicles and the machinery that regulate the fusion and release of neurotransmitter, the synaptic cleft and the postsynaptic side, where proteins of the postsynaptic density core, receptors and ion channels are located. By definition, chemical synapses are specialised structures in which the information is exchanged between a neuron and a targeted cell (Schoch & Gundelfinger, 2006). Furthermore, central synapses are subdivided in three main subtypes, excitatory, inhibitory and modulatory synapses, which together define and regulate neuronal function. Given the focus of the experiments presented in this thesis, I will focus my attention in this introductory section on excitatory (glutamatergic) and inhibitory (gabaergic) synapses.

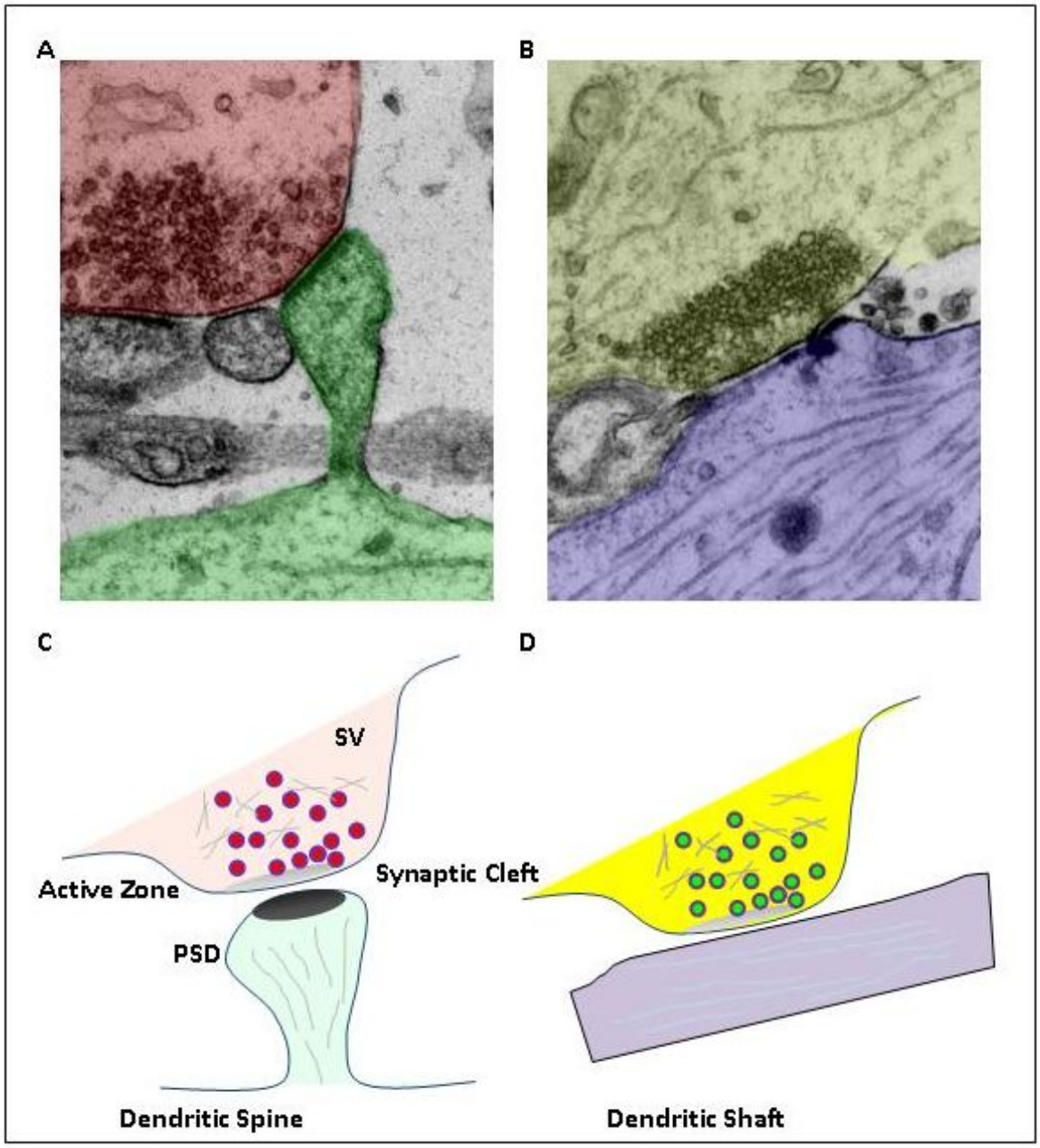

Figure 1.3 - Structure of central excitatory and inhibitory synapses. Electromicrographs and illustrative cartoon representing an excitatory **(A and C)** and inhibitory **(B and D)** synapses. Both types of synapses present similar presynaptic structures, containing numerous synaptic vesicles (SV) associated with an active zone (electron dense area, close to the membrane). Glutamatergic and GABAergic synapses present a clear synaptic cleft - the extracellular space separating the pre- and postsynaptic sides. Excitatory synapses are typically associated with a postsynaptic site, found in a dendritic spine containing an electron dense region (PSD - post synaptic dense region). In contrast, inhibitory presynaptic terminals synapse directly onto the dendritic shaft, with an almost imperceptible PSD.

1.3.1. The structure and function of synapses

Glutamatergic and gabaergic synapses are very abundant in the CNS. Both types of synapses have been extensively studied and characterised, particularly cortical pyramidal neurons (Spruston, 2008). The ultimate goal of both excitatory and inhibitory synapses is to induce neurotransmitter release from the presynaptic terminal once action potential is triggered (B. S. Katz, 1969). Conceptually glutamatergic synapse transmission results in postsynaptic excitation, or depolarization of the nerve cell, whereas gabaergic synapses lead to postsynaptic inhibition, or hyperpolarisation of the neuron. Crucially, brain function is based on a balance between excitation and inhibition. In a healthy brain this balance is essential for proper brain functions, including processing of sensory information, cognition, motor control and balance among others (Cline, 2005; Yizhar et al., 2011). Importantly, it is well accepted that imbalance of excitation and inhibition can lead to CNS disorders such as autism and schizophrenia (Kehrer et al., 2008; Munoz-Yunta et al., 2008; Rubenstein & Merzenich, 2003; Tebartz van Elst et al., 2014). Thus, studying the molecular mechanisms and proteins that are involved in the formation, function as well as maintenance of synapses – which is the main focus of my studies - is essential to understand the CNS, and the implications in health and disease.

Although exerting opposite neural functions in the mature CNS (Ben-Ari, 2002; Kneussel & Loebrich, 2007; K. Li & Xu, 2008; El Mestikawy et al., 2011) excitatory and inhibitory synapses can be considered structurally similar, as these two types of synapses are known for their well-defined presynaptic site containing vesicles in close contact with a postsynaptic area (Schoch & Gundelfinger, 2006) (Fig 1.3). Furthermore, these specialised structures share some common proteins (Evergren et al., 2007; Schoch & Gundelfinger, 2006). For instance, some structural presynaptic core proteins such as synapsin, bassoon and piccolo are present in glutamatergic and gabaergic synapses (Evergren et al., 2007; Schoch & Gundelfinger, 2006). Synapsins, which are the most abundant phosphoprotein present in synapses at the CNS (Evergren et al., 2007), are a family of presynaptic proteins that are a major

substrate for protein kinases as well as for interacting with synaptic vesicles, organising the vesicle pool and its abundance, being therefore important modulators of synaptic function (Bykhovskaia, 2011; Humeau et al., 2011). Piccolo and bassoon, by contrast, are large scaffolding proteins involved in the stabilization, organization and function of the presynaptic active zone (Gundelfinger & Fejtova, 2012; Schoch & Gundelfinger, 2006) – a multifunctional protein rich area responsible for docking and priming of synaptic vesicles, recruitment of Ca^{++} channels, and also stabilization of the presynaptic site (Sigrist & Schmitz, 2011; Sudhof, 2012).

Disruption of different presynaptic proteins leads to distinct outcomes. For instance, loss or disruption of synapsin can lead to defective synaptic function, particularly of glutamatergic synapses (Gitler et al., 2004; Terada et al., 1999). By contrast, loss of piccolo/ bassoon in the hippocampus and in the cortex leads to a surprisingly almost undetectable synaptic transmission phenotype, in both excitatory and inhibitory synapses (Mukherjee et al., 2010). Interestingly, piccolo and bassoon have been reported to be involved in the stabilization and elimination of synapses, and linked to synaptic maintenance (Waites et al., 2013), being therefore tagged as players in synaptic degeneration (Kononenko et al., 2013). In this light, it is important to bear in mind the component parts of inhibitory and excitatory synapses, so their function and architecture can be fully understood.

1.3.2. Glutamatergic synapses

Glutamatergic synapses are specialised structures where the amino acid glutamate functions as the neurotransmitter. Glutamate is the most common neurotransmitter in the brain, accounting for of the majority of neuronal transmission in the cortex (El Mestikawy et al., 2011; Santos et al., 2009). Glutamate neurotransmitter induces fast synaptic communication between neurons, and this process happens through the release of glutamate in the synaptic cleft, where it will act on the postsynaptic nerve cells by binding to different receptors located in the postsynaptic side (Santos

et al., 2009; Takamori, 2006). Glutamate is stored inside synaptic vesicles and the transferred from the cytoplasm into the SV occurs through vesicular glutamate transporters (vGlut) (El Mestikawy et al., 2011; Takamori, 2006). There are three vesicular glutamate transporters (vGlut1, vGlut2 and vGlut3), which are expressed differently according to the subpopulations of neurons (El Mestikawy et al., 2011). For instance, vGlut1 is largely expressed in the cortex and hippocampus, whereas vGlut2 can be found at the thalamus and vGlut3 mRNA can be found at the striatum and raphe nuclei (El Mestikawy et al., 2011). vGlut not only confers glutamate uptake activity to SV but also defines a glutamatergic neuron and the excitatory presynaptic terminal (Santos et al., 2009; Takamori, 2006).

1.3.2.1. Glutamate Receptors and glutamatergic postsynaptic structures

Glutamate can signal through different receptors, classified as ionotropic and metabotropic receptors. There are three major types of ionotropic glutamate receptors, NMDA, AMPA and kainate receptors. Each of these receptors activates a cation channel which is selectively permeable to Na^{+}, Ca^{++} and K^{+}, in a different degree of permeability (Madden, 2002; Mayer & Armstrong, 2004). Metabotropic glutamate receptors (mGluRs), by contrast, are a family of G-coupled transmembrane protein which transduces signals through second messenger signalling cascades (Anwyl, 1999; Collingridge et al., 2004).

N-methyl-D-aspartate receptors (NMDARs) are ionotropic glutamate receptors crucial for synaptic function and plasticity. NMDARs are non-selective cation channels, which are highly permeable to Ca^{++} and Na^{+}, and subject to voltage dependent block by Mg^{++} (Paoletti et al., 2013). NMDARs are assembled as heteromers with different subunit compositions. To date, seven different subunits, which are classified in three distinct subfamilies, were identified: GluN1, GluN2 (subdivided in GluN2A, GluN2B, GluN2C and GluN2D) and GluN3 (GluN3A and GluN3B) (Cull-Candy et al., 2001; Paoletti et al., 2013). The composition of the

tetramers dictates the property of the receptors. For instance, GluN2A has low affinity for glutamate, confers higher channel opening probability than GluN2B (Lau & Zukin, 2007). By contrast, GluN3 appear to have an inhibitory effect to glutamate presence, reducing Ca^{++} permeability and Mg^{++} sensitivity (Cull-Candy et al., 2001). Notably, most NMDARs at central synapses are GluN1/GluN2 assemblies (Lau & Zukin, 2007). The diversity in their molecular (subunit) composition gives NMDARs the unusual property of regulatory sensitivity. Furthermore, NMDARs are highly regarded as capable to convert patterns of synaptic activity, essential for synaptogenesis and crucial mediators for plasticity (long-term depression and long-term potentiation) (Collingridge et al., 2004; Lau & Zukin, 2007).

Interestingly, shortly after the discovery of the involvement of NMDA in LTP plasticity in the induction of LTP at the SC- CA1 synapses in the hippocampus (Collingridge et al., 1983), a study from Morris and colleagues (R. G. Morris et al., 1986) demonstrated the involvement of NMDA receptors in the process or learning. Injection of a specific blocker of NMDA receptors (AP5) in the CA1 area of the hippocampus in rats, led to impaired performance in the Morris water maze, and impaired LTP (R. G. Morris et al., 1986). Since then, many studies demonstrated the crucial role of NMDA receptors for learning and memory, leading to the proposal that modulation of these receptors can represent a target in the context of cognitive impairment (Collingridge et al., 2013). In addition, the use of both genetic and pharmacological manipulations led to the conclusion that NMDA receptors are also mediating some brain dysfunctions such as addiction (Fan et al., 2014; Schilstrom et al., 2006) and psychiatric disorders such as schizophrenia (Paoletti et al., 2013).

AMPARs are a second family of glutamate receptors, widely known as GluA types. Its name comes from its main agonist α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid. There are four AMPA receptors genes, encoding four different subunits (GluA1 to GluA4), which together form homo or hetero-oligomeric assemblies of the receptor that forms channels, allowing the influx of cations

intracellularly (Gan et al., 2014; Madden, 2002; Mayer, 2005; Mayer & Armstrong, 2004). These glutamate receptors are the fastest responding excitatory signalling in the brain (Cheng et al., 2012; Madden, 2002). AMPARs are present throughout the cell, not only at the synaptic site, and when glutamate is present, its binding to AMPA-R result in a fast opening of the channels, allowing the influx of Na^+ in particular, and depolarization of the postsynaptic membrane (Cheng et al., 2012; Mayer, 2005). The arrangement of the subunits confers different properties to the channels. For example, the presence of GluA2 subunit confers a much lower permeability to Ca^{++} to the channel, as most principal neurons in the cortex, amygdale and hippocampus (Brorson et al., 1999; S. J. Liu & Zukin, 2007).

Just as NMDA receptors, the physiological importance of GluA receptors is highly regarded for important brain functions, such as cognition. AMPA receptors are thought to contribute to synaptic plasticity, ability to learn and form memories (Madden, 2002). For instance, it has been demonstrated that during LTP, AMPA receptors modulates synaptic activity by increasing their open probability and changing its conduction properties, in addition to the recruitment of more AMPA receptors to the postsynaptic site, which together contributes to the continuous expression of potentiation (Benke et al., 1998; Collingridge et al., 2004). Supporting the view to the importance of AMPA receptors to plasticity, studies have demonstrated that mice lacking GluR1 (GluR1 -/-) cannot generate LTP (Malinow & Malenka, 2002; Zamanillo et al., 1999). Although surprisingly these animals have an intact spatial reference memory in the MWM (Zamanillo et al., 1999), they are dramatically impaired on spatial working memory (Sanderson et al., 2008). Furthermore, dysfunction of these ionotropic glutamate receptors has also been implicated in a range of neuropathologies such as epilepsy and chronic pain, as well as in Alzheimer's disease (Dingledine et al., 1999; Henley & Wilkinson, 2013).

The third family of ionotropic receptors are the kainate receptors (KA-R). These receptors are formed by homo or hetero arrangement of five different subunits, GluK1 to GluK5 (previously known as GluR5-7), and KA-1 and KA-2 (Dingledine et al., 1999; Lerma & Marques, 2013). Interestingly, KA-1 and KA-2 must be present within

GluA5-7 subunits in order for kainate receptors to be functional (Herb et al., 1992; Sakimura et al., 1992). Similarly to the other ionotropic channels, activation of KA-R leads to the depolarization of neurons, mainly to the influx of Na^+ led ions, which is notably slower than those mediated by AMPA-R (Copits & Swanson, 2012).

Although the dynamics and structure of Kainate receptor family is less understood than NMDA and AMPA receptors, KA-R play a significant role in the CNS at three different levels: mediating postsynaptic depolarization, modulating presynaptic release of both glutamate and GABA neurotransmitters, and playing a role of neuronal circuits during brain development (Collingridge et al., 2004; Lerma & Marques, 2013). Overall, it is suggested the predominant physiological function of kainite receptors is modulate synaptic transmission and plasticity, rather than mediate it, as the other glutamate receptors (Bortolotto et al., 1999; Copits & Swanson, 2012). Given its importance to the brain connectivity, unsurprisingly studies have linked KA-R to a range of brain and mood disorders, including epilepsy, autism, schizophrenia, depression and bipolar disorder (Lerma & Marques, 2013).

Excitatory synapses have morphological and specialised regions at the postsynaptic membrane called postsynaptic density (PSD). These unique sites are exclusive to glutamatergic synapses, located in its majority at the tip of dendritic spines (Sheng & Kim, 2011). The PSD contains not only glutamate receptors, that are activated by glutamate released from the presynaptic terminal, but also a variety of scaffolding proteins and signalling complexes involved in synaptic transmission and plasticity (Harris & Weinberg, 2012; Sheng & Kim, 2011). Proteomic studies have identified over 450 different proteins located at the PSD (Collins et al., 2006; Peng et al., 2004). Although the functional significance of many of these proteins remains elusive, a few of them have been characterised and classified into different functions.

The great majority of these proteins belong to cytoskeleton and scaffolding proteins, protein kinases and GTPases (Sheng & Kim, 2011). Among the list the most common proteins are the PSD-95 family (PSD-95, PSD-93, SAP97 and SAP102), CaMKII family (CaMKIIα and CaMKIIβ) and the Shank family (Shank1, 2 and 3) (Collins et al., 2006;

Peng et al., 2004; Sheng & Kim, 2011). The abundance of synapse scaffolding proteins in the PSD confers their central aspect in synaptic function, due to their structural domains that allow protein-protein interaction, therefore stabilising various membrane proteins and signalling molecules at the PSD (E. Kim & Sheng, 2004).

At the postsynaptic site, glutamate receptors are anchored at particular regions. For instance, NMDA-Rs are anchored to the PSD through a direct interaction with PSD-95 (Lau & Zukin, 2007; Niethammer et al., 1996; Sheng & Kim, 2011). Similarly to NMDA-Rs, AMPA receptors are also anchored at the postsynaptic membrane through a mediated interaction with Stargazing, a postsynaptic transmembrane protein, which directly binds to PSD-95, clustering AMPA receptors at the excitatory synapses (L. Chen et al., 2000; Cuadra et al., 2004). Given its role in synaptic stability and function, the PSD is considered by some authors as a proteinaceous organelle attached to the postsynaptic plasma membrane and held by cytoplasmic actin filaments (E. Kim & Sheng, 2004; Sheng & Kim, 2002).

Postsynaptic densities are usually located at dendritic spines, the primary recipient for excitatory inputs, which provide biochemical compartments that locally control signalling mechanisms of individual synapses. This compartmentalization allows spines to act as electrical and biochemical units (Bourne & Harris, 2008; Sorra & Harris, 2000). Spines are actin-rich protrusions that stem from the dendritic shaft, which vary in size and shape. Typically, spines have an enlarged head, where the PSD containing glutamate receptors, scaffolding proteins and signalling molecules, described above are located. Spines also can have a neck, which connects the spine head to the dendritic shaft (Bourne & Harris, 2008; Sorra & Harris, 2000). For instance, hippocampal spine-heads on average measure 0.6 µm and form long and thin, to mushroom shaped or stubby and neckless structures (Bourne & Harris, 2008). All spines contain a PSD, giving this structure its unique feature of being easily identified by electron microscopy (Sheng & Kim, 2011). Interestingly, spine morphology correlates with their function and maturation. For example, mushroom spines have larger, more complex PSDs, with a higher content of glutamate

receptors (Harris et al., 1992; Matsuzaki et al., 2001), whereas small thin spines are more flexible and responsive to activity (Bourne & Harris, 2008). These features underlie the individuality of each spine to respond to stimuli and change in activity, but more importantly demonstrate their capability of plasticity.

Dendritic spines can undergo morphological changes. The use and the advance of imaging techniques with the combination of in vitro and in vivo studies allowed the characterisation spine morphological changes during LTP and LTD (Alvarez & Sabatini, 2007; A. Holtmaat & Svoboda, 2009; Murakoshi & Yasuda, 2012). For instance, now it is well known that induction of LTP leads to the formation of new dendritic spines, together with the stabilization and a dramatic increase in the volume (up to two fold of the original size) of the already existing ones (Kopec & Malinow, 2006; Matsuzaki et al., 2004; Murakoshi & Yasuda, 2012). Interestingly*, in vivo* studies further confirm these observations (Xu et al., 2009; Ziv & Ahissar, 2009). In contrast, studies show that LTD is associated with spine shrinkage and elimination (Nagerl et al., 2007; Straub & Sabatini, 2014; Q. Zhou et al., 2004). Together these studies demonstrated plastic morphological nature of spines, and how it is orchestrated in an activity dependant manner.

1.3.3. Gabaergic synapses

Inhibitory synaptic transmission in the brain is mediated by the amino-acid neurotransmitter γ-aminobutyric acid, hence the name GABA. GABA is synthesised from glutamate by two glutamic acid decarboxylases, GAD-65 and GAD-67 (Pinal & Tobin, 1998). GABA is loaded into vesicles at the presynaptic side by vesicular GABA transporter (vGAT) (Buddhala et al., 2009; D. L. Martin & Rimvall, 1993). Upon release, GABA leads to hyperpolarisation of the postsynaptic neuron, reducing the probability of cell firing action potentials, being therefore called inhibitory neurotransmitter.

GABA acts on two receptors, GABAa and GABAb (Connelly et al., 2013; Couve et al., 2000; Jacob et al., 2008), which are selectively permeable to Cl^-. GABAa are fast acting ionic receptors. To date, 18 GABAaR subunits have been identified and are classified in six subfamilies (α (1-6), β (1-3), γ (1-3),δ, ε (1-3), ϕ and π), according to its sequence homology (Connelly et al., 2013; Jacob et al., 2008). GABAaR are formed of heteropentameric structures, which despite its variety of subunits, their vast majority are formed of 2α, 2β subunits and one γ or one δ subunits (Amin & Weiss, 1993; Jacob et al., 2008). Unlike GABAaR, GABAbR are metabotropic G-protein coupled receptors. GABAbR play a rather complex role in the neurotransmission, which has just started to be characterised. These receptors are thought to release G$\beta\gamma$ subunits that inhibit Ca^{++} channels and activate K^+ channels (Chalifoux & Carter, 2011; Couve et al., 2000). In addition, GABAbR interact with multiple downstream signalling cascades, including reduction of cAMP levels and decrease in protein kinase A activity (PKA), all in consequence of the release of Gα subunits (Chalifoux & Carter, 2011; Couve et al., 2000).

Despite the intricate machinery involved in transmission, GABAergic synapses have a relative simple structure. Inhibitory synapses form directly onto the dendritic shaft, as opposed to more structurally complex spines of excitatory synapses. In contrast to the PSD of excitatory synapses, which have extensively studied and characterised, much less in known about the postsynaptic aggregations of GABAergic synapses (Fritschy et al., 2008). Although the whole molecular constitution of the postsynaptic side of inhibitory synapses has not been fully studied, it is known that the major component on these synapses is gephyrin (Fritschy et al., 2008; Kneussel & Loebrich, 2007), a scaffolding protein that anchors GABA receptors and interacts with signalling molecules and cytoskeleton proteins (Fritschy et al., 2008; Jacob et al., 2008; Kneussel & Loebrich, 2007; Tretter & Moss, 2008).

Although Gabaergic synapses are classified as inhibitory synapses, the opposite occurs during development. Indeed, GABA is the principal excitatory neurotransmitter until the neonatal period. This phenomenon occurs due to a switch

in the expression of KCC2, a K+/Cl- transporter, leading to the modification of GABA action (Ben-Ari, 2002; K. Li & Xu, 2008). Once adulthood is reached, GABAergic synapses account to approximately 20% of total synapses in the forebrain (Rubenstein & Merzenich, 2003). These unique features of GABA synapses show the importance of their "self-exchangeable" capability for the formation of the neuronal network as well as for the function of neuronal activity. Just as with excitatory synapses, dysfunction in GABAergic synapses can lead to neurological conditions such as epilepsy, memory dysfunction, seizure susceptibility among other behavioural changes, as well as neuro-developmental disorders including autism (Rudolph & Mohler, 2004; Smith-Hicks, 2013).

1.4. Synapse formation

Synapses are complex and highly specialised junctions, formed of thousands of different molecules. The formation of chemical synapses is a process that involves a bi-directional communication between the pre- and postsynaptic partners, so the establishment of a precise connection can be achieved (Cohen-Cory, 2002; Craig et al., 2006). To date, great progress in understanding of synapse formation, including the morphological, cellular and molecular mechanisms, has been made. This process, which was previously mainly based on the knowledge coming from extensive studies at the neuromuscular junction (NMJ), has now been expanded and extended to central nervous system (CNS). The combination of microscopy, real-time live imaging, genetic manipulations and *in vivo* and *in vitro* studies enhanced the understanding of what molecules and processes are orchestrating the process of synaptogenesis, as well as the establishment of neuronal connections.

Extensive studies looking into the formation of central synapses, particularly excitatory synapses, subdivided this process in five key steps: 1) initial contact between the axon and the dendrite; 2) induction of local signals that leads to the next step; 3) synaptic differentiation; 4) structural and functional maturation and 5) maintenance and stabilisation (Garner et al., 2006; Johnson-Venkatesh & Umemori,

2010; Waites et al., 2005), which can be arguably included or not in the process of synaptogenesis. Each of these steps (illustrated in Fig. 1.4) will be individually discussed below (except to synaptic maintenance – separately addressed on Introduction Section 1.5).

Synaptic assembly starts when the axon establishes contact with the target neurons (Fig 1.4) – either their dendrite or soma. Many real-time imaging studies demonstrated that both, axons and dendrites are involved in this first stage of assembly, although still unclear whether the pre- or postsynaptic site starts the process of synaptogenesis (Cohen-Cory, 2002; Jontes & Smith, 2000). At glance, this initial stage occurs in a rather frenetic and promiscuous manner, where the first contacts would be formed in a random and transient fashion, as demonstrated by various studies that simultaneously labelled and observed the pre and postsynaptic sites dynamics over time (Cohen-Cory, 2002; Jontes et al., 2000; Meyer & Smith, 2006; Niell et al., 2004). Importantly, together these studies show that both dendrites and axons are highly dynamic and motile, being both involved in the search for partners during synaptogenesis.

Once the initial contact is established, rapid morphological events in both sides of the "synapse-to-be" take place, a process called differentiation. Studies show that differentiation is triggered and coordinated by bidirectional signals coming from both side of the synapses (Craig et al., 2006; Waites et al., 2005). At the presynaptic site, transport packets containing preassembled synaptic vesicle components, enabling synaptic activity, are rapidly mobilised to the site of contact (Ahmari et al., 2000; Friedman et al., 2000). Interestingly, it has been demonstrated that this process can occur within 15 minutes of the first axon-dendrite contact (Ahmari et al., 2000; Friedman et al., 2000). By contrast, the postsynaptic recruitment of proteins is believed to occur in a slower fashion. Time-lapse microscopy using cultured hippocampal neurons revealed that clusters of postsynaptic molecules – SAP90/PSD95 – and glutamate receptors, may take as long as 45 minutes to reach the newly formed site (Friedman et al., 2000; Ziv & Garner, 2001).

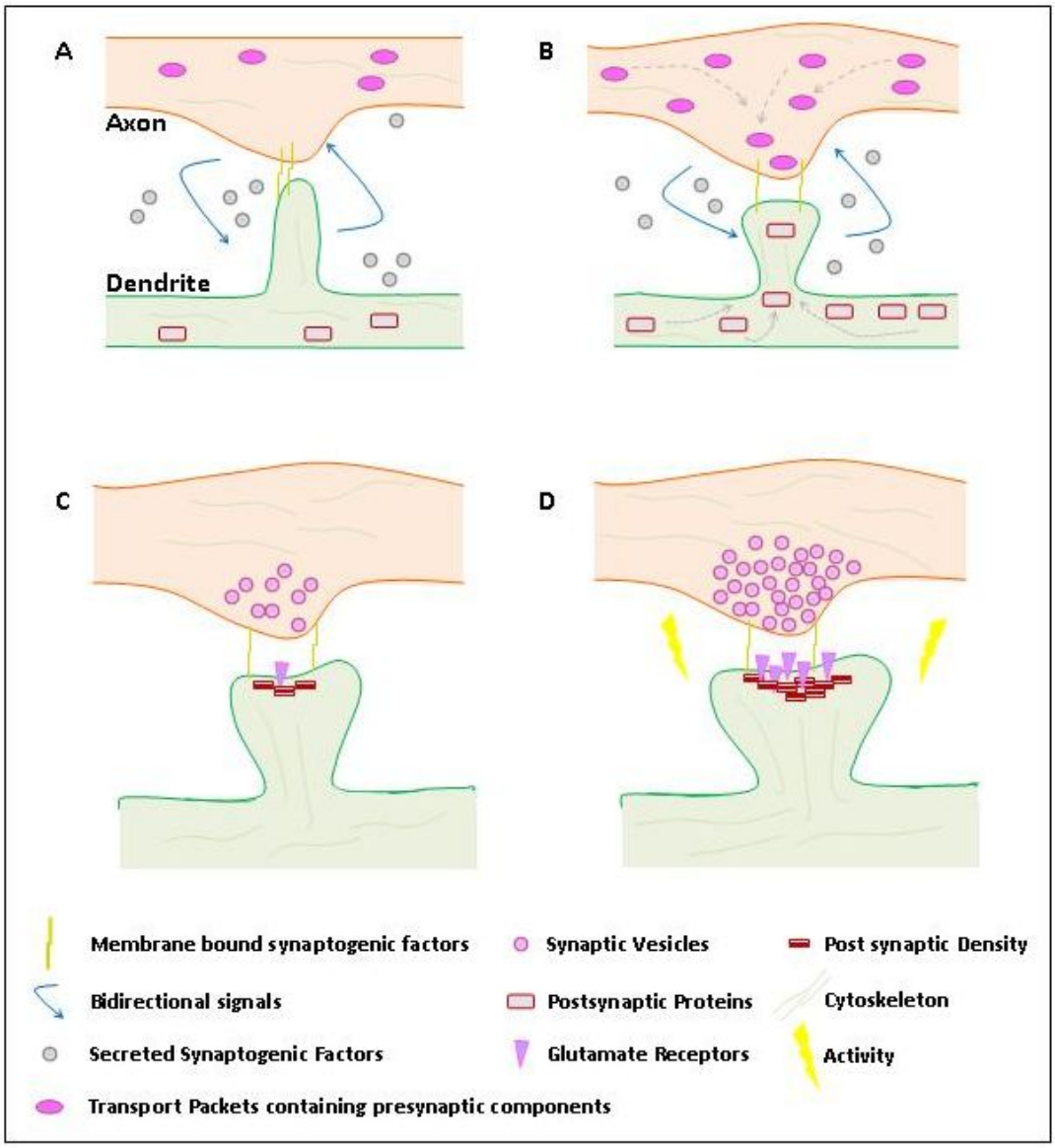

Figure 1.4 - The main steps in the formation of glutamatergic synapses. (A) Initial contact: Synaptic assembly starts when the axon establishes contact with the target neurons, which can be stimulated by either secreted synaptogenic factors such as Wnts and BDNF, or membrane bound proteins such as SynCams. **(B) Induction:** this is the process in which is triggered and coordinated by bidirectional signals coming from both side of the synapses, under the influence of synaptogenic factors, stimulating the transport of proteins to the synaptic sites, leading to the differentiation of the synapse. **(C) Differentiation:** this hallmark of the synaptogenesis is characterised by the mobilisation of transport packets containing preassembled synaptic vesicle components in the axon and the postsynaptic recruitment of proteins and glutamate receptors at the postsynaptic site. **(D) Maturation:** Following differentiation, maturation will be necessary to recruit and assemble the final key synaptic proteins. Presynaptically, vesicles continue to accumulate, and PSD becomes more pronounced. Crucially, this process is activity dependent and is accompanied by changes in the shape of the dendritic spines, which modulated by actin dynamics. *Adapted from Garner et al., 2006.*

Differentiation does not always give rise to a long lasting synapse. Following

differentiation, maturation will be necessary to recruit and assemble the final key synaptic proteins, induce its morphological change and develop its electrical properties (Fox & Umemori, 2006; Scheiffele, 2003; Waites et al., 2005). It has been proposed that the cross-talk between the pre- and postsynaptic site is fundamental for the real establishment and maturation of the synapse. Synapses with "productive signalling", meaning harmonic signals that are responded and influencing both sides of the synapses, will lead to synaptic stabilisation whereas mismatch signalling, will culminate in the elimination of the synapse (Scheiffele, 2003). During the maturation process, whilst some synapses are eliminated, the remaining synapses go under major morphological modifications. At the presynaptic site, vesicles continue to accumulate around the contact site (Ahmari et al., 2000; McAllister, 2007) and at the postsynaptic site, PSD also accumulates and becomes more pronounced (Friedman et al., 2000; Garner et al., 2006). Crucially, this process is accompanied by changes in the shape of the dendritic spines. Young spines go through dramatic changes, maturating and changing their morphology from thin filopodia-like shapes into well-defined heads and constricted necks (Tada & Sheng, 2006; Yuste & Bonhoeffer, 2004; Ziv & Smith, 1996). This maturation stage is highly dependent on the cytoskeleton dynamics, in particular actin (Garner et al., 2006; W. Zhang & Benson, 2001).

Actin is a major component of the postsynaptic densities, where it has a major role anchoring NMDA and AMPA receptors at immature sites (Allison et al., 2000; Allison et al., 1998). Indeed, exposure of young neurons to latrunculin A, an actin depolymerising toxin, leads to the almost complete loss of synapses (W. Zhang & Benson, 2001), demonstrating a key role of this protein in synapse maturation (Garner et al., 2006; W. Zhang & Benson, 2001). Furthermore, studies looking into the role of actin in the maturation of synapses regulated by activity, demonstrated that neuronal stimulation induces the remodelling and appearance of new sites labelled with green-fluorescent actin (Colicos et al., 2001). Interestingly, blockade of glutamate receptors prevents the remodelling of actin and formation of new synaptic sites (Colicos et al., 2001), demonstrating the interdependence of actin and

glutamate receptors in synapse formation, morphological changes and maturation of synapses.

Along with cytoskeleton proteins, shown to be important during synaptogenesis, many other molecules are well regarded to induce synaptogenesis and/or synaptic differentiation. These proteins, which are now known to be involved in different stages – and sometimes all stages – of synaptogenesis, from the initial contact, to induction and throughout the maturation of synapses, are regulated by membrane-bound signalling proteins and secreted factors. Among this broad classification, there are the cell adhesion molecules (CAM), including members of the cadherin family, neurexin, neuroligins and secreted proteins such as Wnts and neurotrophic factors. Given the focus of this thesis, in the next section I will briefly discuss the importance of membrane-bound proteins and give more emphasis to the importance of secreted proteins in synaptogenesis and synaptic maintenance (a summary table with molecules involved in synaptogenesis can be found in the end of this section – Table 1).

1.4.1. Synapse organising molecules

1.4.1.1. Membrane bound proteins – Adhesion systems

SynCAMs and Nectins

Proteins expressed at the synapses and those allow the interaction between the pre and postsynaptic sites, are known to be involved in the organisation and contribution to the formation of synapses. Among these proteins are the SynCAMs (synaptic cell adhesion molecules), a group of proteins that are part of the immunoglobulin (Ig) family, known to be expressed in the central nervous system (Biederer et al., 2002; Robbins et al., 2010; Thomas et al., 2008). SynCAM has 4 distinct isoforms (named SynCAM 1 to 4) and are highly expressed in both the developing and the mature brain and are localised at the pre and postsynaptic sites

(Biederer et al., 2002; Fogel et al., 2007; Thomas et al., 2008). Structurally, SynCAMs contain three Ig domains which allow the specific homophilic interactions via their immunoglobulin domains resulting in strong cell adhesion (Biederer et al., 2002; Missler, 2003).

SynCAM are involved in the formation of synapses (Fig 1.5). It has been shown that SynCAM1 is already expressed in the developing neurons and is localised at their growth-cones during early stages of synapse formation (Stagi et al., 2010). Furthermore, the same study demonstrated that SynCAM1 participates in the axon-dendrite interaction and it is required for the regulation of morphological differentiation of synapses (Stagi et al., 2010), which indicates a role of this protein during the contact-mediated differentiation during synaptogenesis (Robbins et al., 2010; Stagi et al., 2010).

Previous to this finding, an elegant study demonstrated that overexpression of SynCAM promotes the formation of fully functional glutamatergic synaptic sites, including in non neuronal cells co-cultured with hippocampal neurons (Fig 1.5) (Biederer et al., 2002). Corroborating to this finding, a follow up study showed that overexpression of SynCAM in young neuronal cells increase excitatory synaptic function without promoting any changes in inhibitory synapses (Sara et al., 2005). However, the same study demonstrated that overexpression of SyCAM1 in immature neuronal cultures does not promote any further increase in synapse number, suggesting a role of this protein for synapse maturation rather than formation (Sara et al., 2005). Despite the conflictive results, more recently a research group examining the *in vivo* role of SynCAMs demonstrated that indeed overexpression of SynCAM1 has an effect in both formation and maturation of synapses (Robbins et al., 2010). Furthermore, the study suggested the involvement of SynCAM1 not only in synaptogenesis but also in synapse plasticity, memory formation and remodelling of neuronal circuits (Robbins et al., 2010).

Another Ig-like family of cell-cell adhesion molecules are the nectins. Similarly to the SynCAMs, the nectin family has four different isoforms members, named Nectin-1 to

4 (Biederer et al., 2002; Rikitake et al., 2012). These cell-cell transmembrane adhesion molecules, originally identified as virus receptors, are linked to the actin cytoskeleton via an actin-binding protein called afadin (Mandai et al., 1997).

It has been demonstrated that nectins are highly expressed in the CNS, being therefore suggested to play a role in synapse formation (Fig 1.5). Indeed, at the hippocampus CA3 mossy fibers, nectins are present at the synaptic junctions, in both pre- and postsynaptic sites (Mizoguchi et al., 2002), and the nectin-nectin interaction occurs in a heterophilic manner (Mizoguchi et al., 2002). The cited study show that Nectin-1, present at the presynaptic site, binds to Nectin-3 at the postsynaptic site, and this interaction pattern is conserved in both *in vitro* and *in vivo* models (Mizoguchi et al., 2002). Interestingly, the same study yet show that specific inhibition of Nectin-1 in immature hippocampal neuron leads to a decrease in synaptic puncta, labelled with synaptophysin (Mizoguchi et al., 2002), suggesting a role for nectin in synaptic formation. Supporting this finding, a study investigating the temporal and spatial localization showed that Nectin-1 is present in hippocampal neurons even before synaptogenesis (Lim et al., 2008), further supporting the view on the importance of nectins for synaptic formation.

More recently, an in vivo study using an afadin conditional knockout mouse, show a role for this protein for excitatory synapse formation at the hippocampus (Fig 1.5). Inactivation of afadin in the hippocampus before the synaptogenesis period leads to a significant decrease of pre and postsynaptic markers, labelled with vGlut1 and bassoon (Toyoshima et al., 2014), suggesting a role for nectin in excitatory synapse formation. Although it is not clear yet whether nectin is involved in the formation of inhibitory synapses, it has been demonstrated that in young neurons Nectin-1 is colocalised with GAD-67 labelled cluster (Lim et al., 2008), indicating a possible link between nectins and gabaergic synapses.

Neurexins/Neuroligins

Neurexins and neuroligins complexes (NRX – NL) are a family of highly polymorphic neuronal cells surface proteins present trans-synaptically and bridging the synaptic cleft (Craig & Kang, 2007; Sudhof, 2008; Ushkaryov & Sudhof, 1993). Morphologically, NX is present presynaptically binding to NL, present postsynaptically (Ichtchenko et al., 1996; Sudhof, 2008). In vertebrates, NRXs are encoded by three different genes (NRXN1 to 3) under two distinct promoters (α and β), and the alternative splice variants give raise to over 2000 expressed proteins (Baudouin & Scheiffele, 2010; Ullrich et al., 1995). NL, by contrast, are encoded by up to 5 genes in vertebrates (NLCL1 to 4 and NLGL4Y in humans), having up to twelve splice variants (Baudouin & Scheiffele, 2010). The great interest for NRX-NL and their role in the CNS was initiated after the discovery of these proteins as synaptic organisers (Scheiffele et al., 2000; Sudhof, 2008).

NRX and NL are synaptogenic mediators of excitatory and inhibitory synapses (Fig 1.5). When expressed in non-neuronal cells, NL 1 induces clustering of synaptic vesicles proteins and formation of functional presynaptic sites of co-cultured neurons (Scheiffele et al., 2000). Interestingly, when NRX was overexpressed at the presynaptic site in non neuronal cells, it induced the differentiation of postsynaptic proteins and clustering of NMDA glutamate receptors in co-cultured neurons (Graf et al., 2004). Furthermore, overexpression of NRX in non-neuronal cells also induce the clusters of the GABAergic postsynaptic component gephyrin (Graf et al., 2004), demonstrating the involvement of NRX-NL complexes in both excitatory and inhibitory synapses. *In vivo* knockout of NL-1 and NL-2 leads to defects of excitatory and inhibitory synaptic transmission respectively (Chubykin et al., 2007), confirming the importance of NLs for neuronal circuit formation. In addition, these results show the specificity NLs isoforms influencing different types of synapses – NL-1 in excitatory synapses, whereas NL-2 in inhibitory synapses (Chubykin et al., 2007; Graf et al., 2004).

Despite the evidence indicating a role for NRX-NLs in synaptogenesis, they are in fact to be involved in synaptic function and transmission (Sudhof, 2008). The ultimate study supporting this interpretation was shown using mutant mice lacking neuroligin expression (Varoqueaux et al., 2006). Triple NL1-3 knockout die shortly after birth due to respiratory failure (Varoqueaux et al., 2006). However, density of synaptic contacts is unchanged in brains and cultured neurons of mutant mice (Varoqueaux et al., 2006). Interestingly, these mutant animals present a profound deficiency in synaptic transmission, in both excitatory and inhibitory synapses (Varoqueaux et al., 2006). Therefore, it seems that NRX and NL are indeed vital for synapse maturation and function, rather than acting purely as a synaptogenic factor, as previously hypothesised.

Ephrins – Eph Receptors

Eph proteins are a family of postsynaptic tyrosine kinase receptors that interact with the presynaptic ligant ephrins, forming a bidirectional trans-synaptic signalling complex (Kayser et al., 2008; Klein, 2009). The mammalian genome encodes 14 different Eph receptors, nine EphAs (EphA 1-8 and EphA10) and five EphB (EphB1-4 and EphB6). EphAs typically bind to A-type ephrins (five different isoforms), whereas EphBs bind to B-type Ephrins (three isoforms) (Hruska & Dalva, 2012; Klein, 2009). Several Ephs are localised at the spines and dendrites of neurons, suggesting a functional role in synapse formation and/or stability (Hruska & Dalva, 2012).

Ephrins-Eph signalling regulates synapse formation and recruitment of receptors (Fig 1.5). Analysis of triple mutant mice lacking EphB receptors show defective hippocampal spine formation, having defected spine number and morphology (Henkemeyer et al., 2003). Furthermore, it was observed that defective spine formation in triple mutants is associated with a general decrease in the number of NMDA and AMPA receptor clusters (Henkemeyer et al., 2003). In addition, analysis of cortical neurons from animals lacking EphB1-3 show that PSD95 was found to be translocated to the dendritic shaft (Kayser et al., 2006). This demonstrates the critical role of EphB for the localisation and recruitment of PSD-95 to the synapses

and consequently to the anchoring of glutamate receptors at the synaptic sites. Interestingly, post-natal re-expression of EphB2 in triple EphB knockout animals can completely reverse the dendritic phenotype and restore the organisation of PSD95 and associated AMPA receptors (Kayser et al., 2006), demonstrating a crucial role for forward signalling of Ephrin-Eph during synaptic development.

Ephrin-Eph signalling has a dual function. Disruption in the interaction of EphrinA/EphA signalling in a transgenic model leads to a reduction in the number of synaptic terminals projected to the hippocampus from the entorhinal cortex (Martinez et al., 2005), demonstrating the importance of reverse Ephrin-Eph signalling during synaptogenesis (Fig 1.5). Intriguingly, further analysis of the same animal model revealed that mossy fibre terminals form longer and more numerous projections, establishing ectopic contacts during the post natal stages (Martinez et al., 2005). Corroborating to this finding, analysis of EphrinB/EphB3 mutant mice also revealed an increase in hippocampal synaptic density (Rodenas-Ruano et al., 2006), suggesting that Ephrin/Eph reverse signalling can have both pro and anti-synaptogenic properties. Together, these findings demonstrate the importance of forward and reverse Ephrin-Eph signalling for excitatory synapse formation. Given their pro and anti-synaptogenic properties, it raises the question to what extent these molecules can regulate pre and postsynaptic development and whether they can also orchestrate circuitry refinement.

Cadherins

Cadherin is a family of cell adhesion proteins characterised by their large extracellular domain containing five cadherin repeated sequence that mediates calcium-dependent homotypic interactions (Arikkath & Reichardt, 2008; Kwiatkowski et al., 2007). Intracellularly, cadherins contain a cytoplasmatic tail linked to the cytoskeleton actin via the interaction with α and β catenin (Arikkath & Reichardt, 2008; Kwiatkowski et al., 2007). This superfamily of proteins contains at least 80 members, being the vast majority of them classed as transmembrane proteins (Yagi & Takeichi, 2000). Given its structure, this family of proteins provides cell adhesive

function combined with the ability to interact with various intracellular pathways (Arikkath & Reichardt, 2008).

Cadherins are important for synaptogenesis and neuronal connectivity. Multiple cadherins are expressed in the CNS throughout the postnatal development in a different spatial temporal pattern (Bekirov et al., 2002), suggesting their contribution to synapse formation and differentiation. Early studies characterising neural cadherins show that N-cadherin and β-catenin are present in axon and dendrites in cultured hippocampal neurons before synapse formation (Benson & Tanaka, 1998). A complementary study, looking into the localisation of cadherins in relation to synapses during development, has demonstrated that in young neurons, cadherins are distributed evenly along the synaptic clefts and active zones (Elste & Benson, 2006). Interestingly, as the synapse matures, cadherins distribution changes and they are found in clusters within the active zone area (Elste & Benson, 2006).

Loss of function of cadherins during early development leads to defect at the pre and postsynaptic sites as well as abnormal spine morphology (Fig 1.5). Blockade of cadherin function during synaptogenesis in cultured hippocampal neurons result in retarded spine development and altered spine morphology as well as disruption in the distribution of postsynaptic proteins (Togashi et al., 2002). Furthermore, cadherin loss of function also disrupts the organisation of the presynaptic site, where a clear reduction of synapsin puncta site and disruption in vesicle recycling is observed (Togashi et al., 2002). This study demonstrates that indifferent blockade of cadherin has a profound impact in the formation and establishment of excitatory synapses.

Cadherins are also important for inhibitory synapse formation (Fig 1.5). To date different studies have demonstrated the clear involvement of cadherins in excitatory synaptogenesis and spine formation (Saglietti et al., 2007; Togashi et al., 2002) However, until recently the involvement of these family of proteins in GABAergic synapse formation remained unclear. A recent study screening different cadherins in synapse development identified a role of specific cadherins in inhibitory synapse

formation (Paradis et al., 2007). Silencing of Cadherin-11 and 13 using shRNA in young neurons leads to a significant decrease in density of GABAergic synapses, identified by vGAT and GAD67 markers (Paradis et al., 2007). Together, these results indicate that cadherins regulate the formation of both inhibitory and excitatory synapses.

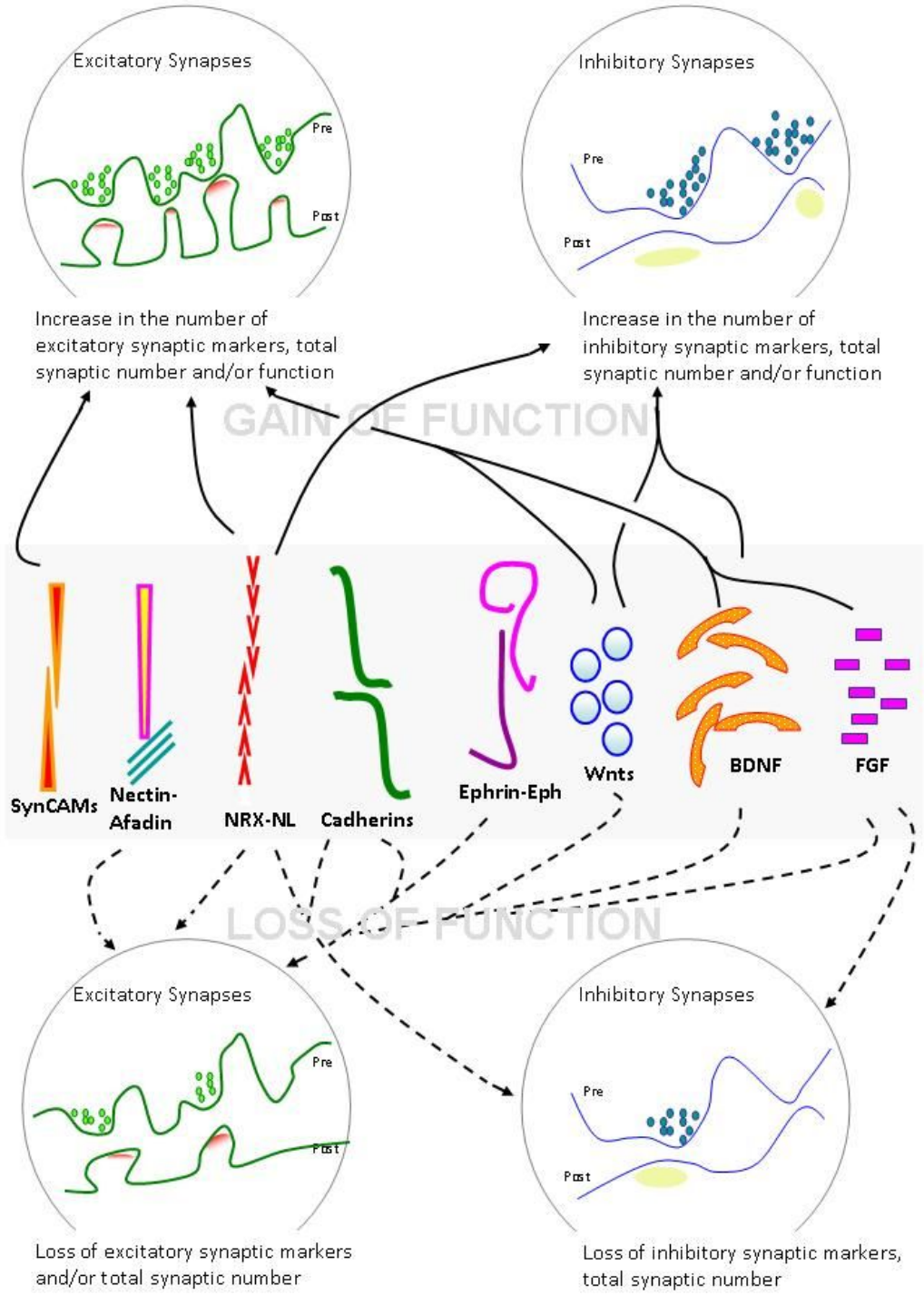

Figure 1.5: Synaptic organising molecules. Gain of function of each individual proteins in the present at the grey panel leads to the phenotypes highlighted at the upper are of the figure (full arrows). Loss of function of the same proteins (dashed arrows), leads to synaptic phenotypes described in the bottom of the figure. Abbreviations: NRX-NL, neurexin-neuroligin.

1.4.1.2. Secreted factors

Neurotrophins

Neurotrophins are a family of closely related proteins firstly identified as promoters of neuronal survival, but now known to be involved in neuronal development and function (Carvalho et al., 2008; Reichardt, 2006). Four different neurotrophins are expressed in mammals: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). Neurotrophins interacts and signal through tyrosine kinase receptors (Trk) activating a variety of intracellular cascades with heavy influence in gene transcription regulation.

NGF led the way as a neurotrophic factor. The first evidence to the importance of NGF in synapses comes from a study that looked into the effect of this factor in the sympathetic nervous system. Treatment of young neonatal neurons with antiserum to NGF causes a reduction in the number of contacts in sympathetic neurons (Nja & Purves, 1978), suggesting a role for this factor during synapse formation. Many subsequent studies demonstrated the importance of NGF signalling for sympathetic neurons, regulating also dendritic and axon growth (Zweifel et al., 2005). Other studies have demonstrated the importance of NGF in the CNS, being expressed during foetal development, but specially regulating the activity of acetylcholine neurons in the adulthood (Pizzuti et al., 1990). Following the characterisation of NGF, the second neurotrophic factor to be described was BDNF, as a factor that allowed the survival of retinal ganglion neurons that were unresponsive to NGF (Johnson et al., 1986). Since its discovery, many studies demonstrated the importance of BDNF for synapse formation in the CNS.

BDNF is capable of promoting synaptogenesis (Fig 1.5). BDNF signals through it receptor tropomyosin kinase-B (TrkB) and both, ligand and receptor, are well characterised by their crucial role during synapse formation (McAllister et al., 1999; Shen & Cowan, 2010). BDNF and TrkB are localised at the synapses and extra-synaptically, at the pre and postsynaptic sites, in both inhibitory and excitatory

synapses – although preferentially at glutamatergic synapses (Gomes et al., 2006; Swanwick et al., 2004).The first evidence of BNDF as a synaptogenic factor comes from a study looking into the formation of the cat visual cortex (Cabelli et al., 1995). This study demonstrated that infusion of BDNF into the cat primary visual cortex causes an imbalance in the formation and elimination of synapses, shifting the balance towards the formation of extra synapses (Cabelli et al., 1995). Supporting this finding, a subsequent study demonstrated that hippocampal neurons exposed to BNDF treatment have a large increase in the number of functional synaptic connection (Vicario-Abejon et al., 1998). Moreover, the study also showed that BDNF induces the formation of inhibitory and excitatory synapses (Vicario-Abejon et al., 1998), suggesting a role for this molecule in glutamatergic and GABAergic synapse formation. Conversely, deletion of TrkB receptor *in vivo* results in lower density of synapses in hippocampus and decrease in the accumulation of presynaptic proteins and reduction in postsynaptic density thickness (Fig 1.5) (Martinez et al., 1998). Although it is yet not clear whether BDNF is also involved in the formation of inhibitory synapses, the cited studies clearly demonstrate importance of BDNF and its receptor for the formation of excitatory synapses.

Fibroblast- growth factor

Fibroblast-growth factors (FGFs) constitute a large family of heparin-binding polypeptide factors found in a range of different multicellular organisms. This family of polypeptides are well known for their essential role during embryonic development and, postnatally, as metabolism regulator (Bottcher & Niehrs, 2005; Itoh & Ornitz, 2011). In mammalians 22 FGFs members have been described, which are grouped in different subfamilies, according to their morphology and mechanism of action (Belov & Mohammadi, 2013; Itoh & Ornitz, 2011). FGFs bind and activate FGF-receptors (FGFR), are a family of 4 different coded tyrosine kinase receptors capable of multiple downstream signalling cascades (Belov & Mohammadi, 2013; Itoh & Ornitz, 2011). Since its first description 40 years ago on the ability to promote fibroblast proliferation (Gospodarowicz, 1974), FGFs have been broadly studied and

found to play multiple roles in every system in the organisms, with much emphasis in the CNS.

FGFs are synaptogenic factors. The first evidence that FGFs were valid candidates for presynaptic organiser and, in fact, they can promote synapse formation, comes from *in vitro* studies using *Xenopus* spinal cord neurons (Dai & Peng, 1995; Johnson-Venkatesh & Umemori, 2010). The study demonstrated that neurons exposed to beads coated with basic FGF, also known as FGF2, have an increased clustering of synaptotagmin and synaptic vesicles at the area in contact with the beads, together with the increase in calcium influx (Dai & Peng, 1995). A similar study investigating the possible role of FGF2 in central synapses, further demonstrate the synaptogenic role for FGF (Fig 1.5) (A. J. Li et al., 2002). The latter demonstrated that young hippocampal neurons exposed to FGF2 have an increased number of synaptophysin and synapsin-I clusters in comparison to control cultures (Johnson-Venkatesh & Umemori, 2010; A. J. Li et al., 2002). Furthermore, the same study shows that FGF2 promotes an increase in the number of PSD-95 clusters and in the number of glutamate receptors GluR1, as well as the number of synaptophysin colocalised with Glur1, suggesting these synapses may be functional (A. J. Li et al., 2002). These findings demonstrate that FGF2 promotes de recruitment and differentiation of excitatory synapses at both pre- and postsynaptic sides, but also indicate the newly formed synaptic sites are functional.

In vivo studies further show the importance of FGF for synapse formation. Virtually all FGF knockout mice have been generated, and a range of phenotypes were observed, from impairment in organ development, to defect in cardiac function and embryonic lethality (Itoh & Ornitz, 2011). Interestingly, FGF7 and FGF22 knockouts mice have an imbalance in excitatory and inhibitory synapses. A recent elegant study demonstrated that disruption of FGF22 expression results in a decrease in both the number of the presynaptic marker vGlut1 and in the number of synaptic vesicles in asymmetric synapses in the hippocampus (Terauchi et al., 2010). FGF7 knockout mice, by contrast, had a specific defect in inhibitory synapses, with impairment in VGAT clusters and decreased number of synaptic vesicles in symmetric synapses

(Terauchi et al., 2010). Conversely, postsynaptic expression of FGF22 and FGF7 promotes the selective differentiation of excitatory and inhibitory presynaptic markers, respectively (Terauchi et al., 2010). Thus this study provides compelling evidence to the role of FGF in the differentiation of synapses, and more importantly it demonstrates for the first time the importance of FGF in inhibitory synapse formation. Together, these studies show the crucial role of FGF for the formation and differentiation of synapses, highlighting the importance of the right levels of their expression to reach the right balance between inhibitory and excitatory synapses for enabling the proper function of the brain.

Wnts

Wnts are a family of secreted molecules which have a crucial role in embryonic development (detailed Wnt pathway and intracellular cascades will be discussed on Section 1.7 of this Introduction). Wnts are also well recognised for their role in synaptic formation (Fig 1.5). The first indication that these molecules could be involved in synaptic formation/organisation comes from a morphological *in situ hybridization* study which shows that Wnt3 is expressed in the developing brain, throughout embryogenesis (Salinas & Nusse, 1992; Speese & Budnik, 2007). Indeed, a few years later, a breakthrough study demonstrated the synaptogenic effect of Wnt (F. R. Lucas & Salinas, 1997). Addition of Wnt7a to granule cells (GC) of cerebellar mouse neurons induced axonal branching, growth cone enlargement and increase in the level of synapsin I (F. R. Lucas & Salinas, 1997). Importantly, this effect was mimicked by blockade of GSK3β, suggesting that canonical Wnt regulate presynaptic differentiation and formation of neural connections, by regulating cytoskeleton and clustering of presynaptic proteins (F. R. Lucas & Salinas, 1997). Yet, in a follow up study, the same group demonstrated that secretion of Wnt7a by the GC act as a retrograde presynaptic organiser, inducing the clustering of synapsin I, but also regulating the formation of synapses between GC and incoming cerebellar mossy fibres (MF) (Hall et al., 2000). The study shows that endogenous Wnt7a modulates the formation of glomerular rosettes - complex interdigitated multi-

synaptic structures formed between GC and MF - a phenotype that is reduced by the addition of the Wnt antagonist Sfrp and in mice lacking Wnt7a (Hall et al., 2000).

Wnts, through Dishevelled-1 (Dvl1) promote the clustering of presynaptic proteins. Young hippocampal neurons treated with Wnt7b have a significant increase in the clustering of several presynaptic proteins, including Synapsin I, VAMP2 and Bassoon (Ahmad-Annuar et al., 2006). Further to the understanding of the mechanism of Wnt in synaptogenesis, the same study shows that overexpression of Dvl1 in hippocampal cultured neurons increases the number of synapsin I clusters by almost two fold (Ahmad-Annuar et al., 2006). In contrast, deficiency of Wnt signalling, demonstrated in the Dvl1 knockout mouse model, leads to a significant reduced number of presynaptic clusters in central synapses (Ahmad-Annuar et al., 2006), an effect that is more pronounced in the Wnt7a/Dvl1 double knockout mice (Ahmad-Annuar et al., 2006). Moreover, the study shows that synapses in Wnt7a/Dvl1 double knockout mice are functionally impaired, a phenotype reflected in the reduced frequency in the electrophysiological spontaneous recordings (Ahmad-Annuar et al., 2006). Following these findings, other studies demonstrated the synaptogenic effect of Wnt3a, Wnt7a and Wnt7b and the positive effect on the formation of new presynaptic clusters (Cerpa et al., 2008; E. K. Davis et al., 2008). Together these findings demonstrate that in central synapses Wnts stimulate the recruitment of presynaptic components to form functional synapses, and mechanistically Wnt7a and b signals through Dvl1 to promote synaptogenesis.

Wnt7a acts on excitatory synapses. A recent study from our group demonstrated that Wnt7a has a positive synaptogenic effect which is exclusive to excitatory synapses, an effect coordinated via postsynaptic signalling (Ciani et al., 2011). Exposure of cultured young hippocampal neurons to Wnt7a leads to an increase in the number and size of excitatory synapses, labelled with vGlut1 and PSD95 (Ciani et al., 2011). Interestingly, Wnt7a does not have any effect on inhibitory synapses (Ciani et al., 2011). The study shows that Wnt7a acts directly on the postsynaptic site via Dvl1 and CaMKII, inducing spine growth and strengthening of synapses (Ciani et al., 2011). Interestingly, Dvl1 overexpression in young neurons does not lead to the

formation of new postsynaptic sites, but induce the change in their morphology, increasing the size of dendritic spines (Ciani et al., 2011). Together with previous evidence on the effect of Wnts on synaptogenesis, these findings lead to the proposal that Wnt7a acts presynaptically via Dvl/Gsk3β to stimulate synaptic assembly, inducing the differentiation and formation of new postsynaptic sites. Postsynaptically, Wnt7a can act directly in the nascent synapse via Dvl1/CaMKII stimulating its growth. Therefore, Wnt7a acts in both sites, signalling bidirectionally, stimulating the formation and then maturation of synapses.

In addition to these studies, recent studies propose the positive action of Wnt5a towards synaptogenesis through alternative pathways. A study showed that hippocampal neurons exposed to Wnt5a in cultures have an increased number of PSD95 clusters, together with an increase in the phosphorylation of JNK (Farias et al., 2009). These observations led to the proposal that Wnt5a signals through a non-canonical Wnt pathway, via JNK, to promote the clustering of postsynaptic protein PSD95 (Farias et al., 2009). Yet, a follow up study, by the same group, suggested that short term exposure to Wnt5a also induce the de novo formation and growth of dendritic spines in dissociated hippocampal neurons (Varela-Nallar et al., 2010). Regrettably, those results were not reproducible (Cerpa et al., 2011). Further to this observation, the same group observed that Wnt5a induces the clustering of GABAergic receptors in mature hippocampal neurons, an effect proposed to be mediated by the activation of CaMKII (Cuitino et al., 2010). Despite the fact that these findings suggest a role for Wnt5a in the assembly and formation of new synapses, both inhibitory and excitatory, a previous study show that in fact exposure to Wnt5a reduces the number of presynaptic puncta in young hippocampal neurons, labelled with vGlut1 (E. K. Davis et al., 2008). These observations highlight the complexity of Wnt signalling and suggest that further work is required to elucidate how and to what extent Wnt5a is regulating the assembly of synapses.

Table 1. Synapse organising molecules and their reported function during synapse formation

Molecule	Location	Function during synaptogenesis	Refs
SynCAM	Pre and postsynaptic	*in vitro* overexpression induces formation, maturation and increased function of glutamatergic synapses no effect in inhibitory synapses *in vitro*	Biederer et al., 2002; Sara et al., 2005; Stagi et al., 2010 Sara et al., 2005
Nectin and Afadin	Pre and postsynaptic	*in vitro* inhibition of nectin-1 decreases synapsin labelled puncta *in vivo* afadin ko decreases the number of excitatory synapses	Mizoguchi et al., 2002 Toyoshima et al., 2014
Neurexins (NX) and Neuroligins (NL)	NRX presynaptic NL postsynaptic	*in vitro* overexpression of NL-1 induces the clustering of SV *in vitro* overexpression of NRX induces clustering of NMDAr and gephyrin puncta *NL-1 ko* mouse present defects in excitatory synaptic transmission; *NL-2* ko mouse present defects in inhibitory synaptic transmission *NL1-3 triple ko:* not viable, but neurons can be cultured and form synapses; presenting defects in synaptic transmission in excitatory *and inhibitory synapses*	Scheiffele et al., 2000 Graf et al., 2004 Chubykin et al., 2007 Varoqueaux et al., 2006
Ephrin and Eph receptors	Pre and postsynaptic	EphB triple ko present defected synapse and spine formation, lower number of glutamate receptor clusters and abnormal recruitment of PSD95 to the synapses	Henkemeyer et al., 2003 Kayser et al., 2006
Cadherins	location changes throughout development	in vitro loss of function disrupts the organisation of excitatory synapses and alters spine morphology shRNA for Cadherin-11 and 13 decreases the number of inhibitory synapses	Togashi et al., 2002 Paradis et al., 2007

***Table 1 continues on the next page

Table 1. Synapse organising molecules and their reported function during synapse formation (cont.)

Molecule	Location	Function during synaptogenesis	Refs
BDNF and TrkB receptor	secreted molecule	*in vitro* exposure to BDNF increases the number of glutamatergic and gabaergic synapses TrkB ko mice present a decrease in excitatory synapses	Cabelli et al., 1995 Vicario-Albejon et al., 1998 Martinez et al., 1998
FGF	secreted molecule	*in vitro* exposure to FGF2 increases the number of pre and postsynaptic sites, SV and number of glutamate receptor clusters *FGF22* ko have a decreased number of excitatory synapses and *FGF7* ko present a decrease in the number of inhibitory synapses	Dai & Peng, 1995; li et al., 2002 Terauchi et al., 2010
Wnts	secreted molecule	*in vitro* exposure to Wnt7a or Wnt7b increases the number of presynaptic proteins Wnt7a acts a synaptogenic factor on excitatory synapses *Wnt7a/Dvl1* ko have a decreased number of synapses and defected synaptic transmission Wnt5a promotes the clustering of postsynaptic excitatory sites and increases the clustering of GABAr	Lucas & Salinas, 1997; Hall et al., 2000 Ciani et al., 2012 Ahmad-Annuar et al., 2006; Ciani et al., 2012 Farias et al., 2009; Cuitino et al., 2010

Summary of molecules involved in the induction, organisation, formation and/or function of new synaptic sites
Abbreviations: ko, knockout; SV, synaptic vesicles

1.5. Synaptic Maintenance

Much evidence suggests that once established synapses can persist for long periods of time, and feasibly throughout the lifespan of the animal. Crucially, synapse stabilisation also influences the whole structure of the neuron. Synaptic formation together with the activation of postsynaptic mechanisms is being shown to promote stabilisation of dendritic arbour, leading to the proposal of the "synaptotrophic model", a model that postulates that synaptic formation can direct dendritic growth and arborisation (Niell et al., 2004; Rajan et al., 1999; G. Y. Wu & Cline, 1998). Supporting this view, *in vivo* studies demonstrate that defects in synaptogenesis, loss of synaptic input or deprivation in neuronal activity leads to the reduction of dendritic branches (P. D. R. Coleman, A.H., 1968; Jones & Thomas, 1962; Y. C. Lin & Koleske, 2010; Sfakianos et al., 2007). This further demonstrates the connection between synapse stability/activity and dendritic maintenance. Although, there is a vast literature that successfully explored and unveiled the various aspects and molecules that regulate synaptic formation and plasticity, very little is known what mechanisms are involved in synaptic and dendritic maintenance.

Given that morphological changes in neurons, synaptic loss and dendritic atrophy are common features in ageing, neuropsychiatric illnesses and neurodegenerative disorders (Burke & Barnes, 2006; Y. C. Lin & Koleske, 2010; Uylings & de Brabander, 2002), understanding how neurons achieve synaptic stability in a healthy brain is a current major goal in the neuroscience field. Therefore, it is essential to identify the molecules that are key regulators in these processes, so that therapies with direct targets can be developed in order to prevent synaptic loss and support synaptic stability. Below I will discuss some of the identified groups of molecules involved in synaptic maintenance (also found in Fig 1.6), as well as cellular aspects involved in this process. For a summary of molecules involved in synaptic maintenance, please refer to Table 2 at the end of this section.

1.5.1. Molecules and cellular processes involved in Synaptic Maintenance

1.5.1.1. Scaffolding Proteins

The PSD is an electron dense region of the synapse, located opposite to the presynaptic site, which concentrate a large quantity of different proteins. PSD-95 is a major component of the postsynaptic site known to bind, tether and stabilise a range of different proteins at excitatory synapses (Sheng & Kim, 2011; Sheng & Kim, 2002). Interestingly, knockdown of PSD95 has been suggested to regulate synaptic stability (Ehrlich et al., 2007). Acute PSD95 knockdown in brain slices cultures by RNAi leads to an increase in the number of unstable spines in the context of activity (LTP induced spine dynamics) (Ehrlich et al., 2007). Although this study showed the importance of PSD95 to the stabilisation of glutamate receptors at the synapses, spine strength and to the stabilisation in the late phase of LTP (Ehrlich et al., 2007); however, it failed to demonstrate the role of PSD95 for the maintenance of synapses in basal condition.

More recently, a study successfully demonstrated a more convincing role to PSD95 to the stabilisation of spines and associated synaptic molecules (X. Chen et al., 2011). Knockdown of PSD95 in mature hippocampal cultures causes disruption in the organisation of the postsynaptic density (PSD material), when evaluated by electron microscopy (X. Chen et al., 2011). More importantly, RNAi of PSD95 causes a major decrease in the levels of GluR1 protein and a decrease in the number of synaptophysin staining (X. Chen et al., 2011). Although the study did not evaluate spines at the morphological level, this was the first clear evidence which suggested the involvement of PSD95 in the molecular organisation of the postsynaptic site and in synaptic maintenance.

Shank and Homer have been studied in the context of synaptic stability. These proteins, which are associated with PSD95 and also localised at the PSD, have been demonstrated to work synergistically promoting spinogenesis, and are essential for the recruitment of synaptic proteins to the synaptic sites (Sala et al., 2001). Importantly, loss of Shank3 causes destabilisation of dendritic spines. Dissociated mature hippocampal neurons treated with small interference RNA (siRNA) to inhibit

the synthesis of Shank3 lead to the decrease in the number of spines and to the increase in filopodia-like shape spines (Roussignol et al., 2005). Given that mutation in Shank3 has been associated with Autism Spectrum Disorder (Durand et al., 2007), and the suggestion of Shank/Homer involvement in synaptic stability, this group of proteins might represent valid and important targets for synapse stabilisation-related diseases.

Recently, two major presynaptic scaffolding proteins have been identified as critical regulators of synaptic integrity. Piccolo and Bassoon are large scaffolding proteins known to be involved in the stabilisation, organisation and function of the active zone (Schoch & Gundelfinger, 2006; Shapira et al., 2003). However, it has been also demonstrated their crucial role in synaptic maintenance (Waites et al., 2013). Knockdown of Piccolo and Bassoon with shRNA lentiviral infection in hippocampal cultures causes a major loss of synaptic proteins (Waites et al., 2013). Importantly, this elegant study shows that the total levels of pre- and postsynaptic proteins are downregulated when Piccolo and Bassoon expression is disrupted (Waites et al., 2013), which makes evident the involvement of these proteins in the degradation of key synaptic proteins such as PSD95, synapsin, VAMP-2, among others.

1.5.1.2. Adhesion molecules

As previously discussed, adhesion molecules mediate interactions between the pre and postsynaptic site as well as provide structure to the synapses. Therefore, it is reasonable to speculate that presence of adhesion molecules after synaptogenesis is also regulating synaptic maintenance. The presence of different cell adhesion molecules at the synapses made difficult to determine the contribution of each individual CAM in synaptic maintenance. However, some studies have demonstrated that loss of certain adhesion molecules after synapse formation causes synaptic loss.

Cadherins and their downstream signalling molecules α and β catenin are expressed throughout development but are also present in mature synapses (Arikkath & Reichardt, 2008; Bekirov et al., 2002; Benson & Tanaka, 1998; Kwiatkowski et al.,

2007; Togashi et al., 2002). Expression of a dominant negative form of N-cadherin after synaptogenesis leads to the destabilisation of synapses and loss of synapsin and PSD95 labelled puncta (Togashi et al., 2002), suggesting a role N-cadherin in the maintenance of excitatory synapses. Additionally, the same study also shows that disruption of N-cadherin signalling in the adulthood causes a minor, but statistically significant, loss of inhibitory postsynaptic puncta (Togashi et al., 2002), implying a role for this molecule in the maintenance of GABAergic synapses. Moreover, reduction in N-cadherin levels by deletion of p120-catenin, a family of proteins that regulate cadherin function, results in a decrease of mature spines and increase in filopodia-like protrusions (Elia et al., 2006), suggesting that cadherin signalling molecules are required for the maintenance of spines in mature neurons (Elia et al., 2006; Y. C. Lin & Koleske, 2010).

Neurexins/Neuroligins might be involved in synaptic maintenance. Despite the fact that mutant mice lacking NL expression does not present any apparent deficiency in synaptic density, but only in synaptic function (Varoqueaux et al., 2006), more recently a study suggested a role for NRX/NL in synaptic and dendritic stability (S. X. Chen et al., 2010). Using the combination of two-photon time lapse microscopy *in vivo* and 3D analysis, this study show that disruption of NRX/NL, via either expression of Neurexin 1β dominant negative or morpholino oligonucleotides targeting Neuroligin-1, results in changes in stability of spines and in the overall architecture of the dendritic arbour (S. X. Chen et al., 2010). Following the synaptotrophic model previously proposed, the loss of synaptic NRX/NL complex reduces the number of stable dendritic filopodia and the complexity of dendritic arbors (S. X. Chen et al., 2010). While the study did not address and evaluate spine stability by knocking down the cell-adhesion complex in mature neurons, it highlighted the importance of synaptic stability and its crucial role in dendritic arborisation as well as dendritic stability. Given the involvement of neuroligins in several cognitive disorders, such as Autism and schizophrenia (Y. C. Lin & Koleske, 2010; Sudhof, 2008), much research investigating how cell adhesion molecules regulate synapse stability is critical to understand the involvement and contribution of these molecules to the stabilisation of neuronal circuits.

1.5.1.3. Actin cytoskeleton and its regulatory proteins

At the synapses actin provides not only structure, but also play a pivotal role in regulating activity and function. At the presynaptic site, actin surrounds and cluster synaptic vesicles, regulating the availability of the vesicle pool (Dillon & Goda, 2005; Sankaranarayanan et al., 2003), whereas postsynaptically, actin cytoskeleton is underlying the size and shape of the spines, as well as organising the postsynaptic density, localisation of neurotransmitter-receptors and molecules involved in the local translation machinery (Bramham, 2008; Hotulainen & Hoogenraad, 2010; Schubert & Dotti, 2007). Given the clear role of actin regulating different aspects of the synapse and their molecular processes, some studies investigated the physiological role of actin and its related proteins in mature synapses and demonstrated their involvement in maintenance.

Long-term stability is dependent on the actin dynamics at the spine. Actin cytoskeleton polymerisation and depolymerisation underlie the mechanism of synaptic assembly and disassembly, a mechanism dependent on the balance between filamentous actin (F-actin) and actin monomers (G–actin) (Dillon & Goda, 2005; Okamoto et al., 2004). Long-term disruption in the polymerisation of actin filaments using latrunculin A in mature hippocampal neurons results in a loss of PSD95 clusters, together with a reduction ad redistribution of NR1 and GluR1 clusters, shifting them to outside of the synapse (Allison et al., 1998). Importantly, the study reports the same treatment has no apparent effect on the presynaptic terminal, labelled with synaptophysin (Allison et al., 1998). Despite the unexpected effect of long-term latrunculin A at the presynaptic site, this was the first study suggesting that actin dynamics is involved in synapse stabilisation. Supporting these findings, a study using fluorescent recovery after fotobleaching (FRAP) revealed a fast loss of postsynaptic markers due to disruption of actin dynamics (Kuriu et al., 2006). Time-lapse analysis demonstrates that the scaffolding proteins Homer, Shank and PSD-95 undergo considerable declustering as a result of actin depolymerisation (Kuriu et al., 2006). Furthermore, the study also demonstrates that latrunculin A indeed induces the disassembly of postsynaptic markers, although in a more rapid

manner (within 5 minutes) (Kuriu et al., 2006), than previously reported. Together, these data demonstrate the importance of actin dynamics and stability for the maintenance of key proteins involved in the maintenance of dendritic spines, under basal conditions.

Disruption of proteins involved in actin dynamics results in loss dendritic spines. Rho/Rac GTPases are a family of guanine nucleotide binding proteins that acts as keys switches integrating extra- and intracellular signals, thus orchestrating actin dynamics and consequently, dendrite and spine structural changes (Nadif Kasri & Van Aelst, 2008; Ramakers, 2002). Mutations in genes associated with Rho GTPases results in abnormal spine morphology and neuronal connectivity, and also have been linked to neurobiological disorders such as mental retardation and amyotropic lateral sclerosis (Nadif Kasri & Van Aelst, 2008; Ramakers, 2002). This raises the question to whether Rho GTPases play a role in synaptic maintenance. Indeed, mutation in Rac1 and RhoA in mature hippocampal and cortical brain slices leads to the loss of spines (Nakayama et al., 2000; Tashiro et al., 2000). Long-term imaging of mature hippocampal neurons transfected with dominant negative Rac1 shows a progressive reduction of spine density, culminating in almost a bare dendritic tree (Nakayama et al., 2000). Although these studies did not evaluate the functional aspect of the synapses after the morphological changes and spine loss, they suggest a role for actin regulatory proteins in synaptic maintenance. Contributing to these findings, a more recent study revealed the downregulation of Kalirin expression, a Rho guanine exchange factor, in brains of AD patients (Youn et al., 2007). Together these studies give evidence to the crucial role of actin cytoskeleton and its related regulatory proteins for synaptic maintenance and their link to synapse loss in disease.

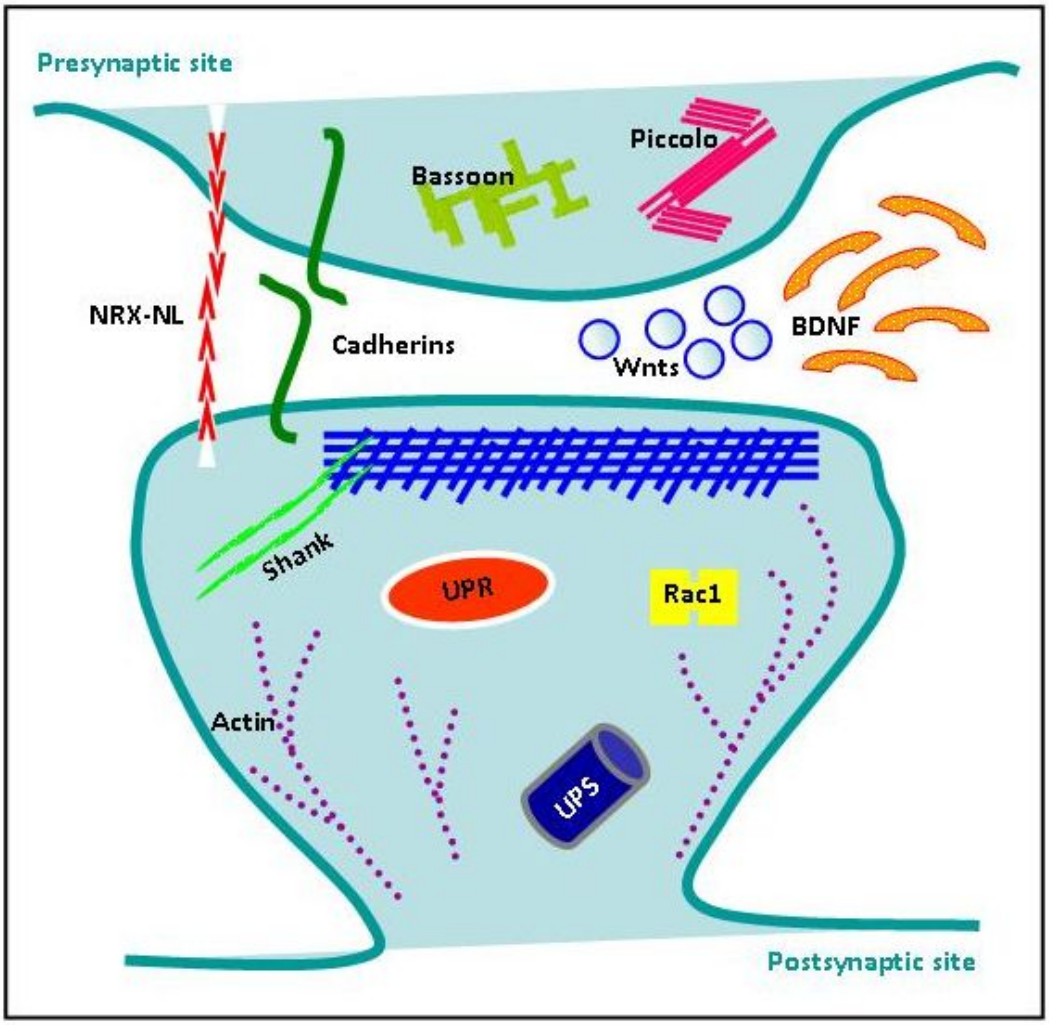

Figure 1.6: Reported molecules and intracellular processes involved in synaptic stability and maintenance. Loss of expression, disruption of the instability or dysregulation of any of the molecules or systems shown in the cartoon, leads to loss of synaptic proteins and/or dendritic spines. Abbreviations: NRX-NL, neurexin-neuroligin; UPR, unfolded protein response; UPS, ubiquitin proteasome system

1.5.1.4. Secreted factors

Secreted factors such as NFG, BDNF, FGF and Wnts are well regarded for their important function during development and synaptic formation. Indeed, as previously discussed manipulation of these factors by either pharmacological blockade or alteration in gene expression, leads to abnormal synaptic formation and clustering of synaptic proteins. Given their expression in the adult brain and their well known role in synaptic function and plasticity (Huang & Reichardt, 2001;

McAllister et al., 1999), it is reasonable to speculate that these factors are also involved in the maintenance of central synapses.

A role for secreted factors in synaptic maintenance is emerging from studies at the NMJ and central synapses. The first evidence that secreted factors are involved synaptic stability come from a study at the muscular junction (Gonzalez et al., 1999). Disruption of TrkB-mediated signalling in the adult muscle of mice, by expression of a dominant–negative form of TrkB receptor, causes the disassembly of AchR clusters at the neuromuscular junction (Gonzalez et al., 1999). Furthermore, disruption of TrkB signalling, by expression of a truncated form of TrkB, leads to synaptic degeneration in facial motor neurons (De Wit et al., 2006), indicating that neurotrophin signalling in the adult is necessary for the maintenance of postsynaptic sites at the peripheral synapses.

In central synapses, secreted factors are also involved in synaptic stability. At the central synapses, *in vivo* studies of cortical neurons in adult mice reveal the requirement of ongoing BNDF expression for the maintenance of dendritic spines (English et al., 2012). Using a Cre-lox recombinant system, to specifically downregulate the expression of BDNF post-natally, this study demonstrated the crucial role of this secreted factor for the maintenance of dendritic spines in cortical neurons (English et al., 2012). Loss of BDNF expression during five weeks, after the end of cortical development, causes a decrease of 30% of dendritic spines (English et al., 2012). The authors strongly suggest the requirement of BDNF expression for synaptic maintenance in the visual cortex, although the study did not reveal whether the spine loss is directly proportional to synapse loss. Together these studies provide compelling evidence to the importance of BNDF and TrkB-mediated signalling for both peripheral and central synaptic stability in adulthood.

BDNF expression in adulthood is required for forebrain circuit stability and function. Anatomical and behavioural analysis of an young-adult forebrain-specific BDNF mutant mouse demonstrate for the first time the functional role of BDNF in the adult brain (Vigers et al., 2012). Knockout of BNDF in the adult forebrain, by CaMKII- Cre

recombinase system, leads to the loss of over 30% in the number of dendritic spines in the visual cortex (Vigers et al., 2012), as previously demonstrated (English et al., 2012). Importantly, the study shows that BDNF mutant mice present profound cognitive defects in the MWM and in contextual-fear conditioning tasks (Vigers et al., 2012). Although the authors did not look at the hippocampal synapses and dendritic spines, given the hippocampal-mediated cognitive defects observed, these finding suggests the importance of BDNF expression for the maintenance of the whole forebrain circuitry. Further studies to clarify whether both pre and postsynaptic sites are equally affected once BDNF signalling is disrupted in the adulthood are yet to be done.

Similarly to BDNF, Wnt proteins are candidates to play a role in synaptic stability. Wnts are expressed in the adult brain (Gogolla et al., 2009; Salinas et al., 1994; Shimogori et al., 2004; Tabatadze et al., 2012), suggesting a potential role of Wnt signalling in synaptic maintenance. Recent studies started to investigate the contribution of Wnt signalling in the adulthood. These studies show that disruption of Wnt signalling has been implicated in memory impairment (Fortress et al., 2013; Jessberger et al., 2009; Tan et al., 2013)(Seib et al., 2013). However, most of these studies suggest a role for Wnt signalling and adult neurogenesis and hippocampal-mediated behaviour (Jang et al., 2013; Lie et al., 2005; Seib et al., 2013), rather than the involvement of Wnts in synaptic maintenance. For instance, gain and loss of function studies i*n vivo* demonstrated that Wnt-β-catenin signalling positively regulates neurogenesis in the adult dentate gyrus (Lie et al., 2005). Furthermore, inhibition of Wnt signalling in the dentate gyrus, using lentivirus expressing a dominant-negative form of Wnts, leads to deficits in long-term retention of spatial memory and object-recognition memory in adult rats (Jessberger et al., 2009). Supported by these findings, a more recent study suggested that by decreasing the levels of the Wnt antagonist Dkk1 in the DG in aged animals, neurogenesis is enhanced, which correlates with improvement in cognition (Seib et al., 2013). Nevertheless, none of these studies analysed whether the impairment in memory observed in these studies was in fact due to a decrease in synapses and synaptic connectivity in the DG and adjacent hippocampal regions.

Recent studies from our lab demonstrate the involvement of Wnt signalling in synaptic maintenance. We show that blockade of endogenous Wnt in dissociated hippocampal neurons, using Wnt antagonist Dkk1 after the peak of synaptogenesis, induces a rapid loss of synapses (Purro et al., 2012). Time-lapse imaging of mature neurons expressing VAMP2-mRFP demonstrated that blockade of endogenous Wnt signalling rapidly induces disassembly of stable synaptic vesicle clusters (Purro et al., 2012). Furthermore, ultrastructural analyses of the remaining synapses by electron microscopy revealed significant shrinkage of postsynaptic density (Purro et al., 2012). This study provides evidence that Dkk1-mediated synapse disassembly is a coordinated process which involves the loss of multiple pre- and postsynaptic proteins and neurotransmitter release sites. However, further work is necessary to fully understand the overall role and mechanisms of Wnts in synaptic maintenance.

1.5.1.5. Translation and protein degradation

Synapses are composed of numerous proteins, present in both pre- and postsynaptic site. Local protein synthesis and degradation underlie neuronal function and changes in connectivity during development and plasticity (Bramham & Wells, 2007; Schuman et al., 2006). Therefore once the synapse is established, the balance among *de novo* formation, stability and degradation of proteins is crucial for synaptic maintenance.

Synapse-associated polyribosome complexes (SPRC) were first identified in the granule cells of dentate gyrus mainly localised beneath the base of the dendritic spine between the spine neck and the shaft (O. L. Steward, W.B., 1982). Quantitative analyses by electron microscopy revealed that in basal conditions the number of SPRC range from 3 to 28, averaging 8 (Schuman et al., 2006; O. Steward & Reeves, 1988). However, evidence suggests that in the context of plasticity, location of SPRC can be shifted towards the spine resulting in an increased number of polyribosome at this site (Ostroff et al., 2002; Schuman et al., 2006). Importantly, under basal condition a variety of mRNA localised at the dendrite of the neurons

have been identified *in vivo* by *in situ* hybridization, including CaMKIIα, MAP2, Arc and NMDAR1 (O. Steward & Schuman, 2001), suggesting a link between the presence of mRNA and SPRC and the maintenance of the postsynaptic site.

At the presynaptic site, axonal mRNA translation is contradictory. Early studies analysing squid giant axon suggest that local protein synthesis is unlikely to occur at these sites, since almost undetectable levels of ribosomal RNA was detected at the axoplasm (Giuditta et al., 2002; Jung et al., 2012; Lasek et al., 1973). However, later studies using more sophisticated techniques demonstrated the presence of ribosomal RNAs, mRNA and translating polysomes in the squid axoplasm (Giuditta et al., 2002; Jung et al., 2012), as well as in mammals (Jung et al., 2012). Local translation in the axon play an important role in axon guidance and synapse formation during development (Jung et al., 2012; A. C. Lin & Holt, 2008), but the presence of ribosomal proteins and RNA, forming ribosomal plaques, in the mature peripheral nervous system (Jung et al., 2012; Koenig & Martin, 1996; Koenig et al., 2000) suggests a role for local translation in synaptic maintenance. Indeed, axon-specific local inhibition of RNA translation in Xenopus retinal ganglion cells disrupts mitochondrial function leading to axon degeneration (Yoon et al., 2012). These evidence suggest that local translation is a reasonable target to investigate what mechanisms are involved in synaptic stability.

Abnormal translation can lead to neurodegeneration. An elegant study has recently shown that accumulation of misfolded proteins in neurodegenerative disorders can lead to repression in one of the pathways involved in translation machinery (Moreno et al., 2012). Using the prion protein (PrP) mouse model for neuronal degeneration, the group studied the cellular mechanisms of unfolded protein response (UPR) in hippocampal neurons. The authors show that infection with PrP leads to a dramatic decline in the levels of two key synaptic proteins (SNAP25 and PSD-95) after nine weeks of prion inoculation (Moreno et al., 2012). Interestingly, the authors demonstrate that prion replication leads to UPR, and increase in the phosphorylation of eukaryotic translation initiation factor eIF2α – resulting in the shutdown in protein translation (Moreno et al., 2012). Furthermore, the study also shows that increase in

the levels of phosphorylation of eIF2α coincides with exacerbated neurotoxicity, which was then correlated with increased synaptic and neuronal loss (Moreno et al., 2012).

Crucially, Moreno et al., 2012 yet demonstrated that prevention in the phosphorylation of eIF2α, by using lentiviruses to target an specific upstream eIF2α phosphatase, could rescue and prevented the phenotype observed in control mice infected with PrP (Moreno et al., 2012). Total levels of synaptic proteins, synaptic number and synaptic transmission were comparable to control groups, as well as an increase of almost two weeks in the survival rates of the animals (Moreno et al., 2012). Furthermore, a follow up study by the same group show that inhibition of the kinase PERK (protein kinase RNA-like endoplasmatic reticulum kinase), another key mediator of the UPR pathway, is neuroprotective for the PrP model system (Moreno et al., 2013). Interestingly, treated PrP mice presented normal burrowing behaviour, and did not appear to develop the disease (Moreno et al., 2013). Curiously, cognitive impairment evaluated by novel object recognition was not prevented (Moreno et al., 2013). Nevertheless, these studies show that UPR can be a potential therapeutic target to prevent and perhaps prevent neurodegenerative diseases triggered by accumulation of misfolded proteins. Further studies investigating whether targeting UPR can rescue, rather than prevent, synapse loss and neuronal degeneration are yet to be carried out.

Protein degradation plays an important role in protein turnover and synaptic maintenance. The ubiquitin proteasome system (UPS) has been described as active in central synapses and studies suggest its importance for synaptic structure and function (Bajic et al., 2012; Bingol & Sheng, 2011). Many studies show that the UPS regulates the size of the presynaptic pool of vesicles and synaptic protein expression during synaptic activity, transmission and plasticity (Bingol & Sheng, 2011; Jiang et al., 2010; Willeumier et al., 2006). Notably, localised protein degradation via UPS has also been demonstrated during the process of synaptic elimination and regulation of spine density (Haas & Broadie, 2008; Hamilton & Zito, 2013). Short-term inhibition of

the proteasome system in mature hippocampal culture neurons increase the number of recycling pool of vesicles, as determined by FM4-64 assay (Willeumier et al., 2006). In addition, this study demonstrates that UPS system is tightly regulated with synaptic activity, as blockade or neuronal stimulation directly correlates with size of the recycled pool, suggesting that protein degradation play as homeostatic role at the presynaptic site (Willeumier et al., 2006).

A recent study investigating the role of proteasome system at the dendritic spines, demonstrate that blockade of UPS has a negative effect on spine number (Hamilton & Zito, 2013). Using a combination of glutamate uncaging and two-photon imaging, this study shows that inhibition of proteasome blocks activity-induced spine growth (Hamilton & Zito, 2013). Importantly, the study shows that not only there is no spine growth, but the overall number of spines is in fact reduced on neurons treated with the UPS blocker (Hamilton & Zito, 2013), suggesting the proteasome system is also regulating spine maintenance. Though these studies support the idea that proteasome degradation plays a role regulating the turnover of proteins at the synapse and indirectly regulates their stability, further studies will be necessary to determine the direct role of the UPS in synapse maintenance

Deregulation of synaptic protein degradation has been linked to neurodegenerative diseases. Studies suggest a link between the deregulation in the UPS, accumulation of proteins and Parkinson's and Alzheimer's disease (Bedford et al., 2008; Haas & Broadie, 2008; McNaught et al., 2003). A study by Smith and collaborators (Smith et al., 2009), shows that increasing the activity of enzymes responsible for deubiquitination, by pharmacological means, can reverse the spine loss effect after Aβ treatment in hippocampal slices (Smith et al., 2009). Conversely, AD mouse models carrying a mutation in the UPS system, and therefore with a disrupted proteasome activity, present lower accumulation of Aβ in the brain (van Tijn et al., 2012). Despite the paradoxical evidence, these studies suggest a contribution of proteasomes to the process of neurodegeneration. Furthermore, some authors propose that protein aggregation might be the original cause of impairment in UPS function (Bence et al., 2001), suggesting that this can potentially be triggering

neuronal degeneration. Whether dysfunction in the proteasome is a cause or a consequence of neurodegeneration, and whether the UPS regulatory mechanism is acting regulating equally the maintenance of the pre- and postsynaptic sites still remains to be studied.

Table 2. Molecules and cellular processes involved in synaptic maintenance ***

	Model and target	Phenotype	Refs
Postsynaptic scaffolding proteins	*ex vivo* RNAi to PSD95	reduction in glutamate receptors content at synapses and reduction in spine stability	Ehrlich et al., 2007
	in vitro knockdown of PSD95	decrease in the number of presynaptic sites and GluR1 receptors	Chen et al., 2011
	in vitro siRNA to Shank3	decrease in total number of dendritic spines and increase in the number of "immature-shaped" spines	Roussignol et al., 2005
Presynaptic scaffolding proteins	*in vitro* shRNA for Piccolo and Bassoon	decrease in total level of key pre and postsynaptic proteins as result of increased protein degradation	Waites et al., 2013
Adhesion Molecules	*in vitro* use of dnCadherin	*loss of pre and postsynaptic excitatory sites and destabilisation of dendritic, with minor effect on inhibitory synapses*	Togashi et al., 2002
	in vitro use of dnNRX or morpholino targeting NL-1	decrease in stability of dendritic spines	Chen et al., 2010
Cytoskeleton and associated proteins	*in vitro* disruption of actin stability with Latrunculin	reduction of PSD95 puncta number and shift in the location of glutamate receptors to extra-synaptic sites rapid declustering of scaffolding proteins Shank and Homer	Allison et al., 1998 Kuriu et al., 2006
	ex vivo use of dnRac1	significant loss of dendritic spines	Nakayama et al., 2000
Secreted Factors	*in vivo* disruption of BDNF expression	loss of around 30% in dendritic spines accompanied by cognitive deficits	English et al., 2012; Vigers et al., 2012
	in vitro blockade of canonical Wnt signalling	loss in the number of synaptic markers, quick dispersal of VAMP-2 and decrease in the size of post-synaptic density	Purro et al., 2012

*** Table 2 continue on the next page

Table 2. Molecules and cellular processes involved in synaptic maintenance (cont.)

	Model and target	Phenotype	Refs
Intracellular processes	*in vivo* disruption in protein translation (UPR)	*decrease in total levels of SNAP25 and PSD9, synapse number and* impaired synaptic transmission	Moreno et al., 2012
		abnormal species-typical behaviour (burrowing in mice)	Moreno et al., 2013
	in vitro disruption in the ubiquitin proteasome system (UPS)	dysregulation in the stability and recycling of presynaptic proteins	Willeumier et al., 2006
		loss of dendritic spines	Hamilton & Zito, 2013

Summary of principal studies demonstrating a link between each molecules and their role in synaptic stability and/or maintenance
Abbreviations: dn, dominant negative; RNAi, RNA interference; shRNA, small hairpin RNA; UPR, unfolded protein response

1.6. Synapse disassembly

Synapse formation, maturation and disassembly are fundamental processes known to happen throughout the life of an organism. Synapse disassembly is an important process that ultimately refine and modulates the connectivity between neurons (Eaton & Davis, 2003; Goda & Davis, 2003; Purves & Lichtman, 1980). Many studies, including long term live imaging, have demonstrate that elimination of synapses is a continuous process, but it is remarkably more apparent during post-natal development, during plasticity (in the young and mature brain), and also in the context of neurodegeneration (Bastrikova et al., 2008; De Paola et al., 2003; A. Holtmaat & Svoboda, 2009; Picconi et al., 2012; Rakic et al., 1986; Selkoe, 2002). Importantly, many studies have demonstrated that synaptic disassembly occurs in both, the peripheral nervous system (neuromuscular junction - NMJ) and in the central nervous system. Although the mechanism of synaptic disassembly is yet to be unravelled, together many studies began to shine a light on the basic principles of the dismounting of synapses, as discussed below.

1.6.1. Synaptic disassembly at the NMJ

The NMJ is one of the most characterised synapses in both invertebrate and mammalian systems. Studies dating as early as 1976 had already started characterising synaptic elimination in this system (Brown et al., 1976). During the formation of the NMJ, acetylcholine receptors are dispersedly distributed along the muscle fibre, which is then approached by axons to form receptors plaque clusters. At early stages of development, multiple axons innervate a single plaque of receptors, which goes through remodelling post development (Colman et al., 1997; Deschenes, 2011).

In the first week of development, after multiple axons had converged to innervate an individual neuromuscular junction, a process that eliminates all these “unnecessary” inputs takes place, leaving a single axon remaining (Barber & Lichtman, 1999; Lichtman & Colman, 2000). This synaptic elimination process is a unique feature of

the peripheral nervous system, where curiously the circuit is created after the redundant connections are eliminated via a competitive process (Fig 1.7).

Interestingly, the competition that leads to this refinement in the circuitry is a result of different patterns or levels of activity between the inputting axons (Barber & Lichtman, 1999; Kasthuri & Lichtman, 2003). Many studies looking into this competitive pattern divided the role of pattern of activity in two main views: more active axons have competitive advantage and the other that postulates that inactive axons have more advantage (Barber & Lichtman, 1999; Kasthuri & Lichtman, 2003). The current view in the field proposes that an active axon releases transmitter locally, which is interpreted by the postsynaptic acetylcholine receptors as a "protective signal", in addition to a long reaching elimination signal that affects the more distal parts of the postsynaptic side. By contrast, inactive synapses do not produce these signals, therefore being susceptible to elimination by the signals coming from the neighbouring axon, resulting in a decrease in the number and area of the acetylcholine receptors and axon withdrawn (Fig 1.7) (Jennings, 1994; Zito, 2003). Furthermore, studies have demonstrated that after the process of axon withdrawn, the strength of the remaining synapse increases, which is associated by the increased amount of neurotransmitter release (Colman et al., 1997). Although this model is largely accepted today, the main target of debate nowadays, which ultimately can modify the current model, is the hierarchy of the disassembly: which synaptic side is eliminated first, the pre or the postsynaptic area?

The cellular mechanisms that mediate synaptic disassembly in the NMJ are not fully understood. Over twenty years ago, an elegant study investigating the mechanism of disassembly led to the proposal that the postsynaptic site is the main player during this process (Rich & Lichtman, 1989). This innovative study imaging the NMJ of mice after inducing damage of the presynaptic terminal fibre demonstrated that denervation leads to the presynaptic terminal retraction but the postsynaptic receptors clusters are rearranged and maintained, suggesting that the postsynaptic terminal is the key structure for the maintenance of a healthy terminal junction (Rich & Lichtman, 1989). Supporting this hypothesis, a study manipulating the activity of

single muscular junctions demonstrated that localised AChR blockade, by local application of α-bungarotoxin, results in a gradual and long-lasting loss of AChR, which is then followed by the presynaptic disassembly (Balice-Gordon & Lichtman, 1994). Interestingly, the same study also shows that blockade of neurotransmission throughout the synaptic junction, as opposed to focal blockade, does not cause elimination of the synapses, suggesting that postsynaptic receptors, via their activity, are the main players in the process of elimination (Balice-Gordon & Lichtman, 1994). Furthermore, a study collaborating electrophysiological recordings with staining techniques show that removal of postsynaptic receptors can precede the retraction of the presynaptic fibre (Colman et al., 1997). Together these studies suggest that the process of synaptic disassembly is driven not only by activity-signals, but more importantly, it is specified postsynaptically.

The puzzle of disassembly at the NMJ is yet not totally resolved. Although studies strongly suggest the postsynaptic side mediating synaptic competition and disassemble, the mechanism of dismantle is still unclear. Live imaging of the mammalian NMJ demonstrates that during the elimination of synapses, the presynaptic axon is retracted before the total loss of the postsynaptic site (Walsh & Lichtman, 2003). Supporting this finding, examination of the Drosophila NMJ demonstrated that elimination of classical presynaptic markers (synapsin and synaptobrevin) precede the disassembly of the postsynaptic apparatus (Eaton & Davis, 2003). In summary, these studies demonstrate that despite evidence are in favour of the postsynaptic cell mediating competition input and elimination. However, further studies would be necessary to clarify the hierarchy of disassembly in the NMJ.

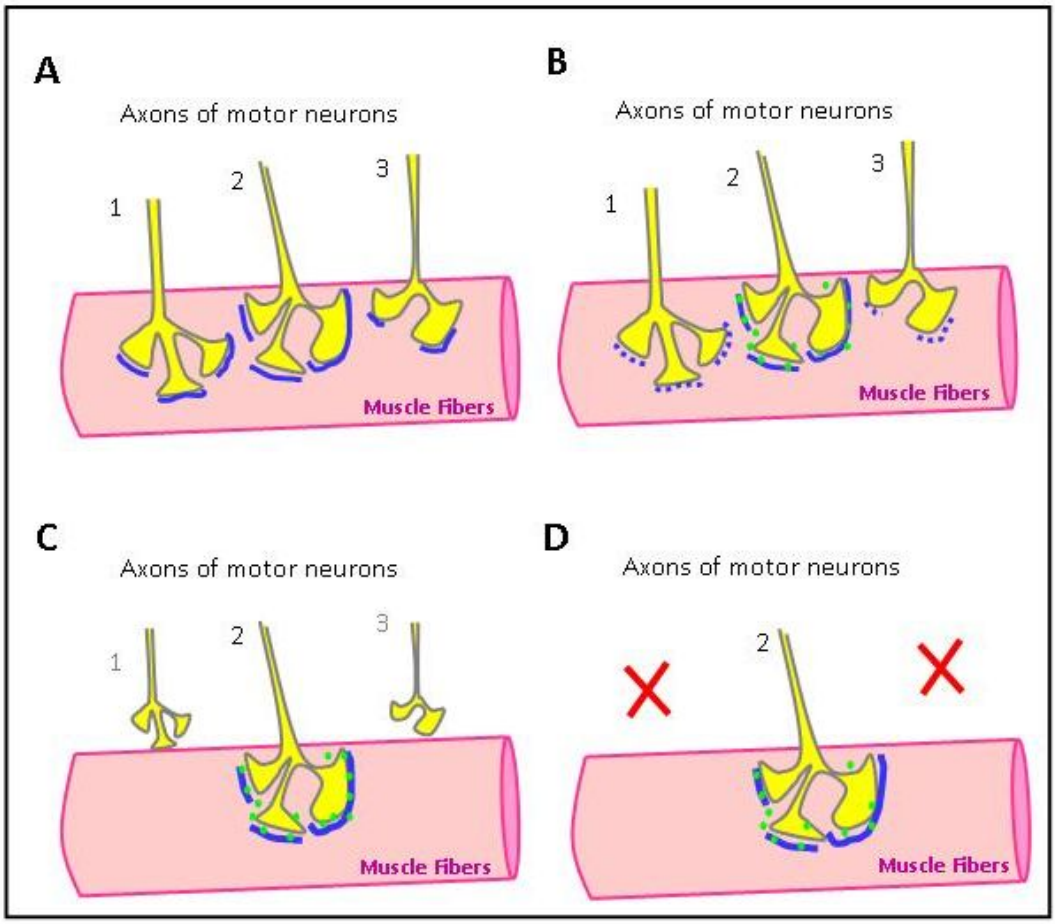

Figure 1.7: Synaptic disassembly at the NMJ. A) During early stages of development, multiple axons - numbered 1, 2 and 3 - innervate a single plaque of acetylcholine receptors, AchR (thick blue lines) at the NMJ. **B)** Elimination of redundant connections start via a competitive process. More active axons release signals (green dots) towards the AchR plaques postsynaptically, which protects the synapses to be disassembled (axon number 2), whereas neighbouring inactive axons (numbered 1 and 3) which do not produce signals cause a decrease in the number and area of AchR. **C)** Lack of activity culminates in the withdraw of "weak and inactive" axons, which eventually are eliminated **(D)**

1.6.2. Disassembly in the developing CNS (cerebellum and cortex)

Throughout and post development the cerebellum goes through a process that leads to the emergence of neuronal circuits by formation and refinement of synaptic connections (Hashimoto & Kano, 2013; van Welie et al., 2011). The cerebellum is formed of a variety of different types of neurons, among them the Purkinje cells (PC), Granule cells (GC) and candelabrum cells, which is anatomically organised in layers (White & Sillitoe, 2013). Importantly, these layered cells receive input from extrinsic

projection. One of the most notable, well studied and anatomically complex microcircuit in this brain region is the connection between the climbing fibres (CF) coming from the medullar area and the PC in the molecular layer of the cerebellum (White & Sillitoe, 2013). In the mature cerebellum, each PC receives input from a single CF, which "climbs" onto the dendrite of the PC (Hashimoto & Kano, 2005, 2013). However, this architectural arrangement during early postnatal development is different. During this period, four or more CFs innervate each single PC and "excessive" synapses are progressively eliminated until mono-innervation is achieved (Crepel et al., 1976; Lohof et al., 1996). Curiously, there is much evidence to suggest that the process of disassembly of synapses and refinement of this circuitry is activity dependent.

Electrophysiological studies have demonstrated that the process of synapse refinement in the cerebellum is a gradual process but that happens in a relatively quick manner. It is well established that at the post natal day 3 (P3), supernumerary CF synapses on PC cells, which is then followed by the differentiation of a single CF cells – causing its selectively strengthening, at around post natal day 7 (P7) (Crepel et al., 1976; Hashimoto & Kano, 2013). Interestingly, at this time point a clear competition is occurring among the different inputs coming from distinct CFs. Electrophysiological recordings show that at this period at least two or more independent CF are functionally innervating a single PC (Crepel et al., 1976). Short after this time point, between the first and second week of post natal development (P8 and P14), the elimination period starts, and supernumerary synaptic contacts of CF upon PC rapidly disappear (Crepel et al., 1976; Hashimoto & Kano, 2005). This process is driven by the weakening of synaptic transmission of the CFs, which progressively loses its evoked excitatory current, in parallel to the increase synaptic strengthening of the "winning" CF (Hashimoto & Kano, 2005, 2013). Progressively, the weaker inputs coming from the CF is eliminated through a strength-competition hierarchy. By the end of the second week of post natal development (approximately P15), the one-to-one relationship between CF and PC has been established, and the mono-innervation CF-PC in maintained throughout the adulthood of the animal (Hashimoto & Kano, 2013).

The process of synaptic elimination is dependent of both pre and postsynaptic mechanisms. The CFs have the intrinsic property of releasing multiple synaptic vesicles containing glutamate to a relatively narrow area simultaneously (multi-vesicular release) (Hashimoto & Kano, 2013). EPSC recording from a single site of release of CFs reveal a strong correlation between multi-vesicular release and synaptic strength (Hashimoto & Kano, 2005). This study suggests that probability of release of multiple vesicles is higher in stronger CFs, whereas weaker CFs have a reduced number of multi-vesicular release, being the cause of synaptic elimination (Hashimoto & Kano, 2005). Furthermore, the same study reveals that at the postsynaptic site, the decline in the abundance of receptors occurs after the decay of presynaptic release (Hashimoto & Kano, 2005). Though this study demonstrated the involvement of the pre and postsynaptic site during elimination and suggests that the process might begin at the presynaptic site, further studies elucidating the mechanism that drives the hierarchy of disassembly in this system is yet to be studied. Following the principle of disassembly at the NMJ, the process of elimination of synapses in the cerebellum is also a competitive process and largely controlled by activity. Interestingly, this feature is shared with another area of the CNS, the visual cortex, as discussed below.

The formation of the cortical connectivity is extremely complex. The human cortex contains about 10 billion neurons, forming an estimated 10^{14} synapses (Wei et al., 2013). Although a great degree of the cortical wiring is genetically orchestrated, a high percentage of these connections are dependant on sensitive experiences, especially those happening during early and post natal development periods (C. C. Chen et al., 2014; Wei et al., 2013). In the mammalian visual system, the patterning of connection that comes from the two eyes must end in separate and non-overlapping areas of the layer IV of the primary visual cortex (Shatz & Sretavan, 1986). This complex patterning must result in an aligned coordinate that ultimately will represent the projection of the visual field coming from the two distinct eyes (Shatz & Sretavan, 1986). This region in the layer IV of the cortex, which receives external inputs from the eyes, is called ocular dominance column (ODC) and

curiously it is not well defined at birth, being rather disorganised in a diffused pattern at this developmental stage.

After birth, during the first days of the eye opening, synapses are gradually eliminated, giving rise to an organised and accurate topographical map representing the coordinate of the visual field, representative of the input coming from the binocular eyes (Fig 1.8) (L. C. Katz & Shatz, 1996; Shatz & Sretavan, 1986). Crucially, this anatomical rearrangement and elimination of synapses are a result of visual stimulation coming from each individual eye, and therefore dependent on activity (L. C. Katz & Shatz, 1996). The first and most classical study that demonstrated the importance of visual experience for the organization of the ODC was performed by the Nobel Prizes winners of Physiology and Medicine, David Hubel and Torsten Wiesel, in 1963 (Barlow, 1982; L. C. Katz & Shatz, 1996). Their work in the developing cortex of cats, showed that if only one eye is deprived (by occlusion) during early post natal development, then cortical neurons are responsive to stimuli coming from the eye that remained open, demonstrating a loss of cortical response from the eye that was occluded (Hubel & Wiesel, 1962). Furthermore, the study shows the degeneration of the connections as a result of visual deprivation (Daw, 2009; Hubel & Wiesel, 1962). Reconstruction of single axonal arbours reveals the rapid withdrawn of branches of the deprived-eye arbour (Antonini & Stryker, 1993), demonstrating quick response of the axons to changes in pattern of neuronal activity. Interestingly, a follow up study looking into the arbors of deprived and non deprived eyes, reveal that reopening of deprived eye can induces the re-grow of some axons (Fig 1.8) (Antonini et al., 1998). Together, these studies further highlight the importance of activity for connection patterning during the postnatal period and also reveal the extent of plasticity in the CNS.

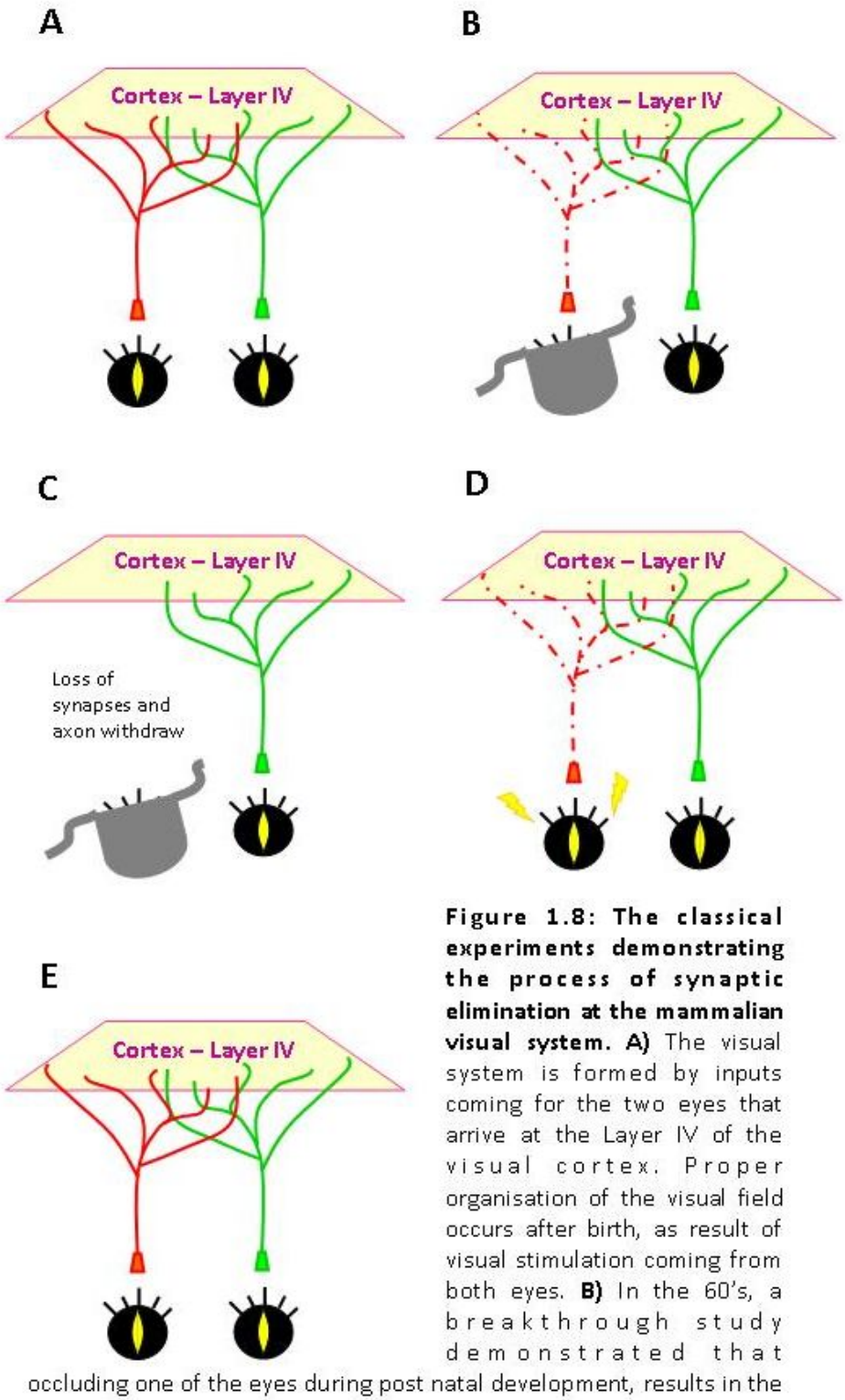

Figure 1.8: The classical experiments demonstrating the process of synaptic elimination at the mammalian visual system. A) The visual system is formed by inputs coming for the two eyes that arrive at the Layer IV of the visual cortex. Proper organisation of the visual field occurs after birth, as result of visual stimulation coming from both eyes. **B)** In the 60's, a breakthrough study demonstrated that occluding one of the eyes during post natal development, results in the decrease of stimuli arriving at the cortex (dashed red neuron). **C)** Loss of activity caused by visual deprivation leads to the loss of synapses and withdraw of axonal branches. **D)** Interestingly, a follow up study showed that if after visual deprivation, the eyes were stimulated, axons could re-grow and establish new connections **(E).**

1.6.2. Synaptic disassembly in the adult brain

The advance of long term in vivo live imaging of dendritic spines allowed neuroscientists to explore the structural changes that might occur in these structures in different scenarios. For instance, at the post developmental stage, cortical spines appear and disappear at a very high rate turnover (C. C. Chen et al., 2014; A. J. Holtmaat et al., 2005). Interestingly, at this period, bigger spines appear to be more resilient, and the overall turnover appears to weight towards the net loss of spines (A. J. Holtmaat et al., 2005). This elimination and establishment of the circuitry reflects the somatosensory experience of the animal (Grutzendler, 2006), but in the adulthood synapses, when the circuit has already been established, are more stable. Indeed, various studies imaging the cortex demonstrated that the turnover of synapses are very low, and over 80% of the spines population is persistent and stable (De Paola et al., 2006; A. J. Holtmaat et al., 2005; Majewska et al., 2006; Zuo et al., 2005). Importantly, just like at early stages, the adult CNS retains the capability to reorganise its network according to the sensory/activity input.

Plasticity orchestrates the turnover of spines. The concept of enlargement of spines and increase in spines density after long term potentiation (LTP) is well accepted. By contrast, induction of long term depression (LTD) by low frequency stimulation is marked by shrinkage and loss of spines, and this phenomenon is observed in mature neurons (N. Becker et al., 2008; Nagerl et al., 2004; Q. Zhou et al., 2004). These studies, recording the dynamics of hippocampal dendritic spines in brain slices, show that LTD induction leads to a reduction of contacts between pre and postsynaptic contacts, and shrinkage of dendritic spines (N. Becker et al., 2008; Nagerl et al., 2004; Q. Zhou et al., 2004). Furthermore, they demonstrate that spine retraction is an NMDAr-dependent activation mechanism, as blockade of these population of receptors prevent the loss of spines (Nagerl et al., 2004). Interestingly, in addition to spine shrinkage and disassemble, it has also been shown that LTD cause a dramatic increase in the turnover of presynaptic boutons, reducing their volume and number upon depression (N. Becker et al., 2008).

Following the principle of stimulation, an elegant study investigated the turnover of spines in an *in vivo* system. This elegant study in young adult mice found that long term sensory deprivation, by trimming the whiskers of the animals, leads to a high turnover of spines, accompanied by a great increase of spine disassembly (Fig 1.9) (Trachtenberg et al., 2002). Although this phenomena is less evident in older animals (Zuo et al., 2005), the fundamental rule that sensory experience plays a crucial role in remodelling the circuitry, in both young and adult brain, is still widely accepted.

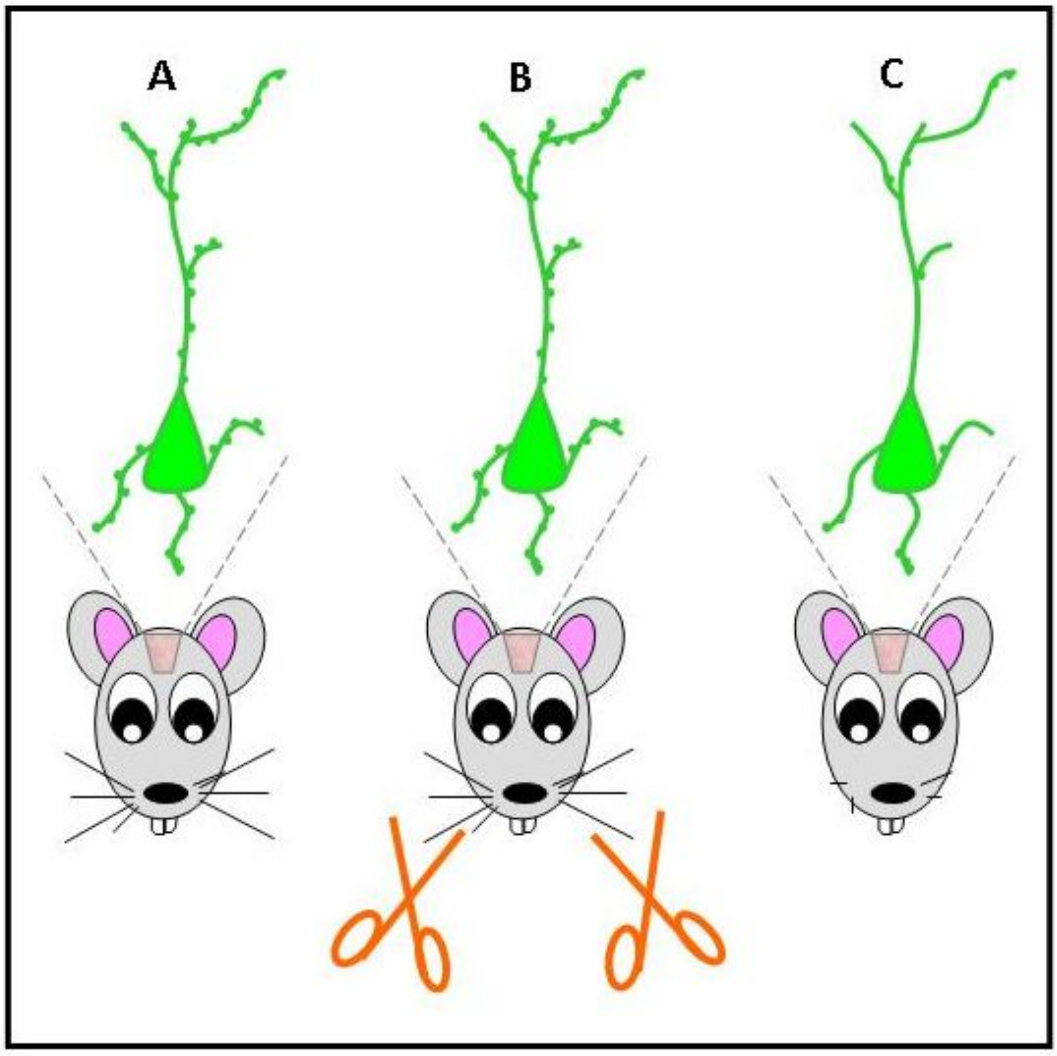

Figure 1.9: Synaptic disassembly in the adulthood due the lack of stimulation. **A)** In vivo time lapse imaging of the cortical area in the adult mouse found that stimulation correlates the remodelling of the circuitry. **B)** An elegant study has demonstrated that trimming the whisker of the rodents, and therefore depriving sensory stimuli, leads to a high turnover dendritic spines accompanied by a great proportion of spine disassemble **(C).**

The existence of long last spine structures in the brain is a clear link between synapse number and memory. Contributing to this hypothesis is the idea that the total number of synapses in the brain increases when learning and stimulation is taking place (Lichtman & Colman, 2000). Furthermore, much evidence suggests that synaptic disassembly is a hallmark for neurodegenerative diseases such as Alzheimer's and Parkinson's disease. In AD, one of the earliest symptoms is the impairment in the cognitive function, manifested as a subtle amnestic symptom (Selkoe, 2002; Shankar & Walsh, 2009). As the disease progresses, patients become unable to encode new memories, both declarative and non-declarative memories become profoundly impaired, as well as the ability of reasoning (Selkoe, 2002; Shankar & Walsh, 2009). The concept that AD is a "synaptopathy" is yet supported by a study which analysed biopsies from human patients, at the early onset stage of the disease, and demonstrated the a loss of up to 35% of synapses per neuron (Terry et al., 1991). Supporting this finding, further studies demonstrated a significant loss of synaptic markers in both patients and animal models of AD (Hsia et al., 1999; Masliah et al., 1991). The correlation between synapses, neurodegeneration and progression of the disease is well supported, but the current scientific challenge is to understand the process of synaptic disassembly in the context of neurodegeneration before neuronal loss.

Studies looking into synaptic changes in the AD field are in their majority derived from *post mortem* brains. Although Aβ accumulation and deposition is correlated to neuronal damage and synaptic loss (Grutzendler, 2006; Selkoe, 2002), in transgenic mouse models of AD, cognitive dysfunction may occur before Aβ aggregation (Hsia et al., 1999; Selkoe, 2002) - suggesting that soluble forms of amyloid oligomers may initiate the disease by targeting the synapses. Recent *in vivo* studies imaging transgenic models of AD, monitoring neuronal circuit disruption in relation to the progression of the disease, successfully demonstrated the degree of neuronal abnormality as a result of Aβ accumulation (Spires et al., 2005; Tsai et al., 2004). The first study, using transcranial two-photon microscopy technique imaging, demonstrated that amyloid deposits affect dendrites and axons, especially those situated at the vicinity of the aggregation (Tsai et al., 2004). Furthermore, the

authors correlated the neuronal damage with the distance of the deposits. Dendrites passing through or nearby the deposits (up to 15µm distance) have a great rate of spine loss, together with shaft atrophy and axon varicosities, which ultimately lead to neurite, break (Tsai et al., 2004). By contrast, axons and dendrites outside the 15µm boundaries, present little or no damage (Tsai et al., 2004).

A follow up study, this time using a different transgenic AD mouse model, further confirmed the previous observations (Spires et al., 2005). Quantification of damage as a result of Aβ deposition demonstrated that spine density are reduced to approximately 50% in dendrites located at the vicinity of the aggregation, and that up to a quarter of spines are lost in dendrites not associated with plaques (Spires et al., 2005). In addition, the same study yet show a parallel loss of pre and postsynaptic sites in this mouse model (Spires et al., 2005). These studies not only show that Aβ accumulation is more detrimental to the circuitry than previously anticipated, but also reinforce the idea that soluble oligomers contribute to the loss of synapses, as previously proposed – given the loss of spines on those dendrites not immediately close to the plaques. Together these studies further suggest that synaptic elimination is partly responsible for the cognitive dysfunction observed in AD patients.

1.7. Wnt proteins

1.7.1. The Wnt family and their secretion

Wnt is a large family of conserved secreted proteins across many animal phyla, and almost all mammalians genome have 19 Wnt genes (Nusse & Varmus, 2012). The term Wnt derives from the Drosophila gene *wingless* (*wg*) and the vertebrate oncogene *Int-1*, being adopted around 1990 due to the large number of genes among *wg* and *int* families, which was becoming confusing and inadequate (Nusse et al., 1991; Nusse & Varmus, 2012). Although Wnts are more famously known for their

link to various types of cancer and other human genetic diseases (Anastas & Moon, 2013; Logan & Nusse, 2004), many studies have demonstrated the crucial role of Wnts in several biological processes such as embryonic stem-cell renewal, muscle development and regeneration, epithelial cell homeostasis and embryonic development and patterning (Arwert et al., 2012; van Amerongen & Nusse, 2009; von Maltzahn et al., 2012). In addition, and more relevant to this study, Wnt proteins also play critical role in the nervous system, regulating neural connectivity by promoting axon guidance, dendritic arborisation, synapse formation and, more recently, have also been implicated in synapse plasticity and maintenance (Budnik & Salinas, 2011; Dickins & Salinas, 2013; Oliva et al., 2013; Salinas & Zou, 2008; Stamatakou & Salinas, 2013).

Wnts are glycoproteins which go through intracellular processing before secretion. After translation, the most characteristic modifications of Wnts are glycosylation and acylation posttranslational modifications necessary for secretion, receptor binding and also to confer functional differences among the Wnt isoforms (Komekado et al., 2007; Kurayoshi et al., 2007; Takada et al., 2006; Willert & Nusse, 2012). The number of glycosylation attachments vary according to the Wnt isotype, nevertheless this process appear to be important for their secretion (Komekado et al., 2007; Kurayoshi et al., 2007; Willert & Nusse, 2012). *In vitro* studies demonstrate that mutation in glycosylation sites of Wnt3a and Wnt5a significantly impairs the secretion of these Wnt isoforms (Komekado et al., 2007; Kurayoshi et al., 2007). Furthermore, mutations at different glycosylation sites of Wnt-1 have minor effects on its activity (Mason et al., 1992). Together, these studies suggest the importance of glycosylation of Wnts for their secretion and appear not to be essential for Wnt signalling.

Glycosylation indirectly influences Wnt activity. Wnts go through palmitoylation at their conserved cysteins within the ER, a process which is dependent on prior glycosylation (Komekado et al., 2007; Takada et al., 2006; Zhai et al., 2004). For instance, mutation of a conserved serine residue (Ser209) of Wnt3a in cultured cells and *Xenopus* embryos leads to an impaired secretion of this Wnt isoform and its accumulation in the ER (Takada et al., 2006). Although this serine residue is a

glycosylation site, the study shows that the phenomenon observed is a consequence of a defective subsequent palmitoylation at this residue, a modification required for the intracellular transport of Wnt3a from the ER (Takada et al., 2006). Interestingly, the same phenotype was mimicked by mutations in *Porcupine*, a putative acyltransferase required for Ser209 palmitoylation, suggesting the importance of palmitoylation of Wnts for their secretion (Komekado et al., 2007; Tada & Sheng, 2006; Zhai et al., 2004). Importantly, analysis of mammalian cells with a mutated Wnt5a at the Cys104 site reveal that post-translational modification at this residue is required for the binding of Wnt5 to the extracellular domain of Frizzled receptor, its internalization, and subsequent activation of intracellular signalling cascade (Kurayoshi et al., 2007). Together, these studies highlight the importance of palmitoylation for Wnt secretion and triggering of Wnt signalling at the cell surface level.

Following posttranslational modifications, Wnts are transported to the cell surface for secretion. A multispan protein receptor called Eveness interrupted/Evi - also known as Wntless (Wls) - encoded by the *Wntless* gene - binds and escorts Wnt to the cell surface (Banziger et al., 2006; Bartscherer et al., 2006; Korkut et al., 2009; Willert & Nusse, 2012). Mutation in Evi in *Drosophila* results is similar phenotypes of *wingless* mutants, including a segment-polarity phenotype at the larval stage and abnormalities in the wing blades in adults, whereas knockdown in the Wntless *in vitro* leads to the accumulation of Wnt in the Golgi apparatus accompanied by the reduction in the secretion of Wnt (Banziger et al., 2006; Bartscherer et al., 2006; Korkut et al., 2009). Furthermore, it has been demonstrated that at the Drosophila NMJ, presynaptic vesicular release of Evi is required for the secretion of Wnts - a process implicated in the release and diffusion of Wnt across synapses - demonstrating a novel function for Evi in both Wnt-producing and receiving cells (Korkut et al., 2009). Together these studies show the crucial role for Evi as a protein that functions facilitating the transit of Wnt to the cell surface and also show its involvement in the Wnt diffusion and signalling cascade. Although it has also been

demonstrated the importance of Evi for Wnt secretion in human cells (HEK293T) *in vitro* (Bartscherer et al., 2006), whether all the described functions of Evi are common among the NMJ, central synapses, invertebrates and vertebrates, remains to be studied.

1.7.2. Wnt signalling

1.7.2.1. Wnt Canonical Pathway

Wnts can activate through their receptor different signalling cascades. The most characterised pathway is the canonical or ß- catenin pathway, which has as one of its main distinctive features the "destruction complex". The destruction complex is composed of the scaffolding protein Axin, the tumour suppressor protein adenomatous polyposis coli complex (APC), and the serine/threonine kinases glycogen synthase kinase-3 (Gsk-3β) and Casein kinase-1 (Ck1) (Clevers & Nusse, 2012; Kimelman & Xu, 2006; MacDonald et al., 2009). In the absence of Wnt, the destruction complex is in its active form, where Gsk-3β and Ck1 phosphorylate Axin and APC, leading to the stabilization of Axin/APC/ß- catenin complex (Clevers & Nusse, 2012; MacDonald et al., 2009; Yamamoto et al., 1999) (Fig 1.10). Importantly, when the complex is stabilized, Gsk-3β phosphorylates ß- catenin, which is consequently targeted for ubiquitination and proteasomal degradation (Kimelman & Xu, 2006; Kitagawa et al., 1999).

In the presence of Wnt however, upon binding of Wnt protein to the Frizzled receptor (Fz) and the recruitment of lipoprotein receptor related-protein class 5 and 6 (LPR5/6), this pathway results in the phosphorylation of LRP5/6, its association with Axin and recruitment of the scaffolding protein Dishevelled (Dvl) to the complex, via the interaction of its PDZ domain with the C-terminal tail of Fz (Gao & Chen, 2010; Rothbacher et al., 2000; Clevers & Nusse, 2012). Following the recruitment of Dvl, CK1 is also recruited and bind to LRP5/6, resulting in the dissociation and inhibition of the destruction complex.

Inhibition of the latter results in a cytoplasmic accumulation of ß-catenin and its subsequent translocation to the nucleus, where in association with TCF/LEF activates the transcription of target genes (Clevers & Nusse, 2012; Gao & Chen, 2010; Rothbacher et al., 2000; Willert & Nusse, 2012) (Fig 1.10).

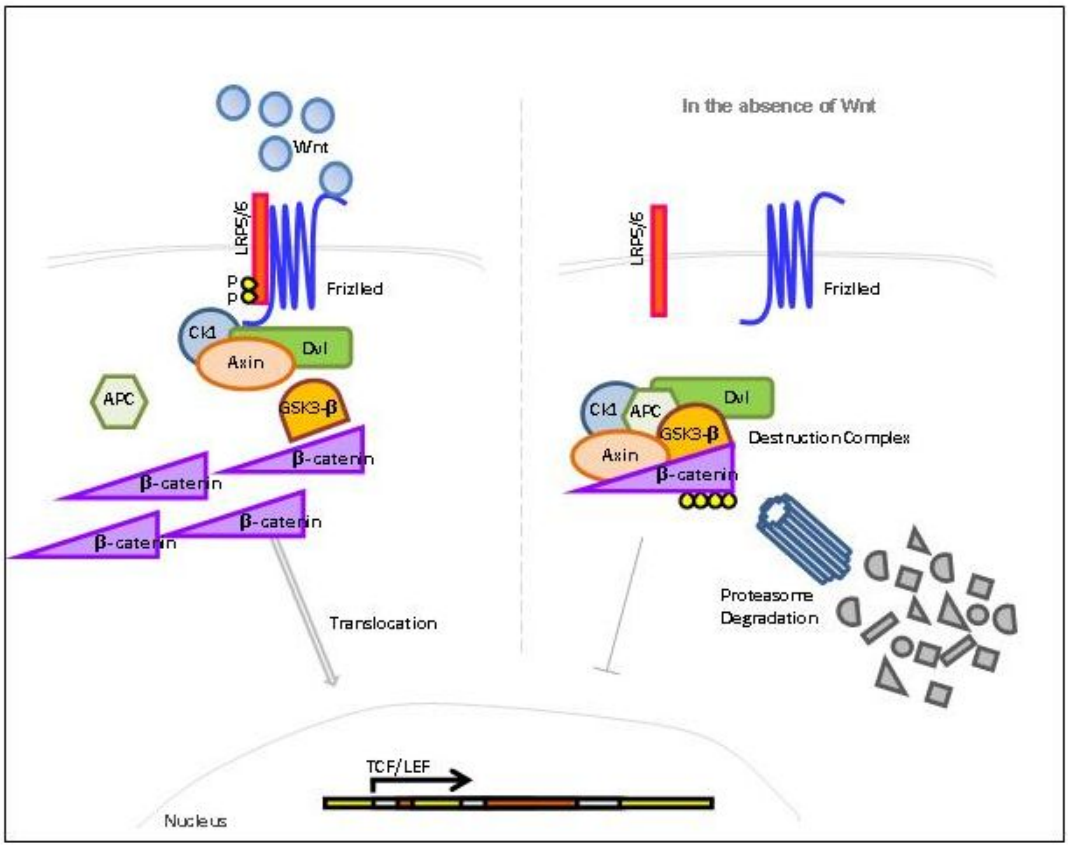

Figure 1.10 - Wnt Canonical Signalling. Scheme of canonical Wnt signalling. Left panel: Binding of Wnts to Frizzled receptors and low-density lipoprotein receptor (LRP5/6) induces association of Axin with phosphorylated LRP6 and recruitment of Dishevelled (Dvl) and Casein Kinase 1 (CK1), disassembling the destruction complex. This event leads to stabilization and accumulation of β-catenin which translocates to the nucleus and initiates the transcription of target genes via TCF/LEF transcription factors. Right panel: In the absence of Wnts, the destruction complex binds to β-catenin, resulting in Gsk3β-mediated phosphorylation of β-catenin, which is then targeted for ubiquitination and proteasome degradation.

Over 120 canonical-Wnt target genes have been described so far (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes - for an updated and comprehensive list), which can be broadly characterised as participating in cell differentiation and proliferation, as well as regulation of Wnt signalling. Notably, it has been reported that disruption of this pathway can lead to

neurodegeneration (H. Kim et al., 2010) and to neurological disorders (Clevers & Nusse, 2012; De Ferrari & Moon, 2006) – the link between Wnts and neurodegenerative diseases will be addressed on Section 1.8 of this chapter.

An alternative diverted canonical Wnt pathway has been described. The divergent canonical pathway, which does not signal to the nucleus but still requires Gsk3β inhibition, has been shown to directly regulate cytoskeleton dynamics and stability (Ciani et al., 2004; Salinas, 2007). A study has shown that in this alternative pathway, Axin are found to be associated directly with microtubules, leading to the stabilization with the latter through Dvl (Ciani et al., 2004). Although the whole divergent intracellular mechanism still remains unknown, this study demonstrates that Dvl stabilizes microtubules by the inhibition of Gsk-3β through a transcription and ß-catenin independent manner (Ciani et al., 2004), suggesting that Axin/Dvl directly interact and inhibit Gsk-3β preventing the phosphorylation of the microtubules, leading to their stabilisation (Ciani et al., 2004; Salinas, 2007). Importantly, since its first description in mammalian cells (Ciani et al., 2004), the divergent canonical pathway has also been shown to occur at the *Drosophila* NMJ (Franco et al., 2004; Gogel et al., 2006), further demonstrating the complexity of Wnt signalling and suggesting this might be a shared mechanism throughout the animal kingdom.

1.7.2.2. Alternative Wnt Cascades - Planar Cell Polarity and Wnt/Ca++ Pathways

Wnt can signal through alternative pathways. The other Wnt signalling pathways are the Planar Cell Polarity (PCP) and the non-canonical Calcium Wnt pathway (Fig 1.11). Similarly to the canonical pathway, the PCP pathway also signals through Wnt/Fz/Dvl complex, and modulates actin cytoskeleton dynamics, but via distinct intracellular molecules. After the binding of Wnt proteins to Fz receptor, Dvl is recruited to the membrane and can signal through two different branches, both of them resulting in GTPases activation (Gordon & Nusse, 2006; van Amerongen & Nusse, 2009) .

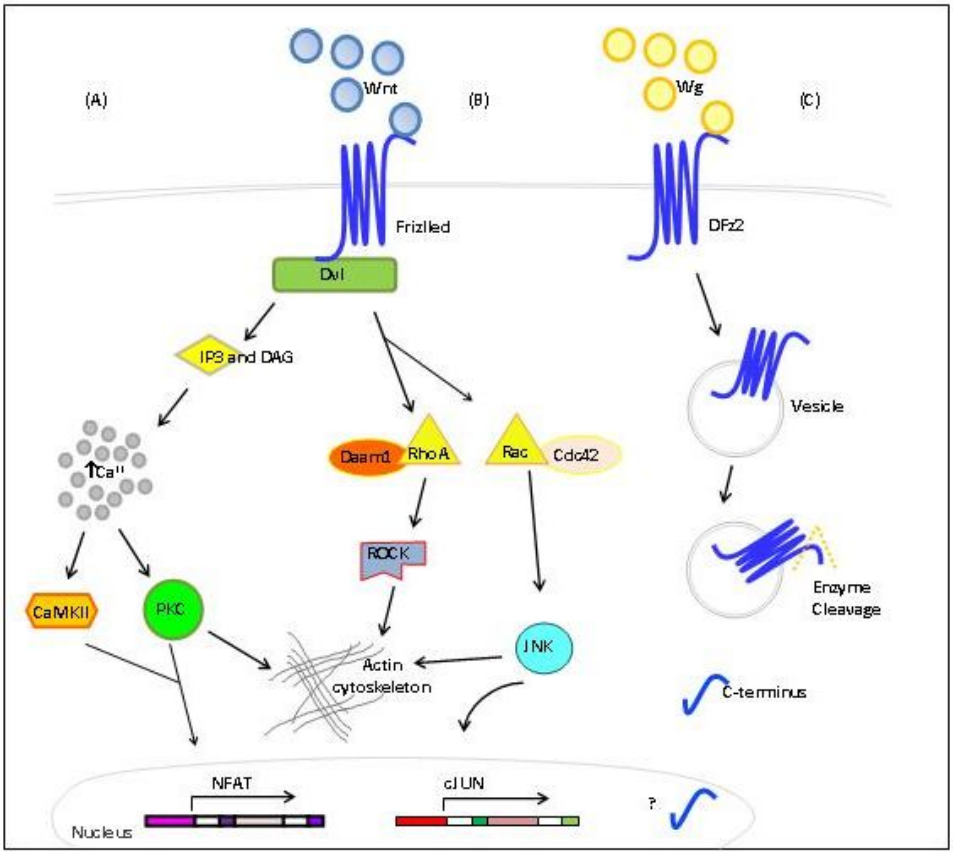

Figure 1.11 - The alternative Wnt Signalling Pathways. (A) Wnt/Ca++ Pathway: Wnt binds to the Fz receptor, activating the scaffolding protein Dvl, which signals through diacylglycerol (DAG) and inositol triphosphatase (IP3) leading to the increase in the intracellular calcium levels. Intracellular concentration of Ca++ activates PKC and CaMKII, which stimulates the transcription factor nuclear factor of activated T-cells (NFAT). PKC can also signal through the small GTPase Cdc42 to regulate actin dynamics. **(B) Planar Cell Polarity Pathway:** After the binding of Wnt proteins to Fz receptor, Dvl is recruited to the membrane and can signal through two different branches, both of them resulting in GTPases activation. Dvl can signal via the activation of c-Jun N-terminal Kinase (JNK) through the small GTPase Cdc42 and Rac. Alternatively, can signal through Damm1 and activate the GTPase RhoA, which culminates in the activation of Rho-associated coiled-coil containing protein kinase (ROCK). Both branches of the PCP pathways converge in actin cytoskeleton regulation. **(C) Frizzled nuclear import pathway:** in this newly described pathway, observed at the NMJ of flies, Fz receptor is endocytosed after Wg/Fz interaction. The vesicle containing the receptor moves towards the nuclear area of the cell, where an enzyme claves the C-terminus of the Fz receptor. Following the cleavage, the fragment is translocated into the nucleus, and transduction of Wg signalling may occur.

Dvl can signal via the activation of c-Jun N-terminal Kinase (JNK) through the small GTPase Cdc42 and Rac (Boutros et al., 1998; Schlessinger et al., 2007). Alternatively, Dvl can signal through Damm1 and activate the GTPase RhoA, which culminates in the activation of Rho-associated coiled-coil containing protein kinase (ROCK)

(McNeill & Woodgett, 2010; Winter et al., 2001). In common, the bifurcation of the pathways converge in the actin cytoskeleton regulation and, as the name determines, PCP is responsible for regulating cell polarity and movement, being crucial for the control of gastrulation and organisation of tissues, in a variety of organisms, from Drosophilas to *Xenopus* and mammals (Fanto & McNeill, 2004; Seifert & Mlodzik, 2007).

In the non-canonical pathway, or Wnt/Ca++ pathway, binding of Wnt to Fz receptor, followed by activation of the scaffolding protein Dvl, leads to the increase in the intracellular calcium levels resulting in the activation of protein kinase C (PKC), CaMKII (calcium/calmodulin dependent protein kinase II) and transcription factor NTFA (nuclear factor associated with T cells) (Kühl, 2000; Robitaille et al., 2002; Saneyoshi et al., 2002; Sheldahl et al., 2003) (Fig 1.11). A few members of the Wnt family have been described to activate the Wnt/Ca++ pathway, among them is Wnt4, Wnt5a and b and Wnt11 (Kühl, 2000; Slusarski et al., 1997; Westfall et al., 2003). Interestingly, it has been reported that CaMKII activation through the Wnt/Ca++ pathway, negatively regulates ß-catenin/TCF transcription, being therefore an antagonist of Wnt canonical signalling, playing a crucial role in the regulation of Xenopus ventral patterning during development (Saneyoshi et al., 2002). Although having different terminologies and mechanisms, studies showed that a crosstalk between various Wnt pathways does happen, as well as the occurrence of additional pathways that mediate the crosstalk between the canonical and non-canonical signalling (McNeill & Woodgett, 2010).

More recently another alternative Wnt pathway has been described at the *Drosophila* NMJ (Mathew et al., 2005). This study demonstrated that in the postsynaptic NMJ of flies, DFz2 is endocytosed from the membrane and subsequently translocated towards the nuclear region of the cell (Mathew et al.,

2005). Immunohistochemistry labelling using specific antibodies for the C- and N-terminal regions of DFz2 demonstrate the distinct intracellular localisation of these domains: the C-terminus is cleaved and translocated into the nucleus, whereas the N-terminus remain at the perinuclear region (Mathew et al., 2005). Furthermore, the authors suggest that the nuclear fragment of DFz2 may act transducing Wg signalling at the postsynaptic site potentially via the regulation of gene transcription. Indeed, a more recent study confirmed the requirement of DFz2-C import for synaptic specialization (Mosca & Schwarz, 2010). Blockade of nuclear translocation of dFz2-C at the NMJ during development, leads to errors in postsynaptic specialisations and increased in the number of "ghost boutons" – synaptic boutons containing vesicles, but lacking active zone proteins (Mosca & Schwarz, 2010). Despite the clear evidence proposing this pathway as a transcriptional regulator of peripheral synapse development, further research is required to determine whether this pathway is common to central synapses.

1.7.3. Endogenous Wnt modulators

Wnt signalling is subject to regulation via the secretion of different endogenous antagonists. A number of different endogenous molecules and families of secreted proteins that antagonise Wnt signalling through different mechanisms have been identified. I will discuss each one of the most well characterised molecules below.

1.7.3.1. Secreted frizzled-related proteins

Secreted frizzled-related proteins (SFRPs) are a family of 5 members (SFRP-1 to 5) of soluble proteins that are structurally similar to Fz, and have been characterised as the largest family of Wnt signalling antagonists (Bovolenta et al., 2008; Cruciat & Niehrs, 2013; Kawano & Kypta, 2003). All SFRP members contain a CRD domain very similar to the CRD extracellular domain of Frizzled receptors, which confers their unique characteristic to directly bind and sequester Wnts away from their receptors,

inhibiting Wnt signalling (Bovolenta et al., 2008; Cruciat & Niehrs, 2013) (Fig 1.12b). The first evidence that SFRP can act as Wnt antagonist comes from a study investigating *Xenopus* embryos and cultured cells (Leyns et al., 1997). This study shows that Frzb/SFRP3 binds to Wnt1 and XWnt8, blocking canonical Wnt signalling, and manifesting the phenotype in embryos as enlarged heads and shortened trunks (Cruciat & Niehrs, 2013; Leyns et al., 1997). Since then, many studies demonstrated the ability of SFRPs to specifically interact with different members of the Wnt family and inhibit both Wnt/β-catenin and Wnt/PCP pathways (Y. Li et al., 2008; Satoh et al., 2008; Sugiyama et al., 2010). Although most of these studies demonstrated a role of SFRPs in embryonic development, curiously single Sfrp1, Sfrp2 and Sfrp5 knockout mice are viable and fertile and have no obvious defective phenotype, suggesting a functional redundancy of SFRP members during embryonic development (Cruciat & Niehrs, 2013; Leaf et al., 2006; Satoh et al., 2006).

SFRPs can activate Wnt signalling. Despite the general consensus and studies demonstrating that SFRPs are negative modulators of Wnt signalling, some recent evidence demonstrates that SFRP can also potentiate intracellular signalling (Bovolenta et al., 2008). For instance, a study demonstrated that SFRP1, via its interaction with Fz2, can stimulate and guide growth cone movement in retinal ganglions of chicks and *Xenopus* (Rodriguez et al., 2005). Likewise, studies using hippocampal cultures demonstrated the pro-synaptogenic effect of SFRP2 when neurons were exposed to this protein (E. K. Davis et al., 2008). Furthermore, a study suggests a biphasic and complex function during kidney development (Yoshino et al., 2001). Immature rats metanephric cultures that were exposed to SFRP1 have impaired tubule formation and branching, a phenotype that was rescued when cultures were treated with a combination of SFRP1 and SFRP2 (Yoshino et al., 2001). Together these studies suggest the ability of SFRP to regulate Wnt signalling through different mechanisms, acting as agonists or antagonists depending on the cellular context.

1.7.3.2. Cerberus

Cerberus is a secreted protein, that similarly to SFRPs inhibits Wnt signalling by binding to Wnt protein and inhibits its interaction with the receptors (Kawano & Kypta, 2003; Piccolo et al., 1999) (Fig 1.12b). This Wnt antagonist was first isolated and described in *Xenopus*, acting as a head organiser and inducing ectopic head in embryos when overexpressed (Bouwmeester et al., 1996). Interestingly, Cerberus has the unique property as a multivalent inhibitor, due to its ability to bind and antagonise to two other factors in addition to Wnt, the bone morphogenic protein (BMP) and Nodal (Kawano & Kypta, 2003; Piccolo et al., 1999). Since its first description in *Xenopus* (Bouwmeester et al., 1996), Cerberus has also been indentified in other vertebrates, including mammals (mouse) (Belo et al., 2009). Curiously, and distinctively to *Xenopus* embryo development, Cerberus has been shown as not essential for mouse head development, rather this Wnt antagonist plays a key role inhibiting Nodal signalling and determining left-right asymmetry in vertebrate embryos (Belo et al., 2009; Cruciat & Niehrs, 2013; Shawlot et al., 2000).

1.7.3.3. Wnt-Inhibitory factor-1

The Wnt-inhibitory factor-1 (WIF1) was first isolated and identified from the human retina, but experiments using *Xenopus* embryos were the initial identification as Wnt antagonist (Hsieh et al., 1999). Overexpression of WIF-1 in this animal model blocked XWnt8 activity, induced abnormal somitogenesis and the formation of a secondary axis (Hsieh et al., 1999). Structurally, this Wnt antagonist has a WIF conserved domain is also found in the extracellular domain of the receptor tyrosine kinase RYK (Cruciat & Niehrs, 2013; Patthy, 2000). Although its mechanisms or action is not well understood, it is believed that similarly to SFRPs and Cerberus, WF1 interacts with Wnt extracellularly, inhibiting its binding to its receptor, tuning its activity in a spatio-temporal manner (Hsieh et al., 1999) (Fig 1.12b). Importantly, a recent study demonstrated that WIF can physically interact and bind to Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt9 and Wnt11, suggesting its impact on both canonical and non canonical Wnt signalling (Surmann-Schmitt et al., 2009). WIF is also present in vertebrates,

including mammals (Hsieh et al., 1999), and broadly expressed in the CNS, retina and cartilage (Y. A. Hu et al., 2008; Hunter et al., 2004; Surmann-Schmitt et al., 2009), playing a role in skeletal development, cardiomyogenesis as well as regulating tumour growth in carcinogenic cells (Buermans et al., 2010; Hunter et al., 2004; Kansara et al., 2009; Yee et al., 2010).

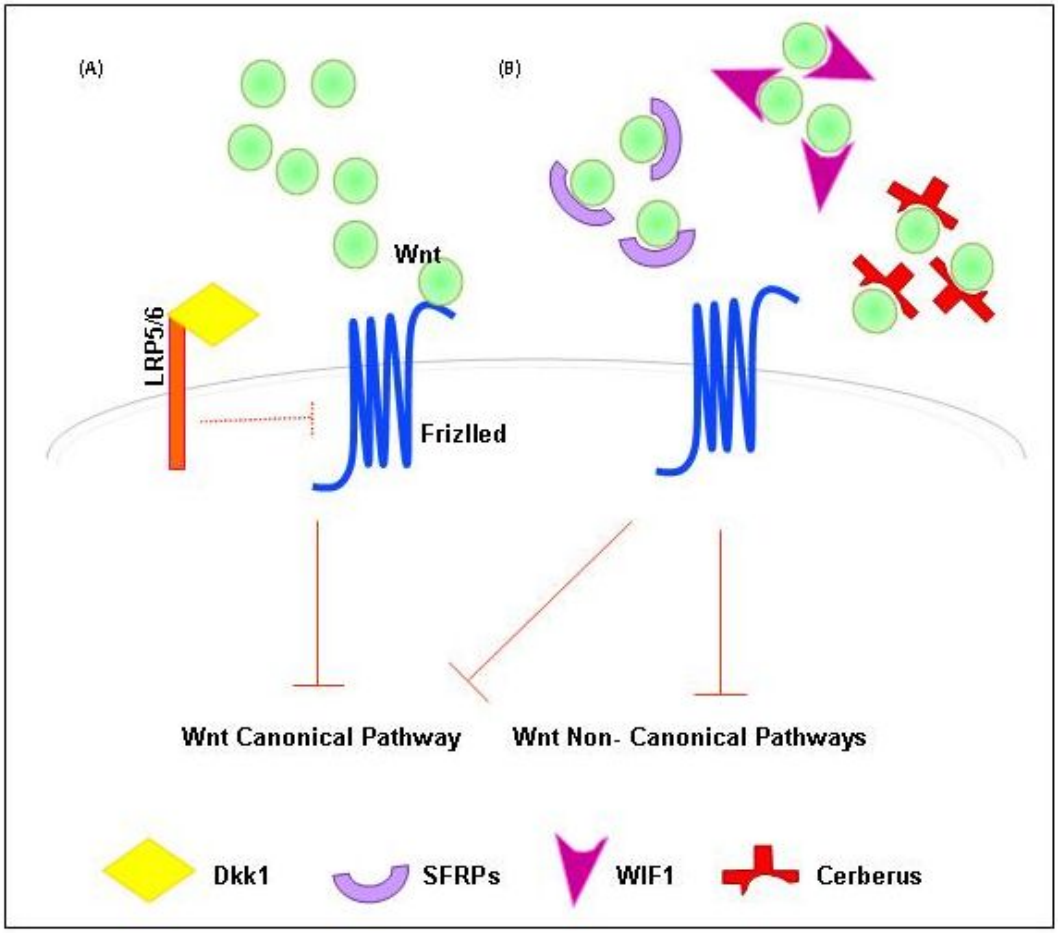

Figure 1.12 - Wnt Signalling is regulated by its endogenous secreted antagonists. Scheme representing the different pathways Wnt antagonists can act. (A) Dkk1 binds to LRP5/6, preventing its interaction with Wnt/Fz complex (red dashed lines) and blocking canonical pathway (detailed representation of Wnt canonical Pathway on Figure X). **(B)** In contrast, presence of Secreted Frizzled Related Proteins (SFRPs), Wnt Inhibitor-factor-1 (WIF1) or Cerberus act sequestering Wnts at the extracellular matrix preventing their binding with the receptors and therefore blocking both canonical and non-canonical pathways

1.7.3.4. The Dickkopf Family

Dickkopf (Dkk) is a family of secreted glycoproteins, present across the animal kingdom, from urochordatas to vertebrates (Cruciat & Niehrs, 2013; Niehrs, 2006). Curiously, Dkk is not found in *Drosophila* and *C. elegans,* possibly due to the divergent evolutionary aspect of the *Dkk* family gene (Niehrs, 2006). In vertebrates, the Dkk family is composed of 4 members: Dkk1 – 4, and with exception to Dkk3, they all contain two conserved cysteins-rich domains (CRD) (Kawano & Kypta, 2003; Krupnik et al., 1999). Dkk1, Dkk2 and Dkk4 act blocking the Wnt signalling by binding to the lipoprotein receptor related-protein class 6 (LPR6), a Wnt co-receptor in the canonical signalling pathway (Brott & Sokol, 2002; Cruciat & Niehrs, 2013; Mao et al., 2001). When present, Dkk1 prevents the formation of the ternary complex Wnt-Frizzled Receptor (Fz) and LRP5/6 (Bourhis et al., 2010; Niehrs, 2006) (Fig 1.12a). Although originally Dkks were described as a potent Wnt signalling inhibitor due to their unique structure that allow their interaction with a distinct extracellular domain to that required for Wnt-Fz interaction (Mao et al., 2001; Zorn, 2001), the viewing on this interaction has changed since its discovery.

Studies using crystallography and mutation at different sites of LRP receptors suggest that the CRD domains at the C-terminus position of Dkk1 and 2 can interact with two different domain of LRP6 receptors, including the site of interaction of most Wnts (Bao et al., 2012; Mao et al., 2001). Unexpectedly, a previously unknown site of LRP5/6– at the top centre of the ectodomain - that interacts with N-terminus end of Dkk1 has been recently identified (Ahn et al., 2011; Bao et al., 2012; Bourhis et al., 2011). Together these studies demonstrate that Dkk1 can bind to different portion of the LRP6 receptor, and therefore inhibit different Wnts in a bipartite manner (Bao et al., 2012). Notably, different studies using luciferase assays in cultured mammalian cells show that, despite the structural homology between LRP5 and LRP6, Dkk1 has a significant lower binding affinity to LRP5 type receptors (Mao et al., 2001; Murrills et al., 2009).

Despite being regarded exclusively as a Wnt inhibitor, Dkk1 can act as a Wnt regulator. A study using a combination of *in silico* screening and luciferase assays in human embryonic kidney cells (HEK293 cells), showed that Dkk1 is a TCF target gene, and its promoter is responsive to β-catenin/TCF mediated transcription elements, suggesting the existence of a negative feedback loop in Wnt signalling (Niida et al., 2004). This finding was further confirmed in a mouse *in vivo* system during development (Diep et al., 2004). Together, these studies indicate that in some cellular contexts, Dkk1 acts as a Wnt signalling modulator.

Dkks can bind to an alternative receptor. In addition to LRP5/6, Dkk1, Dkk2 and Dkk4, can bind with high affinity to the single-pass transmembrane protein receptor Kremen 1 and 2 (Krm1/2) (Cruciat & Niehrs, 2013; Mao & Niehrs, 2003; Mao et al., 2002). Kr1/2 potentiate the ability of Dkks to block Wnt/β-catenin signalling by forming a ternary complex with Dkk-LRP5/6, resulting in the rapid removal and internalization of the LRP5/6 receptor via endocytosis, and the consequently the suppression of Wnt cascade (Mao et al., 2002). Time course analysis of mammalian cells co-transfected with LRP6 and Krm2 and treated with Dkk1, demonstrated that internalisation of the complex occurs within 5 minutes of the exposure to Dkk1 (Mao et al., 2002). Furthermore, the study shows a decrease in the total levels of LRP6 after the treatment with Dkk1, suggesting the protein is degraded after the internalization (Mao et al., 2002). Physiologically, Krm1/2 has been demonstrated to be important in the formation of the CNS during embryogenesis. Antisense morpholino knockdown of Krm1/2 in *Xenopus* embryos result in microcephaly and slight shortening of the trunk (Davidson et al., 2002). However, analysis of Krm1/2 constitutive knockout mice show that double mutant mice are viable and fertile, presenting only a mild ectopic forelimb phenotype (Ellwanger et al., 2008), showing therefore that Kremens are not compulsory for Dkk1 function.

The founder member of the Dkk family is Dkk1, and it was first described over 16 years ago as an embryonic head inducer and potent Wnt inhibitor in *Xenopus* (Glinka et al., 1998; Kawano & Kypta, 2003). This breakthrough study demonstrating the ability of Dkk1 to block Wnt signalling showed that overexpression of Dkk1 during

gastrulation induces the expansion of heads structures and formation of ectopic heads (Glinka et al., 1998). Conversely, injection of antibody against Dkk1 during embryogenesis causes microcephalia and cyclopia (Glinka et al., 1998). The striking phenotype observed in this study named the protein, as Dickkopf is translated as "big head" from German. Indeed, other studies further demonstrated the importance of Dkk1 during embryogenesis and development, especially for embryonic patterning along the antero-posterior axis. For instance, knockout of the Dkk1 gene in mouse leads to embryonic lethality, loss of head structures and fused vertebrae (Mukhopadhyay et al., 2001). Importantly, Dkk2 and Dkk3 null mice are viable and fertile, developing milder phenotypes, presenting with abnormal formation of the cornea and osteoblast, respectively (X. Li et al., 2005; Mukhopadhyay et al., 2006). Furthermore, *in situ* analysis in the expression of the Dkk family members in the forebrain throughout development show that Dkk1, 2 and 3 are expressed at early developmental stages (up to embryonic day 12 – E12) (Diep et al., 2004). Curiously, at later stages (E15.5 onwards), little or no expression of Dkk1, Dkk2 and Dkk4 was found in the mouse forebrain, whereas Dkk3 was detected in different brain areas, including the adult hippocampus (Diep et al., 2004). These studies further highlight the importance of the Dkk family as Wnt signalling inhibitors during development.

The advance of genetic manipulation is allowing the study of Wnts at different stages of development. As previously discussed, dysregulation of Wnt signalling during early development can lead to serious congenital abnormalities and even death (Glinka et al., 1998; Kawano & Kypta, 2003; X. Li et al., 2005; Mukhopadhyay et al., 2006; Mukhopadhyay et al., 2001). To overcome these limitations, a promising model was generated, so blockade of Wnt signalling could be achieved by inducing the expression of the secreted Wnt antagonist Dkk1 using tetracycline inducible system (Chu et al., 2004). Transgenic mice carrying the Dkk1 coding region downstream of a doxycycline responsive promoter (tetO), combined with a *β-globin* addition sequences, was originally generated to study the role of Wnt signalling in the skin throughout development (Chu et al., 2004). By crossing these mice to a second mouse line mice carrying the tetracycline-controlled transactivator (rtTA)

downstream of the keratin 5 promoter (Diamond et al., 2000), the authors successfully demonstrate an inducible suppression of Wnt canonical signalling, and its role for epithelium development and mammary gland morphogenesis (Chu et al., 2004). Given the flexibility for the induction in the expression of Dkk1 in the mouse line above described (Chu et al., 2004), this line represents a great opportunity to study the role of Wnt signalling in the adult brain, without affecting early development – which will be the main focus of my study. An interesting choice is the usage of the tetracycline-controlled transactivator downstream of the forebrain specific CaMKIIα promoter (Michalon et al., 2005). This well established line was generated so any doxycycline induced gene expression could be achieved in the brain (Michalon et al., 2005). Using a LacZ reporter expression, the authors demonstrate the successful generation of a mouse line that allow the inducible and reversible gene expression in the forebrain - in the principal neurons of the hippocampus, striatum and amygdala – during adulthood (Michalon et al., 2005). By crossing the latter with the Dkk1-tetO (Chu et al., 2004), the double transgenic line would allow me to explore the role for Wnt signalling in the adulthood, as demonstrated in the Chapters 4 and 5 of this thesis.

Post-developmental expression of Dkk1 is equally important as cell homeostasis is also dependent of normal levels of transcription of this gene (Niehrs, 2006). Dysregulation in the expression of Dkk was observed in many different types of human carcinomas, which led to speculation that Dkks may be acting as tumour suppressors in these circumstances (Niehrs, 2006). Dkk1-1 gene expression is under control of p53 (J. Wang et al., 2000), a tumour suppressor protein that acts as sensor in response to DNA damage that can ultimately trigger apoptosis. Studies have demonstrated that chronic stress and insults to the CNS can lead to increase in Dkk1 expression and trigger neuronal death (Cappuccio et al., 2005; Mastroiacovo et al., 2009; Matrisciano et al., 2011). Together, these studies shed light on the importance to understand the role of Wnt signalling as well as their antagonist not only during development but also in the adulthood.

1.8. Wnt in the adult brain function and neurodegenerative diseases

Wnt proteins and related molecules are found to be expressed in different areas of the mature brain (De Ferrari et al., 2007; Gogolla et al., 2009; Salinas et al., 1994; Shimogori et al., 2004; Tabatadze et al., 2012) suggesting that Wnt signalling plays a role in different aspects of neuronal circuitry and function in adulthood. Growing evidence points to Wnts as key regulators of long-term plasticity. For instance, in hippocampal brain slices, inhibition of Wnt signalling by blocking either Wnt3a or Fz8 receptor, cause impairment in long-term potentiation (LTP) (J. Chen et al., 2006). Conversely, the same study shows that activation of Wnt signalling using either the GSK3β inhibitor LiCl, or exposing the slices to Wnt3a, increases the magnitude of LTP (J. Chen et al., 2006). Similarly, conditional overexpression of GSK3β in adult mice, leads to impairment in LTP, which can then be rescued by treatment with LiCl (a GSK3β inhibitor) (Hooper et al., 2007). These studies demonstrate that disruption of Wnt signalling impairs plasticity, whereas activation of Wnt signalling facilitates LTP, but can these findings be related to physiological/behaviour situations?

Activity increases the release of Wnts. Supporting previous findings which demonstrated that LTP induces the secretion of Wnt3 in an *ex vivo* system (J. Chen et al., 2006), it has been demonstrated that behavioural experience can positively regulate the secretion of Wnts *in vivo* (Gogolla et al., 2009). Environmental enrichment exposure produces a significant increase in the levels of Wnt7a/b in the CA3 hippocampus area, an event that positively correlates with the size, number and complexity of the synapses in that region (Gogolla et al., 2009). Importantly, this study also show that Wnt7a/b secretion decline with age, accompanied by the loss of complexity and number of synapses, which suggests a link between Wnt secretion and control of synapse number in the adulthood (Gogolla et al., 2009). More recently, a study showed a direct correlation between Wnt secretion and hippocampal learning (Tabatadze et al., 2012). Spatial learning in the Morris water maze selectively increases the levels of Wnt7a/b and Wnt5 in the hippocampus, but

without changing the levels of Wnt3 isoform (Tabatadze et al., 2012). Interestingly, elevation of Wnt7a/b levels is observed only 7 days after training, and upregulation is maintained 30 days after training, suggesting a positive correlation between Wnt secretion and the establishment of long-lasting memories (Tabatadze et al., 2012). These studies provide relevant evidence that support the importance of Wnt signalling for the normal function of the adult brain, and conversely dysregulation of Wnt signalling has been linked to neurological diseases, as discussed in the next section of this chapter,

Wnts are involved in adult neurogenesis. The first study demonstrating the direct role for Wnt signalling in the neurogenesis in the DG region of the hippocampus was published almost ten years ago (Lie et al., 2005). Using viral transduction in the SGZ of the DG the authors show that stimulation of Wnt/β-catenin signalling, by overexpressing Wnt3a, leads to an increase in the number of stem progenitor cells in the area (Lie et al., 2005). By contrast, it was shown blockade of Wnt signalling in the adult DG, using a dominant-negative Wnt viral expressing carrier (dnWnt), hippocampal neurogenesis was almost completely suppressed (Lie et al., 2005). These results were further confirmed and reproduced in the Wnt7a knockout mice, which also present defected neurogenesis in the DG (Qu et al., 2009). Furthermore, a functional follow up study has demonstrated that adult rats injected with dnWnt into the DG, and therefore with impaired neurogenesis, present impaired long-term memory in the Morris water maze as well as poor performance in the novel object recognition (Jessberger et al., 2009), suggesting a link between certain forms of memory, neurogenesis and Wnt signalling. More recently, an *in vivo* study demonstrated that reducing expression of the Wnt antagonist Dkk1 the in DG of aged mice has beneficial outcomes to their memory (Seib et al., 2013). Using a combination of cell biology and behavioural assays, the authors show that age-related decrease in neurogenesis can be rescued if Dkk1 expression is silenced (Seib et al., 2013). Moreover, by suppressing Dkk1 expression in the DG, animals show enhanced spatial working memory in the T-maze test as well as improved memory consolidation in the place avoidance paradigm (Seib et al., 2013). Together these

studies provide sufficient evidence to a positive correlation between Wnt canonical signalling and its role in adult neurogenesis.

1.8.1. A role for Wnts in neurodegenerative diseases

Neurodegenerative diseases are in general associated with destabilisation in the neuronal network, impairment in synaptic transmission, defects in intracellular pathways and synaptic atrophy that ultimately leads to neuronal death (Oliva et al., 2013; Ortiz-Matamoros et al., 2013; Palop & Mucke, 2010). Impaired Wnt signalling is related to synaptopathy and psychiatric disorders, including schizophrenia, depression and autistic spectrum disorder (Duman & Voleti, 2012; P. M. Martin et al., 2013; Miyaoka et al., 1999; Ortiz-Matamoros et al., 2013), but more importantly and in relevance to this study, several studies link the dysregulation of Wnt canonical signalling with Alzheimer's Disease (AD) (Inestrosa et al., 2012; Purro et al., 2014). AD is the most common form of dementia and best characterised by the accumulation of pathogenic amyloid-β (Aβ) in the brain, that leads to synaptic dysfunction, manifested as memory loss and changes in the personality and behaviour (Palop & Mucke, 2010; Wortmann, 2012) and evidence suggests a link between the cellular processing of Aβ and components of Wnt pathway.

Plaque accumulation leads to neuronal degeneration – a process linked to the Wnt pathway. Abnormal processing of amyloid precursor protein (APP) leads to the production, deposition and aggregation of toxic Aβ forms. APP, which can be found at the plasma membrane and intracellularly in vesicles, can go through sequential cleavages by β-secretase and presenilin-γ-secretase, as well as a regulatory secretory cleavage by α-secretase (De Strooper et al., 2012; Selkoe, 2004). Missense mutation in presenilin-1 (PS1) or preselinin-2 (PS2) can cause a imbalance in the cleavage of the transmembrane domain of APP, leading to the accumulation of 42-residue of Aβ, the cleaved form which is capable of oligomerisation (De Strooper et al., 2012; Selkoe, 2004; Selkoe & Wolfe, 2007). In addition, mutation in the human APP gene, that either results in the overexpression of APP or increase in the amyolidogenic

production of APP (in its 42-Aβ peptide form) is also implicated in AD (Haass et al., 2012; Selkoe, 2011). Crucially, studies have demonstrated that components of the canonical Wnt pathway interact with PS1 (Fig 1.13). Immunoprecipitation assays demonstrate the physical interaction between β-catenin and PS1 (Murayama et al., 1998; Soriano et al., 2001; Z. Zhang et al., 1998). Although the significance of this interaction is still not entirely clear, these studies suggest that PS1- β-catenin complexes increases the stability of β-catenin, therefore mutation in PS1 disrupts their interaction and negatively regulates the levels of β-catenin, consequently downregulating the levels Wnt signalling (De Ferrari & Inestrosa, 2000; Murayama et al., 1998; Soriano et al., 2001; Z. Zhang et al., 1998).

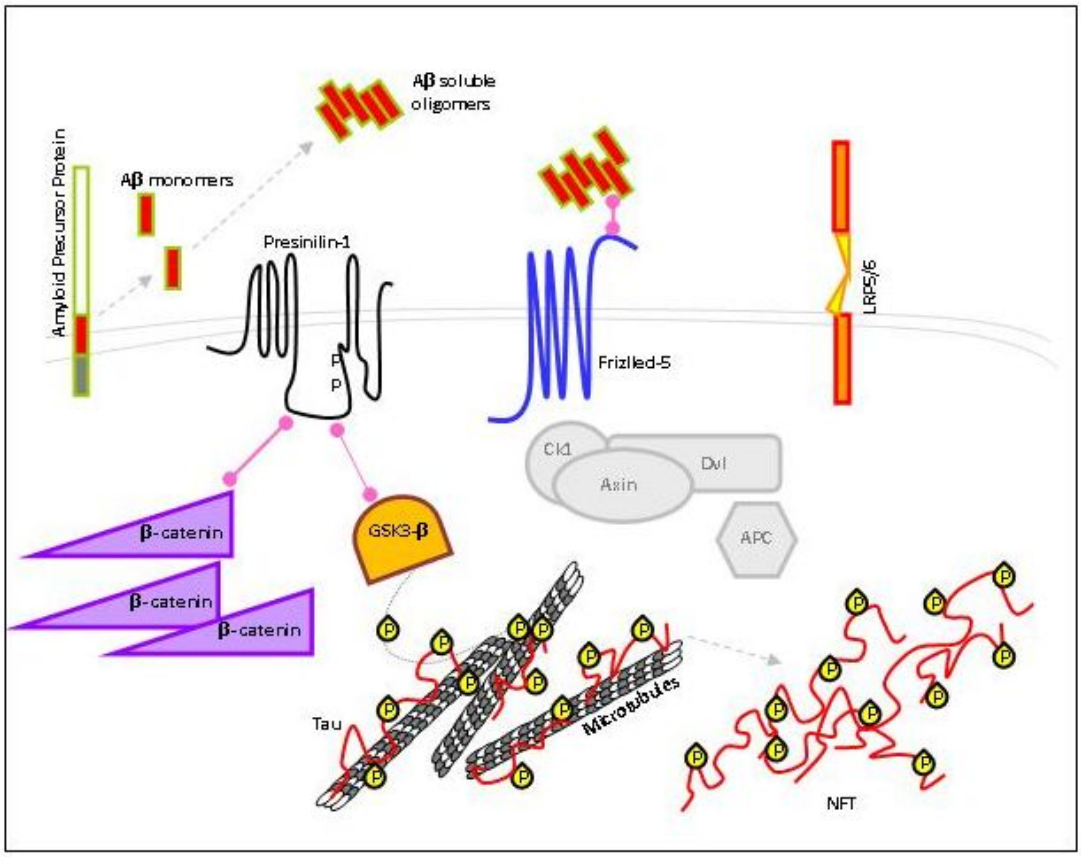

Figure 1.13 – Wnt signalling components and their interaction with AD related proteins. Scheme of canonical Wnt signalling and its components (coloured) that have been reported to physically interact with AD related proteins – interaction shown by the pink barbells. Misprocessing of amyloid precursor protein by presenilin secretase (a multi-pass transmembrane protein, in black) leads to the formation of Aβ monomers, which can accumulate, oligomerise and lead to neuronal toxicity. Aβ oligomers have been demonstrated to directly bind to the extracellular domain of Frizlled-5 receptor. Presenilin-1 interacts intracellularly with two core components of Wnt canonical signalling: β-catenin and Gsk3-β. The significance of this interaction is not fully understood. In addition, is well known that overexpression or over-activation of Gsk3-β leads to hyper-phosphorylation of microtubule-associated protein Tau, causing the destabilisation of microtubules and formation of intra-neuronal neurofibrillary tangles (NFT; bottom right). Furthermore, a genome wide association study demonstrated that genetic variation of the Wnt co-receptor LRP6 (represented as yellow triangles, top right) is linked to late onset of AD. Together these evidence show a clear link between Wnt signalling and AD.

Another core Wnt signalling component has been linked to AD. In addition to β-catenin interaction, PS1 also has been shown to directly interact with GSK3β (Fig 1.13) (Phiel et al., 2003; Takashima et al., 1998). Despite the functional role of this interaction and the molecular mechanism underlying it is also not clear, a study shows that inhibition of GSK3β with lithium chloride (LiCl) in cultured cells

overexpressing APP reduces the secretion and accumulation of Aβ42, in a dose dependent manner (Phiel et al., 2003). Importantly, the same study shows that accumulation of Aβ42 is also prevented by LiCl in a mouse model expressing the pathogenic form of APP (Phiel et al., 2003). Based on these findings, the authors suggest that GSK3β might regulate γ-secretase activity by modulating the access of the enzyme to the substrate (APP), and propose that GSK3β could be a reasonable target to tackle AD (Phiel et al., 2003).

In addition to the proposed function on the APP processing, GSK3β has been linked to neurofibrillar accumulation observed in AD (De Ferrari & Inestrosa, 2000; Hooper et al., 2008; Hooper et al., 2007). Intraneuronal neurofibrillary tangles (NFT) are composed of hyper-phosphorylated forms of microtubule associated protein tau which also leads to neuronal degeneration (Geschwind, 2003), and GSK3β has been suggested to induce hyper-phosphorylation of tau (Fig 1.13) (De Ferrari & Inestrosa, 2000; Hooper et al., 2008). Indeed, transgenic mice overexpressing GSK3β present hyper-phosphorylation of tau, NFT and degeneration, together with the decreased levels of nuclear β-catenin (J. J. Lucas et al., 2001). Furthermore, chronic LiCl administration to an AD mouse model prevents Tau hyper-phosphorylation and the formation of NFT in their brain (Engel et al., 2006). Given the compelling evidence to the involvement of GSK3β in different aspects of the AD pathogenesis, authors introduced the "GSK3 hypothesis of AD" (Hooper et al., 2008), which put this kinase as a causal mediator of AD and propose that this can be a target molecule to treat and perhaps prevent AD pathology.

Further evidence link Wnt signalling, synaptic dysfunction and AD. Although, the molecular mechanisms by which Aß induces synaptic changes remain poorly understood, recent studies suggest that dysfunction in Wnt signalling could contribute synaptic vulnerability. A genome-wide linkage study reported that genetic variation of the Wnt co-receptor LRP6 is linked with late onset-AD (Fig 1.13) (De Ferrari et al., 2007). The study also demonstrates that in heterologous cells, this variant leads to a decreased Wnt/β-catenin signalling and reduced response to Wnt3a condition media (De Ferrari et al., 2007). Furthermore, yet at the receptor

level, a study found that soluble Aβ directly binds to the extracellular CRD domain of the Fz5 receptor, leading to a decrease in β-catenin nuclear translocation and Wnt-targeted gene transcription (Magdesian et al., 2008). Although the significance of this finding remains to be investigated, these studies provide compelling evidence to the direct link between Wnt signalling receptors and AD.

Wnt antagonists are increased in AD – the potential cause of synapse loss in the disease. Dkk3, a secreted Wnt antagonist, has been reported to be elevated in plasma and cerebrospinal fluid (CSF) of AD patients (Zenzmaier et al., 2009). Furthermore, the Wnt antagonist Dkk1 was also found to be elevated in post-mortem brain samples from AD patients (Caricasole et al., 2004), suggesting that Dkk levels can serve as potential biomarkers in the context of AD. Interestingly, elevated Dkk1 is increased in three different AD animal models, an observation that coincided with the localisation of active GSK3β (Caricasole et al., 2004; Rosi et al., 2010). Consistent with these findings, β-amyloid (Aβ) peptides induce Dkk1 expression in cultured neurons and brain slices (Caricasole et al., 2004; Killick et al., 2012; Purro et al., 2012). More importantly, addition of Dkk1 neutralising antibody protects synapses from disassembly by Aß in brain slices, indicating that Dkk1 is required for synaptic loss induced by Aß (Purro et al., 2012). Given that blockade of Wnt signalling with Dkk1 quickly disassemble synapses in mature dissociated hippocampal cultures (Purro et al., 2012), these finding suggests that deficiency in Wnt signalling could contribute to synaptic vulnerability in the adult brain, and can trigger synaptic loss in the context of AD.

Wnt agonist compounds may represent a target for the treatment of AD. A recent study suggests that chronic infusion of either WASP-1 (Wnt-activator small molecule), a canonical Wnt agonist; or FOXY-5 (formylated Wnt5a derived hexa-peptide), a compound that mimics the effects of Wnt5a, and therefore activating the non-canonical Wnt pathway, directly into the brain of APP/PS1 transgenic mice have beneficial outcomes to their synapses and behaviour (Vargas et al., 2014). The authors show that adult APP/PS1 mice treated with either of the compounds have

enhanced basal excitatory synaptic transmission amplitude when compared to control non-treated mice (Vargas et al., 2014). In addition, the study also shows that both Wnt agonists, WASP-1 and FOXY-5, are able to enhance synaptic potentiation and increase LTP magnitude induced by high frequency stimulation in their AD mouse model (Vargas et al., 2014). Furthermore, the authors also demonstrate that the used Wnt activators can rescue non-spatial memory impairment observed in this animal model, as mice chronically treated with either Wnt agonists performed similarly to wild type animals in the novel object recognition task, and better than non treated APP/PS1 mice (Vargas et al., 2014). Importantly, further characterisation of the compounds in regards to their specificity needs to be carried out, as only a very restricted number of scientific studies using these molecules were published so far (Beaumont et al., 2007; Safholm et al., 2006; Safholm et al., 2008). In addition, further studies determining the direct effect of WASP-1 and FOXY-5 on neurons, and synaptic number and morphology are yet to be done.

In summary, these studies demonstrate a function for Wnt signalling in the healthy adult brain. Given that proteins involved in Wnt signalling are expressed throughout life, these findings suggest that endogenous Wnts are regulating synaptic function and stability. The demonstrations that dysregulation of Wnt signalling and overexpression of Wnt antagonists can contribute to the synaptic loss observed in AD, provide strong evidence to focus on the components of Wnt pathway as possible therapeutic targets for treatment of neurodegenerative diseases. Furthermore, these studies further support the proposition that endogenous Wnt signalling is required for the maintenance of synapses – the focus of study of this thesis.

1.9 Thesis Aims:

In this project, I aim to determine the role of Wnt signalling in the synaptic maintenance in the CNS using both *in vivo and ex-vivo* approaches. Wnt proteins are well known to play an important role in the CNS by regulating axon guidance, promoting axonal remodelling, synapse formation and synaptic transmission. Interestingly, Wnt ligands are also expressed in the adult brain. Recent studies investigating the role of Wnt in adulthood showed that blockade or downregulation of Wnt signalling are associated with deficits in memory functions. As none of these studies addressed the cause of the reported phenotypes, I hypothesized that the behavioural phenotypes observed were in fact a consequence of dysfunction in synaptic maintenance affected by disruption of Wnt signalling. Furthermore, I proposed that not all types of synapses might be affected by dysfunction in Wnt canonical signalling. To address these questions I focused on five distinct aims:

1) To investigate the effect of acute blockade of Wnt canonical signalling, using the endogenous antagonist Dkk1, excitatory and inhibitory synapses in the hippocampus area using brain slices
2) To validate a new transgenic mouse model which inducibly expresses the Wnt antagonist Dkk1, and characterise the pattern of Dkk1 expression in the hippocampus
3) To determine the structural changes in hippocampal synapses of adult mice chronically expressing Dkk1 protein.
4) To analyse any hippocampal-dependent behavioural changes in adult animals expressing Dkk1
5) To investigate which intracellular mechanisms underlie the action of Dkk1 on synapses.

2. Materials and methods

2.1. Animals

Heterozygous transgenic tetO-Dkk1 mice (Chu et al., 2004) were crossed with heterozygous transgenic CaMKIIA-rtTA2 mice (Michalon et al., 2005) to obtain double transgenic animals (ind-Dkk1). Litters were genotyped by PCR using samples from ear clippings.

Genotyping were performed using the following primers: CaMKIIα-rtTA: forward 5' TGCCTTTCTCTCCACAGGTGTCC 3', reverse 5' GAGAGCACAGCGGAATGAC 3'; and tetO-Dkk1: forward 5' GCGTCCTTCGGAGATGATGG 3', reverse 5' AAATGGCTGTGGTCAGAGGG 3'.

For evaluation of activated β-catenin/TCF/LEF report, ind-Dkk1 (double transgenic animals, described above) were crossed with heterozygous mice carrying the *LacZ* gene under the control of the regulatory sequence consisting of seven consensus LEF/TCF-binding motifs (Maretto et al., 2003). Genotypes were confirmed by PCR using the primers forward 5' CGTGGCCTGATTCATTCC 3' and reverse 5' ATC CTCTGCATGGTCAGGTC 3'.

All mice were 3 – 6 months of age when doxycycline treatment commenced. Ind-Dkk1 and control littermates were administered food complemented with 6mg/kg of doxycycline (Datesand group) *ad libitum* for 7 or 14 days (specified on each experiment). In all experiments wildtype and single transgenic animals (littermates), also fed with doxycycline, or ind-Dkk1 not fed with doxycycline were used as controls. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and Local Ethical Committee approval.

2.2. RT-PCR analysis of gene expression

RNA was extracted from total hippocampus of adult animals, fed with doxycycline for 14 days, using Trizol (Invitrogen) and treated with DNAse I (Sigma). In brief, tissue was homogenized in 1 ml of Trizol, and incubated for 5 minutes at room temperature. Following incubation, 0.2 ml of chloroform was added and vigorously shaken. The samples where then centrifuged at 12,000x g for 15 minutes (at 4°C). The upper aqueous phase was transferred to a new eppendorf tube, and RNA was precipitated using isopropanol and resuspended in 20 µl of RNAse-free water.
First strand cDNA synthesis was performed with AMV Reverse Transcriptase (Promega) according to manufacturer's instruction. PCR was performed using GoTaq® Polymerase (Promega).

Primers used were:

Gene	**Reverse (5' - 3')**	**Forward (5' - 3')**
Axin2	AGCAGATCCGGGAGGATGAAG	ATTGACAGCCGGGGGTCTTGAGG
Fzd2	GGAGCTCCTGCGCTACTCAC	GCGCTCACCCAGAAACTTAT
LRP6	GGGCCGATGCAAAACTTAAT	CCTCTGTTGGCTGAAAGCAT
SRFP2	ACTTCTCCTACAAGCGCAGCAAC	GCAGGCTTCACACACCTTGG
Wnt11	AACTGATGCGTCTACACAACA	GGTGACGTAGCAGCACCAGTG

2.3. In situ hybridization

To examine the expression of Dkk1 in the adult hippocampus, ind-Dkk1 and control mice were fed with doxycycline for 14 days and in situ hybridization was performed as previously described (Pasterkamp et al., 1998), using the probes described in (Andl et al., 2002). Riboprobes were prepared following manufacture's directions (ROCHE, DIG RNA labelling Kit – SP6/T7). In brief, ~1µg of purified template DNA was diluted in RNAse-free water to a final volume of 13 µl. To this volume, it was added 2 µl of 10X NTP labelling mixture; 2 µl of 10X Transcription buffer; 1 µl of RNAse inhibitor and 2 µl of each RNA polymerase. The mixture was then incubated for 2 hours at 37°C. Following incubation, the mixture was treated with DNAse I to

remove template DNA. RNA was then precipitated by adding 1/20 volumes of 8 M LiCl and 2.5 volumes of 100% Ethanol and incubated overnight at -80 °C. Following incubation, the solution was centrifuged for 20 min at 4°C. Pellet was washed with 70% ethanol, spun for 20 min at 4°C, resuspended in 20 µl of DEPC-water and stored at -80 °C until used.

Once riboprobes were prepared, brains of ind-Dkk1 and control mice brain were taken, snap-frozen in isopentane cooled in liquid nitrogen and stored at –80°C. Sagittal sections (8µm) were cut in a cryostat, air dried and fixed in 4% paraformaldehyde (PFA) in PBS for 20 minutes. Slides were first blocked with 40ug/ml salmon sperm in 5x SSC, 50% formamide, overnight in a humidified chamber. After blocking, slides were incubated with 1ug/ml of Dkk1 cRNA probe diluted in hybridization buffer (5x SSC, formamide), overnight at 55ºC in a humidifying chamber. Next, slides were carefully washed with 5x, 2x and 0.2x SSC and incubated with an antibody anti-DIG-AP, dilute 1:5000 in buffer IS1 for 3.5 hours. After incubation, slides were washed twice with buffer IS1, followed by a quick wash with buffer IS2. Slides were then incubate for 48h in colour solution, washed for 10 minutes in TE buffer to stop the reaction and mounted.

2.4. Acute brain Slices

Mice (p15) were anesthetized and sacrificed by decapitation. Brains were rapidly extracted, hemispheres were separated and placed into ice-cold artificial cerebrospinal fluid (ACSF). Sagittal slices (300 µm) were cut at 4 °C with a vibrotome and transferred into 24-well Costar plates containing aCSF and incubated for a 60-min recovery period at 37°C in an incubator (5% CO_2/95% O_2). After a recovery period (approximately 1 hour), brain slices were treated with recombinant Dkk1, 50ng/ml (R&D systems) prepared in BSA; Bio, 500 nM (Calbiochem) prepared in DMSO or vehicles for 4 hours. DMSO or Bio (BSA or DMSO alone) was used alone or in conjunction with Dkk1. After 4 hours treatment, brain slices were fixed in 4% PFA

with 4% sucrose in PBS for 1 hour at RT. PFA was removed by washing with PBS. Staining of brain sections were carried out as described on section 2.7.

2.5. Drug treatments

For all experiments, acute brains were recovered for at least 30 min., followed by 4 hour drug treatments in the incubator (5% CO_2/95% O_2). Following the recovering period, brain slices were treated with the following pharmacos:

Dkk1 (50ng/ml) (R&D Systems), prepared in 0.1% BSA was used to inhibit Wnt signalling in hippocampal slices. Dkk1 was mixed to pre-warmed aCSF (37°C) before treating the slices.

BIO (Calbiochem) was used as an inhibitor of Gsk3 activity and therefore, mimicking Wnt-canonical signalling (Meijer et al., 2003; Purro et al., 2008; Rosso et al., 2005). Bio was solubilised in DMSO and used at a final concentration of 500nM. For the treatment, Bio was mixed to pre-warmed aCSF (37°C) before treating the slices.

MG-132 (Millipore) is a cell-permeable protein inhibitor which reduces the degradation of ubiquitin-conjugated proteins. MG-132 was solubilised in DMSO (20mg/ml) and used at a final concentration of 10µM. MG132 was mixed to pre-warmed aCSF (37°C) before treating the slices.

Lactacystin (Lact) (Sigma) is also is a cell-permeable protein inhibitor which reduces the degradation of ubiquitin-conjugated proteins. Lact was solubilised in DMSO (20mg/ml) and used at a final concentration of 5µM. Lact was mixed to pre-warmed aCSF (37°C) before addition to the brain slices.

2.6. Immunofluorescence staining

Mice were anesthetized with CO_2 and sacrificed by decapitation. Brains were rapidly dissected and placed into ice-cold artificial ACSF. Sagittal slices (300 µm) were cut at 4 °C with a vibrotome (Campden Instruments) and fixed in 4% PFA/4% sucrose in PBS for 30-60 min at RT. PFA was removed by washing three times with PBS (10 minutes each wash). Slices were incubated with blocking buffer (10% donkey serum, 0.02%

Triton X-100 in PBS), for ~6 hr (over day) at RT. Primary antibodies (for antibody concentrations, refer to the table 2.16) were incubated overnight at 4° C on a shaker. Primary antibodies were removed by washing with PBS, three times (10 minutes each wash). Secondary antibodies conjugated with either Alexa 488, Alexa 568 or Alexa 647 (Invitrogen) were diluted in the blocking buffer (1:600), and slices were incubated for at least 3h at room temperature. Slices were washed three times in PBS (10 minutes each wash) and mounted in Fluoromount-G (SouthernBiotech).

2.7. Confocal microscopy

Confocal images were acquired using an Olympus FV1000 confocal microscope (60× 1.35 NA oil objective). Stacks of 8 equidistant planes (~0.2 um) of 76 x 76 nm/pixel were taken for each field. An average of four fields was taken per brain slice, 3 to 4 slices were analysed per animal or treatment, per staining. Analysis of the maximum projection of the pictures was performed using Volocity software (Perkin Elmer). For each experiment, threshold values were optimised for each channel separately (488, 568 and 647) based on control conditions. Once the threshold was chosen, the same threshold was applied in all the images from all conditions of that given experiment. In addition, puncta volume was also optimised and filtered so objects that were too small or too large would not be considered as synaptic puncta – considered only those 0.1 μm^3 < object < 5 μm^3, with an mean punctum size of ~0.25 μm^3 for vGlut1 and ~0.30 μm^3 for PSD95. For the co-localisation of synaptic puncta (quantification of the number of synapses), after the above described analysis, thresholding of two channels were combined so only those puncta touching each other, within 1 pixel distance, (i.e.: a 488 labelled puncta touching a 568 labelled puncta) were considered as synapses. Punctum density was normalized to volume of the imaged field. This methodology was previously used and described in Purro et al., 2012 and Galli et al., 2014.

2.8. Doublecortin staining

Mice were anesthetized with a combination of fentanyl-fluanisone and benzodiazepine, Hypnorm/Hypnovel respectively - delivered intraperitonially, and perfused with 4% paraformaldehyde (PFA) in PBS. Brains were post-fixed 4 hr at room temperature (RT) or ON at 4 °C. Following fixation, brain were immersed in 30% sucrose/PBS and then frozen in cold isopentane (immersed in dry ice). Serial sagittal sections (20um thick) slices were cut with a microtome and immediately collected onto slides (Superfrost Plus–VWR). Slides were blocked in 10% donkey serum, 0.02% Triton X-100 in PBS for ~4 hr at RT and then incubated with antibody against Dcx (1:200; Santa Cruz) overnight at 4 °C in the same buffer and counterstained with DAPI. Secondary antibody conjugated with Alexa 568 (Invitrogen, 1:600) was used. Single plane images were captured on an Olympus BX60 microscope with a 20× 1.3 NA objective. Images were analysed with counting of positive cells was performed as previously described by (Licht et al., 2011).

2.9. Propidium Iodide Assay

Control and ind-Dkk1 mice were sacrificed and brains were rapidly dissected and placed into ice-cold artificial ACSF. Sagittal slices (300 μm) were cut at 4 °C with a vibrotome (Campden Instruments), recovered for at least 30 min, followed by a 4 hours incubation with Propidium Iodide (PI) (5μg/ml) at 37ºC (5% CO_2/95% O_2). After incubation, slices were fixed in 4% PFA/4% sucrose in PBS for 20-30 min at RT. PFA was removed by washing with PBS. Positive controls were obtained by submerging brain slices of adult wildtype animals in cold aCSF containing PI (5μg/ml), and incubating them for 24h at 4ºC (Frantseva et al., 1999). Slices were washed in PBS and mounted in Fluoromount-G (SouthernBiotech).

2.10. Haematoxylin and Eosin Staining

Brain sections of ind-Dkk1 animals and control littermates were cut with a microtome at 10µm thickness, and immediately collected onto slides (Superfrost Plus–VWR). Slides were then stained with Mayer's Haematoxylin for 2 minutes before washing with acid alcohol and then water. Afterwards slides were left in Eosin for 2 min and then washed twice with 100% IMS. The stained slides were then soaked in xylene and then mounted with DPX. Single plane images were captured on an Olympus BX60 widefield microscope with a 20× 1.3 NA oil objective.

2.11. Golgi staining and spine quantification

Brains from control and ind-Dkk1 mice were quickly dissected, rinsed in phosphate buffer and immersed in Golgi solution (FD Rapid GolgiStain Kit, FD Neuro Technologies) for 7 days. Golgi impregnation solution was replaced after 24h, and then each other day, at room temperature and away from the light. Following impregnation, brains were transferred to 'Solution C' of the Kit, for a further 48 hours. The specimens were then frozen in cold isopentane and cut on a Leica CM3050 cryostat. Serial sagittal sections of 100 µm thickness were collected on slides (Superfrost Plus–VWR), pre-coated with 'Solution C' and left to dry in the dark at RT for at least 48 h. Staining was done as suggested by manufacturer. Slides were then dehydrated in graded ethanol (50%, 75%, 90% and 100%), cleared with Xylene, and mounted with Permount (TAAB Laboratories). Samples were imaged on an Olympus BX60 widefield microscope with a 100× 1.3 NA oil objective. Images were analysed with Volocity software. Spines, defined as dendritic protrusions with a visible head, were measured to their width, counted manually and normalized to dendritic length. Approximately 50 dendrite stretches were counted per animal.

2.12. Electron microscopy

Brains of ind-Dkk1 and control mice were dissected and immersed fixed with 4% PFA, 0.5% Glutaraldehyde in 0.1M Milloning's Phosphate Buffer, pH 7.4 overnight at 4°C. Samples were rinsed in Milloning's Phosphate Buffer and sectioned coronally (200 µm) with a vibrotome. Slices were then post fixed in 1% OsO_4 in Cacodylate buffer for 1h, stained in aqueous Uranyl Acetate for 45 minutes, dehydrated in graded alcohol and embedded in resin. Ultra-thin sections (70nm) were cut and collected on 200 mesh-grid. Photographs were taken at 40,000X, with a JEOL 1010 microscope. Previous to images acquisition, sections were thoroughly scanned for initial check of quality control of the samples (good quality fixation, no holes in the tissue, intact mitochondria structures and membranes). Approximately 40 fields per animal of the CA1 hippocampus were imaged. Asymmetric synapses were considered as those with a clear pre- and postsynaptic membrane, with the presence of a prominent PSD and vesicles in the presynaptic terminal (Bourne & Harris, 2008; Schikorski & Stevens, 1997). Total number of synaptic vesicles was considered as those located up to 200nm of the active zone membrane, where the majority of synaptic vesicles are located (Schikorski & Stevens, 2001; Tao-Cheng, 2006). Number of vesicles was normalized to the PSD length. PSD length was chosen for normalisation as its borders can be identified with accuracy. Statistical analyses were performed using Origin-Pro 9.0 software (OriginLab Data Analysis and Graphing Software).

2.13. Western Blot

Brains from control and ind-Dkk1 mice were removed, quickly washed in ice-cold phosphate buffer solution (PBS). Hippocampi were dissected and immediately immersed in 300µl of RIPA buffer, containing proteases and phosphatase inhibitors (Sigma). Tissue was then triturated using a 25G syringe and centrifuged for 10 minutes at maximum speed (approximately 13,000 rotations per minute) in a bench top pre-cooled centrifuge.

Total levels of protein were determined by Lowry assay. 30µg of protein were run on a SDS gel (8% acrylamide) for approximately 1 hour at 120V. The gel was then transferred to a nitrocellulose membrane (Amersham), overnight at 350mAmp at 4°C. The membrane was rinsed in dH_2O and blocked with 5% milk powder (Marvel) in TBST overday (approximately 6 hours) at room temperature with gentle shaking. Primary antibodies were diluted in 5% milk powder in TBST, and applied overnight at 4C. The membrane was then washed three times in TBST (10 minutes per wash) and secondary antibody, dilute in 5% milk in TBST, was applied for at least 2 hours. The membrane was then washed three times in TBST (10 minutes per wash), and protein levels were detected using chemiluminescence solution (ECL – Amersham) following the manufactures instructions.

Levels of protein were determined by measuring the intensity of each individual band using ImageJ software (http://rsbweb.nih.gov/ij).

2.14. Behavioural paradigms

Previous to all behaviour paradigms, adult animals (3 – 6 moths of age) were handled daily for approximately 2 min. for at least four days before the beginning of the test. Weighting was monitored and annotated periodically. All the experiments were performed and analysed blind to the experimenter.

Open Field

The apparatus consisted of a 45 x 45 x 45 cm wooden box open at the top. Animals were fed for 14d with doxycycline before tested. At the start of the test, the mouse was placed in the centre of the arena, in a position equidistant to the walls, and allowed to explore the field for 300 seconds. The open field was cleaned with 70% ethanol in a damp cloth between trials. The mouse was tracked using a video camera fixed to the ceiling of the room and connected to a digital video tracking system (HVS Image Ltd.). Distance travelled and time spent in the central and peripheral areas were computed by the system.

Plus-maze

Levels of anxiety-like behaviour were evaluated using the elevated-plus maze. Animals were fed for 14d with doxycycline before the test. The apparatus, made of wood laminated with white Formica, consisted of four arms 30 x 5 cm, being two of them surrounded by a 15cm height walls, elevated 40cm above the floor. At the beginning of the test, animals were put in the central area of the maze (neutral area). Tests of 300 sec duration were performed after 14 days of doxycycline feeding. The maze was cleaned with 70% ethanol in a damp cloth between trials. As described above, a digital video tracking system (HVS Image Ltd.) was used to analyse their behaviour.

Novel Object Recognition Task (NOR)

NOR was a modification of a previously described protocol (Mansuy et al., 1998). In brief: before the beginning of the experiment, animals were placed in a neutral arena and allowed to explore it for 10 min. After the initial exploration animals were removed from the arena, which was then cleaned with 70% ethanol and two objects were placed inside, in diagonal position, equidistant to the walls. Mice were allowed to freely explore and habituate to the two identical objects for 10 min. (two plastic bottles, ~15cm height). Following two different retention intervals (24 hr or 6 days after the habituation day), mice were placed back into the environment with two objects in the same locations - one of the familiar objects was replaced with the second novel object being different in shape colour and texture. The mice were then again allowed to explore freely both objects for 10 min. The objects were thoroughly cleaned before each experiment to avoid any odour recognition. A mouse was considered to be exploring the object when its head was facing the object at a distance of 2 centimetres or less or when part of its body except the tail was touching the object. Animals were individually observed through a ceiling mounted camera. Preference index (PI) was calculated based on time the animal spent

exploring each of the objects, (PI= (Time Novel Obj.)/ (Time Familiar + Time Novel Objects).

T-Maze Spontaneous Alternation

For the spontaneous alternation, the test was carried out in an enclosed t-maze shape, each arm measuring 27 x 7 x 10 cm, made of wood laminated with white Formica. A partition extended 7 cm from the back of the T into the start arm. A set of three guillotine doors was used to separate the entrance of each arm. At the beginning of the test all the guillotine doors were raised, except to the one located at the end on the starting arm. A mouse was place in the partition at the end of the starting arm and guillotine door was raised. After it entered a goal arm, guillotine was closed and the animals was confined there for 30 sec, before being returned to the start partition, were it was hold for 30 sec and all guillotine doors raised again. The alternation criterion was for the mouse to enter the opposite arm as the previous choice, for instance if on trial one the animals entered the right arm, on trial two it would be expected to enter the left arm of the maze. Each mouse received eight consecutive trials and results were expressed as number of correct alternations over the total number of possible correct alternations.

Morris Water Maze

Animals were fed for 5 days before the beginning of the task, and continuously fed throughout the test. The test was performed in a circular 120cm diameter pool as previously described (Malleret et al., 2001) with a few modifications. Briefly, the paradigm consisted of two training phases: 4 days with a visible platform followed by 5 days (acquisition phase) with a hidden platform in the training quadrant. For each training phase a four trials/day training protocol with 10 min inter-trial intervals and maximum trial duration of 90 sec was used. In the first phase, mice were trained to a pseudo-randomly located platform with a local cue (flag) protruding out of the water surface. At the visible platform stage, curtains were drawn around the pool to occlude any distal queues present in the room. During the second training phase,

curtains were open and mice were trained to a hidden platform with the extra-maze cues visible around the room. Probe trials - platform removed from the pool - of 90sec of duration were performed before the fourth day of training and after 24h of the last day of training. All trials were videotaped and images were analysed using the dacqTrack-WaterMaze tracking system (Axona Ltd.). Location of the platform and starting position were followed according to Vohrees & Williams, 2006.

Contextual Fear Conditioning

Conditioning took place in a conditioning chamber (Med Associates Inc.) situated in a soundproof box equipped with a video camera fixed inside the door of the apparatus. The conditioning chamber floor was made up of stainless steel grid used for shock delivery and a speaker mounted on one side of the wall for delivery of the tone. Protocol was followed as previously described (Irvine et al., 2011). In brief, prior to training the chamber was cleaned with 70% ethanol. On the conditioning day, mice were brought from the housing room into a holding room where they were allowed to acclimatize. Mice were then placed individually in the conditioning chamber and after a 120-sec introductory period a tone (80 dB, 3.0 kHz) was presented for 30 sec, in which the last 2 sec coincided with a footshock (0.7 mA). Contextual fear memory was tested 24 h after training by re-exposing to the conditioning chamber for 5 min, with no shock delivery. Freezing behaviour (defined as complete lack of movement, except for respiration) was scored for 2 sec in every 5 seconds.

2.15. Statistics

For all set of samples, normality and homogeneity of variance were confirmed by Lilliefords and Chi-square tests, respectively. If samples showed a normal distribution and homogeneity of variance, parametric tests were applied as described below. Otherwise, samples were analysed by a non-parametric test. Statistical analyses were performed using OriginLab software (Origin Group Corp.).

*S*ynaptic puncta and colocalization of synaptic markers was analyzed by 1-way ANOVA with blocking and replication. The number of experiments was considered as blocks; typically 3 independent experiments each of them with 3 mice from each condition. A minimum of 12 images were obtained from 3 different slices per mice.

The number of asymmetric synapses was analyzed in EM micrographs by non-parametric Kruskal-Wallis ANOVA. 30-40 images from each mouse, 3 mice for each condition were analyzed. For the analysis of spine size, data was pooled and analyzed by non-parametric Kruskal-Wallis ANOVA. More than 250 synapses obtained from 3 different mice per condition were analyzed.
Number of spines in Golgi stained images was analyzed by non-parametric Kruskal-Wallis ANOVA. 10 dendrite stretches obtained from 5 different cells from 4 different mice per condition were analyzed.

Elevate plus maze and open field were evaluated by Student's t-test, 15 control animals and 11 ind-Dkk1 mice were used. Object recognition test was evaluated by ANOVA, 16 control animals and 12 ind-Dkk1 mice were used. T-maze was analysed by unpaired Student's *t*-test, 15 control animals and 10 ind-Dkk1 animals were used. Morris water maze was evaluated by ANOVA for repeated measures, 11 control and 12 ind-Dkk1 mice were used. Contextual fear conditioning and Morris water maze were evaluated by ANOVA repeated measures. A total of 8 control animals and 7 ind-Dkk1 animals were used in the fear condition task and 11 control animals and 12 ind-Dkk1 animals were tested in the MWM.

Most of statistical analyses were performed with the help and guidance of Dr. Soledad Galli, a research associate in the lab.

2.16. Antibodies

Antibody	Host	Maker	Concentration		Catalogue Number
			WB*	IHC**	
Cleaved Caspase-3	rabbit	Cell signalling	1 in 1000		9661
Dcx	goat	Santa Cruz		1 in 200	sc-8066
Gephyrin	mouse	Synaptic Systems		1 in 5000	147011
NeuN	rabbit	Millipore		1 in 500	abn78
PSD95	rabbit	Thermo Scientific		1 in 500	MA1-046
PSD95	mouse	AbCam		1 in 500	ab18258
Total Caspase-3	rabbit	Cell Signalling	1 in 1000		14214
Tubulin (DM1A)	mouse	Millipore	1 in 2000		05-829
vGAT	g.p†	Synaptic Systems			131004
vGlut1	g.p. †	AbCam		1 in 2000	ab5905

* Western Blot; ** Immunohistochemistry; *** Immunocytochemistry, † guinea pig.

2.18. Buffers and Solutions

(Columns on the left state final concentration)

Phosphate Buffer Solution (1 litre of 10x PBS)

1.4M NaCl	80 g
26mM KCl	2 g
15mM KH_2HPO_4	2 g
14mM Na_2HPO_4	2 g

(pH the 1x solution to 7.4)

4% Paraformaldehyde (50ml)*

1.3M paraformaldehyde (Sigma)	2 g
4% Sucrose (Sigma)	2 g
0.1mM NaOH (BDH)	200 µl

1x PBS solution	50 ml
(pH the solution to 7.4)	

* PFA was dissolved in a hot water bath (55°C) and stored at 4°C.

2.18.1. In situ Hybridization Solutions:

20x SSC (1L)

3M NaCl	175.3 g
0.3M Sodium Citrate	88.2 g
1L d-H20 treated with DEPC	
pH adjusted to 7.0	

Buffer IS1 (0.2 L)

100mM Tris	2.42 g
250mM NaCl	2.92 g
200ml d-H20 treated with DEPC	
pH adjusted to 7.5	

Buffer IS2 (0.2 L)

100mM Tris	2.42 g
100mM NaCl	1.17 g
5mM $MgCl_2$	0.095 g
200ml d-H20 treated with DEPC	
pH adjusted to 9.5	

Colour Solution

34ul of 100mg/ml NBT (70% DMF)

35ul of 50mg/ml BCIP (100% DMF)

2.4mg of Levamisole

dissolved in 10ml of Buffer IS2

2.18.2. Solution for EM fixation

Milloning's buffer (1L of 1M solution)

1M NaCl (Sigma)	58.4 g
1L dH_2O	
(pH the solution to 7.4)	

EM fixative buffer (50ml)

1.3M paraformaldehyde (Sigma)	2 g
0.5% Glutaraldehyde	250 µl
0.1M Millonig's buffer	50 ml
(pH the solution to 7.4)	

2.18.3. Western Blot Solutions

Tris Buffered Solution (TBST)

1M Tris (ph7.5)	10 ml
5M NaCl	30 mg
Tween (Sigma)	1 ml
dH_20 added up to 1L	

Tris/Glycine Solution (1L of 10x Solution)

Tris (Sigma)	30.3 g
2mM Glycine (Sigma)	144 g
dH_20 added up to 1L	

Running buffer

Tris/Glycine Solution	100 ml
0.35mM Sodium Dodecyl Sulphate (SDS)	100 mg
dH_20 added up to 1L	

Transfer Buffer

Tris/Glycine Solution	100 ml

0.35mM Sodium Dodecyl Sulphate (SDS)	100 mg
Methanol	200 ml
dH_20 added up to 1L	

Chapter 3

3. The importance of Wnt canonical pathway for the maintenance of excitatory synapses and insights to the mechanism of synaptic disassembly

3.1. Introduction

Synapse turnover is essential for brain functions such as perception, cognition and memory (Bhatt et al., 2009; Bourne & Harris, 2008; Henriquez & Salinas, 2012; Meyer et al., 2003). Whilst the molecular mechanisms of synapse formation have been extensively studied (Budnik & Salinas, 2011; Inestrosa & Arenas, 2010; Salinas, 2012; Shen & Cowan, 2010), very little is known about the mechanisms responsible for the maintenance and the disassembly of synapses in the adult brain (Allyson et al., 2012). Therefore, the identification and characterisation of molecules that regulate the maintenance machinery is crucial for understanding synaptic integrity.

Recently, more attention has been given to secreted factors, such as BDNF, and their role in synaptic maintenance (Vigers et al., 2012). Together with neurotrophins, Wnt proteins are one of the main candidates of synaptic integrity, as it has been demonstrated their continuous expression in adulthood (Gogolla et al., 2009; Salinas et al., 1994; Shimogori et al., 2004; Tabatadze et al., 2012), and more importantly due to increasing evidence of a link between dysfunction of Wnt signalling pathway and Alzheimer's disease (AD) (Boonen et al., 2009; Inestrosa & Arenas, 2010; Killick et al., 2012; Kremer et al., 2011; Purro et al., 2012).

Wnts have been linked with neurodegenerative diseases. One of the first evidence comes from a genome-wide association study which showed a link between a variant of the Wnt co-receptor LRP6 with late onset of this disease (De Ferrari et al., 2007). In addition, Dickkopf 3 (Dkk3), an endogenous secreted Wnt antagonist, is elevated in plasma and cerebrospinal fluid (CSF) of AD patients, suggesting a dysregulation of Wnt signalling in AD (Zenzmaier et al., 2009). More importantly, studies have shown that the Wnt antagonist Dkk1 is elevated in post-mortem brain samples from AD

patients and AD animal models (Caricasole et al., 2004; Rosi et al., 2010). In agreement with the latter, β-amyloid (Aβ) oligomers, the main component of amyloid plaques in the brains of AD patients, significantly increased the expression of Dkk1 (Caricasole et al., 2004; Killick et al., 2012; Purro et al., 2012), which ultimately leads to synaptic loss (Purro et al., 2012). Indeed, our lab has recently demonstrated that Dkk1 rapidly reduces the number of synapses in mature hippocampal cultures, suggesting that endogenous Wnts are required for the maintenance of synapses. Importantly, blockade with an anti-Dkk1 neutralising antibody rescued the loss of synapses induced by Aβ (Purro et al., 2012). These results highlight the importance of Wnt signalling for synaptic maintenance and indicate that dysregulation of Wnt signalling can lead to the synaptic loss observed in neurodegenerative diseases. Furthermore, these studies also suggest that Aβ-induced synaptic loss is Dkk1-mediated.

It is well established that Wnt secreted proteins play a critical role in the CNS, and their pathways have been extensively studied. Wnts through their receptor can activate three different signalling cascades (Gordon & Nusse, 2006; McNeill & Woodgett, 2010). The most characterised Wnt pathway is the canonical or ß- catenin pathway. Binding of Wnt protein to the Frizzled receptor and the recruitment of lipoprotein receptor related-protein class 5 and 6 (LPR5/6) activates this pathway through the scaffolding protein Dishevelled (Dvl) resulting in the inhibition of Glycogen Synthase Kinase-3 (Gsk-3β), a serine/threonine kinase (Kremer et al., 2011). Inhibition of Gsk3β results in the cytoplasmic accumulation of ß-catenin and its subsequent translocation to the nucleus; where in association with TCF/LEF activates the transcription of target genes (Archbold et al., 2012). Previous studies from our lab and others revealed that this pathway, via Gsk3β, is involved in the regulation of presynaptic assembly during development (Ahmad-Annuar et al., 2006; E. K. Davis et al., 2008; Hall et al., 2000). Wnt signalling is subject to negative and positive regulation by a range of intra- and extracellular effectors that act by either modulating components of the signal transduction machinery, or by interfering with the ligand receptor interactions. These Wnt modulators play an important role

during development and in the adult homeostasis (Cruciat & Niehrs, 2013; Niehrs, 2006).

Several secreted proteins antagonise Wnts to restrict their function in a time and spatial manner. Dkk1 is a secreted protein that antagonizes the canonical Wnt signalling pathway by binding to the LPR5/6 co-receptor (Mao et al., 2001), required for canonical Wnt signalling. Overall, when present, Dkk1 prevents the formation of the ternary complex Wnt- Frizzled Receptor (Fz) and LRP5/6 (Bourhis et al., 2010; Niehrs, 2006). In addition, Dkk1 also binds to the transmembrane protein receptor Kremen, resulting in the rapid internalization, via endocytosis, of LRP5/6 and the subsequent suppression of Wnt signalling (Mao & Niehrs, 2003; Mao et al., 2002). In this scenario, Gsk3β phosphorylates β-catenin, which then is ubiquitinated and labelled for degradation via proteasome destruction (MacDonald et al., 2009; Willert & Nusse, 1998). Dkk1 is notably important during development especially during embryonic patterning along the antero-posterior axis. Indeed, loss of function of Dkk1 leads to embryonic lethality, loss of head structures and fused vertebras (Mukhopadhyay et al., 2001). Interestingly, in the adult brain Dkk1 expression is almost undetectable (Cappuccio et al., 2005; Purro et al., 2012). However, a very recent study suggested that very low levels of Dkk1 can be found in the hippocampal stem cell niche (Seib et al., 2013).

Based on previous observations from our lab, which suggests that endogenous Wnts are required for the maintenance of synapses (Purro et al., 2012), here I further examine the role of Wnt signalling in synaptic maintenance using the antagonist Dkk1. I found that short-term inhibition of endogenous Wnts in an *ex vivo* model results in the disassembly of excitatory synapses in the CA1 area of the hippocampus, with no effect in inhibitory synapses. Importantly, this effect is prevented by a potent and specific Gsk3 inhibitor Bio, suggesting that canonical Wnt signaling is compromised by Dkk1. These findings demonstrate that Dkk1 can be used as a powerful tool to block and study the role of Wnt canonical signalling in synaptic maintenance. Furthermore, my findings demonstrate that exposure to Dkk1, and blockade of Wnt canonical pathway, leads to the loss of synapses, that ultimately

can be prevented by activation of downstream molecular components of the Wnt pathway. In addition, in order to have an insight to the molecular mechanisms downstream of Dkk1, I used different pharmacological compounds to inhibit downstream targets to the Dkk1 pathway. My data suggests that synaptic disassembly promoted by blockade of Wnt signalling is dependent on proteasome degradation, as inhibition of the latter can override the effect of Dkk1 treatment. These data provide important insight to the molecular mechanism of Dkk1-mediated synaptic disassembly.

3.2. Results

3.2.1. Modulation of excitatory synaptic maintenance by Wnt canonical signalling

Our previous studies have demonstrated that blockade of Wnt signalling with Dkk1 induces the disassembly of synapses in cultured hippocampal neurons (Purro et al., 2012). To determine whether Wnt signalling is involved in the maintenance of excitatory synapses, in an *ex vivo* system, I examined the effect of the Wnt antagonist Dkk1 in mouse hippocampal slices followed by immunohistochemistry examination. The colocalisation of vGlut1 and PSD95, a pre and postsynaptic markers respectively, were used to quantify the number of synapses. In these experiments, brain slices of young (p15) animals were treated for 4 hours with the Wnt antagonist Dkk1 (50 ng/mL) or vehicle (0.1% BSA) before fixation with 4% PFA/ 4% Sucrose, and processing for immunofluorescence microscopy. These pre-optimised conditions were chosen, as previous findings from our lab have demonstrated that lower concentrations of Dkk1 (below 50ng/mL) and shorter exposure to Dkk1 (less than 4 hours) have no effect on brain slices (Galli & Salinas, *unpublished data*). In addition, these conditions closely replicate our previous observations in which Dkk1 induce the disassembly of synaptic markers in cultured neurons (Purro et al., 2012).

Results demonstrate that excitatory presynaptic marker vGlut1 was significantly reduced after treatment with Dkk1 (Fig 3.1a) in the stratum radiatum CA1 area of

the hippocampus. Quantification shows a reduction in the volume of vGlut1 puncta as well as a decrease of ~40% (Fig 3.1b) in the number of presynaptic sites labelled with vGlut1 (Fig 3.1b). In contrast, no changes were observed in the volume or number of postsynaptic sites labelled with PSD95 (Fig 3.1c). However, when the number of vGlut1 apposed to PSD95 was quantified, a significant loss (almost 50%) of excitatory synapses was observed after 4h treatment with Dkk1. Importantly, our laboratory have strong evidence showing that during the period of 4h after brain slicing, there is no increase in the total number of synapses (Lopes & Salinas, *unpublished data*), demonstrating that ultimately Dkk1 treatment leads to the loss of already existing synapses and not the blockade of synaptogenesis. These results indicate that blockade of endogenous Wnts with Dkk1 induce the disassembly of excitatory synapses, demonstrating the role of Wnts in synaptic maintenance.

3.2.2. Acute blockade of Wnt canonical signalling with Dkk1 has no effect on inhibitory synapses

The role of Wnts in central inhibitory synapses has started to be explored. Previous studies from our lab have shown that Wnt7a has no synaptogenic effect on inhibitory synapses (Ciani et al., 2011). Interestingly, a research study suggested that exposure of hippocampal neurons to Wnt5a induces the clustering of GABAergic receptors in mature hippocampal neurons - an effect mediated by a non canonical Wnt pathway (Cuitino et al., 2010). Although some studies started to investigate the role of Wnt in the formation and/or maturation of inhibitory synapses, the role of Wnt pathway in the maintenance of inhibitory synaptic remains elusive.

To test whether blockade of Wnt canonical pathway leads to any change in already existing inhibitory synapses in the hippocampus, I tested the effect of Dkk1 in hippocampal brain slices followed by immunofluorescence microscopy. Antibodies against vGAT and gephyrin, pre- and postsynaptic inhibitory markers respectively, were used. As previously described, for these experiments, brain slices of young (p15) animals were treated for 4 hours with the Wnt antagonist Dkk1 (50 ng/mL), or vehicle (0.1% BSA) before fixation and analysis.

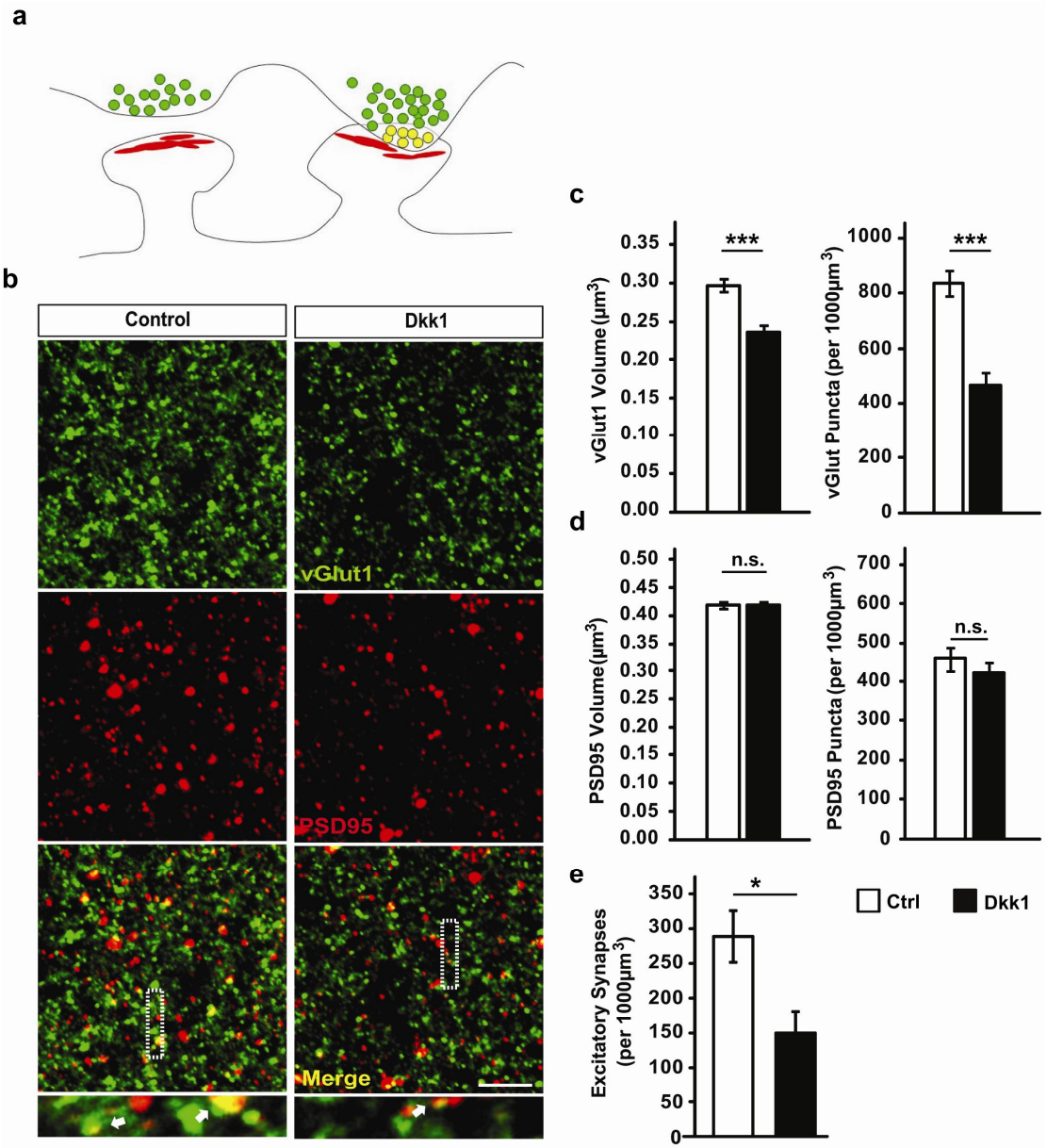

Fig 3.1 – Dkk1 induces the disassembly of excitatory synapses in brain slices. (a) Schematic representation of synapses under the microscope. The presynaptic site is labelled with vGlut1 (green), whereas the postsynaptic site is labelled with PSD-95 (red). When these two markers are opposed to each other, the synapse appears labelled in yellow. **(b)** Representative confocal images of excitatory synapses are shown in the stratum radiatum of the CA1 area of the hippocampus. Acute hippocampal slices were treated with vehicle (control) or Dkk1 (50ng/ml) for 4 hours. Excitatory synapses (white arrows) were defined by the apposition of vGlut1 (green) and PSD95 (red). **(c)** Quantification shows that Dkk1 decreases the volume and number of vGlut1 labelled puncta. **(d)** By contrast, the sites labelled by the postsynaptic marker PSD95, remains unchanged by Dkk1 **(e)** Quantification shows that Dkk1 decreases the total number of excitatory synapses - Dkk1 alone reduces the number of excitatory synapses by almost 50%. Scale bar 5µm. *p< 0.05; **p< 0.005; Student's t-test. Results are shown as mean ± SEM. n= 3 experiments; 3-4 slices per condition/experiment.

My results demonstrate that acute blockade of Wnts with Dkk1, at this particular concentration and length of time, does not have any effect on inhibitory synapses in the CA1 hippocampus area (Fig 3.2a). Quantification shows no changes in the volume or number of sites labelled with vGAT (Fig 3.2b), demonstrating no effect of

Wnt blockade in the presynaptic site. Equally, the puncta volume and puncta density of inhibitory postsynaptic labelled with Gephyrin remained the same as control levels (Fig 3.2c), after the treatment with Dkk1 treatment. Quantification demonstrates that the overall number of inhibitory synaptic sites (Fig 3.2d), shown by the number of vGAT in apposition to Gephyrin, is unchanged after the exposure to Dkk1. These results demonstrate that blockade of canonical Wnt pathway with Dkk1 has no effect on inhibitory synapses. Together my results show for the first time, that Dkk1 induces the specific disassembly of excitatory synapses, with no effect on inhibitory synapses - suggesting a role for Wnt pathway in the maintenance of certain types of synapses.

3.2.3. Dkk1-mediated synaptic disassembly is a result of Wnt canonical pathway blockade

Dkk1 is a strong Wnt antagonist that binds to the LRP5/6 co-receptor to block the canonical Wnt pathway (Glinka et al., 1998; Willert & Nusse, 1998). To determine whether the Dkk1–mediated loss of synapses is due to blockade of canonical Wnt signalling, I evaluated the effect of Dkk1 in the presence of the Gsk3β inhibitor Bio, which mimics canonical Wnt signalling (Meijer et al., 2003; Purro et al., 2008; Rosso et al., 2005). For these experiments brain slices were treated for 4 hours with the Wnt antagonist Dkk1 (50 ng/mL), Gsk3β inhibitor BIO (500nM), Dkk1 in combination with BIO, at the previously stated concentrations - or with vehicle (0.1% BSA or DMSO). This specific dosage of BIO was chosen based on some preliminary results from our lab which demonstrate that at this particular concentration, the Gsk3β inhibitor alone does not have a synaptogenic effect on p15 mouse brain slices (Lopes & Salinas, *unpublished results*).

The results reveal that activation of Wnt canonical pathway blocks the effect of Dkk1 on presynaptic site (Fig 3.3a). Quantification of volume and number of vGlut1 puncta in the CA1 stratum radiatum demonstrates treatment with Dkk1 in combination with the inhibition of Gsk3β prevent the disassembly of the presynaptic site (Fig 3.3b), as both parameters remain at the same level as control. The same treatment (Dkk1 +

BIO) has no effect in the postsynaptic site, as demonstrated by the unchanged number and volume of PSD95 puncta (Fig 3.3c) when compared to control. In addition, when total number of excitatory synapses was quantified, by the apposition of vGlut1 to PSD95, inhibition of Gsk3β totally override the effect of Dkk1, as demonstrated by the conserved number of synapses between control and Dkk1 + Bio treated (Fig 3.3d). Interestingly, Bio alone causes a slight increase in the number of postsynaptic site, without altering the other parameters such as vGlut1 volume and number, PSD95 volume and total number of excitatory synapses. Together these results demonstrate that Dkk1 induces the specific disassembly of excitatory synapses through inhibition of the Wnt canonical pathway.

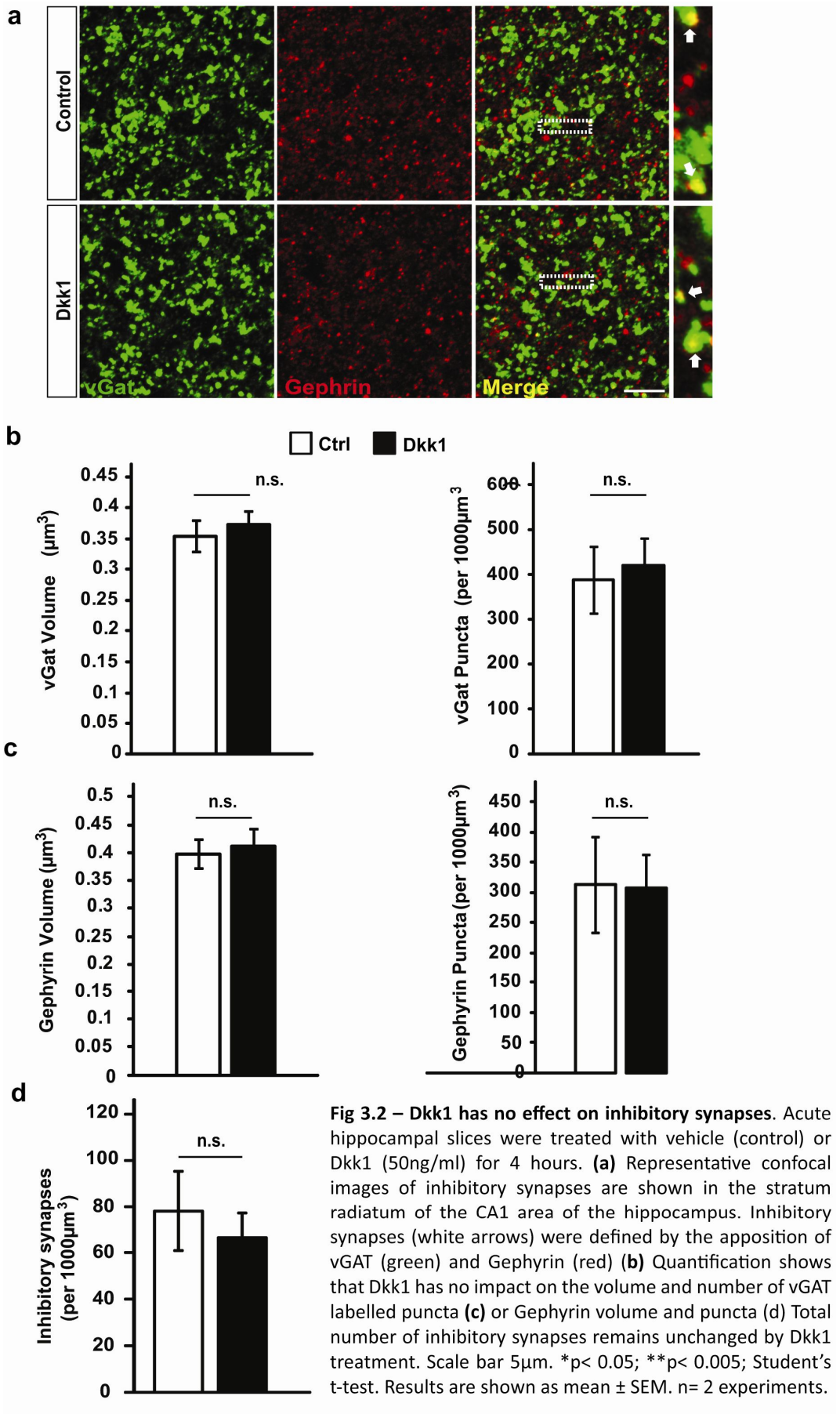

Fig 3.2 – Dkk1 has no effect on inhibitory synapses. Acute hippocampal slices were treated with vehicle (control) or Dkk1 (50ng/ml) for 4 hours. **(a)** Representative confocal images of inhibitory synapses are shown in the stratum radiatum of the CA1 area of the hippocampus. Inhibitory synapses (white arrows) were defined by the apposition of vGAT (green) and Gephyrin (red) (**b)** Quantification shows that Dkk1 has no impact on the volume and number of vGAT labelled puncta **(c)** or Gephyrin volume and puncta (d) Total number of inhibitory synapses remains unchanged by Dkk1 treatment. Scale bar 5µm. *p< 0.05; **p< 0.005; Student's t-test. Results are shown as mean ± SEM. n= 2 experiments.

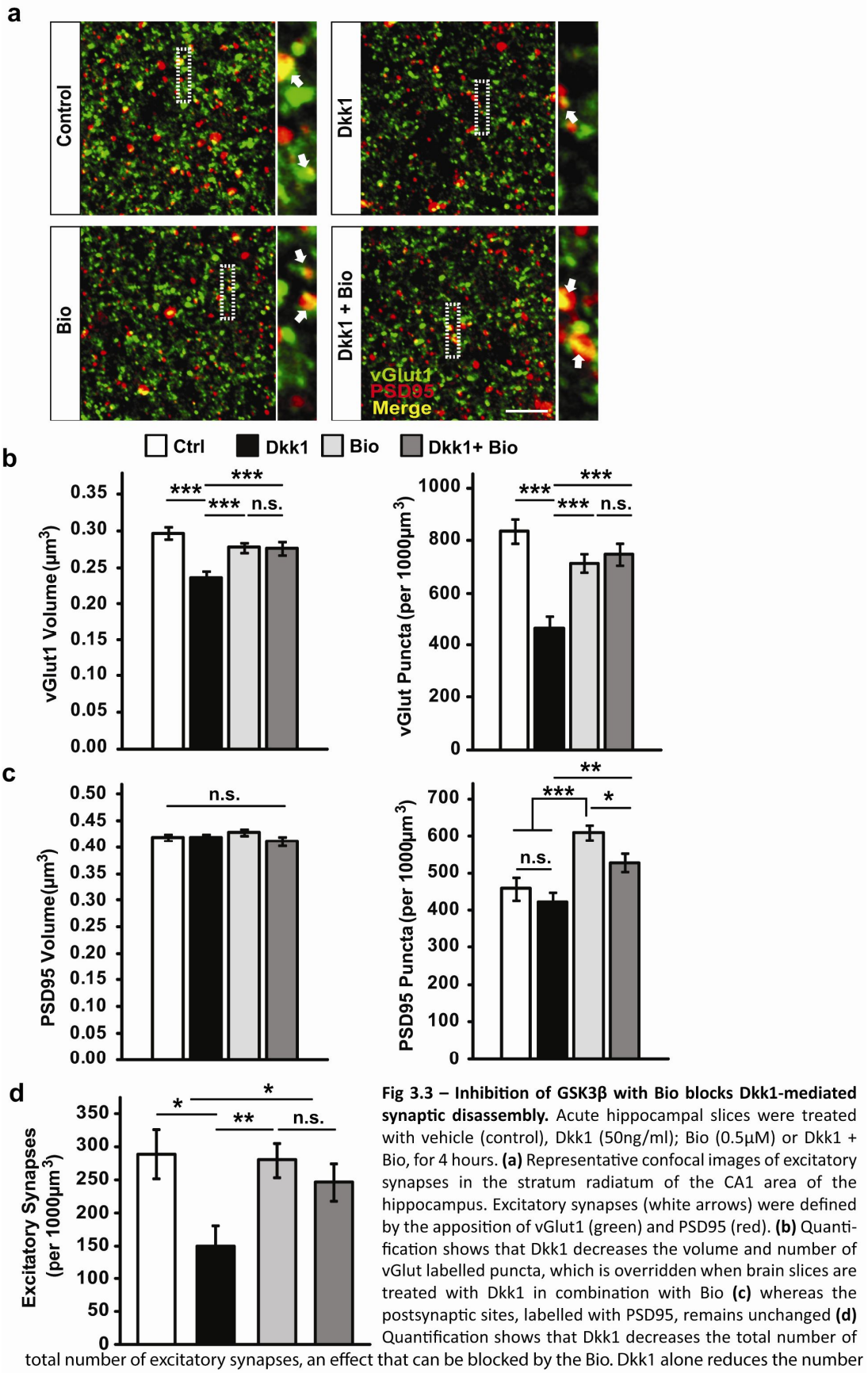

Fig 3.3 – Inhibition of GSK3β with Bio blocks Dkk1-mediated synaptic disassembly. Acute hippocampal slices were treated with vehicle (control), Dkk1 (50ng/ml); Bio (0.5µM) or Dkk1 + Bio, for 4 hours. **(a)** Representative confocal images of excitatory synapses in the stratum radiatum of the CA1 area of the hippocampus. Excitatory synapses (white arrows) were defined by the apposition of vGlut1 (green) and PSD95 (red). **(b)** Quantification shows that Dkk1 decreases the volume and number of vGlut labelled puncta, which is overridden when brain slices are treated with Dkk1 in combination with Bio **(c)** whereas the postsynaptic sites, labelled with PSD95, remains unchanged **(d)** Quantification shows that Dkk1 decreases the total number of total number of excitatory synapses, an effect that can be blocked by the Bio. Dkk1 alone reduces the number of excitatory synapses by almost 50%. Scale bar 5µm. *p< 0.05; **p< 0.005; ANOVA. Results are shown as mean ± SEM. n= 3 experiments.

3.2.4. Proteasome degradation is involved in Dkk1-induced synaptic disassembly

The ubiquitin proteasome system (UPS) has been described as active in central synapses and studies suggest its importance for synaptic structure and function (Bajic et al., 2012; Bingol & Sheng, 2011). Many studies show that the UPS regulates the size of the presynaptic pool of vesicles and synaptic protein expression during synaptic activity, transmission and plasticity (Bingol & Sheng, 2011; Jiang et al., 2010; Willeumier et al., 2006). Localised protein degradation via UPS has also been demonstrated during the process of synaptic elimination and regulation of spine density (Haas & Broadie, 2008; Hamilton & Zito, 2013). Importantly, studies suggest a link between the deregulation in the UPS and Lewi's bodies dementia and Parkinson's disease and also with Alzheimer's disease (Bedford et al., 2008; Haas & Broadie, 2008; McNaught et al., 2003). Given the evidence for UPS in synaptic homeostasis and transmission, and its well characterised role for Wnt canonical signalling, paying a crucial role in ubiquitinated β-catenin degradation, I examined whether by manipulating proteasome-mediated degradation of proteins I could rescue the detrimental effects of Dkk1 on excitatory synapses. To determine whether the loss of synapses is dependent on active UPS, I examined the effect of Dkk1 in the presence of the two widely used proteasome inhibitors, MG132 and Lactacystin. In these experiments, brain slices of young (p15) animals were treated for 4 hours with the Wnt antagonist Dkk1 (50 ng/mL), the proteolitic activity blocker of 26S subunit of UPS, MG132 (10μM) or Lactacystin (5μM), or vehicle (0.1% BSA or DMSO) before fixation with 4% PFA/ 4% Sucrose and processing for immunohistochemical analysis. Minimum MG132 and Lactacystin concentrations were selected based on preliminary data which demonstrate that at these concentrations, the UPS inhibitors used in my studies do not have any effect on synaptic number when used in isolation (evaluated by confocal microscopy), and yet had a effect overriding the detrimental effect of Dkk1 (Lopes & Salinas, *unpublished data*).

Results demonstrate that blockade of proteasome activity, and therefore ubiquitin target degradation, with MG132 reverses the effect of Dkk1 on excitatory synapses

(Fig 3.4a). Quantification of volume and number of vGlut1 puncta in the CA1 stratum radiatum demonstrates treatment with Dkk1 in combination with MG132 prevent the disassembly of the presynaptic site (Fig 3.4b), as both parameters remain at the same level as control. As previously shown, treatment with Dkk1 has no effect in the postsynaptic site, as demonstrated by the unchanged number and volume of PSD95 puncta (Fig 3.4c) when compared to control. Treatment with MG132 also has no effect on PSD95 (Fig 3.4c). Importantly, when total number of excitatory synapses was quantified - vGlut1 apposed to PSD95 - inhibition of UPS with MG132 totally blocks the effect of Dkk1, as demonstrated by the unchanged number of synapses between control and Dkk1 + MG132 treated (Fig 3.4d). These results suggest that Dkk1-mediated disassembly of synapses is dependent on proteasome degradation.

To further confirm the role of UPS in the process of synaptic disassembly mediated by Dkk1, I tested a second blocker of the UPS, Lactacystin. In accordance with previous experiments, brain slices of young (p15) animals were treated for 4 hours with the Wnt antagonist Dkk1 (50 ng/mL), Lactacystin (5µM) or vehicle (0.1% BSA or DMSO) before fixation with 4% PFA/ 4% Sucrose and processing for immunofluorescence microscopy. Analysis of the CA1 stratum radiatum demonstrates similar results to the previously used proteasome blocker (Fig 3.5a).

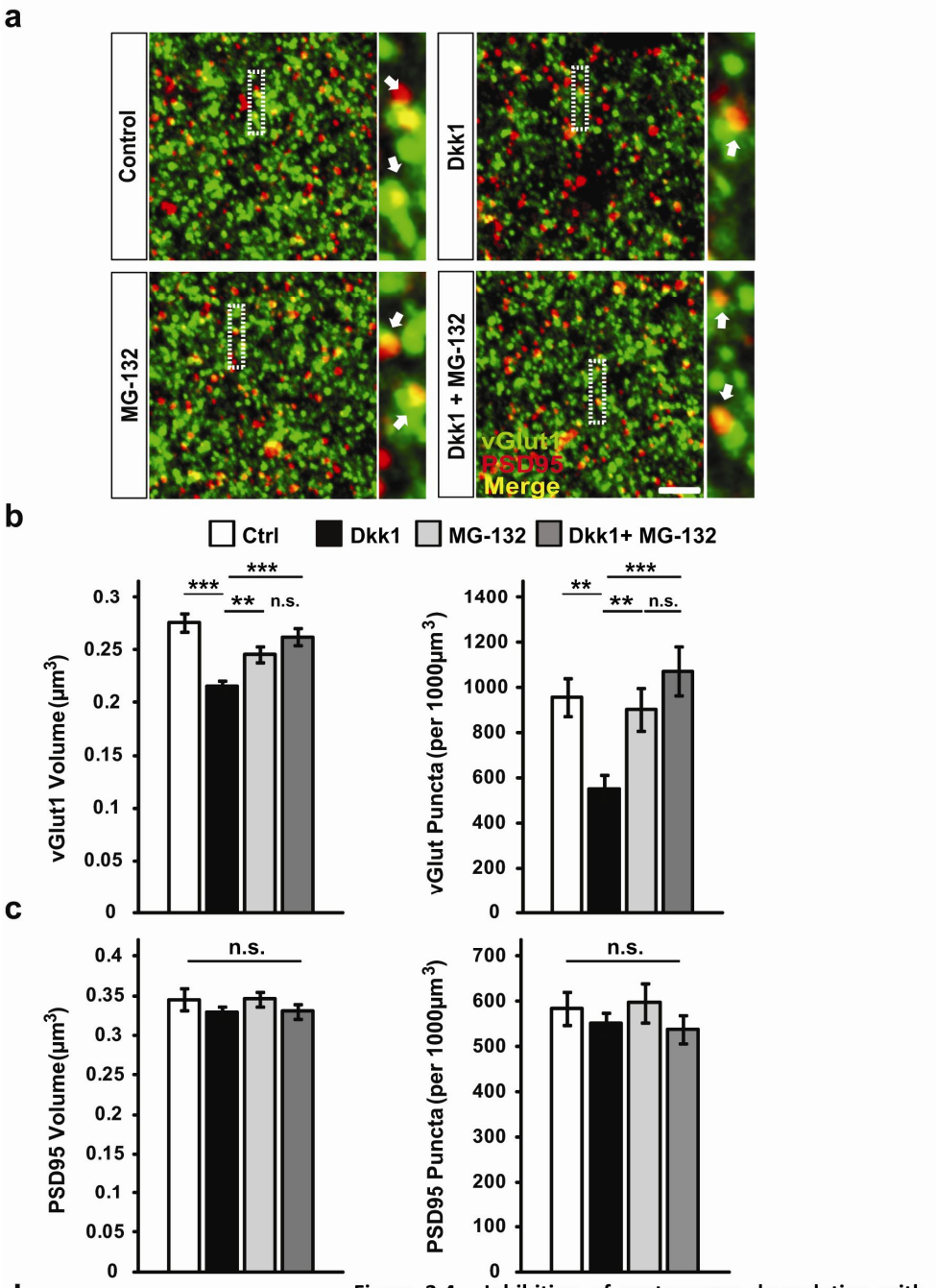

Figure 3.4 - Inhibition of proteasome degradation with MG-132 prevents synapse disassembly promoted by Dkk1. Acute hippocampal slices were treated with vehicle (control), Dkk1 (50ng/ml); MG-132 (10 µM) or Dkk1 + MG-132, for 4 hours. **(a)** Representative images of excitatory synapses (white arrows), defined by the apposition of vGlut1 (green) and PSD95 (red) are shown in the stratum radiatum of the CA1 area of the hippocampus. **(b)** Analysis demonstrates that Dkk1 decreases the volume and number of vGlut1 labelled puncta. This effect is overridden when MG-132 is present **(c)** Neither Dkk1 nor MG-132 treatment causes any changes at the postsynaptic (PSD95) **(d)** Quantification of total number of synapses shows that inhibition of proteasome degradation with MG-132 prevents the synaptic loss induced by Dkk1. Scale bar 2µm. *p< 0.05; **p< 0.005; ANOVA. Results are shown as mean ± SEM. n= 3 experiments.

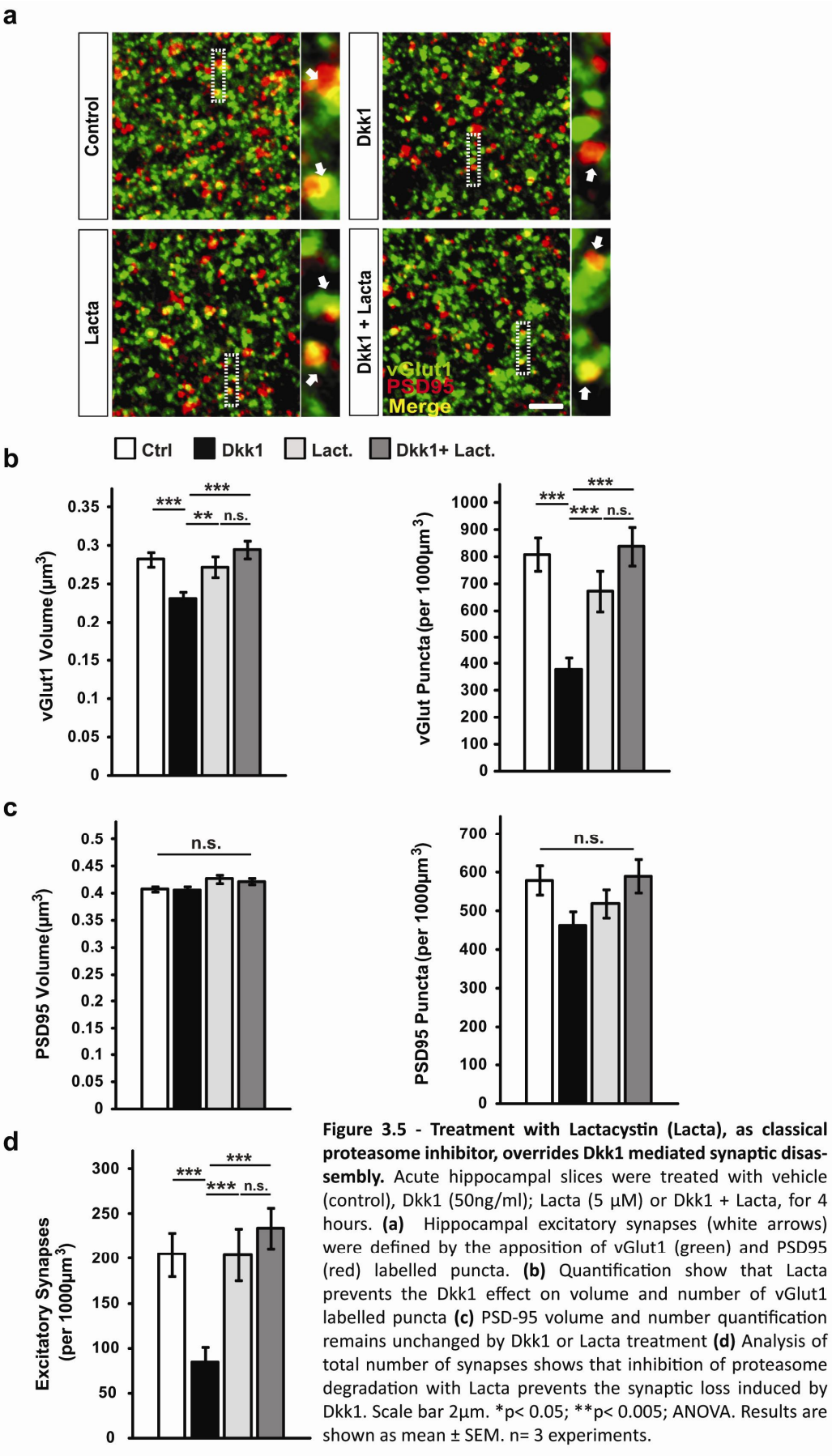

Figure 3.5 - Treatment with Lactacystin (Lacta), as classical proteasome inhibitor, overrides Dkk1 mediated synaptic disassembly. Acute hippocampal slices were treated with vehicle (control), Dkk1 (50ng/ml); Lacta (5 µM) or Dkk1 + Lacta, for 4 hours. **(a)** Hippocampal excitatory synapses (white arrows) were defined by the apposition of vGlut1 (green) and PSD95 (red) labelled puncta. **(b)** Quantification show that Lacta prevents the Dkk1 effect on volume and number of vGlut1 labelled puncta **(c)** PSD-95 volume and number quantification remains unchanged by Dkk1 or Lacta treatment **(d)** Analysis of total number of synapses shows that inhibition of proteasome degradation with Lacta prevents the synaptic loss induced by Dkk1. Scale bar 2µm. *p< 0.05; **p< 0.005; ANOVA. Results are shown as mean ± SEM. n= 3 experiments.

Quantification shows that vGlut1 puncta are maintained at control values after the treatment of Dkk1 in combination with Lactacystin (Fig 3.5b). As previously observed, neither Dkk1 nor proteasome inhibitor manipulation has an effect on the PSD95 volume and puncta number (Fig 3.5c). Notably, inhibition of UPS degradation totally blocks the effect of Dkk1-mediated synaptic disassembly, as shown by the quantification of the total number of excitatory synapses (vGlut1 co-localised with PSD95, Fig 3.5d). Together, these results demonstrate that synaptic elimination is dependent on protein degradation. Furthermore, my results suggest the UPS is a crucial modulatory system during the process of synaptic disassembly in the context of neurodegeneration.

3.3. Discussion

Synapses are dynamic structures: they assemble, remodel and disassemble throughout life. Interestingly, different studies have shown that the majority of the synapses are stable structures which can last throughout the lifetime of the animal (Alvarez & Sabatini, 2007; Bhatt et al., 2009; Meyer et al., 2003). Whilst the mechanisms that control synaptic assembly have been extensively studied (McAllister, 2007; Owald & Sigrist, 2009), the machinery and molecules underlying synaptic maintenance and disassembly in the CNS have not being studied to the same extent. Here I present data that strongly suggests that blockade of endogenous Wnts using Dkk1, for a short period of time, induces the disassembly of synapses in an *ex vivo* system. Given the implication of Wnts in synaptic integrity, I propose Dkk1 as an efficient and powerful tool to study Wnt-mediated synaptic maintenance. In addition, in this Chapter I also presented data that shed new light on the mechanisms underlying Dkk1-mediated synaptic disassembly. My results suggest mechanistically, Dkk1 can only produce an effect in synaptic disassembly if protein degradation, mediated by proteasome system, is active. Although future work establishing the precise mechanism and specific molecules that regulate synaptic maintenance and disassembly, here I report the first evidence to the Dkk1 mechanism of action, downstream of its receptors.

Wnts regulate synaptic maintenance. Many studies have demonstrated that Wnts promote synapse formation by stimulating the recruitment of pre and postsynaptic proteins to future synaptic sites (Ahmad-Annuar et al., 2006; Cerpa et al., 2008; Ciani et al., 2011; E. E. Davis et al., 2006; Farias et al., 2009; Hall et al., 2000). Interestingly, recent studies suggest the importance of Wnt in synaptic assembly and remodelling in the adult brain (Cerpa et al., 2011; J. Chen et al., 2006; Gogolla et al., 2009). A recent study from our lab has shown that blockade of Wnt signalling leads to synaptic shrinkage and disassembly in cultured hippocampal neurons (Purro et al., 2012). Yet, whether Dkk1 indiscriminately targets all types of synapses remained to be elucidated. In this study, I show that inhibition of endogenous Wnt signalling, by the secreted Wnt antagonist Dkk1, induces synaptic disassembly in an *ex vivo* system. Short-term treatment of brain slices with Dkk1 leads to the loss of almost 50% of synapses in the CA1 stratum radiatum. Importantly, Dkk1-mediated synaptic disassemble can be prevented by inhibiting Gsk3β, a signalling molecule downstream of the Wnt canonical pathway (Clevers & Nusse, 2012; D. Wu & Pan, 2010). Although recently it has been suggested that Dkk1 can indirectly modulate Wnt/Ca++ pathway in chondrocytes and in carcinoma cells (Mikheev et al., 2008; Nalesso et al., 2011), my findings strongly support that Dkk1 is indeed a robust and potent Wnt canonical signalling antagonist (Mao et al., 2001; Niehrs, 2006), demonstrating therefore the role for Wnt signalling in synaptic maintenance. However, as it can not be ruled out that Dkk1 might influence alternative signalling pathways, further studies investigating whether Dkk1 can simultaneously influence different Wnt pathways in neuronal cells needs to be carry out.

Dkk1 targets only a subset of synapses. Interestingly, my data shows that Dkk1 specifically disassembles excitatory synapses, having no effect on inhibitory synapses. Although the overall number of synaptic sites decreased after the treatment with Dkk1, notably blockade of Wnt canonical pathway had a stronger effect on the presynaptic site, where the vGlut1 labelled sites were smaller as well as fewer in number, whereas the postsynaptic site remained unchanged. These data bring to light some questions regarding the mechanism of synaptic disassembly: why does Dkk1 target only excitatory synapses and why does it seem to act primarily at the

presynaptic site? A possible reason to Dkk1 specificity could be location of the LRP6 receptors in relation to the synapses, as it has been proposed that these receptors are located in their vast majority at the excitatory sites (K. Sharma et al., 2013) and knockdown of LRP6 has a major effect on excitatory receptors and synaptic markers (K. Sharma et al., 2013; C. C. Liu et al., 2014). Although future work is needed to determine the mechanism of action of Dkk1 in synaptic disassembly, such as location of Kremen receptors in relation to the synapses, my results shed light on the basic, but so far not investigated, function of Dkk1 and its selective targets, as well as the remarkable importance of Wnt on the maintenance of already existing synapses. Further investigation in the presynaptic sites upon presence of Dkk1 will be addressed on Chapter 4 of this study. Moreover, as Dkk1 appears to have no effect on inhibitory synapses however, it can not be ruled out that longer exposure to Dkk1 or higher concentration of recombinant Dkk1 protein can have a detrimental effect towards inhibitory synapses. Chronic exposure to Dkk1 and its possible effect on inhibitory synapses will be addressed in the next chapter of this thesis.

Protein degradation is involved in Dkk1-mediated disassembly. The UPS regulates the turn over short lived proteins, but also ubiquitination is a post-translational modification that can serve as a signal for protein localisation, and other intracellular phenomena such as signal transduction, cell cycle, transcription, DNA repair and endocytosis (Ihara et al., 2012). Protein quality control and proteostasis are crucial processes which take places at the synapses, and this process is tightly associated with synaptic plasticity (Bingol & Sheng, 2011). Key synaptic proteins such as PSD-95, Shank, glutamate transporter, NMDA and GABA receptors are targets for ubiquitin mediated degradation (Bingol & Sheng, 2011; Martinez-Villarreal et al., 2012; Yang et al., 2008). Equally important, and relevant to this study, Dishevelled, GSK3β and β–catenin, the three pivotal components of Wnt canonical pathway, are also targeted for degradation via ubiquitination (Failor et al., 2007; Gao & Chen, 2010; Willert & Nusse, 1998). My data demonstrate that indeed, blockade of proteasome activity using two distinct pharmacological compounds completely prevent the action of Dkk1 disassembling synapses. Interestingly, previous studies have demonstrated that although inhibition of proteasomes increases the accumulation of phospho-β–

catenin, it also increases the total level of this protein (Ghanevati & Miller, 2005; Jullig et al., 2006), suggesting a homeostatic mechanism regulating the intracellular levels of β–catenin. Given that Dishevelled and β–catenin can go through deubiquitination (Jung et al., 2013; Taya et al., 1999), which positively regulates the activity of these proteins, it can not be ruled out the possibility of an increased activity of enzymes responsible for the deubiquitination process when the UPS is obstructed.

Importantly, proteasome degradation and deubiquitination has been studied in the context of neurodegeneration. A recent study showed that increasing deubiquitination activity can reverse the effect of Aβ treatment in spine loss (Smith et al., 2009). In addition, AD mouse models carrying a mutation in the UPS system, and therefore with a disrupted proteasome activity, present lower accumulation of Aβ in the brain (van Tijn et al., 2012). These studies further support my data, which demonstrate the contribution of proteasomes to the process of synaptic and neuronal degeneration. Despite the existence of studies suggesting a link between deregulation of UPS and accumulation of misfolded proteins in the brain, which can lead to PD and AD (Bedford et al., 2008; Haas & Broadie, 2008; McNaught et al., 2003) and, some authors suggest that protein aggregation might be the original cause of impairment in UPS function (Bence et al., 2001). Whether dysfunction in the proteasome is a cause or a consequence of neurodegeneration, my findings provide evidence to a new potential approach to protect synapse from Dkk1.

The work presented here elucidates some aspects of the mechanism underlying Wnt-mediated synaptic maintenance and reveals the importance of Wnt canonical pathway in this process. Although this pathway has been well characterised mechanistically, to date it had not been specifically linked with synaptic maintenance in the CNS. Importantly, for the first time, my studies revealed the specificity of Dkk1 for excitatory synapses in the hippocampus. My work also proposes the use of Dkk1 as a unique tool to understand the mechanism underpinning Wnt-mediated synaptic maintenance. Furthermore, my data also explored the mechanisms of Dkk1 action downstream to the receptor, demonstrating that synaptic disassembly resulted by

the deregulation of Wnt signalling is dependent on proteasome degradation. Moreover my results provide new insight into the mechanism by which Dkk1 induces synapse disassembly and open new possibilities for targets to tackle synaptic loss in neurodegenerative conditions. Notably, some preliminary data produced by our lab implies that acute exposure of brain slices to Dkk1 does not lead cell death – evaluated by qualitative caspase-3 staining (Lopes & Salinas, unpublished data). Although already published data demonstrate that acute exposure to Dkk1 does not lead to cell death (Dickins, 2011; Purro et al., 2012), further experiments looking into detailed neuronal number and time course of caspase-3 activity in acute brain slices would be necessary. This set of experiments would validate even further the use of Dkk1 as a model system to study early stages of synaptic degeneration, before the occurrence of neuronal loss.

Chapter 4

4. Wnt signalling is crucial for synaptic maintenance in the adult brain – an *in vivo* study

4.1. Introduction

Synapse loss is recognised as an early signature in neurodegenerative disease. Importantly, in Alzheimer's disease (AD), the extent of synapse loss strongly correlates with cognitive decline (Naslund et al., 2000; Shankar & Walsh, 2009; Terry et al., 1991). Many studies have demonstrated that synapses are relatively stable structures that can last the lifetime of the individual (Alvarez & Sabatini, 2007; Bhatt et al., 2009; Meyer et al., 2003). However, little is known about the molecular mechanisms involved in synaptic maintenance in the adult brain and what triggers their loss and dysfunction in the context of aging and neurodegenerative diseases (Allyson et al., 2012; Y. C. Lin & Koleske, 2010). Therefore, understanding the mechanisms and what molecules regulate synaptic stability in the adult CNS is crucial for developing therapeutic strategies for the treatment of neurodegenerative diseases at early stages of the condition, before substantial cell loss is evident.

Secreted factors are strong candidates for maintaining synaptic structures. For instance neurotrophins, which are known for their expression in adulthood and their well-established role in synaptic function and plasticity (Huang & Reichardt, 2001; McAllister et al., 1999) have been shown to be important for synaptic maintenance at the neuromuscular junction (Belluardo et al., 2001; Gonzalez et al., 1999). However, only recently some studies have successfully demonstrated the role of brain derived neurotrophic factor (BDNF) in the maintenance of synapses in the CNS (English et al., 2012; B. Hu et al., 2005; Vigers et al., 2012). Disruption of BDNF signalling in adulthood leads to the disassembly of excitatory synapses (English et al., 2012; B. Hu et al., 2005), which ultimately can lead to behavioural changes (Vigers et al., 2012). Together, these findings suggest that factors involved in synaptic dynamics might modulate the stabilization of these structures, and are therefore crucial for the maintenance and normal function of the brain circuitry.

A link between Wnts and its role in the adult CNS is starting to emerge. Studies have demonstrated that Wnt are not only expressed throughout development, but interestingly also during adulthood (Gogolla et al., 2009; Salinas et al., 1994; Shimogori et al., 2004; Tabatadze et al., 2012). Although, the functional aspect of Wnts in the adult brain is not clear, these findings suggest a potential role of Wnt signalling in synaptic maintenance. Contributing to this hypothesis, recent studies demonstrated a possible link between dysfunction of Wnt signalling and neurodegeneration in the context of AD (Boonen et al., 2009; Inestrosa & Arenas, 2010; Killick et al., 2012; Kremer et al., 2011; Purro et al., 2012). Indeed, a recent study from our lab shows that short-term treatment with Aβ induces a rapid increase of the Wnt antagonist Dkk1 expression in parallel with synaptic loss in the hippocampus (Purro et al., 2012). Importantly, in the presence of a Dkk1 neutralising antibody, the effect of Aβ on synapse loss was completely blocked (Purro et al., 2012). These results give an insight for the potential role for Wnt signalling for synaptic maintenance in mature neurons and that abnormal levels of Dkk1 can lead to synaptic loss and/or dysfunction. However, the novel role of Wnt signalling in synaptic maintenance *in vivo* has not been examined.

The data presented in the Chapter 3 of this thesis, together with evidence from previous studies of our lab (Purro et al., 2012), suggest that blockade of Wnt signalling with Dkk1 leads to synaptic disassembly. To further evaluate the role of Wnt signalling in synaptic maintenance I investigated whether presence of Dkk1 protein leads to synaptic disassembly using an *in vivo* system. For this part of the study, I evaluated an inducible transgenic line, which expresses the Wnt antagonist Dkk1 in the hippocampus in a restricted/temporal inducible manner. My analyses demonstrate successful inducible expression of Dkk1 in the hippocampus of adult animals using a tetracycline-controlled system. Dkk1 expression, which suppresses Wnt canonical signalling, did not affect cell viability nor had any major effects in the overall architecture of the hippocampus. These findings demonstrate that this model system is ideal to specifically address the role of Wnt signalling in synaptic maintenance in the adult brain. I found that chronic inhibition (14 days) of Wnt

signalling by Dkk1 reduced the number of excitatory synapses, having no effect on inhibitory synapses. In addition, ultrastructural analyses suggest that Dkk1 primarily targets the presynaptic site, having little effect at the postsynaptic site. These data demonstrate that long-term blockade of Wnt signalling induces synaptic elimination, reinforcing my previous *ex vivo* findings in the role of Wnt signalling in synaptic maintenance (Chapter 3).

4.2. Results

4.2.1. The use of a new mouse model to study Wnt-mediated synaptic maintenance

Based on my results presented on Chapter 3, here I investigated whether Wnt signalling was required for synaptic maintenance in the adult hippocampus using an *in vivo* system. We used a model in which several Wnts could be blocked during adulthood without affecting early development. Blockade of Wnt signalling was achieved by inducing the expression of the secreted Wnt antagonist Dkk1 (Kawano & Kypta, 2003; Mao et al., 2002) in the adult hippocampus using a tetracycline inducible system. Mice carrying the Dkk1 coding region downstream of a doxycycline responsive promoter (tetO) (Chu et al., 2004) were crossed to mice carrying the tetracycline-controlled transactivator (rtTA2S; rtTa hereafter) downstream of the forebrain specific CaMKIIα promoter (Michalon et al., 2005) (Fig. 4.1a). In this system, doxycycline physically interacts with the rtTa protein, causing a conformational change leading to the recognition of the tetO region and finally inducing the expression of Dkk1 gene. Importantly, double transgenic (ind-Dkk1) mice develop normally and have no evident behavioural defects (Fig 4.1b).

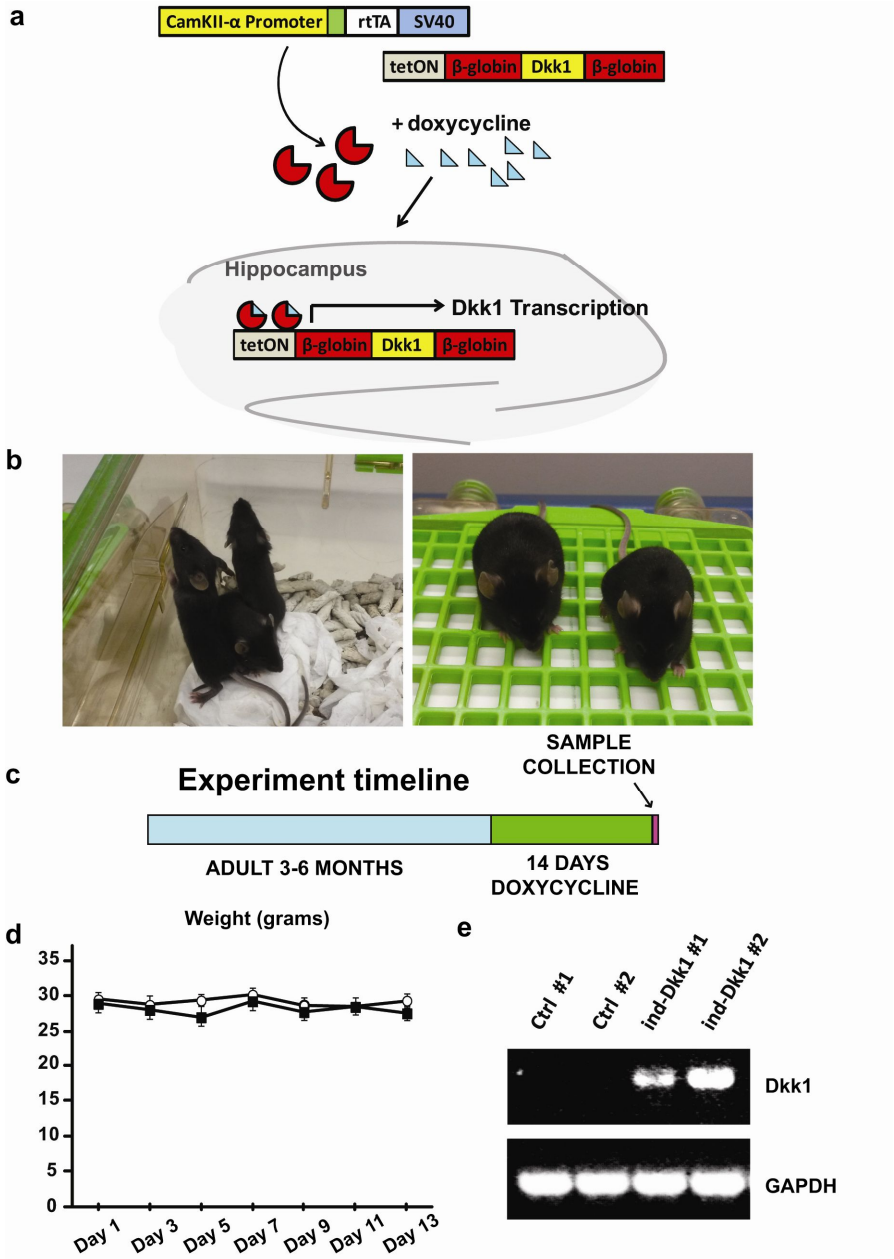

Figure 4.1 - Inducible expression of Dkk1 in the hippocampus. (a) Scheme representing the ind-Dkk1 animal model. Transgenic animals carrying rtTa gene under the control of CaMKIIα were crossed with tetON-Dkk1 transgenic mice. Double transgenic animals (CaMKII-α rtTa; tetON-Dkk1), called ind-Dkk1, were then fed with doxycycline, to induce Dkk1 expression in the hippocampus. **(b)** Transgenic animals have no abnormalities in their development in comparison to control/single transgenic animals (left: animals at postnatal day 20; right: adult ind-Dkk1 and a single transgenic littermate) **(c)** Time scale of the experiments: 3-6 months old ind-Dkk1 and control animals were fed with doxycycline for 14 days before analyses. **(d)** Weight monitoring showed no change in overall body weight of both groups, demonstrating that food intake was equal in control and ind-Dkk1 animals **(e)** RT-PCR shows that Dkk1 expression was induced in the hippocampus after doxycycline feeding, whereas single transgenic (CaMKII – Control #1) or ind-Dkk1 (Control #2) show no Dkk1 expression.

To induce expression of Dkk1, ind-Dkk1 mice were allowed to reach adulthood (3 - 6 months old) and then were fed with doxycycline for 14 days (Fig 4.1c). In all experiments, a combination of wild type (WT) or single transgenic mice (CaMKII-rtTa and tetO-Dkk1) were also fed with doxycycline and used as negative controls.

Positive controls were obtained from genomic DNA from ind-Dkk1 mice (from our lab archive). Notably, both control and ind-Dkk1 groups ate the food pellets containing doxycycline, as demonstrated by their unchanged body weight (Fig. 4.1d). After 2 weeks of doxycycline intake, RT-PCR analyses revealed that Dkk1 is only expressed in the hippocampus of ind-Dkk1 animals fed with doxycycline and not in single transgenic littermates (CaMKII - Ctrl #1), or by ind-Dkk1 mice (Ctrl #2) that were not fed with doxycycline (Fig 4.1e). Furthermore, previous observations demonstrated that ind-Dkk1 animals start expressing Dkk1 as early as 3 days after induction with doxycycline and Dkk1 expression persists for as long as animals are fed doxycycline (Galli & Salinas, unpublished data).

To further characterise our inducible transgenic mice, I examined whether Dkk1 can be ceased by withdrawal of doxycycline from the diet. I therefore performed an On-Off experiment, in which Dkk1 expression was induced for two weeks (On period) followed by a recovery period of two weeks (Off period). RT-PCR analysis show that Dkk1 is expressed while feeding with doxycycline (On), as previously shown, but more importantly, after 2 weeks of the removal of doxycycline, Dkk1 mRNA is not detected in the hippocampus of ind-Dkk1 mice (Fig 4.2a). These results demonstrated successful temporal and reversible induction of Dkk expression when doxycycline was administered in the diet.

I next investigated where Dkk1 is expressed in our transgenic line. Notably, very low or no detectable levels of Dkk1 are normally found in the healthy adult brain (Cappuccio et al., 2005; Mastroiacovo et al., 2009; Matrisciano et al., 2011). Due to lack of a reliable commercially available antibody against Dkk1, I performed *in situ* hybridization on brain sections from animals fed with doxycycline for 14 days. Expression was detected in the DG and CA3 and CA1 regions of the hippocampus (Fig 4.2b). In contrast, no signal was found in the brains of control animals, confirming my previous observation by RT-PCR. Therefore, my *in situ* hybridization revealed that doxycycline induces expression of Dkk1 in the principal neurons of the hippocampus in the transgenic mouse model.

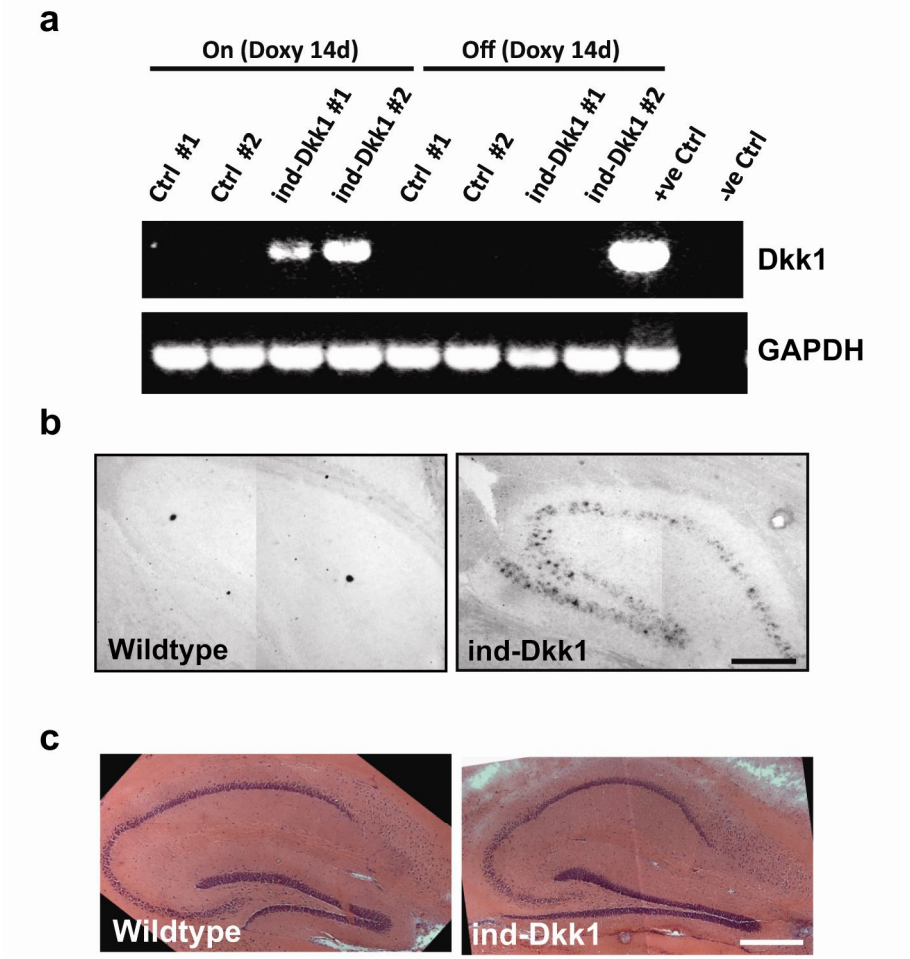

Figure 4.2 – Dkk1 was expressed in the hippocampus in a regulated manner and did not alter hippocampal morphology. (a) RT-PCR shows Dkk1 mRNAs is detected in ind-Dkk1 and control mice after 14 days of doxycycline (On). However, Dkk1 is not expressed after a further 14 days, when doxycycline administration was ceased (Off). (**b)** In situ hybridisation showed that Dkk1 was only expressed by the principal neurons of the hippocampus of ind-Dkk1 but not in control animals **(c)** H&E staining showed no gross architecture abnormalities in the hippocampus of Dkk1-expressing mice. Scale bars: 250µm.

Previous studies have demonstrated that Dkk1 has profound effects on the fate and survival of cells during embryonic development (Grotewold & Ruther, 2002; Watanabe et al., 2005). To examine whether Dkk1 expression affected the overall morphology of the hippocampus, characterization using haematoxylin and eosin (H&E) was carried out. H&E staining of 3-5 months ind-Dkk1 animals, fed with doxycycline for 14 consecutive days, revealed no apparent abnormality in the size, shape and overall architecture of the hippocampus in comparison to control animals (Fig. 4.2c). Overall, gross histological examination demonstrated that ind-Dkk1 animals did not exhibit any hippocampal neurodegeneration upon chronic expression of Dkk1.

I next examined the possible effect of Dkk1 on cell viability. Recent reports suggest that increased Dkk1 expression correlates with neuronal death in models of chronic stress, epilepsy, ischemia and Alzheimer's disease (Busceti et al., 2007; Caricasole et al., 2004; Matrisciano et al., 2011; Rosi et al., 2010). To evaluate whether chronic expression of Dkk1 induces neuronal loss in the hippocampus of our mouse model, I investigated activated caspase-3 protein levels, a classical marker for apoptosis in neuronal cells (Kuida et al., 1996; McIlwain et al., 2013). Quantification from protein extracts of hippocampus demonstrates that levels of cleaved capase-3 in relation to total caspase-3, or total caspase-3 normalised to total level of protein (tubulin) do not change upon Dkk1 expression (Fig 4.3a - graphs). These results demonstrated that induction of Dkk1 in ind-Dkk1 mice did not induce apoptosis.

In addition, to apoptosis, cell loss can be triggered by necrosis. In pathological processes, neurons can die in an unregulated and caspase-independent manner, where rapid swelling, loss of membrane integrity and bursting of the cell content occurs (Pettmann & Henderson, 1998; Yuan et al., 2003). To test whether chronic Dkk1 expression triggered necrosis, I used a membrane-impermeable dye: propidium iodide (PI). This is a nucleic acid stain that binds to DNA and RNA of cells that have had their cell membrane disrupted (Unal Cevik & Dalkara, 2003). For these experiments, brain slices from 14 day fed animals were incubated for 4h with PI (10uM), and as positive control, necrosis was induced by exposing brain slices to cold

(4°C) aCSF containing PI, for 24 hours. My PI experiment demonstrate that Dkk1 expression did not induce necrosis in the hippocampus, as almost undetectable fluorescence signal was found in the ind-Dkk1 brain slices – a level comparable to control animals (Fig 4.3b).

To further confirm that Dkk1 expression does not lead to cell death in the hippocampus, I counted the total number pyramidal cells in the CA1 area of the hippocampus. For these experiments, acute brain slices from adult animals fed for 14d with doxycycline were stained for NeuN and total number of positive cells per area was considered (Fig 4.3c). My quantification demonstrates that that cell death is not triggered upon Dkk1 expression in this area of the hippocampus (Fig 4.3d). Together, my cell biology and biochemistry experiments demonstrate that chronic Dkk1 expression does not lead to apoptosis or neuronal loss in the hippocampus. *Representative figures (4.3c) kindly selected by Dr S. Galli.*

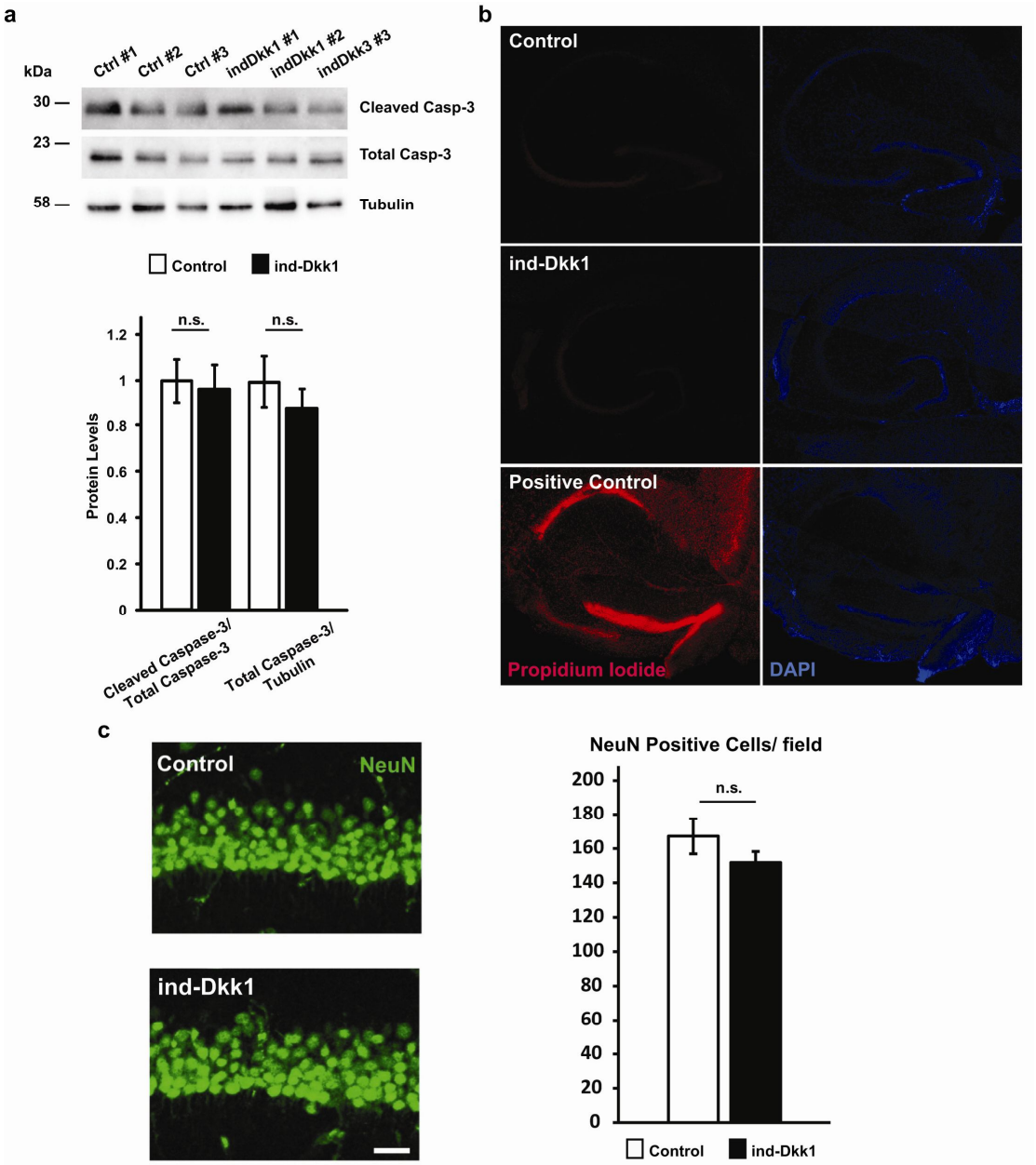

Fig. 4.3 – Dkk1 expression does not lead to increased of activation of Caspase-3, necrosis or neuronal loss. **(a)** Western-blot and quantification (graph, lower panel) demonstrates no change in the levels of cleaved capase-3 in relation to total caspase-3, or total caspase-3 normalised to total level of protein (tubulin) by Dkk1 expression (n=3 animals per group, Student's t-test). **(b)** Evaluation of cell death by propidium iodide (PI). Virtually no fluorescence signal (red) was observed in the control and ind-Dkk1 brain slices, when compared to fluorescence signal found in the positive control slice. **(c)** Representative image and quantification of NeuN staining demonstrate no neuronal loss in the CA1 hippocampus area upon Dkk1 expression after 14 days - ANOVA with replication, 4 mice per genotype). Scale bar: 50 µm. Results are shown as mean ± SEM.

In contrast, a notable level of fluorescence signal was observed in the positive control slices, where necrosis was deliberately induced (Fig 4.3b). Together these findings demonstrate that Dkk1 expression for a period of two weeks in the adult hippocampus, did not affect cell viability.

In summary, I have successfully induced the expression of Dkk1 in the hippocampus of adult animals using a tetracycline-controlled system. Uninterrupted administration of doxycycline led to expression of the Wnt antagonist protein Dkk1 in the hippocampus. Expression of Dkk1 is more noticeable in the main subset of neurons of the hippocampus. Importantly, these animals exhibit neither macroscopic hippocampal abnormalities nor neuronal loss in the hippocampal area, as shown by H&E staining and necropoptosis assays. Therefore, the ind-Dkk1 mouse model comes as a powerful tool to study the role of Wnt signalling, and more specifically Wnt mediated-synaptic maintenance, in the adult brain.

4.2.2. Induction of Dkk1 expression leads to blockade of Wnt signalling

Dkk1 is a strong Wnt antagonist that binds to the LRP5/6 co-receptor to block the canonical Wnt signalling pathway resulting in increased activity of Gsk3β and the degradation of ß-catenin (Glinka et al., 1998; Willert & Nusse, 1998). It is therefore predicted that inducible expression of Dkk1 should decrease ß-Catenin levels and transcription of TCF/LEF target genes in the hippocampus. To address this question and determine which areas of the hippocampus have altered β-catenin signalling upon expression of Dkk1, I crossed ind-Dkk1 mouse line with a β-galactosidase reporter transgenic line (BAT-gal). The BAT-gal expresses the LacZ gene under the control of β-catenin/T cell factors responsive elements (Maretto et al., 2003). The latter has been reported as a successful and reliable reporter of Wnt/ β- catenin activity (Maretto et al., 2003). Indeed, β-galactosidase staining revealed that Dkk1 expression in the hippocampus induced a significant decrease in ß-catenin target genes in the hippocampal CA1 of ind-Dkk1 mice (Fig 4.4a). Notably, a marked decrease in the intensity of the staining was noticeable in the dentate gyrus area (Fig 4.4a), which coincided with the expression of Dkk1, as previously shown by ISH. Curiously, the staining revealed no apparent changes in the CA3 area. Unfortunately due to the low number of triple transgenic mice, I was only able to examine one animal. Therefore, this result should be considered preliminary. However it suggests that expression of Dkk1 blocks canonical Wnt signalling in the adult hippocampus.

To further address the consequence of Wnt canonical blockade, I examined the expression of key targets genes of the Wnt canonical pathway using RT-PCR: Wnt11, Axin 2, Frizzled 2 (Fz2) and Sfrp2 (Fig 4.4b) (Dao et al., 2007; Gudjonsson et al., 2010; Lescher et al., 1998; W. Zhou et al., 2007).

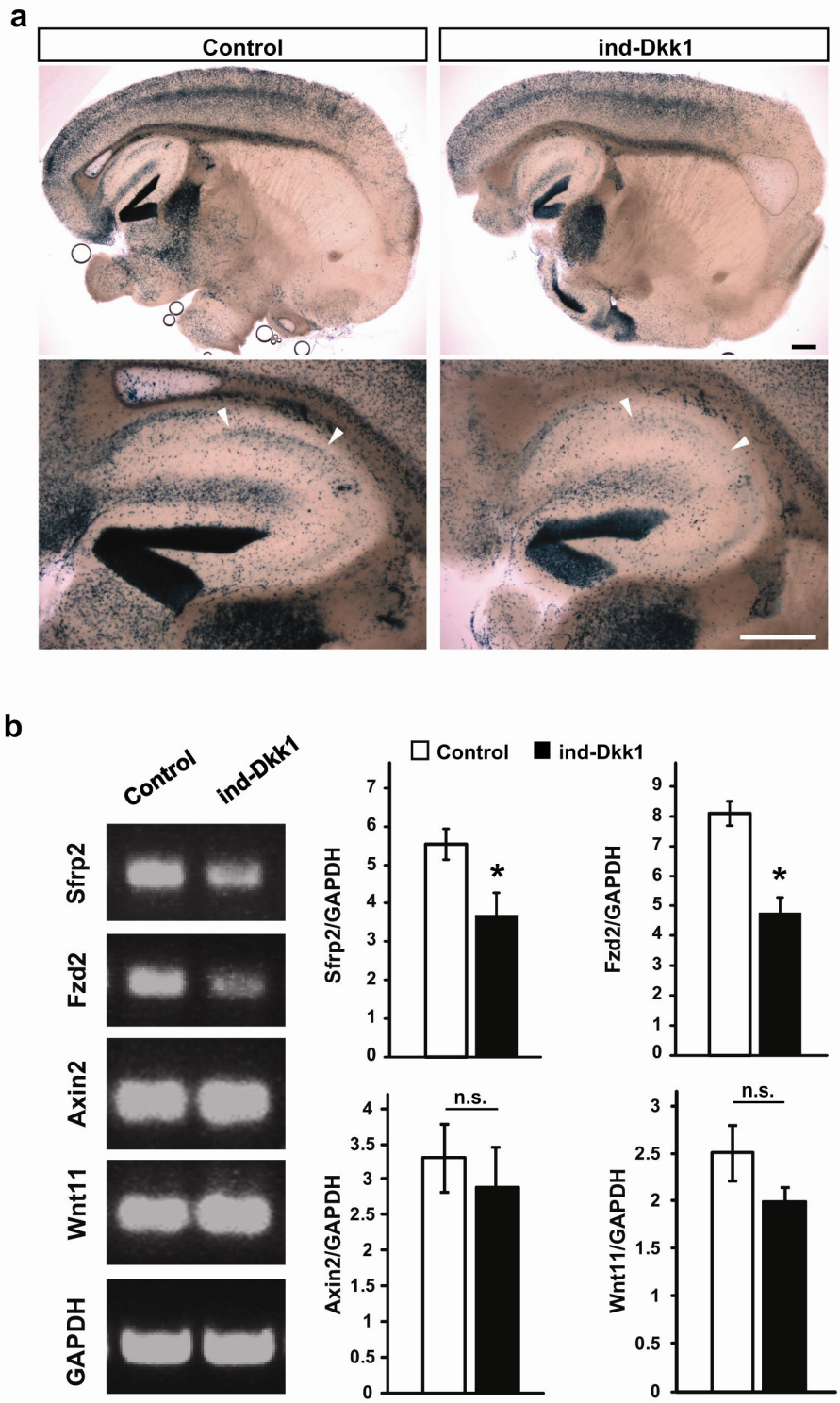

Figure 4.4 - Inducible expression of Dkk1 in the hippocampus reduced Wnt canonical pathway. (a) Sagittal sections of acute brain slices showing β-galactosidase positive areas in a control and in the ind-Dkk1 adult mice. Note that CA1 area of the hippocampus has a distinctive lower β-galactosidase signal (white arrows), when compared to control levels (a – lower panel); n=1 per group. **(b)** RT-PCR quantification show that mRNA levels of Sfrp2 and Fzd2, two genes positively regulated by canonical Wnt signalling, are decreased in ind-Dkk1 animals fed with doxycycline, but Axin2 and Wnt11 remained unchanged. Results are shown as mean ± SEM.*p ≤ 0.05; ***p≤ 0.001, n=3 - 4 animals, Student's t-test. Scale bar: 500um

Semi-quantitative analyses demonstrated a significant down-regulation of Fz2 and Sfrp2 (Fig 4.4b). However, no changes in the expression of Wnt ligand Wnt11 and the β-catenin regulator Axin2 were observed. Together, these results suggested that expression of Dkk1 blocked the canonical Wnt signalling pathway in the adult hippocampus, once again highlighting the uniqueness of this mouse model to study Wnt-mediated synaptic integrity in the adult brain without affecting early development.

4.2.3. Dkk1 expression does affect adult neurogenesis in the dentate gyrus

Recent studies have demonstrated a role for Wnt signalling in the generation of newborn neurons in the adult dentate gyrus (Jessberger et al., 2009; Lie et al., 2005; Qu et al., 2009; Seib et al., 2013). I therefore examined the potential impact of Dkk1 in adult neurogenesis in the subgranular zone of the dentate gyrus (DG) of the ind-Dkk1 animals fed for 14 days with doxycycline. Staining with doublecortin, a standard marker for newly generate neurons (von Bohlen Und Halbach, 2007), demonstrated no differences in the total number of positive cells for these markers between controls and ind-Dkk1 groups (Fig. 4.5a). This result indicated that at basal levels, chronic blockade of Wnt signalling had no impact on the generation of newborn neurons in the DG.

Next, I estimated the number of neurons by counting the total number of DG granule cells. Staining with NeuN, a well-established marker for neuronal nuclei shows that total number of NeuN+ cells did not change in animals expressing Dkk1, when compared to control (Fig. 4.5b). This result demonstrated that cell death is not triggered upon Dkk1 expression in this area of the hippocampus. Together, my results demonstrated that disruption of Wnt signalling in the DG does not lead to any apparent abnormality in the generation of newborn cells and or affect survival of mature cells in this region.

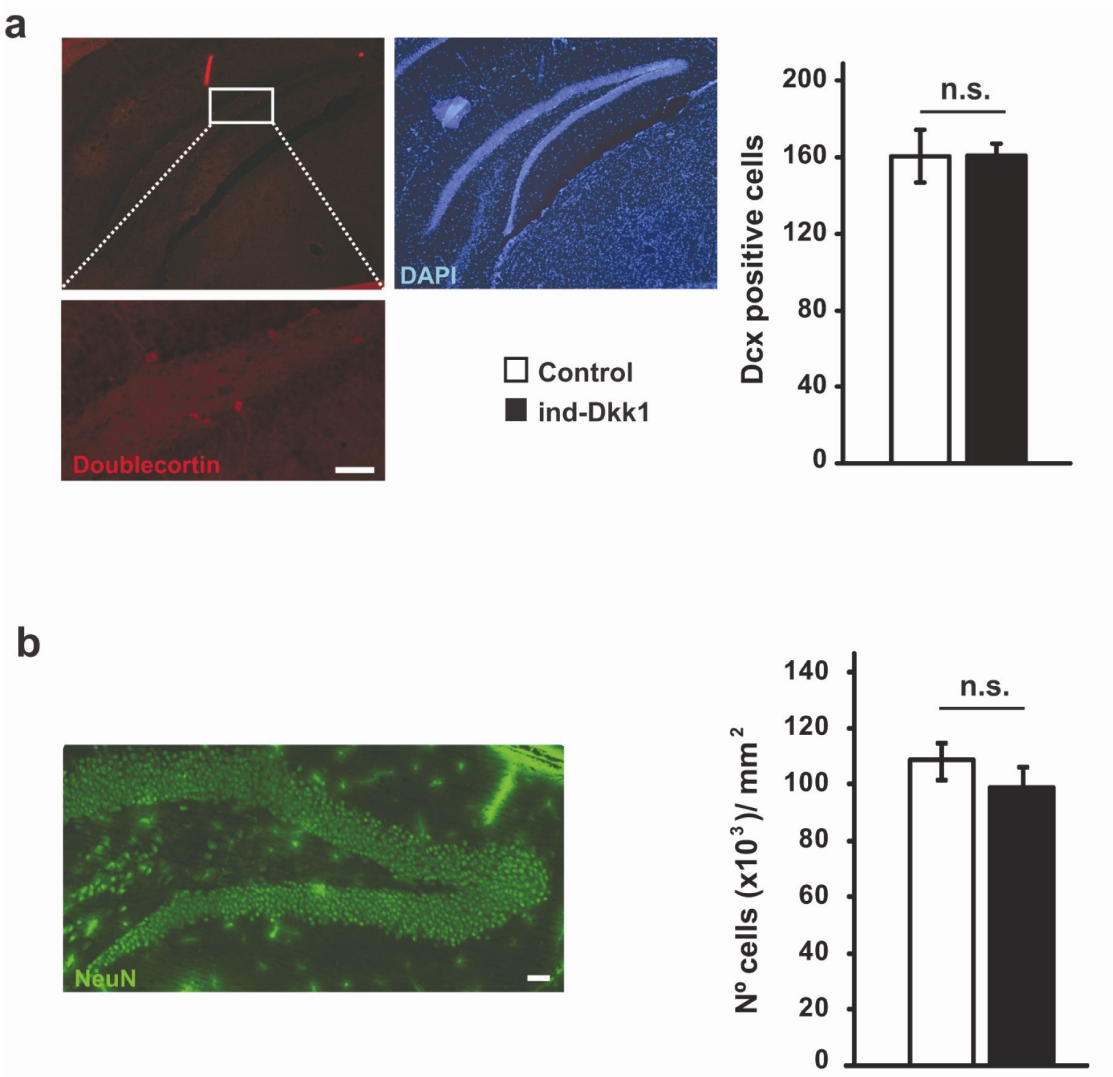

Figure 4.5 – Chronic expression of Dkk1 in the hippocampus does not affect neurogenesis or total number of neurons in the DG. (**a)** Doublecortin staining shows that downregulation of canonical Wnt signalling for 14 days does not affect the number of newborn neurons in the DG. **(b)** Illustrative picture and quantification (graph) of NeuN+ labelled cells in the DG per mm2 area. Results are shown as mean ± SEM, n=3 - 4 animals, Student's t-test. Scale bar: 50µm

4.2.4. Dkk1 specifically induces the disassembly of excitatory, but not inhibitory synapses in the CA1 adult hippocampus

Synaptic disassembly involves the loss of pre- and postsynaptic components. Our previous studies have demonstrated that acute exposure to Dkk1 rapidly disassembles synapses in mature cultured hippocampal cells (Purro et al., 2012), and in acute brain slices, (Chapter 3). Therefore, I examined whether Dkk1 also affected synaptic maintenance *in vivo* using our transgenic mouse line fed with doxycycline

for 14 days. Antibodies against vGlut1 and PSD95, pre and post- synaptic markers respectively, were used to quantify the number and volume of synapses in the stratum radiatum of the CA1 hippocampus (Fig. 4.6a).

Expression of Dkk1 did not affect the number or volume of PSD95-labelled sites (Fig. 4.6c). However, Dkk1 expression was associated with decreased volume as well as density of the glutamatergic presynaptic marker, vGlut1 (Fig 4.6b) and the number of excitatory synapses - determined by the apposition of vGlut1 and PSD95 markers (over 40% decrease, Fig. 4.6d). These results demonstrated that chronic blockade of Wnt canonical pathway with Dkk1 induced the disassembly of excitatory synapses in the CA1 region of the adult hippocampus.

Following my observation in acute brain slices, which demonstrated that Dkk1 treatment targets specifically excitatory synapse, in collaboration with a post-doctorate co-worker from our lab I examined whether chronic expression of Dkk1 has an impact on inhibitory synapses in the hippocampus. Presynaptic vGAT and postsynaptic Gephyrin markers were used, and the volume and number of inhibitory synapses were evaluated, as well as the colocalization of these markers (Fig 4.7a). In accordance with the observation from brain slices (Chapter 3), expression of Dkk1 does not induce the disassembly of inhibitory synapses, or a change in the density of the pre and postsynaptic markers (Fig 4.7b). Taken together, these results show that blockade of Wnt canonical pathway with Dkk1 induces the specific loss of excitatory synapses, with no effect on inhibitory synapses in the CA1 region of the adult hippocampus. *(Images of Figure 4.7 were kindly provided by Dr. Soledad Galli).*

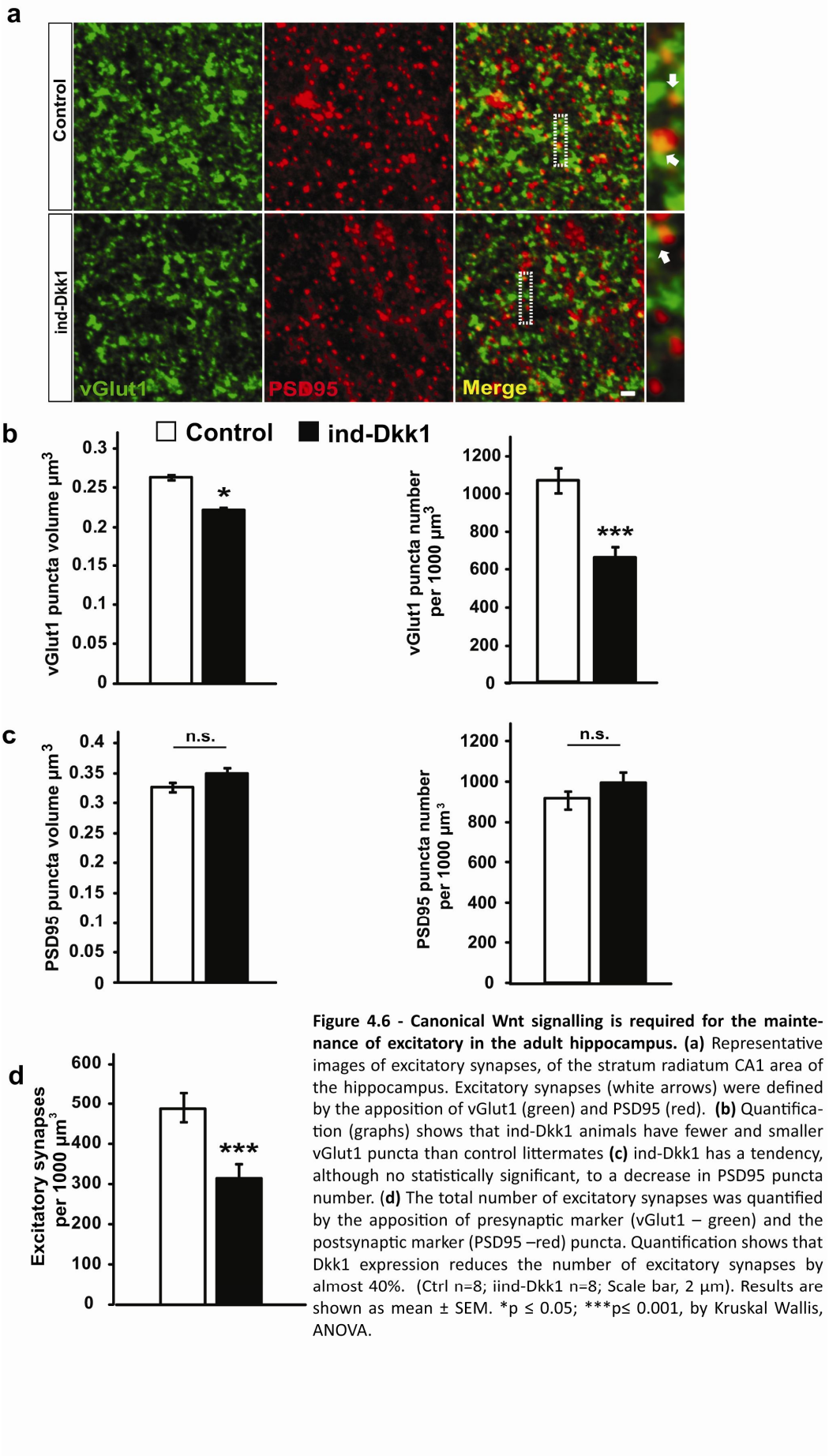

Figure 4.6 - Canonical Wnt signalling is required for the maintenance of excitatory in the adult hippocampus. (a) Representative images of excitatory synapses, of the stratum radiatum CA1 area of the hippocampus. Excitatory synapses (white arrows) were defined by the apposition of vGlut1 (green) and PSD95 (red). **(b)** Quantification (graphs) shows that ind-Dkk1 animals have fewer and smaller vGlut1 puncta than control littermates **(c)** ind-Dkk1 has a tendency, although no statistically significant, to a decrease in PSD95 puncta number. (**d)** The total number of excitatory synapses was quantified by the apposition of presynaptic marker (vGlut1 – green) and the postsynaptic marker (PSD95 –red) puncta. Quantification shows that Dkk1 expression reduces the number of excitatory synapses by almost 40%. (Ctrl n=8; iind-Dkk1 n=8; Scale bar, 2 µm). Results are shown as mean ± SEM. *p ≤ 0.05; ***p≤ 0.001, by Kruskal Wallis, ANOVA.

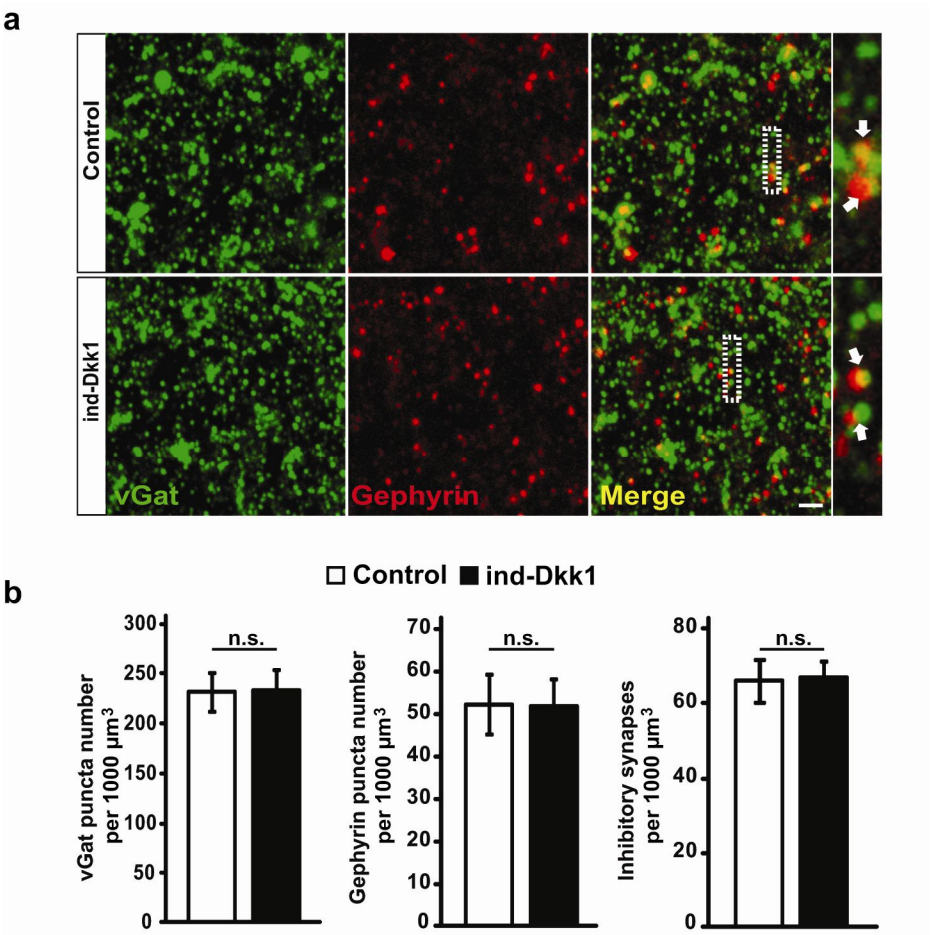

Figure 4.7 - Dkk1 expression in the hippocampus does not have any effect on inhibitory synapses. (a) Representative images of inhibitory synapses of the stratum radiatum of the CA1 area of the hippocampus. Inhibitory synapses (white arrows) were defined by the apposition of vGAT (green) and Gephyrin (red). **(b)** Quantification (graphs) shows that ind-Dkk1 animals have same number of vGAT and PSD95 labelled puncta as control littermates. Total number of inhibitory synapses are unaffected by Dkk1 expression as determined by the apposition (white arrows) of the presynaptic marker vGAT (green), postsynaptic marker Gephyrin (red) (Ctrl n=4; ind-Dkk1 n=4; Scale bar, 2 µm). Results are shown as mean ± SEM. ANOVA

4.2.5. CA3 excitatory synapses remain unaffected in ind-Dkk1 mice

Previously I demonstrated that Dkk1 mRNAs is present throughout the CA1 and CA3 areas of the hippocampus in ind-Dkk1 mice. Therefore I went on to verify whether expression of Dkk1 in the CA3 area of the hippocampus leads to any changes in excitatory synapses, as observed in the CA1 area. As previously stated, antibody against vGlut1 and PSD95, a pre and post- synaptic markers respectively, were used to quantify the number and volume of puncta in the CA3 hippocampus. Surprisingly, Dkk1 expression does not affect excitatory synapses in the CA3 hippocampus (Fig 4.8a). Analyses of presynaptic sites demonstrate that vGlut1 volume (Ctrl= 0.29±0.01 μm^3, ind-Dkk1= 0.28±0.01 μm^3; data not shown) and number the number of vGlut1 labelled puncta remain unchanged between groups. When PSD95 was analysed, data shows that volume remains unchanged (Ctrl= 0.36±0.02 μm^3, ind-Dkk1= 0.38±0.02 μm^3, data not shown), and total number (Fig 3.3b) is also unaffected by the presence of Dkk1. Overall, the number of excitatory synapses determined as the apposition of vGlut1 and PSD95 markers also remains unaffected (Fig 4.8b). This data demonstrates that expression of Dkk1 in the CA3 area of the hippocampus has no effect on excitatory synapses.

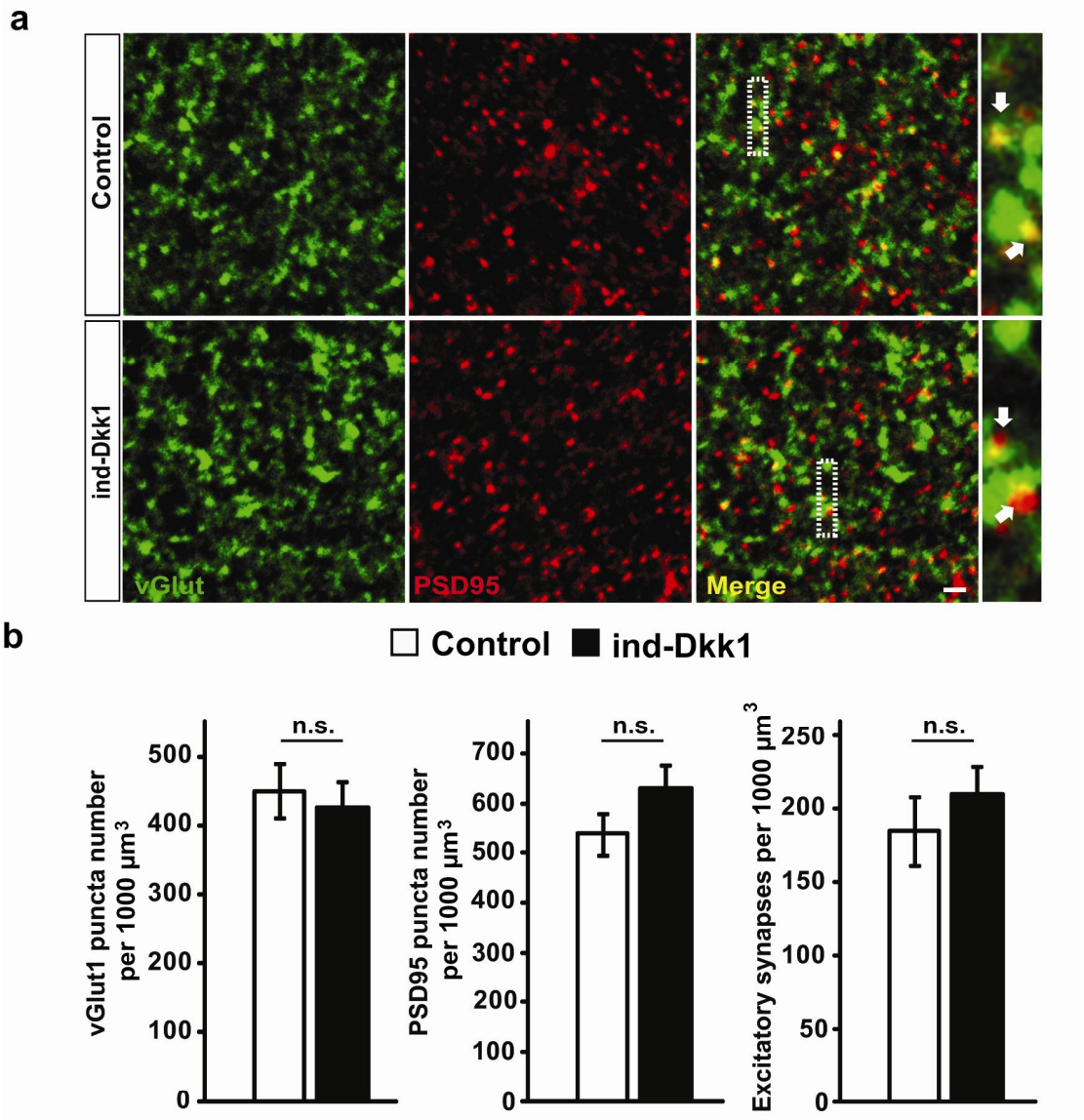

Figure 4.8 – Expression of Dkk1 does not induce the loss of excitatory synapses in the CA3 area of the hippocampus. (a) Representative images of excitatory synapses, of the CA3 area of the hippocampus. Excitatory synapses (white arrows) were defined by the apposition of vGlut1 (green) and PSD95 (red). **(b)** Quantification (graphs) shows that ind-Dkk1 animals have same number of vGlut1 and PSD95 labelled puncta as control littermates. Total number of excitatory synapses was quantified by the apposition of presynaptic marker (vGlut1 – green) and the postsynaptic marker (PSD95 –red) puncta. (Ctrl n=4; ind-Dkk1 n=4; Scale bar, 2 µm). Results are shown as mean ± SEM. ANOVA.

4.2.6. Dkk1 expression affects dendritic spines in the CA1 region

Given the effect of Dkk1 on excitatory synapses, I next examined the role of Dkk1 on dendritic spines, postsynaptic structures that primarily receive excitatory inputs (Bourne & Harris, 2008; Yuste, 2011). To test whether expression of Dkk1 leads to

any change in the overall structure or number of dendritic spines, otherwise not detectable by immunofluorescence, I performed the Golgi impregnation staining technique. This elegant technique is being used for years and allows the study of the complete morphology of isolated labelled neurons (Shankaranarayana, 2004). Two time points were used in this part of the study: adult animals fed with doxycycline for 7 days and animals fed with 14d of doxycycline. For these analyses, the size of the spine head and the total number of spines (normalised by dendritic length) were considered (Fig 4.9a).

Unpublished data from our lab revealed that expression of Dkk1 for 7 days in the hippocampus does not cause any significant change in excitatory synapses at the CA1 hippocampus (Galli & Salinas; *unpublished data*). However, my analyses by Golgi staining reveals that 7 days of Dkk1 expression leads to a small but significant reduction, of approximately 10%, in the size of the dendritic spines CA1 area of the hippocampus (Fig 4.9b). Interestingly, no changes were observed in the number of spines per unit length between ind-Dkk1 and control groups. In contrast, when doxycycline was given to the animals for 14 days, Golgi staining revealed that expression Dkk1 in the CA1 area of the hippocampus decreases dendritic spine size (~20%) and spine number (~10%) (Fig 4.9c).

These results demonstrate that inducible Dkk1 expression in the adult hippocampus for 7 days results in the shrinkage of dendritic spine deficits in the CA1 region of the hippocampus and that this effect is more pronounced with longer expression of Dkk1. The decrease in spine size at early stages of Dkk1 induction also, suggests that shrinkage of spines might precede spine loss.

To investigate whether Dkk1 causes any change in dendritic spines in the CA3 area, otherwise not identified by confocal microscopy due to possible limitations of the technique, I analysed the morphology of the dendritic spines in this region. No changes were observed in the CA3 dendritic spines (Fig 4.9d). Both size and spines number were equal in control and ind-Dkk1 mice. This data further demonstrates that CA3 area of the hippocampus remains unaffected by the expression of Dkk1.

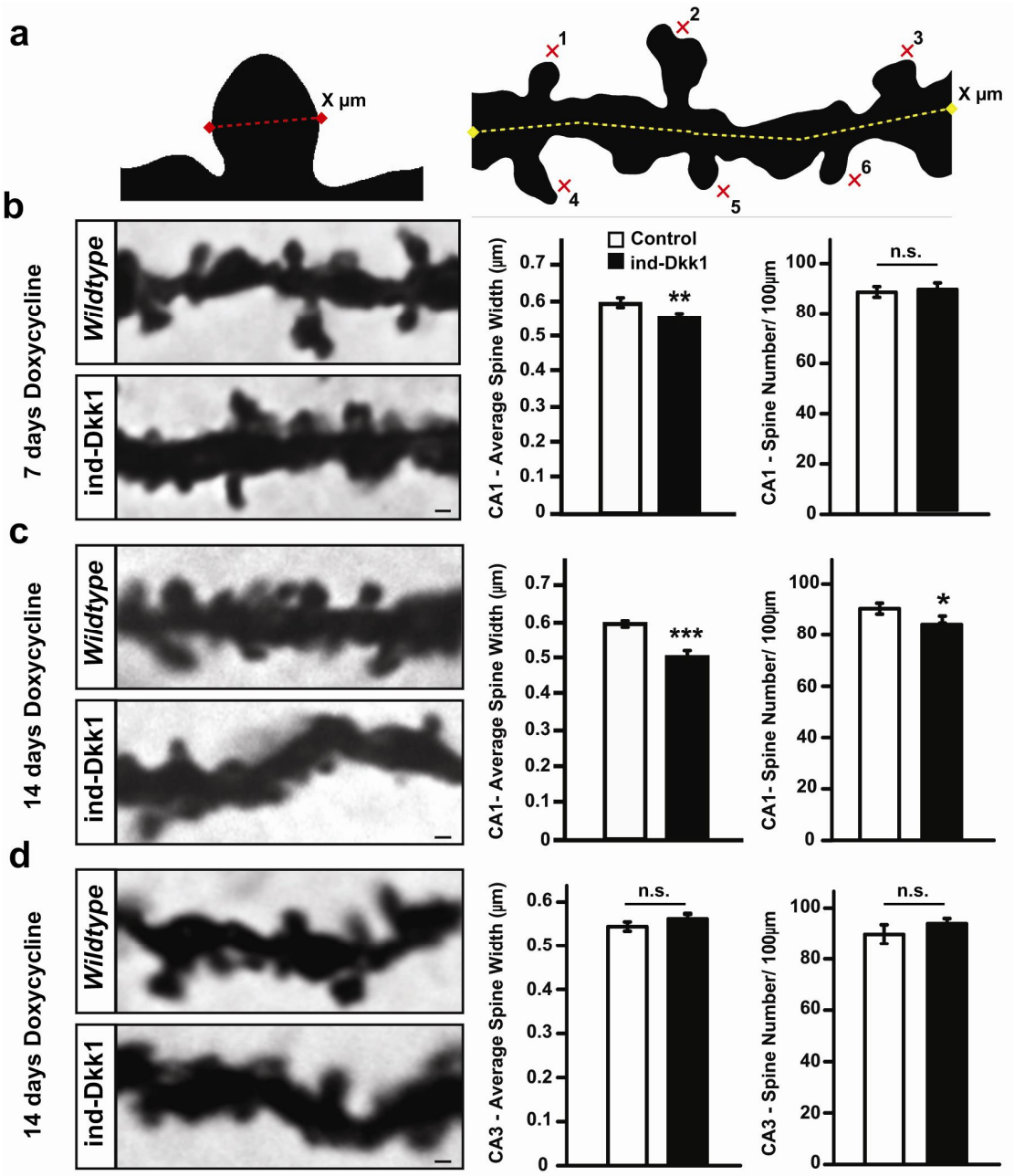

Figure 4.9 - Dkk1 expression in the hippocampus leads to the shrinkage and subsequent loss of hippocampal dendritic spines. (a) Cartoon representing a spine and a dendritic brunch labelled by Golgi staining, demonstrating how the width of the spine was measured (left) and number of spines were quantified (right). **(b)** ind-Dkk1 animals fed for 7 days show no difference in the spine number but with spine size (width) is smaller in the CA1 area of the hippocampus **(c)** Interestingly, ind-Dkk1 animals fed for 14 days with doxycycline show a significant reduction in the width of spine heads and in the density of spines in the CA1 area of the hippocampus **(d)** Quantification show no changes in spine density or spine width were observed in the CA3 area of the hippocampus. Results are shown as mean ± SEM. *p ≤ 0.05; **p≤ 0.01; ***p≤ 0.001. Ctrl n=4; ind-Dkk1 n=4. Student's t test for number, Kruskal Wallis for size. Scale bar, 0.5µm

4.2.7. Dkk1 expression affects the ultra structure of the presynaptic terminal

Given that my previous results in which Dkk1 expression affects excitatory synapses and dendritic spines, as demonstrated by both my immunohistochemistry and Golgi analysis, I went on to further investigate the effect of Dkk1 on excitatory synapses at the ultrastructural level. After feeding both groups of animals - ind-Dkk1 and control littermates - with doxycycline for 14d, I analysed the synapses in the stratum radiatum of the CA1 by electron microscopy. Excitatory synapses were considered as those exhibiting a well-defined postsynaptic density in perfect apposition to a presynaptic bouton containing synaptic vesicles (Fig 4.10a - lower panels). A significant decrease in the number of synapses per area was observed in the ind-Dkk1 mice when compared to control animals (Fig. 4.10b). Interestingly, ind-Dkk1 animals exhibit a dramatic increase in the percentage of synaptic-like structures with a postsynaptic density (PSD) but deprived of synaptic vesicles (Fig 3.5a - red arrows; quantification Fig. 4.10c). These findings further demonstrate that induction of Dkk1 expression in the adult hippocampus induces the disassembly of excitatory synapses in the hippocampus. Moreover, this also suggests that Dkk1 has a stronger effect at the presynaptic terminal as shown by the increased number of synaptic-like structures devoid of vesicles in the ind-Dkk1 mice.

Previous studies suggest the involvement of Wnts in neurotransmitter release at excitatory synapses (Cerpa et al., 2008). Furthermore, unpublished data from our lab demonstrate that Wnt signalling components interacts with molecules of the releasing machinery and modulates excitatory neurotransmission (Ciani, Boyle & Salinas, *unpublished data*). To then characterise any changes in the excitatory synapses triggered by Dkk1 expression, ultra-structure of the remaining asymmetric synapses in the CA1 area of the hippocampus were also analysed by EM. For these analyses, only asymmetric synapses were considered and four parameters were taken into account: PSD length, spine size, and number of synaptic vesicles. My analysis show that on the presynaptic terminal, the number of total vesicles located up to 200nm to the membrane were unchanged by induction of Dkk1 (Fig 4.10d and d, respectively). In contrast, when postsynaptic site was analysed, I found that the

size of the spine head is reduced in ind-Dkk1 mice when compared to controls (Fig 4.10e).

Surprisingly, measurements show that PSD length had a slight increase on ind-Dkk1 animals (Fig 4.10f). These findings suggest that Dkk1 induces the disassembly of a subset of excitatory synapses, but remaining asymmetric synapses appear structurally normal. Furthermore, the results suggest that chronic expression of Dkk1 does not lead to the abnormal recruitment of synaptic vesicles to the synaptic terminal. Together these results demonstrate that remaining, or resilient, asymmetric synapses are unaffected by Dkk1 expression and remain structurally normal.

4.2.8. Continuous expression of Dkk1 does not induce further loss of synapses

The results described above, revealed that expression of Dkk1 for 14 days in the adult hippocampus leads to the loss of excitatory synapses. To test whether long term expression of Dkk1 leads to a further disassembly of synapses, or perhaps any compensatory/homeostatic mechanism is triggered after long term suppression of Wnt signalling, I examined animals that were expressing Dkk1 for a long period of time. For this experiment, animals were fed with doxycycline pellets for 3.5 months and then brain slices were processed for immunofluorescence. Antibody against vGlut1 and PSD95, were used to quantify the number and volume of synapses in the stratum radiatum of the CA1 hippocampus, as previously described.

My confocal imaging analyses (Fig. 4.11a) demonstrate that long term expression of Dkk1 leads to a significant decrease in the volume as well as the density of the presynaptic marker vGlut1 (Fig 4.11b). However, no significant changes in the postsynaptic sites were observed, as demonstrated by the unchanged number and volume of PSD95-labelled sites (Fig. 4.11c). Although the postsynaptic site remains unaffected, the total number of excitatory synapses, determined as the apposition of the pre and postsynaptic markers, decrease in a ration of about 40% in ind-Dkk1

when compared to controls mice (Fig. 4.11d). These results demonstrate that long term expression o Dkk1 does not lead to any further loss of synapses, as the results are compared to animals fed for 14 days with doxycycline. Furthermore, these results suggest that Dkk1 expression for long periods of time does not activate any compensatory cellular mechanism to counterbalance the changes caused by disrupted Wnt signalling.

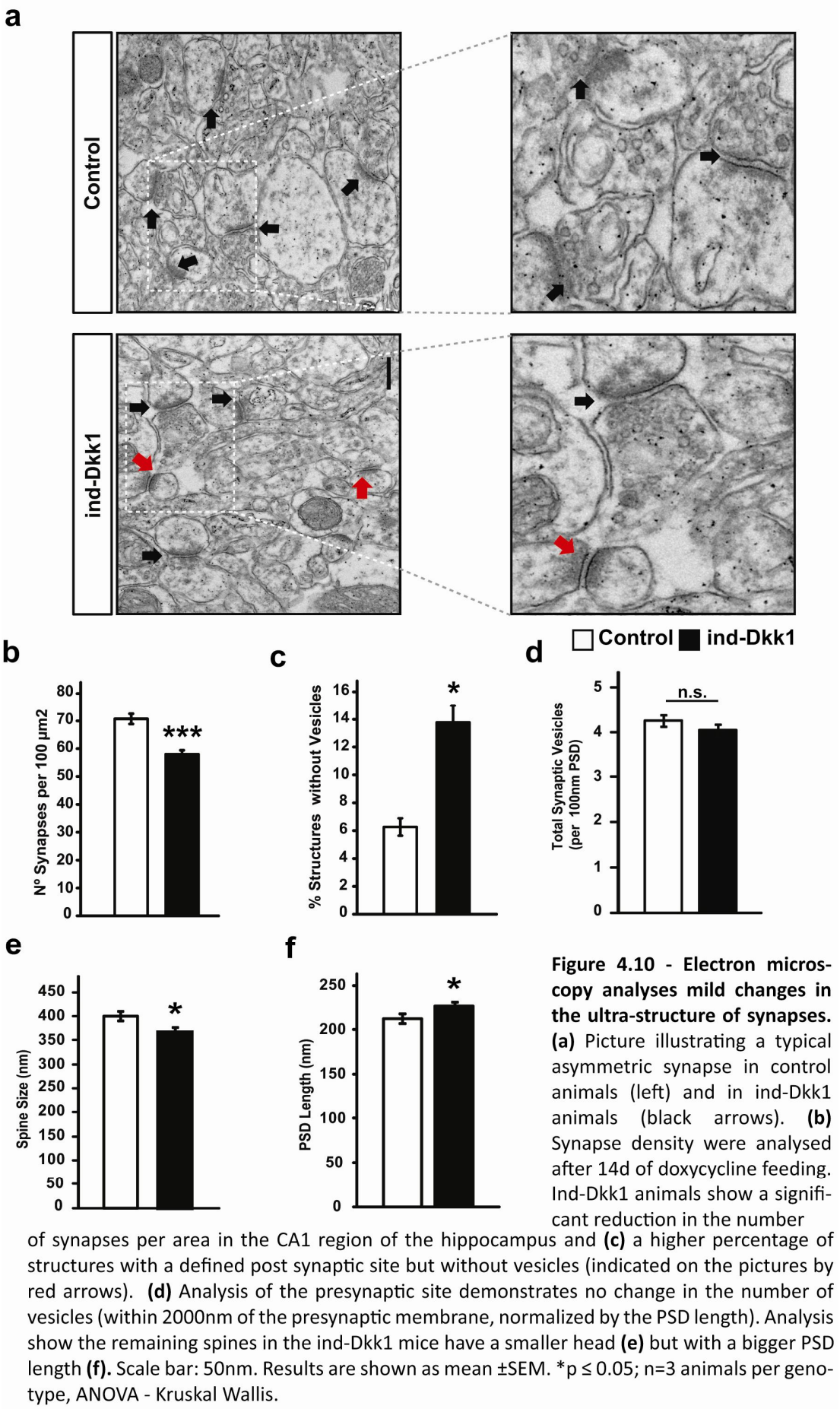

Figure 4.10 - Electron microscopy analyses mild changes in the ultra-structure of synapses. **(a)** Picture illustrating a typical asymmetric synapse in control animals (left) and in ind-Dkk1 animals (black arrows). **(b)** Synapse density were analysed after 14d of doxycycline feeding. Ind-Dkk1 animals show a significant reduction in the number of synapses per area in the CA1 region of the hippocampus and **(c)** a higher percentage of structures with a defined post synaptic site but without vesicles (indicated on the pictures by red arrows). **(d)** Analysis of the presynaptic site demonstrates no change in the number of vesicles (within 2000nm of the presynaptic membrane, normalized by the PSD length). Analysis show the remaining spines in the ind-Dkk1 mice have a smaller head **(e)** but with a bigger PSD length **(f).** Scale bar: 50nm. Results are shown as mean ±SEM. *p ≤ 0.05; n=3 animals per genotype, ANOVA - Kruskal Wallis.

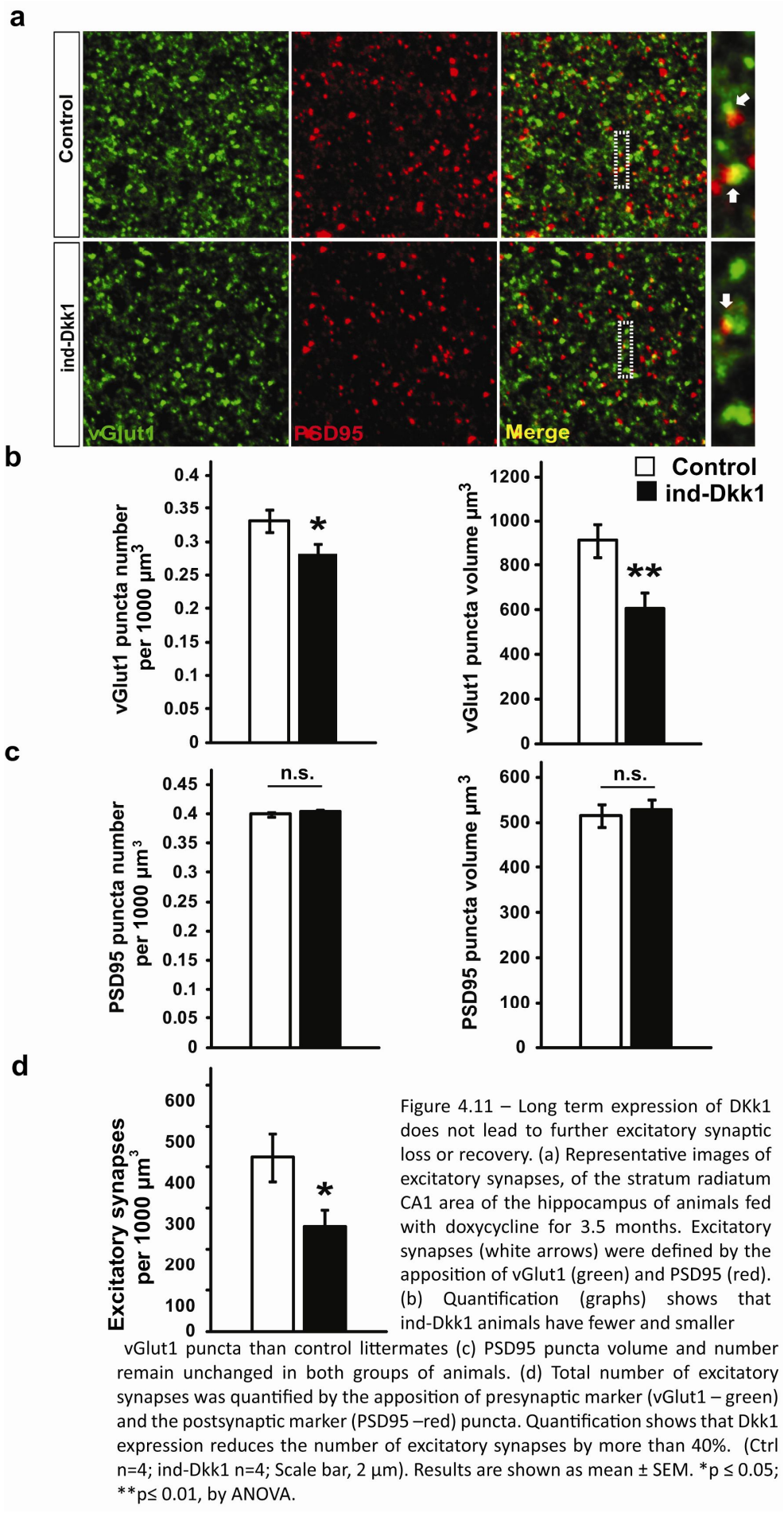

Figure 4.11 – Long term expression of DKk1 does not lead to further excitatory synaptic loss or recovery. (a) Representative images of excitatory synapses, of the stratum radiatum CA1 area of the hippocampus of animals fed with doxycycline for 3.5 months. Excitatory synapses (white arrows) were defined by the apposition of vGlut1 (green) and PSD95 (red). (b) Quantification (graphs) shows that ind-Dkk1 animals have fewer and smaller vGlut1 puncta than control littermates (c) PSD95 puncta volume and number remain unchanged in both groups of animals. (d) Total number of excitatory synapses was quantified by the apposition of presynaptic marker (vGlut1 – green) and the postsynaptic marker (PSD95 –red) puncta. Quantification shows that Dkk1 expression reduces the number of excitatory synapses by more than 40%. (Ctrl n=4; ind-Dkk1 n=4; Scale bar, 2 µm). Results are shown as mean ± SEM. *p ≤ 0.05; **p≤ 0.01, by ANOVA.

4.2.9. Regain of Wnt signalling recovers lost synapses

My data established the importance of Wnt signalling for synapse maintenance, as presence of Dkk1 in the adult hippocampus leads to the disassembly of excitatory synapses. Critically, the next question to be addressed is to whether the loss of synapses is reversible. Investigating whether synapses have the potential for full regeneration is crucial for developing treatments of neurodegenerative diseases, as synapse loss is an early event in the disease (P. Coleman et al., 2004; Mallucci, 2009). On this light, I went on to test whether by restoring Wnt signalling, synapses can be recovered. To test this hypothesis, I performed an On-Off experiment in which Dkk1 expression was induced for two weeks followed by a recovery period of two weeks in which doxycycline were withdrawn from the diet. Following the methodology used in the previous experiments for excitatory synaptic quantification, antibodies against vGlut1 and PSD95, were used to quantify the number and volume of synapses in the stratum radiatum of the CA1 hippocampus. Importantly, as previously shown, after 2 weeks of removal of doxycycline, we detected no Dkk1 mRNA expression in the hippocampus of ind-Dkk1 mice by RT-PCR (Fig 4.2a).

As Wnt signalling was restored, I then examined the impact on excitatory synapses (Fig 4.12a). My data show that two weeks after ceasing the expression of Dkk1, both volume and number of sites labelled with vGlut1 in ind-Dkk1 animals are similar to control levels (Fig 4.12b). Moreover, no significant changes in the volume or number of postsynaptic PSD95 labelled sites were observed (Fig 4.12c). Importantly, based on the colocalization of pre and postsynaptic markers, the number of glutamatergic synapses in the CA1 area is restored to the levels of control mice (Fig 4.12d), demonstrating that ceasing in the expression of Dkk1 leads full synaptic recovery. Overall, these results demonstrate that the effect of Dkk1 on synapse disassembly is reversible in the adult hippocampus and, more importantly, it highlights the role of Wnt signalling in synapse regeneration.

In summary, my results demonstrate a successful induction of Dkk1 in the adult brain upon doxycycline administration - in a spatial restricted and reversible manner.

Furthermore, this proposed animal model has no compromised cell viability, being therefore a powerful tool to study the role of Wnt signalling, and more specifically Wnt mediated-synaptic maintenance, in the adult brain. More importantly, my results demonstrate that blockade of Wnt signalling with Dkk1 results in the loss of excitatory synapses in CA1 area of the hippocampus, without affecting inhibitory synapses. Morphological analyses by Golgi staining, demonstrate that the postsynaptic site is mildly affected by expression of Dkk1. Interestingly, the data also suggests that disassembly of synapses appears to target mainly the presynaptic site.

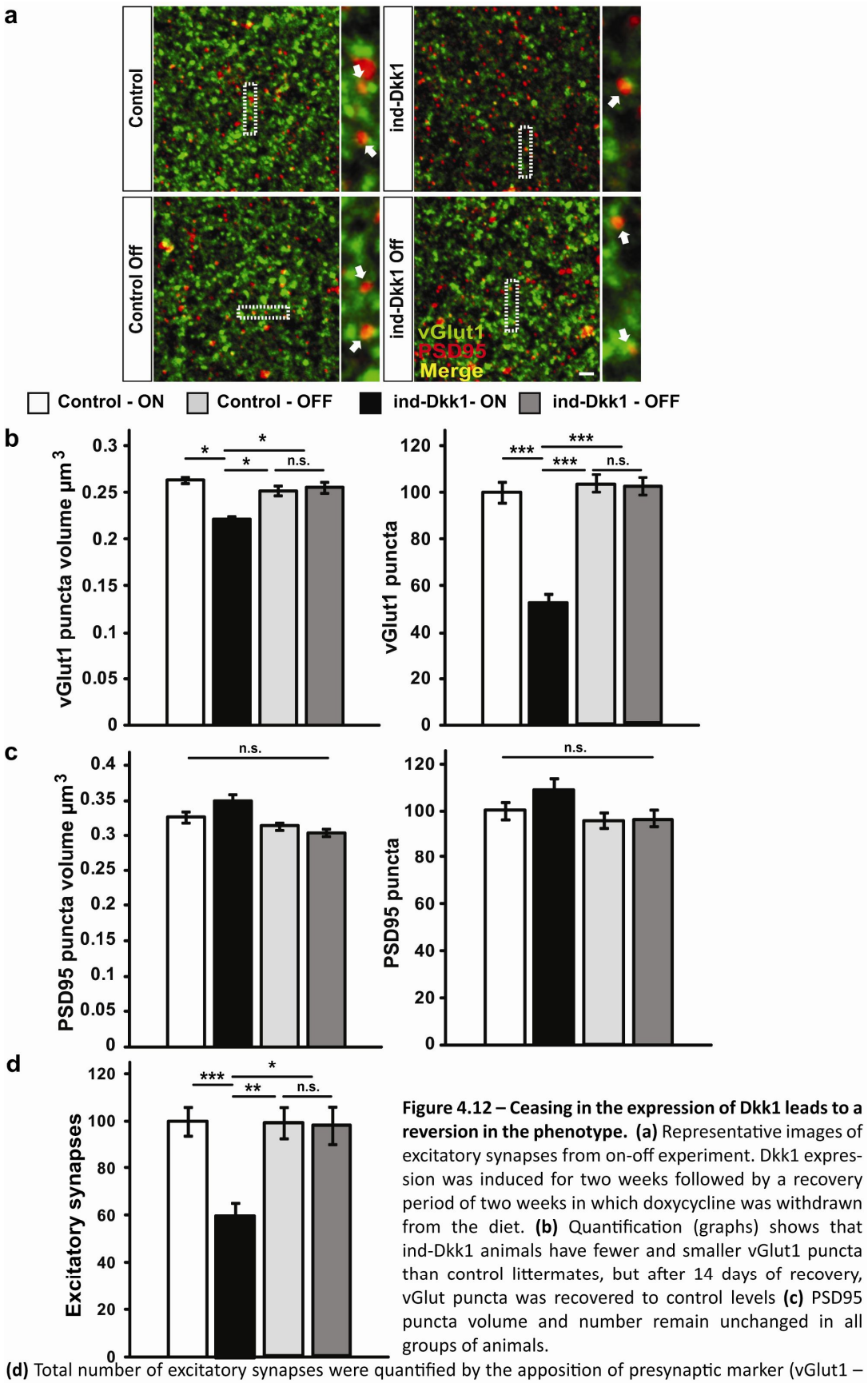

Figure 4.12 – Ceasing in the expression of Dkk1 leads to a reversion in the phenotype. (a) Representative images of excitatory synapses from on-off experiment. Dkk1 expression was induced for two weeks followed by a recovery period of two weeks in which doxycycline was withdrawn from the diet. **(b)** Quantification (graphs) shows that ind-Dkk1 animals have fewer and smaller vGlut1 puncta than control littermates, but after 14 days of recovery, vGlut puncta was recovered to control levels **(c)** PSD95 puncta volume and number remain unchanged in all groups of animals. **(d)** Total number of excitatory synapses were quantified by the apposition of presynaptic marker (vGlut1 – green) and the postsynaptic marker (PSD95 –red) puncta. Colocalisation of pre- and postsynaptic markers demonstrates that ceasing in the expression of Dkk1 leads to a reversion in the previously observed phenotype. Values are expressed in relation to control (ON) levels. (Ctrl n=6; ind-Dkk1 n=6; Scale bar, 2 µm). Results are shown as mean ± SEM. *p ≤ 0.05; **p≤ 0.01; ***p≤ 0.01; Kruskal Wallis, ANOVA.

4.3. Discussion

To date significant progress has been made to understand what molecules and mechanisms are involved in the process of synaptic assembly, but molecules regulating synaptic stability are still to be identified. In neurodegenerative diseases such as AD and PD, synaptic loss and dysfunction represent an early event that might contribute to the subsequent loss of neurons in these diseases. Therefore, it is crucial to identify what molecules and mechanisms underlie synaptic integrity in the adult brain as this could allow the development of therapeutic strategies to target neurodegenerative diseases at early stages, before substantial cell loss. Here I present evidence that strongly suggest that Wnt signalling is involved in synaptic maintenance. Data from my *in vivo* studies demonstrate that chronic blockade of Wnt signalling in the adult hippocampus leads to the loss of synapses and also shed a light on possible mechanisms involved in the process of synapse disassembly.

The role of Wnt signalling in the adult brain was yet to be elucidated. Although Wnts are present in the adult brain (Gogolla et al., 2009; Salinas et al., 1994; Shimogori et al., 2004; Tabatadze et al., 2012), to date very few studies investigated the significance of their expression. The existence of different Wnts, signalling through specific pathways (MacDonald et al., 2009; Willert & Nusse, 2012) adds an immense level of complexity to study this family of secreted proteins. Here I report the successful generation of an animal model that expresses the Wnt antagonist Dkk1 in the hippocampus to address the role of Wnt in synaptic maintenance in the adult brain. This system would allow the understanding of the canonical Wnt canonical signalling role in synaptic maintenance at the CNS in a time-restricted manner.

Dkk1 is not expressed in the healthy adult brain and does not necessarily induce cell death. My study demonstrates that Dkk1 can be induced in the ind-Dkk1 system and, importantly, Dkk1 mRNA is not detected in the brains of control animals fed with doxycycline. Supporting my findings, several studies demonstrated that Dkk1 is not expressed in the adult healthy brain, unless under adverse conditions such as ischemia epilepsy, chronic stress and in AD (Busceti et al., 2007; Cappuccio et al.,

2005; Caricasole et al., 2004; Mastroiacovo et al., 2009; Rosi et al., 2010). These studies also described a potential link between the expression of Dkk1 and neuronal loss in the context pathological circumstances (Busceti et al., 2007; Cappuccio et al., 2005; Caricasole et al., 2004; Mastroiacovo et al., 2009; Rosi et al., 2010). Here I show that induction of Dkk1 in the hippocampus does not cause loss of neurons in this area, a finding supported by other studies showing that Dkk1 does not compromise neuronal viability (Dickins, 2011; Purro et al., 2012). The relatively low level of expression of Dkk1 in the hippocampus of ind-Dkk1 mice, demonstrated by the in situ hybridization assay, may explain this finding. Although my results suggest the presence high levels of endogenous cleaved caspase-3 in control and ind-Dkk1 mice, these findings can be explained by the technique used in this assay. WB exposures were automatically done by the camera software, where the optimum and clearest signal is chosen based on the amount of protein present on the membrane to be analysed. This technique aims to show the best signal of the blot, without causing its overexposure, showing therefore an optimal signal which does not necessarily reflect high amounts of protein – as it averages the best signal for the lowest and highest concentration of proteins. Furthermore, detectable levels of cleaved caspase-3 is expected to be found endogenously at synaptic areas, as previously reported (Z. Li et al., 2010; J. Y. Wang et al., 2014). Thus, the ind-Dkk1 mice comes as a unique model to unravel the role for Wnt signalling in synaptic maintenance and plasticity at early stages of synaptic loss, in the absence of neuronal demise.

Alternative methods have been suggested to detect induction of apoptosis in the ind-Dkk1 system. Many studies have proposed the possibility to detect early stages of apoptosis by using annexin V, a well characterised and potent anticoagulant protein that binds to phospholipids (Niu & Chen, 2010; Rand, 2000). Apoptosis leads to the disruption of membrane phospholipids, which is preceded by the exposure of inner cytosolic phosphatidylserine (PS) at the cell surface, an enzyme with high affinity to annexin V (Koopman et al., 1994; Niu & Chen, 2010). Annexin V coupled with flouroforms is commercially available and promised to efficiently bind to PS and identify cells undergoing apoptosis. Accordingly, further to the experiments

performed in this thesis, evaluating induction of apoptosis and/or necrosis by Dkk1 expression, it has been suggested that annexin V would be a valid and interesting alternative way to evaluate early stages of cell death in the ind-Dkk1 model system, should this phenomenon be happening and not being identified by the techniques used. However, many pitfalls have been pointed out when using the annexin-V staining technique (Niu & Chen, 2010). Among them, high background staining and false positives, unspecific binding to the membrane, as well as the impossibility to distinguish apoptosis and necrosis (Niu & Chen, 2010). Furthermore, it also been proposed that PS exposure might happened after activation of caspase-3 intracellularly (Boersma et al., 2005; Niu & Chen, 2010). Therefore, these studies demonstrate that there is no particular advantage on performing further experiments to evaluate neuronal demise, as my study has fully covered induction of apoptosis at early stages (caspase-3), necrosis (propidium iodide) as well as total neuronal loss (NeuN+ cells). Overall it can be comfortably concluded that Dkk1 expression is not inducing neuronal loss in our mouse model.

The implication of Wnts in adult neurogenesis has started to be explored. Several studies demonstrated that Wnt signalling promotes neurogenesis in the dentate gyrus (DG) (Jessberger et al., 2009; Lie et al., 2005; Qu et al., 2009). Indeed, a recent study suggests that Dkk1, expressed in the DG stem cell niche, acts as a suppressor of neurogenesis in aged mice (Seib et al., 2013). Despite silencing of Dkk1 increases the number of newborn neurons, it also increases cell death of the immature neuronal population in the DG (Seib et al., 2013). Although the specific function of neurogenesis still remains elusive, growing evidence suggests a key role of newly generated neurons in hippocampal circuitry and memory (Deng et al., 2010; Marin-Burgin & Schinder, 2012). Here I show that inducible expression of Dkk1 in adult mice does not affect neurogenesis in the DG. These contrasting results might be due to the low levels of Dkk1 expression, the period of time expressing Dkk1 (14 days), as well as the age of the animals used for the experiments. On this light, the ind-Dkk1 system comes as a strong model to study the effects of Wnt-mediated synaptic maintenance in the hippocampus without compromising neurogenesis.

Wnt canonical signalling is required for the maintenance of excitatory synapses in the adult brain. Secreted factors such as neurotrophins are strong candidates for maintaining synaptic structures, given their expression in adulthood and their well known role in synaptic function and plasticity (Huang & Reichardt, 2001; McAllister et al., 1999). Recently some studies have successfully demonstrated the role of brain derived neurotrophic factor (BDNF) for the maintenance of synapses in the CNS (English et al., 2012; B. Hu et al., 2005; Vigers et al., 2012), however, the *in vivo* role of Wnts in synaptic maintenance had not been explored so far. In this study, I show that blockade of Wnt signalling has no effect in inhibitory synapses. By contrast, downregulation of Wnt canonical signalling *in vivo* leads to the loss of ~40% of excitatory synapses. Curiously, the effect of Dkk1 on excitatory synapses appear to have a stronger, or possibly initial, effect at the presynaptic site, where the number of vGlut1 labelled sites are more affected than those labelled with PSD95 (postsynaptic).

Contributing to these findings, my analyses at the ultrastructural level reveal that Wnt blockade leads to an increase of hemi-synapses, synaptic-like structures with a postsynaptic site but devoid of vesicles in the presynaptic bouton, further suggesting that the presynaptic site is primarily affected by the expression of Dkk1. Although the techniques used in my study can not determine the hierarchy of disassembly, my findings are supported by a study which demonstrate that synaptic loss, induced by long term depression (LTD) and examined by time-lapse imaging, starts at the presynaptic site (N. Becker et al., 2008). Further studies in central synapses also reveal that synaptic elimination is initiated by the dispersal of presynaptic associated proteins (De Paola et al., 2003; Hopf et al., 2002). In addition, a recent elegant study has demonstrated the importance of two major presynaptic scaffolding proteins (Bassoon and Piccolo) for the maintenance of synapses (Waites et al., 2013), giving a further insight to the mechanism and to the importance of the presynaptic terminal for the overall synaptic integrity. Interestingly, studies show that during synapse formation and assembly, some Wnts act by stimulating the recruitment of presynaptic proteins (Budnik & Salinas, 2011; F. R. Lucas & Salinas, 1997). Together the results of my study shed light to the mechanism of Dkk1-mediated synaptic

disassembly, and suggest that perhaps this mechanism shares the same components to the assembly of synapses during development and in the activity-mediated context. Further studies investigating the reason that loss of pre and postsynaptic sites does not occur at a comparable rates and not in a coordinated manner as previously observed *in vitro* (Dickins, 2011), and using alternative pre- and postsynaptic markers, will be necessary to further confirm these findings and to determine the hierarchy of Dkk1-mediated synaptic disassembly.

How can Dkk1 only target a specific subset/ type of synapses? My findings demonstrate that although Dkk1 is expressed throughout the hippocampus in ind-Dkk1 mice, excitatory synapses at the CA3 region remain unaffected. This result could be explained by the pattern of expression of Wnts throughout the hippocampus. Immunohistochemistry analyses from our lab, together with other studies, showed that in adult animals, Wnt7a/b is highly expressed in the CA3 region (Atlas, 2014; Ciani et al., 2011; Gogolla et al., 2009); whereas the levels of these proteins are relatively lower in the CA1 area (Ciani et al., 2011). Although these studies only give insight to the expression of two isoforms of Wnts, they may suggest that higher concentrations of Wnts in the CA3 area of the hippocampus can override the detrimental effect of Dkk1 on excitatory synapses.

Another interesting finding in this Chapter is that only excitatory synapses are affected, and that the continuous expression neither causes further loss of synapses, nor the recovery of the lost one. I speculate that Dkk1 may specifically target excitatory synapses due to the localisation of the Wnt co-receptor LRP6, known to be necessary for Dkk1 action (Mao et al., 2002; Mao et al., 2001). Indeed, a recent study demonstrated that LRP6 is involved in the formation, and localised almost exclusively, at the excitatory synapses (K. Sharma et al., 2013). Interestingly, knockdown of LRP6 with shRNA in young hippocampal cultured neurons leads to the reduction in the number of excitatory synapses, sparing inhibitory synapses (K. Sharma et al., 2013). Furthermore, preliminary data from our lab suggest that not all dendritic spines and synapses contain LRP6 receptors (Stamatakou, Lopes & Salinas, unpublished data). Together, these results suggest that synaptic vulnerability and/or

resilience to Dkk1 may be due to the localisation of LRP6 receptors in relation to the types of synapses. Further studies looking into Kremen receptors in relation to synapses will be necessary to further support this statement.

Blockade of Wnt signalling leads to shrinkage, loss and possible compensatory changes in the remaining dendritic spines. Analyses at the morphological level of dendritic spines by Golgi staining reveal that short term expression of Dkk1 leads to the shrinkage of synapses, and longer exposure to Dkk1 leads to a further decrease in the average spine head followed by total disassembly of spines. Although it can be argued 10% decrease in spine size has no biological relevance, these two different time points analyses strongly suggest that before the occurrence of total disassembly, spines go through structural modification that primarily leads to its shrinkage. This finding is supported by studies showing that synapse shrinkage induced by LTD affects the size of dendritic spines (Bastrikova et al., 2008; Bosch & Hayashi, 2012; Nagerl et al., 2004). Furthermore, my data looking at the ultrastructural level is consistent with the finding that synapses that were not eliminated had a smaller spine head. Surprisingly, when the length of the PSD was analysed, a slight increase in this parameter was observed in the ind-Dkk1 group, suggesting that perhaps those remaining synapses had a compensatory mechanism to counterbalance the lost of some synapses. Given emerging literature which demonstrates that synaptic scaling drives homeostatic regulation of neuronal activity in both *in vivo* and *in vitro* systems (Turrigiano, 2008; Turrigiano & Nelson, 2004) and that synaptic abundance of postsynaptic scaffolding proteins, particularly PSD-95, can be bidirectionally scaled up or down, according to the changes in the circuitry and plasticity (Sun & Turrigiano, 2011), it could not be ruled out that this phenomenon is also observed in my system. Further detailed studies examining whether Dkk1 in a long term can indeed trigger a compensatory mechanism are required to fully characterise the mechanism of Dkk1-mediated disassembly.

Could Wnts be used to restore synaptic loss? It is well recognised that once neurons are lost they do not regenerate. In contrast, increasing evidence suggest that under some circumstances, synaptic loss can be restored. Studies show that

neurotrophic/synaptotrophic factors play a key role counterbalancing and even recovering the loss of synapses observed in early to moderate stages of neurodegenerative diseases (Nagahara et al., 2013; Nagahara et al., 2009; Rockenstein et al., 2003; Rockenstein et al., 2002) as well as the cognitive decline associated with them (De Rosa et al., 2005; Nagahara et al., 2013; Nagahara et al., 2009; Rockenstein et al., 2003; Rockenstein et al., 2002). For instance, long-term treatment with cerebrolysin, a neurotrophic peptide mixture, is capable to rescue the synapse loss observed at early and mid-stages in an AD mouse model (Rockenstein et al., 2003; Rockenstein et al., 2002). Using the same principle, more recent studies have demonstrated that BDNF, either by direct brain infusion or gene delivery, can also rescue the loss of synapses induced by Aβ in animal models (Nagahara et al., 2013; Nagahara et al., 2009). Similarly, here I demonstrate that molecules involved in Wnt pathway could also be used as potential therapeutic targets to revert synaptic loss, as synaptic loss induced by Dkk1 can be recovered once Wnt signalling is restored. More importantly, I demonstrate that those synapses rescued can theoretically be considered as functional synapses, as only synapses with pre- and postsynaptic sites were considered in the analysis. This observation put my results one step further to those cited above, as they only evaluated the loss and rescue of synapses by analysing a single and indiscriminate presynaptic marker (synaptophysin). Together with the previous studies, my findings highlight the potential therapeutic benefits of using secreted factors at early stages of neurodegeneration and put Wnts in the list of new potential targets.

In conclusion, the data presented in this study demonstrate that inhibition of endogenous Wnt signalling *in vivo* leads to the elimination of excitatory synapses having no effect on inhibitory synapses. My data also suggests that the process of disassembly, mediated by Dkk1, appears to target mainly the presynaptic site. Furthermore, the ind-Dkk1 animal model comes an valuable tool to study the mechanisms of synaptic disassembly in the context of neurodegeneration, specially on the light which proposes that synaptic loss precedes, and perhaps is the cause, of neuronal death (Mallucci, 2009; Selkoe, 2002; Yoshiyama et al., 2007). Moreover, my data demonstrates that recover of synaptic loss upon restoration of Wnt

signalling provide novel possible therapy targets to synaptic regeneration at early stages of neurodegenerative diseases.

Chapter 5

5. Dkk1-mediated synaptic loss in the hippocampus leads to memory impairment

5.1. Introduction

Memory is defined in its literal meaning as the "faculty by which the mind stores and remembers information", and/or "something remembered from the past" (Stevenson, 2010). However, scientifically the term 'memory' is used more largely as the capacity of the brain to encode, store and retrieve information (R. G. Morris, 2006). Despite being used in a synonymously manner, learning and memory have yet distinct meanings: learning is the process that modifies subsequent behaviours, whereas memory is the capacity to remember past experiences (Kandel & Squire, 2000; S. Sharma et al., 2010). During the past decades a great deal has been learned about learning and memory, but more importantly multiple evidence points to the hippocampus as a critical player in these processes (Andersen, 2007; Neves et al., 2008; S. Sharma et al., 2010). The breakthrough study that put the hippocampus as a crucial structure for episodic memory comes from the HM patient, who had part of their hippocampus lesioned in an attempt to relieve seizures, and in consequence the patient developed an inability to form new episodic memories (Andersen, 2007; Corkin, 2002; Scoville & Milner, 1957). Since then, many studies using patients and animal models confirmed that subjects with hippocampus lesions suffer from heterograde amnesia (S. J. Martin et al., 2005; Neves et al., 2008; Spiers et al., 2001; Vargha-Khadem et al., 1997). However, more studies are yet to be done to precisely demonstrate which structures and anatomical areas of the hippocampus and connecting areas contribute to the mechanism of episodic memory (Langston et al., 2010).

The hippocampus has a complex network influencing in a variety of functions. Since the anatomic descriptions and speculations regarding the brain connections made by Ramon y Cajal in the late 1800’s and early 1900's, much has been learned about the

hippocampus and its connectivity to understand how memory is processed. Apart from the well described regions (CA1, CA2, CA3 and dentate gyrus), each of these areas receives a variety of inputs from different brain regions which allow the information to flow through the hippocampus (Andersen, 2007; Wojtowicz, 2012). A less known but widely accept function of the hippocampus is its role regulating emotions. A range of studies manipulating this region demonstrate that the ventral hippocampus plays a role in anxiety and conditioned fear, due to the direct connectivity with the amygdala and hypothalamus (Bannerman et al., 2003; Bannerman et al., 2004; Engin & Treit, 2007; Kjelstrup et al., 2002; Moser & Moser, 1998; W. N. Zhang et al., 2013). On the other hand, the most better known function of the hippocampus is as a centre for visuo-spatial processing of information that comes from the dorsal area of the hippocampus and its direct connections to the entorhinal cortex (Andersen, 2007; van Strien et al., 2009). Although decades of studies and sufficient evidence demonstrating the role of the hippocampus and both emotional and cognitive functions, some authors treat the hippocampus as two distinct zones, one as the affective and the other as the cognitive hippocampus (Fanselow & Dong, 2010). Despite different views on the subdivision of the hippocampus, the overwhelming amount of data and studies generated from rodents, to primates and humans demonstrate coherence in the anatomical projection of the hippocampus, and more importantly, its invaluable role in many behavioural functions.

One aspect of the hippocampus which is well established is its contribution for spatial memory. Indeed, according to Nadel & O'Keefe's cognition map theory, spatial memory is dependent of the hippocampus (John O'Keefe & Nadel, 1978). Since the discovery of the "place cells", defined as neurons that fire according to the environmental location (J. O'Keefe, 1979), the hippocampus has been put as a unique and primary region involved in the spatial processing. Along with the cognition map, different tests such as the radial–maze designed by Olton and Samuelson in 1976 (Pick & Yanai, 1983) and Morris water maze (R. Morris, 1984) were developed to evaluate the memory process by obtaining spatial information. These tests have further support the invaluable role of the hippocampus as a key

structure in the processing of spatial memory. Whether the information travels in the hippocampus via the indirect or direct pathway (via EC-DG-CA3 or via the temporoammonic pathway, respectively), the final and ultimate endpoint within the hippocampus proper is the CA1 area (Carr & Frank, 2012; van Strien et al., 2009), being therefore an essential area for the memory processing. Furthermore, it has been reported that the CA1 area is required for the spatial and temporal processing of information, and it also contributes towards others hippocampal-dependent associative recognition tasks (Gilbert et al., 2001; Hunsaker et al., 2006; Kesner, 2005; Langston & Wood, 2010). Among a vast literature demonstrating the different contributions each of the regions of the hippocampus have towards the memory processing, these studies were able to dissect the hippocampus and determine what component of the memory is related to that specific region.

A link between Wnts and memory has started to be explored. Recent studies have begun to investigate the contribution of Wnt signalling in the adulthood and hippocampal function. For instance, an elegant study suggested that Wnts are involved in cognition, as its secretion is increased after specific hippocampal-dependent behavioural tasks (Tabatadze et al., 2012). In addition, some studies have demonstrated the role of Wnt signalling towards adult hippocampal neurogenesis (Jang et al., 2013; Lie et al., 2005; Seib et al., 2013). In fact, by manipulating the levels of Dkk1 in aged animals, neurogenesis can be enhanced, which correlates with improvement in cognition (Seib et al., 2013). However, as it has been demonstrated that Dkk1 promotes the disassembly of synapses in mature hippocampal neurons (Purro et al., 2012), therefore whether the impairment in memory observed in these studies is due to decreased neurogenesis or in fact a decrease in synapses and connectivity remains to be studied.

Given the *in vivo* effects of Dkk1 expression in synaptic maintenance demonstrated in the previous chapters and the emerging evidence to the role of Wnt signalling in hippocampus-mediated memory (Fortress et al., 2013; Tabatadze et al., 2012), in this chapter I investigated the consequences of synapse loss caused by the blockade of canonical Wnt signalling in our animal model. My results show that blockade of Wnt

signalling in the hippocampus does not interfere in the levels of anxiety-like behaviour of ind-Dkk1 animals. Interestingly, expression of Dkk1 in the hippocampus does not have any effect in the spatial working memory. By contrast, chronic blockade of Wnt signalling has profound consequences in different aspects of long - term memory. Furthermore, expression of Dkk1 in the hippocampus leads to defects in spatial memory processing. Together my results demonstrate that blockade of endogenous Wnt signalling in the adult hippocampus does not affect short-term memory and anxiety-like behaviour, but it leads to impaired spatial learning and memory

5.2. Results

5.2.1. Induction of Dkk1 expression has not effect in anxiety-like behaviour

My previous findings demonstrating that blockade of canonical Wnt signalling with Dkk1 in the hippocampus affects synaptic integrity led me to hypothesise that ind-Dkk1 transgenic mouse might exhibit deficits in hippocampal-mediated behaviour. Given the hippocampus plays an important role in anxiety-like behaviour, due to its ventral connections with other areas of the brain involved in anxiety (Adhikari et al., 2010; Bannerman et al., 2014; Bannerman et al., 1999) I first examined anxiety-like behaviour in our transgenic mouse line.

To address these possible behavioural changes, I used ind-Dkk1 and control adult animals after 14 days of feeding with doxycycline and submitted them to the open-field test (OF) (Fig 5.1a, left). The field was subdivided in 4 (virtual) central quadrants and 12 (virtual) quadrants in the periphery (Fig 5.1a). Animal behaviour in the OF was observed during 5 minutes using digital video tracking system (Fig 5.1a; HVS Image Ltd.). OF test showed that ind-Dkk1 exhibit no ambulatory differences when compared to control animals, as demonstrated by the total path length (Fig 5.1b) and the total number of quadrants crossed by the animals (Fig 5.1c). Likewise, measurement of central exploration of the field, regarded as an anxiety-related

index, also show no difference among the two groups of animals (Fig. 5.1d). These results demonstrate that ind-Dkk1 animals do not present any abnormal levels of anxiety-like behaviour in comparison to control animals.

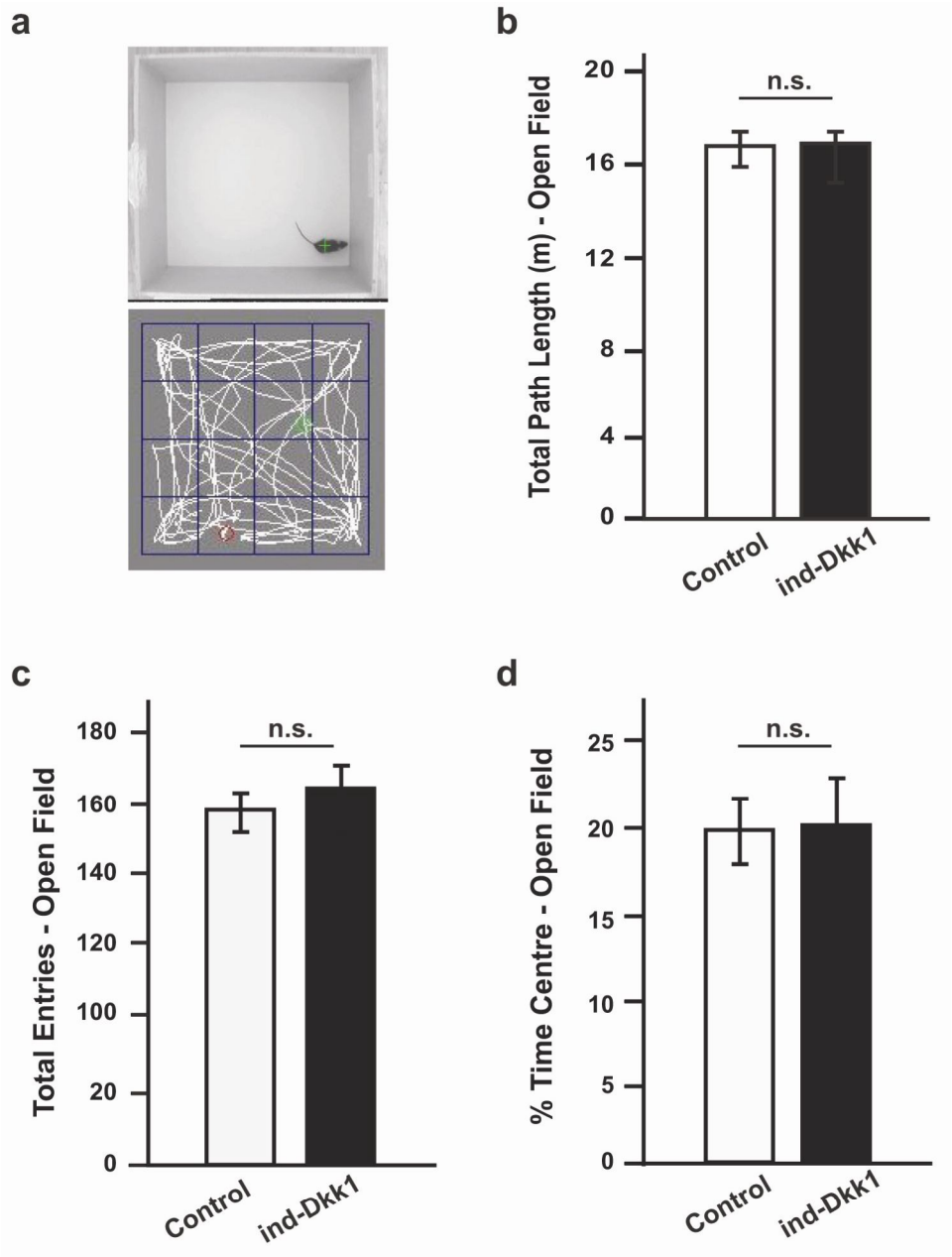

Figure 5.1 - Dkk1 expression does not induce any changes in the anxiety-like behaviour. (a) Snapshot of the Open-Field (OF) apparatus and a representative path designed by the software tracker. **(b)** ind-Dkk1 mice show equal levels of ambulatory behaviour when compared to control animals, as expressed by the total path length in the (OF). **(c)** Equal levels of anxiety in both groups were observed in the OF, as expressed by the number of entries in the different areas of the field and **(d)** percentage of time spent in the centre of the apparatus. Results are shown as mean ± SEM, n=11 ind-Dkk1 and n=15 controls, Student's t-test

To further confirm my findings, the same animals were tested in elevated plus-maze after 24h (Fig 5.2a, left), the most popular test for anxiety in rodents (Ramos, 2008). Following the previous test, I used ind-Dkk1 and control adult animals after 15 days of feeding with doxycycline and observed them for 5 minutes on the maze using digital video tracking system (Fig 5.2a, right; HVS Image Ltd.). Consistent with the OF test, no differences were found in the level of anxiety of our transgenic model, which is reflected by the total time spent in the unprotected arms (Fig 5.2b). Similarly to the OF test, no differences were found in the general locomotion between the control and the ind-Dkk1 groups – expressed by the total number of entries in the enclosed arms (Fig. 5.2c). Together with the OF test, these experiments demonstrate that there is no change in the levels of anxiety-like behaviour or locomotion in our animal model.

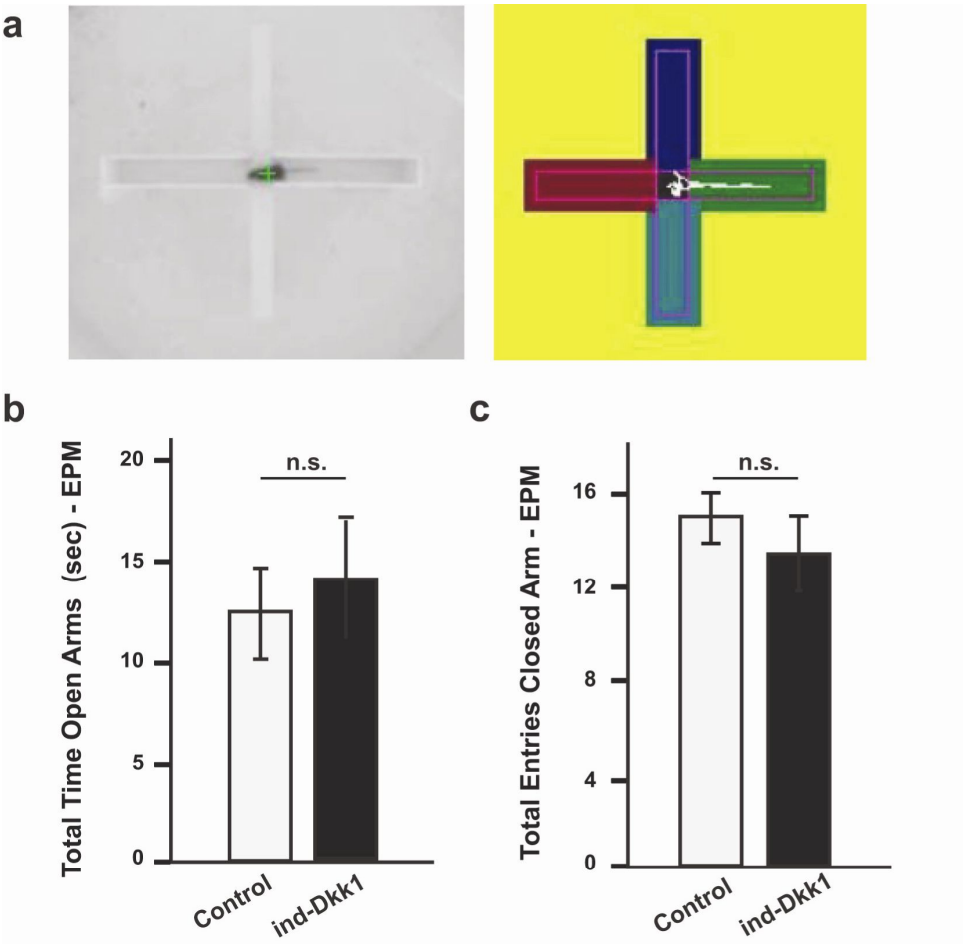

Figure 5.2 – Elevated plus maze (EPM) further confirm no alterations in the levels of anxiety-like behaviour in ind-Dkk1 animals. (a) Snapshot of the EPM apparatus and a representative path drawn by the software tracker. **(b)** ind-Dkk1 mice spend the same overall time in the open arms of the elevated-plus maze (EPM) and **(c)** total number of entries to the closed arms of the EPM than controls. Results are shown as mean ± SEM, n=11 ind-Dkk1 and n=15 controls, Student's t-test.

5.2.2. Disruption of Wnt signalling has no effect on working-memory

The hippocampus plays a crucial role in spatial memory (Bird & Burgess, 2008; Burgess et al., 2002). It has been demonstrated the essential role of the hippocampus in spatial alternation behaviour (Bannerman et al., 2014; Olton & Papas, 1979; Sanderson & Bannerman, 2012). Therefore, I tested whether ind-Dkk1 mice exhibit impairments in spatial memory by testing the animals in the T-maze test. Doxycycline was administered in the food pellets of both control and ind-Dkk1 groups for 14 days, as previously described.

The T-maze test is a widely used task to access spatial working memory, which is regarded as the information used by the rodent during the current experience of the task (S. Sharma et al., 2010). The test relies on basis that the animal has evolved to the optimal instinctive strategy to explore the environment. Short-term memory (working memory) was assessed by the spontaneous alternation protocol in the T-maze test (J. T. Becker & Morris, 1999; Lalonde, 2002; S. Sharma et al., 2010). In the spontaneous alternation protocol, the animal must remember which arm it has entered in the previous trial, to enable to alternate its choice in the following trial (Hughes, 2004).

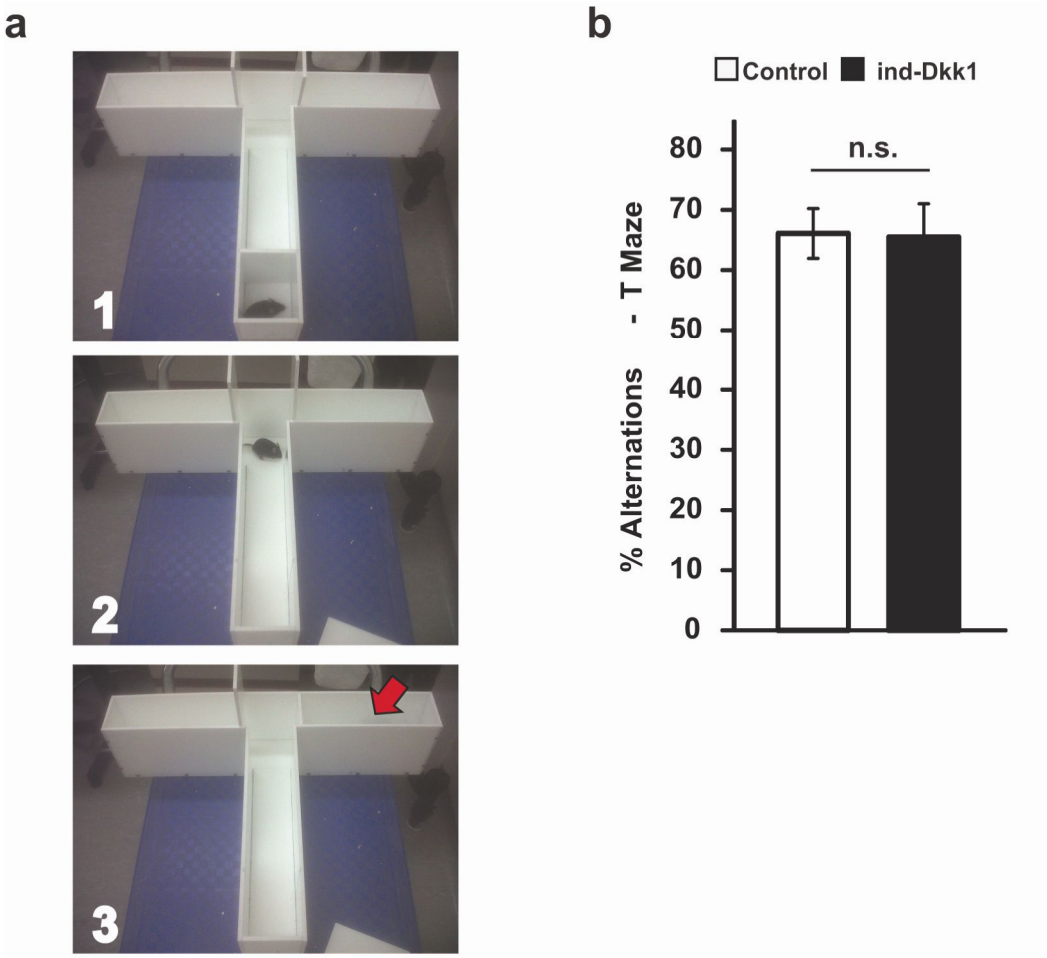

Figure 5.3 - Dkk1 expression does not cause impairment in spatial working memory. **(a)** Representative scheme of the T-maze paradigm. At the start of the trial the animal are held in the starting point (1). After 30 seconds, the guillotine is raised and the animal is allowed to walk down the stem and enter into an arm (2). Once the animal makes a choice the guillotine of the chosen arm is brought down and the animal is confined in the area for a further 30 seconds (3, red arrow). After the delay period, the animal is returned to the starting platform. (**b)** Dkk1 expression does not alter performance in the working-memory task, as demonstrated by unchanged percentage of alternation in the T-maze test. Results are shown as mean ±SEM. n=10 ind-Dkk1 and n=15 controls, Student's t-test.

The T-shaped maze was divided in 2 arms and one main stem, where the starting point was (Fig 5.3a). Each of the partitions had a removable guillotine door. At the start of the trial, the animals were held in the starting point (at the end of the main stem - Fig 5.3a.1). After 30 seconds, the guillotine was raised and the animals were allowed to walk down the stem and enter an arm (Fig 5.3a.2). Once the animal had made a choice (four feet within the boundaries of the arm), the guillotine of the chosen arm was brought down and the animal was confined in the area for a further

30 seconds (Fig 5.3a.3). After the delay period, the animals were returned to the starting platform. The protocol was repeated consecutively for 8 times and percentage of alternation was determined by manually counting the number of alternation between the left and the right arms, over the total number of trials. My analyses show that spontaneous alternation is unaffected in ind-Dkk1 mice, as demonstrated by the consistent percentage of alternation between control and ind-Dkk1 groups, and as both group of animals alternate between the two arms above chance (over 50%) (Fig 5.3b). These results demonstrate that working memory remains unaltered in the ind-Dkk1 animals.

5.2.3. Dkk1 expression leads to deficits in recognition memory

To test whether the loss of synapses and shrinkage of spines caused by the blockage of Wnt signalling in the hippocampus leads to memory impairment, I next tested our transgenic animals in the novel object recognition (NOR) task (Fig. 4.4a). Although still an ongoing debate in the field to which regions are supporting recognition memory (Vann & Albasser, 2011), many scientists believe that this paradigm evaluates recognition memory mediated by the hippocampus (Cohen et al., 2013; Mumby, 2001) and it relies on the fact that subjects will preferentially look and explore the new object, indicating that they remembered the familiar one (Squire et al., 2007). For this paradigm I used ind-Dkk1 and control adult littermates that had been fed with doxycycline for 6 days on their first exposure to the empty arena - habituation session. On the next day (1 week of doxycycline) animals were exposed to two identical objects, of which they were allowed to freely explore for 10 minutes (Fig. 5.4a.1). After 24h delay one of the familiar objects was changed to a new unfamiliar one (with a different shape, colour and texture) and the animals were then allowed to explore the objects for a further 10 minutes (Fig 5.4a.2). After 6 days, when the animals reached 14 days of Dkk1 expression, the unfamiliar object was once more changed to a new one and test was repeated (Fig 5.4a.3).

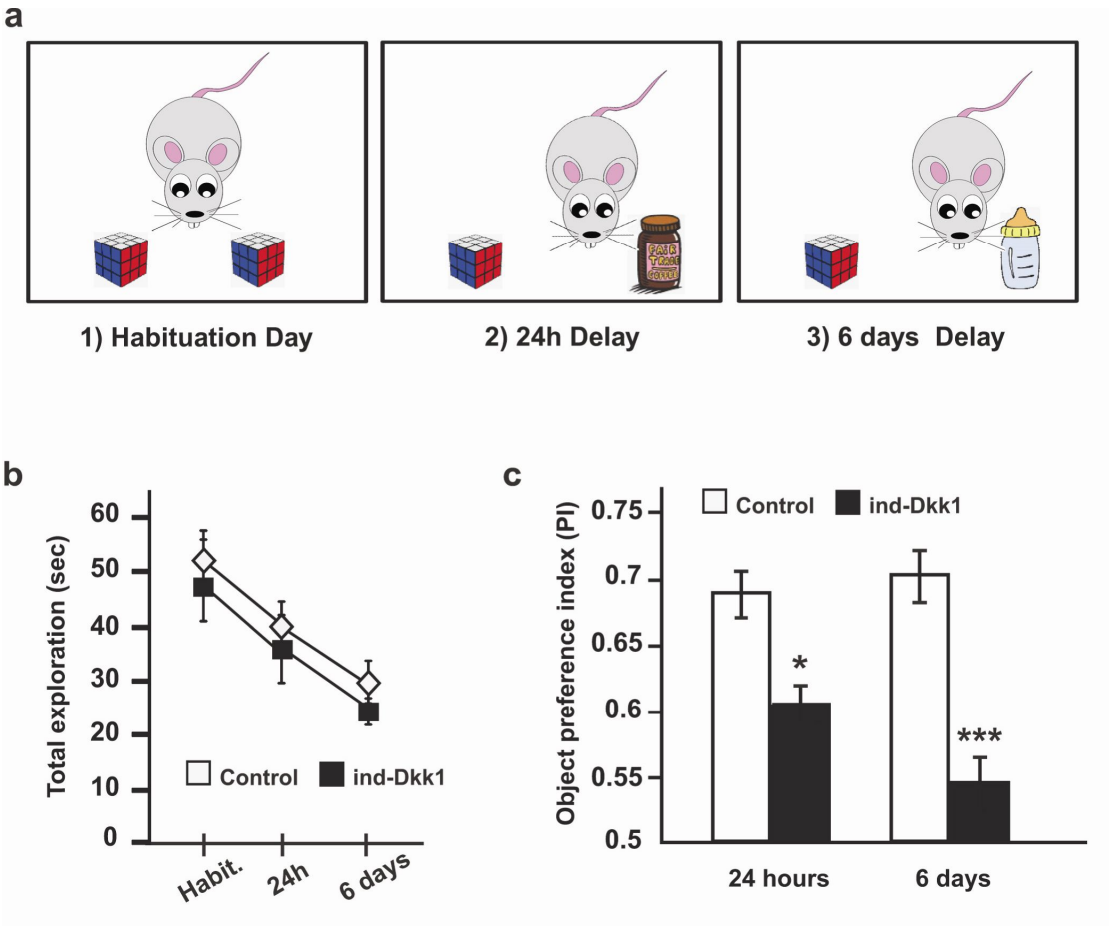

Figure 5.4 – Blockade of canonical Wnt signalling impairs long term recognition memory (a) Schematic representation of the Novel Object Recognition task - on habituation day (1), animals are presented with two identical objects, and after a 24h delay (2) and 6 days (3), one of the objects is swapped to a new unfamiliar object (**b)** Graph expresses the total time the animals spent exploring both objects during the test. Both groups spent similar times exploring the objects and, rate of exploration decreases with time. **(c)** Graph shows object preference index (PI). PI= (Time Novel Obj.)/ (Time Familiar + Time Novel Objects). A PI greater than 0.5 indicates a preference for the novel object. Ind-Dkk1 animals have impaired memory, which decays proportionally to the retention interval. Results are shown as mean ±SEM. n=12 ind-Dkk1 and n=16 controls, Student's t-test.

My analysis showed no differences in the behaviour of animals during the first exposure to the identical objects as demonstrated by the total exploration time exploring both objects (Fig 5.4b). As expected, total object exploration time throughout the task gradually decreased in both groups of animals (Fig 5.4b). On the first trial however, when one of the objects was replaced by an unfamiliar one, the analysis demonstrates that control animals spent significantly more time exploring the new object when compared to ind-Dkk1 animals (Fig. 5.4c). Interestingly, when tested 6 days after the first exposure, wild type animals spent most of their time exploring the unfamiliar, third new object, whereas ind-Dkk1 animals show no strong preference to the unfamiliar object (Fig 5.4c). These results demonstrate the

importance of an intact Wnt signalling for hippocampus normal function in long-term recognition memory.

5.2.4. Blockade of Wnt signalling leads to deficits in the MWM test

To further address the question to whether deficient Wnt signalling results in impaired learning, I performed the Morris Water Maze (MWM) test. The MWM is a reliable and robust paradigm that evaluates hippocampal function, and in particular spatial memory (R. Morris, 1984; Vorhees & Williams, 2006). For the test I used ind-Dkk1 and control adult littermates that had been fed with doxycycline for 5 days at the start of the task, given the known expression of Dkk1 at this time point in the transgenic animals (Galli & Salinas, *unpublished data*). Animals were fed with doxycycline continuously until the end of the experiment. During the whole experiments, animals were given 4 trials sessions per day, with an inter-interval trial of 10 minutes. Importantly no difference in the swim speed was found between control and ind-Dkk1 mice throughout the task (Fig 5.5a). Firstly, to examine whether the expression of Dkk1 in the ind-Dkk1 animals had resulted in any sensorimotor or motivation changes, I tested both groups of mice in the cued version of the task for 4 days. In this initial part of the experiment, which is generally considered as an experimental control condition, no differences were observed between control animals and ind-Dkk1 animals (Fig. 5.5b), demonstrating that both groups of animals had intact basic motor and visual abilities.

Interestingly, when tested with the hidden platform – in which the animals require distal cues in the room to locate the platform – a clear difference was observed. During the first two days of training, both groups took the same time to find the hidden platform. On the third and the fourth day of training however, analysis showed that ind-Dkk1 animals performed significantly worse than their control littermates, reflected on the longer time to reach the escape platform (Fig 5.5b) - visually represented by a longer pathway to reach the platform (Fig. 5.5c).

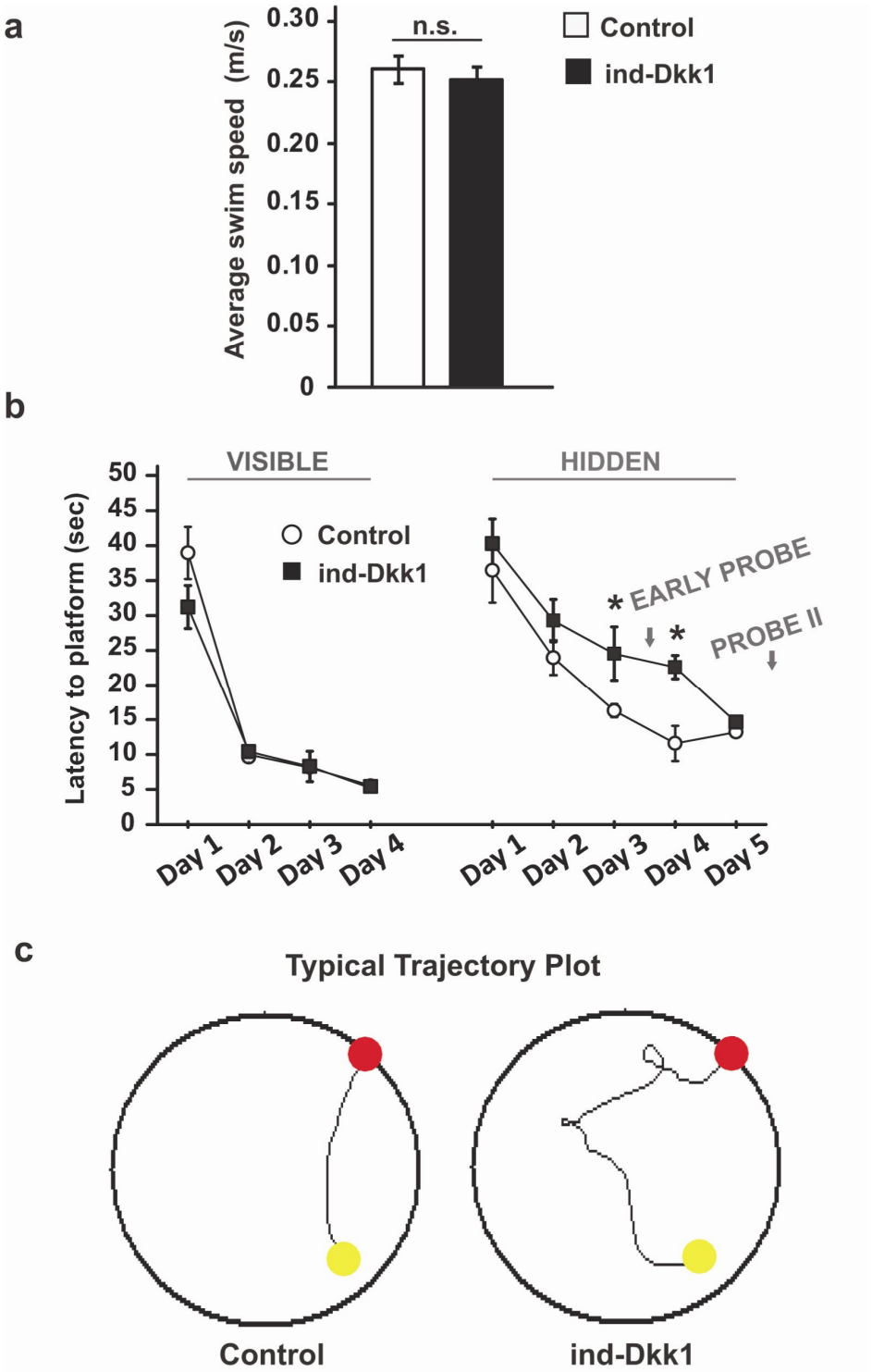

Figure 5.5 – Loss of synapses mediated by expression of Dkk1 causes impairment in spatial memory in the MWM task. (a) Average swim speed of the animals over the days over training remains the same between the control and ind-Dkk1 groups. **(b)** Latency to find the visible platform over a period of 4 days and the hidden platform across 5 days of training in the MWM. No differences between groups were found in the first stage of the task (visible platform). However, ind-Dkk1 animals take longer to locate the hidden platform when compared to controls. **(c)** Schematic representation of swim path drawn by the software tracker demonstrates that ind-Dkk1 animals swim a longer distance from the starting point (red spot) to reach the hidden platform (yellow spot). Results are shown as mean ±SEM. *p ≤ 0.05; **p≤ 0.01; n=12 ind-Dkk1 and n=11 controls, ANOVA repeated measures.

Furthermore, the first probe trial, which was carried out 24h after the 3rd day of training, when the platform was removed from the pool, revealed that Dkk1 expressing animals had worsened spatial memory than control animals. Analyses of the quadrant search times demonstrate that ind-Dkk1 animals spent significantly less time in the target quadrant than control animals (Fig. 5.6a). Furthermore, ind-Dkk1 crossed significantly fewer times the virtual platform than their control littermates (Fig. 5.6a – right panel). Analyses of the 4th day of the acquisition phase, demonstrate that ind-Dkk1 took longer to reach the hidden platform. Interestingly, latencies to reach the platform of ind-Dkk1 and control animals are equal at the 5th day of training (Fig. 5.6b). In the second probe trial, which took place 24h after the 5th day of training, ind-Dkk1 and control animals spent equivalent amount of time in the target quadrant (Fig. 5.6b) and crossed as many times the platform location as their littermates (Fig. 5.6b – right panel). These results demonstrate that blockade of Wnt signalling in the hippocampus leads to impaired spatial reference memory, a deficit that can be overcome when further training is given to Wnt signalling-deficient mice.

5.2.5. Dysregulation of Wnt signalling impairs contextual fear memory

To further determine the effects of Dkk1 expression on hippocampus-dependent cognition, I tested ind-Dkk1 mice in the contextual fear conditioning, a well-established hippocampus dependent task (Maren et al., 2013; Phillips & LeDoux, 1994). In this task, animals learn to associate the aversive stimulus, in this case a mild unexpected foot-shock, with the location in which it occurred (context box). Memory is scored proportionally to their freezing response to the context.

For this experiment, adult ind-Dkk1 and control littermates had been fed with doxycycline for 13 days at the beginning of the task. For the training period, mice were placed individually in the conditioning chamber and after a 2 minutes

habituation period an introductory period tone (80 dB, 3.0 kHz) was presented for 30 seconds, in which the last 2 seconds coincided with a footshock (0.7 mA). Contextual fear memory was tested 24 h after training by re-exposing to the conditioning chamber for 5 min, with no shock delivery and freezing behaviour was scored (Fig. 5.7a).

My analyses show that after 24h of the first exposure to the conditioning box, ind-Dkk1 mice exhibit an inability to associate the context with the foot shock, as demonstrated by the considerable reduced freezing time when compared to control mice (Fig 5.7b). Importantly, Dkk1 expression did not alter the baseline pain sensitivity, as both groups had equal levels of freezing in the first exposure to the shock (Fig 5.7b – baseline), showing that ind-Dkk1 animals do not present any abnormality in response to the aversive stimulus. The reduction in the total freezing time further demonstrates that ind-Dkk1 mice exhibit cognitive impairment, due to the blockade of Wnt canonical signalling, as shown previously in the other hippocampal-dependent tasks.

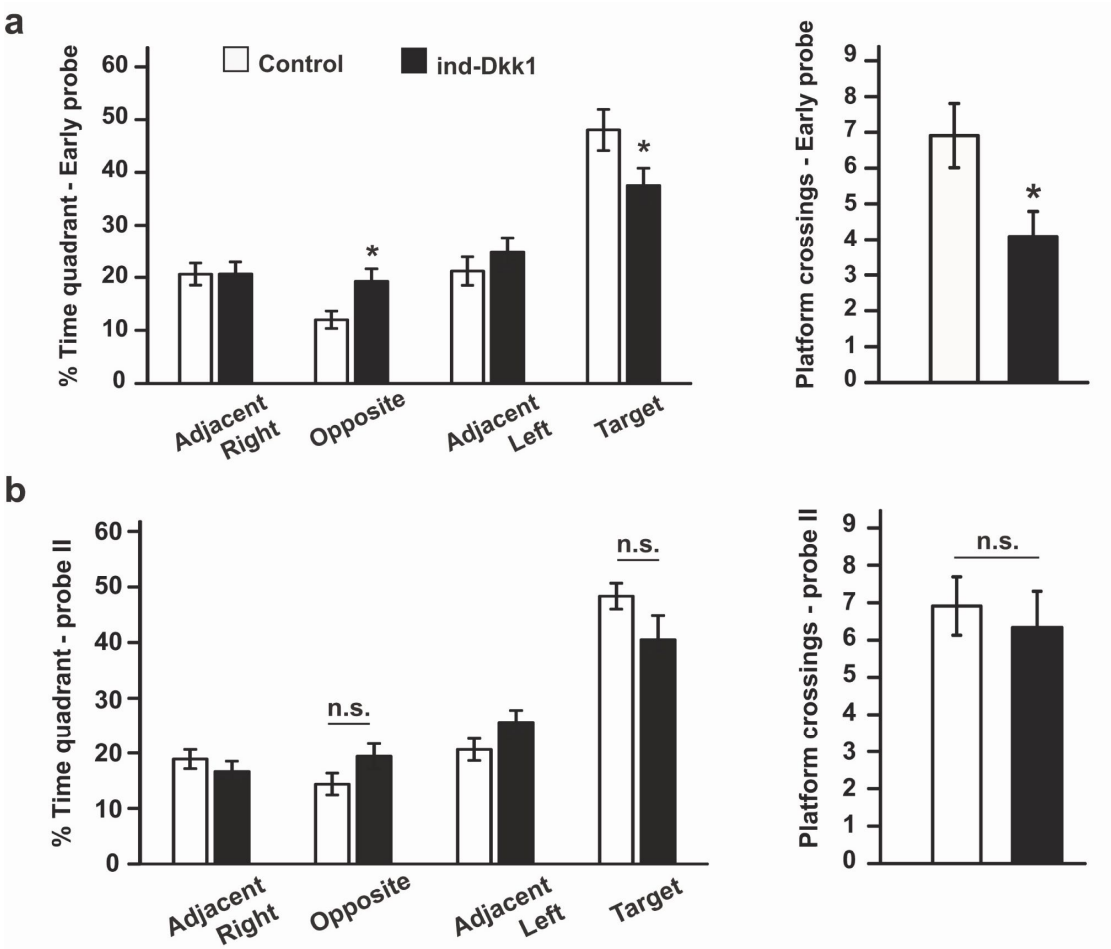

Figure 5.6 – Loss of synapses mediated by expression of Dkk1 causes impairment in spatial memory in the MWM task (cont). Percentage of time spent in each of the quadrants in the absence of platform. **(a)** After 3 days of training (early probe), ind-Dkk1 animals spent significantly less time in the target quadrant and cross less times the exact position where the platform was originally located (a - right panel). (**b)** After further training, ind-Dkk1 animals overcome the cognitive deficits and reach control levels (Probe II) (b- right panel). Results are shown as mean ±SEM. *p ≤ 0.05; **p≤ 0.01; n=12 ind-Dkk1 and n=11 controls, ANOVA.

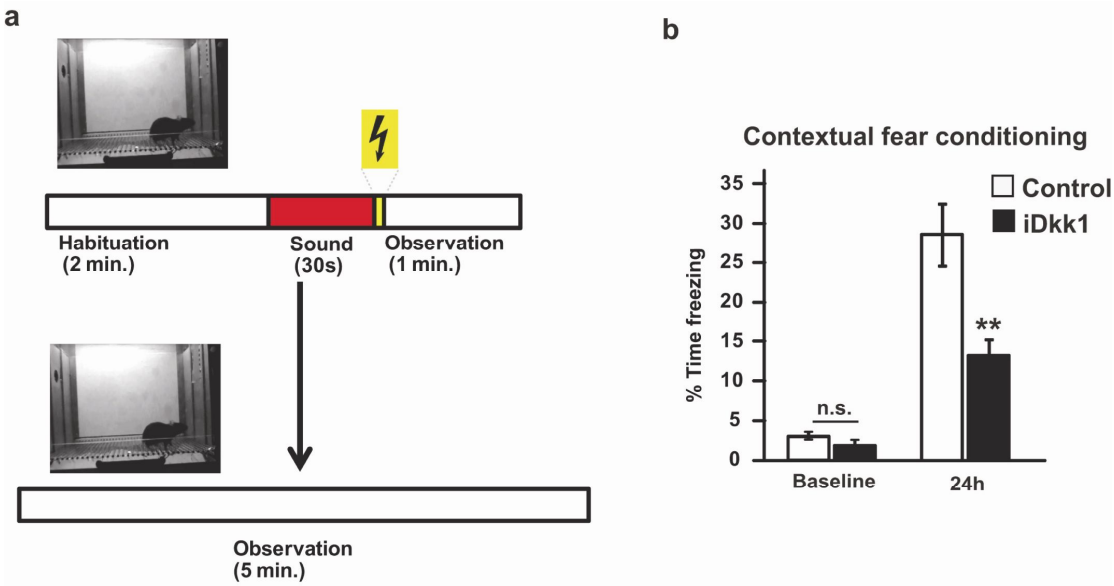

Figure 5.7 – Inducible expression of Dkk1 leads to impairment in contextual fear memory. (a) ind-Dkk1 mice were trained with 1 tone shock-pairing and tested for contextual memory after 24h. Mice were placed individually in the conditioning chamber and after a 2 minutes habituation period an introductory period tone was presented for 30 seconds, in which the last 2 seconds coincided with a footshock. Contextual fear memory was tested 24 h after training by re-exposing to the conditioning chamber for 5 minutes without shock and freezing behaviour was scored. **(b)** Baseline freezing demonstrates equal amount of time between groups during the first exposure to the contextual freezing task. After 24h however, animals expressing Dkk1 spent less time freezing when compared to controls. Results are shown as mean ±SEM. n=7 ind-Dkk1 and n=8 controls, ANOVA.

5.3. Discussion

The search for the role of Wnt signalling in the adulthood has started, particularly into their contribution to learning and memory. Indeed, Wnts are largely expressed in the adult brain (Gogolla et al., 2009; Salinas et al., 1994; Shimogori et al., 2004) and despite several evidence suggesting the involvement of Wnts in hippocampal-mediated behaviour (Fortress et al., 2013; Jessberger et al., 2009; Seib et al., 2013), there is not a clear demonstration that modulation of Wnt signalling affects behaviour. Therefore, the aim of this chapter was to address whether loss of synapses in the hippocampus, mediated by Dkk1 expression, leads to hippocampus-mediated behavioural changes. My studies demonstrate that animals with hippocampus deficient Wnt signalling exhibit cognitive dysfunction without having any affect on anxiety-like and exploratory behaviour. My results provide strong evidence that Wnt signalling is crucial for hippocampus function and, in combination

with the previously presented structural data, I successfully demonstrated for the first time a role of Wnts in synaptic maintenance and the link with the cognitive decline.

The hippocampus has an important role in emotions. Its ventral connections with numerous areas of the brain, such as septum, hypothalamus, pre-frontal cortex and amygdala, are highly implicated in stress and anxiety behaviour (Bannerman et al., 2004; Engin & Treit, 2007; Kjelstrup et al., 2002; Leuner & Shors, 2012; Moser & Moser, 1998; W. N. Zhang et al., 2013). Importantly, it has been demonstrated a link between stress and anxiety-related behaviours and morphological changes in dendritic spines at those areas implicated in these behaviours (Leuner & Shors, 2012). For instance, morphological analyses revealed that the hippocampus is very responsive to stressful events, and such events leads to synaptic alterations and even spines loss (Adamec et al., 2012; Bessa et al., 2009; Donohue et al., 2006; Magarinos et al., 1997; Sandi et al., 2003; Stewart et al., 2005) particularly in the CA3 region (Magarinos et al., 1997; Sandi et al., 2003; Stewart et al., 2005). Interestingly, a recent study demonstrated a strong link between stress and downregulation of Wnt signalling in the hippocampus (Matrisciano et al., 2011). The authors show that mild stress leads to increased expression of Dkk1, decrease in hippocampal volume and subsequent alterations in the neuronal morphology (Matrisciano et al., 2011).

My results however, show that ind-Dkk1 exhibit no alteration in the level of anxiety. The inconsistency with the literature can be explained by regional differences in the expression of Dkk1 in our animal model, as it is not clear whether the expression of Dkk1 occurs in the ventral hippocampus and, crucially, if the connections with the other key areas modulating stress and anxiety behaviours are affected by its expression. More importantly, one key observation in the literature suggests that spine shrinkage and loss in the hippocampus area is a consequence of stress/anxiety rather than the cause of it (Magarinos et al., 1997; Matrisciano et al., 2011; Sandi et al., 2003; Stewart et al., 2005). Overall, my results come as an essential piece of information, as these animals were tested in other behavioural paradigms, and any

changes at their anxiety levels could have negative impacts in the other tests, specially those evaluating memory.

Disruption of Wnt signalling in the CA1 hippocampus does not affect working memory. It has been demonstrated the hippocampus is essential for working memory tasks in rodents, and notably this immediate memory processing in rodents is very distinct to humans (Sanderson & Bannerman, 2012). In humans, working memory is independent of the medium temporal lobe (MTL) (Baddeley et al., 2011; Jeneson & Squire, 2012), whereas cytotoxic lesion in the dorsal hippocampus of rodents leads to poor performance in the T maze task (Bannerman et al., 1999). In addition, it has been proposed that in rodents spatial working memory is also dependent on hippocampal glutamate receptors (Lee & Kesner, 2002). Blockade of NMDA receptors, specifically in the CA3 subregion, results in poor performance in spatial working memory in the radial maze (Lee & Kesner, 2002). Furthermore, studies looking at AMPA receptors also demonstrated that constitutive GluR1 knockout mice have specific spatial working memory impairment when compared to control mice (Reisel et al., 2002; Sanderson & Bannerman, 2012). Here I showed that although ind-Dkk1 mice present loss of synapses in the hippocampus CA1, it does not present impairment in working memory. Importantly, in the previous chapter, I also showed that CA3 synapses remain unaffected by Dkk1 expression. Given the importance of CA3 for working memory (Lee & Kesner, 2002), I speculate that ind-Dkk1 mice does not present any defect in working memory due to the preservation of the synapses in the CA3 area. Although further investigation is needed to determine whether glutamate receptors are affected by expression of Dkk1, my data suggests that these receptors seem to be spared in the CA3 region, explaining my results on short-term memory.

Increasing evidence suggest a role for neurogenesis in some forms of memory. As previously discussed in Chapter 3, a role for Wnt signalling in adult neurogenesis has been demonstrated. Indeed, Wnt signalling promotes neurogenesis in the dentate gyrus (DG) and contributes to hippocampal dependent behaviours (Jessberger et al., 2009; Lie et al., 2005; Qu et al., 2009; Seib et al., 2013). Although controversial, a full

body of evidence demonstrate that spatial working memory is a neurogenesis dependent process (Corsini et al., 2009; Farioli-Vecchioli et al., 2008; Madsen et al., 2003; Saxe et al., 2007). In my study I demonstrated that inducible expression of Dkk1 in adult mice does not affect neurogenesis (Chapter 3), further supporting my findings that working memory would not be affected in ind-Dkk1 mice. Thus, my histological, structural and behaviour analyses consistently demonstrate that neither the CA3 regions, nor neurogenesis in the DG are affected by Dkk1 expression, reflected on the sound performance of the ind-Dkk1 animals in the working memory task.

Dkk1 expression has profound effects on recognition memory. It has been proposed that recognition memory is dependent of the medial temporal lobe, where the hippocampus is essential to recollection, whereas the perirhinal cortex is involved in the familiarity component of the task (Squire et al., 2007). My results show that ind-Dkk1 mice present a significant impairment in the OR task after long-term delays. Similarly, a recent study has demonstrated that hippocampal infusion of recombinant Dkk1 protein result in impairment in the OR consolidation (Fortress et al., 2013). Despite the involvement of the hippocampus in recognition memory being controversial, as many studies show that hippocampal lesion produce no effect in OR (Barker & Warburton, 2011; Mumby, 2001), a number of studies show that hippocampal lesions do have a negative impact in the OR task (Broadbent et al., 2004; Clark et al., 2000; Clarke et al., 2013). Although as it can not be ruled out that Wnt signalling is defected in the perirhinal cortex in ind-Dkk1 mice, a region that is believed to be crucial for OR (Barker & Warburton, 2011; Ennaceur et al., 1996; Mumby & Pinel, 1994; Norman & Eacott, 2004), yet my studies demonstrate a link between Wnt canonical signalling and recognition memory. Furthermore, my study shows that the behavioural impairment observed strongly correlates to deficits in the maintenance of synapses – a phenomenon never reported before.

Wnt mediated synaptic maintenance is important for other aspects of memory. Despite the evidence in the literature, to date only one study has investigated the involvement of Wnt signalling in hippocampal spatial learning (Tabatadze et al.,

2012). My result shows that deficiency in Wnt signalling in the hippocampus leads to deficits in memory acquisition in the MWM. Supporting my findings, a study has demonstrated that increased activity in Gsk3-β, a key component for Wnt canonical signalling, leads to similar defects in the MWM (Hernandez et al., 2002). Animals conditionally over-expressing Gsk3-β also present longer latency to reach the hidden platform from day 3 of training in the MWM (Hernandez et al., 2002), a phenotype resembled in the ind-Dkk1 mice. Interestingly, given that ind-Dkk1 animals eventually learn to locate the platform in the MWM, my data also suggests that the memory retrieval and encoding processes may remain intact.

Memory impairment in the ind-Dkk1 mice was further confirmed by the contextual fear conditioning. This paradigm is dependent on the intact hippocampus to associate the training context with a mild foot shock (Anagnostaras et al., 1999; J. J. Kim et al., 1993; Phillips & LeDoux, 1992, 1994). Indeed, my results demonstrate that loss of synapses in the hippocampus, as consequence of disrupted Wnt signalling, leads to an impaired learn ability in the task. Importantly, it has been shown that constitutive expression of Dkk1 in the brain results in deficit in the same task (Killick et al., 2012). In addition, a recent study demonstrated that mice that has LRP6 receptors conditionally deleted at the CA1 hippocampus, also present a similar phenotype (C. C. Liu et al., 2014). However, the cited studies did not clarify the cause of the impairment - for instance if it was due the loss of synapses, loss of neurons or, in the case where Dkk1 was constitutively expressed (Killick et al., 2012), due to any early any developmental abnormality. Here, I show a clear link between Dkk1 expression, loss of synapses and the consolidation of new memories during the learning process.

In conclusion, the results presented in this chapter demonstrate for the first time a clear link between the loss of synapses induced by expression of Dkk1 and cognitive impairment. Given the emerging link of Wnt signalling and that increased expression of Dkk1 has been recently demonstrated to be associated in neurodegenerative diseases (Caricasole et al., 2004; De Ferrari & Inestrosa, 2000; Purro et al., 2012), my

data was the ultimate demonstration that increased levels of Dkk1 in the adult hippocampus induce loss of excitatory synapses which leads to cognitive impairment.

Chapter 6

6. Discussion

6.1. Summary of results

Recent studies have started to investigate a role for Wnt signalling in the adult brain as several Wnts, their receptors and signalling components are expressed in many brain areas (Coyle-Rink et al., 2002; De Ferrari et al., 2007; Gogolla et al., 2009; Salinas et al., 1994; Shimogori et al., 2004). Studies from different labs have demonstrated the role of Wnt signalling in synaptic plasticity (Avila et al., 2010; Budnik & Salinas, 2011; Cerpa et al., 2008; J. Chen et al., 2006). However, the function of Wnt signalling in synaptic integrity in the adult brain has not been addressed so far. Here, using a combination of cell biology, molecular biology, biochemistry and behavioural assays, I demonstrated a novel role for Wnt signalling in synaptic maintenance and in spatial memory in the adult hippocampus.

Inducible expression of Dkk1, a Wnt antagonist, in a subset of principal neurons in adult mice has allowed me study the contribution of canonical Wnt signalling to adult synaptic maintenance in the adult brain without affecting early developmental stages. Induction of Dkk1 expression for 14 days led to the loss of excitatory synapses without affecting inhibitory synapses. Importantly, the effect of Dkk1 on synapses occurs in the absence of neuronal death or changes in the stem cell niche, demonstrating that Dkk1 directly affects the integrity of mature synapses. Moreover, I also demonstrate that blockade of Wnt signalling impairs spatial and recognition memory. Furthermore, my studies also provide some insights to the mechanisms by which Dkk1 induces synapse disassembly. Given the recent link between dysregulation of Wnt signalling and AD and PD (Berwick & Harvey, 2014; Caricasole et al., 2004; De Ferrari & Inestrosa, 2000; Killick et al., 2012; Purro et al., 2012), my results suggest new possible targets to tackle synaptic degeneration, before neuronal death.

6.2. ind-Dkk1: a new model to study degeneration

The use of transgenic models to study the mechanism of neurodegeneration and develop new targets to prevent neurodegenerative diseases continues to raise and gain credibility in the neuroscience field. Since the first report of a transgenic model that successfully replicates Alzheimer-type neuropathology (Games et al., 1995), numerous animal models to address AD have been created and help to explain the pathology. However, by replicating human AD, most of these animal models also have extensive neuronal death due to the toxicity of Aβ overproduction and accumulation, a phenomenon accompanied by the reduction of several structures in the brain (Duff, 2001). Indeed, the existence of neuronal demise is a feature of many other existent animal models for neurological diseases, including stroke and Huntington's disease (Leist & Nicotera, 1998; Nicotera et al., 1999). To this extent, cell death represents a significant limiting factor to study the mechanisms that lead to synapse loss and dysfunction, as the loss of synapses could be secondary effect from the loss of cell viability.

The data presented in Chapter 4 gives a strong argument to the use of the ind-Dkk1 animal model to study synaptic degeneration without cell death. Although previous studies have suggested that increased Dkk1 expression correlates with neuronal death in models of chronic stress, epilepsy, ischemia and Alzheimer's disease (Busceti et al., 2007; Caricasole et al., 2004; Matrisciano et al., 2011; Rosi et al., 2010), the thorough examination of ind-Dkk1 mice shows that induction of Dkk1 expression for 2 weeks does not lead to neuronal apoptosis in the hippocampus. To further support these findings, unpublished data from our lab show that long term expression of Dkk1 (3.5 months) does not induce cell loss in the hippocampus (Galli & Salinas*, unpublished data*). Furthermore, in the case of neurodegenerative diseases such as AD and PD, much evidence demonstrates that cell loss is an invariable feature, but it is not clear which form of cell death – either apoptosis or necrosis - is prevalent and more relevant to the progression of the disease (Nicotera et al., 1999). In the ind-Dkk1 model, my analyses demonstrate no occurrence of necrosis upon Dkk1 expression. Together, my data demonstrate that Dkk1

expression *per se* do not lead to any form of cell death in the hippocampus. However, given the published work on Dkk1 and cell death, it can not be ruled out that presence of Dkk1 increases the vulnerability of neurons to insults, such as hypoxia and ischemia, therefore placing Dkk1 as a risk factor under certain conditions. For instance, it would be interesting to examine whether ind-Dkk1 animals, or neurons treated with recombinant Dkk1, would be more vulnerable to ischemic insults or other challenges. Further studies to examine the contribution of Dkk1 to neuronal vulnerability are necessary to answer this question.

ind-Dkk1 is a unique model to study early stages of synaptic disassembly. Given my observations that Dkk1-mediated synaptic loss, the ind-Dkk1 model comes as a new system to understand synaptic disassembly in a time-scale manner. The possibility of crossing the ind-Dkk1 mice with a transgenic line expressing fluorescent proteins which label a specific subset of neurons or proteins, in combination with the technology of long-term live imaging (De Paola et al., 2003; A. J. Holtmaat et al., 2005), could allow a detailed and accurate investigation in the process of disassembly. For instance, this technique could reveal whether the location or shape of dendritic spines influence their stability in the context of disassembly. Furthermore, it could also allow the investigation into the hierarchy of the disassembly, and confirm whether loss of vGlut occurs prior to the loss of glutamate receptors and other postsynaptic proteins, as suggested by my results. In summary, my findings demonstrate the uniqueness of this animal model to study Wnt-mediated synapse stability, and also support the use of the ind-Dkk1 line for further studies in the investigation of synapse disassembly.

6.3. Wnt signalling in synaptic stability – a role in the maintenance of specific synapses

Wnt canonical signalling is required for the maintenance of excitatory synapses in the adult brain. Studies have demonstrated that Wnt are expressed in adulthood (Gogolla et al., 2009; Salinas et al., 1994; Shimogori et al., 2004; Tabatadze et al., 2012), but despite the evidence, there is no clear justification to the presence of

Wnts in the adult brain – could the expression represent a role for Wnt signalling in synaptic maintenance? Here I demonstrate that indeed, endogenous Wnt regulate synaptic stability in the adult hippocampus.

Recent studies from our lab suggested that endogenous Wnts regulate the maintenance of synapses in cultured neurons (Dickins, 2011; Purro et al., 2012). Our previous *in vitro* studies showed short-term exposure to Dkk1 leads to dispersal of synaptic vesicles clusters, in a relative short period of time (20 min.) (Purro et al., 2012). Furthermore, data from our lab has also showed that blockade of Wnt signalling with Dkk1 induces the disassembly and loss of a number of puncta for pre- and postsynaptic protein markers such as Bassoon, Cask, Synapsin1, PSD95 and Neuroligin-2 suggesting full synaptic disassembly (Dickins, 2011; Purro et al., 2012). Importantly, the study also show that Dkk1 treatment increases in de-localization of two presynaptic markers VAMP2/Bassoon suggesting presynaptic disassembly (Dickins, 2011). In addition, ultrastructural analyses revealed that Wnt blockade significantly reduces the length of both the AZ and PSD at remaining synapses (Dickins, 2011). However, the *in vivo* role of Wnts in synapse integrity has not been demonstrated. Moreover, it was not demonstrated whether the Dkk1-mediated disassembly was a "general" effect to subtypes of synapses, or canonical Wnt pathway is involved in the maintenance of a specific type of synapse.

To explore these questions, I evaluated the effect of Dkk1 in an *in vivo* system and examined different subtypes of synapses. My analyses show that in a physiological context, inhibition of Wnt signalling leads to the loss of excitatory synapses, and have no effect on inhibitory synapses (Fig 6.1). In addition, my studies show that in both *ex vivo* and *in vivo* models, the effect of Dkk1 on excitatory synapses appears to have a stronger, or possibly initial, effect at the presynaptic site. Furthermore, my analyses at the ultrastructural level reveal that Wnt blockade leads to an increase in the number of hemi-synapses – further suggesting that the presynaptic site is primarily affected by the expression of Dkk1. My findings are supported by studies which demonstrate that synaptic elimination is initiated by the dispersal of presynaptic associated proteins (De Paola et al., 2003; Hopf et al., 2002). Despite the

fact that the techniques used in my study could not determine the ultimate hierarchy of disassembly, my findings suggest that structurally, Dkk1-mediated synaptic disassembly has a notorious effect, which may be initiated, at the presynaptic site, which in some cases is followed by the loss of the postsynaptic site (Fig 6.2).

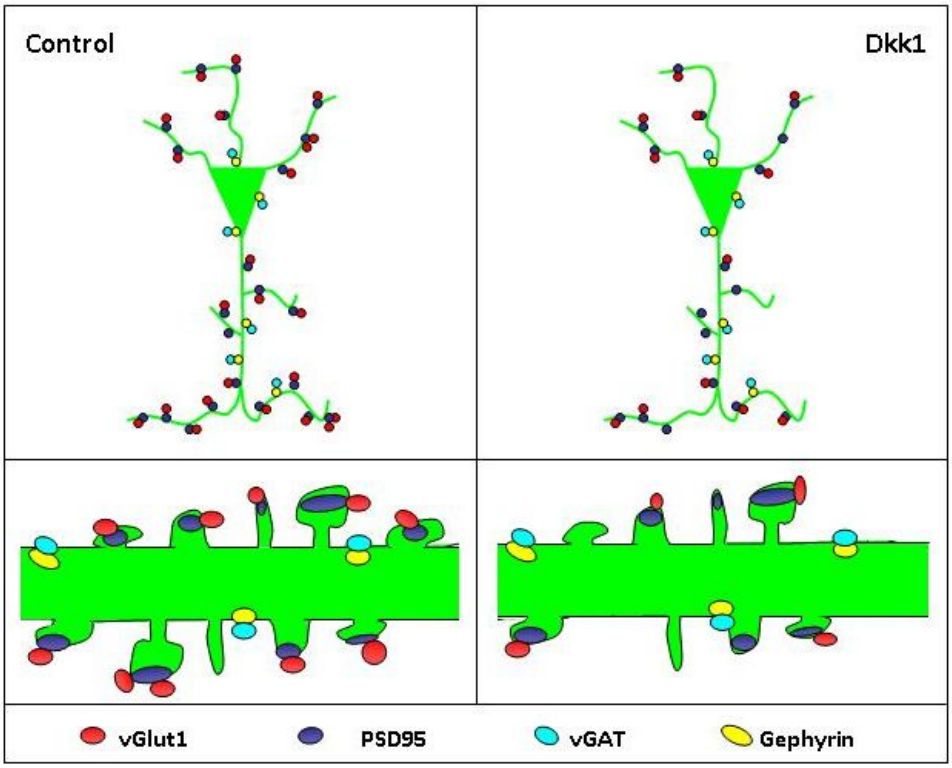

Figure 6.1: Blockade of Wnt canonical signalling leads to the disassembly of specific types of synapses. Presence of Dkk1 (right) causes the loss of vGlut1 puncta (red), with no change in the number of PSD-95 labelled sites and a small reduction in the number of dendritic spines. In ind-Dkk1 mice and ex vivo brain slices treated with recombinant Dkk1, there is almost a 50% reduction in the number of excitatory synapses based on the colocalisaton of vGlut1 with PSD95 (left). In contrast, Dkk1 has no effect on inhibitory synapses, shown by vGAT (light blue) and Gephyrin (yellow) labelled puncta and their unchanged percentage of colocalisation upon Dkk1 presence.

Synapses are not indiscriminately affected in neurodegenerative diseases. It has been proposed that amyloid pathology progresses in a time and neurotransmitter-specific manner (Bell et al., 2003). Studies using transgenic mouse models for AD have suggested that cholinergic synapses are the most vulnerable to disassembly in AD, followed by the glutamatergic synaptic boutons, and then gabaergic synapses (Bell et al., 2003; Bell et al., 2006). Although the reason to the resilience of inhibitory synapses remains elusive, these studies propose that “synaptic targeting” to degeneration, in the context of amyloidosis, is directly correlated to the progression

of the disease. Interestingly, this theory was recently reproduced and further supported by another research group using a different transgenic model for AD (Mitew et al., 2013) and, more importantly, also demonstrated in AD patients at late stages of the disease (Mitew et al., 2013). According to their findings, GABAergic synapses – identified by the presence of VGAT - are more resilient and remain unaffected in human pre-clinical and end stages of AD (Mitew et al., 2013).

Yet, supporting the neurotransmitter-specific targeting, a more recent study showed that intra-cerebral administration of soluble Aβ leads to a decrease in the content of vesicular glutamate transporter (vGlut1), and unchanged levels in vesicular GABA transporter (Canas et al., 2014). Furthermore, a transgenic mouse line (TgCRND8) which has previously been reported to express Dkk1 in the brain (Rosi et al., 2010), also have unchanged numbers of GABAergic synapses, despite the substantial Aβ load (Krantic et al., 2012). Given our previous findings which demonstrate that Aβ treatment leads to an increased expression of Dkk1, and correlate to the disassembly of synapses (Purro et al., 2012), these studies strongly support my findings which show the resilience of GABAergic synapses in the context of degeneration. Further studies will be necessary to determine whether cholinergic synapses are also more susceptible to Dkk1-mediated disassembly in relation to the other subtypes of synapses.

Wnt signalling-mediated maintenance: is canonical Wnt exclusively mediating the stability of a subtype of synapses? Recently, increased attention to the hippocampal dopaminergic system is being given due to its role in memory (Chowdhury et al., 2012; S. Lewis, 2012); in addition to its role mediating the interaction between hippocampus and other brain areas such ventral tegmental area and pre-frontal cortex (Rossato et al., 2009; Takahashi et al., 2008). Furthermore, it has been proposed a strong and direct correlation between loss of dopamine receptors and memory function in AD (Kemppainen et al., 2003).

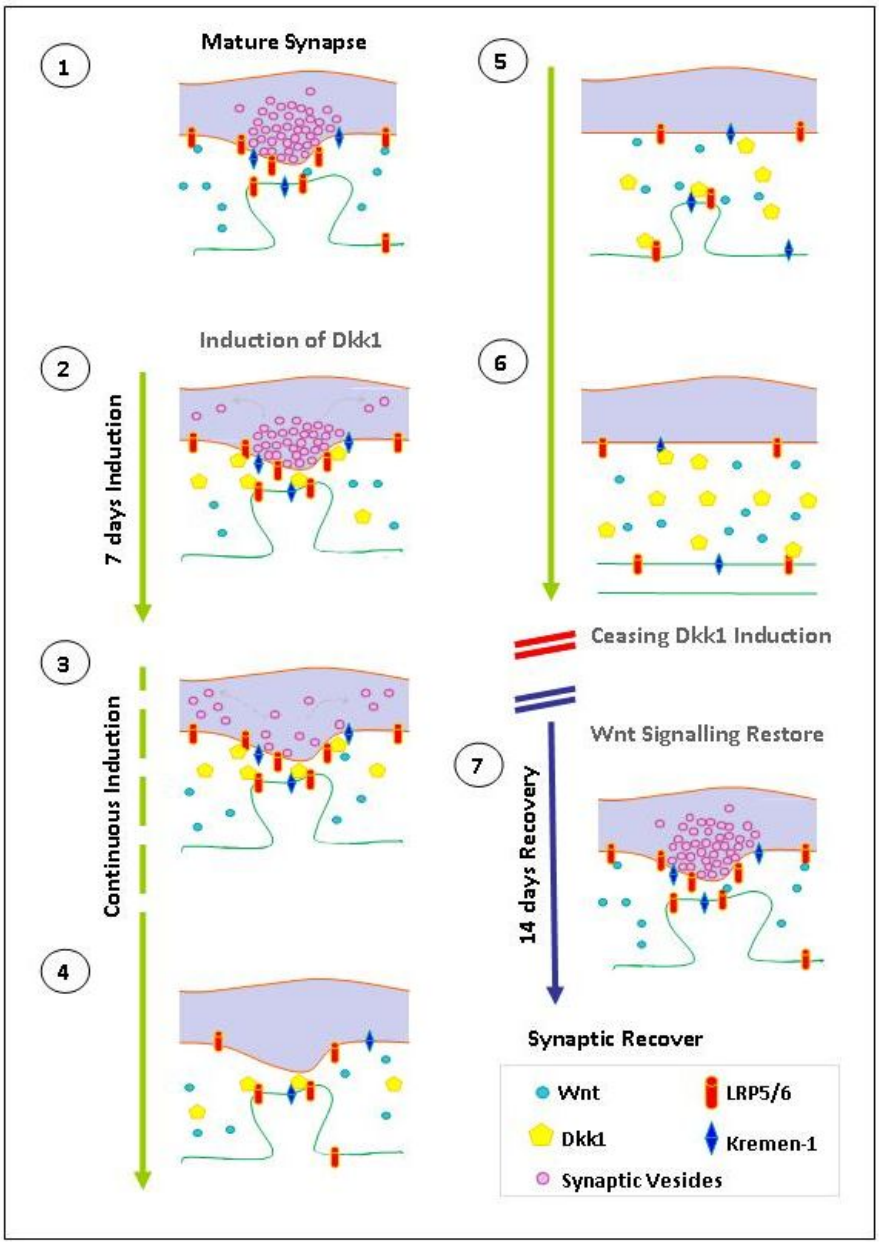

Fig 6.2 – A model for Dkk1-mediated synaptic disassembly. The cartoon outlines the main steps during synaptic disassembly, observed in this study. 1) Endogenous Wts are crucial for the maintenance of mature synapses. 2) 7 days of Dkk1 expression leads to undetectable changes at the synapses by immunohistochemistry. Possibly, at this stage synaptic vesicles start to disperse from the presynaptic site; at the postsynaptic very little change is observed at the dendritic spine size. 3) Continuous expression of Dkk1 (14days) culminates in the dispersal of the presynaptic vesicles, 4) leading to the increase of hemy-synapses; 5) shrinkage of spines and 6) total disassemble of synapses. 7) Interestingly, once Wnt signalling is restored, the synapses lost are recovered. Whether the recovered synapses reform at the same site of the previously lost one, and the order of events during the re-formation is yet to be studied.

Given the clear evidence to the importance of dopaminergic synapses in the hippocampus, I briefly investigated the role of Wnt signalling to dopaminergic

synapses in the CA1 area of the hippocampus, where dopamine receptors are highly concentrated (Amenta et al., 2001; Goldsmith & Joyce, 1994; Otmakhova & Lisman, 1999). A significant decrease in the number of dopamine receptor was observed in the ind-Dkk1 animal model, resulting in a decrease of the total number of modulatory synapses in our animal model (*Lopes & Salinas, unpublished preliminary data*). Supporting these preliminary findings, a recent study from our lab also demonstrated the importance of Wnt canonical signalling for the maintenance of dopaminergic synapses in the striatum (Galli, 2014). Although preliminary, my data suggest that maintenance of dopaminergic synapses in the hippocampus is also Wnt-signalling mediated and emphasize the importance of Wnt canonical signalling for specific subtypes of synapses.

In summary, my results demonstrate for the first time the role of Wnt-canonical signalling for the maintenance excitatory synapses. In addition, my preliminary results also suggest the involvement of Wnts in dopaminergic synaptic stability. Together my data show the involvement of Wnts in the maintenance of a specific subset of synapses and, given the similarity with the progression of AD, it also advocates that some types of synapses might need prioritising when looking into pharmacological manipulations that aim to protect synapses from degeneration – depending on the context and advance of the disease. Furthermore, my results demonstrate that lost synapses can be recovered by restoring Wnt signalling, putting Wnts and its associated signalling molecules as strong candidates to battle neurodegeneration.

6.4. Wnts and hippocampal function

A link between Wnt signalling, hippocampal function and memory is starting to emerge. Growing evidence points towards Wnts as key regulators of synaptic plasticity. Indeed, studies show that inhibition of Wnt signalling leads to impairment of long term potentiation (LTP) in the hippocampus (Cerpa et al., 2011; J. Chen et al., 2006). Similarly, conditional overexpression of GSK3β in adult mice also causes

defects in LTP (Hooper et al., 2007). By contrast, studies show that activation of Wnt signalling, by inhibiting GSK3β, increases the magnitude of LTP (J. Chen et al., 2006; Hooper et al., 2007). In addition, it has been shown that LTP in brain slices induces the secretion of Wnt3 (J. Chen et al., 2006), further suggesting the involvement of Wnts in hippocampus plasticity. Furthermore, electrophysiological studies in the ind-Dkk1 recently performed by a colleague working in our lab (Dr. Aude Marzo), demonstrated that chronic blockade of Wnt signalling by Dkk1 expression has profound impact in hippocampal induced LTP (Fig Supplementary 1). Induction of LTP is profoundly impaired in ind-Dkk1 mice, demonstrating that endogenous Wnt signalling is required for synaptic plasticity in the hippocampus.

Adding to these findings, a study correlated the secretion of Wnts with brain activity, as behavioural experience increased significantly the levels of Wnt7a/b in the hippocampus (Gogolla et al., 2009). Interestingly, this study also shows that Wnt7a/b secretion decline with age, which is accompanied by the loss of complexity and number of synapses (Gogolla et al., 2009). Given the clear evidence of the importance of endogenous Wnts in plasticity in the hippocampus and now, demonstrated in this thesis, for synaptic maintenance, it also creates a strong link to the importance of Wnt for hippocampus-mediated memory.

Recently, a study showed that disruption of canonical Wnt signalling in adult flies impairs long term memory (Tan et al., 2013), a phenotype that can be rescued by the overexpression of β-catenin (Tan et al., 2013). In mammals, Wnt secretion has also been correlated with learning (Tabatadze et al., 2012). Spatial learning in the Morris water maze (MWM) selectively increases the levels of Wnt7a/b and Wnt5 in the hippocampus (Tabatadze et al., 2012). Interestingly, the elevation in Wnt secretion levels is observed only 7 days after training, and is maintained after long periods of time, further suggesting a role for Wnt signalling in log-term memories (Tabatadze et al., 2012). In addition to the above evidence, a recent study has demonstrated a further link between Wnt signalling in adulthood and memory (C. C. Liu et al., 2014). The latter demonstrates that deletion of neuronal LRP6 in the mouse hippocampus,

by using Cre-lox recombination technology, leads to impairment in the contextual fear conditioning (C. C. Liu et al., 2014). Although the cited studies do not show a clear relationship between Wnt signalling and synaptic maintenance in the adult CNS, collectively these studies suggest a strong association between Wnt signalling and learning behaviour.

Following these observations, my results clearly demonstrate a link between Dkk1-mediated synaptic disassembly in the hippocampus and memory impairment. The behavioural data presented in this study demonstrated that expression of Dkk1 in the hippocampus leads to long term memory dysfunction, as shown by the learning defects in the fear context task and in the MWM. Interestingly, blockade of Wnt signalling with Dkk1 does not have an effect on short term memory (working memory). Adding to these results, a pilot experiment training the animals in the MWM using an alternative protocol (two training sessions a day, 4 hours apart – Figure Supplementary 2) suggests that ind-Dkk1 mice have defects in memory consolidation, as they are able to learn in between sessions, but unable to consolidate the information newly encoded. Supporting my findings, a recent study showed that blockade of Wnt canonical signalling with Dkk1 impairs memory consolidation in mice (Fortress et al., 2013). Bilateral hippocampal infusion of Dkk1 leads to the impairment in the novel object recognition task (Fortress et al., 2013), similarly to the results of the object recognition task found in my study. Together, these data provide strong evidence of the role of Wnt signalling in long term memory.

In summary, my data in combination with the already published studies provide relevant evidence that support the importance of Wnt signalling for the normal function of the adult brain. Interestingly these findings can be correlated to the amnestic symptoms of AD patients, who present with deficits in the acquisition of new memories. Although significant impairment in short-term memory is a hallmark of the symptoms in AD patients (Britton & Rao, 2011; Tarawneh & Holtzman, 2012), a phenotype not observed in the ind-Dkk1 model, nonetheless this animal model provides a valid tool to study cognitive decline related to the loss of synapses. Thus,

my results give the ultimate link between Wnt-mediated synaptic stability and cognitive dysfunction observed by the loss of Wnt signalling, further reasoning in support of the use of this molecule to protect synaptic loss and memory decline.

6.5. Wnts in neurodegenerative diseases

Wnts are known for their well established link with cancer and other human genetic diseases (Anastas & Moon, 2013; Logan & Nusse, 2004) and other biological processes including embryonic stem-cell renewal, muscle development and regeneration, epithelial cell homeostasis and embryonic development and patterning (Arwert et al., 2012; van Amerongen & Nusse, 2009; von Maltzahn et al., 2012). In the nervous system, Wnts are also established to play a critical role regulating neural connectivity by promoting axon guidance, dendritic arborisation, synapse formation, and more recently also been implicated in adult hippocampal neurogenesis and synapse plasticity (Budnik & Salinas, 2011; Dickins & Salinas, 2013; Jessberger et al., 2009; Lie et al., 2005; Oliva et al., 2013; Qu et al., 2009; Salinas & Zou, 2008; Seib et al., 2013; Stamatakou & Salinas, 2013). In addition, Wnt signalling have been emphasised in the context of synaptic loss in neurodegenerative disease (Berwick & Harvey, 2014; Purro et al., 2014), as discussed below.

6.5.1. Alzheimer's Disease

AD and other forms of dementia not only have an impact to people suffering with the condition, but also on their family, their careers and the society as whole, having therefore huge economic impact on our society. In 2010, it was estimated that more than 35 million people were living with dementia, a number that is increasing exponentially, and predicted to reach the 66 million mark in 15 years (Wortmann, 2012). The impact and challenges of this epidemic became the driving force to many

research groups and pharmaceutical companies, which are not only trying to find ways to improve the lives of individuals with AD, but also studying the basics of the disease – which ultimately aim to allow its prevention, before the manifestation of the symptoms. Despite the efforts, to date no cure has been found to AD, and only a few medications are available to temporarily reduce some symptoms and slow down the progression in some people (*Font: NHS UK*).

In the past years, a number of studies have suggested a role for Wnt signalling in AD. Abnormal processing of amyloid precursor protein (APP) leads to the production, deposition and aggregation of toxic Aβ forms. Crucially, studies have demonstrated that components of the canonical Wnt pathway interact with PS1. In addition, studies have demonstrated the physical interaction between preselinin-1 (PS1) and β-catenin (Murayama et al., 1998; Soriano et al., 2001; Z. Zhang et al., 1998), and GSK3β (Phiel et al., 2003; Takashima et al., 1998). Furthermore, a genome-wide linkage study reported that genetic variation of the Wnt co-receptor LRP6 is linked with late- onset of AD (De Ferrari et al., 2007). More recently, a study found that soluble Aβ directly binds to the extracellular CRD domain of the Fz5 receptor (Magdesian et al., 2008). Although the significance of these findings remains to be investigated, these studies provide compelling evidence of the direct link between Wnt signalling receptors and AD.

Adding to the above evidence, a recent study demonstrated a clear link among Wnt canonical signalling, APP processing and AD progression. This elegant study shows that neuronal LRP6 deficiency *in vivo* increases the levels of Aβ42 in the cortical area of the brain, when compared to control animals (C. C. Liu et al., 2014). Furthermore, if LRP6-deficient animals were crossed with an AD (APP/PS1 mutant) mouse line, the accumulation of amyloid plaque load observed throughout the brain was increased by around 50% in comparison to the control APP/PS1 transgenic animals (C. C. Liu et

al., 2014). In addition, the same study shows that LRP6 overexpression in cultured neurons dramatically increases the levels of non amyloidogenic APP at the cell surface (C. C. Liu et al., 2014), whereas knockdown of LRP6 increases the surface levels of Aβ (C. C. Liu et al., 2014). Although the study does not clarify the mechanism by which Wnt is involved in the Aβ processing, it suggests an interaction between LRP6 and APP trafficking, as well as the favouring of non-amyloidogenic APP processing by the "healthy" state of Wnt canonical signalling.

Can Wnt signalling components represent a target for AD therapy and its prevention? The discovery that Wnt antagonists are elevated in AD patients and in AD mouse models was a hallmark in the Wnt – AD field. Dkk3 and Dkk1 have been reported to be elevated in plasma and cerebrospinal fluid (CSF) and in post-mortem brain of AD patients, respectively (Caricasole et al., 2004; Zenzmaier et al., 2009), not only suggesting that these molecules can be potential biomarkers to diagnose of AD, but also opening a range of new possible targets to the disease. Consistent with these findings, β-amyloid (Aβ) treatment peptides induce Dkk1 expression in cultured neurons and brain slices (Caricasole et al., 2004; Killick et al., 2012; Purro et al., 2012), suggesting that Dkk1 expression is triggered by the presence of Aß, being therefore downstream to the latter. Crucially, our lab demonstrated that addition of Dkk1 neutralising antibody protects synapses from disassembly by Aß in brain slices, indicating that Dkk1 is required for synaptic loss induced by Aß (Purro et al., 2012). My results presented in this thesis are the ultimate demonstration to the role of Dkk1 mediating synaptic disassembly. Here I show that the presence of Dkk1 leads to the loss of excitatory synapses and, importantly, this loss could be prevented by specific inhibition of Gsk3β, using BIO. Furthermore, I show that Dkk1-mediated synaptic disassembly could be rescued after two weeks of restoring Wnt signalling in our animal model. Together, my findings create a further link between dysregulation of Wnt canonical signalling and AD, as previously discussed, and also demonstrate that synapse loss can be reversed by manipulating the Wnt pathway.

6.5.2. Parkinson's Disease

Parkinson's disease (PD) is a neurological disorder that, similarly to AD, is characterised by its progressive synaptic dysfunction, culminating in synaptic and neuronal loss (Janezic et al., 2013; Villalba & Smith, 2010). It is believed that over 125,000 people live with PD in the UK today, making its prevalence approximately 1 in 500 people (*Font: Parkinson's UK)*. Since its first description back in 1817 as an involuntary tremulous motion or "Shaking Palsy" (Kempster et al., 2007; Parkinson, 2002), much has been learned about this disease, including the genetics underlying it. Nowadays, it is well acknowledged that PD results from the degeneration and death of dopaminergic neurons of the substantia nigra of the brain, resulting in a loss of dopamine in the striatal projection area (Dauer & Przedborski, 2003; Gasser, 2009). However, besides the well characterised symptom and treatments to ameliorate them, very little is known about the mechanism triggering the loss of these synapses and neurons.

Evidence to the involvement of Wnt signalling in PD is growing. In the last two decades many genes, which segregate in a Mendelian model of inheritance, have been identified as genetic risk factors to the development of PD (Gasser, 2009). Interestingly, some of these genes have been linked to the canonical Wnt signalling, suggesting the involvement of the latter with the pathogenesis of PD (Berwick & Harvey, 2014). Mutation in the leucine-rich repeated kinase 2 gene (LRRK2) – a protein kinase, GTPase and more recently suggested to be functional as scaffolding protein (Berwick & Harvey, 2012; P. A. Lewis & Manzoni, 2012) - has been reported to be the highest contributor to cases of sporadic PD worldwide (up to 5%) (Berwick & Harvey, 2014; Kumari & Tan, 2009). A study has demonstrated that LRRK2 directly interacts with members of the scaffolding protein Dvl (Dvl1 – 3) and disruption or overexpression of Dvls in cultured cells shifts the localisation of LRRK2, suggesting a link between Dvls and LRRK2 activity (Sancho et al., 2009). Supplementing these findings, a study using Drosophila as a model for neurodegeneration, demonstrated that LRRK2 interacts with Gsk3β, and mutation of LRRK2 can lead to hyperphosphorylation of Tau protein and dendrite degeneration (C. H. Lin et al.,

2010). Furthermore, a more recent report has demonstrated that, in addition to interacting with Dvls, LRRK2 also binds to the cytoplasmatic domain of LRP6, and interacts with key components of the canonical Wnt pathway, being therefore speculated as a key regulator of Wnt signalling (Berwick & Harvey, 2012). Indeed, the study shows that pathogenic LRRK2 mutation reduces Wnt canonical pathway activity (Berwick & Harvey, 2012). Together, these studies give strong evidence for the connection between Wnt signalling and PD.

PD genes and Wnt signalling: a growing link. In addition to the LRRK2 mutation discussed above, a range of *PARK* gene mutations, implicated in more rare causes of PD, were reported to be linked to Wnt canonical pathway and its dysregulation - among them Parkin, Gsk3β and Wnt3 (Berwick & Harvey, 2014). For example, polymorphism in the Gsk3β gene is implicated in a higher susceptibility to develop PD (Kalinderi et al., 2011; Kwok et al., 2005), a phenotype speculated to be the result of abnormal phosphorylation of microtubule proteins, ultimately affecting their dynamics and function (Berwick & Harvey, 2014). In addition to the growing evidence, a recent study from our lab has demonstrated that blockade of Wnt signalling with Dkk1 *in vivo*, leads to the loss of glutamatergic synapses and decreases the number of dopaminergic receptors clusters in the striatum (Galli, 2014). Furthermore, the study demonstrated that Dkk1-mediated synaptic loss led to the impairment in striatal-regulated behaviour, including deficient performance in the rotarod (Galli, 2014), a classic behavioural phenotype observed in some PD animal models. Notably, studies evaluating whether Dkk1 is expressed in brains of PD patients are yet to be performed. In summary, these studies bring to light exciting information about the involvement of Wnt signalling in PD, provide some basic information to the elucidation of molecular mechanisms involved in PD and promising new targets to the treatment, and perhaps prevention of synaptic loss.

6.6. Dkk1 mechanism of action

Many aspects to the mechanism of action of Dkk1 is yet to be elucidated, in particular the intracellular events resulted by its presence. Dkk1 blocks Wnt signalling by binding to the lipoprotein receptor related-protein class 6 (LPR6), a Wnt co-receptor in the canonical signalling pathway (Brott & Sokol, 2002; Cruciat & Niehrs, 2013; Mao et al., 2001). It is well established that when present, Dkk1 prevents the formation of the ternary complex Wnt- Frizzled Receptor (Fz) and LRP5/6 (Bourhis et al., 2010; Niehrs, 2006). In addition to LRP5/6, Dkk1 can bind with high affinity to the single-pass transmembrane protein receptor Kremen 1 and 2 (Krm1/2) (Cruciat & Niehrs, 2013; Mao & Niehrs, 2003; Mao et al., 2002) resulting in the rapid removal and internalization of the LRP5/6 receptor via endocytosis, and consequently the suppression of Wnt cascade (Mao et al., 2002). Here I demonstrate that blockade of Dkk1 leads to the loss of synapses – but what are the mechanisms downstream to Dkk1 in the context of synaptic disassembly?

My data demonstrates that Dkk1 action on synaptic disassembly can be prevented by modulating a specific intracellular process: protein degradation. Here I show that blockade of the UPS can override the loss of synapses caused by Dkk1. Given that this intracellular process has been shown to be crucial in the context of amyloidosis and spine loss (Bedford et al., 2008; Haas & Broadie, 2008; McNaught et al., 2003; Smith et al., 2009; van Tijn et al., 2012), my results not only demonstrate for the first time what mechanisms are downstream to Dkk1, but also suggest that manipulation of proteasome degradation can be a potential target to prevent synaptic degeneration (Fig 6.3). Furthermore, my results create a further link between synapse loss in the context of amyloidosis and disruption in Wnt signalling, highlighting the importance of these molecules to the maintenance of synapses in the adult brain. In addition, my results provide new exciting information to the understanding of intracellular events triggered by Dkk1, based on this information, genes and proteins – whether ubiquitinated or not - can potentially be identified and tested to overcome synapse vulnerability and loss, and prevent the cognitive impairment associated with neuronal degeneration.

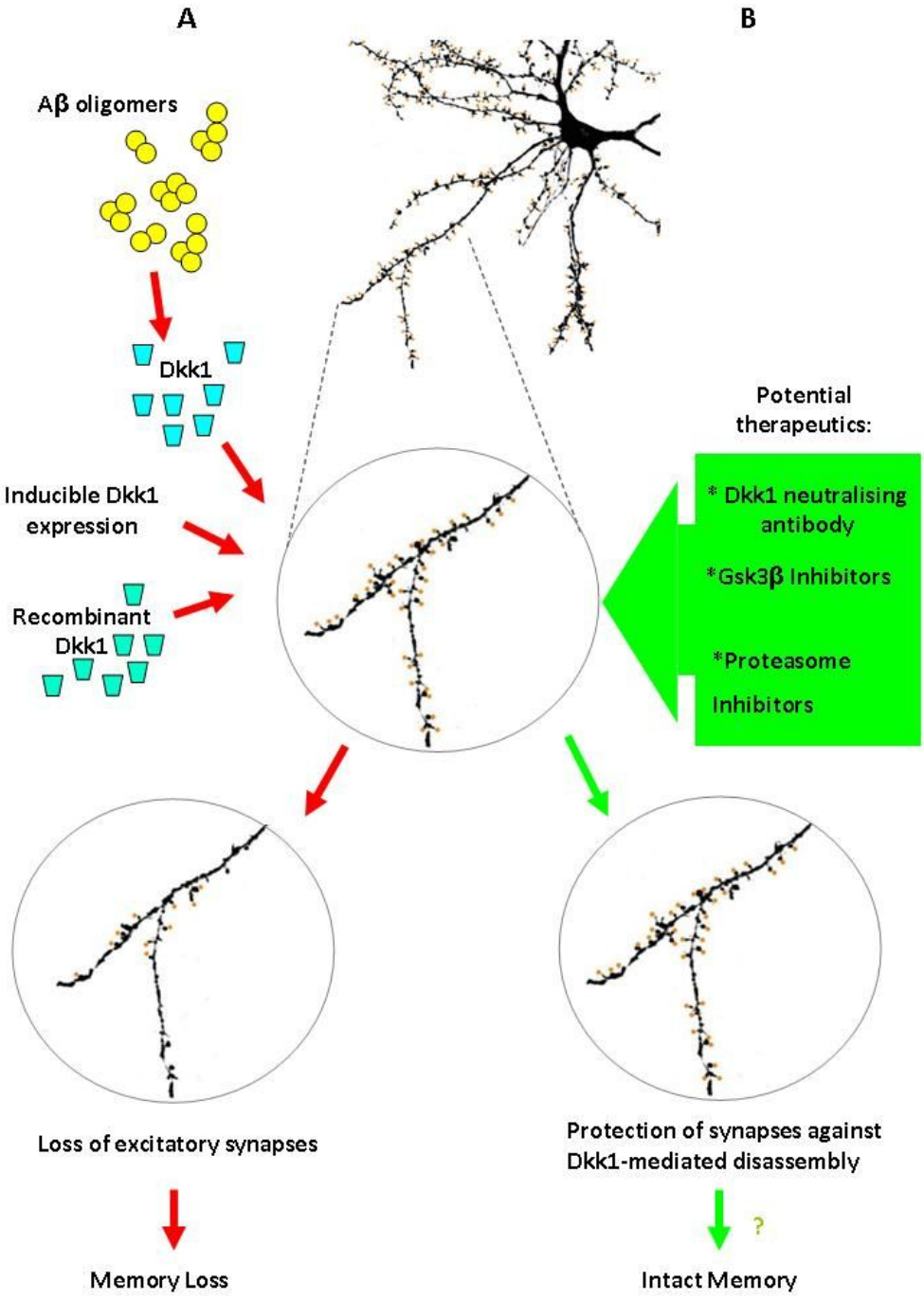

Figure 6.3: Potential therapeutic targets for early stages of synaptic degeneration. (A) Soluble amyloid-β oligomers (Aβ), stimulate the expression of Dkk1 protein (Caricasole, et. al., 2004; Purro et. al., 2012). Presence of Dkk1, induced either by presence of Aβ, by genetic manipulation or other external source, leads to the loss of excitatory synapses (less dendritic spines and vGlut puncta, in orange) – which I showed to correlate to cognitive decline. **(B)** Dkk1-mediated synaptic loss and subsequent cognitive decline can potentially be prevented by presence of anti-Dkk1 neutralising antibody (Purro et al., 2012); by stimulating Wnt signalling downstream of the receptors (using Gsk3β inhibitor); or by pharmacological compounds that target the UPS system.

6.7. Conclusions and future directions

This work demonstrates three main aspects of Wnt signalling for the maintenance of synapses. First, I showed that Wnt canonical signalling is crucial for the maintenance of excitatory synapses, as blockade of Wnts with Dkk1 leads to the loss of excitatory synapses but inhibitory ones are spared. Secondly, I propose that acute or chronic Dkk1-mediated synaptic loss, using an *ex vivo* or *in vivo* approach respectively, has a similar effect in neurons, appearing to have a more pronounced effect on disassembling the presynaptic excitatory sites. Furthermore, my findings show that the loss of synapses *in vivo*, in consequence of Wnt blockade, results in memory impairment. Moreover, I also investigate the mechanisms downstream to the Dkk1 action and suggest potential targets to overcome synapse loss. Together, these findings give a direct link between Dkk1-mediated synaptic disassembly and cognitive impairment, in addition to providing new insights to mechanisms involved in synapse loss upon disruption of Wnt signalling.

6.7.1. Future research plans

My study opened new avenues not only to investigate the cellular and molecular mechanism underlying Dkk1 action but also to clarify the direct link between Dkk1 and AD. Many scientific questions were raised during the execution of this project and are yet to be answered; therefore I propose a list of experiments to follow up my studies (below):

1) To investigate further whether any other types of synapses are specifically targeted by the presence of Dkk1 in the hippocampus (dopaminergic synapses and cholinergic synapses)
2) To characterise the effect of Dkk1 on the vulnerability of synapses/spines in relation to their position at the dendritic tree as well as their structural shape – for instance, are distal synapses and smaller spines more vulnerable to synapse disassembly?

3) To determine the hierarchy of synaptic disassembly triggered by the presence of Dkk1 – by using a combination of time lapse microscopy and labelling pre and postsynaptic sites

4) To show the physical position of Kremen receptor in relation to different types of synapses in the hippocampus (inhibitory, excitatory and modulatory synapses)

5) To investigate whether there is any change in the non-canonical Wnt pathways upon Dkk1 expression at neuronal cells at the molecular level– i.e.: upregulation, downregulation or any compensatory mechanisms

6) To test functionally if after the ceasing of Dkk1 expression (On - Off experiments), ind-Dkk1 animals also recover their cognitive function

7) To identify what molecules and genes and underpinning Dkk1 mechanism of action by performing proteomics and/or RNAseq, and validate the relevant candidates

8) To further study the contribution of DKK1 to AD by evaluating whether Dkk1 expression can accelerate, or perhaps enhance, AD symptoms I propose to cross the ind-Dkk1 line with a mouse model for AD and study any synaptic and behavioural changes

9) To test molecules that directly target Dkk1 and evaluate their potential as therapeutic treatment to protect or reverse synaptic loss in AD mouse models (i.e.: newly developed compounds)

10) To validate the targets identified so for (Dkk1 neutralising antibody, UPS inhibitors, and GSK3β inhibitor) to protect synapses in an *in vivo* system

Appendices

Supplementary Figures

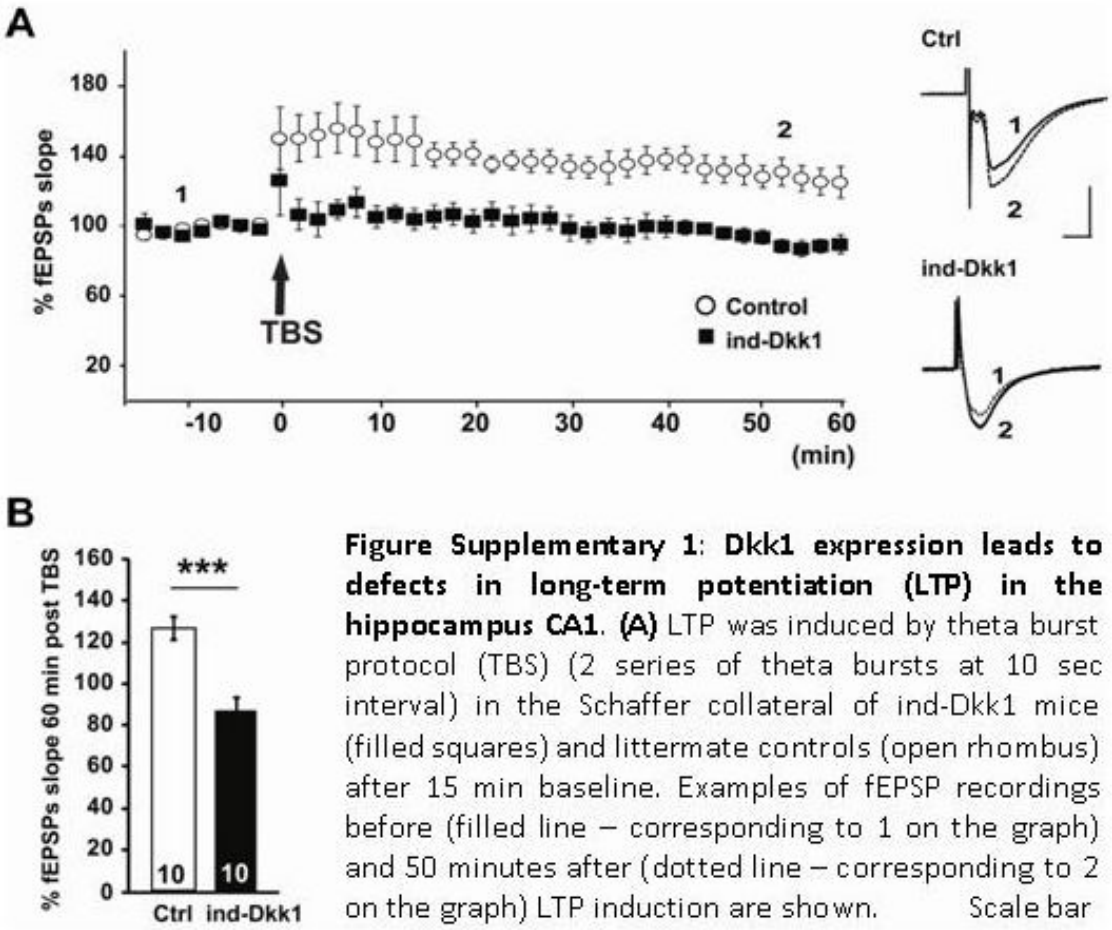

Figure Supplementary 1: **Dkk1 expression leads to defects in long-term potentiation (LTP) in the hippocampus CA1**. **(A)** LTP was induced by theta burst protocol (TBS) (2 series of theta bursts at 10 sec interval) in the Schaffer collateral of ind-Dkk1 mice (filled squares) and littermate controls (open rhombus) after 15 min baseline. Examples of fEPSP recordings before (filled line – corresponding to 1 on the graph) and 50 minutes after (dotted line – corresponding to 2 on the graph) LTP induction are shown. Scale bar represents 10 ms; 0.1mV. **(B)** Percentage of fEPSPs slope 60 min after TBS (n=10 slices from 6 control mice; n=8 slices from 7 ind-Dkk1 mice). *Figures and legends kindly provided by Dr. Aude Marzo.*

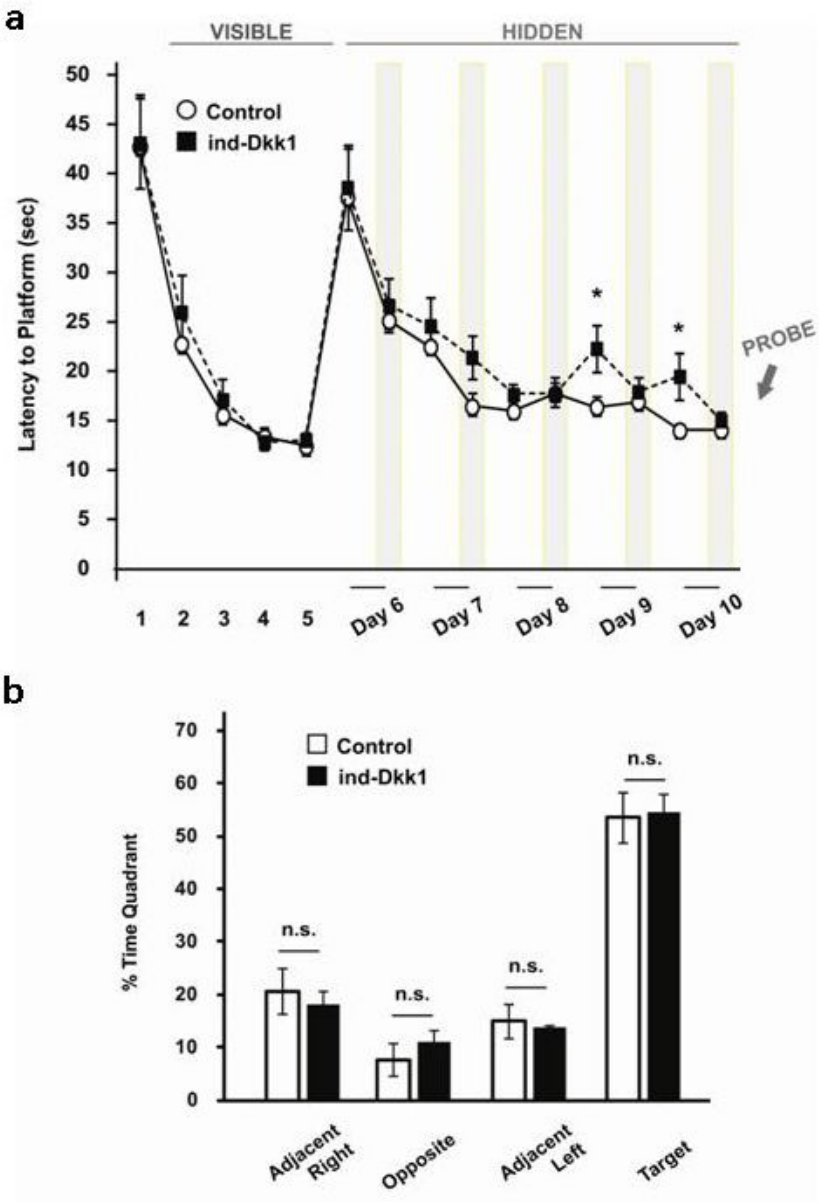

Figure Supplementary 2 – Loss of synapses mediated by expression of Dkk1 causes impairment in spatial memory in the MWM task. (a) Latency to find the visible platform over a period of 5 days and the hidden platform across 5 days of training in the MWM. During the hidden platform training phase, mice received 2 sessions of 4 trials/day, each session took place ~4 hours apart. No differences between groups were found in the first stage of the task (visible platform). However, ind-Dkk1 animals take longer to locate the hidden platform when compared to controls. Notably, during all second sessions (light grey) the ind-Dkk1 animals performed as well as controls mice. **(b)** After five days of training, ind-Dkk1 animals did not present any noticeable cognitive deficits and performed equal to control animals. Results are shown as mean ±SEM. *p ≤ 0.05; **p≤ 0.01; n=7 ind-Dkk1 and n=8 ctrls, ANOVA repeated measures.

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