THE ROLE OF β-CATENIN AND THE CADHERIN ADHESION COMPLEX IN SYNAPTIC PLASTICITY, LEARNING AND ADDICTION

by

Fergil Mills

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Abstract

A fundamental property of synapses is their ability to change in response to activity, termed 'synaptic plasticity'. Synaptic activity can cause long-lasting increases in the strength of synapses (long-term potentiation, or 'LTP'), as well as decreases in synapse strength (long-term depression or 'LTD'), both of which are believed to be important for learning and memory. The synaptic adhesion molecules 'cadherins' and their intracellular binding partner β-catenin have been identified as key mediators of plasticity at synapses. The cadherin adhesion complex is important for maintaining the strength and stability of synapses, and disruption of cadherin function has been shown to impair long-term potentiation (LTP). However, it remains unclear how *increases* in cadherin adhesion can affect synaptic function and cognition. This is important in light of studies showing that increases in β-catenin levels and mutations in cadherin adhesion complex proteins are associated with many different neurodegenerative diseases, as well as psychiatric disorders such as drug abuse, raising the possibility that aberrant increases in cadherin adhesion may contribute to cognitive impairments in these disorders.

In this dissertation, I examine the effects of increases in cadherin adhesion on different forms of synaptic plasticity in the brain. I demonstrate that increases in β-catenin in the hippocampus can stabilize cadherin at the synaptic membrane and abolish long-term depression (LTD) at synapses, leading to significant impairments in spatial memory flexibility and reversal learning. I also demonstrate a role for cadherin in activity- and drug-induced plasticity in the ventral tegmental area (VTA), a region of the brain important for reward learning which is implicated in addiction, and show that cocaine-mediated conditioned place preference results in redistribution of cadherin and AMPA receptors to excitatory synapses onto dopaminergic neurons in the VTA.

Together, these results demonstrate that the β-catenin/cadherin adhesion complex plays an important role in several forms of learning and memory, and that aberrant increases in synaptic adhesion can have a detrimental effect on synaptic plasticity and cognitive function.

Preface

The work in Chapter 2, entitled: "Cognitive flexibility and LTD are impaired following β-catenin stabilization *in vivo*", has been published as:

"**Mills F**, Bartlett T, Dissing-Olessen L,Wisniewska M, Kuznicki J, Wang YT, Bamji SX (2014) *Cognitive flexibility and LTD are impaired following β-catenin stabilization* in vivo. ***Proceedings of the National Academy of Sciences*** 111(23):8631-6. doi: 10.1073."

All experiments were conceived by FM and SXB, and conducted by FM with the following exceptions: electrophysiological experiments were conducted by TEB, quantitative RTPCR was conducted by MBW, and LDO assisted with slice preparation and NMDA treatment for endocytosis assay experiments. FM and SXB wrote the manuscript.

The work in Chapter 3, entitled: "N-cadherin mediates cocaine- and activity-induced plasticity in the ventral tegmental area" will be published as:

"**Mills F**[1], Globa A[1], Liu S, Cowan CM, Phillips A, Borgland S, Bamji SX (2014) *N-cadherin mediates cocaine- and activity-induced plasticity in the ventral tegmental area.* (manuscript in preparation)." [1]These authors contributed equally to this work

All experiments were conceived by FM, AG and SXB, and all experiments were jointly conducted by FM and AG, with the following exceptions: electrophysiological experiments were conducted by SL, and tissue processing and data analysis from immunogold electron microscopy experiments was assisted by CMC and MM. **Experiments by FM and AG were done in equal partnership, and equal intellectual contribution**. FM, AG and SXB wrote the manuscript.

Experimental procedures and animal housing conditions were approved by the UBC Animal Care Committee under protocols A10-0316, A10-0319 and A09-0805.

Table of Contents

List of Figures

List of Symbols and Abbreviations

aCSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPAR	AMPA receptor
ANOVA	analysis of variance
AP5	(2R)-amino-5-phosphonovaleric acid
APC	adenomatous polyposis coli
ARM	armadillo repeats
$\beta\text{-}cat^{\Delta ex3/+}$	*Ctnnb1*$^{lox(ex3)/+}$*;CaMKIIα:Cre/+* mice
BDNF	brain-derived neurotrophic factor
BNST	bed nucleus of the stria terminalis
CA1	cornu ammonis 1
CA3	cornu ammonis 3
CaMKII	Ca2+/calmodulin dependent protein kinase II
cAMP	cyclic adenosine monophosphate
cdc42	cell division control protein 42
CNS	central nervous system
CP-AMPAR	calcium-permeable AMPA receptor
CPP	conditioned place preference
CPu	caudoputamen
D1	dopamine receptor 1
D2	dopamine receptor 2
DA	dopamine
DAPI	4',6-diamidino-2-phenylindole
DAT	dopamine transporter
ddH_20	double-distilled water
DG	dentate gyrus
DNMTP	delayed non-match to place
DSH	disheveled
EC	entorhinal cortex
EC(1-5)	extracellular cadherin repeat (1-5)
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
EM	electron microscopy
fEPSP	field excitatory postsynaptic potential
GABA	gamma-aminobutyric acid
GDP	guanosine diphosphate
GluA(1-4)	glutamate receptor subunits (1-4)
G-protein	guanine nucleotide-binding protein
GRIP1	Glutamate receptor-interacting protein 1
GSK3β	glycogen synthase kinase 3β
GTP	guanosine triphosphate

HAV	Histidine-Alanine-Valine
HB	homogenization buffer
HD	Huntington's disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFS	high-frequency stimulation
Hz	hertz
JMD	juxtamembrane domain
K	potassium
LDTg	lateral dorsal tegmental nucleus
LFS	low-frequency stimulation
LG	laminin G
LH	lateral habenula
LPP	lateral perforant pathway
LTD	long-term depression
LTP	long-term potentiation
mA	milliamp
Mg	magnesium
mGluR	metabotropic glutamate receptor
mGluR-LTD	metabotropic glutamate receptor-dependent long-term depression
MPP	medial perforant pathway
mRNA	messenger ribonucleic acid
mV	millivolt
MWM	Morris water maze
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NOR	novel object recognition
P	p-value
P2	crude synaptosomal fraction
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDZ	post synaptic density protein, Drosophila disc large tumor suppressor, and zonula occludens-1 protein domain
PFA	paraformaldehyde
PFC	prefrontal cortex
polyQ	poly-glutamine
PP	perforant pathway
PS1	presenilin-1
PSD	postsynaptic density
RM ANOVA	repeated measures analysis of variance
RNA	ribonucleic acid
RP	reserve pool
RRP	readily-releasable pool
RT	room temperature
RTPCR	reverse transcription polymerase chain reaction
S1	soluble fraction

S2	soluble/small compartments fraction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SN	substania nigra
SNP	single nucleotide polymorphism
sr	stratum radiatum
STDP	spike timing-dependent plasticity
TA	temporoammonic
TBOA	threo-β-benzylaspartic acid
TBS	tris-buffered saline
TBST	tris-buffered saline and tween
TCF/LEF	T-cell factor/lymphoid enhancing factor
TH	tyrosine hydroxylase
TK	tyrosine kinase
VTA	ventral tegmental area
Wnt	wingless/int1
X-gal	x-galactose (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

Acknowledgements

First and foremost, I would like to thank Shernaz Bamji. Her mentorship, patience and unfailing support have been the bedrock of my PhD, and I feel incredibly fortunate to have had such a phenomenal supervisor. Indeed/Moreover, she has been a great friend to me and patiently looked out for me from New York to Hong Kong. I know that as I move forward in life I will often ask myself 'What Would Shernaz Do?' and be inspired to do twice as much, twice as fast, to twice as high a standard. Thanks, Shernaz.

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Dedication

To my family –

I love you Mom,

I love you Dad,

I love you Hannah.

Chapter 1: Introduction

1.1 GENERAL STRUCTURE AND FUNCTION OF SYNAPSES IN THE CENTRAL NERVOUS SYSTEM (CNS)

Synapses are specialized adhesive junctions between neurons that act as points of communication allowing signals to be transferred from one cell to another. Signaling at synapses is primarily accomplished by release of neurotransmitter-filled synaptic vesicles by the pre-synaptic neuron, allowing neurotransmitter to diffuse across the synaptic cleft between cells and bind to post-synaptic receptors, resulting in an influx of extracellular ions to the postsynaptic neuron (reviewed in Sudhof, 2004). Synapses are typically formed between presynaptic sites (also called 'boutons') located along axonal projections from one neuron, and postsynaptic sites located along the dendrites and cell body of other neurons. Signaling at synapses is also inherently directional, as extensive molecular machinery for neurotransmitter release must be assembled on the presynaptic side of the synapse, and molecular machinery for postsynaptic responses must be assembled directly on the postsynaptic side. The study of synapse function has been an important area for neuroscience research, and understanding the molecular mechanisms underlying synaptic transmission has provided many insights into how neurons receive and integrate information in the brain.

Synapses in the CNS can be broadly categorized into two different types; excitatory synapses and inhibitory synapses, which are defined by the effects that they exert on the electrochemical potential of the postsynaptic neuron. Individual neurons are typically polarized and have a resting potential of -70 mV relative to the surrounding intercellular space (Bean,

2007). When a neuron reaches a potential of -55 mV, voltage-gated ion channels open which result in an influx of ions to the cell and a wave of depolarization called an ‘action potential’ that propagates throughout the neuron and triggers the release of neurotransmitter at presynaptic boutons along its axon (reviewed in Bean, 2007). Excitatory synapses are synapses which, following the release of neurotransmitter and activation of postsynaptic receptors, cause a depolarization of the postsynaptic neuron, moving the potential of the neuron closer to the threshold of initiating an action potential. Conversely, inhibitory synapses are those whose activity causes further polarization of the postsynaptic neuron, making it less likely to initiate an action potential. Individual neurons then integrate inputs from thousands of synapses of different types, which is believed to give rise to the ability of the brain to compute and process information (Stein and Stanford, 2008).

There are many different types of individual excitatory and inhibitory synapses in the CNS, and they can be further categorized based on the type of neurotransmitter that is released at the synapse. The primary excitatory neurotransmitter in the brain is glutamate, which is present at an estimated 80% of all synapses in the brain (Harris and Kater, 1994) (**Fig 1.1**), while the most common inhibitory neurotransmitter is gamma-aminobutyric acid (GABA) (Bormann, 2000). Another important neurotransmitter is dopamine, which can act as both an excitatory or inhibitory neurotransmitter and plays a major role in the motivational circuit underlying reward learning and motivated behaviour (Wise, 2004). Neurons typically only form synapses with a single type of neurotransmitter along their axonal projections, and so individual neurons can be classified based on the types of synapses they form onto other neurons; for example ‘excitatory glutamatergic’ neurons only form excitatory, glutamate-releasing synapses along their axons.

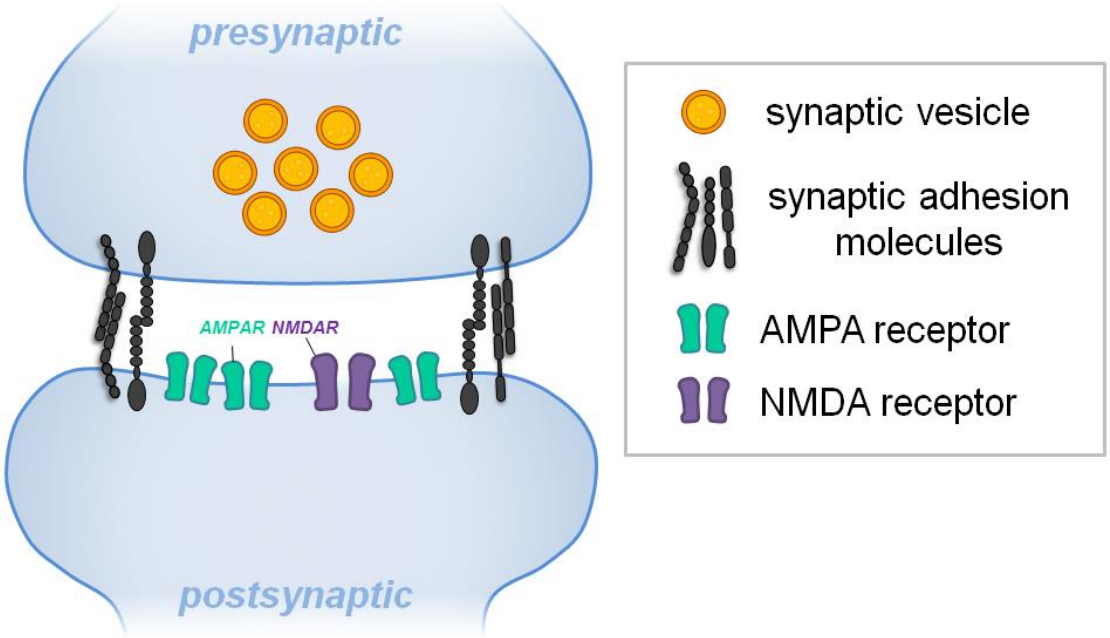


Figure 1.1 General structure of a glutamatergic synapse in the central nervous system. Glutamatergic synapses are typically formed between a presynaptic bouton along an axon (top) which contains synaptic vesicles filled with the neurotransmitter glutamate, and a postsynaptic dendritic spine protruding from a dendrite (bottom). Synaptic vesicles fuse with the presynaptic membrane and release neurotransmitter molecules across the synaptic cleft, where they bind to specialized postsynaptic receptors such as AMPA and NMDA receptors primarily located in a protein-dense region called the postsynaptic density (PSD). Upon binding of neurotransmitter, these postsynaptic receptors change conformation, allowing them to open and conduct ions, resulting in depolarization of the postsynaptic cell and transmission of a signal from the presynaptic to postsynaptic neuron. Many different types of synaptic adhesion molecules are localized to both sides of the synapse (Image made by Fergil Mills).

1.2 GLUTAMATERGIC SYNAPSES: STRUCTURE AND FUNCTION

Though neurons typically form only one type of synapse along their axons, they receive inputs from many different types of synapses, and at each of these types of presynaptic input neurons must assemble the appropriate corresponding proteins at postsynaptic specializations to allow for neurotransmission. Some of the most critical of these proteins are the specific

postsynaptic ligand-gated ion channels which are a major binding targets of neurotransmitters released at each synapse.

1.2.1 AMPA receptors

At excitatory synapses, there are two major classes of postsynaptic glutamatergic ion channels. The first of these are AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate receptors, also called AMPA receptors or 'AMPARs'. AMPA receptors are the primary mediators of fast postsynaptic responses at glutamatergic synapses; in response to glutamate binding, AMPA receptors at the synaptic cleft rapidly change in conformation and allow extracellular positively-charged ions to enter the postsynaptic compartment, resulting in a local depolarization of the postsynaptic neuron. The amount of AMPA receptors localized to the postsynaptic membrane at individual synapses is one of the major determinants of the 'strength' of the synapse; in response to a single quantal event of glutamate release by a presynaptic vesicle, a greater number of AMPA receptors present will result in a greater influx of ions and increased depolarization of the postsynaptic neuron (reviewed in Malenka and Bear, 2004).

AMPA receptors are typically tetramers made from a combination of four types of subunits ('GluA1-4', also called 'GluR1-4') which can be arranged in different combinations. Though structurally similar, there are considerable variations in the function of each subunit type, resulting in AMPA receptors with different properties based on their subunit composition (**Fig. 1.2**). The presence or absence of different AMPA receptor subunits can affect the ion permeability of the individual AMPA receptors that they compose. The GluA2 AMPA receptor subunit contains a residue within the channel pore region that determines the ion selectivity of

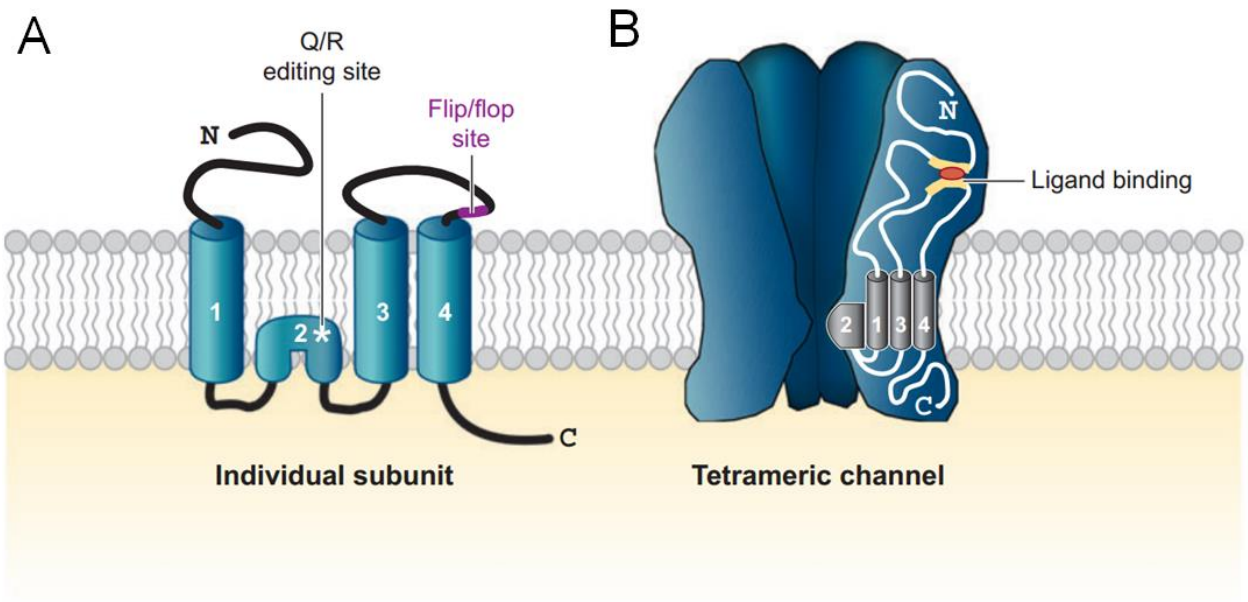


Figure 1.2 Structure of AMPA receptor subunits and tetramers.
A) Structure of AMPA receptor subunits. Each subunit has four transmembrane domains, an extracellular N-terminal region, and a short or long intracellular C-terminal tail. The second transmembrane domain contains the 'Q/R editing site' - a single amino acid that can be post-transcriptionally modified from glutamine (Q) to arginine (R), preventing Ca^{2+} ions from passing through the AMPA receptor. The flip/flop site is a region of the subunit where one of two different exons can be inserted, which results in a longer (flip) or shorter (flop) activation of the AMPA receptor in response to glutamate binding (Eastwood et al., 1997). B) Tetrameric structure of AMPA receptor. The channel pore region is formed by the second transmembrane domain, positioning the Q/R site to determine ion selectivity. The ligand binding site of the AMPA receptor is formed between the two extracellular domains (Adapted with permission from Shepherd and Huganir, 2007).

the AMPA receptor. The mRNA for GluA2 is modified by post-transcriptional RNA editing, replacing the uncharged glutamine amino acid at that site to a positively-charged arginine. This arginine residue allows Na^{+} and K^{+} ions to pass through the AMPA receptor but excludes Ca^{2+} ions, which are a critical intracellular signal that activate many downstream signaling pathways in neurons (Lynch, 2004). As most AMPA receptors are heteromers that include GluA2 subunits, this means that most AMPA receptors are calcium impermeable. However, the GluA1 AMPA

receptor subunit lacks this motif, and consequently AMPA receptors composed only of the GluA1 subunits are calcium-permeable (reviewed in Cull-Candy et al., 2006). The different AMPA receptor subunits have specific differences in binding sites that allow them to interact with other proteins, as well as phosphorylation sites which can affect the function, trafficking and stability of the AMPA receptors at the synapse (Shepherd and Huganir, 2007). Because of the importance of AMPA receptors in determining the strength of synapses, many pathways converge on the common point of changing the distribution and activity of AMPA receptors to mediate long-lasting changes at the synapse. Synaptic adhesion molecules which affect AMPA receptor trafficking and stability can therefore regulate synapse strength and plasticity.

1.2.2 NMDA receptors

The second major type of receptor at excitatory glutamatergic synapses are N-methyl-D-aspartate (NMDA) receptors (also called 'NMDARs'). NMDA receptors are also activated by binding of glutamate at extracellular sites, but have several important features which distinguish them from AMPA receptors. First, NMDA receptors do not selectively exclude specific cations from the ion channel, allowing calcium ion (Ca^{2+}) influx to the postsynaptic neuron in response to activation. Intracellular Ca^{2+} ions are an important intracellular signal in neurons, and NMDA receptors are therefore a critical point of control for activity-dependent changes in synaptic strength or 'plasticity' of synapses. A second distinguishing feature of NMDA receptors is that in addition to requiring glutamate binding for activation NMDA receptor function is also voltage-dependent. At normal resting membrane potentials, the NMDA receptor ion channel is blocked by Mg^{2+} ion, preventing the influx of extracellular ions even following binding of glutamate to

the NMDA receptor, and depolarization of the postsynaptic neuron results in the removal of the Mg^{2+} block from the channel pore (Malenka and Nicoll, 1993). This combination of requirements for activation has led many to describe NMDA receptor as a molecular 'coincidence detector' as its activity requires both synaptic activity to facilitate the removal of the Mg^{2+} block and glutamate release at the specific synapse where the NMDA receptor is localized.

1.2.3 Synaptic pasticity of glutamatergic synapses

The term 'synaptic plasticity' refers to the ability of synapses to undergo changes in structure and function in response to synaptic activity, which is believed to be a major mechanism underlying the ability of the brain to learn and store memories (Benfenati, 2007). At glutamatergic synapses, a major effector of changes in synaptic strength is the insertion and removal of AMPA receptors from the postsynaptic membrane.

One of the best-studied forms of synaptic plasticity is long-term potentiation (LTP). LTP is a long-lasting increase in the magnitude of postsynaptic response or 'synapse strength' resulting from a period of intense stimulation of that synapse, or from simultaneous activity at the synapse and depolarization of the postsynaptic neuron. Though several different forms of LTP have been characterized, the most common form of LTP is dependent on NMDA receptor function at glutamatergic synapses (**Fig. 1.3A**). NMDAR-dependent LTP can be induced by brief tetanic stimulation of presynaptic inputs to a postsynaptic neuron, resulting in depolarization of the postsynaptic neuron to remove the Mg^{2+} block of NMDAR receptors, and further release of neurotransmitter to allow an influx of Ca^{2+} ions (Malenka and Nicoll, 1993). This increase in

intracellular Ca^{2+} results in activation of many signaling pathways including the protein kinase CaMKII (Ca^{2+}/calmodulin dependent protein kinase II), which lead to increases in the number and activity of AMPA receptors at the synapse through several different mechanisms. First, in early-phase LTP, AMPA receptors are phosphorylated at sites which result in conformational changes that increase in the unitary channel conductance of individual receptors (Benke et al., 1998). Second, phosphorylation of AMPA receptors and other proteins result in increased trafficking of AMPA receptors located perisynaptically or in recycling endosomes to the postsynaptic membrane, where they are situated to respond to neurotransmitter release from the presynaptic terminal. Finally, late-phase LTP has also been shown to require the synthesis and insertion of new AMPA receptors at the synapse (Abraham and Williams, 2003, Lynch, 2004). Induction of LTP has also been shown to increase the size and stability of the potentiated synapse (Matsuzaki et al., 2004, Mendez et al., 2010), and cause a concomitant increase size of the presynaptic active zone where synaptic vesicles are released (Applegate et al., 1987, Toni et al., 2001).

A complimentary form of synaptic plasticity is long-term depression, or LTD, a form of synaptic plasticity in which synaptic activity causes a long-lasting decrease in the strength of synapses. Interestingly, despite their opposing effects on synapse strength LTD and LTP share many common mechanisms. One form of LTD (NMDAR-dependent LTD) requires NMDA receptor activation, and is mediated by modification and redistribution of AMPA receptors at the synapse (**Fig. 1.3B**). However, in contrast with the high-frequency stimulation required for LTD induction, LTD is elicited by low frequency stimulation (LFS, 0.5-3 Hz) which causes a relatively lower level of intracellular Ca^{2+} in the postsynaptic neuron (Malenka and Bear, 2004). Expression of LTD is mediated by the

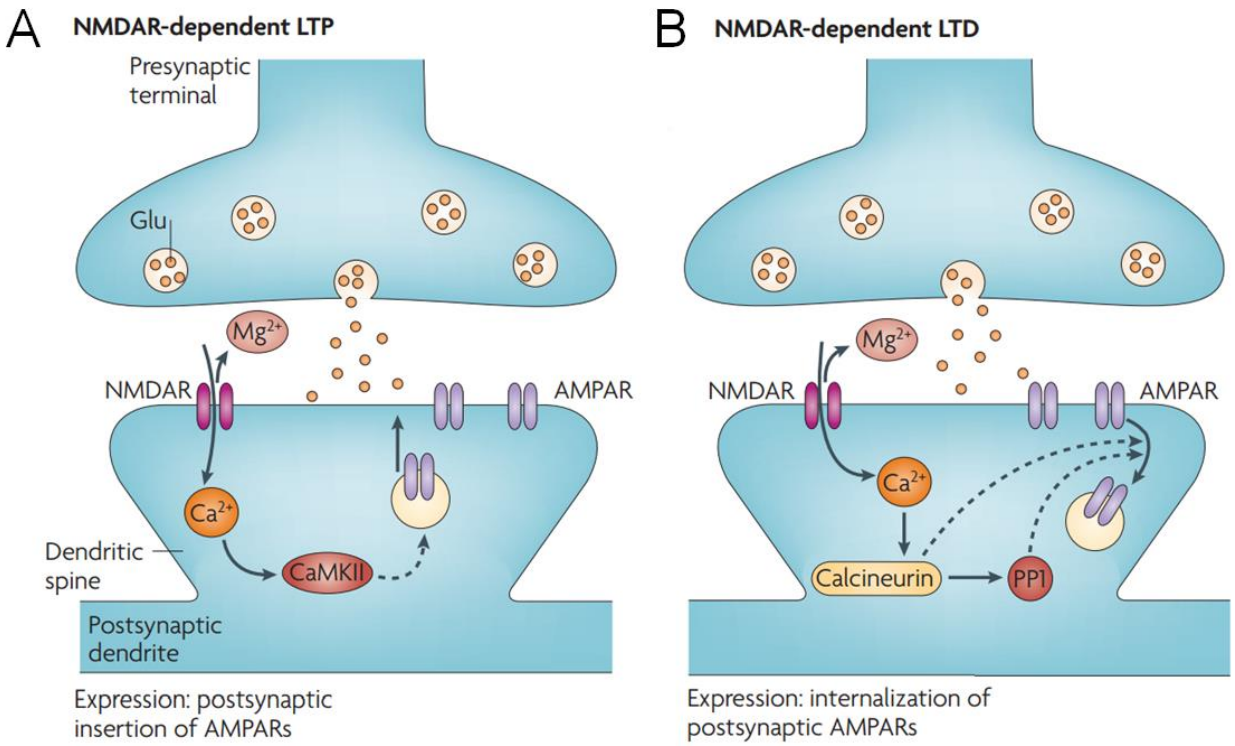


Figure 1.3 LTP and LTD at glutamatergic synapses
A) NMDAR-dependent LTP. Following depolarization of the postsynaptic neuron, the Mg^{2+} block is removed from NMDA receptors. Subsequent glutamate release from the presynaptic neuron activates NMDA receptors, allowing calcium influx to the postsynaptic compartment. This leads to downstream signaling cascades through kinases including CaMKII, resulting in insertion of AMPA receptors to the postsynaptic membrane. The increase in postsynaptic AMPA receptors results in greater influx of ions and greater responses to presynaptic neurotransmitter release, resulting in 'potentiation' of the synapse. B) NMDAR-dependent LTD. LTD works through similar mechanisms as LTP, requiring removal of the Mg^{2+} block from postsynaptic NMDA receptors. Activity which leads to LTD typically results in a prolonged, low concentration of Ca^{2+} postsynaptically, which activates calcineurin and protein phosphatase 1 (PP1), which promotes the endocytosis and removal of AMPA receptors from the synapse, resulting in decreased magnitude of depolarization in response to presynaptic inputs (Adapted with permission from Kauer and Malenka, 2007).

rapid internalization of AMPA receptors from synapses, which is mediated in part by dephosphorylating of the GluA1 subunits of AMPA receptors, reducing the number of receptors available to respond to presynaptic release of neurotransmitter (Lee et al., 1998). Since altering the number and activity of AMPA receptors at the synapse is a critical common mechanism for

both forms of plasticity, adhesion molecules and signaling pathways that affect AMPA receptor trafficking have an important influence on synaptic plasticity. Additionally, both forms of LTP and LTD have been shown to play important roles in learning and memory. In the hippocampus, LTP is critical for acquisition of spatial memory (Grant et al., 1992, Tsien et al., 1996b), while LTD has been associated with tasks involving behavioural flexibility such as reversal learning (Duffy et al., 2008, Nicholls et al., 2008, Dong et al., 2013).

1.3 DOPAMINERGIC SYNAPSES: STRUCTURE AND FUNCTION

Dopaminergic synapses are similar to glutamatergic in the principles of their organization. Specific postsynaptic receptor proteins corresponding to the type of neurotransmitter released (dopamine) are localized to postsynaptic sites, and alterations in the number, composition and posttranslational modification of those receptors is a major locus for activity-dependent changes in synaptic strength.

At dopaminergic synapses, the primary postsynaptic receptors are dopamine receptors, a family of G-coupled protein receptors. Rather than acting as ion channels in response to neurotransmitter release, binding of dopamine to these receptors facilitates the activation and release of G-proteins associated with the receptor, which then activate further downstream signaling pathways (**Fig 1.4**) (reviewed in Beaulieu and Gainetdinov, 2011). There are many different types of dopamine receptors which can be grouped into two broad categories; D1-like receptors and D2-like receptors. D1-like receptors, which have an excitatory or potentiating effect at synapses, stimulate adenylate cyclase, resulting an increase in the intracellular ‘second messenger’ cyclic AMP, which in turn activate cyclic AMP-dependent protein kinases at the synapse. Conversely, activation of D2-like receptors, which primarily have an inhibitory or

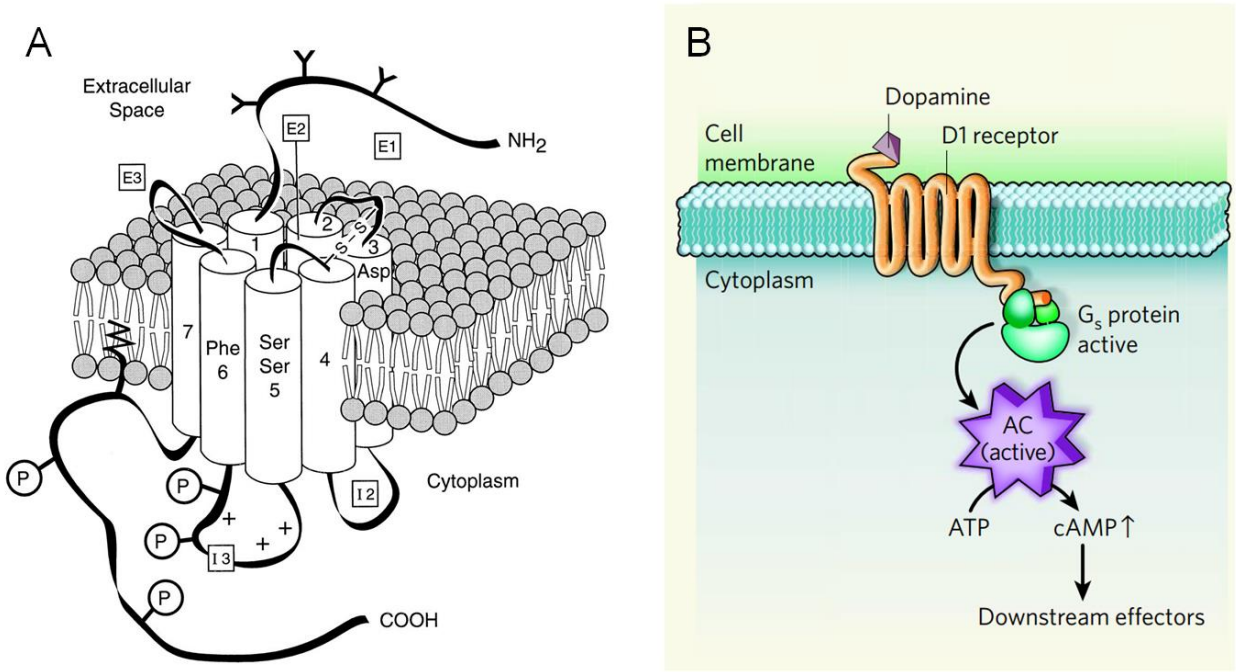


Figure 1.4 Dopamine receptor activation
A) Structure of dopamine receptor (D1 receptor shown). Dopamine receptors are G-protein coupled receptors, and are composed of several transmembrane domains (numbered 1-7 in schematic), an extracellular N-terminal head region and an intracellular C-terminal tail. B) Upon binding of dopamine, a conformation change causes facilitates the activation of the associated G-protein by substitution of GDP for GTP. The activated G-protein then activates the enzyme adenylyl cyclase, catalyzing the formation of cyclic AMP (cAMP) which acts as a second messenger and activates various downstream effectors (Adapted with permission from Missale et al., 1998, Weinshenker and Warren, 2008).

depotentiating effect at synapses, inhibits adenyl cyclase and reduces intracellular cyclic AMP levels. Because of the ability of dopamine to influence the activity and strength of other synapses, and cause both excitation and inhibition of the postsynaptic neuron, dopamine is often described as a ‘neuromodulatory’ neurotransmitter. The majority of dopaminergic neurons in the brain are spatially restricted to the mesolimbic system, including the ventral tegmental area (VTA), the substania nigra (SN). However, neurons in this region project to neurons throughout the brain, including the nucleus accumbens and throughout the prefrontal cortex, and constitute a

critical circuit involved ing motivated behaviour and reward learning in the brain (Johnson and Kenny, 2010, Beaulieu and Gainetdinov, 2011).

1.4 THE CADHERIN ADHESION COMPLEX

1.4.1 Cadherins

The sophisticated assembly of postsynaptic receptors to specific types of presynaptic inputs requires a wide variety of synaptic adhesion molecules. One of the most important and best-studied and most important families of adhesion molecules are cadherins, which mediate adhesion throughout the body at adherens junctions between cells and have many specialized roles at synapses. The cadherin superfamily can be divided into a number of different subcategories, including classical cadherins, protocadherins and desmosomal cadherins (**Fig. 1.5**). Structurally, most cadherins are transmembrane proteins with an intracellular tail and commonly have extracellular 'cadherin' repeats, specialized domains that become structurally rigid in the presence of calcium ions (Takeichi, 1995). Other cadherins have unique structural features, such as Cadherin 13 (also called T- of H-cadherin) which lacks a transmembrane region and is instead anchored at the synaptic membrane by a glycosyl-phosphatidylinositol glycolipid anchor (Ranscht and Dours-Zimmermann, 1991).

1.4.2 Classical cadherins

Classical cadherins have several common structural features which are critical to their function. All classical cadherins have five extracellular cadherin repeats, forming a large extracellular domain that becomes structurally rigid in the presence of Ca^{2+}, and allows cadherins to interact in *trans* with other cadherins and provide strong adhesion between cells

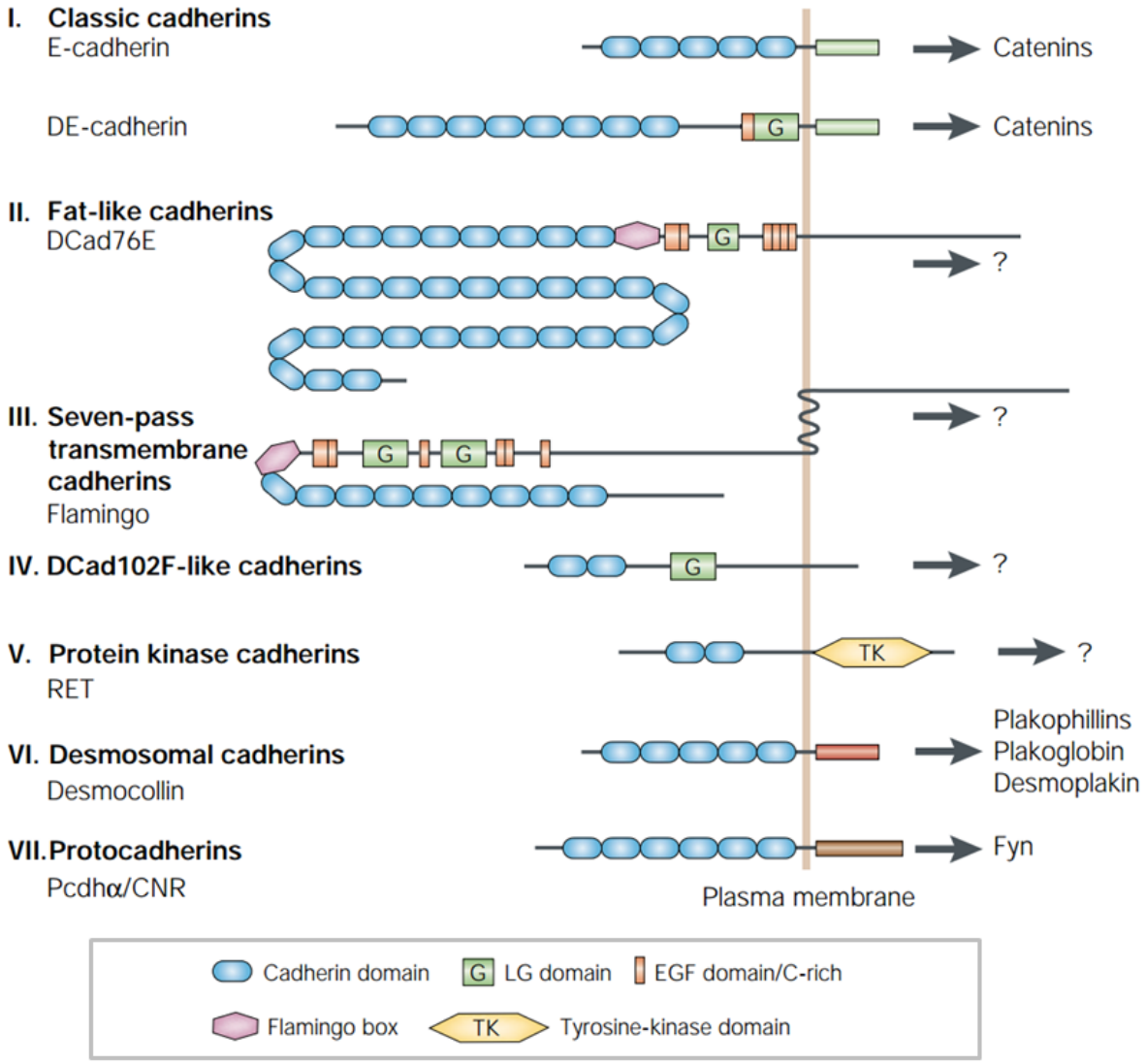


Figure 1.5 The cadherin superfamily.
Representative examples of different cadherin subfamilies. All cadherins have at least one 'cadherin' domain; an extracellular domain which co-ordinates calcium ions. Other common structural features include a laminin G (LG) domains and epidermal growth factor (EGF) domains. 'Fat-like' and 'seven-pass transmembrane' cadherins are considerably larger than classical cadherins, and often have a 'flamingo box' domain in their extracellular region. Protein kinase cadherins also have tyrosine kinase domains located in their intracellular C-terminal tail region. The 'protocadherin' family consists of 52 different proteins produced from alternative splicing of 3 protocadherin genes. Intracellular binding partners for some cadherin families have also been identified, though many remain unknown. Classical cadherins and DE-cadherin interact with the catenin family of proteins, while desmosomal cadherins interact with plakophilins, plakoglobin and desmoplakin, and protocadherins interact with the tyrosine kinase Fyn via their intracellular C-terminal tails (Adapted with permission from Tepass et al., 2000).

(Takeichi, 1995). Classical cadherins also have a single transmembrane domain and an intracellular C-terminal tail region, both of which have many binding sites that mediate interactions with other proteins at the synapse. Classical cadherins can be further divided into type I cadherins (including N-cadherin, E-cadherin, P-cadherin), which have a highly conserved His-Ala-Val (HAV) adhesion recognition sequence, and type II cadherins (including cadherin 7, 8, and 11), which have similar overall structure but lack this HAV motif. Type I cadherins primarily form homophilic interactions with cadherins of the same type, while Type II cadherins frequently form both homophilic associations and heterophilic associations with other types of cadherins (Shimoyama et al., 2000). The highly conserved N-terminal extracellular cadherin repeat (EC1) appears to confer the adhesive properties and binding specificity of cadherins. Analysis of the interaction between cadherins EC1 domains by protein crystallography has shown that type I cadherins exchange a single N-terminal β strand between EC1 domains, resulting in strong and specific adhesion between the two proteins, while type II cadherins exchange two β strands between their EC1 domains. (Patel et al., 2006). In addition to interacting in *trans* to provide adhesion between cells, cadherins have also been shown to interact in *cis* with adjacent cadherins at the cellular membrane (**Fig 1.6**). These interactions in *cis* are mediated by an interaction between a different region of the EC1 domain with the EC2-EC3 linker region of adjacent cadherins, which promotes the clustering and organization of large groups of cadherins into a lattice-like structure at sites of adhesive contact (Wu et al., 2010).

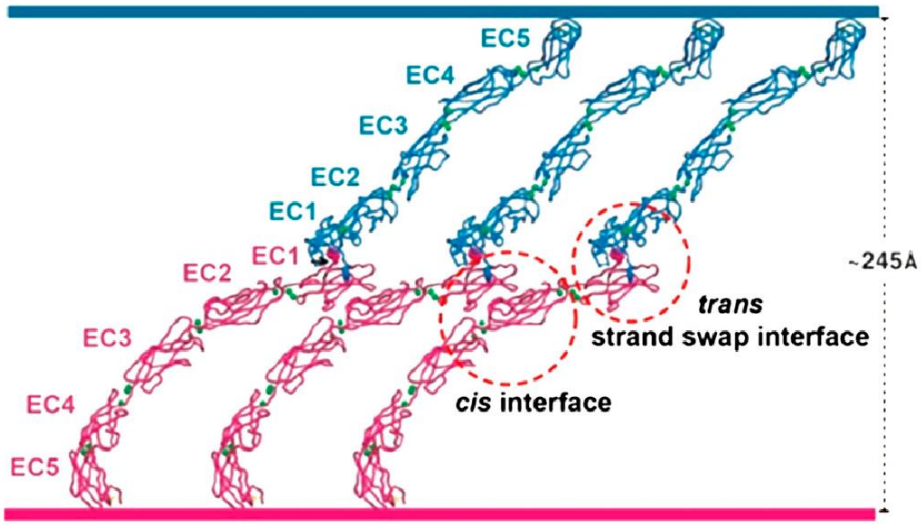


Figure 1.6 Cadherin interactions in *cis* and *trans*.
X-ray crystallography studies of cadherin interactions have identified different sites which mediate binding with other cadherins. Interactions in *trans* are mediated by the exchange of a single N-terminal beta strand between the N-terminal extracellular cadherin (EC) repeat domain. A second interaction can occur in *cis* between a different region of the EC1 domain and the linker region between the EC2 and EC3 domains of a neighbouring cadherin, creating a more stable lattice structure between cadherins (Adapted with permission from Wu et al., 2010).

At synapses, cadherins are enriched at pre- and post-synaptic compartments (Uchida et al., 1996), and mediate adhesion by homophilic binding across the synaptic cleft (reviewed in Arikkath and Reichardt, 2008). Indeed, the size of the synaptic cleft is appears to closely match the dimensions of the cadherin extracellular domains interacting in *trans*, suggesting cadherins play a central role in organizing the fundamental architecture of the synapse (Tai et al., 2008). Cadherins also interact with many other proteins at the synapse, forming a multi-protein structure called the 'cadherin adhesion complex' (**Fig 1.7**). Among the most important proteins associated with cadherin are the catenins, which are grouped into three subfamilies; 1) the α-catenin family, including αN-catenin, αE-catenin, and αT-catenin, 2) the β-catenin family, which includes β-catenin and plakoglobin, and 3) the P120 catenin family, which includes P120 catenin, δ-catenin, ARVCF (armadillo repeat gene deleted in velocardiofacial syndrome), and P0071 (Arikkath,

2009, Keil et al., 2013). P120 catenin and δ-catenin bind to the juxtamembrane domain of cadherin, and are believed to stabilize cadherin at the synaptic membrane and promote its clustering in *cis*, increasing the stability of cadherin adhesion at synapses (Brigidi et al., 2014).

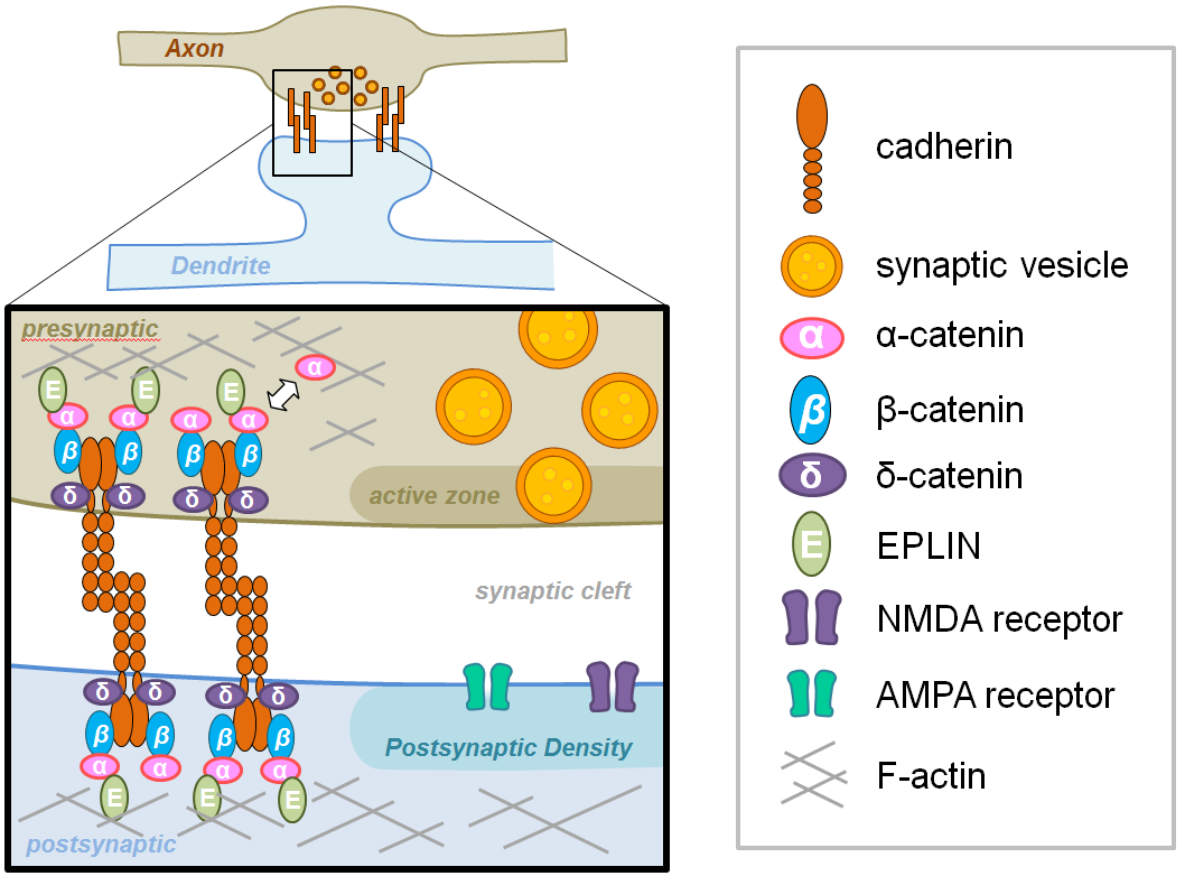


Figure 1.7 The cadherin adhesion complex at the synapse.
Cadherins are enriched both pre-and post-synaptically, and interact with a number of different proteins at the synapse and play an important role in synapse assembly. On both sides of the synapse cadherins interact with a series of intracellular binding partners (β-catenin → α-catenin → EPLIN → actin filaments) which form a link between cadherins and the actin cytoskeleton. β-catenin also recruits the proteins scribble and β-pix to synapses (not shown), which promotes actin polymerization. These direct and indirect associations with the actin cytoskeleton are important for the recruitment of synaptic vesicles presynaptically. Postsynaptically, β-catenin has also been shown to regulate AMPA receptor localization to synapses, though the precise mechanisms behind this remain unclear (Image made by Fergil Mills).

Cadherins form many direct and indirect associations with AMPA receptors at synapse, providing a link between the structural adhesion and stability mediated by cadherins with the

physiological strength of the synapses (Fig 1.8). The extracellular N-terminal region of the AMPA receptor GluA2 interacts directly with N-cadherin in *cis* and in *trans*, which is critical for the normal morphological and physiological development of synapses (Saglietti et al., 2007). The GluA1 subunit of AMPA receptors also interacts with N-cadherin in *cis*, (Nuriya and Huganir, 2006). Cadherins can interact with the protein AKAP79/150 at synapses, which also associates with AMPA receptors through the protein PDZ proteins such as SAP97 (Gorski et al., 2005). Cadherins and AMPA receptors also interact simultaneously with the scaffold protein GRIP1 in transport vesicles, and have recently been shown to be trafficked together to postsynaptic sites in dendrites (Heisler et al., 2014).

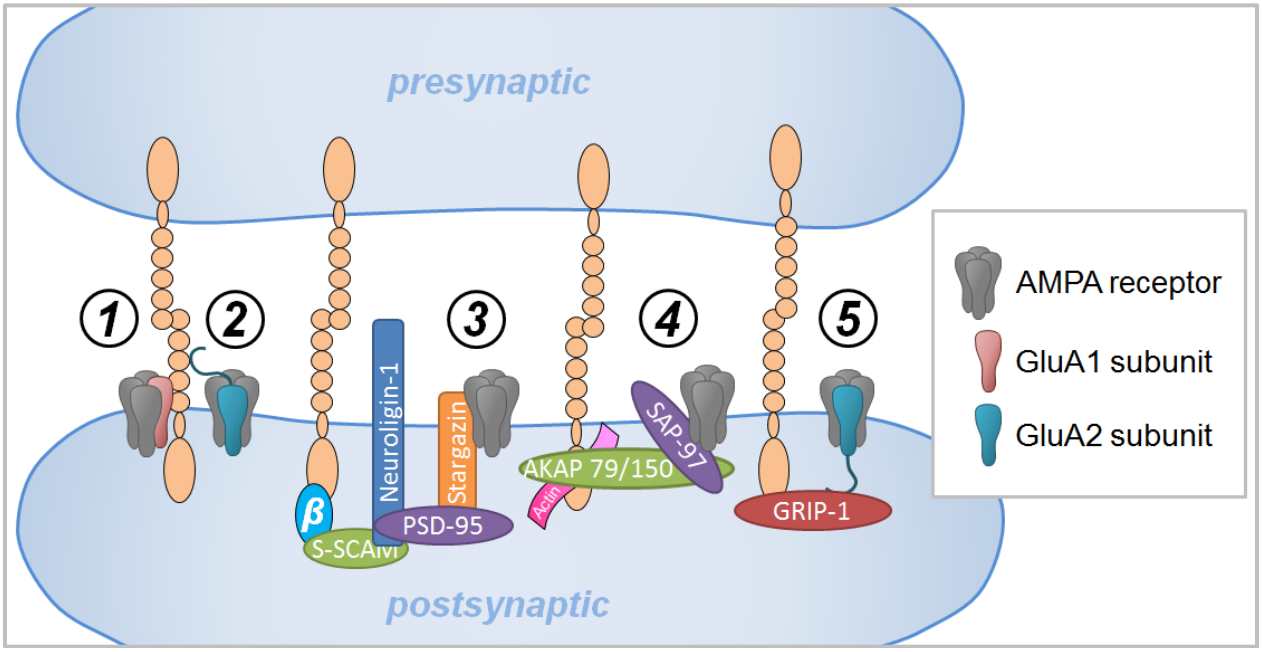


Figure 1.8 Interactions between cadherin and AMPA receptors at synapses.
(1,2) Cadherins form direct associations with both GluA1 and GluA2 subunits of AMPA receptors at synapses. (3) Cadherins interact with neuroligins at the synapse through the scaffold protein S-SCAM, and as a consequence are situated to stabilize AMPA receptors associated with PSD-95, another binding partner of neuroligins. (4) Cadherins can also associate with AMPA receptors through the protein AKAP 79/150, as well as (5) GRIP-1, which simultaneously associations with both cadherins and AMPA receptors and facilitates their delivery together to synapses (Image made by Fergil Mills).

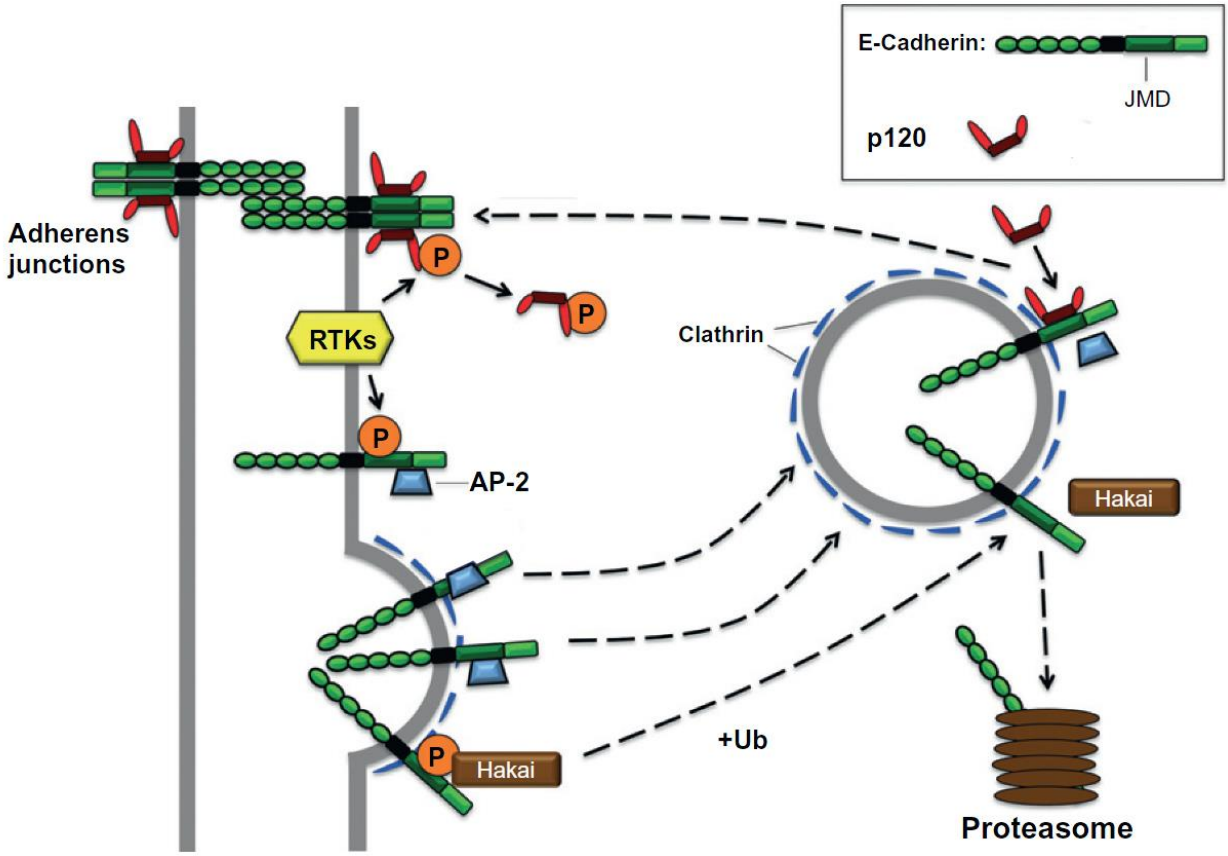


Figure 1.9 Mechanisms of cadherin endocytosis.
In the absence of p120 catenin, adaptor protein 2 (AP-2) can associate with a binding motif in the juxtamembrane domain (JMD) of cadherin, which promotes its endocytosis via clathrin-coated pits. The dissociation of p120 catenin from cadherin can be facilitated by phosphorylation of p120 catenin or cadherin by receptor tyrosine kinases (RTKs). Additionally, the protein Hakai can associate with cadherin and promote its ubiquitinylation, leading to its degradation in the proteasome. (Adapted with permission from Kourtidis et al., 2013).

Removal of cadherin from the synapse is primarily achieved by clathrin-mediated endocytosis (**Fig. 1.9**). The juxtamembrane region of cadherin contains an endocytic signal motif (DEE) that is blocked by the association of p120 with cadherin (Nanes et al., 2012). In the absence of p120 binding this motif is exposed, allowing the protein adaptor protein 2 (AP-2) to associate with cadherin and promote its removal from the synaptic membrane in clathrin-coated endosomes (Chiasson et al., 2009).

1.5 β-CATENIN

1.5.1 β-catenin structure and function

β-catenin is a soluble protein that is a major intracellular binding partner of all classical cadherins (**Fig 1.10**). β-catenin binds to the C-terminal tail of cadherin via a series of 'armadillo' or 'ARM' repeats, resulting in the stabilization of cadherin at the membrane. In the absence of β-catenin the cytoplasmic tail of cadherin is unstructured, and motifs that facilitate the proteolysis of cadherins are exposed (Huber et al., 2001). In addition to these ARM repeats, β-catenin also has a PDZ binding domain near its C-terminal tail (Perego et al., 2000), and an N-terminal α-catenin binding domain (Aberle et al., 1996, Pokutta and Weis, 2000).

Cadherin, β-catenin, α-catenin and actin have all been found to localize together to adherens junctions in epithelial cells, suggesting they form a complex that links cadherin to the actin cytoskeleton. Indeed, the name 'catenin' is derived from the Latin *catena*, meaning chain (Ozawa et al., 1989). Investigations into the interactions between these proteins have resulted in several revisions in our understanding of the structure of this complex. β-catenin was initially believed to simultaneously bind to both cadherin and α-catenin, which in turn bound to actin filaments, providing a direct link between cadherins and the actin cytoskeleton. However, detailed *in vitro* studies of the interactions between these proteins demonstrated that α-catenin

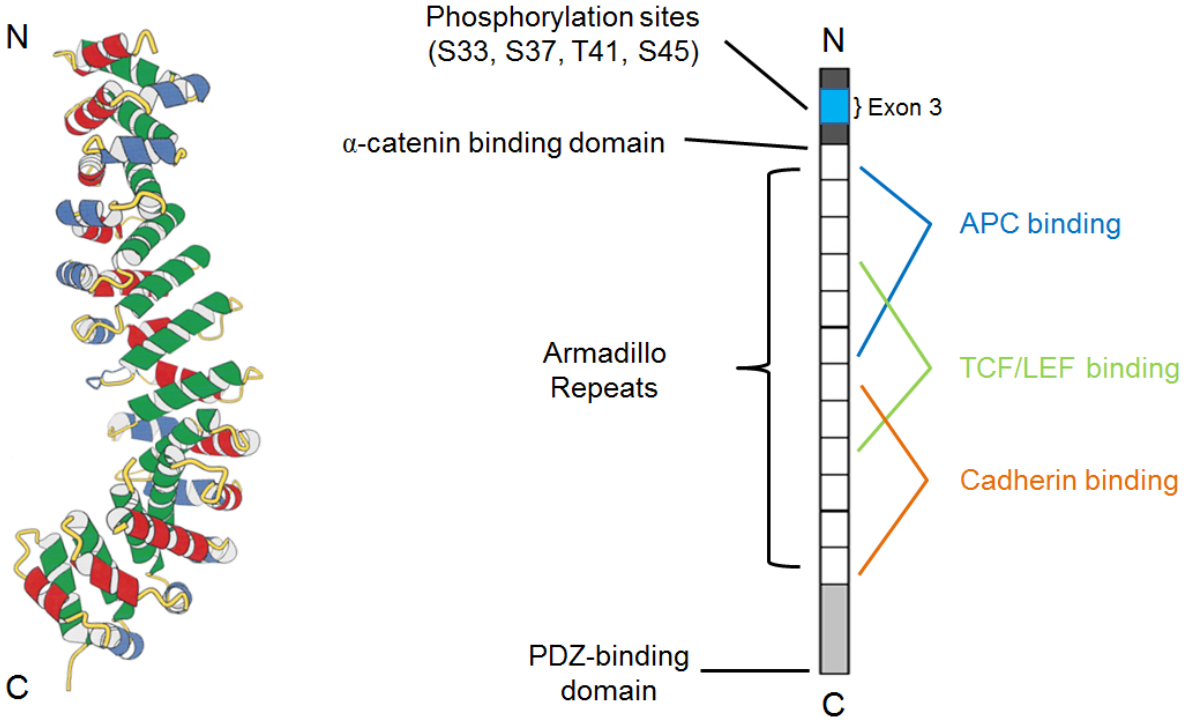


Figure 1.10 Structure of β-catenin.
β-catenin has several important structural features which mediate its roles in cell adhesion and signaling. The primary structural feature of β-catenin is a series of coiled a-helices called 'armadillo' repeats. The repeats mediate β-catenin's interaction with cadherins, its binding to TCF/LEF (T-cell factor/lymphoid enhancing factor) transcription factors, which promote the transcription of Wnt target genes. Armadillo repeats also facilitate β-catenin's interaction with APC (adenomatous polyposis coli) and other components of the 'β-catenin destruction complex' which phosphorylates β-catenin and target it for degradation. β-catenin also has a PDZ binding domain at its C-terminal tail end, and an α-catenin binding domain near its N-terminal head. Finally, of particular importance for regulating β-catenin's stability are a series of phosphorylation sites (S33,S37,T41,S45) located near the N-terminal of β-catenin, in a region encoded by exon 3 of the β-catenin gene (*Ctnnb1*) (Adapted with permission from Huber et al., 1997).

could not simultaneously bind β-catenin and actin (Yamada et al., 2005), and it was instead proposed that α-catenin dynamically switched between interacting with β-catenin (Drees et al., 2005), despite the ordered co-localization of α-catenin with cadherin at adherens junctions in epithelial cells. Further investigation revealed that an additional protein, EPLIN (epithelial

protein lost in neoplasm), was localized to these sites and could interact with both actin and α-catenin bound to β-catenin, indicating that all these proteins simultaneously be present in a single multi-protein complex (Abe and Takeichi, 2008). Mouse genome-wide atlas projects have identified that EPLIN is widely expressed throughout the brain (Lein et al., 2007), suggesting it plays a similar role at synapses in linking cadherin with the actin cytoskeleton.

Through its interactions with cadherins, actin cytoskeleton proteins and many other binding partners at the synapse, β-catenin also plays an important role in synapse assembly. Presynaptically, β-catenin is important for the localization of synaptic vesicles to synapses; deletion of β-catenin results in dispersion of synaptic vesicles along axons and a reduction of the number of reserve pool localized to presynaptic terminals (Bamji et al., 2003). Factors that disrupt cadherin/β-catenin interactions result in the mobilization of synaptic vesicles from synapses (Bamji et al., 2006), whereas factors that promote their association enhance the localization of vesicles to synapses (Lee et al., 2008). Additionally, β-catenin can regulate actin polymerization and specifically regulate synaptic vesicle localization through its recruitment of scribble (Sun et al., 2009) and the Rac/cdc42 guanine nucleotide exchange factor, β-pix (Sun and Bamji, 2011), promoting the formation of localized clusters of filamentous actin within presynaptic compartments which are thought to act as a structural tether for the reserve pool of synaptic vesicles (Dillon and Goda, 2005). Though less is known about the contribution of β-catenin to postsynaptic assembly, targeted deletion of β-catenin has been shown to reduce quantal AMPA receptor responses and activity-dependent scaling of synapse strength, suggesting that β-catenin may also play a role in the trafficking or recruitment of a functional pool of AMPA receptors at synapses (Okuda et al., 2007).

1.5.2 β-catenin degradation and signaling

In addition to its roles in synapse assembly and adhesion, β-catenin is also a key component of the Wnt signaling pathway, a highly conserved signaling pathway that regulates gene expression and plays a critical role in morphogenesis during development (**Fig 1.11**). Under basal conditions, levels of β-catenin are carefully regulated in cells; β-catenin is phosphorylated at Ser-33/Ser-37/Thr-41 by glycogen synthase kinase 3β (GSK3β) (Yost et al., 1996) targeting it for ubiquitinylation and degradation, a process mediated by a 'β-catenin destruction complex' including the scaffold proteins Axin2 and adenomatous polyposis coli (APC) (Aberle et al., 1997, Hart et al., 1999). However, in the presence of Wnt ligands, Wnt receptors sequester Axin2, preventing the formation of this destruction complex and allowing β-catenin to accumulate in cells. Following an increase in cytoplasmic β-catenin, β-catenin can be translocated to the nucleus where it interacts with TCF/LEF family transcription factors to initiate transcription of Wnt target genes (Clevers, 2006)

β-catenin can also mediate activity-dependent gene expression in neurons. NMDA receptor activation during synaptic activity has been shown to activate the protease calpain and promote cleavage of β-catenin, producing a truncated fragment of β-catenin lacking its N-terminal region, which translocates to the nucleus and induces TCF/LEF-depended gene transcription, including the immediate-early response gene *Fosl1* (Abe and Takeichi, 2007). Remarkably, this N-terminal fragment lacks the phosphorylation sites Ser-33/Ser-37/Thr-41 which typically target β-catenin for degradation, suggesting that other as-yet undiscovered pathways for control of β-catenin levels exist in neurons.

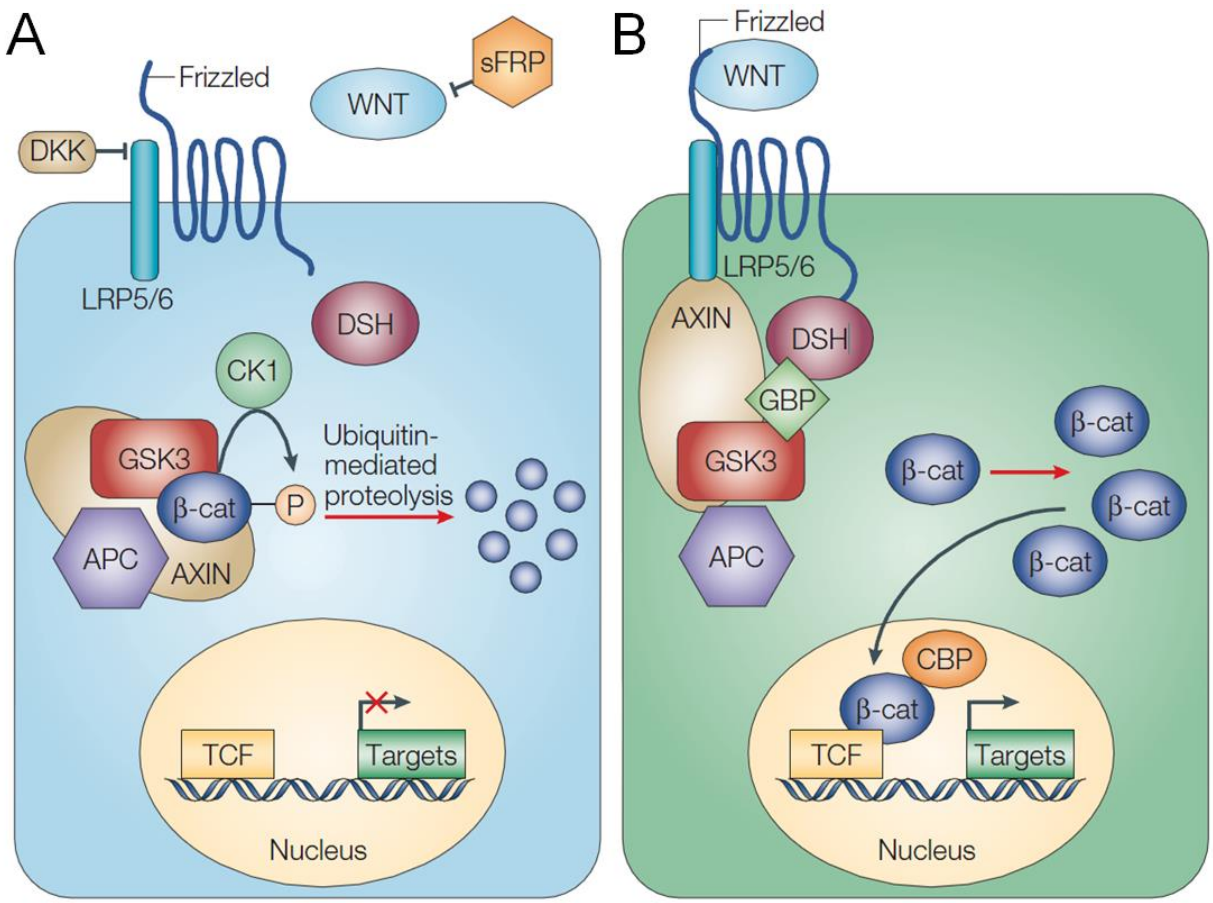


Figure 1.11 β-catenin and Wnt signaling.
A) In the absence of Wnt ligands, cytoplasmic β-catenin can associate with a multi-protein 'β-catenin destruction complex' which includes GSK3β and the scaffold proteins axin and APC (adenomatous polyposis coli). β-catenin is then phosphorylated at sites near its N-terminal which targets it for degradation via the ubiquitin-proteasome degradation pathway. B) When Wnt ligands bind to the receptor frizzled, the β-catenin destruction complex is sequestered by the binding of axin and disheveled (DSH), allowing β-catenin to accumulate in cells. β-catenin can then translocate to the nucleus and associate with TCF/LEF (T-cell factor/lymphoid enhancing factor) transcription factors to initiate transcription of Wnt target genes.

1.6 THE CADHERIN ADHESION COMPLEX IN THE FORMATION AND PLASTICITY OF CNS SYNAPSES

1.6.1 The cadherin adhesion complex in synapse formation

The cadherin adhesion complex plays an important role in synapse formation. Cadherins are present throughout developing neurons, and are among the first proteins localized to sites of contact between axons and dendrites (Benson and Tanaka, 1998). Cadherins are believed to help recruit and organize other components to and pre and postsynaptic sites. In addition to the essential role of β-catenin in presynaptic assembly of synaptic vesicles and postsynaptic recruitment of AMPA receptors (Bamji et al., 2003, Okuda et al., 2007), disruption of cadherin function has also been shown impair the recruitment of the cell adhesion molecules neuroligins (Aiga et al., 2011) and the postsynaptic scaffold protein PSD-95 to synapses (Togashi et al., 2002). N-cadherin has been shown to work cooperatively with neuroligin-1 and S-SCAM to promote the clustering of synaptic vesicles to synapses *in vitro* (Stan et al., 2010). Disruption of N-cadherin or different catenin proteins in cultured neurons has been shown to disrupt the structure of dendritic spines, typically causing a reduction in the density of dendritic spines as well as a phenotype of immature elongated spine morphology (Abe et al., 2004, Elia et al., 2006, Okuda et al., 2007).

However, the effects of cadherin manipulation are more pronounced at earlier developmental points *in vitro*, and have less impact at mature synapses (Togashi et al., 2002). Consistent with this, *in vivo* studies have shown that ablation of N-cadherin or β-catenin in late postnatal excitatory neurons did not affect basal synapse density or morphology (Bamji et al.,

2003, Bozdagi et al., 2004, Nikitczuk et al., 2014). Similarly, knockout of N-cadherin or β-catenin in adult neurons in vivo has not been found to affect basal synapse strength (Bamji et al., 2003, Bozdagi et al., 2010), though the possible functional redundancy of different cadherins to maintain synaptic adhesion may mask the effects that disruption of individual cadherins on synapse density. The pattern of expression of cadherin also varies with respect to different types of synapses and stages of their development. N-cadherin is initially localized to both nascent excitatory and inhibitory synapses, however as these synapses mature N-cadherin is found only at a subpopulation of excitatory synapses (Benson and Tanaka, 1998), while E-cadherin plays an important role at inhibitory synapses (Fiederling et al., 2011), suggesting that different cadherins may specialized functional roles at different synapse types.

1.6.2 The cadherin adhesion complex in synaptic plasticity

Many studies have demonstrated that the β-catenin/cadherin adhesion complex is important for synaptic plasticity. Unlike its role in synaptogenesis, which is most important at earlier developmental stages, cadherin has been shown to play a critical role mediating changes in synaptic strength at mature synapses *in vitro* and in the adult brain *in vivo*. First, cadherin adhesion in *trans* is critical for LTP at synapses. Treatment with antibodies against the extracellular domains of N-cadherin and E-cadherin or treatment with an HAV (His-Ala-Val) peptide which blocks adhesion between the EC1 groups of classical cadherins both disrupt the induction of LTP at synapses in acute hippocampal slices, but neither treatment affects the strength of previously potentiated synapses (Tang et al., 1998). Following enhanced synaptic activity, protein levels of N-cadherin and number of N-cadherin puncta are increased in neurons,

which is correlated with the onset of late-phase LTP (L-LTP) (Bozdagi et al., 2000). Cadherin is also required for increases in the size and stability of synapses and dendritic spines following activity (Okamura et al., 2004), and ablation of N-cadherin impairs the persistent enlargement of dendritic spines associated with LTP (Bozdagi et al., 2010). The most detailed analysis to date of N-cadherin trafficking following enhanced activity has directly demonstrated that N-cadherin is synthesized *de novo* and specifically recruited to synapses potentiated by tetanic stimulation, and that this recruitment of cadherin is essential for increases in spine size and stability at these synapses following activity (Mendez et al., 2010).

While many of these studies have focused on N-cadherin due to its prevalence at well-studied excitatory synapses in the hippocampus (**Fig 1.12**), other cadherins may have distinct roles in different forms of synaptic plasticity. In conditional knockout mice lacking cadherin-11, a type-II classical cadherin, LTP was found to be significantly enhanced at synapses in the hippocampus (Manabe et al., 2000). This surprising result suggests considerable functional differences exist between different cadherins, and further study is required to understand the contribution of each type of cadherin to synapse function.

In contrast to LTP, the role of cadherin in LTD is currently less well understood. LTD at excitatory synapses is mediated by the internalization of AMPA receptors from synapses, reducing synapse strength, and is accompanied by decrease in the size of synapses and associated dendritic spines, as well as synapse elimination. Unlike LTP, conditional knockout of N-cadherin in hippocampal neurons *in vivo* did not impair LTD (Bozdagi et al., 2010), indicating that cadherin-based adhesion is non-essential with this form of plasticity. However, cadherin has been shown to be important for LTD mediated by metabotropic glutamate receptors (mGluR) (Zhou et al., 2011)

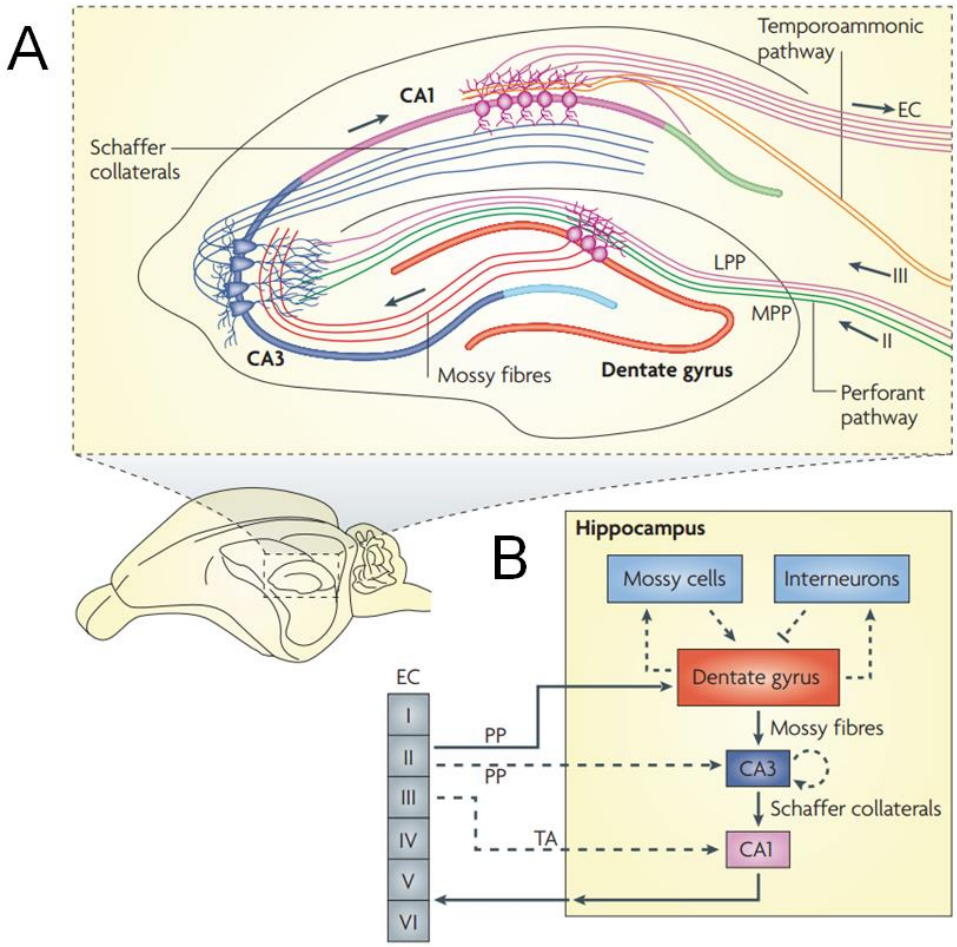


Figure 1.12 The connectivity of the hippocampus

A) A major pathway within the hippocampus is the 'trisynaptic circuit', a series of three synaptic connections between different groups of hippocampal neurons. First, perforant pathway inputs from layers II and III of the entorhinal cortex synapse onto neurons in the dentate gyrus (DG) of the hippocampus. DG neurons then project to neurons in the CA3 region via mossy cell fibers, and CA3 neurons project to neurons in the CA1 region via Schaffer collateral pathway axons. CA1 neurons then project out of the hippocampus to layer 5 of the entorhinal cortex. B) Schematic showing connectivity between different cell groups and layers in the hippocampus and entorhinal cortex. In addition to the trisynaptic circuit (solid lines), several other projections exist within the hippocampus (dashed lines), including direct inputs to CA3 neurons from performant pathway (PP) inputs and projections from layer III of the EC to the CA1 region via the temporoammonic (TA) pathway (Adapted with permission from Deng et al., 2010).

Regulation of β-catenin's association with cadherin has emerged as a key modulator of cadherin-based adhesion during different forms of synaptic plasticity. Dephosphorylation of β-catenin at tyrosine residue 654 (Y654) has been shown to increase its binding affinity for cadherin (Roura et al., 1999), and depolarization of neurons promotes β-catenin Y654 dephosphorylation leading to increased translocation of β-catenin into dendritic spines, and increased β-catenin/cadherin interaction at synapses (Murase et al., 2002). Cyclin-dependent kinase 5 (Cdk5) has been identified as a key kinase of this phosphorylation site, and depolarization of neurons inhibits the activity of this kinase and results in decreased phosphorylation of β-catenin at Tyr-654, and increased β-catenin /cadherin interaction (Schuman and Murase, 2003). However, forcibly maintaining β-catenin/cadherin interaction by expressing a mutant form of β-catenin which cannot be phosphorylated at this site (β-catenin Y654F) has been shown *in vitro* to impair the formation of new synapses following BDNF treatment (Bamji et al., 2006).

Expression of the β-catenin Y654F mutant has been shown to impair NMDAR-dependent LTD in cultured neurons *in vitro* (Tai et al., 2007). The form of NMDAR-dependent LTD measured in this study was expressed only as a decrease in frequency of mEPSCs, rather than a decrease in mEPSC amplitude which is the typical physiological consequence of AMPA receptor endocytosis at synapses (Luscher and Malenka, 2012). This study also found that NMDA treatment resulted in increased interaction between β-catenin and cadherin, and increased cadherin stability at the synaptic membrane, though increased recruitment of cadherin to synapses has since been found to be associated with LTP (Mendez et al., 2010). Thus, while the association between β-catenin and cadherin is dynamically regulated and affects cadherin

turnover, there is some ambiguity as to the contribution of changes in cadherin-based adhesion during different forms of plasticity.

The requirement of Ca^{2+} for cadherin adhesion may also be an important feature of cadherin's role in synaptic plasticity. Cadherin structure and adhesion can change in response to alterations in extracellular Ca^{2+} which occur in the synaptic cleft in response to synaptic activity and NMDA receptor activation. Consequently, changes in cadherin structure may act as intercellular signals by altering interactions with other cadherins in *trans* (Tai et al., 2008). Increasing extracellular Ca^{2+} has been found to prevent the impairment of LTP in response to disruption of cadherin with HAV peptides or anti-cadherin antibodies (Tang et al., 1998). This suggests that enhanced Ca^{2+} maintained interactions between EC1 binding domains of cadherin in *trans*, preventing antibodies and peptides from accessing these regions and disrupting cadherin adhesion (Tai et al., 2008).

Given that cadherin adhesion at synapses appears to be highly dynamic in response to synaptic activity, it has been hypothesized that transient disruption of cadherin adhesion is important for different forms of synaptic plasticity. At synapses, both the insertion and removal of AMPA receptors may require temporary disassembly of components of the cadherin adhesion complex (**Fig 1.13**) (Tai et al., 2008). This transient disruption may also be important to allow the formation of new synapses at sites, which may require redistribution of synaptic components from existing synapses (Bamji et al., 2006). In particular, the finding that enhanced β-catenin/cadherin interaction can impair LTD at synapses *in vitro* suggests that excessive cadherin based adhesion may be detrimental for certain forms of plasticity (Tai et al., 2007), may also affect certain forms of learning and memory.

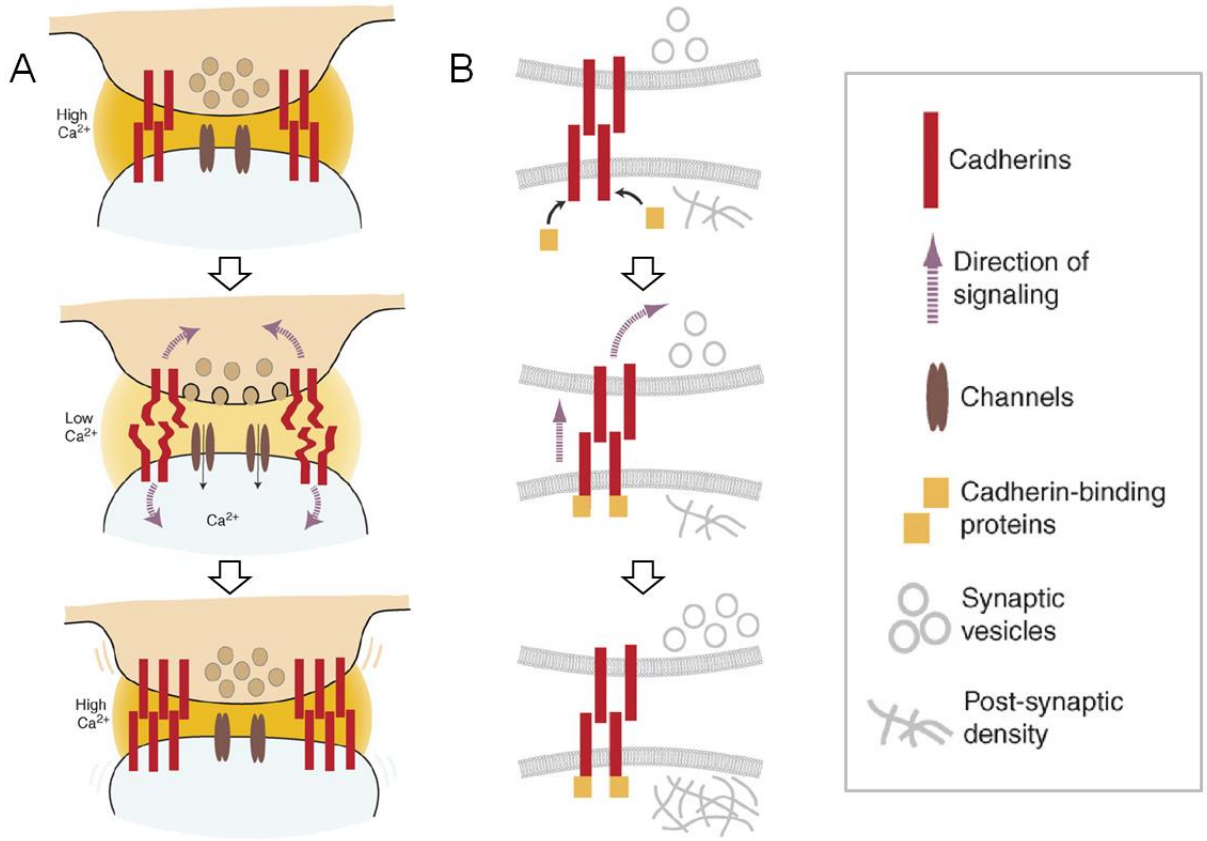


Figure 1.13 Trans-synaptic communication through cadherins.
A) Cadherin adhesion is dependent on high extracellular Ca^{2+}, to maintain structural rigidity of the extracellular 'cadherin' domain repeats (top). Changes in extracellular calcium concentration following synaptic activity could therefore disrupt cadherin-based adhesion, and potentially also propagate intracellular signals by affecting proteins bound to the C-terminal tail of cadherins within pre- and post-synaptic compartments (middle). It is also possible that transient destabilization of cadherins may be important for increases in synapse strength and adhesion at the synapse (bottom). B) The interactions between cadherins in *trans* may allow trans-synaptic signaling between the intracellular proteins on both sides of the synapse. Various different intracellular proteins regulate the stability and localization of cadherins at the synapse (top), which in turn affects the clustering of cadherins on adjacent cells through their homophilic interactions in *trans* (middle). Organization of these cadherins can then affect the intracellular localization of proteins in the adjacent cell (bottom) (Adapted with permission from Tai et al., 2008).

1.6.3 The cadherin adhesion complex in learning and memory

Experience-driven changes in synapse strength and function are believed to be one of the major mechanisms underlying learning and memory in the brain. Consequently, the importance of cadherins in different forms of synaptic plasticity strongly suggests the cadherin adhesion complex is important for learning, and studies investigating the effects of *in vivo* manipulation of components of the cadherin adhesion complex have begun to demonstrate this directly. Disruption of cadherin adhesion with HAV peptides in the hippocampus has been shown to cause deficits in long-term memory of contextual fear conditioning, which depends on intact hippocampal LTP, though short-term fear memory was unaffected (Schrick et al., 2007). This is consistent with studies of cadherin's role in LTP, which show that following disruption of cadherin function high-frequency stimulation can cause short-term potentiation of synapses, but longer-lasting changes in synapse strength are impaired (Tang et al., 1998, Bozdagi et al., 2010). Similarly, conditional deletion of N-cadherin *in vivo* was shown to cause an impairment in LTP and LTP-dependent behavioral tasks, specifically a radial arm version of the Morris water maze (Nikitczuk et al., 2014). Consequently, cadherin appears to be required to stabilize potentiated synapses which are involved in the encoding of memories in the brain.

Other components of the cadherin adhesion complex have also been implicated in learning and memory as well. A study investigating the role of β-catenin in learning and memory found that β-catenin, and the interaction between β-catenin and cadherin, may play an important role in in the consolidation of memory in the adult brain (Maguschak and Ressler, 2008). First, contextual fear conditioning was shown to cause post-translational modification of β-catenin in vivo. Immediately following the conditioned foot-shock stimulus there was significant increase

in the phosphorylation of β-catenin at the Tyr-654 residue (Maguschak and Ressler, 2008), which has been shown to *decrease* the binding affinity of β-catenin for cadherin (Roura et al., 1999). Consistent with this, immunoprecipitation experiments showed that the interaction between β-catenin and cadherin is transiently disrupted immediately following fear conditioning, and then re-established during fear memory consolidation (Maguschak and Ressler, 2008). Additionally, specific deletion of β-catenin in the amygdala was shown to prevent the transfer of newly acquired fear learning into long-term memory showing that this re-establishment of β-catenin/cadherin interactions was crucial to memory consolidation. However, despite the intriguing implication that de-stabilization of cadherin is important during learning, little is known about the role of the cadherin adhesion complex in behavioural flexibility, including tasks which may involve de-potentiation of synapses and the modification or elimination of information in the brain. Many *in vivo* studies of the β-catenin/cadherin adhesion complex have been limited to their role in fear conditioning, as it is a simple and powerful form of associative learning requiring low time investment. and involves well-studied regions of the brain - primarily the hippocampus and amygdala. However, the role of cadherin in other forms of learning has not yet been studied, and much less is known about the role of cadherin in plasticity or learning in non-hippocampal brain regions.

1.7 THE CADHERIN ADHESION COMPLEX IN DYSFUNCTION OF THE NERVOUS SYSTEM

1.7.1 Alterations in β-catenin levels in different diseases

As the cadherin adhesion complex plays many important roles at the synapse, alterations in cadherin function may have an impact on synapse function and cognition, and have been implicated in a number of neurological disorders. Several studies have examined as possible role for β-catenin and cadherin in the etiology of Alzheimer's disease (AD). Cadherins have been shown to interact with presenilin-1 (PS1) (Baki et al., 2001), one of the major molecules involved in the proteolytic cleavage of amyloid beta precursor protein (APP) to generate amyloid beta protein. PS1 is mutated in many cases of familial Alzheimer's disease (FAD) and is believed to be an important part of AD pathology (Vetrivel et al., 2006). As a component of the cadherin adhesion complex, PS1 is recruited to sites of cadherin adhesion between cells, including at synapses (Georgakopoulos et al., 1999), and has been shown to regulate the stability of both cadherin and β-catenin in cells. Indeed, many studies have investigated a possible role for β-catenin in AD. Aggregations of β-catenin phosphorylated at the Ser-33/37/Thr41 sites have been reported in the brains of AD patients, indicating that impairment in proteasome function may prevent the degradation of phosphorylated β-catenin in this disease (Ghanevati and Miller, 2005). β-catenin has also been shown to associate in a molecular complex with PS1 (Yu et al., 1998), and PS1 has been identified as a regulator of β-catenin stability and trafficking in cells (Zhang et al., 1998, Nishimura et al., 1999). However, different studies have reported conflicting results regarding the effect of mutations in PS1 on overall β-catenin stability. Some studies have shown that mutations in PS1 result in a destabilization of β-catenin in cultured cells,

resulting in impaired Wnt/β-catenin signaling and increased neuronal apoptosis (Zhang et al., 1998). In contrast, other studies have found that PS1 mutations increase the stability and overall levels and signaling activity of β-catenin in the brain (Kang et al., 1999, Soriano et al., 2001, Chevallier et al., 2005). Additionally, there appears to be a functional relationship between β-catenin and Tau, a microtubule-associated protein which has also been linked to AD pathology. Hyperphosphorylation of tau in cells has been shown to antagonize the phosphorylation of β-catenin in cells by GSK-3β, resulting in increased β-catenin levels and signaling which provides anti-apoptotic protection to neurons and may therefore disrupt the normal elimination of damaged cells in AD (Li et al., 2007). In sum, there appears to be greater evidence for an increase of β-catenin in AD, rather than the decrease which was initially reported (Zhang et al., 1998). However, to date studies of the effects of different AD-linked mutations and pathologies on β-catenin stability have not investigated the functional consequences of changes in β-catenin levels on cadherin-based adhesion or plasticity at the synapse.

The most substantive evidence of dysregulation of β-catenin in a neurodegenerative disorder is in Huntington's disease (HD). Brain samples from both human patients and animal models of HD have shown significant increase in β-catenin, which is believed to be due to the disruption of β-catenin's association with the β-catenin destruction complex by accumulated mutant polyQ-huntingtin protein (Godin et al., 2010). Targeted reduction of β-catenin was also found to have a therapeutic effect on polyQ-huntingtin-induced toxicity and mortality in *Drosophila* models of HD. However, similar to studies of β-catenin in Alzheimer's disease discussed above, the potential effects of this increase in β-catenin on synapse function in HD have not been investigated.

1.7.2 The cadherin adhesion complex in addiction

Changes in cadherin adhesion complex proteins have been associated with different forms of substance addiction. A genome-wide study of mutations associated with methamphetamine dependence in substance abusers identified several cell adhesion genes, including cadherin 13, as risk factors for addiction (Uhl et al., 2008). Single nucleotide polymorphisms (SNPs) in cadherin 12 have also been identified as risk factors for increased susceptibility to alcoholism (Lydall et al., 2011).

Increases in β-catenin have also been linked to various forms of substance abuse. Analysis of mRNA levels in the brains of primates following self-administration of cocaine found a significant increase (<150%) of β-catenin expression in the nucleus accumbens (NAc), an important region in the mesolimbic system which mediates reward and reinforcement learning (Freeman et al., 2001). In rodents, cocaine exposure has been shown to cause an increase in β-catenin protein levels in the NAc and caudoputamen (CPu), which is associated with an increase in downstream transcription of β-catenin target genes (Zhang et al., 2002). Increased levels of β-catenin have also been reported in the brains of patients with chronic alcoholism (Al-Housseini et al., 2008). However, the role of increased levels of β-catenin in the brain is unknown; while it appears to be a consequence of substance abuse, it may be a causative factor leading to addiction or some sort of compensatory response in the brains of chronic substance abusers. Additionally, the consequences of increased β-catenin at the synapse have not been investigated in any study to date.

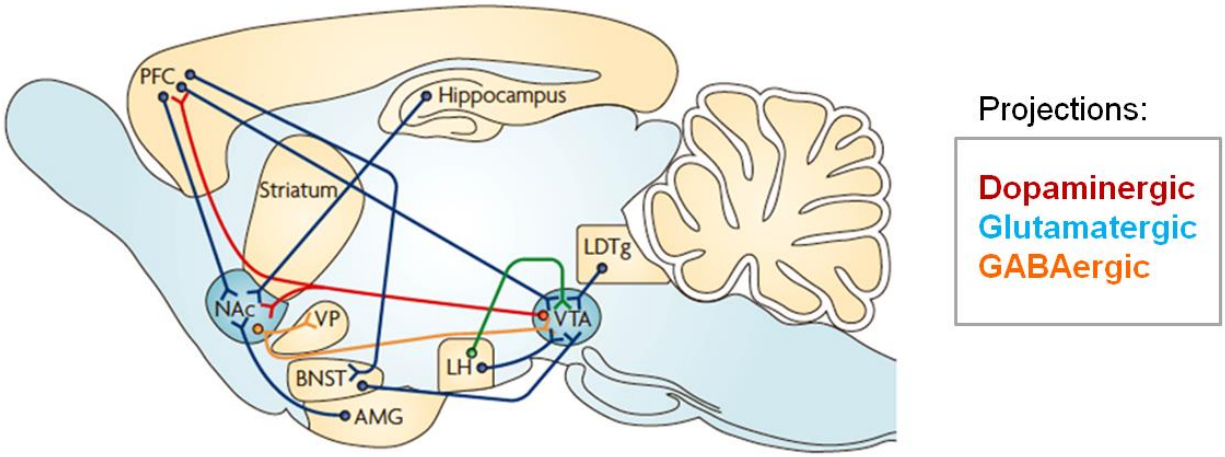


Figure 1.14 Inputs and projections of the ventral tegmental area (VTA)
Dopaminergic neurons in the VTA have been identified as a key site of importance for the formation and expression of addiction-related behaviours. These neurons send dopaminergic projections (red) from the VTA to the prefrontal cortex (PFC) and the nucleus accumbens (NAc). The activity of these DA neurons is in turn regulated by inputs from many different brain regions. VTA neurons receive excitatory glutamatergic inputs (blue) from many different brain regions, including the prefrontal cortex, the bed nucleus of the striata terminalis (BNST), the lateral habenula (LH) and the lateral dorsal tegmental nucleus (LDTg). They also receive inhibitory inputs (orange) from the nucleus accumbens and orexinergic inputs from the lateral habenula (Adapted with permission from Kauer and Malenka, 2007).

The changes in cadherin and β-catenin reported in these studies are of interest as the cadherin adhesion complex is well-situated to mediate changes in synaptic plasticity in regions of the brain associated with addiction. Drugs of abuse have been shown to cause an increase in dopamine release from dopaminergic neurons (DA neurons) in the VTA which project to the prefrontal cortex and nucleus accumbens, a critical circuit for reward learning in the brain. (**Fig 1.14**) (reviewed in Mameli and Lüscher, 2011, Lüscher, 2013). Excitatory synapses formed onto these DA neurons are key sites which regulate the activity of this circuit, and changes in the trafficking of AMPA receptors at these synapses (processes known to be affected by changes in cadherin function) have been implicated in the establishment and expression of drug-associated behaviour. Several studies have shown that cocaine exposure leads to changes in the composition

of AMPA receptors at these synapses. Electrophysiological recordings have demonstrated that exposure to cocaine causes a rapid (1-2 hours) and significant increase in the AMPAR-to-NMDAR ratio at excitatory synapses onto DA neurons in the VTA, which last up to 10 days after cocaine administration (Borgland et al., 2004). Additionally, cocaine has been shown to cause insertion of calcium-permeable AMPA receptors (CP-AMPARs) composed of homomeric associations of GluA1 AMPA receptor subunits to the excitatory synapses in the VTA (**Fig 1.15**). The increase in postsynaptic Ca^{2+} influx caused by the presence of these CP-AMPARs is believed to contribute to the changes in synaptic plasticity caused by cocaine which lead to addiction (Wolf and Tseng, 2012). Interestingly, self-administration paradigms of cocaine exposure have been shown to cause more robust changes in plasticity at these synapses, including increased recruitment of CP-AMPARs and increased timescales for changes in plasticity. In some cases, self-administration of cocaine was shown to cause persistent LTP at synapses in the VTA lasting up to several months (Chen et al., 2008). This suggests that the changes in plasticity at these synapses are due to an interaction between the effects of cocaine and a form of association learning involving the animal's drug-seeking behaviour and the drug's effects.

The reversal of cocaine-mediated CP-AMPAR insertion relies on form of LTD mediated by metabotropic glutamate receptor 1 (mGluR1). Activation of mGluR1 selectively removes GluA2-lacking AMPA receptors from the excitatory synapses in the VTA, restoring these synapses to their original state with calcium-impermeable AMPA receptors composed of GluA1/2 heteromers present at the synapse (Bellone and Lüscher, 2006). This form of plasticity can be blocked by administration of mGluR1 antagonists and enhanced by positive modulators of mGluR1 function, and the magnitude of LTD evoked by mGluR1 activation was found to be

greater in cocaine-treated mice due to the increased amount of GluA1 homomers at synapses in the animals (Bellone and Lüscher, 2006).

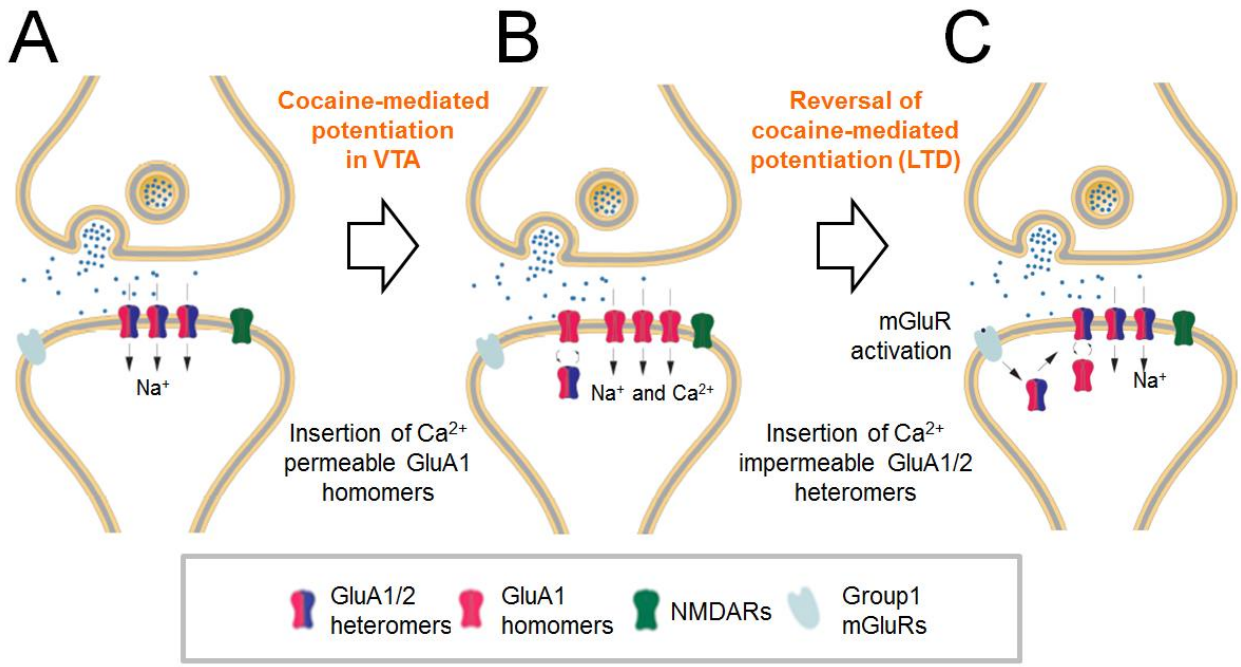


Figure 1.15 Changes in AMPA receptor composition at excitatory synapses in the VTA following cocaine administration.
A) Under basal conditions, AMAP receptors at excitatory synapses onto dopaminergic neurons in the VTA are primarily GluA1/2 heteromers, which are impermeable to calcium. B) Following cocaine exposure, calcium-permeable GluA1 homomers are inserted at the synapses, allowing Ca^{2+} influx postsynaptically in response to glutamate release, a critical intercellular signal which promotes plasticity and potentiation of synapses. C) The reversal of these cocaine-mediated changes in AMPA receptor composition is mediated by a form of LTD through metabotropic glutamate receptors (mGluRs), leading to endocytosis of GluA1 homomers and restoration of GluA1/2 heteromers at the synapse (Adapted with permission from Shepherd and Huganir, 2007).

As cadherins interact directly and indirectly with AMPA receptors at the synaptic membrane, changes in cadherin adhesion and trafficking may have an important effect on these drug-induced changes in AMPA receptor localization. The finding that forcibly maintaining β-catenin/cadherin interactions can impair LTD *in vitro* (Tai et al., 2007) provides some insight into possible mechanisms by which changes in cadherin could affect plasticity at these synapses.

Association of cadherins with calcium-permeable GluA1 homomers at synapses could help maintain their stability at synapses and prolong the effects of cocaine on synaptic plasticity, and impair the mGluR-LTD-mediated endocytosis of GluA1 homomers required to restore normal AMPA receptor composition at these synapses. Increases in cadherin adhesion and stability at the synaptic membrane, due to mutations within cadherins themselves of increased association of binding partners such as β-catenin, would exacerbate this effect. However, while *in situ* hybridization has identified the mRNA expression of several classical cadherins in the mesolimbic system, including the VTA (Hertel et al., 2008), the expression levels and cell-type specificity of different cadherins, and their function at synapses in the VTA, remain unknown.

1.8 RATIONALE AND HYPOTHESIS

Intact cadherin-based adhesion is extremely important for synapse formation and plasticity, processes which form the biological basis for learning and memory in the brain. Additionally, interaction between cadherin and intracellular binding partners is important for synapse assembly and maintaining the strength and stability of cadherin adhesion. However, due to the importance of the cadherin adhesion complex in many different aspects of synapse function, it is possible that excessive amounts of cadherin adhesion could impair synaptic plasticity, potentially leading to deficits in learning and memory. Previous studies have demonstrated that disruption of cadherin adhesion can impair memory formation (Schrick et al., 2007), and targeted deletion of β-catenin can impair memory consolidation (Maguschak and Ressler, 2008). However, to date the consequences of increased cadherin adhesion have not been investigated. This is particularly important in light of the many neurological disorders which

have been associated with alterations in cadherin adhesion complex proteins. Increases in β-catenin levels have been reported in disorders such as Alzheimer's disease (Kang et al., 1999, Soriano et al., 2001, Chevallier et al., 2005) and Huntington's disease (Godin et al., 2010). Because mutations in cadherins and associated proteins have been identified by genome-wide association studies as risk factors for substance abuse (Uhl et al., 2008, Lydall et al., 2011), it is possible that changes in cadherin adhesion plays a role in the formation and expression of addiction-related behaviours, possibly mediated by their effects on the trafficking of different subtypes of AMPA receptors at synapses in the VTA. Based on these lines of evidence, it is probable that aberrant increases in cadherin adhesion may result in impairments in synaptic plasticity and lead to deficits in learning and memory, which could be a possible mechanism that contributes to cognitive impairments associated with many different neurodegenerative and psychiatric disorders.

I hypothesize that 1) increases in β-catenin in neurons will result in aberrant stabilization of cadherin at the synaptic membrane, resulting in impairments in synaptic plasticity and behavioural flexibility, and that 2) increases in cadherin-based adhesion will be associated with enhanced drug-mediated associative learning or 'conditioned place preference', indicating a role for the cadherin adhesion complex in plasticity at synapses implicated in addiction. In chapter 2, I investigate the effects of increased stabilization of β-catenin in the mouse hippocampus, and determine the effects of increased levels of β-catenin on synaptic plasticity and behavioural flexibility. In chapter 3, I examine the role of cadherins in synaptic plasticity at synapses in the ventral tegmental area (VTA), and how cadherin distribution at these synapses is altered by the acquisition and extinction of drug-mediated conditioned preference for a novel environment. Together, these studies illuminate the effects of increases in cadherin adhesion at the synapse,

and provide new insights into how alterations in the cadherin adhesion complex can impair synaptic plasticity and cognitive functions.

Chapter 2: Cognitive flexibility and LTD are impaired following β-catenin stabilization *in vivo*

The cadherin/β-catenin adhesion complex is a key mediator of the bidirectional changes in synapse strength which are believed to underlie complex learning and memory. In the present study, we demonstrate that stabilization of β-catenin in the hippocampus of adult mice results in significant impairments in cognitive flexibility and spatial reversal learning, including impaired extinction during the reversal phase of the Morris Water maze and deficits in a delayed non-match to place T-maze task. In accordance with this, β-catenin stabilization was found to abolish long-term depression (LTD) by stabilizing cadherin at the synaptic membrane and impairing AMPA receptor endocytosis, while leaving basal synaptic transmission and long-term potentiation (LTP) unaffected. These results demonstrate that the β-catenin/cadherin adhesion complex plays an important role in learning and memory, and that aberrant increases in synaptic adhesion can have deleterious effects on cognitive function.

2.1 INTRODUCTION

Activity-driven increases and decreases in synapse efficacy, termed long-term potentiation (LTP) and long-term depression (LTD), respectively, are believed to underlie learning and memory in the brain. Although LTP has been widely studied as a physiological correlate of learning (Lynch, 2004), LTD has emerged as a critical and complimentary form of synaptic plasticity in tasks involving the modification or elimination of previously learned

information. Pharmacological disruption of LTD impairs reversal learning and behavioral flexibility (Nicholls et al., 2008, Dong et al., 2013), and it has been suggested that LTD is required to depotentiate synapses from earlier memory traces to allow the storage of new memories in overlapping sets of synapses (Nicholls et al., 2008).

Cadherins are homophilic adhesion molecules that play a central role in regulating changes in synapse strength and stability during LTP and LTD (Tai et al., 2007, Brigidi and Bamji, 2011). The recruitment of cadherin to synapses is essential for the maintenance of long-term potentiation (LTP) and memory consolidation (Tang et al., 1998, Bozdagi et al., 2000, Schrick et al., 2007), while patterns of activity that induce long-term depression (LTD) have been shown cause cadherin internalization (Mills et al., 2014).

A major regulator of cadherin stability is its intracellular binding partner β-catenin. (Huber et al., 2001). *In vitro* evidence demonstrates that β-catenin/cadherin interactions are dynamically regulated in response to activity (Murase et al., 2002, Bamji et al., 2006), allowing β-catenin to modify cadherin stability during different forms of synaptic plasticity. Enhanced neural activity increases β-catenin/cadherin interaction in dendritic spines, stabilizing cadherin at synapses (Murase et al., 2002). Targeted deletion of β-catenin has been shown to block the consolidation of fear memory, indicating β-catenin is important for long-term memory formation (Maguschak and Ressler, 2008). However, the role of β-catenin in regulating cadherin stability during the modification or elimination of memory remains unknown.

Understanding the role of β-catenin in synaptic plasticity and learning is critical in light of reports implicating deregulation of β-catenin in neurological disorders. Significant increases in levels of β-catenin have been reported in the brains of patients with Huntington's disease (HD) (Godin et al., 2010). Alzheimer's disease (AD)-linked mutations in presenilin-1 (Soriano et al.,

2001) and hyperphosphorylation of tau (Li et al., 2007) have been shown to increase the stability of β-catenin in cells. Given the importance of β-catenin in regulating adhesion and synaptic plasticity, it is likely that alterations in β-catenin levels have significant ramifications for neuronal function and cognition. In this study we characterize the effects of β-catenin stabilization on hippocampal synapses, and demonstrate that *in vivo* stabilization of β-catenin impairs behavioral flexibility by aberrantly stabilizing cadherin and AMPA receptors at the synaptic membrane and abolishing LTD.

2.2 MATERIALS AND METHODS

Animals. Female *Ctnnb1* $^{lox(ex3)/lox(ex3)}$ mice (Harada et al., 1999) were crossed with male *CaMKIIα:Cre*$^{+/-}$ mice (Xu et al., 2000) resulting in an equal proportion of *Ctnnb1*$^{lox(ex3)/+}$*;CaMKIIα:Cre*$^{-/-}$ (control) and *Ctnnb1*$^{lox(ex3)/+}$*;CaMKIIα:Cre*$^{+/-}$ (*β-cat*$^{\Delta ex3/+}$) mice. All mice were in a C57BL/6 background and heterozygous for the R26R LacZ reporter. Unless otherwise specified, all experimental animals used were 1 year old. Experimental procedures and animal housing conditions were approved by The UBC Animal Care Committee, and were in accordance with Canadian Council on Animal Care (CCAC) guidelines.

Behaviour.

Morris Water Maze. Mice were tested in a pool 90 cm in diameter surrounded by distal extra-maze visual cues. A circular platform (10 cm diameter) was used as the goal platform. Room and water temperature were held at 22-23 °C. Each trial, animals were placed into the pool at start positions that were randomly determined prior to testing, and the swim path to locate the goal

platform was recorded. Mice were tested four trials per day, with a ten minute interval between successive trials. Testing was conducted in 3 phases: Visible Platform Training (1 day, goal platform visible and in a different quadrant for each trial), Acquisition (4 days, goal platform not visible, in fixed location), and Reversal (5 days, goal platform not visible, moved to a different fixed location). All behavioral experiments were performed and analyzed blind to the genotype of each mouse.

Delayed non-match to place T-maze. Mice were placed on restricted feeding hours (2-3 hours once per day after testing/handling) and habituated to a condensed milk reward in their home cage, and then in the T-maze apparatus. The T-maze consisted of a start arm (10 cm x 30 cm) and two goal arms (10 cm x 20 cm), with a central partition extending into the start arm that separated the goal arms (Deacon and Rawlins, 2006). On each trial, both goal arms were baited with reward (30 μL milk) and mice were placed in start arm. Each trial consisted of a 'forced' run, in which one arm of the maze was blocked, and a 'choice' run, in which both arms were open. A correct choice was scored when animal entered the previously-blocked goal arm to locate remaining reward. Mice were tested in 4 trials per day, with an interval of 5s between 'forced' and 'choice' runs and an inter-trial interval of 15 minutes. Throughout testing animals were monitored to ensure they did not fall below 85% starting weight.

Y-maze. Mice were placed in a Y-maze consisting of a 20 cm start arm and two 15 cm choice arms joined at 120° angles from one another, and allowed to explore the maze for 7 minutes. Number of entries to each arm and sequence of entries were recorded and analyzed. A successful series of alternation entries was scored when the mice sequentially visited each arm (i.e. ABC or CBA, but not ABA).

Context-dependent Fear Conditioning and Extinction. Mice were placed in the conditioning chamber for 5 minutes, and after 3 minutes received an unconditioned foot shock stimulus (1mA, 50Hz) lasting 3 seconds. On each subsequent day of testing, mice were placed in the same chamber for 4 minutes, but did not receive an additional foot shock. Freezing behavior was recorded and analyzed on a second-by-second basis using FreezeFrame (Actmetrics Software) and total time % freezing was compared between groups.

Novel Object Recognition. Novel object recognition (NOR) was tested in a square box (30 cm x 30 cm, walls 40 cm high) coated with plastic adhesive to allow cleaning of scent traces between trials. The objects used were approximately 4 cm in diameter and with distinct shapes, but similar size and material composition. Mice were placed in the test chamber for 5 minutes with a pair of identical objects, then one hour later placed in the test chamber for ten minutes with a familiar and novel object. All trials were recorded and manually scored for time spent investigating the novel and familiar objects. The test apparatus and objects were wiped down with ethanol between trials to remove any odor cues.

Statistical analysis. Unless otherwise noted, statistical analysis was done using Student's t-test (two tailed) and two-way ANOVA. Data from the Morris water maze and T-maze was analyzed by two-way repeated measures (RM) ANOVA with genotype as the between-subjects factor and days as the within-subjects factor. For comparisons between genotypes and within days, one-way ANOVA was used. Data from the LTP, LTD and repetitive stimulation experiments were analyzed by two-way repeated measures (RM) ANOVA with genotype as the between-subjects factor, and *post-hoc* analysis was done using Bonferroni's test. Results were considered

significant when $p<0.05$. Analysis was performed using IBM SPSS software (IBM Corporation, New York) and GraphPad Prism (GraphPad Software, San Diego).

***Immunohistochemistry*.** Adult male mice were deeply anesthetized by intraperitoneal injection of 150 mg/kg ketamine and 15 mg/kg xylazine, and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were cryopreserved with sucrose-PBS solutions (15%–30%) and sliced into 30 μm thick coronal sections by cryostat. Immunohistochemistry on free-floating sections was performed as previously described (Bamji et al., 2003).

Immunoblot Analysis. Hippocampal lysates were homogenized in ice cold lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 0.5% NP-40, 10% glycerol with protease and phosphatase inhibitor tablets (Roche) and centrifuged at 14,000 x g for 40 min at 4°C. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL). Lysates were separated by SDS–PAGE and analyzed by means of immunoblotting with antibodies against β-catenin (rabbit, 1:250, Santa Cruz Biotechnology), pan-cadherin (rabbit, 1:250, Zymed), β-actin (mouse, 1:10000, Sigma), N-cadherin (mouse, 1:500, BD Transduction), p120 catenin (mouse, 1:500, Invitrogen), Synaptophysin (rabbit, 1:500, Abcam Inc.), Synaptotagmin (mouse, 1:500, Synaptic Systems), SynapsinI (rabbit, 1:1000, Abcam), PSD-95 (rabbit, 1:500, Abcam), Axin2 (rabbit, 1:500, Abcam), c-Myc (mouse, 1:500, Sigma), c-Jun (rabbit, 1:500, Abcam) and Lef1 (rabbit, 1:500, Cell Signaling). Proteins were visualized using enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL) on a Bio-Rad Versadoc 4000 (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON). For all target proteins, bands in each lane were first normalized to actin to account for any variation on protein loading, and the

relative intensity of bands were individually normalized to the average intensity of bands in control mice. In the case of β-catenin, both full-length and Δexon3 forms of β-catenin were normalized to the average intensity of full-length β-catenin in control mice. The brightness and contrast of entire images was moderately adjusted using Photoshop (Adobe Systems Canada, Toronto, ON) after recommended, scientifically acceptable procedures, and no information was obscured or eliminated from the original.

Synaptosomal Fractionation. Hippocampi from adult mice were homogenized in ice cold homogenization buffer (HB) (320 mM sucrose, 4 mM HEPES, 1 mM EGTA). The homogenate was centrifuged at 1312 x *g* for 10 min to remove nuclei and cell debris. This pellet was resuspended in lysis buffer (P1 – crude nuclear fraction) and the resulting supernatant (S1) was removed and centrifuged at 14,418 x *g* for 15 min. The resulting supernatant (S2 - Soluble/small compartments fraction) was removed, and the resulting pellet (P2 - Crude synaptosomal fraction) was resuspended in lysis buffer and used for immunoblot analysis as described above.

Co-immunoprecipitation. For each animal, 1000 μg (500 μL) of hippocampal lysate was incubated with 5 μg of antibodies against pan-cadherin (rabbit, Zymed) and mixed gently overnight at 4°C. A mixture of Protein A/G Sepharose beads (50 μl of each, GE Healthcare, Chicago, IL) was then added to the lysates and mixed gently at 4°C for 2 hours. Beads were gently precipitated by centrifugation (1000 x g, 30 seconds) and washed three times with 1 ml lysis buffer. Bead-bound immunocomplexes were solubilized at 95°C in 30 μl lysis buffer with SDS sample buffer and analyzed by immunoblot as described above.

Electron Microscopy. Mice were anesthetized as described above and perfused with PBS followed by fixative solution (4% formaldehyde, 1.25% glutaraldehyde in PBS). The brain was removed and fixed overnight at 4°C. A 1 mm^3 section of hippocampus including the CA1 region and stratum radiatum was dissected. For morphological analysis, tissue was dehydrated in an ethanol series and embedded in JEMBED/Spurr's resin. Ultrathin sections (70 nm) were cut and stained with 2% uranylacetate and Reynold's lead and imaged on a Hitachi H7600 TEM (Hitachi). For each mouse, the same region of the stratum radiatum directly underlying the CA1 pyramidal cell layer was imaged to analyze synapse density (15000X magnification, 50+ images per mouse) or morphology (60000X magnification, 200+ images per mouse). All images were acquired and analyzed blind to the genotype of each mouse.

NMDA evoked endocytosis assay. Mice were anaesthetized using halothane (Sigma) and decapitated. The brains were removed and sliced horizontally (300 µm, Leica VT1200S vibratome) in an ice-cold oxygenated (95% O_2 and 5% CO_2) solution containing (in mM): N-Methyl-D-glucamine (120), KCl (2.5), $NaHCO_3$ (25), $CaCl_2$ (1), $MgCl_2$ (7), NaH_2PO_4 (1.2), D-glucose (2), sodium pyruvate (2.4), and sodium L-ascorbate (1.3). The slices were transferred to oxygenated artificial cerebrospinal fluid (aCSF) containing (in mM): NaCl (126), KCl (2.5), $NaHCO_3$ (26), $CaCl_2$ (2.0), $MgCl_2$ (2), NaH_2PO_4 (1.25), and D-glucose (10) (pH at 7.3-7.4, osmolarity 300 mOsm) and allowed to recover for 1 hour at room temperature (RT). Slices from the same mouse were transferred to parallel perfusion chambers and continuously perfused (3 ml / min) with oxygenated aCSF containing glycine (10 µM) and reduced $MgCl_2$ (0.6 mM, NaCl was increased to 128 mM to balance osmolarity). The temperature was kept constant at 30°C in each chamber by individual inline heaters (Warner instruments SH-27B). After 20 min in the

perfusion chambers NMDA (20 µM) was bath applied for 3 min to half of the slices while the other slices served as controls. After 3 mins NMDA was allowed to wash off by reperfusion with aCSF and slices allowed to recover for 80 mins. Slices were then rapidly fixed by immersion for 2 min in 4% paraformaldehyde that was pre-heated to 80°C. The slices were rinsed and stored in 0.1 M PBS.

Immunogold electron Microscopy. For immunogold labeling, fixed tissue from acute hippocampal slices was cryoprotected in glycerol (10-30%) in PBS and plunge-frozen in ethane at -180°C. Tissue was then fixed *en bloc* with 1.5% uranyl acetate in methanol (-90°C, 30 h), followed by a progressive series of HM-20 resin (Electron Microscopy Sciences) in methanol (-45°C, 48 h), and finally UV polymerized in pure HM-20 (48 h). Ultrathin sections were cut and collected on formvar-coated grids (Electron Microscopy Sciences) for immunogold labeling. Grids were etched with a solution of 0.1% sodium borohydride followed by 50 mM glycine, washed in TBST, and blocked in a solution of 2% BSA in TBST. Grids were then incubated overnight in primary antibodies (mouse pan-Cadherin, Sigma, rabbit GluA1, Millipore), washed in TBST, and incubated for 2 hrs in gold bead-conjugated secondary antibodies (mouse 15 nm, rabbit 10 nm, Aurion), washed in TBST and ddH_2O, dried, and briefly stained with 2% uranylacetate and Reynold's lead. To quantify immunogold labeling, the distance of all 15 nm gold particles (cadherin) from the synaptic membrane were measured, and all 10 nm gold particles (GluA1) to the postsynaptic active zone membrane were measured. Due to the sizes of proteins and reagents involved (Mathiisen et al., 2006, see also Results section), cadherin-labeled gold particles within 40 nm of the synaptic membrane and GluA1-labeled gold particles within 30 nm of the active zone were considered to be associated with the synaptic membrane or active

zone, respectively. The percentage of immungold particles localized to these regions was determined by [# immungold beads at target region]/[total # immunogold beads at pre and post-synaptic compartments]. All images were acquired and analyzed blind to the genotype of each mouse.

Electrophysiology. Electrophysiological recordings were made from parasagittal hippocampal brain slices (300 µM thick) prepared from 1-year old adult male mice. Animals were fatally anaesthetized according to approved policy, decapitated, and the brain placed in oxygenated (5% CO_2 95% O_2) ice cold artificial cerebrospinal fluid (aCSF) which contained (in mM) NaCl, 124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1.25; $MgSO_4$, 10; Glucose, 10; $CaCl_2$, 1. Slices were cut using a Vibratome (Leica) and allowed to recover for at least 4 hours in oxygenated aCSF (as above except 1 mM $MgSO_4$, 2 mM $CaCl_2$) maintained at room temperature. Slices were transferred one by one to a room temperature recording chamber continuously superfused with aCSF at 2.5 ml/minute. Standard extracellular recording techniques were used to monitor evoked fEPSPs from CA1 stratum radiatum. Baseline stimuli were delivered at 0.03 Hz with twisted bipolar NiCr electrodes and responses recorded with a glass micropipette of resistance 1-2 MΩ when filled with aCSF. Data was collected using Multiclamp 700A and Digidata 1322A hardware, then plotted and analyzed using WinLTP software (Anderson and Collingridge, 2001). Before long-term plasticity experiments a 30 minute baseline was recorded at 50% of the maximal fEPSP slope. 14 Hz and 100 Hz stimulation was done in the presence of 50 µM D-AP5 to block NMDAR-mediated effects that would mask pure presynaptic effects (Bamji et al., 2003). fEPSP slope data in the RP and RRP experiments was plotted normalized to the slope of the first

response of the train. Statistical significance of the difference between the genotypes in all electrophysiological experiments was determined by Student's t-test.

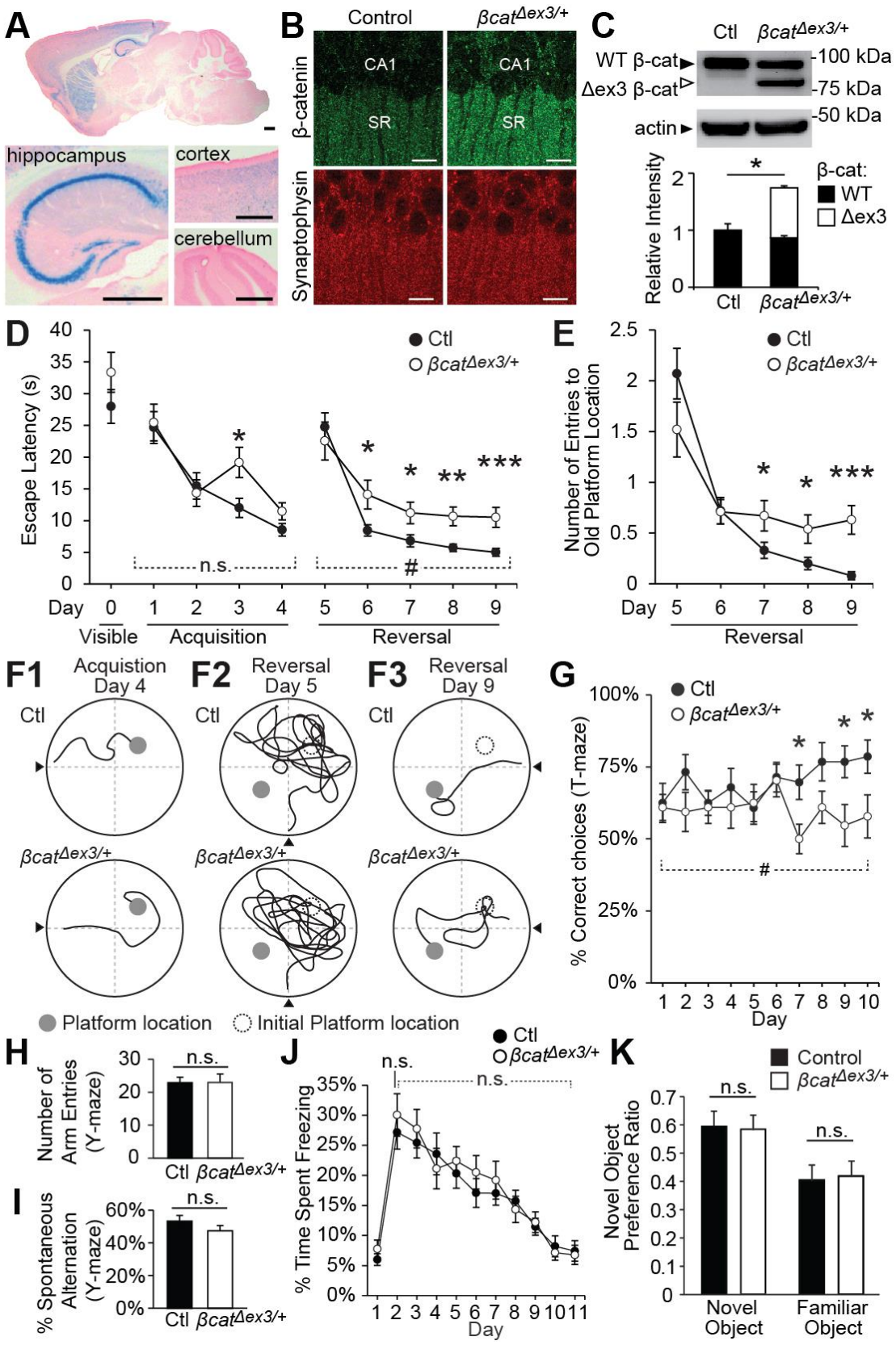
RNA isolation and real-time PCR. Mice were euthanized with CO_2/O_2, and brains quickly removed and frozen in liquid nitrogen and stored at -80°C. RNA was isolated with the RNeasy kit from QIAGEN (RNeasy for Lipid Tissue with additional DNase treatment). cDNA was synthesized (SuperScript III RNase H-Reverse Transcriptase; Invitrogen) and examined by real-time PCR in a 7500 Real-Time PCR System using SYBR Green dye (Applied Biosystems). The results were analyzed by absolute quantification with a relative standard curve. Primers used were as follows; *Lef1*: sense, 5′-CCCACACGGACAGTGACCTA-3′; antisense, 5′–TGGGCTCCTGCTCCTTTCT-3′. *Axin2*: sense, 5′- AAGCCCGCCACCAAGACCTACATA-3′; antisense, 5′– TTTCCTCCATCACTGCCTGGATCT-3′. *GAPDH*: sense, 5′-CAGTGGCAAAGTGGAGATTG-3′; antisense, 5′- AATTTGCCGTGAGTGGAGTC-3′.

2.3 RESULTS

2.3.1 Conditional stabilization of β-catenin in hippocampal neurons in vivo.

To investigate the effects of increased β-catenin levels on synapses, we generated mice that expressed a stabilized form of β-catenin in a subset of neurons in the brain. β-catenin is degraded following phosphorylation of serine/threonine residues at its N-terminus, and mutation of these residues leads to the stabilization of β-catenin (Aberle et al., 1997). Using the Cre/loxP system, β-catenin N-terminal phosphorylation sites can be ablated by excision of exon 3, resulting in a

stabilized, active form of β-catenin (Harada et al., 1999). Importantly, this mutant form of β-catenin still retains all its binding domains, including its α-catenin binding region (Aberle et al., 1996) and internal armadillo repeats which mediate binding to cadherin and TCF/LEF (Huber and Weis, 2001), and is competent for Wnt signaling (Li et al., 2009). Thus, this stabilized form of β-catenin can mediate the same functions as wildtype β-catenin, and is widely used as a tool to increase overall β-catenin levels *in vivo (Harada et al., 1999, Heiser et al., 2008, Li et al., 2009).* Mice homozygous for this loxP-flanked exon 3 transgene (*Ctnnb1*$^{lox(ex3)/lox(ex3)}$ mice) (Harada et al., 1999), were crossed with *CaMKIIα:Cre/+* mice (Xu et al., 2000, Bamji et al., 2003), generating heterozygous *Ctnnb1*$^{lox(ex3)/+}$*;CaMKIIα:Cre/+* mice (termed *β-cat*$^{\Delta ex3/+}$ mice for brevity), as well as *Ctnnb1*$^{lox(ex3)/+}$*;+/+* littermates, which were used as controls. Cre recombinase is expressed in *CaMKIIα:Cre/+* mice from P17, allowing the effects of increased β-catenin levels to be examined in adult animals while leaving earlier neuronal development to progress normally. Cre expression was determined by X-gal staining (**Fig. 2.1A**), which was observed throughout the hippocampus (including the CA1 region, CA3 region and the dentate gyrus), in a subset of cortical and striatal neurons, and was absent from the cerebellum. This pattern of Cre expression is consistent with a previous study which reports Cre expression in 99.5% of CA1 neurons, ~9% of cortical neurons, ~29% of striatal neurons, and no cerebellar neurons (Tsien et al., 1996a). Previous studies have shown that expression of this transgene is restricted to excitatory neurons that express endogenous CamKII (Xu et al., 2000). *β-cat*$^{\Delta ex3/+}$ mice exhibited an increase in overall β-catenin levels in the hippocampus, including the stratum radiatum region where CA3 pyramidal neurons synapse onto CA1 neurons (**Fig. 2.1B**).


A
hippocampus
cortex
cerebellum
B
Control
βcatΔex3/+
β-catenin
Synaptophysin
CA1
SR
C
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WT β-cat
Δex3 β-cat
actin
100 kDa
75 kDa
50 kDa
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β-cat:
WT
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Escape Latency (s)
Ctl
βcatΔex3/+
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#
Day
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E
Number of Entries to Old Platform Location
Reversal
F1
Acquistion Day 4
F2
Reversal Day 5
F3
Reversal Day 9
Platform location
Initial Platform location
G
% Correct choices (T-maze)
Day
H
Number of Arm Entries (Y-maze)
I
% Spontaneous Alternation (Y-maze)
J
% Time Spent Freezing
K
Control
Novel Object Preference Ratio
Novel Object
Familiar Object

Figure 2.1 Reversal learning, spatial memory extinction and behavioral flexibility are impaired in *β-cat*$^{\Delta ex3/+}$ mice.
(**A**) X-gal staining in sagittal sections of 1 year old *β-cat*$^{\Delta ex3/+}$ mice, heterozygous for both *CaMKIIα:Cre* and the R26R lacZ reporter. X-gal staining was detected in the CA1 region, CA3 region and dentate gyrus of the hippocampus, and a subset of cortical and striatal neurons. Staining was absent in the cerebellum. Counterstaining with Fast Red. (All scale bars = 0.5 mm) (**B**) Confocal images of brain sections from 1 year old male control and *β-cat*$^{\Delta ex3/+}$ mice immunostained for β-catenin (s.r. = stratum radiatum, Scale bar = 10 µm). (**C**) Total levels of β-catenin (sum of wildtype β-catenin and Δexon3 β-catenin) were significantly increased in *β-cat*$^{\Delta ex3/+}$ mice compared to controls (*n*=4 blots from 4 animals per group, p=0.017). (**D**) During initial acquisition of a hidden platform location, escape latencies were similar between *β-cat*$^{\Delta ex3/+}$ and control mice (RM ANOVA, days 1-4, main effect of genotype, p=0.16). Following reversal of platform location, *β-cat*$^{\Delta ex3/+}$ mice showed significantly increased escape latencies compared to controls (p=0.028, main effect of genotype days 5-9, RM ANOVA). (**E**) *β-cat*$^{\Delta ex3/+}$ mice made significantly more entries to the initial platform location over time following learning reversal, indicating impaired spatial memory extinction (RM ANOVA, significant interaction between day and genotype, p=0.008). (**F1-3**) Representative traces of swim paths. (*n*=15 mice control, 11 mice *βcat*$^{\Delta ex3}$). (**G**) Percent correct choices in a delayed non-match to place T-maze task. Control mice were significantly better than *βcat*$^{\Delta ex3}$ mice following 10 days of training (p=0.026, main effect of genotype days 1-10, RM ANOVA, *n*=15 mice control, 16 mice *β-cat*$^{\Delta ex3/+}$). In the Y-maze task, the number of arm entries (**H**) and percentage spontaneous alternation (**I**) were similar between control and *β-cat*$^{\Delta ex3/+}$ mice (p=0.98 and p=0.21, respectively, *n*=15 mice control, 16 mice *β-cat*$^{\Delta ex3/+}$). (**J**) No significant difference in acquisition or extinction of context-dependent fear conditioning was observed (day 2, p=0.70, ANOVA within days, days 2-10, p=0.65, RM ANOVA *n*=19 mice control, 13 mice *β-cat*$^{\Delta ex3/+}$). (**K**) No difference in novel object preference was observed in *β-cat*$^{\Delta ex3/+}$ mice (p=0.86, *n*=17 mice control, 14 mice *β-cat*$^{\Delta ex3/+}$). Data shown as mean ± SEM. $^{\#}$ p<0.05, RM ANOVA; * p<0.05, ** p<0.01, *** p<0.001 ANOVA within days.

Using western blot analysis, we confirmed the expression of both wildtype and stabilized β-catenin in the hippocampus of 1-year-old *β-cat*$^{\Delta ex3/+}$ mice, the latter of which was slightly smaller due to the deletion of exon 3 (**Fig. 2.1C**). The total amount of β-catenin in hippocampal lysates (sum of wildtype and stabilized β-catenin) was significantly increased in *β-cat*$^{\Delta ex3/+}$ mice compared to age-matched controls (64.0% ± 6.2% increase). Subcellular fractionation of hippocampal lysates showed β-catenin levels were increased in the synaptosomal (P2) and soluble (S1) fractions from *β-cat*$^{\Delta ex3/+}$ mice, however total β-catenin levels remained unchanged in the crude nuclear fraction (P1) (**Fig 2.2**).

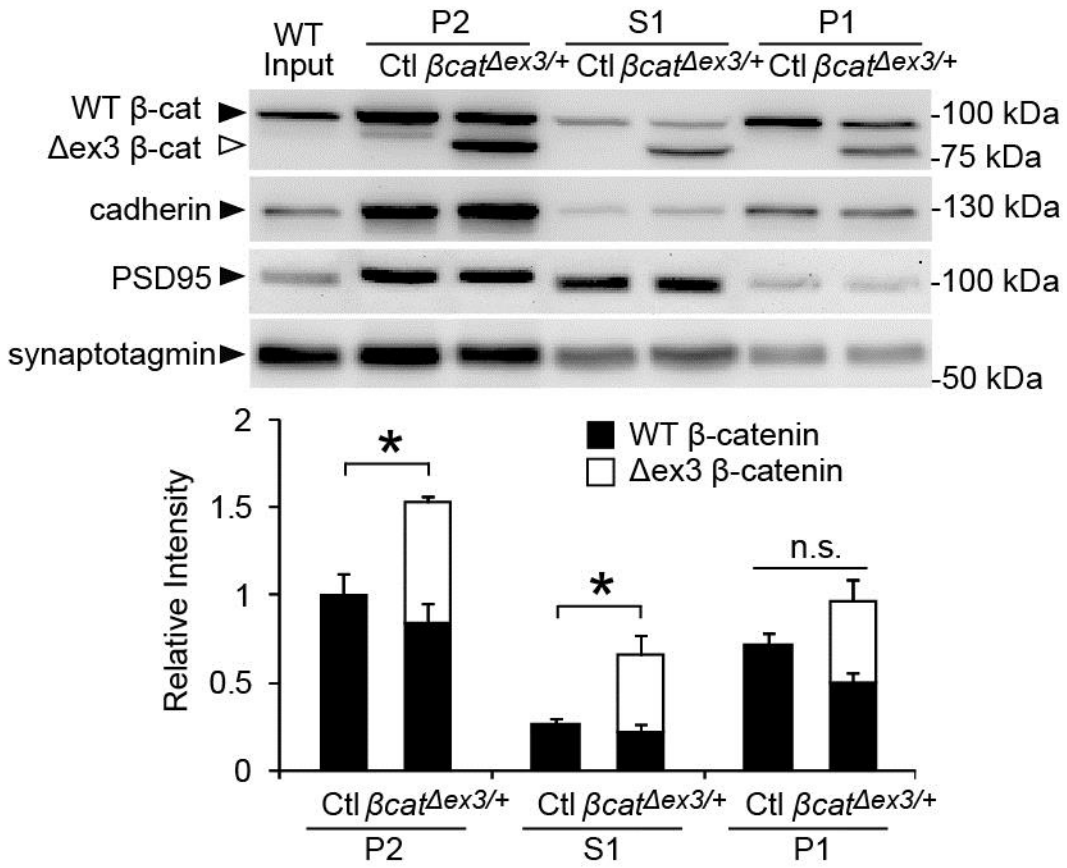

Figure 2.2 Subcellular fractions in *β-cat*$^{\Delta ex3/+}$ mice.
There was an increase in total β-catenin levels in synaptosomal (P2, p=0.030) and soluble (S1, p=0.037) fractions from *β-cat*$^{\Delta ex3/+}$ hippocampal lysates, whereas the level of β-catenin in the crude nuclear (P1) fraction was unchanged (*n*=3 blots from 3 separate animals per group). Data shown as mean ± SEM. *p< 0.05.

2.3.2 *β-cat*$^{\Delta ex3/+}$ mice exhibit deficits in reversal learning and spatial memory extinction in the Morris water maze.

We first tested memory in *β-cat*$^{\Delta ex3/+}$ mice using the Morris water maze (MWM), a test of spatial learning and reference memory which is dependent on intact hippocampal function (Vorhees and Williams, 2006). During initial visible platform training we found that *β-cat*$^{\Delta ex3/+}$ mice exhibited similar escape latency times (**Fig. 2.1D**) and average swim speed (control = 13.7 ± 0.67 cm/s, *β*-

cat$^{\Delta ex3/+}$= 14.9 ± 0.22 cm/s, p=0.12) compared to controls, indicating no gross physical impairments in these mice. Initial acquisition of a spatial memory of a hidden platform location was also similar in control and *β-cat*$^{\Delta ex3/+}$ mice (**Fig. 2.1D, days 1-4**). An impairment in escape latency was observed on day 3 of training in *β-cat*$^{\Delta ex3/+}$ mice, but by day 4 there were no significant differences compared to controls indicating effective learning of the initial platform location (see also sample traces in **Fig. 2.1F1**). We then sought to determine if reversal learning was impaired in *β-cat*$^{\Delta ex3/+}$ mice. On day 5 of MWM testing we moved the hidden platform to the opposite quadrant of the pool (**Fig. 2.1D, days 5-9**). Both control and *β-cat*$^{\Delta ex3/+}$ mice displayed greatly increased escape latency times after the platform was moved from the initial location (**Fig. 2.1D, day 5**, see samples traces in **2.1F2**). Several days after the platform switch (days 6-9), control mice could quickly locate the new platform (**Fig. 2.1D**), and had almost completely eliminated entries to the old platform location (**Fig. 2.1E; 2.1F3,** top). In contrast, *β-cat*$^{\Delta ex3/+}$ mice showed significantly impaired escape latency times, taking over twice as long to locate the new platform even after five days of training (**Fig. 2.1D, days 5-9**), Remarkably, *β-cat*$^{\Delta ex3/+}$ mice also showed a striking persistence of entries to the old platform location up to 5 days after the platform location switch, indicating an impairment in the extinction of the initial spatial memory (**Fig. 2.1E**, see also sample trace **2.1F3,** bottom).

2.3.3 *β-cat*$^{\Delta ex3/+}$ mice exhibit impaired behavioral flexibility in delayed non-match to place T-Maze task.

We next examined the performance of *β-cat*$^{\Delta ex3/+}$ mice in a delayed non-match to place (DNMTP) version of the T-maze, another hippocampal-dependent spatial task that tests behavioral flexibility. On each trial, both goal arms of the T-maze were baited with reward and

mice were placed in the start arm of the maze. Testing consisted of two phases; a ‘forced’ run, in which one goal arm of the maze was blocked and the open arm contained a reward, and a ‘choice’ run, in which both goal arms were open. To receive a reward, the animal had to choose the previously-blocked arm which had not yet been visited (delayed non-match to place). Thus, mice must learn new spatial information in each trial and use that information to correctly locate the food reward, thereby requiring the suppression or elimination of previously learned spatial information similar to the reversal phase of the MWM. Control animals gradually improved at this task, reaching a plateau of 77.4% ± 3.4% average correct choices for the last 3 days. However, *β-cat*$^{\Delta ex3/+}$ mice showed significantly impaired performance on this task, frequently making errors in the ‘choice’ run and reaching a plateau of only 57.8% ± 3.7% average correct choices (**Fig. 2.1G**). The deficit we observed in DNMTP T-maze performance was not associated with differences in exploratory behavior when on a similar Y-maze apparatus (**Fig. 2.1H, I**). We also observed no differences in contextual fear conditioning (**Fig. 2.1J**) or novel object recognition in *β-cat*$^{\Delta ex3/+}$ mice (**Fig. 2.1K**), two tasks which have both been shown to involve hippocampal LTP for acquisition of contextual and object location memory. The lack of impairment in these tasks, together with the normal acquisition of initial platform location in the MWM, indicated that stabilization of β-catenin specifically disrupted cognitive flexibility on hippocampal-dependent spatial memory tasks.

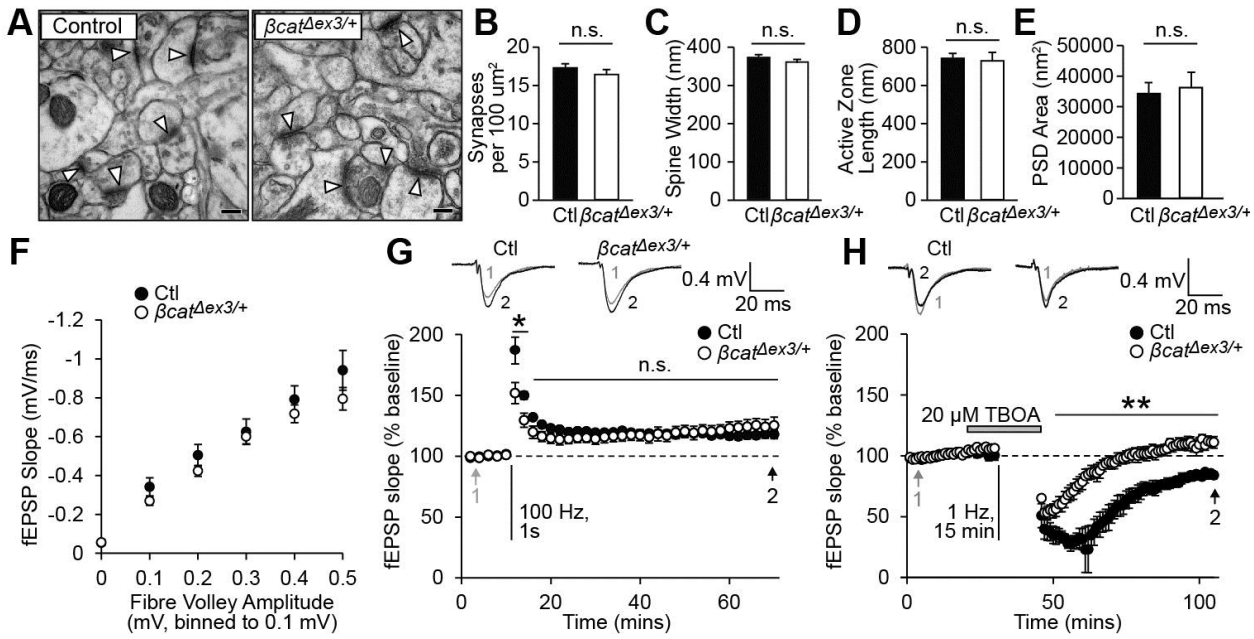

Figure 2.3 Basal synaptic transmission and LTP are unchanged but LTD is abolished in *β-cat*$^{\Delta ex3/+}$ mice.
(**A**) Electron micrographs of hippocampal synapses (scale bar = 100 nm). No differences in (**B**) synapse density, (**C**) dendritic spine head width (**D**) active zone length or (**E**) PSD area were observed in in *β-cat*$^{\Delta ex3/+}$ mice (*n*=3 mice, >200 synapses). (**F**) Basal synaptic transmission was unaffected in *β-cat*$^{\Delta ex3/+}$ mice (control: *n*=9 slices/5 mice; *β-cat*$^{\Delta ex3/+}$: *n*=10 slices/6 mice). (**G**) LTP was intact in *β-cat*$^{\Delta ex3/+}$ mice (p=0.60, RM ANOVA; n=6 slices/6 mice control and *β-cat*$^{\Delta ex3/+}$). (**H**) 1 Hz stimulation induced LTD in controls, but LTD was abolished in *β-cat*$^{\Delta ex3/+}$ mice (p=0.012, RM ANOVA; n=3 slices/3 mice control, 5 slices/5 mice *β-cat*$^{\Delta ex3/+}$). Data shown as mean ± SEM. # p<0.05, RM ANOVA; *p< 0.05, **p< 0.01, Bonferroni's test *post hoc*.

2.3.4 LTP is intact but LTD abolished in *β-cat*$^{\Delta ex3/+}$ mice.

Impairments in spatial learning have frequently been linked to deficits in synaptic plasticity in the hippocampus, so we next determined whether different forms of long-term plasticity were affected by β-catenin stabilization. We first determined whether synapse density and basal synaptic transmission were altered in *β-cat*$^{\Delta ex3/+}$ mice. We examined synapse ultrastructure in a defined region of the stratum radiatum directly below CA1 pyramidal neurons (**Fig. 2.3A**). As Cre recombinase is expressed in both CA3 and CA1 pyramidal neurons in *β-cat*$^{\Delta ex3/+}$ mice, β-catenin levels are elevated both pre and postsynaptically at synapses in this region. However, no

changes in synapse density (**Fig. 2.3B**), dendritic spine head width (**Fig. 2.3C**), active zone length (**Fig. 2.3D**) or PSD area (**Fig. 2.3E**) were detected in *β-cat*$^{\Delta ex3/+}$ mice. We also observed no difference in the input-output relationship of fEPSP slopes to evoked fiber volley amplitudes at synapses in this region, indicating basal synaptic transmission was not affected by β-catenin stabilization (**Fig. 2.3F**). We observed some deficits in presynaptic responses during repetitive stimulation and an overall increase in synaptic vesicles localized to synapses in *β-cat*$^{\Delta ex3/+}$ mice (**Fig 2.4**). Since no perturbations in basal synaptic transmission or postsynaptic strength were detected in in *β-cat*$^{\Delta ex3/+}$ mice, this suggests that the impaired responses to repetitive stimulation indicate a mild presynaptic impairment in the mobilization, replenishment or release of synaptic vesicles due to increased levels of β-catenin at the synapse.

We assayed long term potentiation (LTP) induced using brief high-frequency stimulation (HFS; 100 Hz, 1s). In slices from control and *β-cat*$^{\Delta ex3/+}$ mice we observed a persistent increase in synaptic strength up to 60 minutes after HFS, with no significant differences detected between the two groups (**Fig. 2.3G**). The LTP observed (~20%) was consistent with previous studies showing reduced magnitude of LTP in aged mice (Rosenzweig and Barnes, 2003). After HFS there was a transient impairment in responses *β-cat*$^{\Delta ex3/+}$ mice, but no significant differences in LTP were detected 50-60 minutes after HFS, indicating that long-term increases in postsynaptic strength were not affected in *β-cat*$^{\Delta ex3/+}$ mice. We then examined long-term depression (LTD), as disruption of LTD has previously been shown to produce impairments in reversal learning and behavioral flexibility similar to the deficits observed in *β-cat*$^{\Delta ex3/+}$ mice (Nicholls et al., 2008, Dong et al., 2013). We induced LTD by low-frequency stimulation (1 Hz, 900 stimuli) in the presence of 20 µm threo-β-benzylaspartic acid (TBOA), a competitive blocker of glutamate transporters (Massey et al., 2004). In slices from control mice we observed a decrease in synaptic

strength that persisted over 1 hour after stimulation, but in *β-cat*$^{\Delta ex3/+}$ mice this long-lasting depression in synaptic strength was completely abolished (**Fig. 2.3H**).

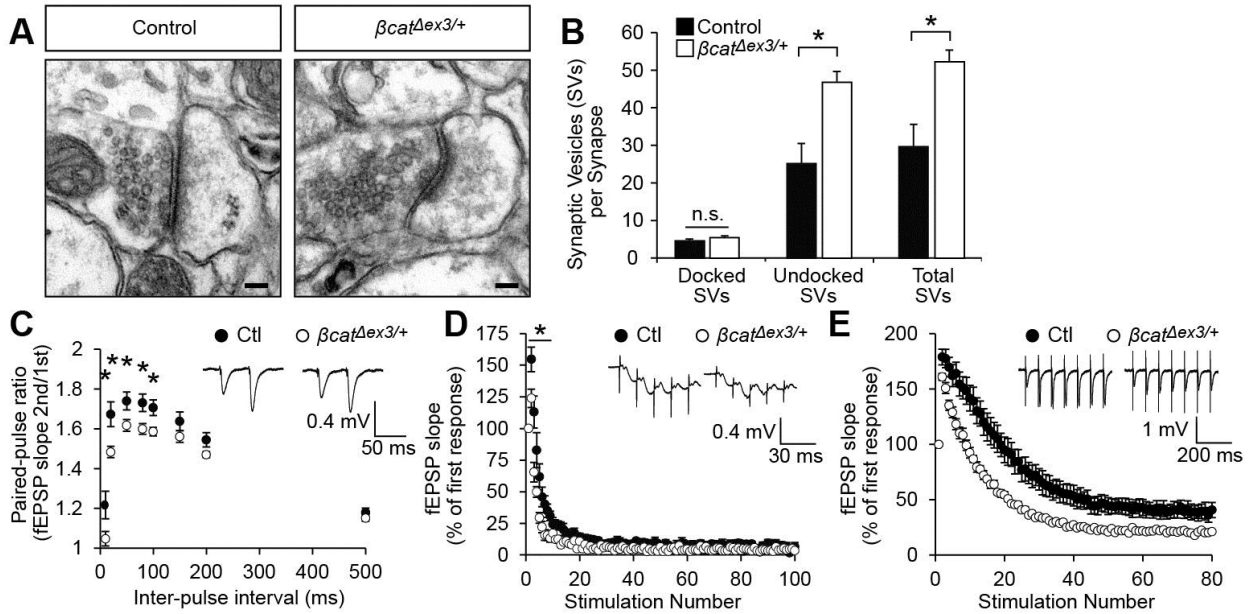

Figure 2.4 Altered synaptic vesicle localization and responses to repetitive stimulation in *β-cat*$^{\Delta ex3/+}$ mice.
(A) Electron micrographs of hippocampal synapses from the stratum radiatum region of the hippocampus (Scale bar = 100 nm). (B) There is an increase in the number of undocked synaptic vesicles (p=0.037) and total synaptic vesicles (0.036) per synapse in *β-cat*$^{\Delta ex3/+}$ mice compared to controls (n=3 mice, >200 synapses). (C) Paired-pulse facilitation was significantly impaired in *β-cat*$^{\Delta ex3/+}$ mice at inter-pulse intervals of 10 ms, 20 ms, 50 ms, 80 ms, and 100 ms (p=0.049, p=0.018, p=0.038, p=0.022, p=0.018, respectively; n=9 slices/5 mice control, 10 slices/6 mice *β-cat*$^{\Delta ex3/+}$). (D) Initial responses to 100 Hz high-frequency stimulation were impaired in *β-cat*$^{\Delta ex3/+}$ mice (p=0.045, significant effect of genotype, RM ANOVA, n=6 slices/4 mice control, 7 slices/4 mice *β-cat*$^{\Delta ex3/+}$). After the first 9 pulses no significant difference in response was observed between control and *β-cat*$^{\Delta ex3/+}$ mice. (E) Responses to 14 Hz low-frequency stimulation were significantly impaired in *β-cat*$^{\Delta ex3/+}$ mice throughout the stimulus train (p=0.0047, significant effect of genotype, RM ANOVA, n=7 slices/4 mice Control, 7 slices/4 mice *β-cat*$^{\Delta ex3/+}$). All repetitive stimulation experiments were done in the presence of NMDA receptor antagonist D-AP5 (50 µM) to isolate differences in short-term presynaptic plasticity. Data shown as mean ± SEM. *p < 0.05, post-hoc ANOVA for B,C. *p < 0.05, post-hoc Bonferroni's test for D,E.

2.3.5 Activity-dependent endocytosis of cadherin and GluA1 is impaired in in *β-cat*$^{\Delta ex3/+}$ mice.

To elucidate the mechanism underlying deficits in LTD and cognitive flexibility in *β-cat*$^{\Delta ex3/+}$ mice, we sought to determine whether enhanced β-catenin stabilization could be impairing the activity-induced internalization of cadherin and associated AMPA receptors. We treated acute hippocampal slices with 20 μM NMDA for 3 minutes ('chemical LTD'), which has been shown to induce widespread downscaling of synapse strength (Lee et al., 1998) and endocytosis of AMPA receptors (Beattie et al., 2000), and then used immunogold electron microscopy to quantify the resulting changes in the distribution of cadherin and GluA1 subunits in these slices. Immunogold labelling allows for extremely precise spatial resolution of targets at the synapse; empirical studies have shown that antibody-conjugated immunogold particles are localized to within approximately 30 nm of epitopes identified (Mathiisen et al., 2006). Consequently, to analyze the amount of GluA1 situated to respond to presynaptic neurotransmitter release, we quantified the proportion of immunogold-labelled GluA1 (10 nm beads) within only 30 nm of the postsynaptic active zone membrane. Similarly, as the C-terminal tail of cadherin could be located up to 10 nm from the synaptic membrane, we have considered immungold particles (15 nm beads) within 40 nm from the synaptic membrane to represent cadherin molecules that are situated to participate in trans-synaptic adhesion (**Fig. 2.5A, B**).

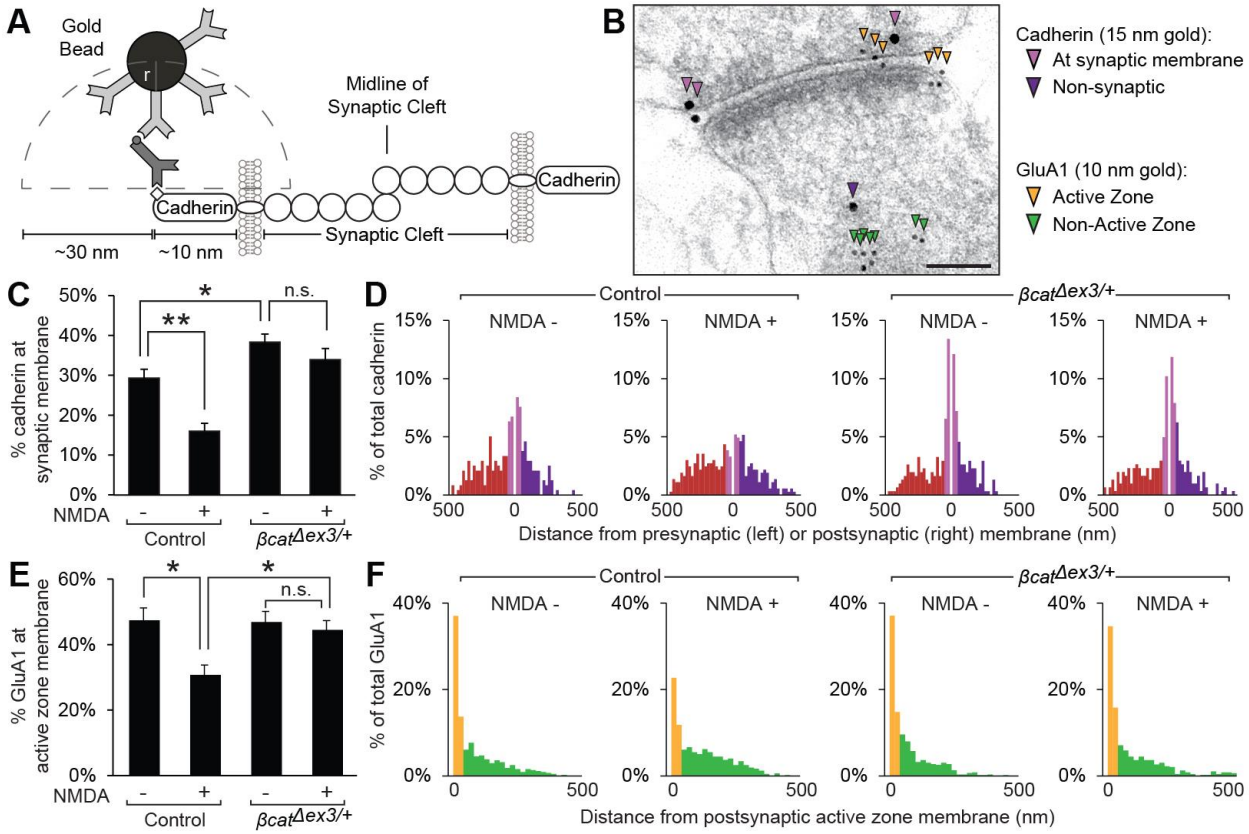

Figure 2.5 Cadherin and GluA1 endocytosis following NMDA treatment is significantly impaired in *β-cat*$^{\Delta ex3/+}$ mice.
(**A**) Estimated sizes of immunogold reagents (adapted from Mathiisen et al., 2006). (**B**) Electron micrograph of hippocampal synapse from control mice showing immunogold labelled cadherin and GluA1 (Scale bar = 100 nm). (**C**) The percentage of cadherin at the synaptic membrane was significantly decreased in control mice following NMDA treatment, but not *β-cat*$^{\Delta ex3/+}$ mice, indicating stabilization of cadherin at synapses ($p<0.0001$, ANOVA, n=3 mice, 4 sections per cond., >100 synapses per group). (**D**) Histograms of immungold labelled cadherin distances from synaptic membranes. (**E**) The percentage of GluA1 localized to the postsynaptic active zone membrane was significantly decreased in control mice following NMDA treatment, but not *β-cat*$^{\Delta ex3/+}$ mice. ($p=0.079$, ANOVA, n=3 mice, 4 sections per cond., >100 synapses per group). (**F**) Histograms of immungold labelled GluA1 distances from postsynaptic membrane. Data shown as mean ± SEM. *$p< 0.05$, **$p< 0.01$, Tukey's test *post hoc*.

Although we observed no difference in total amount of cadherin and GluA1, the proportion of cadherin localized to the synaptic membrane under basal conditions (within 40 nm) was significantly increased in *β-cat*$^{\Delta ex3/+}$ (**Fig. 2.5C,D**; control = 29.3% ± 2.23%, *β-cat*$^{\Delta ex3/+}$= 38.3% ± 2.03%**),** whereas the proportion of GluA1 localized to the active zone was similar in

control and *β-cat*$^{\Delta ex3/+}$*mice* (**Fig 2.5E**; control = 47.2% ± 5.1%, *β-cat*$^{\Delta ex3/+}$= 46.7% ± 2.4%). The lack of change in GluA1 under basal conditions was consistent with data showing similar postsynaptic strength under basal conditions in control and *β-cat*$^{\Delta ex3/+}$ mice (**Fig. 2.3A**). Following NMDA treatment there was a striking reduction in the proportion of immunogold-labelled cadherin at the synaptic membrane (**Fig. 2.5C,D**) and GluA1 at the active zone (**Fig. 2.5E,F**) in acute hippocampal slices from control mice, consistent with endocytosis of AMPAR and cadherin. In contrast, in *β-cat*$^{\Delta ex3/+}$ mice we observed no significant change in the proportion of immunolabelled cadherin at the synaptic membrane following NMDA treatment (**Fig. 2.5C,D**) or GluA1 at the active zone (**Fig. 2.5E,F**). Analysis of immunogold particle localization by frequency distribution (**Fig. 2.5D,F**) further demonstrated that following NMDA treatment in control mice there was a redistribution of cadherin and GluA1 to the non-synaptic or 'recycling' population, indicating activity-dependent endocytosis, but that this redistribution was impaired in *β-cat*$^{\Delta ex3/+}$ mice.

2.3.6 Cadherin/β-catenin interactions at synapses are increased in *β-cat*$^{\Delta ex3/+}$ mice.

In order to confirm the mechanism by which stabilization of β-catenin was responsible for the impairment in cadherin and AMPA receptor endocytosis observed in *β-cat*$^{\Delta ex3/+}$ mice, we next examined β-catenin/cadherin interactions directly by immunoprecipitation. Synaptosomal fractions from hippocampal lysates were immunoprecipitated with anti-cadherin antibodies and analyzed by western blot. Both wildtype and stabilized forms of β-catenin immunoprecipitated with cadherin, and a significant increase in total β-catenin associated with cadherin was observed in *β-cat*$^{\Delta ex3/+}$ mice (**Fig. 2.6A,** 56.5% ± 13.0% increase), indicating enhanced β-catenin/cadherin association at synapses.

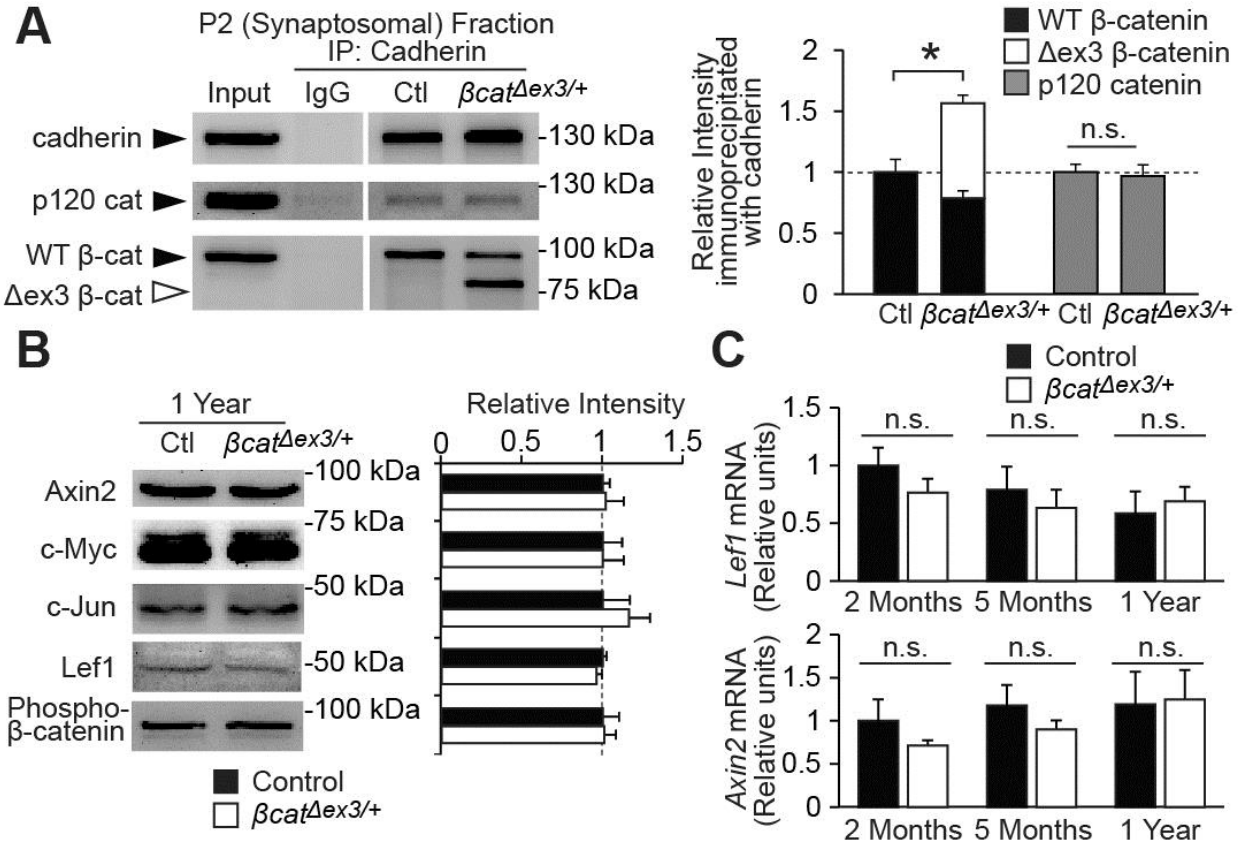

Figure 2.6 β-catenin/cadherin interaction is significantly increased and expression of Wnt pathway targets is unchanged in *β-cat*$^{\Delta ex3/+}$ mice.
(**A**) Representative immunoblots and quantification of synaptosomal fractions from hippocampal lysates immunoprecipitated with anti-pan-cadherin. There was an increase in total cadherin-associated β-catenin (wildtype β-catenin plus Δex3 β-catenin, p=0.029), but not p120 catenin. (**B**) Representative immunoblots and quantification for Wnt pathway targets in hippocampal lysates from 1-year *β-cat*$^{\Delta ex3/+}$ mice and controls (*n*=3 blots, 3 separate mice for each cond.). No significant differences in Wnt target expression or N-terminal phosphorylation (Ser-33/37, Thr-41) of wildtype β-catenin was observed in *β-cat*$^{\Delta ex3/+}$ mice. (**C**) RTPCR analysis of relative *Lef1* and *Axin2* mRNA levels from the hippocampus of 2-month, 5-month and 1-year mice. Expression reported in relative units normalized to GAPDH expression (2 months: *n*=4 *β-cat*$^{\Delta ex3/+}$, 4 control; 5 months: *n*=4 *β-cat*$^{\Delta ex3/+}$, 4 control; 1 year: *n*=3 *β-cat*$^{\Delta ex3/+}$, 4 control). Data shown as mean ± SEM. *p< 0.05.

Increasing β-catenin levels in the brain did not significantly impact cadherin's association with p120 catenin, another component of the cadherin adhesion complex (**Fig. 2.6A**). There were

also no differences in expression of components of the cadherin adhesion complex (cadherin, p120 catenin), postsynaptic proteins (PSD-95, GluA2, NR1) or synaptic vesicle proteins (synaptophysin, synaptotagmin and synapsin I) (**Fig 2.7**). Thus, stabilization β-catenin enhances β-catenin's association with cadherin at synapses but does not perturb overall synaptic protein expression in the hippocampus.

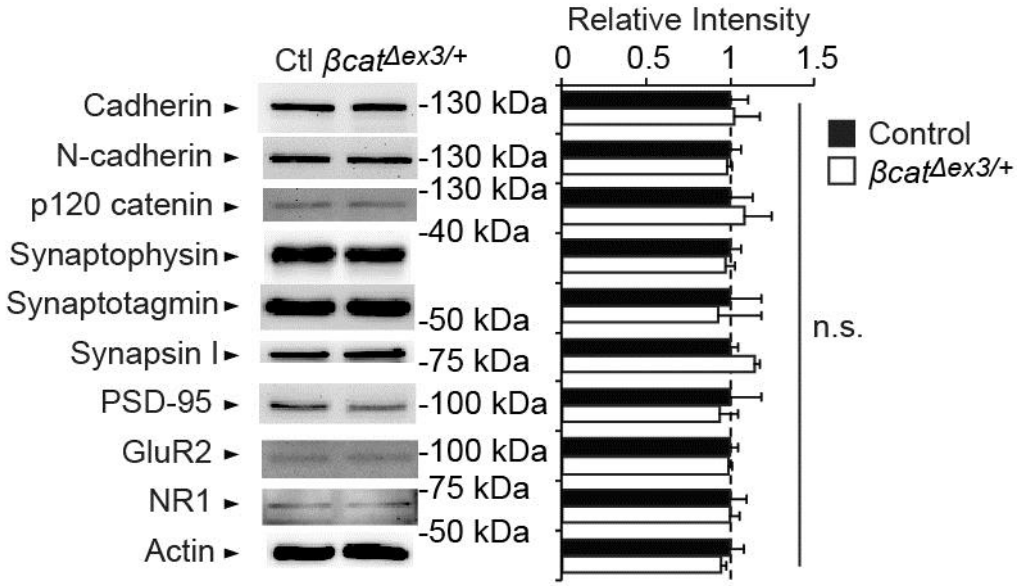

Figure 2.7 Expression of cadherin adhesion complex proteins and other synaptic proteins in unaffected in *β-cat*$^{\Delta ex3/+}$ mice.
Representative immunoblots for cadherin adhesion complex proteins and synaptic proteins in hippocampal lysates from 1-year-old mice. There were no observable changes in the expression of any proteins analyzed. (n=3 blots from 3 separate animals). Data shown as mean ± SEM.

2.3.7 Wnt signaling is not affected in *β-cat*$^{\Delta ex3/+}$ mice.

We then determined whether any changes in Wnt signaling may have contributed to the impairments observed in *β-cat*$^{\Delta ex3/+}$ mice. We examined the expression of several known Wnt targets by western blot analysis (**Fig. 2.6B**) and RTPCR (**Fig. 2.6C**), but found no changes in Wnt target expression in the hippocampus of *β-cat*$^{\Delta ex3/+}$ mice. We also examined Wnt target expression by immunohistochemistry, but saw no changes in expression in the dentate gyrus or

other subregions of the hippocampus (**Fig 2.8**). These findings were in accord with the observations that β-catenin levels in the P1 crude nuclear fraction were similar between *β-cat*$^{\Delta ex3/+}$ mice and controls and expression of synaptic proteins was unchanged (**Fig 2.2, 2.7**). We therefore concluded that Wnt signaling was not augmented in *β-cat*$^{\Delta ex3/+}$ mice, providing further support that impaired cadherin and AMPA receptor endocytosis following β-catenin stabilization was responsible for the deficits in LTD and cognitive flexibility observed in *β-cat*$^{\Delta ex3/+}$ mice.

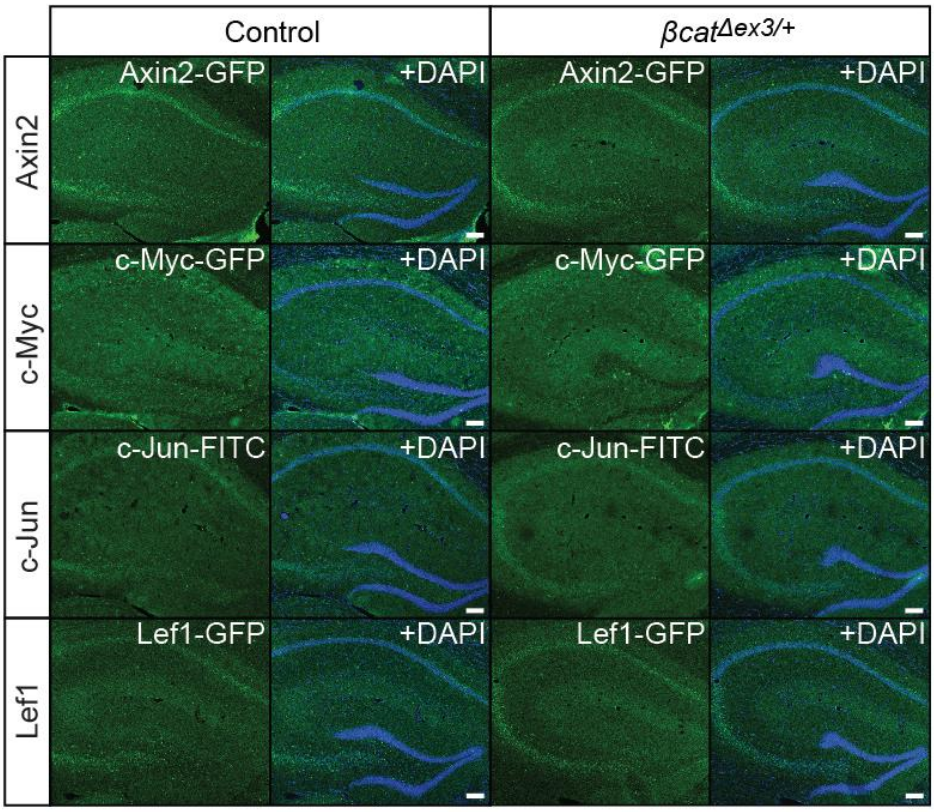

Figure 2.8 Expression of Wnt targets is unchanged throughout the hippocampus in *β-cat*$^{\Delta ex3/+}$ mice.
Confocal images of brain sections from *β-cat*$^{\Delta ex3/+}$ mice and controls co-immunostained for Wnt pathway targets. No change in Wnt target expression was observed in the dentate gyrus or other regions of the hippocampus (Scale bar = 100 µm).

2.4 DISCUSSION

In the present study, we demonstrate that stabilization of β-catenin in the adult hippocampus is sufficient to cause significant disruption of synaptic plasticity and cognitive flexibility. The results from this study support three main conclusions. First, increased levels of β-catenin in the hippocampus leads to significant deficits in spatial memory flexibility and reversal learning. Second, β-catenin stabilization is sufficient to abolish LTD at hippocampal synapses while leaving basal synapse function and LTP intact. Third, increased β-catenin/cadherin interaction at synapses results in impaired activity-dependent endocytosis of cadherin and AMPAR at synapses, which appears to be the primary mechanism responsible for these synaptic and cognitive impairments as Wnt signaling was not perturbed β-cat$^{\Delta ex3/+}$ mice. Together, these results indicate that aberrant increases in the stability of synaptic adhesion molecules can have a negative effect on synaptic plasticity and cognitive function.

The behavioral deficits we observed in *β-cat*$^{\Delta ex3/+}$ mice are all consistent with a specific impairment in hippocampal LTD and a subsequent impairment in spatial memory plasticity. Behavioral tasks which have been shown to involve hippocampal LTP or non-hippocampal brain regions were largely unaffected in *β-cat*$^{\Delta ex3/+}$ mice, including contextual fear conditioning, novel object recognition and initial acquisition of spatial memory in the MWM. Indeed, deletion of N-cadherin in vivo has been shown to cause impairment in spatial memory acquisition, but fewer perseverative errors on a radial arm version of the MWM (Nikitczuk et al., 2014), which is almost precisely the opposite and complimentary phenotype to that observed in *β-cat*$^{\Delta ex3/+}$ mice (intact acquisition, but more perseverative errors). Our data support the hypothesis that LTD acts to depotentiate synapses in order to facilitate the acquisition of novel information (Nicholls et al., 2008); we observed that *β-cat*$^{\Delta ex3/+}$ mice exhibited impairments in reversal learning in the MWM

and behavioral flexibility on the DNMTP T-maze, two tasks which involve the modification or elimination of spatial memory rather than simply memory acquisition. The most intriguing result from the present study was the persistence of *β-cat*$^{\Delta ex3/+}$ mice in entries to the old learned platform location in the reversal phase of the MWM, indicating that the deficit in acquiring novel spatial information was due to an inability to eliminate previously learned spatial information. This suggests that transient changes in β-catenin/cadherin-mediated adhesion and stability are critical for the restructuring of synapses underlying cognitive and behavioral flexibility. Indeed, previous studies have shown that following enhanced activity a transient disruption of β-catenin association with cadherin precedes increased association of the two proteins (Maguschak and Ressler, 2008), suggesting a window of structural plasticity exists that facilitates normal experience-induced changes in synapse strength.

Our data indicate the impairments in LTD observed in *β-cat*$^{\Delta ex3/+}$ mice were due to increased association of β-catenin with cadherin at hippocampal synapses, resulting in impaired activity-dependent endocytosis of both cadherin and associated AMPA receptors. Cadherins form both direct and indirect associations with AMPA receptors (Nuriya and Huganir, 2006, Saglietti et al., 2007, Zhou et al., 2011), and stabilizing β-catenin/cadherin interactions *in vitro* has been shown to prevent the internalization of cadherin following NMDA treatment (Tai et al., 2007). Interestingly, mGluR-dependent LTD has also been shown to require interaction between N-cadherin and the GluA2 subunit of AMPA receptors, providing further evidence of an important functional relationship between cadherin and AMPA receptors at synapses (Zhou et al., 2011). As LTD is achieved through removal of AMPA receptors from the postsynaptic membrane (Collingridge et al., 2004), the physiological impairment in LTD observed in *β-cat*$^{\Delta ex3/+}$ mice is consistent with the deficits in AMPA receptor endocytosis observed by

immunogold electron microscopy. This model of synaptic dysfunction is also consistent with the lack of impairment in hippocampal LTP in *β-cat*$^{\Delta ex3/+}$ mice; enhanced cadherin stability at the synaptic membrane is unlikely to affect the insertion of additional AMPARs to the synaptic membrane, which is the primary mechanism responsible for LTP (Collingridge et al., 2004). Interestingly, basal synaptic transmission and postsynaptic strength were unaffected by the stabilization of cadherin in *β-cat*$^{\Delta ex3/+}$ mice, and previous studies have shown that knockdown of N-cadherin (Jungling et al., 2006, Bozdagi et al., 2010) or β-catenin (Bamji et al., 2003) *in vivo* also did not significantly impact synapse number or basal synaptic transmission. This suggests that while cadherin is important for activity-dependent plasticity, other mechanisms play a more dominant role in determining basal synapse density and synaptic strength. Furthermore, ablation of N-cadherin *in vivo* has been shown to impair spine enlargement and LTP, but does not affect LTD (Bozdagi et al., 2010), as compared to our study in which cadherin stabilization results in intact LTP but impaired LTD. Together, these studies suggest a consistent model where N-cadherin must be present to stabilize synapses and AMPA receptors to mediate LTP, but must be transiently destabilized or removed from synapses to facilitate LTD.

We observed no difference in Wnt target expression in *β-cat*$^{\Delta ex3/+}$ mice, indicating that changes in Wnt signaling did not contribute to the physiological and behavioral changes observed in these animals, though the stabilized form of β-catenin lacking exon 3 is competent for Wnt signaling (Harada et al., 1999, Li et al., 2009). Why was Wnt signaling unaffected in the hippocampus of *β-cat*$^{\Delta ex3/+}$ mice? β-catenin stabilization was also restricted to adult, differentiated neurons, which are less sensitive to changes in β-catenin levels (Kratz et al., 2002), and much of the available β-catenin was localized to synapses due to its association with cadherin. Finally, nuclear transport of β-catenin may also be more carefully regulated in neurons

(Schmeisser et al., 2009) and levels of Lef1 are relatively low in the hippocampus compared to other brain regions (Wisniewska et al., 2010), reducing the sensitivity of Wnt signaling to changes in levels of cytoplasmic β-catenin.

The findings of the present study indicate that changes in β-catenin and cadherin stability can have pronounced effects on synapse function and, more generally, demonstrate that *increases* in synaptic adhesion can have a detrimental effect on normal synaptic plasticity and cognition. Increased levels of β-catenin have been reported in a wide variety of neurological disorders, including Alzheimer's disease (Soriano et al., 2001, Li et al., 2007), Huntington's disease (Godin et al., 2010), and alcoholism (Al-Housseini et al., 2008), but the contribution of β-catenin to these disorders remains unclear. In Huntington's disease (HD), β-catenin levels are significantly increased in the brains of HD patients as well as in mouse and *Drosophila* models of HD, and targeted reduction of β-catenin was shown to have a therapeutic effects (Godin et al., 2010). Intriguingly, deficits in reversal learning due to perseveration similar to those observed in $\beta\text{-}cat^{\Delta ex3/+}$ mice have been reported in both mouse models of HD and in HD patients (Lione et al., 1999). Taken together with these studies, our work supports the hypothesis that enhanced levels of β-catenin may contribute to the pathology of HD. Also, lithium, a widely used antipsychotic, is known to inhibit GSK3β, stabilizing β-catenin in neurons (Gould et al., 2004). Increased Wnt signaling is believed to exert a therapeutic effect in these cases, but data from the present study leads us to speculate that enhanced structural stability at synapses may also contribute. Since alterations in β-catenin levels can have significant effects on synaptic plasticity and memory, understanding the contribution of β-catenin and synaptic adhesion in individual neurological disorders may provide important insights into disease pathology and therapeutic approaches.

Chapter 3: N-cadherin mediates cocaine- and activity-induced plasticity in the ventral tegmental area.

Dopaminergic (DA) neurons in the ventral tegmental area (VTA) are a key part of the reward circuitry of the brain. The activity of DA neurons is influenced by excitatory and inhibitory synapses they receive, and it has been hypothesized that alterations in the normal mechanisms of plasticity at these synapses may underlie the formation and expression of addiction-related behaviours caused by drugs of abuse. Cadherin adhesion molecules may play an important role in both normal and pathological plasticity at synapses in the VTA; cadherins have been shown to mediate activity-induced changes in synaptic strength and stability, and recent studies have identified mutations in cadherins and associated proteins as risk factors for addiction. In the present study, we demonstrate that several classical cadherins are expressed throughout the VTA, and that cocaine-mediated conditioned place preference is correlated with increased recruitment of cadherin to excitatory synapses onto dopaminergic neurons, suggesting an increase in synaptic adhesion at these synapses. Furthermore, we demonstrate that disruption of cadherin homophilic interactions abolishes spike-timing-dependent increases in synaptic strength at synapses in the VTA. These results show that cadherins play an important role in synaptic plasticity in the VTA and may also be involved in structural changes at synapses caused by cocaine use.

As stated in the preface, all work and experiments presented in this chapter were done in equal partnership by Fergil Mills and Andrea Globa, who will be co-first authors on the paper publishing these results.

3.1 INTRODUCTION

Changes in synaptic strength in response to experience are thought to underlie learning and memory, with these processes being "hijacked" in the context of drug addiction. Drugs of abuse have been shown to cause increased dopamine release by dopaminergic (DA) neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) and prefrontal cortex, which induces widespread alterations to this crucial part of the motivational circuit for normal reward learning (reviewed in Mameli and Lüscher, 2011, Lüscher, 2013). Excitatory synapses onto these DA neurons are a key point of control which regulates their activity, and drugs of abuse have also been shown to cause major changes in plasticity and potentiation at these sites as well.

Changes in the composition of postsynaptic receptors have been shown to play an important role in drug-induced changes in plasticity at synapses in the VTA. Even a single exposure to cocaine or other drugs of abuse results in the potentiation of glutamatergic synapses onto DA neurons within the VTA (Ungless et al., 2001, Saal et al., 2003), a process which requires NMDA receptor activation (Engblom et al., 2008), and is mediated by the trafficking of AMPA receptors. Specifically, GluA2-lacking calcium-permeable AMPA receptors (CP-AMPARs) are inserted, while GluA2-containing calcium-impermeable AMPA receptors (CI-AMPARs) are removed from these synapses, resulting in increased calcium influx and potentiation constituting a pathological form of plasticity (Bellone and Lüscher, 2006). This change in AMPA receptor composition is reversed by long-term depression (LTD) mediated by metabotropic glutamate receptor (mGluR) activation (Bellone and Lüscher, 2006), and requires the synthesis and insertion of GluA2 subunits (Mameli et al., 2007). When mGluR1 function is

disrupted, cocaine-mediated potentiation of synapses in the VTA becomes persistent, driving increases in synaptic strength in the NAc which are normally observed after repeated cocaine exposure (Mameli et al., 2009). Importantly, these drug-induced changes in receptor composition at synapses onto dopaminergic neurons of the VTA may constitute the initial mechanism leading to compulsive drug seeking in addicted individuals. Indeed, when plasticity is impaired by knocking out NMDA receptor subunit NR1 at these synapses, the reinstatement of drug preference is abolished in rodent models (Engblom et al., 2008). However, although a great deal of attention has focused on the changes in receptor composition that occur after drug exposure, much is still unknown about the specific mechanisms of plasticity controlling the insertion and removal of these AMPA receptors implicated in drug addiction.

Cadherin adhesion molecules are important regulators of synapse plasticity, and have been shown to play a vital role in the trafficking and stability of AMPA receptors underlying different forms of long-lasting synaptic plasticity. Cadherins and AMPA receptors are transported together in neurons (Heisler et al., 2014), and N-cadherin interacts with GluA1 and GluA2 AMPA receptor subunits (Nuriya and Huganir, 2006, Saglietti et al., 2007, Zhou et al., 2011). Cadherin is also essential for long-term potentiation (LTP) in hippocampal neurons (Tang et al., 1998, Bozdagi et al., 2010), and the activity-mediated enlargement of dendritic spines (Mendez et al., 2010). Additionally, the internalization of cadherin is essential for hippocampal LTD; if N-cadherin internalization is prevented, then NMDAR-dependent LTD is abolished (Tai et al., 2007). The interaction between GluA2 and N-cadherin is essential for mGluR-LTD in the hippocampus(Zhou et al., 2011), which is the same type of LTD responsible for the reversal of cocaine-induced potentiation at synapses in the VTA (Mameli et al., 2011). For these reasons, cadherins are strong candidate molecules for mediating changes in synaptic plasticity induced by

drugs of abuse. However, very little is known about the expression of cadherins in the VTA, and their potential function in this region has not been examined.

In the present study, we find that classical cadherins are expressed in the VTA, and establish that cadherin adhesion is essential for spike-timing-dependent long-term potentiation (LTP) at excitatory synapses onto DA neurons in the VTA. Additionally, we characterize the changes in localization of cadherins at these synapses following cocaine-mediated conditioned place preference (CPP) in a novel environment, a behavioural paradigm which allows drug preference to be quantified based on the preference created for an unconditioned context associated with the drug's effects, and mimics the associative learning in addiction which leads to sensitization of previously unrelated environmental cues due to repeated association with drug use. We find that cocaine-mediated CPP is correlated with increased recruitment of cadherin to excitatory inputs onto dopaminergic neurons, which is reversed by extinction of CPP. We also demonstrate that administration of cocaine in a familiar environment does not cause the same changes in cadherin localization at synapses, indicating the increase in synaptic cadherin is due to the association between the drug's effects with the novel environment during CPP. Finally, we find that GluA1, a key component of calcium-permeable AMPA receptors, shows highly similar patterns of insertion and removal from the synaptic membrane as cadherin. These results indicate that cadherins play an important role in synaptic plasticity in the VTA and may also be involved in mediating structural changes induced by cocaine use.

3.2 MATERIALS AND METHODS

Animals. Male C57BL/6 mice between 6-12 weeks old were used in all experiments. Mice were housed in reverse day/night cycle and given *ad libitum* access to food and water. Experimental procedures and animal housing conditions were approved by The UBC Animal Care Committee, and were in accordance with Canadian Council on Animal Care (CCAC) guidelines.

*Immunoblot Analysis***.** Mice were anesthetized by cervical dislocation and their brains quickly removed, then sliced into 300 µm thick horizontal sections by vibratome. The VTA was dissected from these slices and homogenized in lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 0.5% NP-40, 10% glycerol) with protease and phosphatase inhibitor tablets (Roche) and cleared by centrifugation at 14,000 x g for 40 min at 4°C. VTA lysates were separated by SDS-PAGE and probed with antibodies against N-cadherin (mouse, BD Transduction), R-cadherin (rabbit, Novus), cadherin-7 (rabbit, Santa Cruz), cadherin-8 (rabbit, Abcam), cadherin-11 (mouse, Invitrogen) and pan-cadherin (rabbit, Zymed). Proteins were visualized by chemiluminesence on a Bio-Rad Versadoc 4000 (Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON).

*Immunohistochemistry***.** Mice were anesthetized with sodium pentobarbital (120 mg/kg), transcardially perfused by PBS followed by 4% paraformaldehyde (PFA) in PBS. Brains were removed and post-fixed in 4% PFA for two hours, then cryoprotected by saturation with 30% sucrose, frozen, and sliced into 20 µm thick coronal sections by cryostat. For immunolabelling of target proteins, sections were first placed in a blocking buffer containing 10% goat serum, 0.1% bovine serum albumin and 0.1% Triton-X-100 in PBS. Primary antibodies against DAT

(dopamine transporter) (rat, Millipore), N-cadherin (mouse, BD Transduction), R-cadherin (rabbit, Novus), cadherin-7 (rabbit, Santa Cruz), cadherin-8 (rabbit, Abcam), cadherin-11 (mouse, Invitrogen) were diluted in this buffer were then added to sections and incubated overnight at 4°C. The following day, samples were washed three times with PBS, and secondary antibodies diluted in the blocking buffer were added to sections and incubated for 1-2 hours at room temperature. Slides were washed again with PBS and stained with DAPI (0.5 μg/mL). Sections were mounted with ProLong (Life Technologies, Carlsbad, CA), and were imaged on an Olympus Fluoview 1000 confocal microscope using Fluoview software (Olympus, Melville, NY). The brightness and contrast of entire images was moderately adjusted using Photoshop (Adobe Systems Canada, Toronto, ON) following recommended, scientifically acceptable procedures, and no information was obscured or eliminated from the original images.

Electrophysiology. Electrophysiological recordings were taken from dopaminergic cells within the VTA. Horizontal slices of mouse brain were cut with by vibratome, and slices were equilibrated in artificial cerebrospinal fluid (aCSF). Slices were preincubated in 200 μM HAV peptide (Ala-Arg-Phe-His-Leu-Arg-Ala-<u>His-Ala-Val</u>-Asp-Ile-Asn-Gly-Asn-Gln-Val), 200 μM scrambled control peptide (Val-Ala-Val-Leu-Tyr-Glu-Lys-Ser-Gly-Ile-Ala-Tyr-His-Asn-Ser-Ala-Ser), or vehicle. The spike-timing protocol for LTP induction was carried out as previously described (Liu and Poo, 2005). Briefly, the protocol consisted of 20 bursts of EPSP-spike pairs, with each burst consisting of 5 paired stimuli at 10 Hz (100 ms intervals), with an interburst interval of 5 s. Postsynaptic spikes were evoked by injection of depolarizing current pulses, with the onset of EPSPs preceding the peak of postsynaptic spikes by 5 ms. Evoked EPSPs were sampled once per minute before and after LTP induction.

Conditioned Place Preference. Conditioned place preference (CPP) was induced using a standard three-chambered apparatus, consisting of two conditioning compartments and a middle connecting compartment (Stoelting Co., Wood Dale, IL). The two conditioning compartments had distinct wall patterns and floor textures (cloth, felt) to allow mice to distinguish between them. Mice were first allowed to habituate to the entire apparatus during two 30 minute sessions over two days. Following habituation, mice in the conditioned group received a 15 mg/kg injection of cocaine, and placed in a 'conditioned' chamber for 15 minutes. Mice were assigned to receive cocaine in one compartment or the other using an unbiased design. The following day, these mice received an equivalent volume of saline, and were placed in the opposite 'non-conditioned' compartment. This alternating pattern of conditioning was repeated three times (6 days total). On test day, place preference was assayed by giving each mouse with a priming injection of saline, placing them in the middle connecting compartment, and recording the amount of time spent in the two conditioning compartments over a 30 minute period. If mice underwent the extinction of CPP, the test day protocol was repeated each day until the drug group's preference for the conditioned chamber had returned to habituation levels.

Electron Microscopy Sample Preparation. Mice were anesthetized with sodium pentobarbital (120 mg/kg), transcardially perfused by PBS followed by 4% paraformaldehyde (PFA) as described above, then post-fixed in 4% PFA overnight. Brains were then cut into 250 µm thick horizontal sections by vibratome. Small pieces of VTA tissue (<1mm in any dimension) were dissected from these slices and cryoprotected in 30% glycerol overnight at 4 °C. Samples were then plunge frozen in liquid ethane at -170 °C in an EM cryopreparation chamber (Leica), and transferred to a 1.5% Uranyl Acetate solution in 100% Methanol, kept at -90 °C in a Leica EM

AFS for 30 hours. The temperature was increased to -45 °C over 11 hours. Next, samples were rinsed in 100% Methanol, and infiltrated with HM-20 acrylic resin (Electron Microscopy Sciences, Hatfield, PA) by increasing the resin to methanol ratio in 2-hour steps while maintaining the temperature at -45 °C. Samples were set up in capsules containing pure resin and polymerized under UV light for 24 hours at -45 °C, after which the temperature was slowly increased to 0 °C. Tissue sections were cut at 85 nm using a Diatome diamond knife and a Leica Ultramicrotome. Sections were collected on 300-mesh, formvar-coated Nickel grids.

Immunogold Electron Microscopy. Grids were rinsed with distilled water and subsequently immersed in a bead of TTBS with 0.1% Triton-X with 0.1% Sodium Borohydride and 50 mM glycine. The grids were then rinsed with TTBS with 0.1% Triton-X three times. Following this, nonspecific binding was blocked by immersing grids in a bead of 2% BSA in TTBS with 0.1% Triton-X for 10 minutes. Primary antibodies against DAT (dopamine transporter) (Rat, Millipore), PSD-95 (rabbit, Sigma), pan-cadherin (species, company), GluA1 (species, company), Gephyrin (species, company) and Tyrosine Hydroxylase (TH) were diluted in 2% BSA in TTBS with 0.1% Triton-X. Grids were immersed in 15 µl beads of diluted primary antibody overnight, at room temperature. The following day, grids were thoroughly rinsed by immersion in vials of TTBS with 0.1% Triton-X three times. Secondary antibodies were diluted in 2% BSA in TTBS with 0.1% Triton-X, and 0.05% Polyethyleneglycol (PEG) was added to prevent aggregation of gold beads. Grids were immersed in 15 µl beads of secondary antibody for 1.5 hours. Following this step, grids were immersed in a vial of TTBS with 0.1% Triton-X, and then thoroughly rinsed in vials of Milli-Q H_2O and dried. Grids were then lightly counterstained with 2% uranylacetate and Reynold's lead citrate. Images were collected at

98000X magnification on a Tecnai G2 Spirit transmission electron microscope (FEI Company, Eindhoven, Netherlands). To quantify immunogold labeling, different synapse and cell types were identified by DAT and PSD-95 markers, and the distance of all immungold-labelled cadherin from the synaptic membrane, or all immungold labelled GluA1 to the postsynaptic active zone membrane were measured. Due to the sizes of proteins and reagents involved (Mathiisen et al., 2006, see also Results section and Figure 4.3), cadherin-labeled gold particles within 40 nm of the synaptic membrane and GluA1-labeled gold particles within 30 nm of the active zone were considered to be associated with the synaptic membrane or active zone, respectively. The percentage of immungold particles localized to these regions was determined by [# immungold beads at target region]/[total # immunogold beads at pre and post-synaptic compartments]. All images were acquired and analyzed blind to the genotype of each mouse.

Statistical analysis. Unless otherwise noted, statistical analysis was done using Student's t-test (two tailed) and two-way ANOVA. Data from electrophysiology experiments was analyzed by two way repeated measures (RM) ANOVA, and *post-hoc* analysis was done using Bonferroni's test. Data from CPP experiments was also analyzed by two-way RM ANOVA with genotype as the between-subjects factor and time as the within-subjects factor. For comparisons between genotypes and within days, one-way ANOVA was used. Results were considered significant when p=0.05. Analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA).

3.3 RESULTS

3.3.1 Cadherins are widely expressed throughout the VTA.

We first examined the expression of cadherin proteins in the VTA. mRNA for several cadherins and protocadherins had been detected in the VTA by *in situ* hybridization (Hertel et al., 2008), but levels of protein expression and cell type specificity had not been examined. We confirmed the expression of N-cadherin, R-cadherin, cadherin-7, cadherin-8 and cadherin-11 proteins in the VTA using western blot analysis (**Fig. 3.1A**), and immunohistochemistry (**Fig. 3.1B**). We found that these classical cadherins were present throughout the VTA and expressed in both dopaminergic and non-dopaminergic neurons. Indeed, over 95% of DA cells in the VTA were immunopositive for N-cadherin, cadherin-7, and cadherin-8, and over 80% were immunopositive for R-cadherin and cadherin-11.

3.3.2 Spike-timing dependent potentiation is abolished by disruption of extracellular cadherin adhesion in the VTA.

We then sought to determine whether cadherins are required for activity-induced potentiation of synapses formed onto DA neurons in the VTA. Previous studies of cadherin in other brain regions have shown that N-cadherin homophilic interactions are critically important for mediating long-lasting changes in synaptic strength, including LTP in the hippocampus (Tang et al., 1998, Bozdagi et al., 2010). To determine whether cadherins are similarly essential for changes in synaptic strength in the VTA, we induced LTP in dopaminergic cells of the VTA using a spike-timing dependent plasticity (STDP) protocol that replicates the burst pattern of firing observed in VTA dopaminergic neurons in response to rewarding stimuli (Liu and Poo, 2005) . Following stimulation, we observed a robust increase in synaptic strength (~60%

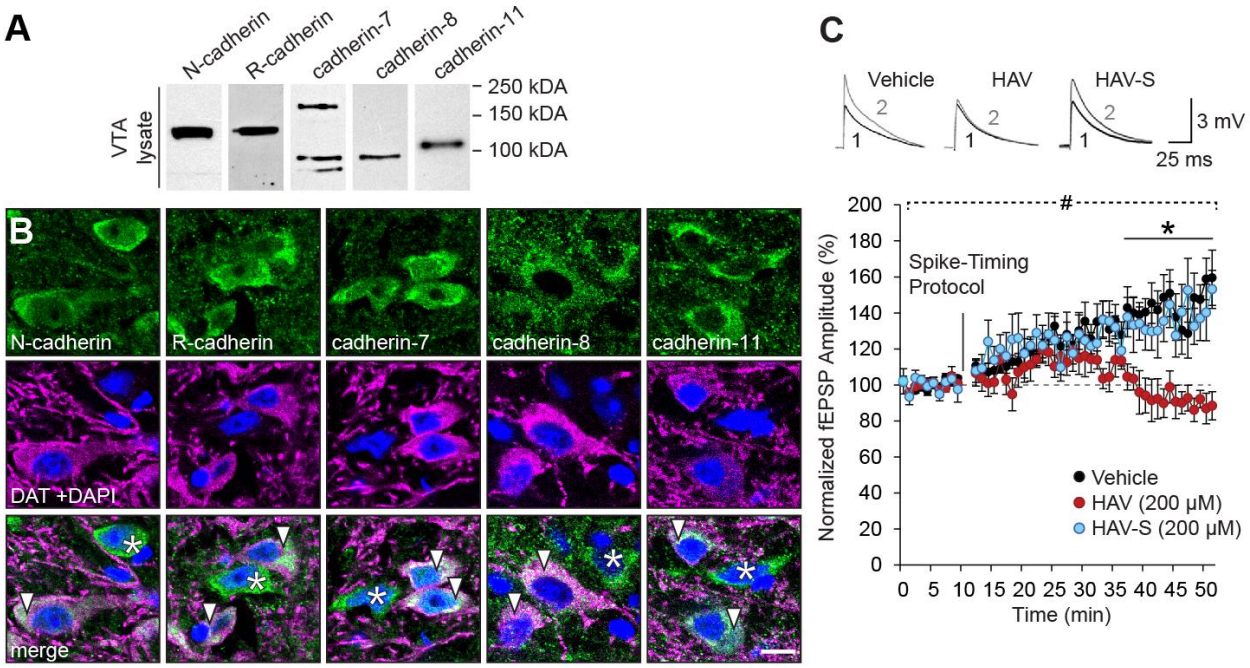

Figure 3.1 Cadherins are expressed throughout the VTA and required for spike-timing dependent potentiation.
(**A**) Representative immunoblots identifying expression of cadherins in the VTA. (**B**) Confocal images of VTA neurons co-immunostained for different cadherins (green), dopamine transporter (DAT; magenta) and DAPI (blue). Arrowheads indicate neurons positive for both cadherin and DAT, asterisks indicate neurons which are positive for cadherin but do not express DAT. Scale bar = 10 μm. (C) Spike timing dependent plasticity (STDP) in the VTA was abolished by treatment with a peptide containing the His-Ala-Val motif (HAV) that disrupts N-cadherin extracellular interactions. Vehicle-only and scramble peptide (HAV-S) controls had no effect on STDP (p=0.048, RM ANOVA; n=8 cells vehicle, 6 cells HAV, 5 cells HAV-S). Data shown as mean ± SEM. # p<0.05, RM ANOVA, *p< 0.05, Bonferroni's test post hoc.

increase) up to 40 minutes later (**Fig. 3.1C**), indicating effective potentiation of excitatory inputs onto dopaminergic cells.

We then repeated this LTP protocol in slices which had been pre-incubated with an antagonistic peptide 17 amino acids long containing a His-Ala-Val (HAV) motif, which has been shown to block extracellular N-cadherin interaction (Tang et al., 1998, Schrick et al., 2007, Bozdagi et al., 2010). Pre-incubation with the HAV peptide significantly abolished spike-timing dependent LTP at glutamatergic inputs to DA neurons in the VTA (**Fig. 3.1C,** p<0.05 two-way RM ANOVA), whereas pre-incubation of slice s with a control scrambled peptide (HAV-S) had

no effect. This strongly indicates that disruption of extracellular N-cadherin interactions impairs the activity-induced potentiation of synapses formed onto DA neurons within the VTA.

3.3.3 Cocaine-induced conditioned place preference increases the membrane localization of cadherin specifically at excitatory synapses formed onto dopaminergic neurons in the VTA.

The disruption of LTP induction by the HAV peptide indicated that cadherins have an important role mediating activity-induced plasticity at excitatory inputs onto DA neurons, a population of synapses shown to exhibit increases in synaptic strength in response to addictive drugs (Ungless et al., 2001, Chen et al., 2010), and believed to be of critical importance for the establishing and expression of both normal reward-related learning and drug-induced addictive behaviours (Engblom et al., 2008). We therefore sought to determine whether alterations to excitatory synapses formed onto DA neurons by cocaine-mediated CPP might be correlated with alterations in cadherin localization at these synapses. We first examined the effects of cocaine-induced conditioned place preference (CPP) on the localization of cadherins at synapses in the VTA. We induced CPP in a standard three-chamber apparatus; on each day of training, mice received either 15 mg/kg of cocaine (15 mg/kg; days 1, 3, 5) and were placed in the ‘conditioned’ chamber, or received an equivalent volume of saline (days 2, 4, 6) and were placed in the ‘unconditioned’ chamber (**Fig 3.2A**). In conditioned mice, we observed a robust increase in total time spent in the drug-paired chamber compared with time spent in the same chamber prior to drug administration (**Fig. 32B**). In contrast, we observed no change in place preference in the control group that received only saline in both chambers (**Fig. 3.2B**). Following quantification of place preference, mice were immediately sacrificed and the VTA isolated by microdissection,

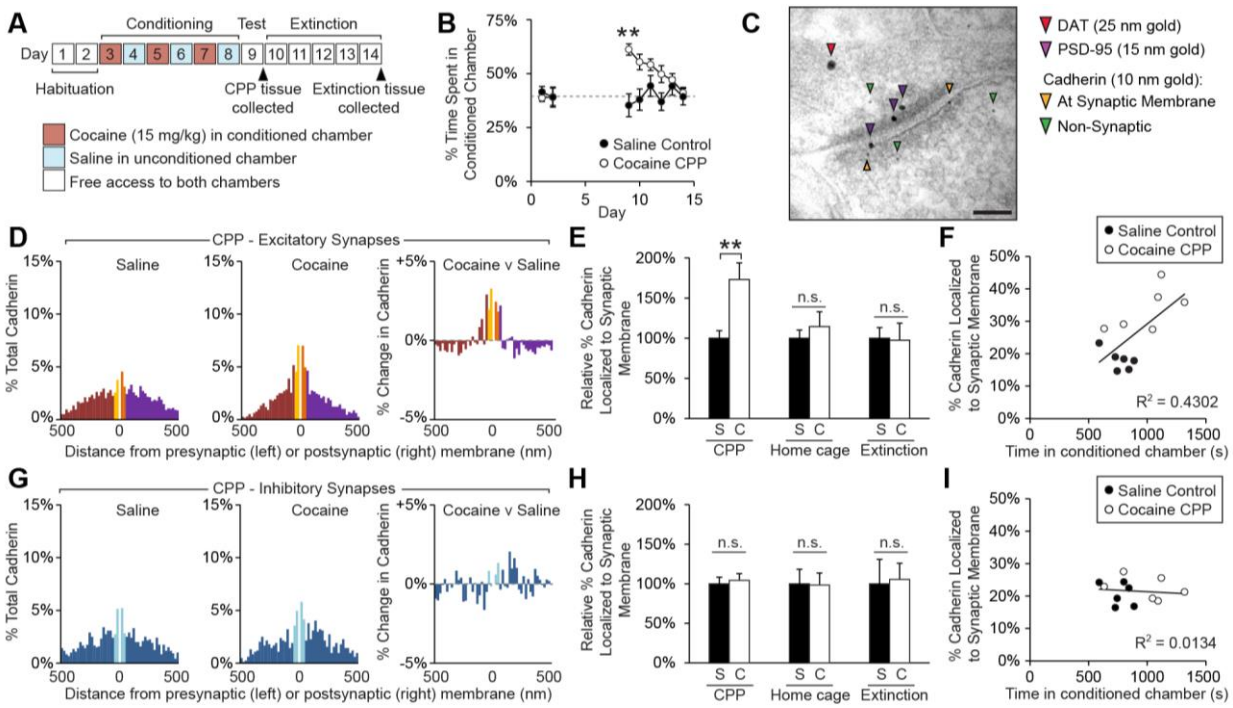

Figure 3.2 Cocaine-mediated conditioned place preference (CPP) leads to recruitment of cadherin to excitatory synapses onto DA neurons in the VTA.
(**A**) Experimental schedule for induction of CPP and extinction experiments. Home cage controls followed the same schedule of cocaine and saline administration as CPP mice, but animals were returned to their home cages instead of being placed in novel contexts. (**B**) Representative data showing acquisition and extinction of cocaine-induced place preference in two-chamber apparatus. Cocaine paired with the 'conditioned' chamber produced robust CPP (Day 9, $p<0.01$, Bonferroni's test *post hoc*) that gradually extinguished over 5 days of unpaired re-introduction to the CPP apparatus (n=6 mice saline, 6 mice cocaine). (**C**) Representative electron micrograph of VTA synapse from control mice showing immunogold labelled DAT, PSD-95 and cadherin (Scale bar = 100 nm). (**D**) Histograms of immungold labelled cadherin distances from presynaptic (left of 0) and postsynaptic (right of 0) membranes at excitatory synapses in cocaine CPP group and saline controls. (**E**) The percentage of cadherin at the synaptic membrane at excitatory synapses was significantly increased following cocaine-mediated CPP, indicating increased cadherin-based adhesion at synapses ($p<0.01$, ANOVA), but not in home cage controls or following extinction of CPP (n=6 mice saline, 6 mice cocaine, >100 synapses per group). **(F)** Correlation of percentage of cadherin localized to synaptic membrane at excitatory synapses with total time spent in conditioned chamber following cocaine-mediated CPP. **(G)** Histograms of immungold labelled cadherin distances from presynaptic (left of 0) and postsynaptic (right of 0) membranes at inhibitory synapses in cocaine CPP group and saline controls. **(H)** The percentage of cadherin at the synaptic membrane at inhibitory synapses was not changed following cocaine-mediated CPP, as well as in home cage controls and following extinction of CPP, indicating no alteration in cadherin distribution or cadherin-based adhesion at inhibitory synapses (ANOVA, n=6 mice saline, 6 mice cocaine, >100 synapses per group). **(I)** Correlation of percentage of cadherin localized to synaptic membrane at inhibitory synapses with total time spent in conditioned chamber following cocaine-mediated CPP. Data shown as mean ± SEM. **$p< 0.01$, Bonferroni's test *post hoc*.

and we analyzed the ultrastructural distribution of cadherin at synapses in the VTA using immunogold electron microscopy. As there are many different cell types and synapse types in the VTA, we used immunogold labels of different sizes to identify dopamine transporter (DAT)-positive neurons (25 nm gold, dopaminergic neurons) and PSD-95-positive synapses (15 nm gold, excitatory synapses), allowing for the reliable identification of excitatory inputs onto dopaminergic synapses (**Fig. 3.2C**). All primary antibodies used in immunogold experiments were validated to confirm their specificity (**Fig 3.3**).

Following CPP, analysis of immungold-labelled cadherin revealed a striking redistribution of cadherin at excitatory synapses onto DA neurons with a significant increase in the relative proportion of immunogold-labelled cadherin localized to the synaptic membrane in cocaine-conditioned mice compared to saline controls (73% increase, **Fig 3.2D, 3.2E**). Cadherin mediates synaptic adhesion by homophilic binding across the synaptic cleft, and the increase in membrane-associated cadherin suggests a significant increase in adhesion and stability at these synapses following cocaine-mediated CPP. Our data also suggested increased potentiation of synapses due to the enhanced stability of AMPA receptors associated with cadherin (Nuriya and Huganir, 2006, Saglietti et al., 2007). Increases in cadherin stability at the synaptic membrane have also been shown to impair activity-dependent endocytosis of cadherins and impair LTD at synapses (Tai et al., 2007, Mills et al., 2014). To examine the relationship between cadherin at these synapses and the acquisition of cocaine preference, we compared the proportion of cadherin localized to the synaptic membrane from each mouse with their individual behavioural preference for the cocaine-paired chamber. We found that the proportion of cadherin localized to the synaptic

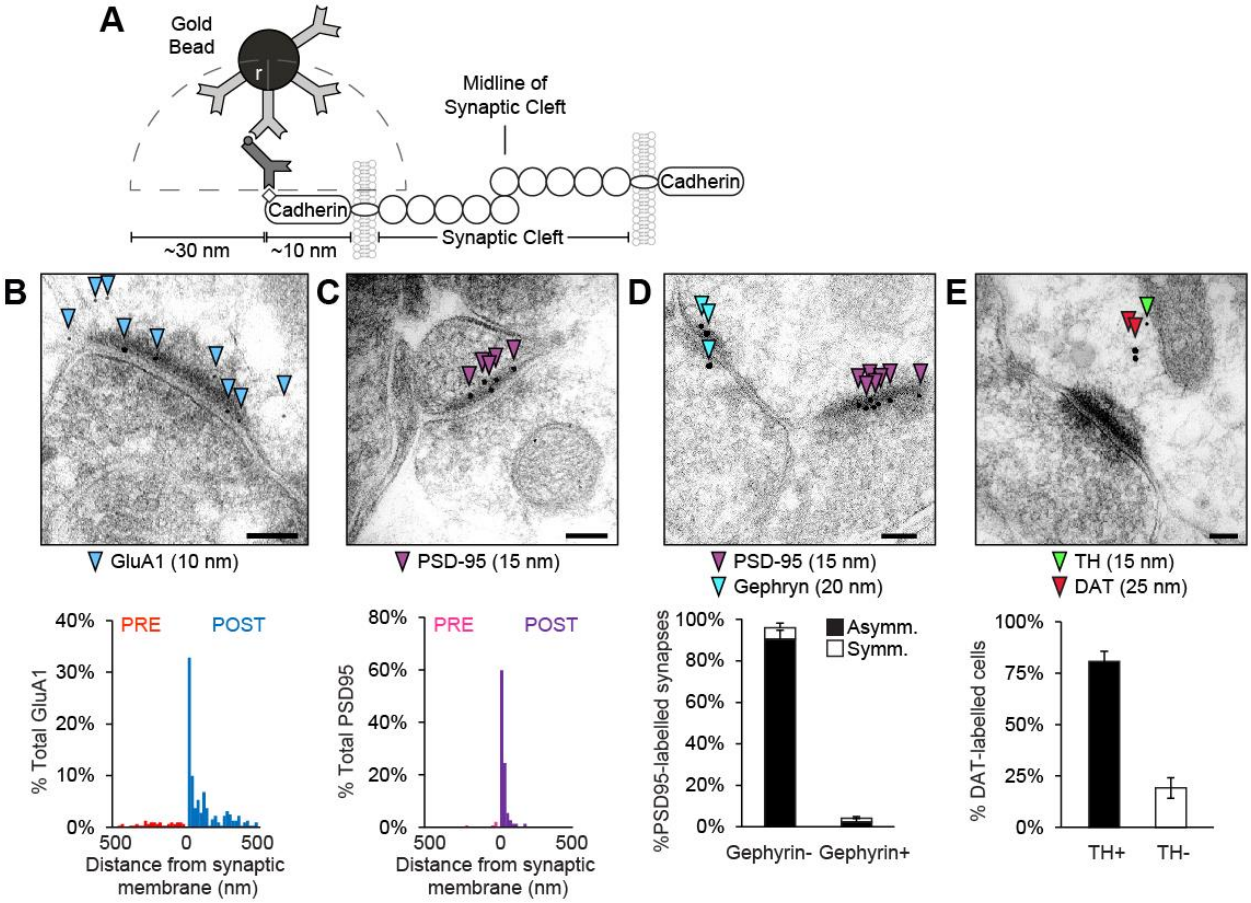

Figure 3.3 Validation of immunogold EM reagents.
(**A**) Diagram of estimated sizes of immunogold reagents (adapted from Mathiisen et al., 2006). (**B**) Top: Representative image of GluA1 antibody labelling at synapses in the VTA. Bottom: Histogram of immunogold-labelled GluA1 distances from the synaptic membrane. A majority of GluA1 immunogold labelling is found postsynaptically, both directly at the synaptic membrane and within the postsynaptic compartment (n= >150 synapses). (**C**) Top: Representative image of PSD-95 antibody labelling at synapses in the VTA. Bottom: Histogram of immunogold-labelled PSD-95 distances from the synaptic membrane. Almost all PSD-95 immunogold labelling is localized directly to the postsynaptic membrane (n= >300 synapses). (**D**) Top: Representative image of PSD 95 and Gephyrin antibody labelling at synapses in the VTA. Bottom: Quantification of PSD-95-labelled synapse morphology and Gephyrin co-labelling. PSD-95 labelling was primarily localized (>93%) to synapses which did were not labelled by the inhibitory synapse marker Gephryin. Some PSD-95 positive synapses did contain Gephyrin (<7%), however, the many of these synapses were morphologically identifiable as 'symmetric' inhibitory synapses. Thus, we could reliably analyze excitatory synapses by restricting our analysis to PSD-95 labelled synapses with asymmetric morphology (n=4 mice, >150 synapses). (**E**) Representative image and quantification of Dopamine Transporter (DAT) and Tyrosine Hydroxlase (TH) immunogold labelling at synapses in VTA. Over 80% of cells labelled with DAT were also co-labelled for TH, indicating that the DAT antibody used was accurately identifies dopaminergic neurons (n=>50 synapses). Data shown as mean ± SEM.

membrane in individual mice was well-correlated with the degree of place preference for the drug-paired chamber following CPP (**Fig 3.2F**), suggesting the increase in cadherin at these excitatory synapses played an important role contributing to the formation and expression of place preference due to association with the rewarding effects of cocaine.

Though excitatory synapses are believed to play a central role in enhancing DA neuron activity, we also analyzed the distribution of cadherin at inhibitory synapses formed onto DA neurons to determine if cocaine CPP caused any changes in cadherin localization at these synapses. Because cocaine has been shown cause an increase in dopamine release from neurons in the VTA, we did not anticipate major changes in cadherin at synapses which would inhibit DA neuron activity. Indeed, we saw that no changes in cadherin localization (**Fig 3.2G**) or the proportion of cadherin at the synaptic membrane were observed at these synapses (**Fig 3.2H; 'CPP'**). Additionally, there was no apparent relationship or correlation between the proportion of cadherin localized to the synaptic membrane at inhibitory synapses and the total amount of place preference for the drug-paired chamber in the CPP group mice (**Fig 3.2I**). These results indicate that cocaine-mediated CPP causes a significant increase in cadherin localization to the synaptic membrane specifically at excitatory synapses on dopaminergic neurons, while inhibitory synapses remained unaffected.

3.3.4 Cocaine administration in the absence of contextual association does not affect cadherin localization at synapses in the VTA.

Even in the absence of contextual conditioning or self-administration paradigms, single doses of cocaine have been shown to cause changes in postsynaptic receptor composition (Ungless et al., 2001, Mameli et al., 2009, Mameli et al., 2011). We therefore sought to determine if the changes

we observed in the localization of cadherin were due to entirely cocaine exposure, or were the result of the contextual association of the drug reward with the conditioned chamber. To test this, we examined mice in a home cage control group, which underwent the same schedule of cocaine and saline administration as in the CPP training but were returned to their home cages following drug administration instead of being placed in the distinct chambers of the CPP apparatus. Analysis of cadherin distribution at excitatory synapses in these mice showed that unlike the CPP group, there was no significant increase in the proportion of immunogold labelled cadherin localized to the synapse (**Fig 3.2E; 'Home cage'**). We also observed no changes in cadherin distribution at inhibitory synapses in these mice (**Fig 4.2H; 'Home cage'**).

3.3.5 Extinction of cocaine-mediated CPP results in reversal of CPP-induced increases in localization of cadherin to the synaptic membrane at VTA synapses.

Following establishment of drug-induced CPP, re-exposure to the conditioned context in the absence of further reinforcement with drug administration causes a gradual extinction of the preference for that context over time (Mueller and Stewart, 2000). Due to the correlation between CPP in individual mice with increases in cadherin localized to synaptic membrane, we sought to determine if extinction of place preference would correspond with reversal of these changes in cadherin distribution at excitatory synapses in the VTA. First, we trained mice using the same schedule of cocaine and saline administration paired with individual chambers as in the CPP training, and then quantified the resulting place preference in each group following re-introduction to the CPP box without barriers between the conditioned and unconditioned chambers (**Fig 3.2B**). These mice were then re-introduced to the CPP apparatus each day with free access to both chambers while receiving only injections of saline, and the change in

preference for the conditioned chamber over time was quantified. When the average place preference in the cocaine group had returned to pre-CPP levels (over 6 days), we collected VTA tissue from the mice and analyzed cadherin distribution at synapses as in the previous CPP groups. We found that following extinction of CPP, and mice in the cocaine extinction group showed similar levels of synaptic cadherin at the synaptic membrane of excitatory synapses onto DA neurons compared to saline controls which had undergone the same schedule of CPP and extinction (**Fig 3.2E, 'Extinction'**), indicating that the increase in localization of cadherin to the synaptic membrane observed following CPP had been reversed. Again, no changes in cadherin distribution at inhibitory synapses were observed in the extinction group (**Fig 3.2H; 'Extinction'**).

3.3.6 GluA1 re-distribution following drug-induced conditioned place preference and extinction parallels changes in cadherin localization at synapses in the VTA.

In addition to providing adhesive stability at synapses, cadherins form direct and indirect associations with AMPA receptors at synapses (Nuriya and Huganir, 2006, Saglietti et al., 2007, Zhou et al., 2011), and impairment of cadherin endocytosis has been shown to also LTD at synapses (Tai et al., 2007, Mills et al., 2014). The insertion and removal of GluA1-containing AMPA receptors has also been shown to play an important role in the establishment and reversal of drug-mediated plasticity (Bellone and Lüscher, 2006, Mameli et al., 2011) We therefore examined the distribution of GluA1 at excitatory synapses on DA neurons in the VTA, to determine how the ultrastructural distribution of this protein was affected by drug-mediated CPP, and to see if changes in the localization of GluA1 were similar to that of cadherin. We saw that

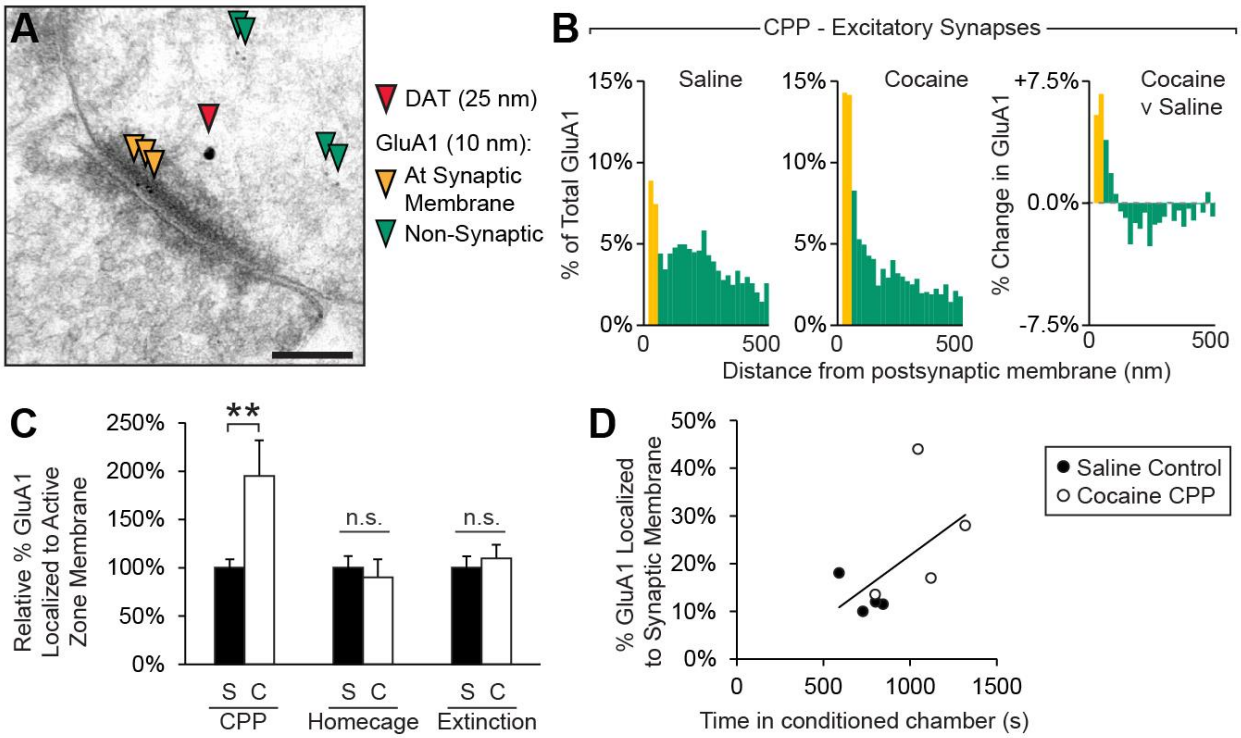

Figure 3.4 Cocaine-mediated conditioned place preference (CPP) leads to recruitment of GluA1 to excitatory synapses onto DA neurons in the VTA.
(**A**) Representative electron micrograph of VTA synapse from control mice showing immunogold labelled DAT and GluA1 (Scale bar = 100 nm). (**B**) Histograms of immungold labelled GluA1 distances from postsynaptic active zone in cocaine CPP group and saline controls. (**C**) The percentage of GluA1at the synaptic membrane at excitatory synapses was significantly increased following cocaine-mediated CPP ($p<0.01$, ANOVA), but not in home cage controls or following extinction of CPP (n=6 mice saline, 6 mice cocaine, >100 synapses per group). **(F)** Correlation of percentage of GluA1 localized to synaptic membrane at excitatory synapses with total time spent in conditioned chamber following cocaine-mediated CPP. Data shown as mean ± SEM. **$p< 0.01$, Bonferroni's test *post hoc*.

following CPP, there was a redistribution of GluA1 at excitatory synapses onto DA neurons (**Fig 3.4B**), with a significant increase (95% increase) in the proportion of GluA1 localized to the synaptic membrane (**Fig. 3.4C, 'CPP'**). We also saw that cocaine administration in the absence of contextual conditioning did not cause a similar redistribution of GluA1 to the synaptic membrane (**Fig. 3.4C, 'Home Cage'**), and that extinction of CPP resulted in the reversal of the localization of GluA1 to the synapse observed in the CPP group (**Fig. 3.4C, 'Extinction'**). Together, these data show that drug-mediated CPP causes major changes in the ultrastructural

distribution of GluA1 at synapses, and that the redistribution of GluA1 following induction and extinction of CPP is similar to that observed in cadherin at excitatory synapses in the VTA.

3.4 DISCUSSION

The results from this study demonstrate that cadherins are present in both dopaminergic and non-dopaminergic neurons in the VTA, and are critical for LTP at excitatory synapses onto DA neurons. In addition to this role in activity-dependent plasticity, cocaine-mediated CPP results in a major redistribution of cadherin to the synaptic membrane at these synapses, and this redistribution is reversed by extinction of CPP. Finally, we show that GluA1 AMPA receptor subunits, which have been shown to traffic with cadherins, show similar changes in distribution at synapses as cadherins following acquisition and extinction of CPP.

The disruption of spike-timing dependent LTP by treatment with the HAV peptide indicates that activity-dependent increases in cadherin adhesion are necessary for potentiation of synapses in the VTA, but since the HAV sequence is found in many different classical cadherins the specific cadherin molecules involved remain unclear. Previous studies have found that treatment with the HAV peptide prevents activity-induced increases in synaptic strength (LTP), but does not decrease increases in basal synapse strength at previously potentiated synapses (Tang et al., 1998). This suggests that when cadherins are bound in *trans* the HAV peptide cannot access the homophilic binding site and therefore does not disrupt existing cadherin interactions, but during transient dissociation of cadherins during synaptic plasticity the peptide can then access and block these sites. Consistent with this, treatment with HAV has been found to have acute effects on processes such as the cadherin-mediated targeting neurite outgrowth

(Poskanzer et al., 2003), where high cadherin turnover would allow the HAV peptide to have greater access to homophilic binding region of cadherins.

A defining feature of addiction is the extreme difficulty in altering addiction-related behaviours. Understanding the drug-induced changes in the circuitry of reward and motivation which can cause these behaviours to become so intractable has been a focus of addiction research. Due to the role of cadherins regulating the strength and stability of synapses, the increase in cadherin localization to the synaptic membrane of excitatory synapses onto DA neurons observed following cocaine-mediated CPP has important implications for how these synapses are altered by drugs of abuse. Cadherins recruited to these synapses may act to stabilize synapses potentiated by drug-induced synaptic plasticity, strengthening association between conditioned cues and contexts and increasing DA neuron activity leading to addiction. Additionally, cadherin adhesion has been shown to act as a negative regulator of LTD, which may be of importance to extinction of drug-induced CPP. Indeed, mGluR-LTD has been shown to remove calcium-permeable AMPA receptors from excitatory synapses in the VTA following their insertion after cocaine exposure (Bellone and Lüscher, 2006), and cadherins have been identified as an important component in mGluR-LTD (Zhou et al., 2011). The LTD-like reversal of cadherin localization to the synaptic cleft following extinction observed in the present study also indicates that cadherin must be removed from the synapses as part of the extinction process. Intriguingly, D-serine, an NMDA receptor agonist which has been shown to enhance LTD in hippocampal neurons (Duffy et al., 2008), has also been shown to accelerate the extinction of cocaine-mediated CPP in mice (Thanos et al., 2009).

Analysis of GluA1 at excitatory synapses in the VTA showed the redistribution of GluA1 following induction and extinction of CPP is very similar to the redistribution of cadherin at

these synapses. This is also consistent with evidence that the insertion and removal of AMPA receptors is dependent on cadherin during different forms of activity-dependent synaptic plasticity such as LTP and LTD (Tang et al., 1998, Bozdagi et al., 2000, Tai et al., 2007, Mills et al., 2014), and that the association between cadherin and AMPA receptors provides an important link between physical adhesion and electrochemical strength at the synapse. The increase in GluA1 at the synapse is also consistent with data suggesting that GluA1-containing CP-AMPARs are inserted at the postsynaptic membrane following cocaine exposure, and are responsible for changes in plasticity at these synapses (Wolf and Tseng, 2012). Interestingly, no changes in the total amounts of immunolabelled cadherin or GluA1 were detected in cocaine group mice relative to saline controls in any of the CPP experiments, which suggest that the synaptic alterations associated with CPP are primarily due to redistribution of existing proteins rather than changes in protein expression.

One intriguing finding was the lack of alteration in the localization of cadherin and GluA1 in home cage control mice, which received the same schedule of cocaine administration as the CPP group but were not placed in novel contexts to establish conditioned place preference. This suggests that the changes in cadherin and GluA1 localization see in the CPP group were not solely due to cocaine administration alone, but rather due to an interaction between the novel environments and the conditioned association of those environments with the drug effects. Previous studies have shown that single doses of cocaine are sufficient to cause alterations in the physiology of excitatory synapses onto DA neurons, including an increase in the AMPA/NMDA ratio and increase in CP-AMPARs composed of GluA1 subunits, and (enhanced LTP), and these changes have been shown to persist up to 5-10 days after cocaine administration (Ungless et al., 2001, Saal et al., 2003). However, analysis of excitatory synapses onto DA neurons showed no

changes in distribution of GluA1 in home cage controls, which received three doses of cocaine on the same schedule as the CPP group mice. Methodological differences between these studies and our own, may account for the lack of change of GluA1 in the home cage group. In our experiments, extinction of CPP was affected by repeated exposure to the conditioned environment in the absence of the drug. Thus, the home cage controls experienced extinction-like training events on days they received saline in the same context where they had previously received cocaine. This may have prevented the associative pairing of drug exposure with the environment, and thus mitigated the potentiation of excitatory inputs to DA neurons. Other studies have shown that drug-paired associative learning or self-administration paradigms cause greater changes in AMPA receptor expression and more persistent increases in synaptic strength than 'yoked' controls which are simply administered the same amounts of cocaine on the same schedule. Indeed, changes in AMPA receptor composition in the NAc that occur during withdrawal from cocaine are significantly more robust after cocaine pairings than after noncontingent drug treatment (McCutcheon et al., 2011).

The importance of cadherin to plasticity of these synapses, and the redistribution of cadherins and GluA1 by cocaine-mediated CPP suggests a mechanism of action by which mutations in cadherins could affect the susceptibility of individuals to drug addiction. If DA neuron firing influences compulsive drug taking behaviours, mutations which result in enhanced cadherin adhesion could predispose excitatory inputs to DA neurons to be potentiated following exposure to drugs of abuse, resulting in increased DA neuron activity. Enhanced cadherin-based adhesion could also impair the subsequent depotentiation of these synapses, by inhibiting NMDAR-dependent LTD (Tai et al., 2007, Mills et al., 2014) or

mGluR-LTD (Zhou et al., 2011), resulting in greater perseveration of the drug-induced changes or addictive behaviour. These alterations in cadherin function could be due to mutations within the cadherins themselves, resulting in altered homophilic binding affinity in *cis* or *trans*, or mutations in binding sites of regulatory proteins such as catenins, or in changes in these binding proteins themselves (Zhang et al., 2002, Uhl et al., 2009, Lydall et al., 2011). Together, the data from this study show that the cadherin adhesion complex plays an important role at this critical population of synapses, and further study of cadherin's role in the neural circuitry of reward may shed light onto the mechanisms of addiction.

Chapter 4: General discussion and conclusions

The work presented in this thesis provides many new insights into the role of β-catenin and the cadherin adhesion complex in synaptic plasticity. Our findings show that β-catenin is an important regulator of the stability of cadherins and AMPA receptors at synapses, which is crucial point for long-term depression at synapses and tasks which demand cognitive flexibility. Additionally, we demonstrate that the cadherin adhesion complex is important for synaptic plasticity in the reward learning circuitry of the brain, and that cadherin localization is affected by drug-mediated associative learning, suggesting the cadherin adhesion complex may play an important role in the neurological changes underlying addiction. Together, these studies demonstrate how bidirectional changes in cadherin-based adhesion are important for different forms of learning and memory in the brain.

4.1 THE ROLE OF β-CATENIN IN PLASTICITY AT THE SYNAPSE

Initial studies of the cadherin adhesion complex at adherens junctions identified β-catenin as an essential component for cadherin interaction in *trans* between cells. However, while there is turnover of proteins at adherens junctions in non-neuronal cells, the overall connections between cells remain relatively static over time. In neurons, synaptic contacts are constantly being formed and eliminated in response to experience-driven synaptic plasticity, which necessitates mechanisms for dynamically increasing and decreasing adhesion between neurons when required. In Chapter 2, we examined the effects of stabilization of β-catenin on synapse function and plasticity, and the impacts this had on hippocampal-dependent learning and memory. We found that increases in β-catenin at synapses (in '$\beta\text{-}cat^{\Delta ex3/+}$' mice) impaired long-

term depression (LTD) and activity-dependent endocytosis of cadherin and AMPA receptors at these synapses, which led to deficits in hippocampal-dependent learning and memory on tasks which required behavioural flexibility (**Fig 4.1**). This demonstrates that transient disruption of β-catenin/cadherin interactions is necessary to allow changes at synapses during normal learning and memory, and supports the idea that dynamic regulation of β-catenin's interaction with cadherin is a critical point of control of activity-dependent changes in synapse strength and adhesion.

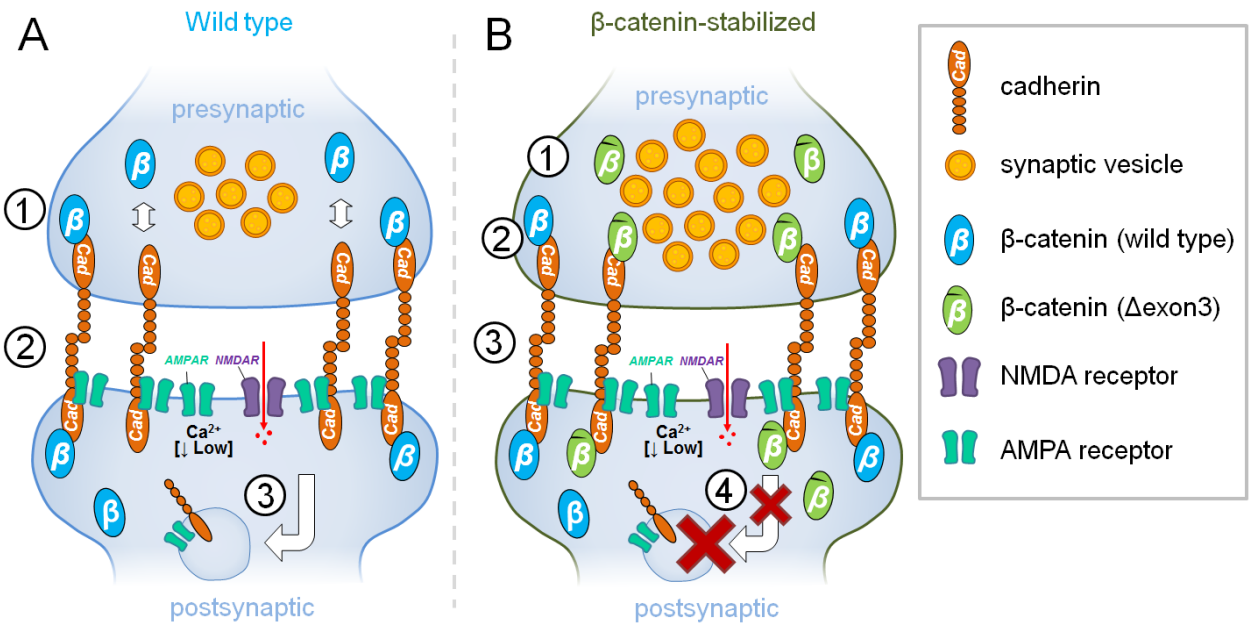

Figure 4.1 Model of the effects of β-catenin stabilization at hippocampal synapses. A) In wildtype mice, there is a dynamic interaction between β-catenin and cadherin (1), allowing cadherin stability at the synaptic membrane to be regulated by activity-driven changes in β-catenin/cadherin interaction (2). Following certain patterns of activity (such as low-frequency stimulation or NMDA treatment), β-catenin binding to cadherin is disrupted, allowing weakening of cadherin interactions in trans and endocytosis of cadherin and associated AMPA receptors (3), leading to a decrease in overall synapse strength. B) Following β-catenin stabilization, there is a significant accumulation of β-catenin at synapses (1) and an increase in β-catenin bound to cadherin at synapses (2). This results in increased stabilization of cadherins at the synapse (3), which prevents the endocytosis of cadherin and AMPA receptors from the synaptic membrane (4). This impaired normal synaptic plasticity and led to deficits in cognitive flexibility (Image made by Fergil Mills).

Other studies have previously shown that disruption of cadherin-based adhesion can impair increases in synapse strength and stability during long-term potentiation (LTP).Deletion of N-cadherin *in vivo* has been shown to cause deficits in LTP and performance on spatial memory tasks (Nikitczuk et al., 2014), indicating that a lack of cadherin adhesion disrupts the encoding of memory. However, the impairments in reversal learning observed in $\beta\text{-}cat^{\Delta ex3/+}$ mice demonstrate that the reverse is also true; *excessive* synaptic adhesion can also be detrimental to the normal synaptic plasticity, and can impact cognitive function. This finding is of broad importance to the study of all adhesion molecules at synapses, as it demonstrates that the consequences of excessive adhesion should be considered when evaluating the normal function of different adhesion proteins. Additionally, mutations in adhesion molecules linked to different neurological disorders should also be considered in terms of their potential to cause aberrant gain of adhesive function.

In $\beta\text{-}cat^{\Delta ex3/+}$ mice, there was no direct manipulation of AMPA receptors, yet the enhanced cadherin stability at synapses due to β-catenin stabilization prevented AMPA receptor endocytosis. Though AMPA receptors at the synapse are a key determinant of synapse strength, this suggests that changes in adhesion may be equally important, or in some cases more important, than AMPA receptors themselves on the normal plasticity of synapses. The fact that enhanced adhesion resulted in impairments in reversal learning and behavioural flexibility also demonstrates that increases in synaptic adhesion are sufficient to cause cognitive impairments, and that control of adhesion at synapses is an important consideration for future studies of the mechanisms of learning and memory.

4.2 THE ROLE OF CADHERIN IN PLASTICITY IN THE VENTRAL TEGMENTAL AREA

In Chapter 3, we demonstrate a role for cadherin in plasticity at synapses in the ventral tegmental area (VTA). Dopaminergic neurons in the VTA are a key part of the reward circuitry of the brain, and are implicated in the formation and expression of addiction-related behaviour. We show that classical cadherins widely expressed in the VTA, and are important for LTP at excitatory synapses onto dopaminergic neurons, as disruption of cadherin adhesion using an HAV peptide blocked the long-lasting increase in strength caused by stimulation in a spike-timing dependent plasticity protocol. Additionally, our analysis of ultrastructural localization of cadherin and GluA1 using immunogold electron microscopy revealed that cocaine-mediated conditioned place preference (CPP) causes a significant redistribution of these proteins to the synaptic membrane (**Fig 4.2**). These changes were also limited to excitatory synapses; no changes in the localization of cadherin or GluA1 were observed at inhibitory synapses onto dopaminergic neurons following CPP. The increase of cadherin at the synaptic membrane indicates enhanced synaptic adhesion and strength at these synapses, which may contribute to the persistent enhancement of synaptic potentiation that underlies addiction.

Two other findings from this study are also of interest for the study of drug-mediated changes in synaptic plasticity which may contribute to addiction. First, home cage controls for cocaine CPP, which received the same schedule of drug administration but were not placed in the novel environments of the CPP apparatus, did not show any increased in synaptic localization of cadherin or GluA1 at synapses in the VTA. This lack of increase in synaptic cadherin in the home cage controls indicates that cadherin localization to the synaptic membrane is not simply a

consequence of the action of cocaine in the VTA, but due to an interaction between associative learning in conditioned place preference, in which a novel environment is associated with the rewarding effects of the drug. Thus, our findings are consistent with the 'cue-sensitization' hypothesis of addiction, which suggests that compulsive drug-seeking and drug-taking behaviours are triggered by exposure to environmental cues that have been associated with the rewarding effects of the drug.

Second, following extinction of CPP we observed that the increase in cadherin and GluA1 at the synaptic membrane was reversed, with the extinction of preference for the drug-paired chamber coinciding with a return of cadherin localization similar to that seen under basal conditions. The removal of cadherin from synaptic membrane during extinction of CPP is particularly interesting in light of our findings from Chapter 2 demonstrating that aberrant stabilization of cadherin at the synaptic membrane led to increased perseveration of spatial memories during the reversal phase of the Morris water maze. There is an interesting parallel between the inability to change or eliminate spatial memories in the water maze due to enhanced cadherin-based adhesion, and the increase in cadherin adhesion observed following cocaine CPP in regions of the brain involved in addiction, a condition which is extremely intractable and resistant to change. Previous electrophysiological studies have shown an increase in the relative proportion of calcium-permeable GluA1 homomeric AMPA receptors (CP-AMPARs) at these synapses following cocaine treatment, and that a form of LTD is required to remove these AMPA receptors from synapses. The insertion and removal of cadherin from the synaptic membrane with GluA1 suggests that cadherins traffic together with these CP-AMPARs and may have a similar function as in plasticity at hippocampal synapses. We demonstrate that cadherin is essential for potentiation at these synapses, which is supported by its trafficking to the

membrane, but its reversal during extinction suggests that, similar to hippocampal LTD, removal of cadherin is required for the removal of calcium-permeable homomers and reversing cocaine-mediated changes in synaptic plasticity.

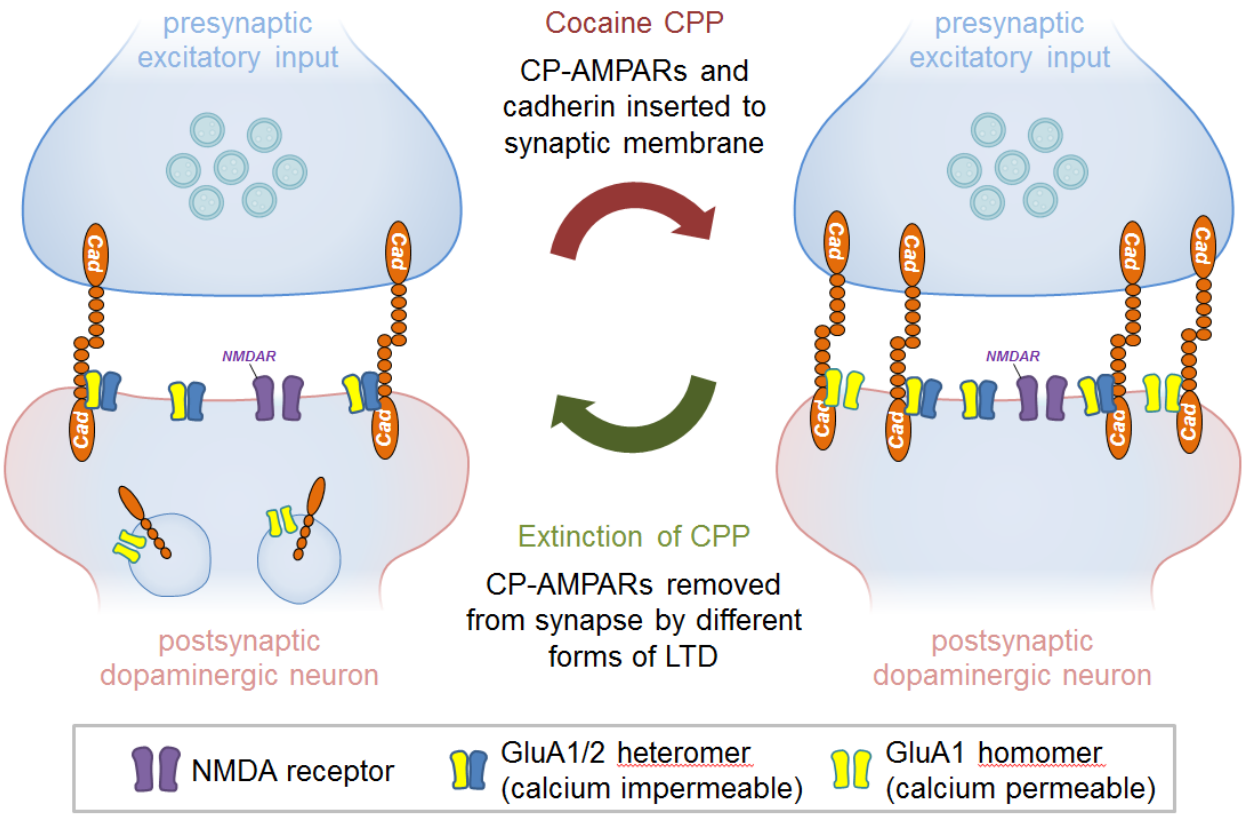

Figure 4.2 Model of changes in cadherin and AMPA receptor localization following acquisition and extinction of cocaine-mediated CPP.
Following cocaine-mediated conditioned place preference, there is a significant increase in cadherin and GluA1 localization to the synaptic membrane at excitatory synapses onto dopaminergic neurons in the VTA. The potentiation of these synapses is due to the effects of cocaine on the postsynaptic dopaminergic neuron, combined with the enhanced activity of specific excitatory inputs which represent associative connections with the novel environment. We hypothesize that the increase in GluA1 is due to trafficking of calcium-permeable GluA1 homomers to the synaptic membrane, which has been reported in other studies. Extinction of CPP is mediated by the endocytosis and removal of cadherin and these associated AMPA receptors from the synaptic membrane (Image made by Fergil Mills).

A key common feature of the studies in Chapter 2 and 3 is the concept of ‘excessive’ adhesion at synapses -- how *increases* in synaptic adhesion can affect synaptic plasticity, and how this can negatively impact normal learning and memory. Both studies also examine the importance of removal of cadherin from the synapse as an important part of certain forms of plasticity. In the hippocampus, endocytosis of cadherin was shown to be essential for LTD at hippocampal synapses, and aberrant increases in synaptic adhesion. In the VTA, cocaine mediated CPP caused an increase in cadherin at the synaptic membrane of excitatory synapses onto dopaminergic neurons, suggesting an increase of adhesion at these synapses which was then reversed during extinction of CPP.

Overall, these findings join a small number of new studies examining the role of the cadherins in complex learning and memory. Initial studies of cadherin established the basic properties of this class of molecules and its role in strong, calcium-dependent adhesion between cells, and the binding partners required for this adhesion, which include the different catenin proteins. Then, cadherin adhesion was demonstrated to be important for LTP, and mediating increases in the size and stability of synapses caused by synaptic activity. Now, we are beginning to understand the role of cadherin in more complex forms of learning, and different forms of plasticity besides LTP, including LTD and drug-mediated associative learning.

4.3 β-CATENIN AND WNT SIGNALING IN THE BRAIN

Though Wnt signaling was not the primary focus of this thesis, the findings presented in Chapter 2 provide several useful insights into the relationship between β-catenin levels and Wnt signaling in adult neurons in the brain. We found that an increase in β-catenin in adult,

differentiated excitatory neurons in the hippocampus did not result in any changes in Wnt signaling detectable by Western blot analysis, RTPCR or immunohistochemistry. We also saw no changes in the overall morphology of the hippocampus where β-catenin was stabilized, though increases in β-catenin levels in many other tissues have been shown to cause increases in Wnt signaling leading to widespread tumor and polyp formation. These differences in the effects of increased levels of β-catenin indicate that there are important differences in the canonical Wnt/β-catenin signaling pathway in neurons. Many of the assumptions about Wnt signaling which were developed in studies of other cell types are a useful starting point for understanding this system, but do not provide a complete picture of how this pathway functions in neurons.

First, there are several reasons why neurons in the adult brain would have finer control over activation of Wnt target genes, and be less responsive to changes in overall β-catenin levels. Wnt signaling is extremely important during development for both the morphogenesis of individual neurons and the overall organization of the brain including patterning of cell layers and differentiation of specific neuronal cell types. However, after this organization is established, it could be detrimental to brain function to reactivate the expression of downstream Wnt targets required for development in response to experience-driven synaptic activity which accompanies normal learning and memory formation. Additionally, *in vitro* studies have shown that dramatic changes in β-catenin localization are a normal part of responses to different forms of synaptic activity; depolarization of neurons causes a major redistribution of β-catenin into dendritic spines (Murase et al., 2002), while treatment with BDNF or NMDA has the opposite effect, and promotes the dispersal of β-catenin into the dendritic shaft. Since fluctuations in the amount of cytoplasmic β-catenin is a very common consequence of normal neuronal activity, it is therefore

much less surprising that increases in β-catenin levels in adult, differentiated neurons in transgenic mice (as in Chapter 2) did not have a dramatic impact on Wnt target expression.

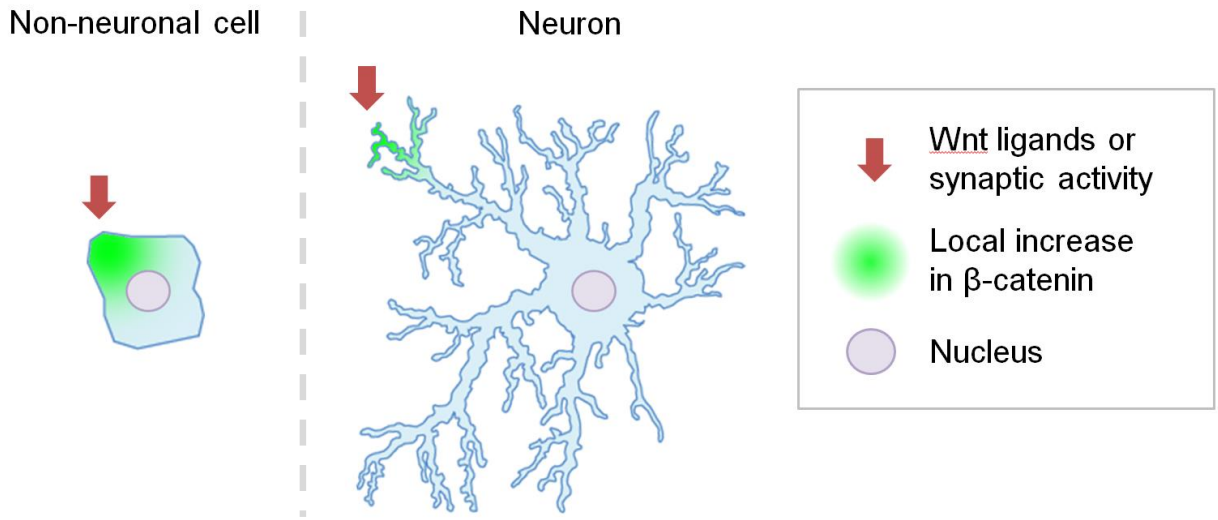

Figure 4.3 Comparison of the neurons and non-neuronal cells with respect to changes in β-catenin levels and Wnt signaling.
Non-neuronal cells have a relatively simple structure and unified cytoplasmic environment, and as a consequence an increase in cytoplasmic β-catenin caused by either Wnt ligands or disruption of β-catenin/cadherin interactions would immediately increase β-catenin levels near the nucleus of the cell, which would facilitate translocation of β-catenin into the nucleus and activation of Wnt target genes. In neurons, distal increases in β-catenin levels along axons or dendritic branches would likely only cause a local increase in β-catenin, which would readily become either degraded by proteolytic machinery or would associate with binding partners such as cadherin at other sites along the neuronal process. These local increases in β-catenin levels would consequently have little or no effect on cytoplasmic β-catenin near the nucleus, and would be less likely to result in downstream Wnt signaling. Neurons also have additional mechanisms controlling nuclear import and export of β-catenin, and may have alternate degradation pathways to control levels of cytoplasmic β-catenin (Image made by Fergil Mills).

There are several mechanisms which may contribute to mitigate the effects of changes in β-catenin levels on Wnt signaling. First, the physical structure of neurons may play a role; neurons have an extremely complex structure, and appear designed to compartmentalize the effects of individual inputs before integrating them into the overall output of the neuron. Indeed, the morphology of dendritic spines seems optimized to create a biochemically isolated

compartment at sites of neuronal input, so that local activity has input-specific effects on synaptic plasticity. Local increases in cytoplasmic β-catenin would therefore have less effect on β-catenin levels in the cell body or near the nucleus (**Fig. 4.3**).

There also appears to be more sophisticated control of trafficking of β-catenin in neurons, including its nuclear import and export. In neurons, the transport protein LAPSER-1 has been shown to bind to β-catenin and co-migrate to the nucleus together to induce the expression of known β-catenin target genes (Schmeisser et al., 2009). The requirement for additional co-factors for nuclear import may help distinguish genuine 'signal' from Wnt ligands from normal fluctuations in cytoplasmic β-catenin levels due to neuronal activity.

A related point to the lack of change in Wnt signaling observed following in β-catenin-stabilized mice ($\beta\text{-}cat^{\Delta ex3/+}$ mice) is the relatively modest increase in overall β-catenin levels observed in the hippocampus of these animals. In $\beta\text{-}cat^{\Delta ex3/+}$ mice, the Cre/loxP system was used to cause a heterozygous deletion of exon 3 of the β-catenin gene, which contains the phosphorylation sites (Ser-33/Ser-37/Thr-41) which target β-catenin for degradation via the ubiquitin/proteasome degradation pathway. This resulted in a 64% increase in total β-catenin levels in the hippocampus, a smaller overall increase than seen in non-neuronal cells following expression of Cre recombinase in other using this exact same transgenic mouse line (Li et al., 2009). A possible explanation for the difference in these effects can be drawn from a study which found that β-catenin is a target of the protease 'calpain' in neurons (Abe and Takeichi, 2007). This study found that calpain can be activated in response to NMDA receptor activity at neurons and cleave β-catenin, creating an N-terminal truncated version of β-catenin which was then translocated to the nucleus and activates TCF/LEF-dependent gene expression. The existence of this calpain-mediated signaling pathway implies that a form of β-catenin which

lacks the degradation sites in exon 3 is produced endogenously by neurons. Consequently, alternate 'non-canonical' pathways must exist in neurons to allow the degradation of these N-terminal-truncated β-catenin fragments. It is therefore possible that in *β-cat*$^{\Delta ex3/+}$ mice the mutant Δexon3 form of β-catenin, though more stable than wildtype β-catenin, could still be degraded by this alternate pathway, which is consistent with the amount of total β-catenin observed in hippocampal lysate from *β-cat*$^{\Delta ex3/+}$ mice. This hypothetical alternate pathway may have helped limit the effects of β-catenin stabilization on Wnt signaling, and the identities of proteins and kinases involved will need to be identified in future studies. This could have important implications for oncological studies as well; increases in β-catenin signaling have been shown to cause different forms of cancer, and different degradation pathways might have important effects on the development or prevention of cancer by cells in the body. Overall, the findings presented in Chapter 2 help to integrate findings from many different studies and provide supporting evidence for increased control over Wnt signaling in adult neurons in the brain.

4.4 METHODOLOGICAL STRENGTHS AND LIMITATIONS OF THE THESIS RESEARCH

In both Chapters 2 and 3, quantitative analysis of immuogold electron microscopy (EM) was used to analyze the localization of GluA1 and cadherin at synapses. Immunogold EM had several important strengths for our research: it allowed for the nanometer-scale resolution of the ultrastructural localization of these proteins at synapses, and allowed us to distinguish changes in the proportion of GluA1 and cadherin localized directly to the synaptic membranes on either side of the synaptic cleft. This fine level of detail was crucial, because the presence of these proteins at the synaptic membrane indicated that they were situated to actively participate in either

postsynaptic responses to neurotransmitter release (as in the case of GluA1, a component of AMPA receptors), or transsynaptic adhesion between pre- and post-synaptic compartments (as in the case of cadherin). Conventional approaches such as confocal microscopy would not have been able to distinguish between proteins directly at the synaptic membrane versus proteins localized close to the synapse but not situated to make a functional contribution to the synapse strength or adhesion (i.e. proteins in recycling endosomes within the pre- and post-synaptic compartments). The work in this thesis demonstrates that quantitative analysis of immunogold EM can provide useful insights into changes in protein localization during different forms of synaptic plasticity, and will be a fruitful approach for future studies of cadherins and other proteins at the synapse.

However, one potential limitation of this technique is that we cannot ascertain the interactions or binding partners of individual proteins detected. For instance, though cadherin localized to the synaptic membrane is very likely to be participating in synaptic adhesion, we cannot be certain which molecules are actively interacting in *trans* with cadherins from the opposing synaptic membrane. By comparing the relative proportion of cadherin at the synaptic membrane between mice which had undergone cocaine-mediated CPP and saline controls, we can infer that a significant increase in cadherin-based adhesion is likely, but it is conceivable that the adhesive properties of individual cadherins was affected by other variables such as post-translational modification of cadherins or association of different binding partners which regulate cadherin adhesion. Thus, though we can draw upon the results of other studies to infer that physical adhesion at these synapses is increased, the actual amount of adhesion at synapses is increased is not directly testable by the immunogold EM approach.

A similar problem applies to interpretation of the re-distribution of GluA1 to the synaptic

membrane observed at synapses in the VTA following cocaine-mediated CPP. Following fixation of tissue and immunogold EM analysis, we can determine the spatial distribution of GluA1 with precision, but have no information with respect to which other proteins may be associated with the GluA1 monomers, particularly the identities of the other glutamate receptor subunits with which these GluA monomers are associated to form functional AMPA receptors. As outlined in the introduction (see section 1.3.1) the composition of AMPA receptors has major impact on their function. GluA2 subunits have a motif which excludes Ca2+ from the channel pore, making AMPA receptors that contain this subunit impermeable to calcium, while GluA1 subunits lack this motif and AMPA receptors composed entirely of GluA1 are calcium-permeable.

Determining the distribution of GluA1 homomers is therefore of importance to understanding changes in synaptic plasticity caused by drug-induced associative learning. However, while anti-GluA1 antibodies used in immunogold labelling are more likely to detect GluA1 homomers (since all of their subunits will have GluA1 epitopes), we cannot definitively determine whether the GluA1 subunits detected by immunogold EM are associated with either GluA2 or GluA1 subunits in AMPA receptors. Thus, we cannot distinguish between GluA1-containing homomers and heteromers by this approach, and cannot detect differences in the trafficking of these AMPA receptor subtypes, which is potentially of importance at these synapses. However, our findings that GluA1 is localized to synapse following cocaine-mediated CPP are consistent with electrophysiological analysis showing an increase in physiologically-defined calcium-permeable AMPA receptors at synapses (Bellone and Lüscher, 2006), and it is therefore likely that much of the GluA1 localized to the synapses observed in our studies represents GluA1 homomers at the synapse. The data from our experiments demonstrate that

immunogold EM can effectively complement electrophysiological experiments, and the two techniques can be applied in combination to provide a more complete understanding of structural and functional changes at synapses.

4.5 APPLICATIONS AND FUTURE DIRECTIONS

The work in the present thesis helps illuminate the role of the cadherin adhesion complex in different forms of synaptic plasticity, and suggests several new directions for future research into neurodegenerative disorders and addiction-related plasticity and behaviour.

The finding that β-catenin stabilization can impair synaptic plasticity and behavior is potentially of importance to the study of Huntington's disease (HD), where over 4-fold increases in total β-catenin have been reported in the brain (Godin et al., 2010), significantly more than the 64% increase in hippocampal β-catenin which we found was sufficient to cause synaptic and cognitive impairments. Intriguingly, cognitive deficits including increased perseveration have been reported in human patients with HD (Lione et al., 1999). Genetic knockdown of β-catenin was shown to have a neuroprotective effect on poly-glutamine huntingtin toxicity in mouse and fly models of HD, but the effects of β-catenin on synapse function have not been investigated. Targeted disruption of β-catenin/cadherin interactions might therefore alleviate some of the cognitive impairments caused by this disorder, and should be investigated in animal models of HD.

Our findings may also be of importance to pharmaceutical research into the function and design of mood stabilizers and anti-psychotic drugs. Lithium, a widely used anti-psychotic, is known to inhibit GSK3β, a kinase which phosphorylates β-catenin and targets it for degradation

(Gould et al., 2004). Though enhanced Wnt signaling is believed to mediate the therapeutic effects of lithium, the findings from this thesis suggest that the possible contribution of increased cadherin-based adhesion should be investigated. The effects of lithium treatment on β-catenin/cadherin interaction, dendritic spine stability and synaptic plasticity could be examined in wildtype mice or mouse models of schizophrenia, either *in vivo* or in acute slices brain slices. While our data showed that excessive β-catenin was detrimental to cognitive function in mice, if a deficit in synaptic adhesion and stability was a contributing factor in schizophrenia then increased cadherin stability could help restore normal synapse function and connectivity. This could be a potentially valuable avenue of research, as lithium has many negative side-effects and off-target effects, and better understanding of the downstream targets of this drug could help tailor more specific and effective medications in the future.

The findings presented in Chapter 3 also have several important implications for the neurological mechanisms underlying drug addiction. Our immunogold EM experiments demonstrate that cocaine-mediated CPP causes a redistribution of cadherin and GluA1 to the synaptic membrane at excitatory synapses onto DA neurons in the VTA. An important future direction for this work will be analyzing changes in cadherin localization in response to non-drug natural rewards such as sweetened or high-calorie foods. If the redistribution of cadherins is also present following CPP using such food rewards, it would indicate that increased cadherin adhesion is a part of normal reward learning. However, if we did not observe similar increases in cadherin at the synaptic membrane in response to non-drug rewards, it would provide strong evidence that this change in cadherin distribution is a unique consequence of drug-mediated synaptic plasticity and may be highly significant to the formation and expression of addiction-related behaviours. Additionally, if increased cadherin adhesion helps mediate the plastic

changes caused by drug-mediated conditioning, then targeted disruption of cadherin interactions in this region could therefore represent a therapeutic approach to disrupt the association between environmental cues and drug-seeking or drug-taking behaviours.

Based on the findings of our study, SNPs and other mutations linked with drug abuse should be considered in light of the effects they may have on synaptic adhesion. Specifically, mutations at sites that act regulators of adhesion function could have an important effect on synaptic plasticity. Examples of these sites on would include phosphorylation sites that target adhesion molecules for degradation, phosphorylation sites which affect their binding to other proteins, or binding sites for other proteins at the synapse. The need to evaluate the potential of mutations to cause enhanced synaptic adhesion applies not only to association with substance abuse, but also in genome-wide screens for mutations associated with other neurodegenerative disorders and schizophrenia.

Finally, the work presented in this thesis underscores the importance of studying the cadherin adhesion complex in different brain regions and functions. A majority of the studies investigating the function of cadherin have been limited to *in vitro* studies of primary hippocampal cultures, or within the hippocampus itself. The relatively well-understood connectivity and function of the hippocampus is certainly an asset for initial studies of adhesion molecules, but cadherins may have important and unexpected roles in other brain regions as well. The large diversity of cadherin family proteins suggests functional specializations for each individual cadherin that remain poorly understood. These may include specialized function and responses to different forms of activity, assembly of different components at synapses due to interactions with different binding partners, or ‘tuning’ of physical properties of the synapse such as width of the synaptic cleft, which facilitate activity at different synapse types. Differential

expression of cadherins and protocadherins also appears to be involved in establishing patterning of cell layers in different regions of the brain (Frank et al., 2005, Hertel et al., 2008, Krishna et al., 2009). Overall, different cadherins may make unique contributions to both the development and function of synapses, and understanding the role of each type of cadherin will likely lead to new insights into the mechanisms of synaptic plasticity and the etiology of different neurological disorders.

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