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Kimberly A. Maguschak Date

The Role of β-Catenin in Long-Term Memory Formation

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Doctor of Philosophy

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The Role of β-Catenin in Long-Term Memory Formation

By

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B.S., The University of Scranton, 2001

Advisor: Kerry J. Ressler, M.D., Ph.D.

An abstract of

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Graduate Division of Biological and Biomedical Science

Neuroscience

2010

Abstract

The Role of β-Catenin in Long-Term Memory Formation

By Kimberly A. Maguschak

The ability of connections between neurons to change as a function of learning is commonly referred to as synaptic plasticity. Such changes in synaptic morphology and organization are thought to underpin long term memory formation. Among the most studied structural changes has been the elaboration of new synaptic architecture following a learning event. This process has been postulated by many investigators to be a physiologically-relevant means of synaptic potentiation. The processes governing dendritic morphogenesis are many and varied, but much work has focused on the role of β-catenin in allowing the remodeling of synapses in an activity-dependent way. β-Catenin is expressed broadly in the adult mammalian brain and plays a role in both cadherin-mediated cell-cell adhesion and Wnt signaling. Although β-catenin has been implicated in neuronal synapse regulation and plasticity, all of this work has been done *in vitro*, and not in intact animals. Here we have examined the role of β-catenin in the adult mouse brain and its role in amygdala- and hippocampal-dependent learning and memory. We found that β-catenin is highly expressed in these brain regions and is dynamically regulated at the post-translational level with fear learning. Such alterations correlated with a change in the association of β-catenin with cadherin. Genetically, the role of β-catenin was confirmed with site-specific deletions of *loxP*-flanked *Ctnnb1* (encoding β-catenin) in the amygdala or dorsal hippocampus. In both cases, the manipulation affected the consolidation, but not acquisition, of memory. Notably, *Ctnnb1* deletion did not affect a number of other behaviors, including locomotor or anxiety-related behavior. Furthermore, we found that Wnt signaling may play a role in mediating β-catenin dependent memory formation. Interfering with Wnt signaling prevented consolidation and altered the transient destabilization and re-stabilization of β-catenin-cadherin interactions during memory formation. Therefore, our findings suggest a general role for β-catenin in the synaptic remodeling and stabilization underlying long-term memory in adults.

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Acknowledgments

I would like to thank my advisor, Kerry Ressler, for being a wonderful mentor and friend. I cannot thank him enough for his patience and support throughout my graduate studies. He is one of the most intelligent people I have ever met, and has played a significant role in shaping my career as a scientist. I am honored to have been given the opportunity to pursue my research interests in his laboratory.

I would like to thank my committee members, Randy Hall, Allan Levey, David Sweatt, and Larry Young for their support and guidance over these past few years. They are all highly respected scientists in their fields and I feel very privileged to have had each one of them on my committee.

I would also like to thank my collaborators, Tig Rainnie, Chris Muly, and Marcelia Maddox. Tig has been a great colleague and friend throughout my studies. I have been consistently impressed by his depth of knowledge and am very appreciative of all the help and support he has given me along the way. Chris Muly has opened up his laboratory to me and provided me with the rare opportunity to learn electron microscopy. I am incredibly grateful for all the help I have received from both him, and his technician, Marcelia Maddox.

I would also like to acknowledge my dear friends and colleagues in the Ressler laboratory, past and present, especially Jasmeer Chhatwal, Dennis Choi, Scott Heldt, Karyn Myers, and Lisa Stanek. They have all offered constant support and encouragement throughout my studies.

Last, but not least, I would like to thank all my friends and family for their unconditional love and support.

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Chapter 1

General Introduction

INTRODUCTION

The search to understand the mechanisms underlying learning and memory in the brain has been a topic of interest for more than a century. Dating back to the nineteenth century, Cajal proposed that learning requires the formation of new neuronal connections[1]. Later, based on these and other early speculations, Konorski and Hebb independently suggested that alterations in synaptic strength, as well as formation of new synapses, are responsible for information storage[2, 3]. The ability of connections between neurons to change as a function of learning is commonly referred to as synaptic plasticity, and is accepted today as a mechanism underlying memory formation. Such alterations in synaptic connections can be produced through artificial processes, such as long-term potentiation (LTP), or more natural processes, such as behavioral learning in animals.

Molecular mechanisms of synaptic plasticity

Synaptic plasticity in mature neurons is often initiated by neural activity and an influx of calcium. Calcium influx can lead to alterations in synapse structure and function through a process involving post-translational modification, protein synthesis and gene transcription. Following presynaptic activity and postsynaptic depolarization, calcium enters the postsynaptic neuron through N-methyl-D-aspartic acid (NMDA)-type glutamate receptors (NMDARs) and voltage-gated calcium channels (VGCCs)[4-6]. Elevated intracellular calcium levels then activate additional signaling pathways, including calmodulin (CaM)-dependent protein kinases (CaMKs)[7, 8]. Activation of these protein kinases, subsequently leads to the phosphorylation of AMPA-type glutamate

receptors (AMPARs), along with the activation of protein synthesis, and initiation of new gene transcription. These cellular processes, which contribute to synaptic plasticity, begin within a few minutes following neural activity, but can persist for several hours.

Morphological changes associated with synaptic plasticity

Rapid structural changes, involving the rearrangement of the cytoskeleton at the synapse, can also occur with synaptic plasticity. Most excitatory synapses in the brain terminate on dendritic spines[9]. Dendritic spines are tiny protrusions on the shaft of dendrites, that represent sites where new contacts between cells can be created, and existing contacts strengthened. The cytoskeleton of dendritic spines contains high concentrations of filamentous actin (F-actin)[10]. The dynamic nature of the actin permits the spine to change shape within seconds to minutes, thereby contributing to synaptic plasticity. The resulting change in spine shape can last for hours or even days, and may influence synaptic transmission.

In addition to actin, spines also contain a multi-protein complex, the postsynaptic density (PSD), which includes receptors, channels, cell adhesion proteins, and other signaling molecules[11]. All of these components play a role in mediating spine changes. Such changes may be characterized by an increase in the number of spines, or in the overall shape of the spine[12]. Changes in spine number and form have been observed following many different experimental and behavioral conditions. For example, induction of LTP in hippocampal slice cultures has been shown to produce new spine formation, increase the size of the spine head, and shorten the length of the spine neck[13-15].

Similarly, changes in spine morphology have been shown *in vivo* with trace eyeblink conditioning[16] and fear conditioning[17].

Cell adhesion molecules and synaptic plasticity

Along with structural changes in dendritic spines, synaptic plasticity also results in the formation of new synaptic contacts. During this process, the adhesion between the pre- and postsynaptic neurons is altered. The strengthening and weakening of these contacts can be modulated by cell adhesion molecules. These molecules are bound to the membrane and contain an extracellular domain that engages in either homophilic or heterophilic interactions with similar cell adhesion molecules, or other proteins in the extracellular matrix, respectively. They also contain an intracellular domain that interacts with the cytoskeleton and triggers signaling pathways that can regulate spine and synapse formation[18].

One class of cell adhesion molecules that has been well studied over the years are cadherins. Cadherins are homophilic, calcium-dependent cell adhesion molecules that have been shown to play a role in synapse assembly, synaptic plasticity, and memory formation[19, 20]. They contain an extracellular domain which provides a link between opposing cells, promoting structural stability, and an intracellular domain which provides a link to the actin cytoskeleton, promoting spine dynamics. Considerable attention has been given to the latter. The intracellular domain of cadherins binds to β-catenin which then links cadherins to the actin cytoskeleton via α-catenins (Figure 1.1). This cadherin-catenin complex is localized in synaptic junctions, and alterations in this complex are

thought to influence not only synapse development, but also synaptic connectivity and activity[21]. Although α-catenin links to the actin cytoskeleton, the role of β-catenin in this cadherin-catenin complex is more pronounced and has been shown to be a prerequisite for adhesion.

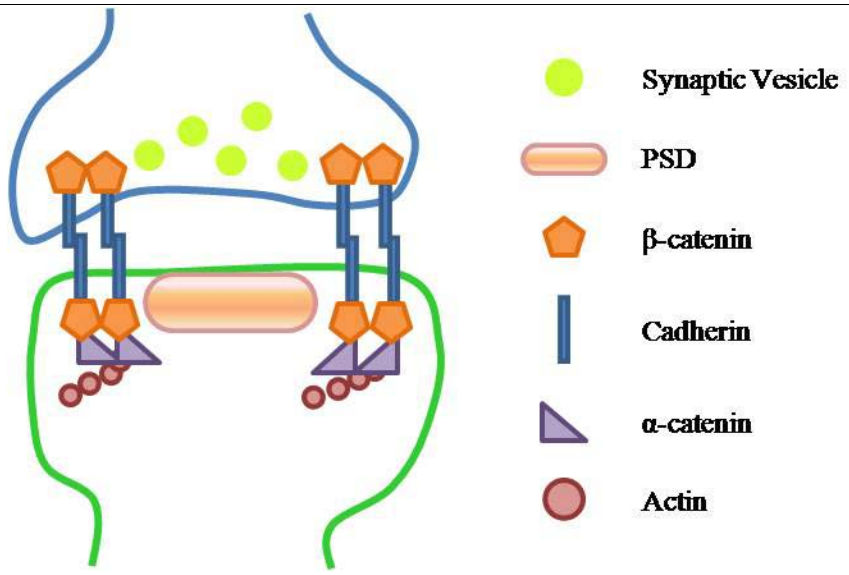

Figure 1.1 - β-catenin at the synapse

The intracellular domain of cadherin binds to β-catenin which then links cadherin to the actin cytoskeleton via α-catenin.

β-CATENIN STRUCTURE AND FUNCTION

Structure of β-catenin

β-Catenin belongs to the armadillo family of proteins, and is composed of three domains: an N-terminal domain, a central domain, and a C-terminal domain[22]. The central domain is the core region and contains 12 copies of a 42 amino acid sequence

motif known as an armadillo repeat. Originally identified in *Drosophila*, this armadillo repeat domain is specialized for protein-protein binding, and forms a superhelix of helices that features a long, positively charged groove[23]. β-catenin's binding partners, including cadherins, adenomatosis polyposis coli (APC) , and T-cell factor (TCF), are negatively charged, and are proposed to interact with this groove.

Functional roles of β-catenin

As mentioned earlier, β-catenin provides an essential, structural component of the cadherin/catenin adhesion complex. It is necessary to prevent the rapid degradation of the cadherin cytoplasmic domain[24] and to recruit α-catenin to sites of cell-cell contact[25, 26]. Without the interaction between β-catenin and cadherin, cell-cell adhesion would be compromised.

In addition to β-catenin's role in cell adhesion, it also is a central player in the Wnt/β-catenin signal transduction pathway. Wnts are highly conserved secreted glycoproteins that regulate cell-cell communication, and are involved in a diverse array of cellular processes including development. When Wnt proteins bind to Frizzled (Fz) and low-density lipoprotein-related protein (LRP) receptors, disheveled (Dsh) is recruited to the membrane. Activation of Dsh by Fz inhibits glycogen synthase kinase-3β (GSK-3β), a kinase that phosphorylates β-catenin and marks it for degradation by the proteasome pathway. Through the inhibition of GSK-3β, β-catenin is stabilized, enters the nucleus, and then forms a complex with the TCF/lymphocyte enhancer factor (LEF) family of transcription factors to regulate the expression of Wnt target genes[27, 28]. This

process is important for cell proliferation, survival, migration, and differentiation, and as suggested by recent research, modulation of synaptic plasticity[29]. Despite a wealth of developmental and *in vitro* data, prior to our work there was no evidence of a role for β-catenin in learning and memory processes in animals. Figure 1.2 illustrates these different functional roles of β-catenin.

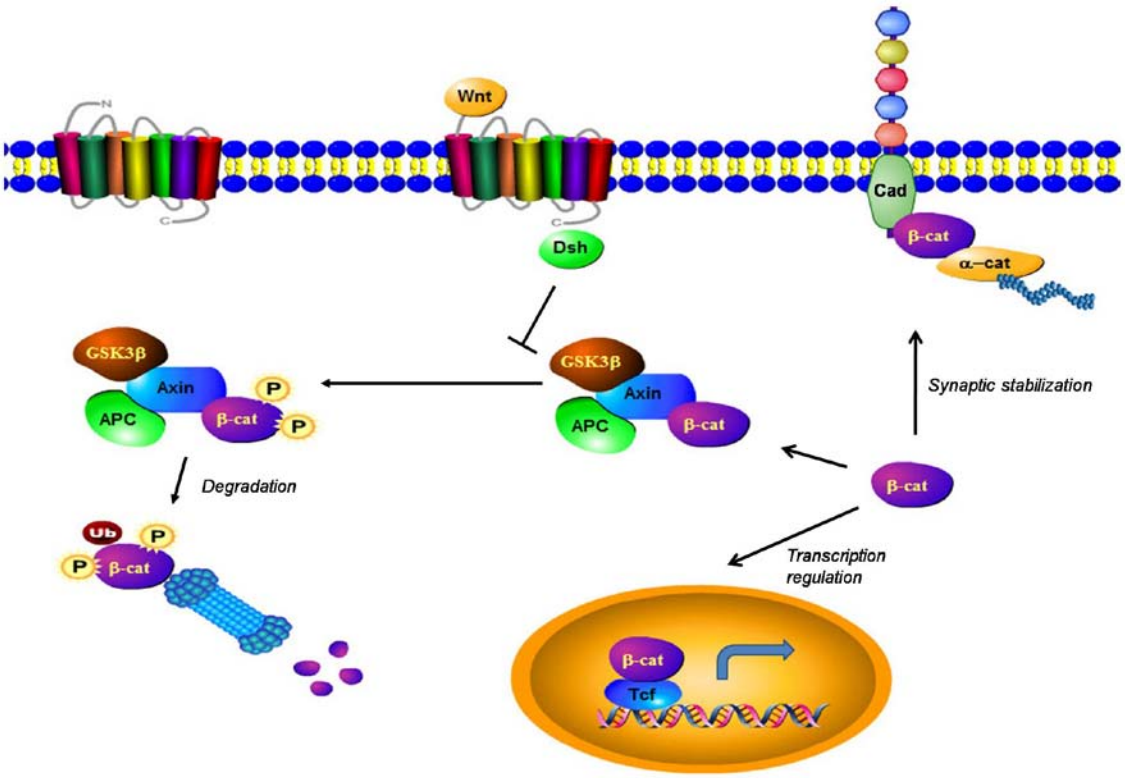

Figure 1.2 – β-Catenin in Wnt signaling and the cadherin complex

In the resting state, β-catenin is phosphorylated by GSK3β and rapidly degraded by the proteasome pathway. Upon activation of Wnt signaling, β-catenin is stabilized through the inhibition of GSK3β and translocates to the nucleus to regulate the expression of Wnt target genes. β-Catenin also associates with the cytoplasmic domain of cadherin and directly links to the actin cytoskeleton through α-catenin.

There has been much discussion as to whether there is crosstalk between cadherin-mediated cell adhesion and canonical Wnt signaling through β-catenin. Experiments involving genetic modification or overexpression in embryos have suggested that the same pool of β-catenin is involved in both cell adhesion at the plasma membrane and signal transduction in the nucleus. However, others have shown that there are different pools of β-catenin: 1) a monomeric, intramolecularly folded-back form that is generated by Wnt signaling and binds only to TCF transcriptional complexes, and 2) a separate pool that exists as a heterodimer with α-catenin, and preferentially binds to cadherins[30]. One explanation for this potential selectivity may be different requirements for cadherin and TCF to bind β-catenin. X-ray crystallography studies have shown that cadherins require all 12 armadillo repeats of β-catenin, while TCF only requires the central eight armadillo repeats[31]. Since Wnt signaling generates a folded-back form of β-catenin, it is possible that some of the repeats may no longer be exposed, thus blocking the binding site for cadherin. Although many of the details of these differential interactions and pathways remain unknown, it is clear that β-catenin plays a critical function as a 'hub' of neuronal plasticity, mediating intracellular signaling that results both in structural changes underlying synaptic strength and in regulating expression of activity-related genes.

Posttranslational modifications regulate β-catenin function

In addition to structural changes in β-catenin, posttranslational modifications have also been reported to affect the interaction between β-catenin and its binding partners. For

example, phosphorylation of β-catenin at tyrosine 654 (located on the 12th armadillo repeat) by src and EGFR, decreases its affinity for cadherin binding, and reduces its adhesive functions[32-34]. Cadherin/β-catenin binding may also be influenced by kinases involved in the signaling form of β-catenin, including casein kinase 1 (CK1), GSK3β, and CK2.

Aside from being bound to cadherin at the membrane, β-catenin can also be found in the cytosol. This less stable pool of β-catenin is continuously phosphorylated by a dual kinase mechanism. First, CK1α phosphorylates β-catenin at serine 45. This event primes β-catenin for further phosphorylation by GSK3β at residues serine 33, serine 37, and threonine 41[35, 36]. Upon phosphorylation at these sites, β-catenin is ubiquitinated and rapidly degraded by the 26S proteasome. As mentioned earlier, Wnt signaling can inhibit this phosphorylation-dependent degradation by inhibiting GSK3β activity. Consequently, there is an increase in the unphosphorylated form of β-catenin, which has been shown to accumulate more readily in the nucleus, and is associated with increased transcriptional activity. [37]

Once β-catenin enters the nucleus, it binds to TCF, and recruits complexes that promote transcriptional activation. These complexes can be recruited by both the N- and C-termini of β-catenin. Phosphorylation of β-catenin by the Met receptor at N-terminal residue tyrosine 142 (Y142), promotes the association between BCL9-2 and β-catenin. BCL9-2 has been shown to increase nuclear location of β-catenin, and as a consequence, increase signaling[38]. Since BCL9-2 cannot co-localize with the stable pool of β-catenin bound to cadherin at the plasma membrane, BCL9-2 may play a role in determining whether β-catenin molecules favor the cell adhesion or Wnt signaling pathways.

Phosphorylation of β-catenin at the C-terminal can also affect transcriptional activation. It has been shown that AKT phosphorylates β-catenin at residue serine 552, resulting in its dissociation from cell-cell contacts and an accumulation of β-catenin in both the cytosol and nucleus[39]. Similarly, PKA-mediated phosphorylation of β-catenin at residue serine 675 has been shown to enhance the transcriptional activity of β-catenin[40].

The aforementioned studies suggest that posttranslational modifications can impact the interaction of β-catenin with both cadherin and TCF, or other transcriptional co-activators. In turn, alterations in the affinity of β-catenin for these binding partners may influence β-catenin's ability to take part in cell adhesion, Wnt signaling, or both. Nevertheless, both processes require β-catenin, and have been shown to modulate synaptic plasticity, thus suggesting that β-catenin may be a candidate molecule to study learning and memory.

Role of β-catenin in presynaptic structure and function

β-Catenin is expressed in the developing and adult CNS. It can be found in both pre- and postsynaptic cells and appears to be present before a synapse becomes functional. Due to the localization of this protein prior to synapse formation, β-catenin stands out as a protein that may play a role in synapse assembly. Over the past several years, there has been much evidence suggesting that β-catenin may be acting both pre- and postsynaptically to regulate synapse formation and function (see Table 1 for an overview).

Table 1.1 Effects of loss of function or overexpression of β-catenin on presynaptic and postsynaptic function

Manipulation	Effect	Reference
Presynaptic Role		
LOF	Increase in diffusion of vesicles along synapse	[47]
LOF	Decrease in number of synaptic vesicles per synapse	[47]
LOF	Impairment in response to prolonged repetitive stimulation	[47]
LOF	No change in PSD95	[47]
Postsynaptic Role		
LOF	Increase in thin, elongated spines	[51]
LOF	Decrease in dendritic arborization	[53, 137]
LOF	Decrease in mEPSC amplitude	[51]
O/E	Increase in dendritic arborization	[37, 52, 53]
O/E	Decrease in mEPSC amplitude	[52]
O/E	Decrease in AMPAR density	[52]

LOF: Loss of function, O/E: Overexpression

The presynaptic axon contains an active zone, where synaptic vesicles dock and fuse to the plasma membrane[41]. Presynaptic molecules associated with synaptic vesicle proteins are also recruited to the active zone, and aid in the transformation of nascent presynaptic sites to functional presynaptic structures[42]. Originally synaptic proteins were thought to be relatively stable in mature synapses; however, more recent studies have suggested that these proteins are highly mobile and shuffle into and out of individual synapses[43, 44]. The cadherin/β-catenin complex has previously been shown to play a role in the recruitment and localization of synaptic vesicles to synapses[45, 46]. More recently, it has been determined that interfering with β-catenin itself can affect synapse assembly[43, 47].

β-Catenin is important for controlling the size and localization of vesicle clusters. Deletion of β-catenin in hippocampal pyramidal neurons has been associated with a decrease in the number of synaptic vesicles per synapse, and an increase in the diffusion of these vesicles along the synapse[43, 47]. This effect does not appear to be due to postsynaptic components since examination of the shape and distribution of postsynaptic density (PSD)-95, a marker of postsynaptic densities, remains unaffected[47]. The decrease in the number of synaptic vesicles seems to be specific to undocked vesicles, those vesicles in the reserved/resting pool, as opposed to docked vesicles, those corresponding to the readily releasable pool. A decrease in the reserved/resting pool is complemented by an impaired response to prolonged repetitive stimulation, and corresponds to a dispersion of vesicles along the axon. The C-terminal domain of β-catenin, which contains a PDZ target sequence, may be responsible for presynaptic vesicle localization[47]. Evidence

suggests that the PDZ binding motif of β-catenin allows it to act as a scaffolding protein to link cadherins to PDZ domain-containing proteins, retaining vesicles at discrete sites.

In addition to the PDZ binding motif, the phosphorylation state of β-catenin at tyrosine 654 (Y654) may also affect synaptic vesicle localization. As stated earlier, phosphorylation of β-catenin at Y654 decreases its affinity for cadherin[33]. Recent evidence has shown that phosphorylation of β-catenin at this residue can be promoted by application of brain-derived neurotrophic factor (BDNF) to cultured hippocampal neurons[43]. BDNF is a neurotrophin that is well known to function in synaptic plasticity and regulate synaptic morphology[48]. Upon application of BDNF, the cadherin-β-catenin complex is disrupted, and an enhancement in synaptic vesicle mobility is observed. The dispersal of synaptic vesicles into perisynaptic regions can be abolished by preventing the phosphorylation at this residue by a β-catenin point mutation[43].

An effect on synaptic vesicle localization by phosphorylation of β-catenin at Y654 can also be observed following manipulation of Fer[49]. Fer is a cytoplasmic tyrosine kinase that is known to act in several signaling pathways, including cell adhesion molecule-regulated signaling[50]. Depletion of Fer using small hairpin RNAs (shRNAs) in cultured hippocampal neurons results in an increase in the motility of presynaptic clusters, along with an increase in tyrosine phosphorylated β-catenin[49]. This dispersion of synaptic vesicle clusters could, once again, be prevented by overexpressing a mutant form of β-catenin that prevents phosphorylation at Y654. Overall, these results suggest that β-catenin acts presynaptically to control synaptic vesicle localization.

Role of β-catenin in postsynaptic structure and function

The role of β-catenin in postsynaptic shape and function has also been studied. As mentioned earlier, the dendritic spines on the postsynaptic neuron are the sites where most excitatory synapses take place. Spines can generally be classified by their shape and volume as thin, stubby, or mushroom-like. The different shapes of dendritic spines are thought to represent strength and maturity, where thin spines are immature, and mushroom shaped are mature. β-Catenin has been shown to be important in regulating spine shape and size. Without postsynaptic β-catenin, there is an increase in thin, elongated spines and subsequent decrease in short mushroom-shaped spines[51]. Although ablation of postsynaptic β-catenin alters spine morphology, no changes in density of presynaptic markers were observed. Thus, these results suggest that poststynaptic β-catenin ablation does not prevent neurons from maintaining presynaptic inputs.

Alterations in the dendrites themselves have also been observed following β-catenin manipulation. Overexpression of β-catenin in hippocampal neuronal cultures increases dendritic growth and arborization, while decreasing endogenous β-catenin prevents dendritic morphogenesis[37, 52, 53]. This function of β-catenin in regulating dendritic growth appears to be due to its role in cell adhesion, and is not dependent on its transcriptional actions.

The above changes have been reported at baseline; however, a similar effect can be observed following neural activation. Neural activity is known to induce changes in dendrite morphology, and this remodeling is critical for neural circuit formation and synaptic function. The effects of neural activity can be mimicked by chronically

depolarizing neurons with extracellular potassium, and has been shown to increase dendritic arborization[52, 53].

Interestingly, the effect reported following overexpression of β-catenin is remarkably similar to the observed increase in dendritic arborization following treatment with high potassium, thus suggesting that the two treatments may function through a common signaling pathway. Indeed, depolarization induced by elevated potassium increases Wnt secretion, and one way to increase intracellular β-catenin is through activation of the Wnt pathway. Therefore, it may be that neuronal depolarization increases Wnt activity, which then stabilizes the intracellular pool of β-catenin, ultimately leading to an enhancement in dendritic arborization.

β-Catenin has also been shown to regulate postsynaptic strength. Miniature excitatory postsynaptic currents (mEPSCs) are events generated in dendrites and generally arise from the spontaneous release of single vesicles. Measuring mEPSCs in β-catenin-ablated neurons gives insight into the effect of β-catenin loss on glutamatergic quantal responses. Loss of β-catenin in hippocampal neurons results in a decrease in the amplitude of mEPSCs, which is largely dependent on AMPA receptors, without affecting the frequency of mEPSCs, which is dependent on the density of functional presynaptic boutons[51]. Following application of wild-type β-catenin to these neurons, the mean mEPSC amplitude is restored to levels comparable to control neurons, suggesting that β-catenin plays a role in modulating AMPA-mediated synaptic currents. Similar to the presynaptic role of β-catenin in controlling synaptic vesicle localization, the central armadillo repeats, which bind to cadherins and TCF/LEF transcription factors, along with the C-terminal PDZ-binding motif, are important for regulating synaptic AMPARs[51].

Overexpression of β-catenin also reduces mEPSC amplitudes and is accompanied by a decrease in surface AMPAR cluster size and density. However, the decrease in AMPA receptor density does not appear to coincide with a decrease in synapse density[52]. This incongruity can be explained by an observed increase in the NMDAR/AMPAR ratio, which is indicative of silent, or inactive, synapses. Furthermore, the physiological changes occur in parallel with the aforementioned increases in dendritic arborization following neural activity[52]. These results suggest that changes in dendritic morphology may coordinate with excitatory synaptic strength to regulate synaptic scaling. Altogether, these results show that β-catenin may act postsynaptically to couple the structure and function of excitatory synapses.

Activity-dependent regulation of β-catenin

Neural activity alters the localization of β-catenin at synapses, thus providing more evidence that β-catenin plays a role in synaptic regulation. Following depolarization with a solution containing a high concentration of KCl, there is an NMDA-dependent redistribution of preexisting β-catenin from dendritic shafts to spines in cultured hippocampal neurons. This redistribution of β-catenin to the spines coincides with an increase in the association of β-catenin with cadherin, and can be mimicked or prevented by application of a tyrosine kinase or phosphatase inhibitor, respectively[54].

Similar results were obtained by studying β-catenin point mutations at site, tyrosine 654 (Y654). Point mutations that prevent phosphorylation of β-catenin at this specific site result in a redistribution of β-catenin to the spine, and an increase in the size

and density of synapsin-1 and PSD-95, markers of presynaptic and postsynaptic proteins. In addition to changes in synaptic proteins, preventing phosphorylation at Y654 alters synaptic function by increasing the frequency of mEPSCs[54]. An increase in the frequency of mEPSCs, with no change in the amplitude, may reflect an increase in the probability of neurotransmitter release, or a conversion of silent, or inactive synapses to active synapses[55, 56]. Nonetheless, manipulating tyrosine phosphorylation and dephosphorylation of β-catenin produces changes in synaptic size and strength. More recent evidence suggests that cyclin-dependent kinase 5 (Cdk5) activity is responsible for this activity-dependent phosphorylation of β-catenin at Y654[57]. Together, these results suggest that activity-induced changes in the localization of β-catenin, along with the regulation of tyrosine phosphorylation, are important for synaptic regulation.

β-Catenin regulation has also been shown to play a role in activity-dependent gene expression, an important component of synaptic plasticity[58, 59]. In response to NMDAR-dependent activation of calpain, β-catenin is cleaved at the N terminus, making it resistant to GSK-3β mediated degradation[60]. This results in an increase in the stabilized form of β-catenin which then translocates to the nucleus to regulate gene transcription. Fosl 1 has been identified as one gene that is upregulated following the NMDAR-mediated β-catenin signaling cascade, and interestingly, Fosl 1 has also been shown to be upregulated following behavioral learning in rats[61]. Therefore, β-catenin regulation may be important for the transcription of genes following synaptic activity.

β-Catenin and memory related pathology

Recent evidence supports a role for β-catenin in pathological states such as Alzheimer's disease (AD). Alzheimer's disease is a neurodegenerative disorder characterized by progressive memory loss and cognitive impairment, and at the molecular level, by the presence of neurofibrillary tangles (NFTs) and senile plaques[62, 63]. NFTs are intraneuronal aggregates formed by an accumulation of hyperphosphorylated tau, a microtubule-associated protein, while plaques are extracellular deposits comprised of the insoluble β-amyloid peptide (Aβ)[64]. It has been suggested that these neuropathological hallmarks may be related, such that over-production of Aβ leads to the hyperphosphorylation of tau, resulting in the formation of NFTs[64]. Consistent with this hypothesis, the three mutations identified in families affected by Familial Alzheimer's disease (FAD), presenilin-1 (PS-1), presenilin-2 (PS-2), and the amyloid precursor protein (APP), result in dysfunctional Aβ production[65, 66].

There is indirect evidence from AD brains supporting a role for β-catenin in disease. AD patients with PS-1 mutations have reduced levels of β-catenin[67]. PS-1 is a transmembrane protein located at synaptic cell-cell contact sites. It is involved in the processing of APP, and has been shown to form complexes with β-catenin[68-71]. This interaction is thought to increase β-catenin stability[67]. Mutations in PS-1 decrease β-catenin stability and are associated with an overproduction and aggregation of Aβ peptide[72].

PS-1 is also thought to inactivate GSK3, a negative regulator of β-catenin. Mutations in PS-1 facilitate GSK3 activity, resulting in an increase in the hyperphosphorylation of tau[73, 74]. In agreement with these findings, there is evidence

suggesting that GSK3 activity may be increased in AD. GSK3 expression has been shown to be upregulated in the hippocampus of AD patients[75]. Similarly, an increase in phosphorylated GSK3 has also been reported in the frontal cortex in AD[76]. Since increased GSK3 activity is associated with a decrease in β-catenin stability, these findings suggest that impairments in β-catenin regulation may be linked to AD pathology. Indeed, over the past several years, there has been substantial evidence supporting this link.

Aβ neurotoxicity can be produced by the addition of Aβ to neuronal cultures, and results in effects that are similar to AD pathology, including an increase in the hyperphosphorylation of tau protein[77]. Under these conditions, lower levels of cytoplasmic β-catenin have been reported, suggesting that Aβ neurotoxicity may compromise the stability of β-catenin. Lithium, which acts as a positive regulator in the Wnt signaling pathway, by inactivating GSK-3β, can prevent the cytotoxic effects of Aβ[78, 79]. Lithium has also been shown to prevent neurodegeneration and behavioral impairments induced by injections of Aβ fibrils into the dorsal hippocampus of rats[77]. Along with preventing the cytotoxic effects of Aβ, lithium increases β-catenin levels, thus suggesting that the mechanism by which lithium treatment may be acting is due, at least in part, to the stabilization of β-catenin[77].

It has been suggested that AD manifests itself as a perturbation of neuroplasticity prior to overt Aβ –dependent neural degeneration and toxicity[80, 81], which could hypothetically be a result of early β-catenin dysfunction. Considerable evidence has shown that β-catenin is required for synaptic plasticity; thus, understanding the

physiological role of β-catenin may be critical to inform new treatment and prevention approaches in AD.

β-Catenin and learning and memory in the adult brain

Thus far, there has been substantial evidence suggesting that β-catenin is involved in neuronal synapse regulation and plasticity; however, all of this work has been done *in vitro*, and not in intact animals. Since knockouts of β-catenin are embryonic lethal[82], there have been no studies on the role of this intriguing protein in standard behavioral learning and memory assays. This thesis aims to fill that gap, by examining the role of β-catenin in long-term memory formation in adults.

In Chapter 2, I examine the role of β-catenin and its interaction with cadherin in the amygdala during the consolidation phase of fear-memory formation. In Chapter 3, I aim to identify a role for Wnt/β-catenin signaling in mediating β-catenin-dependent memory formation in the amygdala. Then, in Chapter 4, I explore the possibility that β-catenin may also play a role in hippocampal-dependent learning and memory. Finally, In Chapter 5, I review the main findings and discuses how understanding the role of β-catenin memory formation may provide insight into the functional deficits underlying the cognitive impairments associated with AD.

CHAPTER 2

β-Catenin is required for memory consolidation

Adapted from:

Maguschak, K.A. & Ressler, K.J. β-Catenin is required for memory consolidation. *Nat Neurosci* 11, 1319-1326 (2008).

ABSTRACT

β-Catenin has been implicated in neuronal synapse regulation and remodeling. Here we have examined β-catenin expression in the adult mouse brain and its role in amygdala-dependent learning and memory. We found alterations in β-catenin mRNA and protein phosphorylation during fear-memory consolidation. Such alterations correlated with a change in the association of β-catenin with cadherin. Pharmacologically, this consolidation was enhanced with lithium-mediated facilitation of β-catenin. Genetically, the role of β-catenin was confirmed with site-specific deletions of *loxP*-flanked *Ctnnb1* (encoding β-catenin) in the amygdala. Baseline locomotion, anxiety-related behaviors, and acquisition or expression of conditioned fear were normal. However, amygdala-specific deletion of *Ctnnb1* prevented the normal transfer of newly formed fear learning into long-term memory. Thus, β-catenin may be required in the amygdala for the normal consolidation, but not acquisition, of fear memory. This suggests a general role for β-catenin in the synaptic remodeling and stabilization underlying long-term memory in adults.

INTRODUCTION

Structural changes at synapses are thought to underpin long-term memory formation. Dendritic spines, where most excitatory synapses terminate[83, 84], show alterations in motility and morphology after a learning event[85-87]. The processes governing dendritic morphogenesis are many and varied, but recent work has focused on the role of cell adhesion molecules in mediating activity-dependent changes at synapses.

β-Catenin is a candidate molecule that may function in mediating the structural changes associated with long-term memory formation. It associates with the cytoplasmic domain of cadherin and directly links to the actin cytoskeleton through α-catenin[88]. This cadherin-catenin complex is localized in synaptic junctions, and alterations in this complex are thought to influence synaptic size and strength[54]. Recent work has suggested that the cadherin-catenin complex is involved not only in synapse development, but also in modulation of synaptic connectivity and activity[21, 89].

In addition to its role in cadherin-mediated cell-cell adhesion, β-catenin has an important role in the Wnt signal transduction pathway. In the resting state, β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK-3β) and rapidly degraded by the proteasome pathway. Upon activation of Wnt signaling, β-catenin is stabilized through the inhibition of GSK-3β, and translocates to the nucleus, where it binds the TCF/LEF family of transcription factors to regulate the expression of Wnt target genes[27, 28]. This signaling pathway has recently been shown to be involved in the regulation of synaptic plasticity in a hippocampal slice preparation[29].

Thus β-catenin seems to be an important 'hub' protein in synaptic plasticity, with involvement in regulating both activity-dependent synaptic remodeling and gene transcription. Taken together, there is tremendous face validity to the hypothesis that β-catenin is directly involved in crucial events that mediate learning and memory. However, because knockouts of β-catenin are embryonic lethal[82], it has not been possible to examine the potentially crucial role of this protein in learning and memory assays in animals. Also, no specific pharmacological agents that target β-catenin have yet been

identified, so no pharmacological studies have directly examined learning and memory modulation by β-catenin.

These experiments described here outline a role for the regulation of β-catenin regulation and its interaction with cadherin during the consolidation phase of fear memory formation. We showed that memory formation is enhanced by acute administration of lithium, which acts in part by stabilizing β-catenin through the inhibition of GSK-3β. We used an inducible genetic approach to examine whether β-catenin is required for the consolidation of fear memories *in vivo*. When examining the effects of temporal- and region-specific deletion, we found that β-catenin within the amygdala is required for the consolidation, but not the acquisition or expression, of fear memory.

RESULTS

β-Catenin mRNA expression in the adult mouse brain

T he heavy emphasis on the role of β-catenin in development has resulted in a scarcity of data on the expression of β-catenin in adult animals. We therefore examined β-catenin expression in the brains of wild-type adult (8-10 week old) C57Bl/6J mice. *In situ* hybridization analyses using an antisense probe spanning exons 2 through 6 of *Ctnnb1* revealed very dense expression of this gene throughout the adult brain, particularly in regions associated with synaptic plasticity (Figure 2.1). A sense probe spanning the same region was used as a negative control, resulting in no significant

labeling above background (data not shown). These data indicate that β-catenin is present in the adult brain and may be required for normal neuronal functioning in adults.

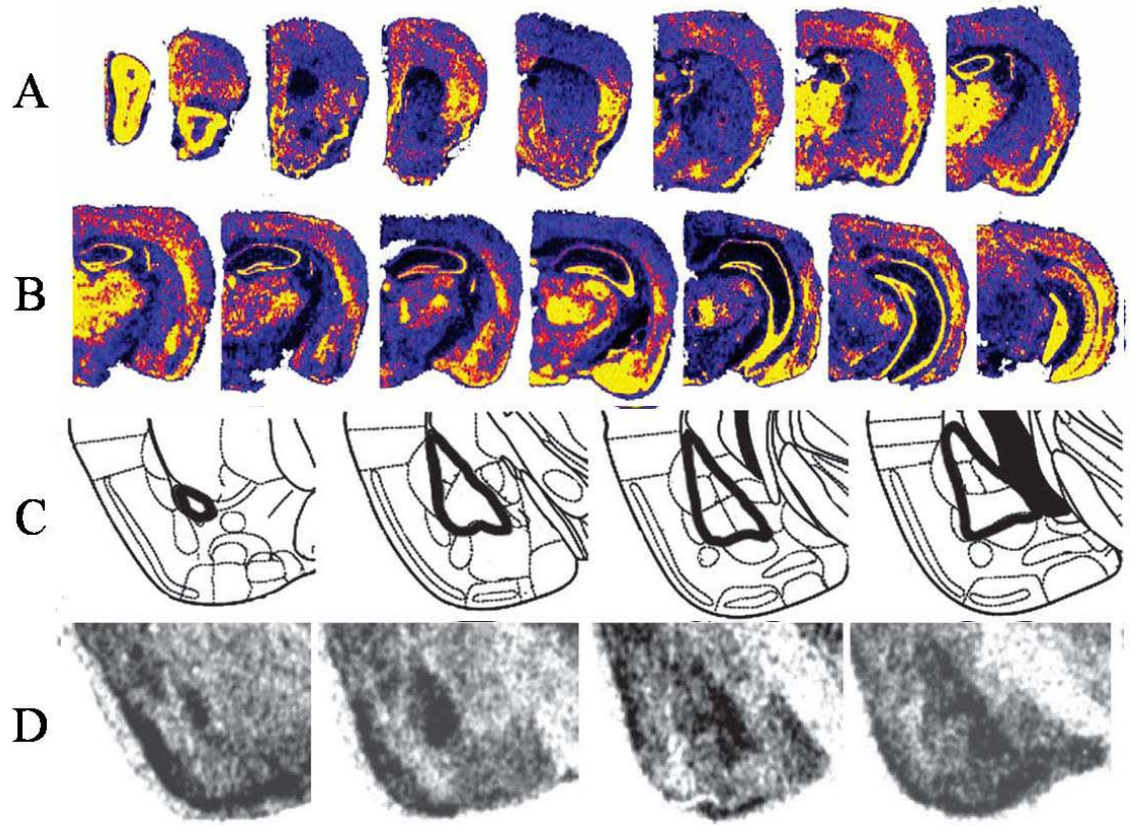

Figure 2.1 - β-Catenin expression in the adult mouse brain

(A,B) Pseudocolored *in situ* hybridization photomicrographs showing high β-catenin mRNA throughout the brain, particularly within the amygdala, some cortical regions, thalamus and hippocampus. Arrows, basolateral amygdala (BLA). Yellow, highest expression; blue-black, lowest expression. (C) Schematic diagram from Paxinos and Watson[90] showing the location of the amygdala and its subdivisions in the temporal lobe (BLA is outlined). (D) β-Catenin mRNA is present at high levels spanning the basolateral nuclei of the amygdala, as outlined in C.

β-Catenin mRNA increases in the amygdala following fear conditioning

We next examined the hypothesis that β-catenin is involved in the synaptic plasticity underlying learning and memory in adults, specifically fear conditioning in the amygdala. After 3 d of habituation to the conditioning chambers, mice received five tone-shock pairings. A context control group was placed in the conditioning chambers for the same amount of time, but no stimuli were presented. We collected brains from the control mice 2 h after context exposure; brains from the trained mice were collected immediately, 0.5 or 2 hr after conditioning. Mice that had received the five tone-shock pairings were able to acquire and express fear, as shown by increased freezing throughout the training (Figure 2.2 A). We then measured β-catenin mRNA in various brain regions at different time points after fear conditioning. β-Catenin mRNA in the basolateral amygdala (BLA) was altered with fear conditioning, with a significant increase at 2 hr after training (control, 1.00 ± 0.06 versus 2 hr, 1.27 ± 0.08; $t_{14} = 2.764$, $P < 0.05$; Figure 2.2 B-C). We did not find any significant differences in β-catenin mRNA in the somatosensory cortex or striatum (Figure 2.2 C; $P > 0.05$).

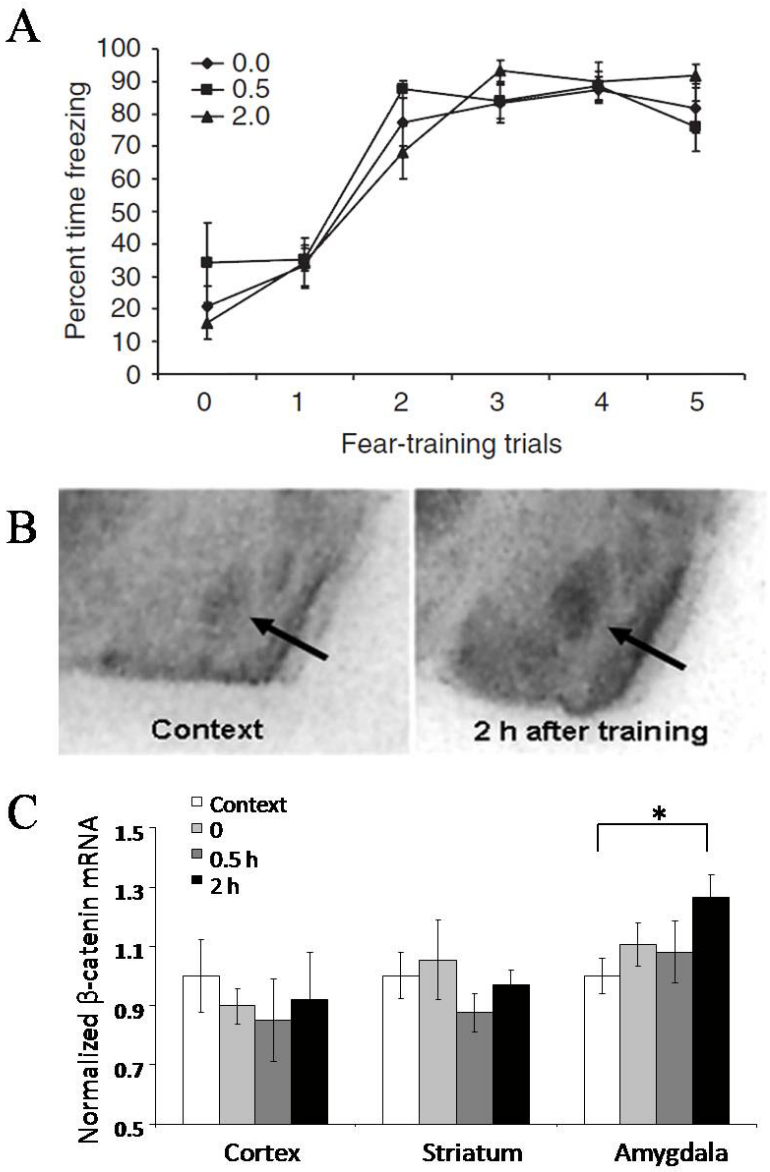

Figure 2.2 – β-Catenin gene expression in the amygdala is increased after fear conditioning

Mice were exposed to five tone–shock pairings and then killed 0, 0.5 or 2 h after training. (A) Acquisition curve showing the percentage of time spent freezing during each tone before the presentation of the footshock. Mice in all groups showed similar levels of freezing before the presentation of any tones (0) and then showed increased freezing

during the tone trials throughout training (trials 1–5). Arrows, the presentation of footshock. (B) Qualitative *in situ* hybridization analysis of β-catenin mRNA in the amygdala in context-exposed mice (left) and mice killed 2 h after training (right). (C) Relative expression of β-catenin mRNA in the somatosensory cortex, striatum and amygdala, normalized to expression in context-exposed mice. Only β-catenin mRNA expression in the amygdala was significantly increased 2 h after fear conditioning. n = 8 for context, 0 h and 2 h; n = 7 for 0.5 h. Error bars, s.e.m. *P < 0.05.

β-Catenin is post-translationally regulated following fear conditioning

We next examined whether the increase in β-catenin mRNA with fear learning is the result of altered expression or altered post-translational modification .We used western blot analyses to examine β-catenin expression in mice exposed to the context alone, mice exposed to unpaired tone and shock presentations, and mice trained and killed 0, 0.5, 2, 4, 12, or 24 hr after fear conditioning with five tone-shock trials (Figure 2.3 A). In contrast to the observed increase in β-catenin mRNA expression, total β-catenin protein in the amygdala did not change with training (ANOVA, $P > 0.05$; Figure 2.3 B), suggesting that protein modification or degradation occurs in concert with the increases in gene transcription.

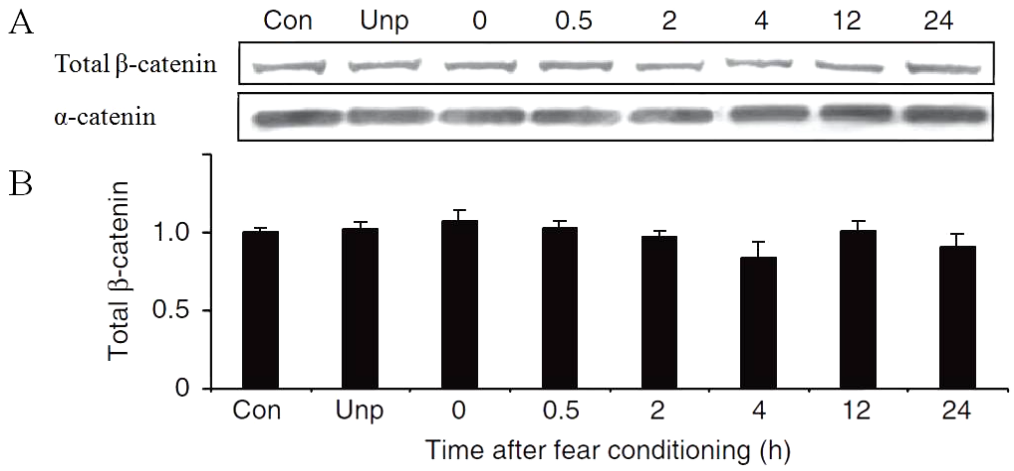

Figure 2.3 – β-Catenin expression in mice

(A) Qualitative western blot data. Protein amounts are expressed relative to the α-tubulin loading control. (B) Total β-catenin did not change with fear conditioning. n = 31 for context; n = 13 for unpaired; n = 14 for 0 h; n = 27 for 0.5 and 2 h; n = 8 for 4 h; n = 6 for 12 h; n = 7 for 24 h. Con, context control group; Unp, unpaired shock control group. Error bars, s.e.m.

Thus, we wanted to determine whether post-translational modifications of β-catenin occur with learning. We first measured changes in the stabilization of β-catenin after GSK-3β inactivation. GSK-3β destabilizes β-catenin by phosphorylating it at Ser33/37/Thr41. However, when GSK-3β is phosphorylated, it becomes unable to destabilize β-catenin. We measured phosphorylated GSK-3β after learning and found a significant main effect of time ($F_{7, 132} = 3.943$, $P \leq 0.001$ by ANOVA). *Post hoc* least-square difference analyses indicated that phosphorylated GSK-3β was significantly higher in trained mice 2hr after fear conditioning (1.71 ± 0.18) than in unpaired control

mice (1.03 ± 0.16; $P \leq 0.005$), context exposed mice (1.00 ± 0.10; $P \leq 0.001$) and trained mice killed immediately after conditioning (0-hr time point; 0.68 ± 0.11; $P \leq 0.001$; Figure 2.4 A,B). Notably the amount at the 0-hr time point was also significantly different from the 4-hr time point (1.34 ± 0.28; $P \leq 0.001$). This significant increase in phosphorylated GSK-3β after fear conditioning is consistent with enhanced stabilization of β-catenin during fear consolidation.

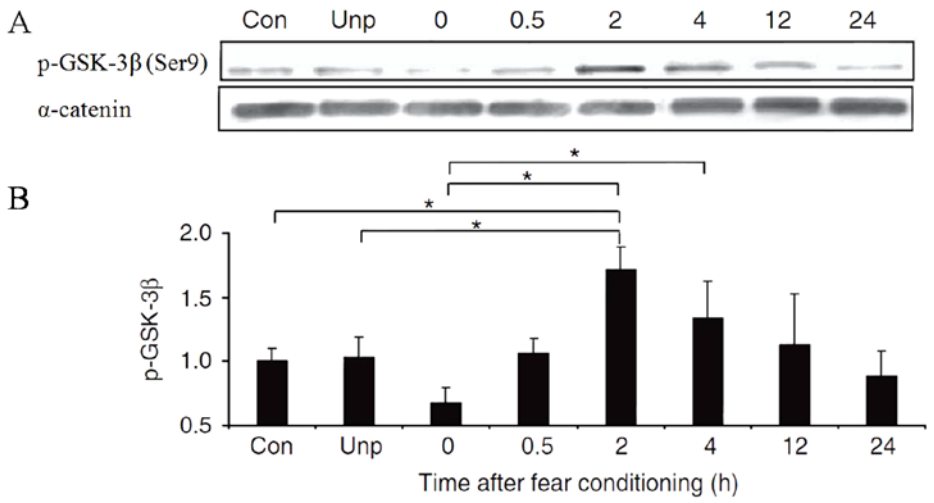

Figure 2.3- Phosphorylation state of GSK-3β is altered after fear conditioning

Qualitative western blot data. Protein amounts are expressed relative to the α-tubulin loading control. (B) Phosphorylated GSK-3β (p-GSK-3β) changed significantly over time. n = 31 for context; n = 13 for unpaired; n = 14 for 0 h; n = 27 for 0.5 and 2 h; n = 8 for 4 h; n = 6 for 12 h; n = 7 for 24 h. Con, context control group; Unp, unpaired shock control group. Error bars, s.e.m. *P≤ 0.05.

We then examined phosphorylation of β-catenin at Tyr654, which decreases its affinity for cadherin[33]. Overall, ANOVA indicated a significant main effect for time ($F_{7,132} = 2.107$, $P \leq 0.05$). *Post hoc* tests revealed that Tyr654-phosphorylated β-catenin was significantly higher in trained mice 12 hr after conditioning (1.63 ± 0.26) than in unpaired controlled mice (1.14 ± 0.14; $P \leq 0.05$) and context exposed animals (1.00 ± 0.08; $P \leq 0.01$; Figure 2.5 A,B). Notably, compared to the context-exposed group, Tyr654-phosphorylated β-catenin was also significantly increased at 0.5 hr after conditioning (1.28 ± 0.12; $P \leq 0.05$). Thus the affinity of β-catenin for cadherin within the amygdala seems to be dynamically regulated during fear consolidation.

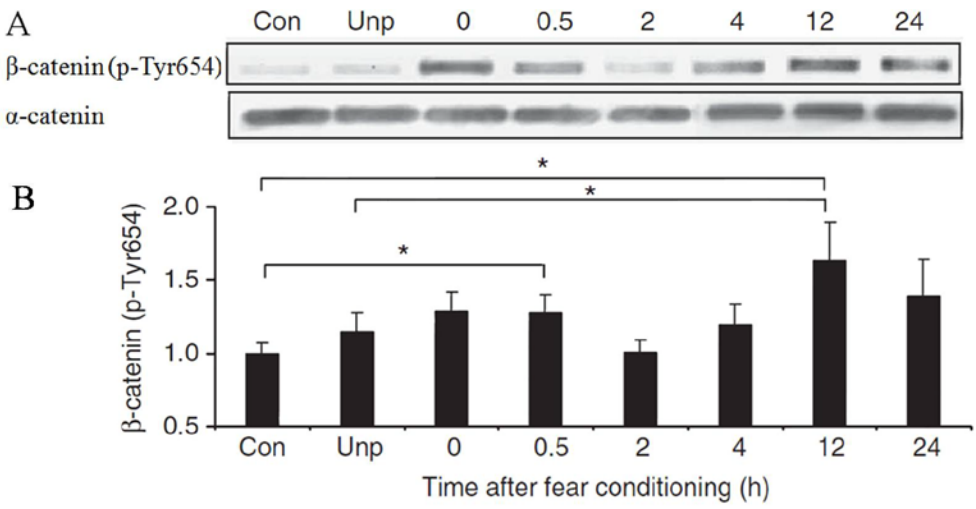

Figure 2.4 – Phosphorylation state of β-catenin is altered after fear conditioning

(A) Qualitative western blot data. Protein amounts are expressed relative to the α-tubulin loading control. (B) Tyr654-phosphorylated β-catenin (p-Tyr654) changed significantly over time. n = 31 for context; n = 13 for unpaired; n = 14 for 0 h; n = 27 for 0.5 and 2 h; n = 8 for 4 h; n = 6 for 12 h; n = 7 for 24 h. Con, context control group; Unp, unpaired shock control group. Error bars, s.e.m. *P ≤ 0.05.

Given these results, we wanted to determine whether these changes in Tyr654-phosphorylated β-catenin abundance significantly affect the association of β-catenin with cadherin. We immunoprecipitated β-catenin from the amygdala of the above mice, and then probed with an antibody to pan-cadherin antibody. ANOVA indicated a significant main effect for time ($F_{7,132} = 2.320$, $P \leq 0.05$; Figure 2.5 A,B). *Post hoc* analyses revealed that the amount of cadherin coimmunoprecipitated with β-catenin was significantly lower in trained mice immediately after conditioning (0.60 ± 0.08) than in unpaired mice (1.05 ± 0.12; $P \leq 0.01$) and context control mice (1.00 ± 0.06; $P \leq 0.01$). Notably, this immediate decrease was followed by a significant increase in binding at 2 h (1.02 ± 0.10; $P \leq 0.01$) and 4 h (1.17 ± 0.24; $P \leq 0.01$) after conditioning, returning cadherin binding to normal. We also found a significant negative correlation between the amount of cadherin coimmunoprecipitated with β-catenin and the amount of Tyr654-phosphorylated β-catenin ($r_{133} = - 0.184$, $P \leq 0.05$), confirming a significant relationship between these measures.

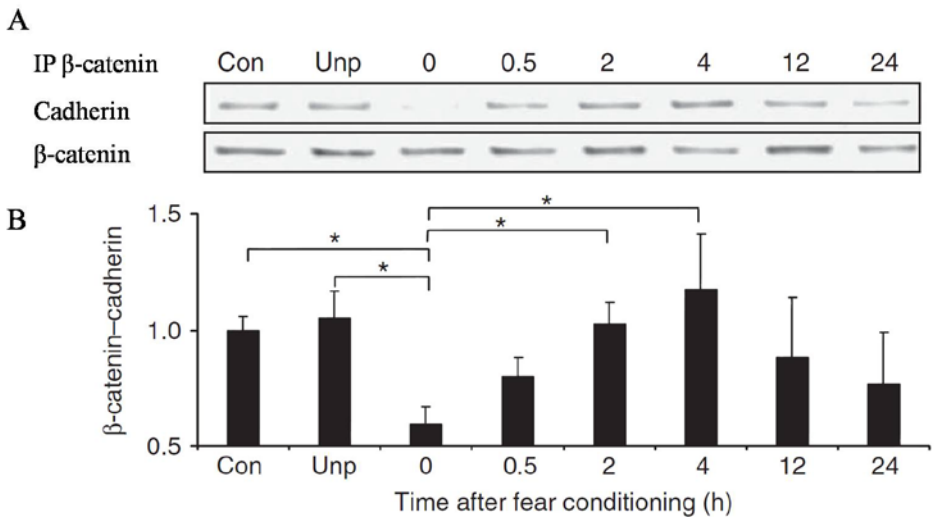

Figure 2.5 – Coimmunoprecipitation of cadherin and β-catenin

(A) Qualitative western blot data. (B) Cadherin interaction with β-catenin changed significantly over time. n = 31 for context; n = 13 for unpaired; n = 14 for 0 h; n = 27 for 0.5 and 2 h; n = 8 for 4 h; n = 6 for 12 h; n = 7 for 24 h. Con, context control group; Unp, unpaired shock control group. Error bars, s.e.m. *$P \leq 0.05$.

None of the blot analyses showed significant differences between context-exposed mice and mice receiving unpaired tones and shocks ($P > 0.05$). Thus, the time-dependent differences we observed in β-catenin modulation are likely to result from associative learning and not from the stress of shock alone.

Increasing β-catenin functional stability results in an enhancement in learning

Because β-catenin regulation in the BLA is correlated with fear conditioning, we examined whether manipulating β-catenin function would affect this learning process. No specific pharmacological agents that target β-catenin have yet been identified, making it difficult to directly examine the effect of β-catenin function on learning. However, lithium chloride (LiCl), though not as specific as we would like, is widely accepted as a modulator of β-catenin. LiCl inhibits GSK-3β, decreasing its ability to phosphorylate β-catenin at its Ser33, Ser37 and Thr41. As a consequence, the unphosphorylated β-catenin is more stable and less prone to degradation[36, 91, 92]. The temporal changes we observed in phosphorylated GSK-3β suggested that it is a good target for pharmacological manipulation of β-catenin with learning.

To examine the effects of acute LiCl administration on learning, we first confirmed that systemic administration of LiCl inhibits GSK-3β in the amygdala. We injected mice intraperitoneally with either saline or LiCl (100 mg kg^{-1}) and killed them 30 min later. As expected, acute administration of LiCl significantly increased phosphorylated GSK-3β in the amygdala (1.43 ± 0.30) compared to controls (0.64 ± 0.13; $t_{17} = 2.344$, $P \leq 0.05$; Figure 2.6 A). We then examined whether LiCl alters GSK-3β-dependent phosphorylation of β-catenin after fear conditioning. We injected mice with either saline or LiCl 30 min before training, and then killed them 0.5 or 2 hr after fear conditioning. Total β-catenin in the amygdala was higher, although not significantly, in LiCl treated mice compared to saline-treated mice (Figure 2.6 B). Notably, in agreement with the model of LiCl inhibiting GSK-3β, the ratio of GSK-3β phosphorylated β-catenin to total β-catenin was significantly lower at the 0.5- and 2- h time points than the ratio in

saline-treated mice ($F_{1, 28} = 11.931$, $P < 0.01$; Figure 2.6 C). Together, these results suggest that acute LiCl administration inhibits GSK-3β-mediated phosphorylation of β-catenin, potentially enhancing its overall stability, during the consolidation period after fear conditioning.

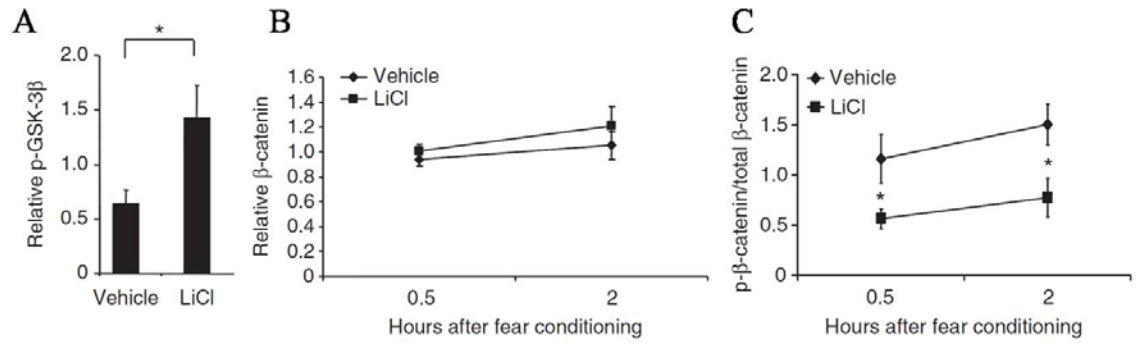

Figure 2.6 - LiCl decreases GSK-3β-mediated β-catenin phosphorylation in the amygdala

(A) Mice in their home cages were killed 30 min after systemic injections of either vehicle or LiCl (100 mg kg–1). There was significantly more phosphorylated GSK-3β in the amygdala in LiCl-treated mice than in vehicle treated mice (n = 9 for vehicle group, n =10 for vehicle group). (B,C) A separate group of animals was injected with either vehicle or LiCl 30 min before fear conditioning and then killed 0.5 or 2 h after training (n = 8 per group). (B) Total β-catenin in the amygdala was increased, although not significantly, at the 0.5- and 2-h time points in LiCl-treated mice. (C) The ratio of β-catenin phosphorylated at the GSK-3β dependent sites (Ser33, Ser37 and Thr41) to total β-catenin was significantly decreased in LiCl-treated mice at the 0.5- and 2-h time points after fear conditioning. In A-C, protein amounts are expressed relative to α-tubulin loading control. Error bars, s.e.m. *P < 0.05.

We next determined whether decreasing GSK-3β-mediated phosphorylation of β-catenin through acute LiCl administration could affect learning. We injected mice with either saline or LiCl and then fear-conditioned them 30 min later. The intensity of the unconditioned stimulus was lowered to 0.6 mA to prevent ceiling effects on fear expression. Throughout this training paradigm, we measured freezing behavior during each tone presentation (conditioned stimulus) before the presentation of footshock (Figure 2.7). We found a significant main effect of time across all mice ($F_{5,180}$ = 111.495, $P \leq 0.01$); however, there was no main effect of LiCl treatment ($F_{1,36}$ = .167, P = 0.685).

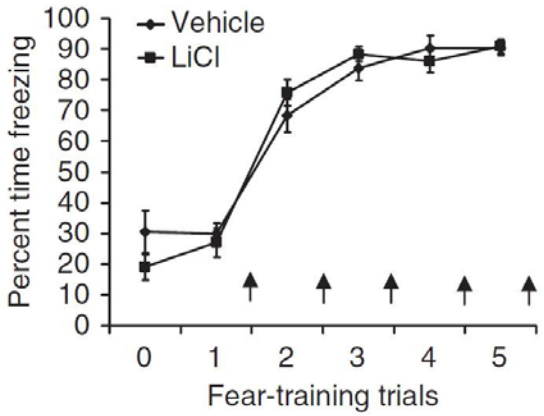

Figure 2.7 - LiCl treatment enhances learning

An additional group of mice was injected with either vehicle or LiCl 30 min before fear conditioning and then tested 48 h later (n = 19 per group). (A) Acquisition curve showing percentage time spent freezing during each tone before presentation of the footshock. All mice were able to acquire and express equal levels of fear. Arrows, the presentation of footshock. Error bars, s.e.m. *P < 0.05.

Forty-eight hours after fear conditioning, in the absence of drug, mice were placed in a new chamber and presented with 15 conditioned-stimulus tones. The mean percent time spent freezing during these tones was recorded and used as a measure of conditioned fear (Figure 2.8 A). Mice that received LiCl before the fear training 2 d earlier now showed significantly more fear (57.00 ± 4.64) than did mice that had received saline (43.07 ± 4.99; $t_{38} = 2.044$, $P < 0.05$). This increase in fear retention was present throughout the testing session but most notable toward the middle and end of the session (Figure 2.8 B). Percent time spent freezing was recorded in blocks of five trials each as follows (vehicle versus lithium): block 1, 39.5 ± 5.5 versus 47.6 ± 5.3 ($t_{38} = 1.06$, not significant); block 2, 46.2 ± 6.4 versus 63.0 ± 5.7 ($t_{38} = 1.95$, $P \leq 0.05$); block 3, 43.7 ± 5.6 versus 61.2 ± 6.5($t_{38} = 2.05$, $P \leq 0.05$). These data suggest that the difference in retention of fear memory was not caused by differences in extinction within testing. The enhancement of fear memory also did not seem to be caused by effects on locomotor behavior, as the mice did not show any significant differences across groups in activity level or freezing behavior before the first conditioned stimulus. Similarly, previous reports have shown that this specific dose of LiCl does not produce locomotor effects[93].

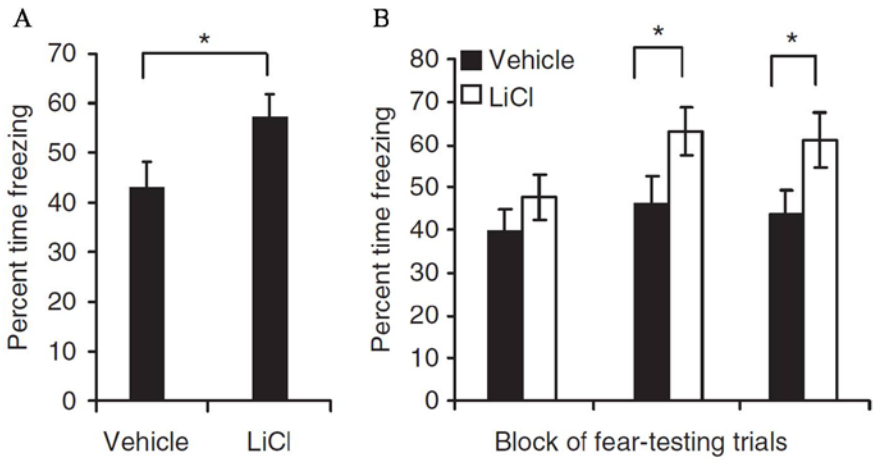

Figure 2.8 - LiCl treatment enhances learning

An additional group of mice was injected with either vehicle or LiCl 30 min before fear conditioning and then tested 48 h later (n = 19 per group). (A) Mice that received LiCl before fear training showed significantly more fear than did mice that received saline upon retesting 48 h later in the absence of drug. (B) Freezing data from A were grouped in blocks of five, revealing that this difference in fear retention is maintained across the testing session. Error bars, s.e.m. *P < 0.05.

Together, these data suggest that a single, albeit nonspecific, pharmacological manipulation that increases functional β-catenin during or soon after fear conditioning leads to relatively specific increases in the expression of fear behavior 48 h later. This is consistent with the hypothesis that increasing functional β-catenin enhances consolidation of new memories.

Ctnnb1 deletion in the BLA does not alter baseline measures

As stated above, LiCl is somewhat non-specific[94], so we sought to examine the effect of β-catenin on learning and memory through a more direct genetic mechanism: 'floxed' β-catenin mice[95], which possess *loxP* sites located in introns 1 and 6 of *Ctnnb1*. Injection of these mice with a Cre recombinase-expressing lentivirus (LV-Cre), resulted in region-specific deletion of the floxed *Ctnnb1* allele (Figure 2.9). Ten days after unilateral infection with LV-Cre virus, we probed adjacent brain sections with radiolabeled *Ctnnb1* antisense mRNA or Cre recombinase. These experiments showed that relatively specific deletion of *Ctnnb1* in the amygdala can be achieved with LV-Cre injection. When mice were injected with a control lentivirus expressing green fluorescent protein (LV-GFP), the abundance of β-catenin remained similar to that in wild-type mice. Notably, there were no effects of Cre-mediated *Ctnnb1* deletion or LV-GFP injection on the cellular or anatomical structure of the amygdala, as shown with a Nissl stain (Figure 2.10).

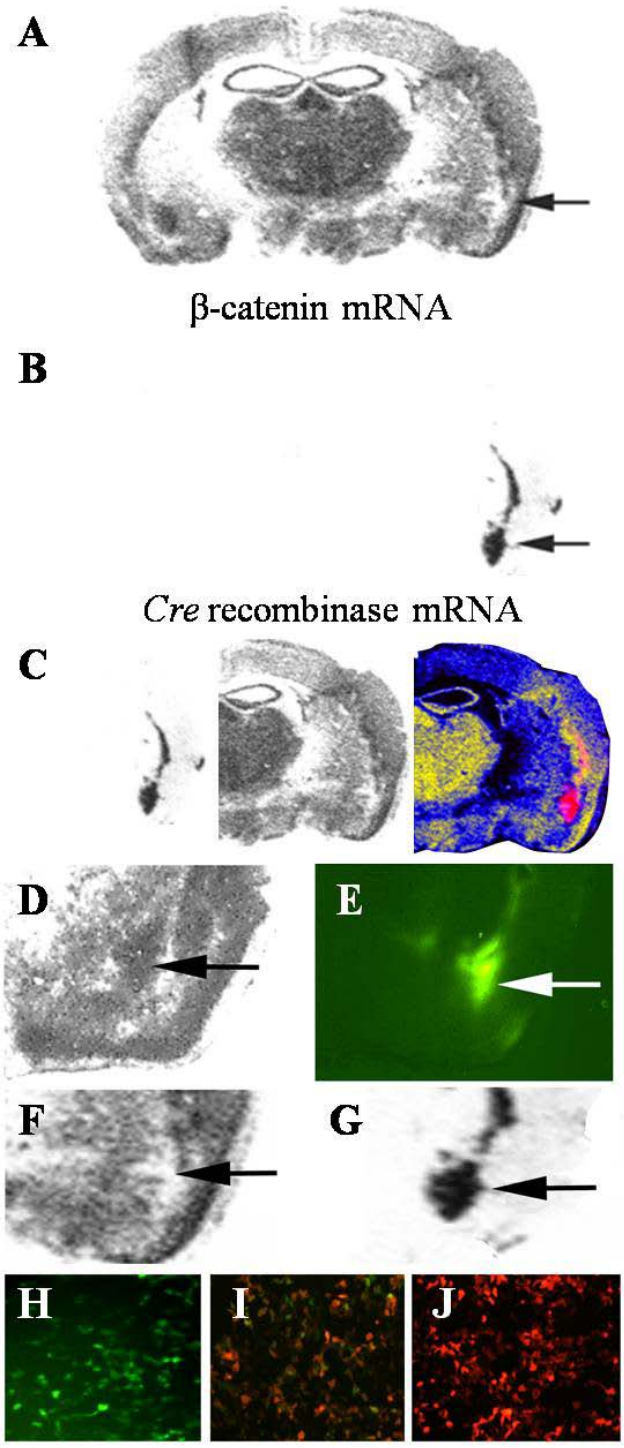

Figure 2.9 - Region-specific deletion of β-catenin in the adult brain

(A, B) β-Catenin–floxed mice were injected with either LV-Cre or LV-GFP and killed 10 d later. Unilateral injection of LV-Cre resulted in site-specific loss of expression. Adjacent sections were probed with radiolabeled antisense β-catenin mRNA (A) or Cre recombinase (B). A pseudocolor overlay of these two adjacent sections (C) shows the regional specificity. (D-F) Mice that that received LV-GFP injections into the amygdala had normal β-catenin abundance (D) where the LV-GFP was injected (e). This is in contrast to β-catenin mRNA expression (F) in the amygdala of a mouse injected with LV-Cre (G). (H-J) *In vitro* functional assay of lentivirus-expressed Cre recombinase. HEK293T cells were transiently transfected with a vector containing a floxed GFP reporter, pLoxpGFP-DsRed, in the absence (H) or presence (I) of LV-Cre and visualized using a green filter. (J) HEK293T cells transfected with pLoxpGFP-DsRed in the presence of LV-Cre, visualized using a red filter.

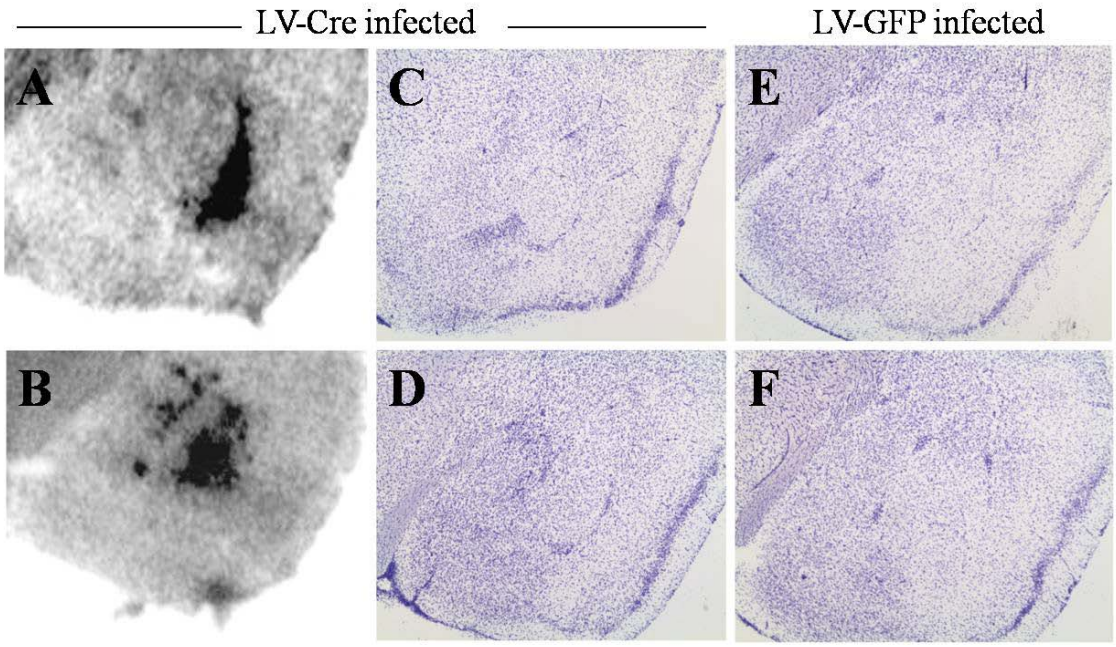

Figure 2.10 – Cresyl Violet (Nissl) staining of infected basolateral amygdala

(A, B) *In situ* hybridization for Cre recombinase performed on parallel sections to (C,D) which have been Nissl stained. These Cre-infected sections showed similar Nissl cellular patterns to LV-GFP infected amygdala as shown in (E,F). No evidence of scarring or significant histological abnormalities was found.

Having confirmed our ability to locally delete *Ctnnb1* in a temporally specific, inducible manner, w examined whether amygdala-specific deletion affects baseline anxiety or activity measures. We injected the mice with either LV-GFP or LV-Cre bilaterally in the amygdala at 6-8 weeks of age. Ten days later, we examined the mice in a series of basic behavioral tasks. In these baseline measures, we found no difference ($P > 0.1$) in anxiety as measured by baseline startle, elevated plus-maze (time in open or

closed arms) and open-field (distance traveled in center, time at rest, and average velocity; Figure 2.11 A-E). These data suggest that amygdala-specific deletions of *Ctnnb1* do not alter motor activity or anxiety levels.

We then wanted to quantitatively confirm that injections of LV-Cre into the amygdala decrease β-catenin mRNA. We processed brains for *in situ* hybridization and measured β-catenin mRNA. Mice injected with LV-Cre had significantly less β-catenin mRNA (26.73 ± 7.20) than did mice injected with LV-GFP (54.60 ± 2.96; $t_{6.88} = 3.580$, $P \leq 0.01$; Figure 2.11 F).

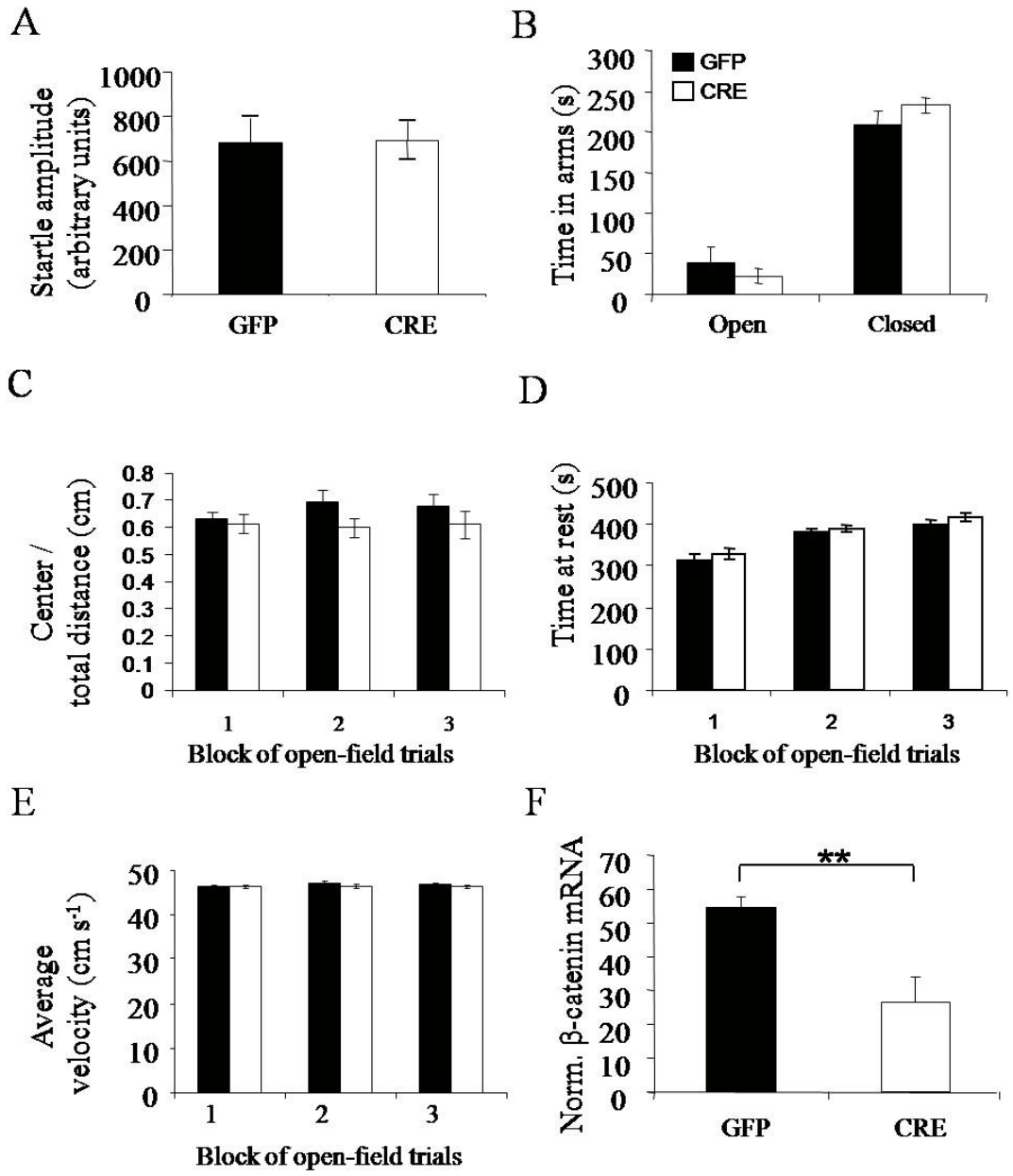

Figure 2.11 - Amygdala-specific β-catenin deletions do not affect baseline anxiety or activity measures

Mice received bilateral injections of LV-GFP or LV-Cre into the amygdala and were allowed 10 d to recover. (A) Baseline startle for mice injected with LV-GFP or LV-Cre. (B) Time spent in the open and closed arms of the elevated plus-maze. (C-E) Activity

measures, recorded in three blocks of 10 min, for mice placed in an open-field apparatus for 30 min. There were no differences between mice injected with LV-GFP or LV-Cre in terms of distance traveled in the center compared to total distance (C), time at rest (D) or average velocity (E). (F) β-Catenin mRNA, normalized to local non-amygdala background. n = 7 for LV-GFP group; n = 6 for LV-Cre group. Error bars, s.e.m. **P ≤ 0.01.

β-Catenin is required for fear-memory consolidation

We then examined whether amygdala specific deletion of *Ctnnb1* affects amygdala-dependent learning, as outlined in Figure 2.12 A. We fear-conditioned the mice and obtained freezing measures during fear acquisition (Figure 2.12 B). As with acute LiCl administration, there was a significant main effect of trial ($F_{5,125} = 104.698$, $P \leq 0.01$) but no effect of virus ($F_{1,25} = 1.964$, $P = 0.173$). The similar acquisition curves for LV-GFP and LV-Cre mice suggest that mice with *Ctnnb1* deletions are initially able to encode and express fear memories normally.

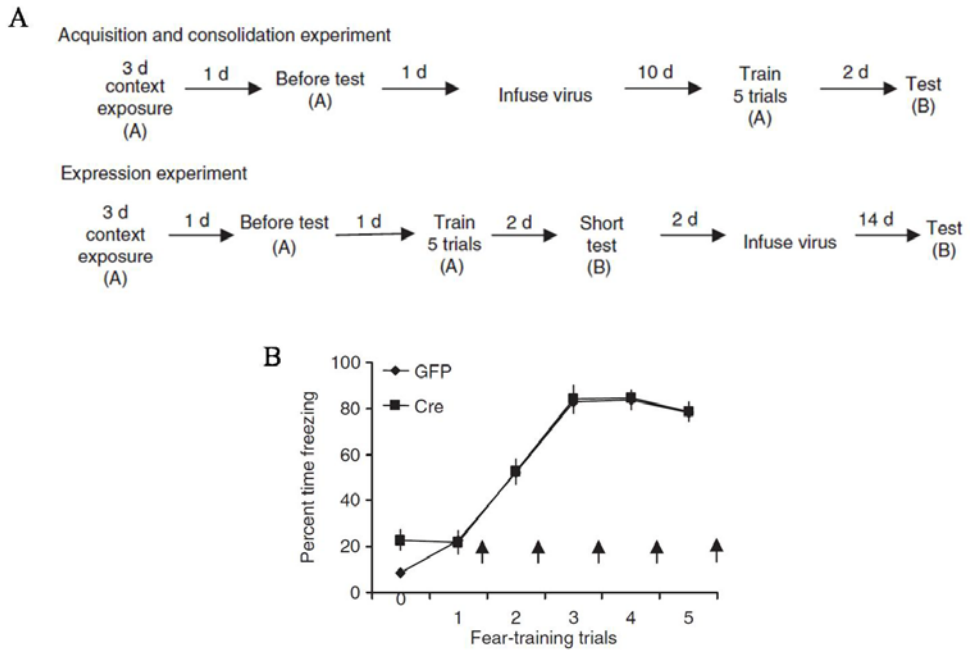

Figure 2.12 - Amygdala-specific *Ctnnb1* deletion does not prevent the expression, of conditioned fear

(A) Timeline for acquisition, consolidation and expression experiments. 'A' and 'B' indicate the context used. (B) Acquisition curve for LV-GFP and LV-Cre mice during training. Arrows, the presentation of footshock. n = 15 for LV-GFP group; n = 12 for LV-Cre group. Error bars, s.e.m.

Forty-eight hours after the first five trials, we tested mice for cue fear conditioning in a new context (Figure 2.13 A). In contrast to the acquisition data above, animals infected with LV-Cre - and thus, those with the *Ctnnb1*deletions - showed over 40% less freezing averaged across all sessions (35.00 ± 7.26) compared to mice infected with LV-GFP (60.00 ± 4.94; $t_{25} = 2.935$, $P < 0.01$). Notably, even in the first freezing trial of the test, LV-Cre-infected mice froze less than LV-GFP-infected mice, which froze

at rates near those seen during acquisition. The data for within-session freezing across the full testing session (Figure 2.13 C) were in grouped blocks of five trials each as follows (LV-GFP versus LV-Cre): block 1, 66.33 ± 6.24 versus 38.33 ± 7.67 (t_{25} = 2.863, P < 0.01); block 2, 58.67 ± 6.35 versus 35.00 ± 8.77 (t_{25} = 2.239, P < 0.05); block 3, 55.00 ± 6.45 versus 31.67 ± 7.11 (t_{25} = 2.426, P < 0.05). These data confirm that the decrease in fear is most likely to be a function of decreased consolidation at, or soon after, the initial learning event, as mice were able to acquire and express fear normally (Figure 2.12 B) and did not show decreased average fear resulting from increased within-session extinction (Figure 2.13. 7B). In addition, there was a positive correlation between β-catenin mRNA and freezing as a measure of fear (r_{13} = 0.752, P < 0.01; Figure 2.13 C).

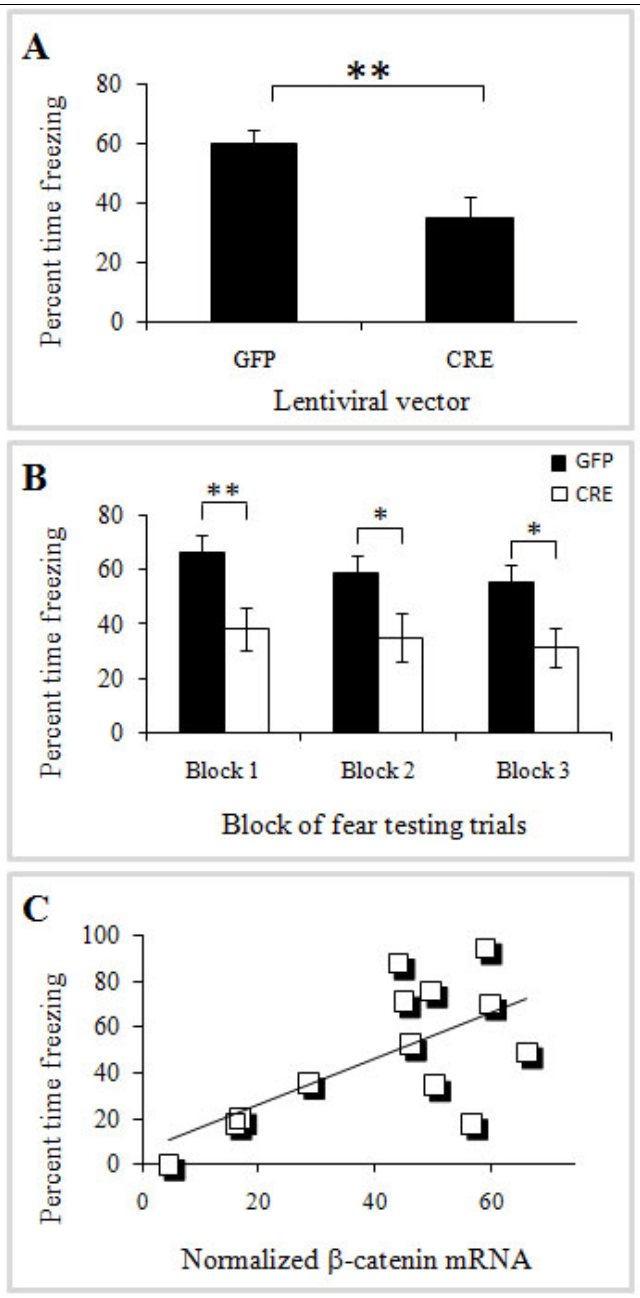

Figure 2.13 - Amygdala-specific *Ctnnb1* deletion prevents the consolidation of conditioned fear

(A) Percentage time spent freezing during the 48-h post-training test in LV-GFP and LV-Cre mice in response to the tone presented in a new context. (B) Freezing data in A

grouped in blocks of five trials (n = 15 for LV-GFP group; n = 12 for LV-Cre group). (e) Correlation between β-catenin mRNA expression and freezing behavior (n = 7 for LV-GFP group; n = 6 for LV-Cre group). Error bars, s.e.m. *P < 0.05; **P ≤ 0.01.

Together, these data suggest that β-catenin expression in amygdala is not required for normal anxiety-related behaviors or for the acquisition of fear, an amygdala-dependent task. However, consistent with the dynamic regulation of β-catenin abundance during the consolidation period after fear acquisition, these data suggest that β-catenin is required for the normal consolidation of fear memory. In the absence of β-catenin, newly formed memories do not seem to be stabilized and thus cannot be expressed 48 h later.

β-Catenin is not required for expression of fear memory

Thus far, our pharmacological and genetic manipulations of β-catenin have shown that β-catenin is not involved in the acquisition of fear but rather in the stabilization of fear memory. We therefore examined whether deletion of *Ctnnb1* after the consolidation of fear memory would affect further expression of conditioned fear (outlined in Figure 2.12 A). To determine the effect of *Ctnnb1* deletion on expression, we trained mice and then presented them 48 h later with a three- trial 'short-test' for freezing to confirm that they had acquired and consolidated the fear memory (Figures 2.14 A, B). We did not administer the full 15 trials in order to reduce the likelihood of extinction processes. We then injected the mice with either LV-GFP or LV-Cre bilaterally into the amygdala and allowed the mice to recover for 14 d. The mice were then tested again for fear memory

after this delay (2-3 weeks after training, Figures 2.14 A , C). Although the levels of freezing in this 21-day fear expression test were lower than those in the 48-h experiments, both groups of mice showed significantly more freezing during the tone than before the conditioned stimulus during this expression test ($F_{1,17} = 14.786$, $P \leq 0.01$). Nissl staining of infected amygdala showed that the observed decrease in freezing in both groups was not caused by damage to the amygdala. Thus, it is more likely that the decrease in freezing resulted from the passage of time between training and testing. Notably, upon testing the animals after this delay, we found that mice receiving LV-Cre showed similar levels of freezing (14.58 ± 6.34) to mice receiving LV-GFP (13.91 ± 3.45; $t_{17} = 0.095$, $P > 0.05$). Because both LV-Cre and LV-GFP groups showed comparable, and statistically significant, levels of freezing when tested for fear expression while Cre recombinase and GFP protein are being expressed in the amygdala, these data suggest that β-catenin in the amygdala is not required for fear expression after the memory has been consolidated.

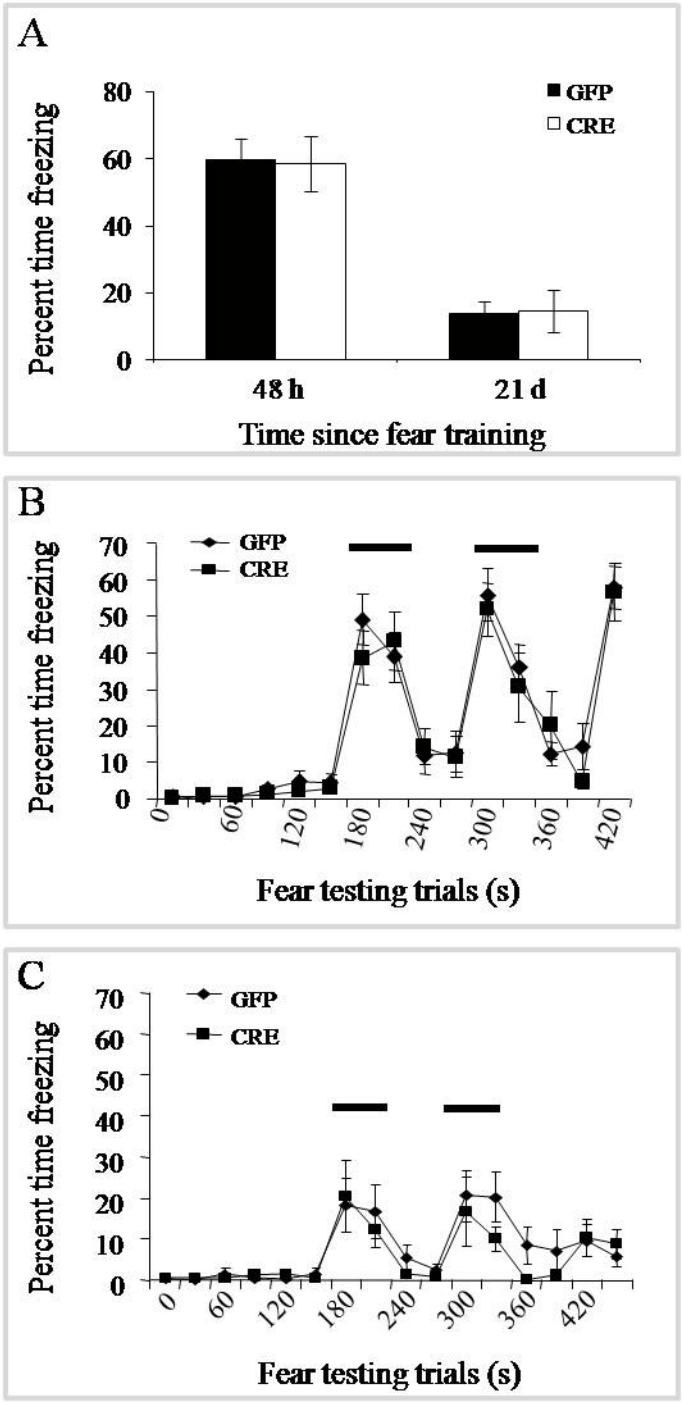

Figure 2.14 - Amygdala-specific *Ctnnb1* deletion does not affect the expression of conditioned fear

(A) Percentage time spent freezing by LV-GFP and LV-Cre mice at 48 h (before infusion) and 21 d (after infusion) after training. (B) Freezing behavior 48 h after training, shown in 30-s intervals. (C) Freezing behavior 21 d after training, shown in 30-s intervals. Horizontal bars in f–h indicate periods during which the conditioned stimulus was present. n = 10 for LV-GFP group; n = 9 for LV-Cre group. Error bars, s.e.m. *$P < 0.05$; **$P \leq 0.01$.

DISCUSSION

Our data suggest that β-catenin has a role in long-term memory formation in adults. We showed that β-catenin is highly expressed in the adult mouse amygdala and is dynamically regulated at both the transcriptional and post-translational levels with fear learning. Pharmacological stabilization of β-catenin with LiCl resulted in enhanced learning, whereas genetic deletion of *Ctnnb1* in the amygdala resulted in deficient learning. By studying the effects of *Ctnnb1* deletion in adult mice, we have identified a role for β-catenin in learning and memory

Our data also suggest that β-catenin is required for the consolidation, not the acquisition, of fear memory. However, once the memory has been consolidated, we found that β-catenin is no longer required to express the memory. During this consolidation period, the interaction between β-catenin and cadherin is dynamically regulated, suggesting that β-catenin is involved in the structural conversion of short-term labile to long-term stable memory traces.

We found that β-catenin mRNA expression was increased in the BLA, but not the somatosensory cortex and striatum, after fear training. To our knowledge, this is the first

study to examine β-catenin *in vivo* with learning, but this result is consistent with previous *in vitro* studies of hippocampal slices showing an increase in nuclear β-catenin with tetanic stimulation[29]. Wnt target genes have also been shown to be upregulated with long-term potentiation in hippocampal slices, anywhere from 15 to 120 min after stimulation[29].

Notably, when we measured total β-catenin protein levels, we did not see any alterations with training. It has been shown that depolarization of hippocampal neurons with KCl does not change the total amount of β-catenin at the synapse, but instead, causes a redistribution from dendritic shafts to spines[54]. It is possible that rapid dynamic changes in breakdown, redistribution, and replacement do not result in apparent change in total protein visualized with immunoblots.

We observed biochemical changes suggesting that the roles of β-catenin in both cell-cell adhesion and Wnt signaling are affected by fear conditioning. Phosphorylation of β-catenin Tyr654 has been shown to decrease the affinity of β-catenin for cadherin[33, 96]. In addition, inhibiting the phosphorylation of Tyr654 with a point mutation redistributes β-catenin from dendritic shafts to spines, thereby increasing the β-catenin-cadherin interaction[54]. In our study, Tyr654-phosphorylated β-catenin was dynamically regulated after training. Our coimmunoprecipitation experiments show a very rapid period of β-catenin-cadherin destabilization, followed by a period of stabilization during consolidation. Overall, these findings suggest that the affinity of β-catenin for cadherin initially weakens to allow for modifications of the synapse, and then strengthen to stabilize the synapse, which we hypothesize to be a molecular and cellular correlate of memory consolidation.

Such dynamic regulation of β-catenin phosphorylation on Tyr654 has previously been proposed. Treatment with brain-derived neurotrophic factor has been shown to induce synaptic vesicle dispersion in hippocampal cultures, which is associated with an increase in β-catenin tyrosine phosphorylation and decrease in β-catenin-cadherin interactions. Soon after this dispersion, phosphorylation decreases, and the β-catenin-cadherin interaction is restored[43]. Notably, we previously showed that brain-derived neurotrophic factor activation of the TrkB receptor is required in the amygdala for consolidation of fear memories[97]. Thus, a similar mechanism may be taking place in this *in vivo* learning paradigm, such that when new memories are formed, pre-existing synapses must become destabilized transiently before the stabilization of synapses involved in memory formation.

We have provided both biochemical and behavioral evidence suggesting that increased stabilization of β-catenin, through the inhibition of GSK-3β, is important for learning and memory. Normally, GSK-3β phosphorylates β-catenin at Ser33, Ser37, and Thr41, marking the protein for degradation. However, when GSK-3β is inactivated by phosphorylation at Ser9, β-catenin becomes stabilized[27]. In our study, there was an increase in phosphorylated GSK-3β in the amygdala 2 h after fear conditioning. In addition, increasing the inhibition of GSK-3β with LiCl decreased β-catenin phosphorylation. Acute administration of LiCl 30 min before training resulted in an enhancement in learning measured 48 h later, without any effect on acquisition.

Although administration of LiCl has been shown to produce behaviors similar to those resulting from overexpression of β-catenin in the mouse brain[98],the actions of LiCl are not necessarily specific to β-catenin. To more definitively identify the role of β-

catenin in long-term memory formation, we used genetic manipulations to delete *Ctnnb1* from the adult amygdala. We found that deletion of *Ctnnb1* before training does not affect the acquisition or immediate expression of fear but does produce deficits in learning when measured 48 h after training. In addition, deletion of *Ctnnb1* after consolidation has occurred does not affect the expression of learned fear. These findings provide further support that normal β-catenin expression is necessary to consolidate the newly acquired memory.

One limitation of this study is our inability to specifically inhibit or delete *Ctnnb1* immediately after training. Previous work has elegantly shown, using consolidation of inhibitory avoidance, that post-training manipulations are the gold-standard for demonstrating disruption of fear consolidation[99, 100]. Although the data on consolidation of amygdala-dependent classical conditioning paradigms have been less clear, this is an important manipulation for interpretation of consolidation effects. Unfortunately, there are no drugs currently available that selectively inhibit β-catenin. Additionally, a minimum of 7-10 d is required for optimal lentiviral gene expression, so we are unable to delete *Ctnnb1* shortly after training. However, we feel that our current powerful method of genetic manipulation is an important approach to specifically examine the role of *Ctnnb1* in the amygdala during learning. Furthermore, we feel that the lack of an effect of *Ctnnb1* deletion on acquisition and expression of fear makes a strong case for its role during the consolidation period.

Given the results obtained thus far, we propose that synapses weaken during the acquisition of fear and immediately afterwards (as indicated by decreased β-catenin-cadherin association immediately and 0.5 h after training), thereby alleviating the

requirement for β-catenin. Once the synapses have been modified during the consolidation process, β-catenin is required to convert that memory trace into long-term memory. These proposed changes in synaptic strength will need to be further explored. Additional studies are also needed to determine whether it is the role of β-catenin in cell-cell adhesion, Wnt signaling or both, that contributes to its observed effects on learning and memory.

In summary, our results suggest that β-catenin, a 'hub protein' involved in both transcriptional regulation and stabilization of cell-cell contacts and synaptogenesis, is required for normal consolidation of new memories in adult mice. This finding adds to the body of knowledge describing the role of β-catenin in normal cell functioning, tumor regulation, and development. Although β-catenin has been implicated with *in vitro* approaches in synaptogenesis and synaptic plasticity, our results provide definitive support for its function in learning and memory processes. Further understanding of its role may provide important insights into the nature of the molecular mechanisms underlying memory consolidation. In humans, the development of new small molecule specific inhibitors of β-catenin function may eventually provide a powerful clinical approach to transiently inhibit the consolidation of newly formed trauma memories and thus prevent fear-related disorders, such as post-traumatic stress disorder. Similarly, enhancing β-catenin function may be helpful in disorders of memory such as Alzheimer's disease.

METHODS

Animals

Adult male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were used for immunoblotting and drug treatment experiments. All other experiments were performed using homozygous β-catenin floxed mice. These mice were obtained from Jackson Labs (B6.129-Ctnnb1tm2Kem/KnwJ[95]) and bred within our facility. Originally generated by Kemler and colleagues, these mice possess loxP sites located in introns 1 and 6 of the β-catenin gene[95]. All mice were housed in groups of four in a temperature-controlled (24°C) animal colony, with *ad libitum* access to food and water. They were maintained on a 12 hr light/dark cycle, with all behavioral procedures being performed during the light cycle. All procedures used were approved by the Institutional Animal Care and Use Committee of Emory University and in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Immunoblotting

For Western blotting studies, mice were habituated to handling and to the conditioning chambers for 3d before training. On the day of training, mice received 5 trials consisting of a 30 sec, 6 kHz, 85 db tone, co-terminating with a 0.5 s, 1.0 mA shock (5 min inter-trial interval). The mice in the unpaired training group were given the same number of tone and shock presentations, but the stimuli were explicitly unpaired, with a variable interstimulus interval. A second group of control mice were habituated and exposed to the conditioning chambers for the same amount of time but in the absence of

any tones or shocks (context control), At the appropriate time following training, mice were quickly anesthetized with isoflurane and decapitated. Brains were blocked rapidly over ice and kept frozen at -80^{o}C until processed. Bilateral amygdala punches were obtained with a 1 mm brain punch tool, pooled, and then homogenized in buffer (5 mM HEPES, 1 μM EDTA, and protease inhibitors). Whole-cell lysed samples were analyzed for protein concentration using a BCA assay (Pierce, Rockford, IL). Twenty micrograms of protein per animal were loaded onto SDS-polyacrylamide gels, separated electrophoretically, blotted onto nitrocellulose membranes (BioRad, Hercules, CA), and blocked for 1 hr in 2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM HEPES, pH 7.4 (NDM-HEPES). Membranes were incubated in primary antibody overnight at 4^{o}C [1:100, β-catenin (phospho-Y654) (Abcam, Inc, Cambridge, MA); 1:500, β-catenin (BD Biosciences, San Jose, CA); 1:1000, phospho-β-catenin (Cell Signal, Danvers, MA); 1:1000, phospho-GSK-3β (Cell Signal, Danvers, MA); 1:000, Pan-Cadherin (Cell Signal, Danvers, MA)]. The membranes were then washed three times in blocking buffer and incubated with an HRP-labeled secondary antibody for 60 min. Bound antibody was detected by SuperSignal West Chemiluminescence (Pierce, Rockford, IL) in an Alpha Innotech Fluorchem imaging system (Alpha Innotech, San Leandro, CA). Total blotted protein levels were normalized to levels of alpha-tubulin (1:5000; Sigma), detection of which was used to control for variations in protein loading. Thus, the relative values are expressed as the protein of interest divided by the loading control.

Immunoprecipitation

The same samples from above were used for immunoprecipitation studies. The solubilized proteins were first centrifuged at 10000 x g for 10 min at 4^{o}C. Following centrifugation, the supernatants were incubated with Protein A/G PLUS- Agarose beads (Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 30 min at 4^{o}C with end-over-end agitation. The samples were then centrifuged for 5 min at 2500 rpm. β-Catenin antibody (Cell Signal, Danvers, MA) and Protein A/G PLUS-Agarose beads were then added to 25 µg of each protein sample and incubated with gentle rocking overnight at 4^{o}C. The beads were washed 4 times with homogenization buffer prior to Western blot analyses.

***In situ* hybridization**

In situ hybridization was performed as previously described[101]. The full-length clone used for the β-catenin *in situ* probe was obtained from the NIH IMAGE database (ATCC, Manassas, VA) (GI Accession #31419847). After sequence verification, the cloned cDNA was used to amplify and subclone the regions between exons 2 and 6 of β-catenin, the area flanked by loxP sites in the mutant mouse. This loxP-flanked subclone was then linearized and both antisense and sense riboprobes were generated using the appropriate RNA polymerase and [35S]-UTP in the reaction. After a stringent wash protocol, slides were apposed to Biomax MR autoradiography film (Eastman Kodak Co., Rochester, NY) for 1-3 days. Hybridization density of β-catenin mRNA in the BLA, somatosensory cortex, and striatum were assessed using the mean luminosity function of Adobe Photoshop.

Lentiviral constructs and virus production

Viral vectors were produced and concentrated as previously described[101-106]. Briefly, a Cre-recombinase expressing vector (referred to as 'LV-Cre') or a green-fluorescent protein (GFP)-expressing control vector (referred to as 'LV-GFP'), delta8.9, and VSV-g were co-transfected into HEK293T producer cells to produce replication-incompetent but highly infective virus. Media containing the unconcentrated packaged virus was then concentrated by ultracentrifugation. Virus was titered with infections of exponentially growing HEK293T cells with serial dilutions of concentrated virus, followed by quantification of number of detectable cells with native GFP fluorescence (for LV-GFP) or Red Fluorescent Protein (for LV-Cre, see Figure 5H-J, also see[102]. Virus with a final infectious unit titer of 10^9 IU/ml was used for stereotaxic injections into the amygdala. A green fluorescent protein (GFP) -expressing virus was used to control for variables associated with surgery, stereotaxic injection, and lentiviral infection.

Surgery and injection of virus

Mice were anesthetized by intraperitoneal injections of a ketamine-dormator mixture and placed in a stereotaxic apparatus. Small holes were drilled in the skull above the injection site; BLA coordinates were as follows: AP = -1.8, DV = -4.9, ML = + 3.2 relative to bregma. A 10 µl BSA pre-coated Hamilton microsyringe was used to deliver bilateral intra-amygdala injections of lentiviral vectors expressing GFP or Cre. 0.2 µl of virus/side were injected at a rate of 0.025 µl/min. The needle was left in place for 10 minutes following the injection and the skin was closed using a 6-0 Vicryl suture. All

animals were allowed to recover for 10-14 days before testing. Visualization of injection sites was performed to verify the location of virus infusion.

Behavior

Elevated plus maze

Mice were moved to holding area just prior to testing. The animal was then placed quickly onto the center square between the plus-maze arms. The mouse was left to explore the plus maze for 5 min, and then returned to its cage. Total time spent in closed verses open arms was recorded.

Open field

The open field consisted of a box (27.9 cm x 27.9 cm) made of Plexiglass. The mice were placed in the periphery of the arena at the start of the 30-min test period. At the end of the test, the animal was returned to its home cage. All testing was conducted under standard room lighting. Activity data was obtained and analyzed using the Open Field Activity Software (Med Associates Inc., St. Albans, VT).

Fear Conditioning Apparatus

Mice were fear-conditioned in eight identical startle response systems (SR-LAB, San Diego Instruments, San Diego, CA). Each system consisted of a nonrestrictive Plexiglas cylinder (5.5 cm in diameter and 13 cm long) mounted on a Plexiglas platform and located in a ventilated, sound-attenuated chamber. Cylinder movements were sampled each millisecond by a piezoelectric accelerometer mounted under each platform.

Startle amplitude was defined as the peak accelerometer voltage that occurred during the first 100 ms after the onset of the startle stimulus. The output sensitivity of all response systems was calibrated to be nearly identical (SR-LAB Startle Calibration System, San Diego, CA). Startle and background stimuli were presented through a high-frequency speaker located 15 cm above the chambers. Startle was elicited by a 110 dB, 50 ms white noise burst. A continuous 65 dB white noise background was delivered through chamber speakers during training and testing. Sound intensities were measured by an audiometer (Radio Shack, #33-2055, Ft. Worth, TX). The footshock unconditioned stimulus (US) was generated by a programmable animal shocker (San Diego Instruments) located outside the isolation chambers and was delivered through the cage floor bars. Footshock intensity was 1.0 mA, except where noted. Stimuli presentation and data acquisition were controlled, digitized, and stored by an interfacing IBM PC-compatible computer using SR-LAB software.

Fear conditioning

After 3 d of exposure to the conditioning chambers, mice were given a pre-training test to examine baseline levels of startle in the presence of the tone conditioned stimulus (CS), to ensure that they did not display significant unconditioned excitatory effect to the tone before tone-shock pairing. Twenty-four hours after the pretest, mice were placed in the conditioning chamber, and after 5 min presented with 5 tone-shock pairings at an inter-trial interval (ITI) of 5 min. Each pairing consisted of a 30 s tone (6 kHz, 85 db) CS, which co-terminated with a 0.5 s footshock US (1.0 mA, except where noted). Freezing in startle reflex chambers was assessed as described previously[101, 107].

Briefly, activity measurements during the presentation of the CS were first converted to the average voltage output for each second of the 5-s activity window.
Based on the voltage output, each mouse was give an immobility score of 1 or 0 (0, moving; 1, immobile) for each 1 s of the 5-s activity window. A mean percent immobility score was computed by averaging the five immobility scores and multiplying by 100. The percent immobility score was used as an index of freezing, and in pilot work has demonstrated a high correlation with observational ratings of freezing (rs > 0.89).

Freezing apparatus

Forty-eight hours after training (see above), mice were tested for freezing in rodent modular test chambers (ENV-008-VP; Med Associates Inc., St. Albans, VT) with an inside area of 30.5 cm x 24.1 cm x 21.0 cm. Three minutes later, 15
CS tones (6 kHz, 85 db) with an ITI of 1.5 min were delivered through a high-frequency speaker (Motorola, Model 948, Shaumburg, IL) attached to the side of each chamber. Percentage time spent freezing during the CS presentations was calculated for each mouse using FreezeFrame (Coulbourn Instruments, #ACT-100, Allentown, PA). Note that although the freezing measured within the training (startle) chambers and the testing (freezing) chambers is highly correlated with observer methods of freezing, the y-axis measures of freezing during acquisition (e.g. Figure 2.7) are not directly comparable to the y-axis measures of freezing during testing (e.g. Figure 2.8 A).

Data analysis

Statistically significant differences were determined by Student's t test, or ANOVA, with *post hoc* least squares difference (LSD) tests for multiple comparisons. The results are presented as mean ± SEM.

CHAPTER 3

Wnt signaling in amygdala-dependent learning and memory

ABSTRACT

In addition to its role in cellular development, proliferation, fate, and motility, there is emerging *in vitro* data for the Wnt/β-catenin pathway in synaptic plasticity. Yet *in vivo* studies have not examined if Wnt is required for learning and memory. We infused a Wnt antagonist, Dickkopf-1 (Dkk-1) or vehicle, bilaterally into adult mouse amygdalae prior to fear conditioning. Although locomotor, anxiety, and fear acquisition were equivalent across groups, Dkk-1 prevented long-term fear memory consolidation. mRNA RT PCR arrays demonstrated that many (>50) Wnt-signaling genes were dynamically regulated during fear memory consolidation, with most amygdala Wnt mRNAs being down-regulated. This rapid decrease in Wnt mRNA was confirmed with individual quantitative PCR and *in situ* hybridization. Amygdala-specific infusion of Wnt1 prevented the decrease in Wnt, its effect on β-catenin-cadherin destabilization, and fear memory consolidation. These data suggest that Wnt/β-catenin signaling may be critical for the structural basis of long-term memory formation and stability in adults.

INTRODUCTION

Wnt/β-catenin signaling has been shown to be important for a wide variety of cellular processes including development, cell proliferation, cell fate, and motility[27, 28]. Wnt proteins constitute a large family of secreted molecules that can bind to several distinct receptors, activating different signaling pathways. One such pathway is the Wnt/β-catenin signaling pathway. When Wnts bind to a member of the Frizzled family of cell surface receptors, the transmembrane lipoprotein receptor related proteins 5 and 6 (LRP5/6) are recruited, and the cytoplasmic protein Disheveled is activated[108]. The

activation of Disheveled results in the inhibition of glycogen synthase kinase-3 (GSK3), an enzyme which phosphorylates β-catenin, resulting in the rapid degradation of the protein by the proteasome pathway. The inhibition of this degradation increases the stability of β-catenin, which can then translocate to the nucleus, bind to the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, and regulate the expression of Wnt target genes[27, 28, 109, 110].

Alternatively, β-catenin can be located in a complex with the cytoplasmic domain of cadherin, and upon differential phosphorylation, dissociate from the β-catenin-cadherin complex, and lead to the transient destabilization of synapses required for new synapse formation[43, 111].

The role of Wnt and Wnt/β-catenin signaling in neuronal and synaptic development is increasingly well-established. Previous *in vitro* work has shown that the addition of Wnt7 to both cultured cerebellar granule cells and hippocampal neurons increases axonal spreading and branching, as well as promoting presynaptic assembly and synaptic vesicle accumulation[112-114]. A similar role for Wnt3 has been shown in motor-sensory neurons. The addition of Wnt3 to sensory neurons in culture results in growth cone enlargement and increased axonal branching[115]. This evidence suggests that Wnt peptides function in a retrograde manner to regulate presynaptic remodeling during the development of synapses. In adults, burgeoning evidence suggests that deregulation of Wnt signaling is associated with a number of cognitive disorders, including schizophrenia and Alzheimer's[116]. Together, these data suggest that the Wnt family may play a role in adults beyond initial brain development.

Although there is emerging data suggesting that it may be involved in structural and functional plasticity of mature synapses, the functional role of the Wnt signaling pathway in adult neural circuits remains uncertain. Recent *in vitro* and slice physiology approaches have shown that Wnts may function in synaptic transmission and activity-dependent synaptic plasticity[29, 117]. A microarray analysis of a hippocampal slice preparation following the induction of LTP, identified several members of the Wnt signaling pathway[29]. Furthermore, when Wnt function was suppressed, the magnitude of late LTP was reduced.

There is no evidence to date on the role of Wnt signaling *in vivo* underlying learning and memory formation in animals. We have previously shown that β-catenin signaling is required for fear memory formation *in vivo*, and transient dissociation of the β-catenin-cadherin complex is associated with the consolidation of new fear memories[118]. Given this recent evidence that β-catenin regulation is critical for memory formation, and that Wnt signaling is integral for β-catenin functioning, we hypothesized that Wnt regulation is required for β-catenin stability and regulation of synaptic plasticity *in vivo*.

Here, we examine the possible role of Wnt signaling in mediating β-catenin-dependent memory formation in adults. We first identified a role for Wnts in adult fear learning and memory by blocking Wnt signaling with Dickkopf-1. Then, using real-time PCR arrays, we analyzed the effect of Pavlovian fear conditioning on the expression of a number of genes related to Wnt-mediated signaling in the amygdala of adult mice. We found that the mRNA levels of multiple genes encoding Wnt signaling proteins were dynamically regulated in a time-dependent manner following fear conditioning. We also showed that altering the function of Wnt signaling, via direct infusion of an agonist into

the amygdala prior to fear conditioning, produced deficits in the ability to transfer newly formed fear learning into long-term memory. Together, these data suggest that Wnt/β-catenin signaling is an important pathway regulating long-term memory formation in adults.

RESULTS

Decreasing Wnt mediated signaling impairs memory formation

Most research on the role of Wnt mediated signaling in synaptic plasticity has been based on *in vitro* data. Therefore, we sought to examine the role of the Wnt/β-catenin pathway in the amygdala of adult mice, using Pavlovian cue-dependent fear conditioning as a model of learning and memory. Our first aim was to determine if blocking the Wnt/β-catenin pathway would affect memory formation. Dickkopf-1 (Dkk-1) is a known extracellular antagonist of the Wnt pathway[119]. It is expressed at low levels in the adult brain, but when present, promotes the internalization of LRP6, thereby, preventing Wnt from binding to its co-receptor.

In order to examine the effect of deregulating normal Wnt signaling in the amygdala, cannula were bilaterally implanted in the basolateral amygdala, and either Dkk-1 (100 ng/side) or saline were infused into mice. The presence and spread of the Dkk-1 peptide was verified by immunohistochemistry (Figure 3.1A,B), and there did not appear to be any histological abnormalities as assessed by Nissl stain (Figure 3.1C,D) due to the infusion.

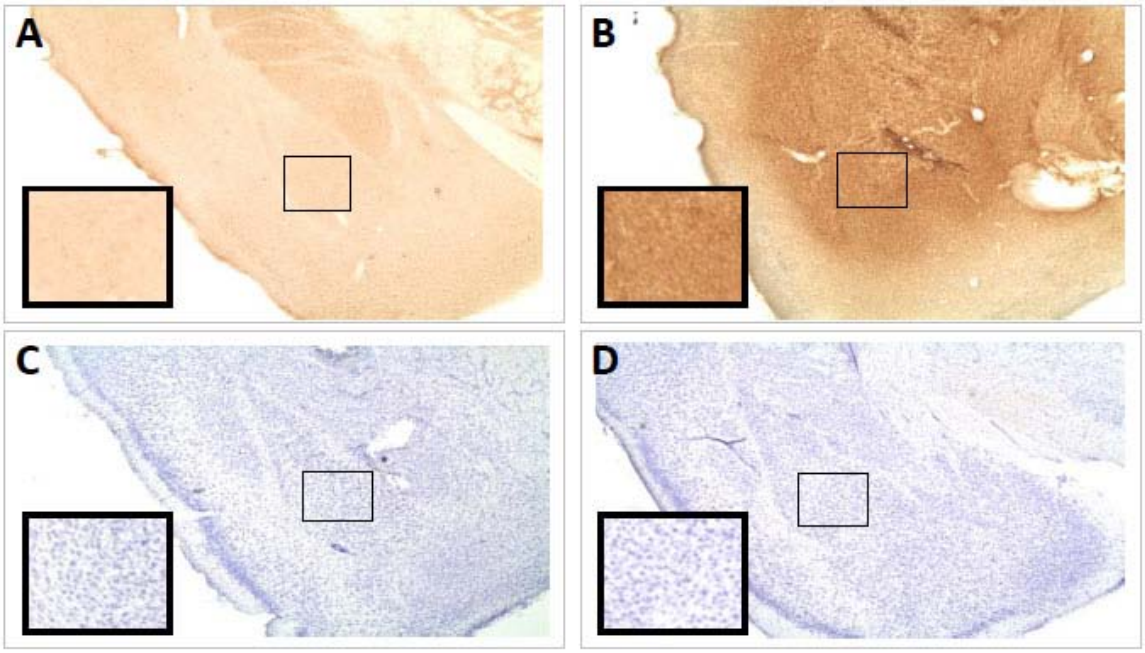

Figure 3.1 - Expression of Dkk-1 in the amygdala

(A,B) Immunohistochemical analyses of Dkk-1 in animals receiving bilateral amygdala injections of either vehicle (A) or Dkk-1 (B). (C,D) Cresyl violet staining of parallel sections showing the absence of damage in vehicle (C) or Dkk-1 (D) infected regions.

After confirming that we could increase the expression of Dkk-1 within the amygdala, we examined whether Dkk-1 affected baseline emotional behavior or locomotion. We injected Dkk-1 prior to examining open-field behavior, and found that there was no difference ($P > 0.1$) between Dkk-1 on mouse locomotion or anxiety-related behavior as measured by distance traveled and time spent in the center compared to time in the surround (Figure 3.2).

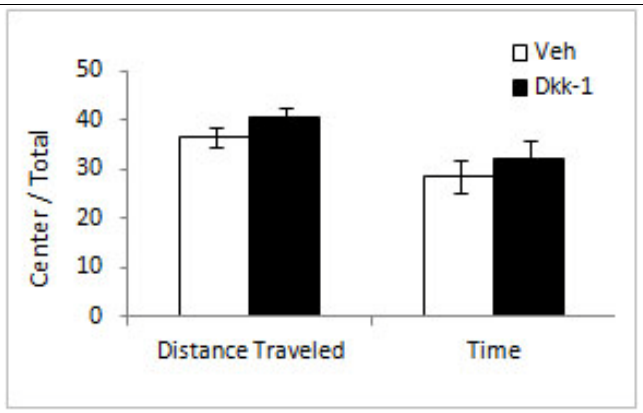

Figure 3.2 - The effect of Dkk-1 in the amygdala on baseline emotional behavior. Activity measures of mice receiving injections of vehicle or Dkk-1 and placed in an open field apparatus for 10 m. n = 10 per group. Mean ± s.e.m.

We then tested whether the presence of this peptide interfered with memory formation. After 5 days of habituation to the conditioning chambers to minimize context effects, mice received infusions of either Dkk-1 or saline bilaterally into the amygdala, and were fear conditioned 15 minutes after infusion with five tone-shock pairings. Throughout the training paradigm, we measured freezing behavior during each tone presentation (conditioned stimulus) before the presentation of footshock. We found a significant main effect of time across all mice ($F_{5,90} = 35.75$, $P < 0.01$, Figure 3.3); however, there was no main effect of treatment ($P > 0.1$). These data demonstrate that the Dkk-1 peptide does not alter baseline locomotion or anxiety-like behavior, nor does it affect the acquisition of conditioned fear memory.

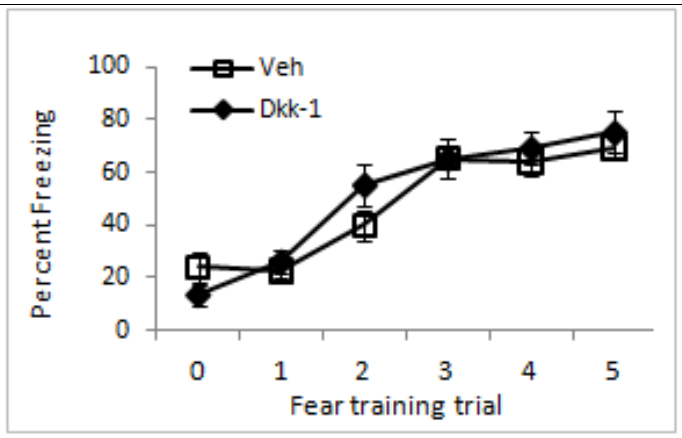

Figure 3.3 - The effect of Dkk-1 in the amygdala on fear learning and memory

Acquisition curve showing percent time spent freezing during each tone prior to footshock presentation. n = 10 per group. Mean ± s.e.m.

Forty-eight hours after cue fear conditioning, mice were placed in a different chamber and presented with 15 conditioned stimulus tones. The mean percent time spent freezing during these tones was recorded and used as a measure of conditioned fear. Mice that received Dkk-1 prior to fear training now showed significantly less fear than did mice that had received saline ($t_{10.10} = 3.62$, $P < .01$, Figure 3.4A). This deficit in fear retention was present throughout the testing session ($F_{1,18} = 13.08$, $P < 0.01$, Figure 3.4B), as measured by percent time spent freezing in three blocks of five trials: block 1 ($t_{10.43} = 3.33$, $P < 0.01$), block 2 ($t_{10.27} = 3.76$, $P < 0.01$), block 3 ($t_{10.98} = 3.38$, $P < 0.01$). Furthermore, the deficit did not seem to be caused by effects on locomotor behavior, as the mice did not show any significant differences across groups in activity level or freezing behavior before the first conditioned stimulus.

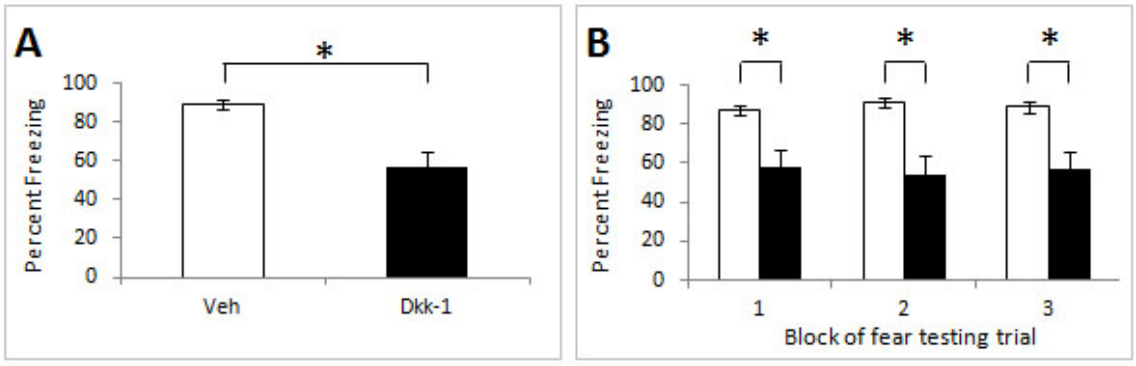

Figure 3.4 - The effect of Dkk-1 in the amygdala on fear learning and memory when given prior to acquisition

(A) Percent time spent freezing when tested 48 h after fear conditioning, and presented with 15 tone presentations. (B) Freezing data from A, presented in 3 blocks of 5 tone presentations. n = 10 per group. Mean ± s.e.m. * $P < 0.01$.

Although we found deficits in the consolidation, but not acquisition, of fear memory when Dkk-1 was injected prior to conditioning, we wanted to confirm that the effect we observed was indeed a consolidation effect. Therefore, we trained a separate group of animals, and injected either vehicle or Dkk-1 immediately following training. The mice were then tested for fear memory 48 hours later.ha We found that mice receiving Dkk-1 following fear training showed significantly less fear than did mice that had received saline ($t_{19} = 3.565$, $P < 0.01$, Figure 3.5 A). Once again, this deficit in fear retention was present throughout the testing session ($F_{1,19} = 12.09$, $P < 0.01$, Figure 3.5 B), as measured by percent time spent freezing in three blocks of five trials: block 1 ($t_{19} = 4.70$, $P < 0.01$), block 2 ($t_{19} = 2.10$, $P < 0.05$), block 3 ($t_{19} = 3.47$, $P < 0.01$). These data

suggest that a nonspecific manipulation that decreased Wnt functioning soon after fear conditioning leads to decreases in the expression of fear behavior 48 h later, consistent with a role for Wnt signaling within the amygdala in memory formation or consolidation.

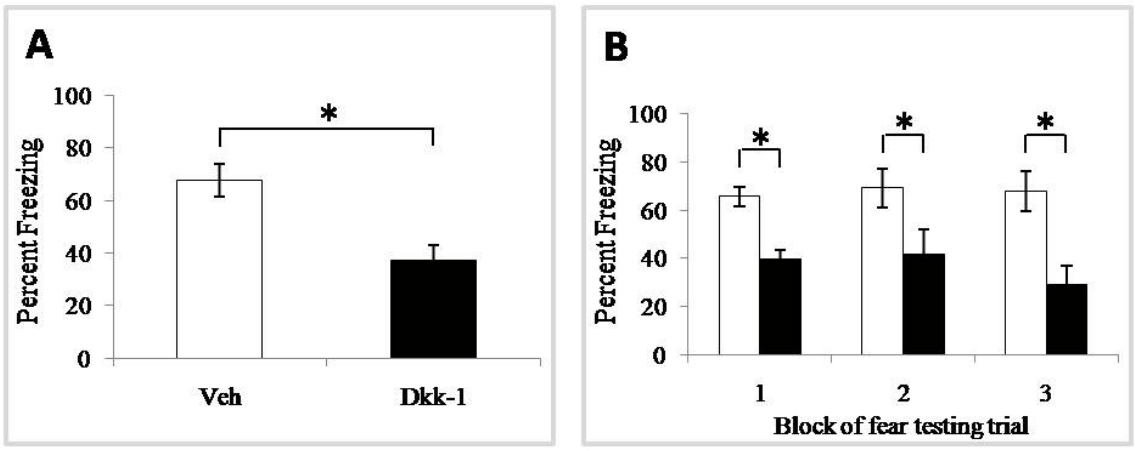

Figure 3.5 - The effect of Dkk-1 in the amygdala on fear learning and memory when given immediately post-acquisition

(A) Percent time spent freezing when tested 48 h after fear conditioning, and presented with 15 tone presentations. (B) Freezing data from A, presented in 3 blocks of 5 tone presentations. n = 11 for Veh, 10 for Dkk-1. Mean ± s.e.m. * $P < 0.05$.

Dkk-1 prevents learning-dependent re-stabilization between β-catenin and cadherin

We have shown that inhibiting Wnt signaling immediately following learning impairs memory formation. Next, we wanted to examine the biochemical changes that may be affected by interfering with Wnt signaling during the initial consolidation period

following learning. Previous *in vitro* research has shown that Dkk-1 decreases the accumulation of β-catenin and cadherin at the cell membrane, resulting in a decrease in cell-cell adhesion[120]. Since we have previously shown that the association between β-catenin and cadherin is decreased immediately following learning, which then returns to baseline at 2 h, we wanted to examine if inhibiting Wnt signaling would prevent the re-association of the β-catenin/cadherin complex at the 2 h time point.

We infused either saline or Dkk-1 bilaterally into the amygdala prior to context exposure or fear conditioning, and sacrificed the animals 2 h later. We then collected the brains, immunoprecipitated β-catenin from the amygdalae and probed with an antibody to pan-cadherin. We found a significant main effect for treatment ($F_{3,35} = 2.86$, $P \leq 0.05$, Figure 3.6). *Post hoc* least-square difference analyses indicated that the amount of cadherin coimmunoprecipated with β-catenin was significantly lower in trained mice receiving injections of Dkk-1 prior to conditioning than in context control mice ($P < 0.01$) as well as fear conditioned mice ($P < 0.05$) receiving vehicle injections. These results suggest that exogenous Dkk-1 may impair memory formation by preventing the re-stabilization of the β-catenin-cadherin interaction during the early consolidation period.

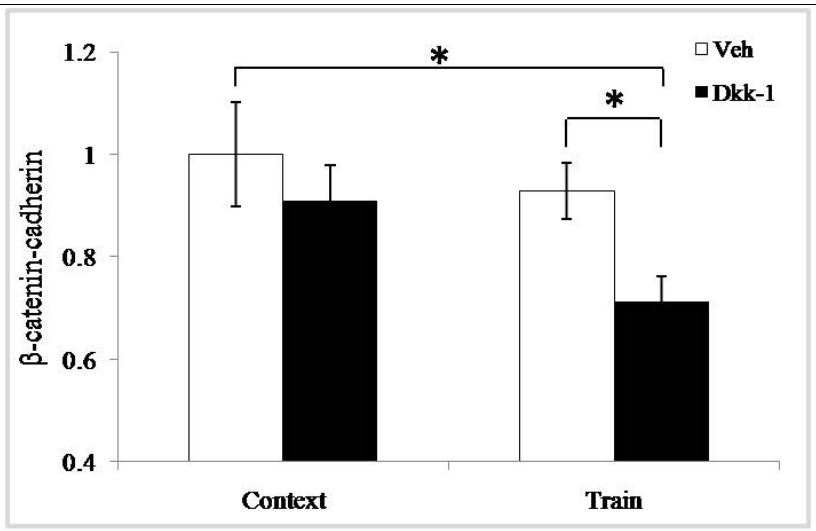

Figure 3.6 - The effect of fear conditioning and Dkk-1 administration on the interaction between β-catenin and cadherin

Quantitative western blot data showing a coimmunoprecipitation of cadherin from β-catenin. n = 9 for Veh_Context, n = 11 for Veh_Train, n = 10 for Dkk-1_Context, n = 9 for Dkk-1_Train. Mean ± s.e.m., * $P < 0.05$.

Genes in Wnt-mediated signaling are altered during memory formation

As shown above, inhibiting Wnt signaling via Dkk-1 interferes with fear memory. However, since Dkk-1 acts as an antagonist to the Wnt peptide receptors, we wanted to examine the role of specific Wnts and other related genes in fear learning and memory. Thus we performed a series of mRNA expression arrays focusing on 84 Wnt-related genes, to identify which genes were endogenously regulated within the amygdala during the memory consolidation period following fear learning.

Following five days of habituation to the conditioning chambers, behaviorally naïve mice received five tone-shock pairings. A context control group was placed in the conditioning chambers for the same amount of time, but no stimuli were presented. We collected brains from the control mice 2 h after context exposure; brains from the trained mice were collected immediately, 0.5, 2, 4, 12, or 24 h after conditioning.

The amygdalae from the above mice were dissected and RNA was isolated. Equal amounts of RNA from each animal were then pooled into seven groups, one group for each time point, including context control. Total RNA was converted to cDNA, and the samples were then subjected to quantitative gene expression analysis using rtPCR arrays containing genes involved in Wnt signaling, as well as housekeeping genes.

The results from the PCR arrays showed multiple patterns and amplitudes of modulation for the expression of Wnt genes following learning (Table 3.1). We did not find any changes in the control housekeeping genes across groups. We found that 15 out of 17 Wnts (and over 50 of all Wnt-related genes examined) decrease relative to control, immediately following fear conditioning (Figure 3.7 and Table 3.1). Furthermore, the expression patterns for most of the Wnts could be assigned to one of two categories at the 0 and 0.5 h time points, the 2 and 4 h time points, and the 12 and 24 h time points (Figure 3.7). Together these data suggest that transcriptional regulation or mRNA stability of Wnt genes are highly correlated with new memory formation within the amygdala.

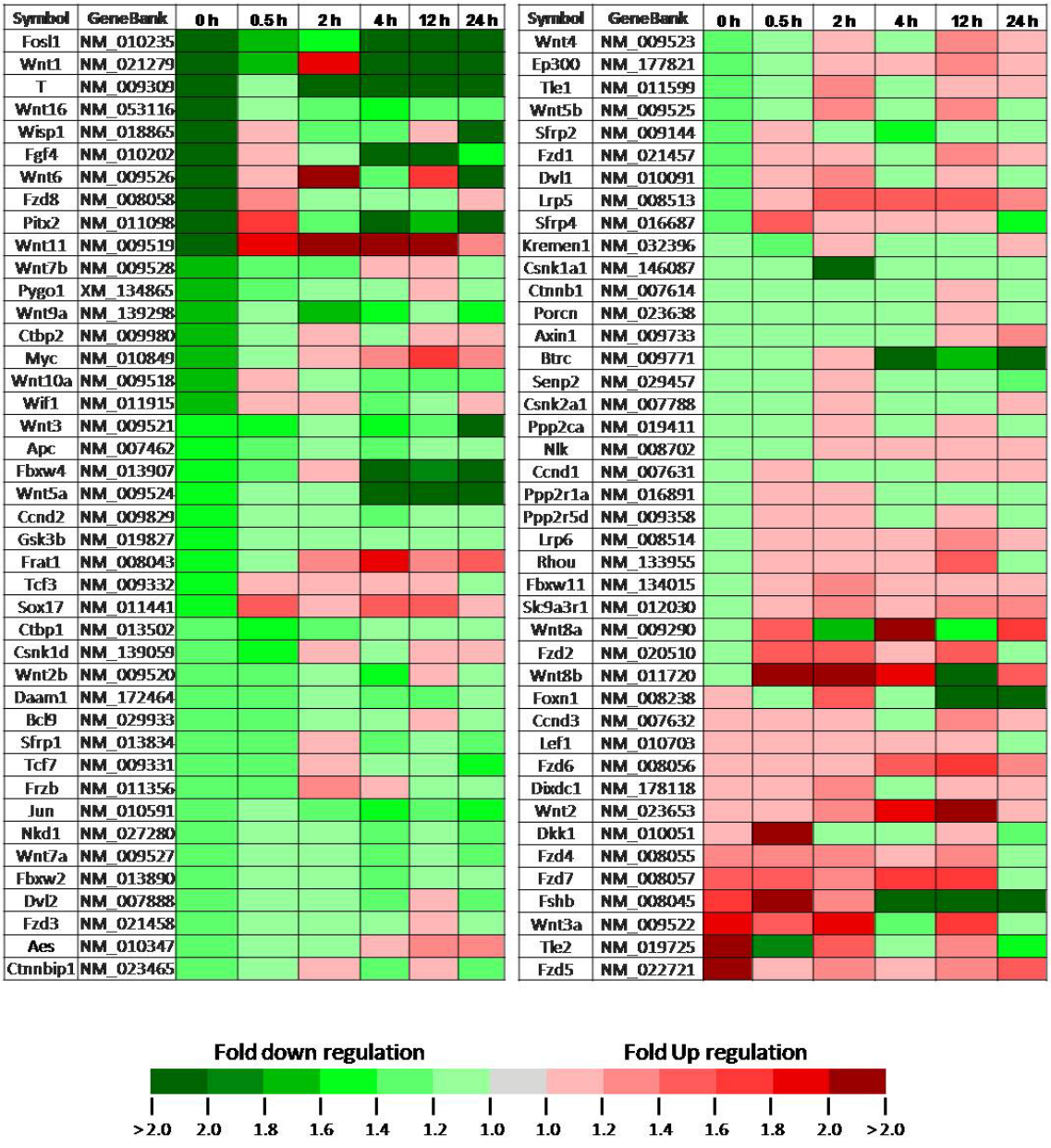

Table 3.1 - Temporal changes, expressed as fold regulation, in the expression of 84 genes related to Wnt-mediated signaling following fear conditioning.

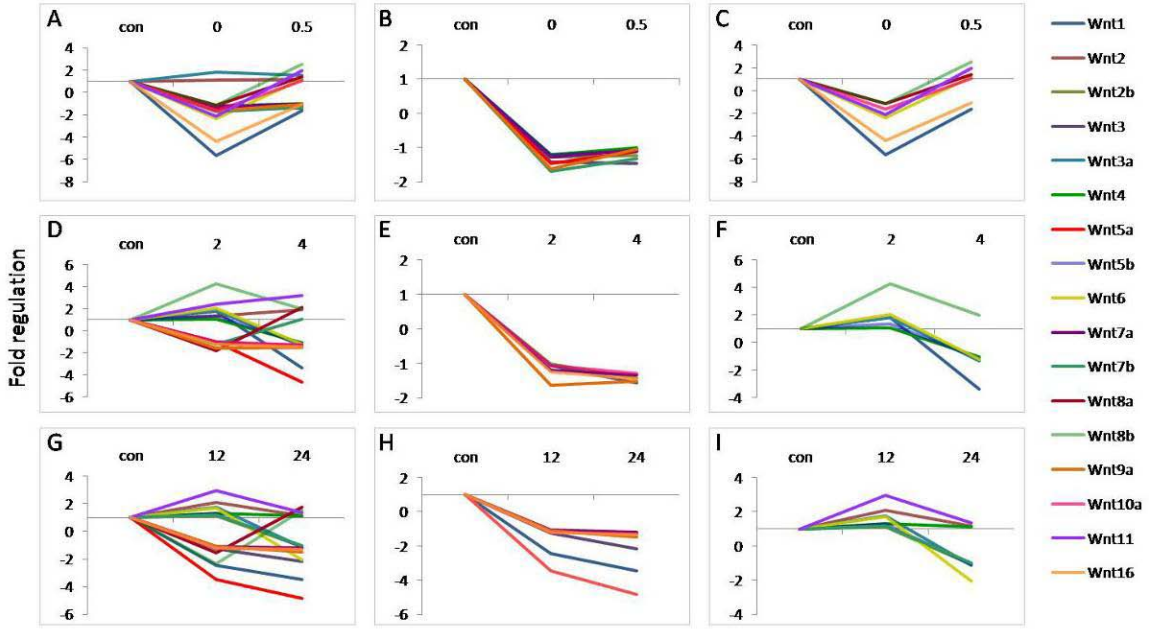

Figure 3.7 - Temporal changes in Wnt gene expression after fear conditioning

All genes are expressed as fold up or down regulation from context exposed animals. (A) The expression of 17 Wnt genes at 0 and 0.5 h after conditioning. (B) 8 of the 17 Wnt genes go down immediately after conditioning, and stay down at 0.5 h (C) 7 of the Wnt genes go down immediately after conditioning, and go back up at 0.5 h. (D) The expression of 17 Wnt genes at 2 and 4 h after conditioning. (E) 6 of the Wnts go down at 2 h after conditioning and stay down at 4 h. (F) 6 of the Wnts either stay the same or go up at 2 h and then decrease at 4 h post conditioning. (G) The expression of Wnt genes at 12 and 24 h after conditioning. (H) 7 of the Wnt genes go down at 12 h and stay down at 24 h. (I) 8 of the Wnt genes either stay the same or go up at 12 h and down at 24 h.

Wnt1 gene expression changes with learning

Among all the Wnts in the PCR array, Wnt1 showed the greatest fold change following fear conditioning. Therefore, we wanted to confirm and replicate this initial finding based on PCR array. We performed another experiment with a new series of naïve mice using quantitative real-time PCR (qPCR) specifically focused on the Wnt1 gene. After five days of habituation to the conditioning chambers, mice were either exposed to the context alone, to unpaired tone and shock presentations, or to five paired tone and shock presentations. The mice were then sacrificed as above. RNA from both amygdalae of each mouse was prepared, and then analyzed by qPCR. We found that the pattern of expression from the qPCR results closely resembled the pattern observed from the PCR array (Figure 3.8 A,B). More specifically, we observed a significant decrease in Wnt1 gene expression immediately following fear conditioning, compared to animals exposed to the context, without any stimuli presentations (ANOVA, $F_{7,83} = 3.18$, $P < 0.01$. *Post hoc pairwise comparisons:* 0 h is significantly decreased from context, unpaired, 2, 4, 12, and 24 h). This effect does not appear to be due to the presentation of the tone or the stress of shock alone, since the unpaired group did not differ significantly from the context group ($P > 0.1$).

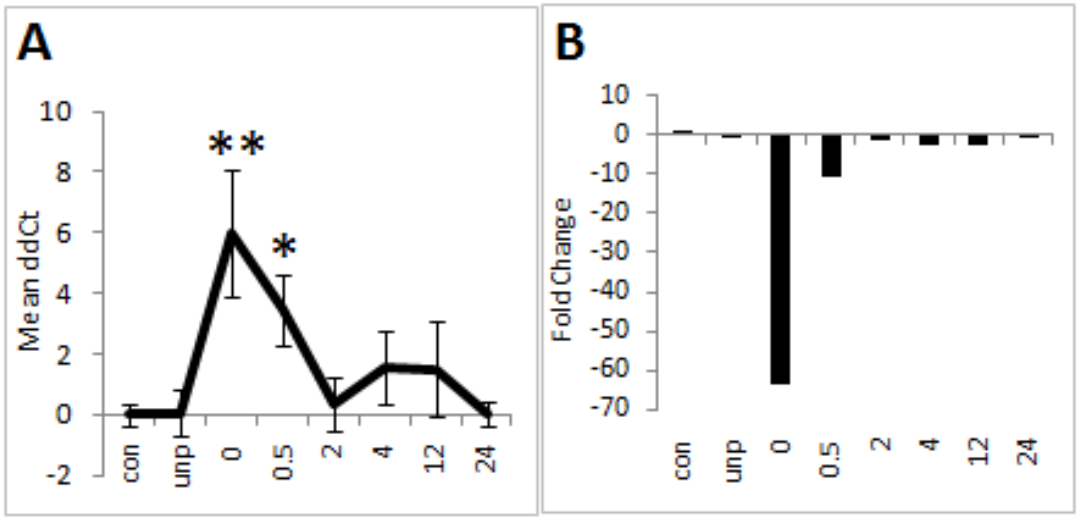

Figure 3.8 - Wnt1 mRNA expression in the amygdala

(A) Quantitative RT-PCR data showing the mean ΔΔCt values ± s.e.m. across different conditions. Note that at the 0 (immediate) timepoint post-fear conditioning, detecting the Wnt mRNA signal took on average 6 PCR cycles more than in the control and unpaired conditions. (B) The same data as in (A) presented as mean fold up or down regulation of Wnt1 mRNA compared to context-exposed mice. n = 14 for context, 7 for unpaired, 11 for 0 and 0.5 h, 12 for 2, 4, 12, and 24 h. * $P < 0.05$, ** $P < 0.01$.

To further validate our Wnt1 results, we fear conditioned a separate group of animals, and sacrificed the mice at the same time points as above. This time, however, we analyzed the expression of Wnt1 by *in situ* hybridization (Figure 3.9 A-D). This method allowed us to look more specifically at the regional expression pattern of Wnt1, focusing on the basolateral amygdala (BLA). Again, we found that Wnt1 mRNA in the BLA was altered with fear conditioning, similar to the pattern observed in both the array and the

qPCR ($F_{5,47} = 18.36$, $P < 0.01$, Figure 3.9 E). *Post hoc* least-squares difference analyses indicated a significant decrease in Wnt1 mRNA expression immediately after, 0.5, 2, and 4 h after fear conditioning ($P < 0.01$) compared to context animals and animals sacrificed 24 h after conditioning. We did not find any significant differences in Wnt1 mRNA in the striatum ($P > 0.1$), a brain region not involved in cue-dependent fear conditioning. Interestingly, the pattern that was observed was remarkably similar to the β-catenin/cadherin destabilization time course that we have previously reported[118], suggesting that Wnt1 regulation within the amygdala during fear consolidation may be highly correlated with the previous β-catenin effects we found to be required for normal memory formation.

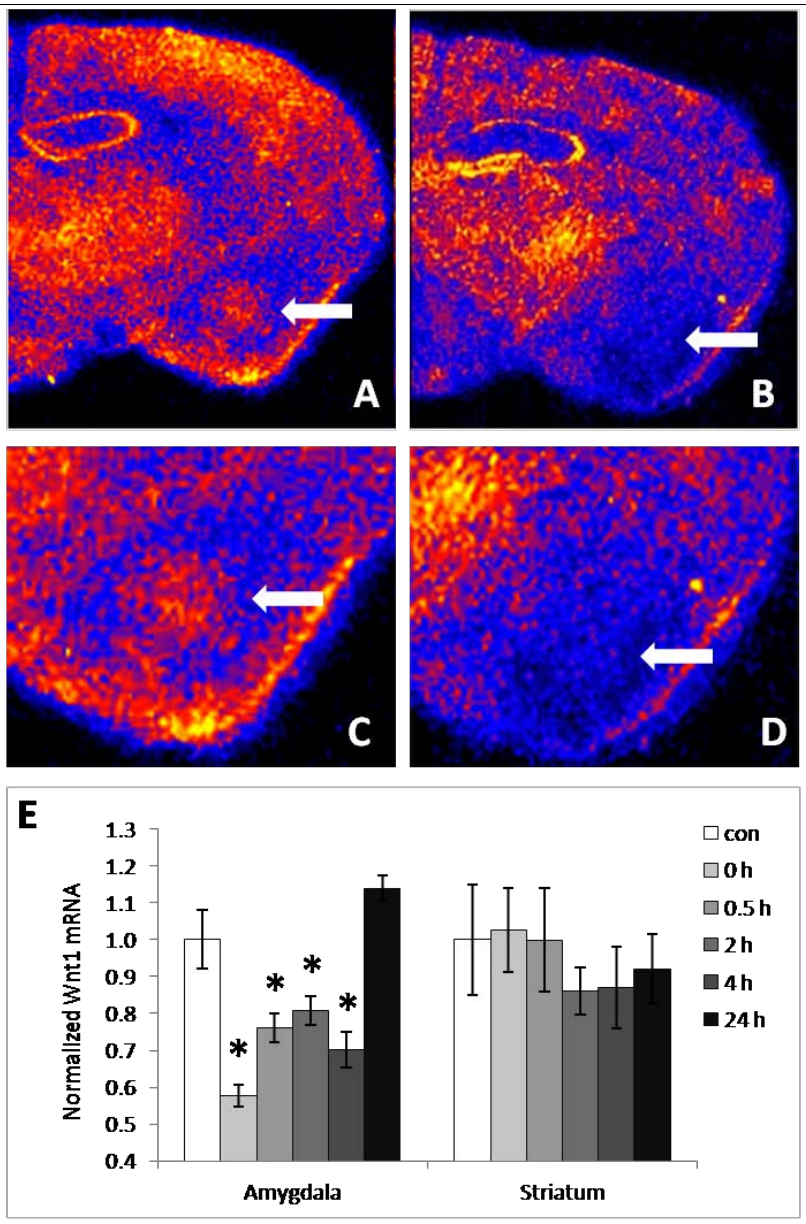

Figure 3.9 - Wnt1 mRNA expression in the amygdala

(A-D) Pseudolocolored *in situ* hybridization of Wnt1 in context exposed animals (C,E) and animals sacrificed immediately after training (D,F). Yellow, highest expression; blue-black, lowest expression. Arrow points to amygdala. (G) Relative expression of Wnt1 mRNA in the amygdala and striatum, normalized to expression in context-exposed mice. n = 8 per group. Mean ± s.e.m., *P < 0.01.

Increasing Wnt1 impairs fear memory formation

The above data suggest that the transient decrease in Wnt1 expression immediately following fear conditioning is important for memory formation. Therefore, we examined if preventing this transient decrease by replacing Wnt1 in the amygdala would produce deficits in memory formation. First, we infused either a Wnt1 peptide (100 ng/side) or saline bilaterally into the amygdala of adult mice, and processed the tissue for immunohistochemistry. We found that infusion of the peptide led to an increase in the detectable expression levels of Wnt1 in the amygdala, compared to control animals (Figure 3.10 A,B). Furthermore, the infusion did not produce any structural damage to the amygdala, as visualized by Nissl staining (Figure 3.10 C,D).

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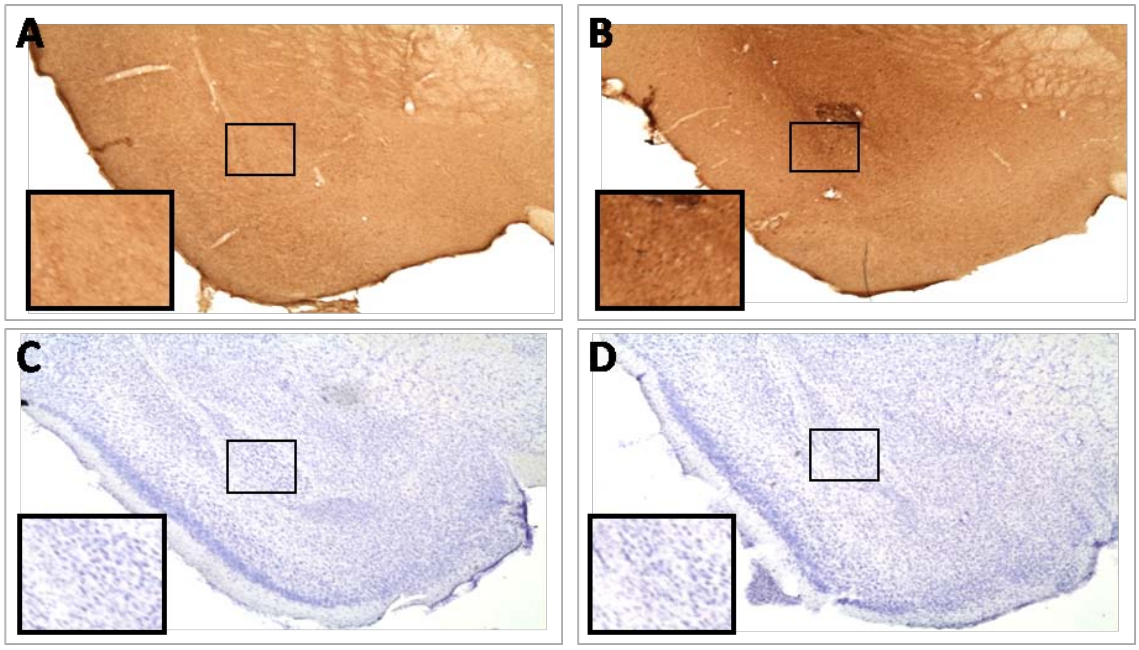

Figure 3.10 - Wnt1 expression in the amygdala

(A,B) Immunohistochemical analyses of Wnt1 in animals receiving bilateral amygdala injections of either vehicle (A) or Wnt1 (B). (C,D) Cresyl violet staining of parallel sections showing the absence of damage in vehicle (C) or Wnt1 (D) infected regions.

We next examined whether Wnt1 would affect baseline locomotion or anxiety-like behaviors. We injected Wnt1 prior to open-field behavior and found that there was no difference ($P > 0.1$) between Wnt1 and vehicle on mouse locomotion (distance traveled) or anxiety-related behavior (time spent in the center compared to time in the surround) (Figure 3.11).

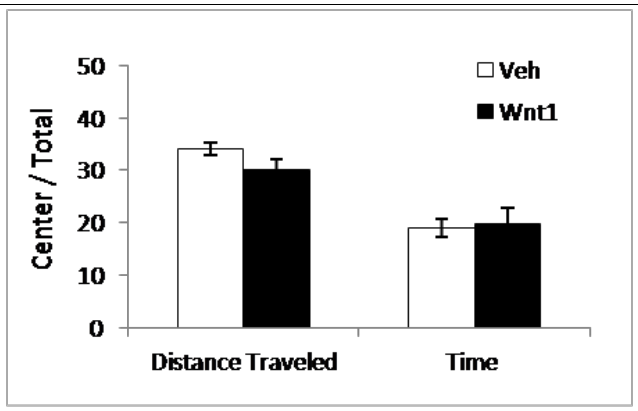

Figure 3.11 - The effect of Wnt1 in the amygdala on baseline anxiety

Activity measures of mice receiving injections of vehicle or Wnt1 and placed in an open field apparatus for 10 m. n = 13 for vehicle, n = 15 for Wnt1.

We then infused Wnt1 or saline immediately before and after fear conditioning to determine if increasing Wnt1 affects memory formation. The intensity of the unconditioned stimulus was lowered to 0.6 mA to prevent ceiling effects on fear expression. We found that all mice were able to acquire fear equally ($F_{5,125} = 35.36$, $P < 0.01$, Figure 3.12), irrespective of whether they had saline or Wnt1 infused into the amygdala prior to conditioning ($P > 0.1$).

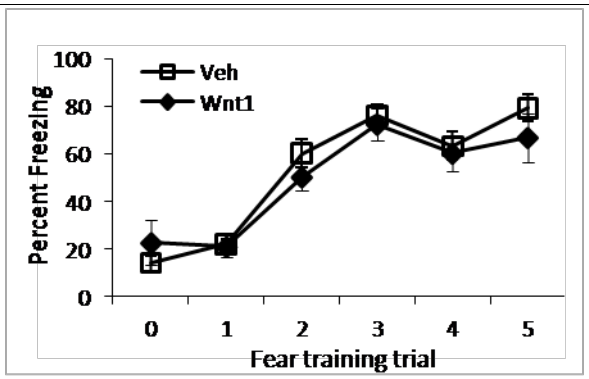

Figure 3.12 - The effect of Wnt1 in the amygdala on fear learning and memory

Acquisition curve showing percent time spent freezing during each tone prior to footshock presentation. n = 15 for vehicle, n = 23 for Wnt1.

However, when tested 48 h later, there was a significant difference between animals that had received vehicle or Wnt1 prior to training ($F_{2,26} = 10.73$, $P < 0.01$). *Post hoc* analyses revealed that animals that had received Wnt1 prior to training froze significantly less compared to animals that had received either saline prior to training ($P < 0.01$), or Wnt1 post training ($P < 0.01$, Figure 3.13 A). This difference in fear retention across treatment was apparent throughout the testing session ($F_{2,24} = 10.73$, $P < 0.01$, Figure 3.13 B). When examined in three blocks of five tone presentations each, *post hoc* analyses indicated that animals receiving Wnt1 prior to training froze significantly less than animals receiving vehicle prior to and after training during block 1 ($P < 0.01$), block 2 ($P < 0.01$) and block 3 ($P < 0.05$). In addition, animals receiving Wnt1 prior to training froze significantly less than animals receiving vehicle before and Wnt1 after training for all blocks ($P < 0.01$). These data suggest that infusing Wnt1 into the amygdala, counteracting the normal rapid decrease in Wnt1, prevents fear memory formation. If

Wnt1 is given following fear conditioning, however, after this critical period of transient endogenous Wnt decrease, then normal learning occurs. Thus, the rapid decrease in endogenous Wnt1 mRNA which likely begins at the end of fear training and is observed immediately after conditioning may be critical for the formation of fear memory.

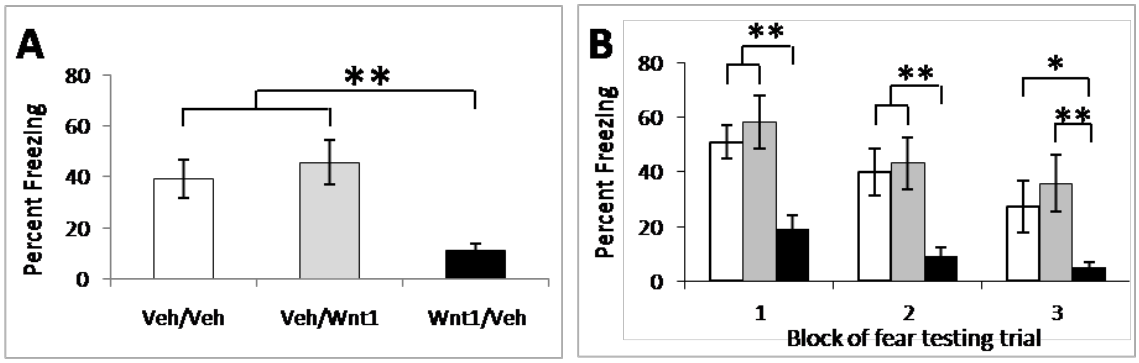

Figure 3.13 - The effect of Wnt1 in the amygdala on fear learning and memory

(A) Percent time spent freezing when tested 48 h after fear conditioning, and presented with 15 tone presentations. (B) Freezing data from A, presented in 3 blocks of 5 tone presentations. Each condition denotes the amygdala infusion prior / immediately after fear conditioning. White bars are Veh/Veh, Grey bars are Veh/Wnt1, and black bars are Wnt1/Veh animals. For both A and B, n = 8 for Veh/Veh, n = 7 for Veh/Wnt1, n = 12 for Wnt1/Veh. Mean ± s.e.m. **P < 0.01, * P < 0.05.

Wnt1 prevents learning-dependent disassociation between β-catenin and cadherin

We have shown that preventing the Wnt1 decrease during or immediately following learning impairs memory formation. Next, we wanted to examine the biochemical changes that may be occurring at this time point. Previous *in vitro* research has shown

that Wnt1 increases cell-cell adhesion by promoting the binding of β-catenin to cadherin[121]. Our finding of a decrease in Wnt1 gene expression appears to be correlated with the rapid pattern of β-catenin-cadherin dissociation following learning. Since we have previously shown that the association between β-catenin and cadherin is decreased immediately following learning, we wanted to examine if increasing Wnt1 prior to or soon after fear conditioning would prevent this observed dissociation.

In training group (T) of animals, we infused either Wnt1 (Wnt1_T) or saline (Veh_T) bilaterally into the amygdala prior to fear conditioning. A third control group was included, which received saline prior to context exposure, but did not receive any stimulus presentations (no training: Veh_NT). In agreement with our previous results, all trained animals were able to equally acquire the fear during the conditioning ($F_{5,145}$ = 26.59, $P < 0.01$). In addition, animals receiving training froze significantly more across time compared to the untrained control group ($F_{1,37} = 27.57$, $P < 0.01$, Figure 3.14 A). A subset of these animals was sacrificed immediately after training or exposure to the context, and brains were collected. We then immunoprecipitated β-catenin from the amygdalae and probed with an antibody to pan-cadherin. We found a significant main effect for treatment ($F_{2,25} = 3.35$, $P \leq 0.05$). The amount of cadherin coimmunoprecipitated with β-catenin was significantly lower in trained mice immediately after conditioning than in context control mice as we had previously demonstrated ($t_{13} = 2.68$, $P < 0.5$, Figure 3.14 B). Notably, we found that this decrease was abolished when Wnt1 was injected into the amygdala prior to training ($t_{12.37} = -2.89$, $P \leq 0.01$), preventing the normal learning-dependent transient decrease in Wnt1 expression. These results suggest that exogenous Wnt1 may impair memory formation by

stabilizing β-catenin-cadherin interactions during the early consolidation period, thus preventing the normal transient β-catenin-cadherin disassociation that occurs with transient Wnt1 depletion.

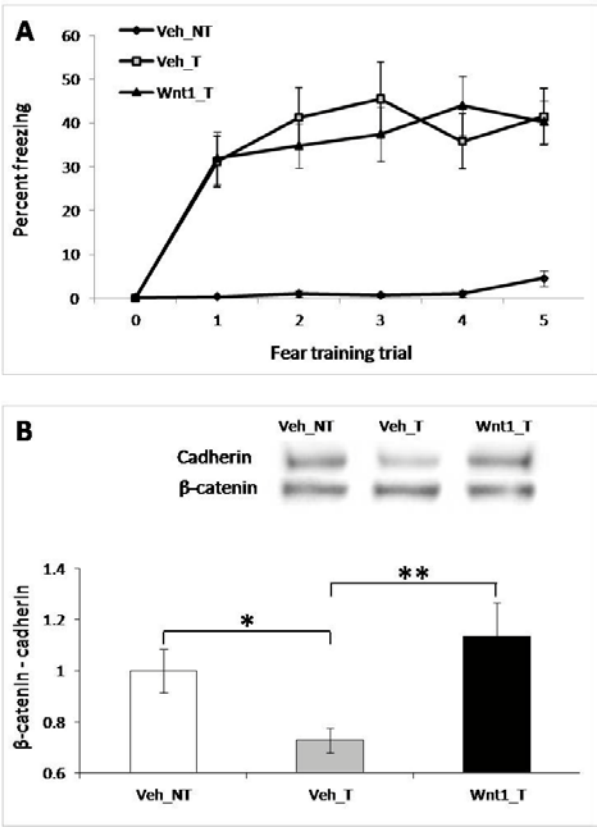

Figure 3.14 - The effect of fear conditioning and Wnt1 administration on the interaction between β-catenin and cadherin.

(A) Acquisition curve showing percent time spent freezing in untrained animals (Veh_NT) compared to trained animals receiving either vehicle (Veh_T) or Wnt1 (Wnt1_T) prior to fear conditioning. n = 8 for Veh_NT, n = 12 for Veh_T, n = 19 for Wnt1_T. (B) Qualitative and quantitative western blot data showing a co-immunoprecipation of cadherin from β-catenin. n = 8 for Veh_NT, n = 7 for Veh_T, n = 11 for Wnt1_T. Mean ± s.e.m., * $P < 0.05$, ** $P \leq 0.01$.

DISCUSSION

Our data suggest that Wnt signaling in the amygdala plays an important role in long term memory formation. We showed that interfering with Wnt signaling, by infusing the antagonist, Dkk-1, into the amygdala of adult mice impaired long-term memory formation. This impairment may be due to the ability of Dkk-1 to prevent cell-cell adhesion, and thus the stabilization of memory.

Further examination of Wnt signaling during the early consolidation of memory revealed that a number of genes in this pathway are dynamically regulated with fear conditioning. The most striking difference was a rapid downregulation of most Wnt genes, with Wnt1 having the most robust effect, immediately after conditioning. This was found across several replication studies using different methodologies. Increasing Wnt1 levels, by infusing Wnt1 peptide directly into the amygdala, not only impaired memory consolidation, but also prevented the transient decrease in β-catenin-cadherin interaction that may be required for memory consolidation and long-term memory formation.

Dkk-1 is a known negative modulator of Wnt signaling, and abnormalities in Wnt signaling have been associated with human memory disorders such as Alzheimer's disease[78, 122]. Previous *in vitro* work has shown that overexpression of Dkk-1 prevents activity-dependent dendritic branching[53], and decreases cell-cell adhesion[120]. Our results show that overexpression of Dkk-1 *in vivo* prevents memory formation. We found that infusing Dkk-1 into the amygdala before fear conditioning does not affect the acquisition or immediate expression of fear, but does produce deficits in learning when measured 48 h after training. Similar effects were observed when Dkk-1 was injected immediately following fear conditioning. The observed behavioral effect does not appear to be a result

of neurotoxicity or alterations in baseline anxiety or locomotor activity. Notably, as shown in Table 3.1, we find that endogenous Dkk-1 is transiently increased during fear consolidation. This is consistent with a transient decrease in Wnt signaling playing a role in plasticity and memory formation. We think that disrupting the normal temporal control of Wnt inhibition, as well as the likely more profound effect of exogenous Dkk-1 beyond endogenous levels, led to abnormal Wnt regulation in the first experiment. Thus, the Dkk-1 behavioral results suggest that dynamic regulation of Wnt signaling is required for the consolidation of new memory.

The inhibition of Wnt signaling can lead to alterations in a variety of genes. The results from our PCR array allowed us to examine alterations in individual genes related to Wnt-mediated signaling. We found that over 50 Wnt signaling genes within the amygdala are dynamically regulated during the memory consolidation period following fear learning. This finding is consistent with other research showing that fear conditioning can differentially affect the expression of various genes in the amygdala[123-126]. However, to our knowledge, this is the first evidence that rapid, dynamic regulation of Wnt signaling is involved in memory formation or in neural functioning *in vivo* in adult animals.

We found that many of the Wnts show rapid regulation (15 of 17 are immediately decreased) during memory consolidation. Activity-dependent changes in Wnt expression have also been shown as a result of LTP induction in a mouse hippocampal slice model[29]. In the current studies, we showed that Wnt1, compared to the other Wnt genes, was found to have the most substantial changes immediately following conditioning. Therefore, we focused our remaining studies on the role of Wnt1 in memory formation.

To further examine its role in memory formation, *in vivo*, we infused Wnt1 into the amygdala either before or after fear conditioning. We found that infusing Wnt1 in the amygdala prior to training did not affect acquisition, but resulted in deficits in the expression of fear as measured 48 h after training. The infusion of Wnt1 after training did not have an effect on learning. Given that acquisition of fear appeared to be normal in the presence of Wnt1, these data suggest that we may be preventing the normal rapid decrease in Wnt signaling that occurs at the end of training and immediately during the early period of consolidation. These data suggest that an immediate decrease in Wnt1 with learning may be required for memory formation. Thus, the infusion of Wnt1 prior to training may allow just enough time to reverse that downregulation of Wnt1 gene expression, preventing consolidation. Conversely, infusing Wnt1 after training may not increase expression of Wnt1 until after a critical period of early consolidation has passed. Therefore, our results suggest that specifically inhibiting the decrease in Wnt1 expression which occurs with training produces memory deficits.

Interestingly, the observed downregulation of endogenous Wnt1 immediately following training closely resembles the alterations seen in the interaction of β-catenin with cadherin following fear conditioning[118]. There has been much discussion regarding the crosstalk between Wnt signaling and β-catenin–mediated cell adhesion. Previous *in vitro* work in nonneuronal cells has shown that Wnt stimulation leads to an increase in cell-cell adhesion[121, 127]. Furthermore, there are many lines of convergent data suggesting that Wnts may both directly and indirectly regulate β-catenin interactions with cadherin[110]. Our results showed that Wnt1 may have a similar effect in promoting β-catenin/cadherin stability *in vivo* within the adult brain, and conversely that Wnt

inhibition or downregulation may destabilize these complexes. We found that infusing Wnt1 into the amygdala prior to training prevented the destabilization of the β-catenin/cadherin interaction that occurs immediately following fear conditioning. This lack of transient destabilization may prevent synaptic modifications from taking place which are required for new memory formation.

We have previously shown that β-catenin in the amygdala is required for the normal consolidation of fear memory, and that the interaction between β-catenin and cadherin is altered during this process[118]. Those data suggest that β-catenin/cadherin complexes are involved in normal synaptic stability, and that transient disruption of stable synapses may be required for new synapse formation to occur. Here, we show that Wnt signaling may contribute to β-catenin's effect on learning and memory. We propose that during the acquisition of fear memory and immediately after, normal Wnt signaling is significantly reduced, which may allow for phosphorylation of the Y654 site on β-catenin, contributing to β-catenin–cadherin destabilization[54], and subsequent synaptic weakening[111]. Then, once the synapses have been modified during the consolidation process, Wnt signaling is normalized, the β-catenin Y654 site is dephosphorylated, and β-catenin-cadherin interaction is subsequently restored, stabilizing the new synapses[43]. These processes of Wnt-dependent β-catenin/cadherin mediated synapse stability, coupled with transient destabilization and re-stabilization during memory consolidation, may provide for a structural mechanism underlying long-term memory formation and stability.

METHODS

Animals

Adult male C57BL/6J mice (Jackson Labs) were used for all experiments. Mice were housed four per cage in a temperature-controlled (24 ^{o}C) animal colony, with *ad libitum* access to food and water, on a 12-h light-dark cycle, with all behavioral procedures done during the light cycle. All procedures used were approved by the Institutional Animal Care and Use Committee of Emory University and in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

Stereotaxic surgery and infusion of peptides

Mice were anesthetized by intraperitoneal injections of a ketamine-dormitor (medetomidine) mixture and placed in a stereotaxic apparatus. Small holes were drilled into the skull and 6 mm stainless-steel guide cannulae (Plastics One) were lowered bilaterally into the basolateral amygdala (BLA). BLA coordinates were as follows: anteriorposterior, -1.8; dorsoventral, -4.9; mediolateral, ± 3.2 relative to bregma. Dorsoventral coordinates were measured from the skull surface with the internal cannula extending 2 mm beyond the end of the guide cannula. Coordinates were based on the mouse brain atlas of Paxinos and Watston[90]. The guide cannula was fixed to the skull using dental acrylic and jeweler's screws and dummy cannulae (Plastics One) were inserted into each guide cannula to prevent clogging. All animals were allowed to recover

for 7 days before testing. During this time, mice were handled daily for acclimation and inspection of cannula fixture.

Animals were infused with either Dickkopf1 (R&D Systems) (100 ng/side), Wnt1 (AbD Serotec) (100 ng/side), or PBS (vehicle). 1.0 µl infusions were made using an injection cannula (33 gauge cannula, Plastics One), which extended 2.0 mm beyond the tip of the guide cannula. Peptide was delivered manually with a 5 µl Hamilton syringe attached to the injection cannula via polyethylene tubing (PE-10). Administration of a volume of 1.0 µl/side was delivered over a period of 60s by slowly turning the microsyringe plunger. After each infusion, the injection cannula was allowed to remain for 2 min. Visualization of injection sites was performed postmortem to verify the location of peptide infusion.

Immunoprecipitation and Immunoblotting

After behavioral procedures, brains were blocked rapidly and kept frozen at -80 ^{o}C. Bilateral amygdala punches were obtained and homogenized. For immunoprecipitation experiments, solubilized proteins were incubated with Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) and centrifuged. Protein A/G PLUS-Agarose beads and antibody to β-catenin (1:200, Cell Signaling) were then added to the supernatant of each protein sample, incubated overnight at 4 ^{o}C, and washed prior to western blot analyses. For immunoblotting, twenty five micrograms of protein per mouse were electrophoretically separated on SDS-PAGE, transferred onto nitrocellulose membranes (BioRad), blocked for 1 h in 2% nonfat dry milk, 0.1% Tween 20, 50 mM

NaCl, 10 mM HEPES, pH 7.4. Membranes were incubated in primary antibody overnight at 4 ^{o}C. Antibodies to the following proteins were used: β-catenin (1:500, BD Biosciences), pan-cadherin (1:1000, Cell Signaling). Membranes were washed and incubated with a horseradish peroxidase-labeled secondary antibody (1:5000, Vector), then detected by SuperSignal West Chemiluminescence (Pierce) in an Alpha Innotech Fluorchem imaging system (Alpha Innotech).

Immunohistochemistry

Brain sections (40 µm) were blocked with 0.5 M PBS and 0.5 % Triton X-100, and incubated in a 1:100 dilution of primary Dkk1 (Santa Cruz) or Wnt1 (Santa Cruz) antibodies overnight at 4 ^{o}C. Sections were then washed with PBS and bathed in a 1:500 dilution of secondary antibody for 2 hr. Avidin-biotin complexes were amplified using a standard Vectastain Elite ABC kit and visualized with diaminobenzidine (DAB) peroxidase staining.

Behavioral studies

Open Field Behavior

The open field consisted of a box (27.9 cm x 27.9 cm) made of Plexiglass. The mice were placed in the periphery of the arena at the start of the 10-m test period. At the end of the test, the animal was returned to its home cage. The anxiolytic-like effects were evaluated by computing percentage of time mice spent in the central zone of the open

field. The central zone was defined as the central compartment of the floor centrally located 6 cm from the perimeter of the chamber walls. All testing was conducted under standard room lighting. Activity data was obtained and analyzed using the Open Field Activity Software (Med Associates Inc.)

Fear Conditioning Apparatus

Mice were fear conditioned in eight identical startle response systems (SR-LAB, San Diego Instruments). Each system consisted of a nonrestrictive Plexiglas cylinder (5.5 cm in diameter and 13 cm long) mounted on a Plexiglas platform and located in a ventilated, sound-attenuated chamber. Cylinder movements were sampled each millisecond by a piezoelectric accelerometer mounted under each platform. The footshock unconditioned stimulus (US) was generated by a programmable animal shocker (San Diego Instruments) located outside the isolation chambers and was delivered through the cage floor bars. The conditioned stimulus (CS) was a tone delivered by a speaker located about 15 cm above the chambers. Sound intensities were measured by an audiometer (Radio Shack). Stimuli presentation and data acquisition were controlled, digitized, and stored by a Dell computer using SR-LAB software.

Fear Conditioning

After five days of exposure to the conditioning chambers, mice were placed in the chamber, and after 5 min presented with five tone-shock pairings at an inter-trial interval (ITI) of 5 min. Each pairing consisted of a 30-s tone (6 kHz, 85 db, CS) that terminated with a 0.5 s footshock (1.0 mA, except where noted; US). Freezing in startle-reflex chambers during fear acquisition was assessed as described previously[118]. Forty-eight

hours after training, mice were tested for freezing in a separate context: Med Associates rodent modular test chambers with an inside volume of 30.5 cm x 24.1 cm x 21.0 cm. Three minutes later, 15 conditioned stimulus tones (6 kHz, 85 db) with an ITI of 1.5 min were delivered through a high-frequency speaker attached to the side of each chamber. Percentage time spent freezing during the conditioned stimulus presentation was calculated for each mouse using FreezeFrame video monitoring software (ACT-100; Coulbourn Instruments), using settings which were previously calibrated to levels of observed freezing.

RNA extraction and cDNA synthesis

RNA isolation was carried out using the Qiagen RNeasy Micro Kit for animal tissues (Qiagen). In short, amygdala punches were lysed and then homogenized. After centrifugation, ethanol was added to the lysates, and the samples were loaded onto the column, followed by DNase treatment. The columns were then washed to remove DNase and any other contaminants, and the remaining pure, concentrated RNA was eluted in RNase-free water. 1.0 µg of total RNA was reverse transcribed in a final reaction mix of 20 µl using RT2 First Strand Kit (SuperArray Bioscience) according to manufacturer's instructions. cDNA was diluted by adding RNase free water.

Real-time PCR

Real-time PCR was performed using 2×SuperArray RT qPCR Master Mix (SA Biosciences) and RT^2 Profiler^{TM} PCR Array System (PAMM-043, SA Biosciences) or

mouse Wnt1 PCR primer stock (SA Biosciences). The Wnt Pathway array consisted of 84 genes related to Wnt-mediated signal transduction, along with appropriate RNA quality controls, in 96-well plates. For each array, amygdala samples from each experimental group were pooled together. For the Wnt1 PCRs, amygdala samples were analyzed individually. Thermal cycling parameters were 10 m at 95 ℃, followed by 40 cycles of amplifications for 15 s at 95 ℃, 1 m at 60 ℃. A dissociation stage, consisting of 15 s at 95 ℃, 1 m at 60 ℃, and 15 s at 95 ℃, was added at the end. Quantification of mRNA was performed using the Applied Biosystems 7500 Real-Time PCR System. Relative levels of mRNA expression were normalized in all the samples with expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For the Wnt1 PCRs, the ΔΔCts were compared across groups. Figure 3.8 A shows the mean +/- s.e.m. of the ΔΔCt values, and Figure 3.8 B shows the mean fold change following previously described methods[128].

In situ hybridization

In situ hybridization was performed and quantified as previously described[118, 125]. The Wnt1 clone was obtained by inserting the 144 bp Wnt1 RT PCR product from above into the pCR2.1-TOPO vector (Invitrogen). After sequence verification, the cloned cDNA was linearized and antisense riboprobes were generated using T7 RNA polymerase and [35S]-UTP in the reaction. After a stringent wash protocol, slides were apposed to Biomax MR autoradiography film (Eastman Kodak Co.). Hybridization

density of Wnt1 mRNA in the BLA was assessed using the mean luminosity function of Adobe Photoshop.

Data analysis

Statistically significant differences were determined by Student's *t*-test or ANOVA, with *post hoc* least-squares difference tests for multiple comparisons. The results are presented as mean ± s.e.m.

CHAPTER 4

β-Catenin is required for hippocampal-dependent learning in adult mice

ABSTRACT

Although it was initially identified for its role in development, β-catenin is expressed broadly in the adult mammalian brain and is implicated in neuronal synapse regulation and plasticity. Additionally, some disorders of memory impairment, such as Alzheimer's disease, have implicated β-catenin dysfunction. Several lines of evidence suggest that β-catenin plays a critical function in the synaptic remodeling underlying new memory formation, however no studies have demonstrated that β-catenin is required for hippocampal-dependent learning. We examine this process in the present study. Following virally-mediated deletion of β-catenin specifically within the hippocampus of adult mice, we examined a variety of learning and memory paradigms. Our results show that hippocampal-specific β-catenin deletions do not affect baseline anxiety, measures of locomotor activity, or cue-dependent fear learning. However, we found that deletion of β-catenin in the hippocampus produced deficits in the consolidation of context-dependent fear, object recognition and spatial memories. These data suggest that β-catenin may play an active role in facilitating the structural changes that occur during the consolidation period of hippocampal-dependent memory formation.

INTRODUCTION

Structural changes in the nervous system are thought to underlie synaptic plasticity and memory. Such changes may involve the establishment of new synaptic connections or the remodeling of existing synapses[2, 3]. Although structural plasticity is typically associated with development, there is emerging evidence suggesting that

structural plasticity is a lifelong process[129]. One brain region that has been well studied in this area is the hippocampus.

The hippocampus is an ideal brain region to study structural plasticity for several reasons. 1) The majority of physiology evidence for synaptic plasticity is based on the model of long-term potentiation (LTP), a mechanism that relies on changes in strength of synaptic connections. The hippocampus is particularly good for examining this process due to the laminar nature of its cellular organization. 2) The structural changes that are thought to underlie LTP as well as many forms of learning and memory include alterations in dendritic morphology, as well as synapse formation and elimination. All of these processes occur within preexisting neurons in the hippocampus. 3) Additionally, the hippocampus has the capability of producing new neurons through neurogenesis, a process involving the growth of axons, dendrites, and synapses. Therefore, the hippocampus is regarded as a very dynamic and plastic region of the brain. Structural plasticity in hippocampal neuronal connections is thought to be modulated by experience, including learning.

A variety of hippocampal-dependent learning tasks have been shown to produce changes in spine number and morphology, including olfactory learning[130], trace eyeblink conditioning[16], water maze training[131-133], and avoidance conditioning[134]. Alterations in the number and distribution of synapses along dendrites have also been reported following water maze training[135, 136]. Therefore, structural plasticity appears to be important for learning and memory processes that result from hippocampal function.

One molecule that may mediate structural plasticity in the hippocampus is β-catenin, a protein involved in both cell adhesion and gene transcription. It is highly

expressed in the hippocampus, and has been shown to be required for morphological changes in both newborn and preexisting neurons *in vivo*. Postnatal born neurons lacking β-catenin show significant defects in dendritic arborization. These neurons have short, but very few branches[137]. Similarly, β-catenin deletion in hippocampal neuronal cultures has been shown to produce decreases in dendritic growth and arborization[53] as well as changes in the shape and size of dendritic spines[51]. In contrast, overexpression of β-catenin has been shown to increase dendritic growth and arborization[52]. These findings suggest that β-catenin is important for dendrite development and arborization in hippocampal neurons. Since changes in dendritic morphology are associated with learning and memory, β-catenin may be important for hippocampal-dependent memory formation, however this question has never been addressed experimentally.

We have previously shown that β-catenin is required in amygdala-dependent learning and memory. Here we examine if β-catenin also mediates hippocampal-dependent memories. We used an inducible genetic approach to examine whether β-catenin in the adult hippocampus is required for context, object, and spatial memories *in vivo*. We found that β-catenin is required for the consolidation, but not the acquisition, of these hippocampal-dependent memories.

RESULTS

β-Catenin deletion in the hippocampus does not alter baseline activity or anxiety measures

β-Catenin was deleted within the hippocampus by performing bilateral injections of a lentivirus expressing Cre recombinase (LV-Cre) into the dorsal hippocampus of β-

catenin 'floxed' mice[95]. These mice possess *loxP* sites located in introns 1 and 6 of *Ctnnb1*, and following injection of LV-Cre, β-catenin is deleted specifically within the dorsal hippocampus. Mice injected with a lentivirus expressing green fluorescent protein (LV-GFP), which does not affect β-catenin expression, served as controls. Examination of the infected area by Nissl staining revealed that the cellular and anatomical structure of the dorsal hippocampus remained intact following injections of either LV-Cre of LV-GFP.

Following confirmation that lentivirus injections into the dorsal hippocampus do not produce damage to the area, we wanted to determine if deletion of β-catenin within this same area affects baseline locomotor or anxiety measures. We injected either LV-GFP or LV-Cre bilaterally in the dorsal hippocampus of 6-8 week old mice. Ten days later, after a time delay which we had previously shown results in robust gene deletion, we examined behavior. We first placed the mice in an open field apparatus, and measured their total distance traveled in a ten minute time period. In addition, we also calculated the distance traveled and time spent in the center verses the surround as a measure of baseline anxiety. We found no differences ($P > 0.1$) in locomotor or anxiety measures as assessed by the open field apparatus (Figure 4.1).

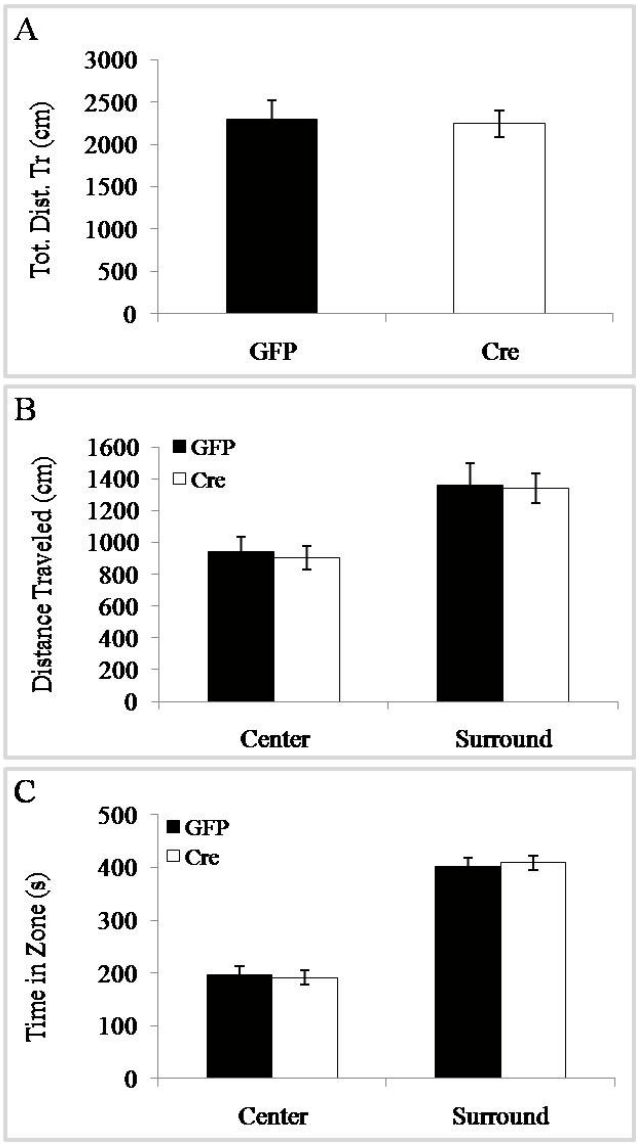

Figure 4.1 – Hippocampal-specific β-catenin deletions do not affect baseline activity or anxiety measures as measured by open field

Activity measures for mice placed in an open-field apparatus for 10 min. There were no differences between mice injected with LV-GFP or LV-Cre in terms of total distance traveled (A), distanced traveled in the center compared to surround (B), or time spent in the center compared to surround (C). n = 10 for LV-GFP, n = 8 for LV-Cre. Error bars, s.e.m.

We also examined the performance of these mice in an elevated plus maze, another measure of anxiety. Similar to the open field test, we did not find any differences between LV-GFP and LV-Cre infected animals in terms of time spent in the open arms verses the closed arms (Figure 4.2). These data suggest that hippocampus-specific deletions of *Ctnnb1* do not alter motor activity or baseline anxiety levels.

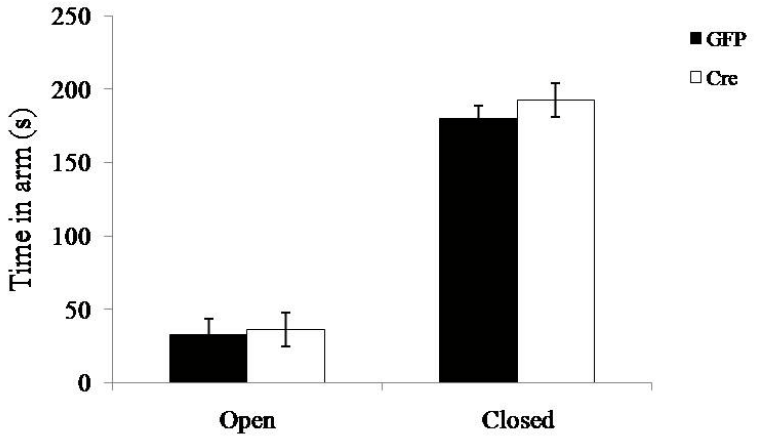

Figure 4.2 - Hippocampal-specific β-catenin deletions do not affect baseline activity as measured by elevated plus maze

Activity measures for mice placed in an elevated plus maze for 5 min. There were no differences between mice injected with LV-GFP or LV-Cre in terms of time spent in open arms compared to closed arms. n = 10 for LV-GFP, n = 8 for LV-Cre. Error bars, s.e.m.

β-Catenin in the hippocampus is required for context-dependent, but not cue-dependent fear memory consolidation

Hippocampal-specific deletion of *Ctnnb1* affects context-dependent fear memory. We fear-conditioned the mice using five shock presentations and obtained freezing measures during fear acquisition. We found a significant main effect of trial ($F_{5,80}$ = 94.04, $P \leq 0.01$) but no effect of virus ($F_{1,16}$ = 0.145, P = 0.708) (Figure 4.3 A). Forty-eight hours after the training session, we returned the mice to the training context for ten minutes and tested them for expression of context-dependent fear. In contrast to the acquisition data, we found a main effect of virus ($F_{1,16}$ = 8.550, $P \leq 0.01$) on the expression of fear memories. During this fear memory test, the data for within-session freezing were analyzed in five blocks of two minutes. Mice infected with LV-Cre showed significantly less freezing than mice infected with LV-GFP during three of the five blocks (Figure 4.3 B). The data for within-session freezing across these blocks are as follows (LV-GFP versus LV-Cre): block 2, 45.45 ± 6.25 versus 24.92 ± 6.23 (t_{16} = 2.295, $P < 0.05$); block 3, 43.76 ± 4.45 versus 21.12 ± 5.01 (t_{16} = 3.380, $P < 0.01$); block 4, 37.86 ± 6.11 versus 19.52 ± 3.25 (t_{16} = 2.335, $P < 0.05$).

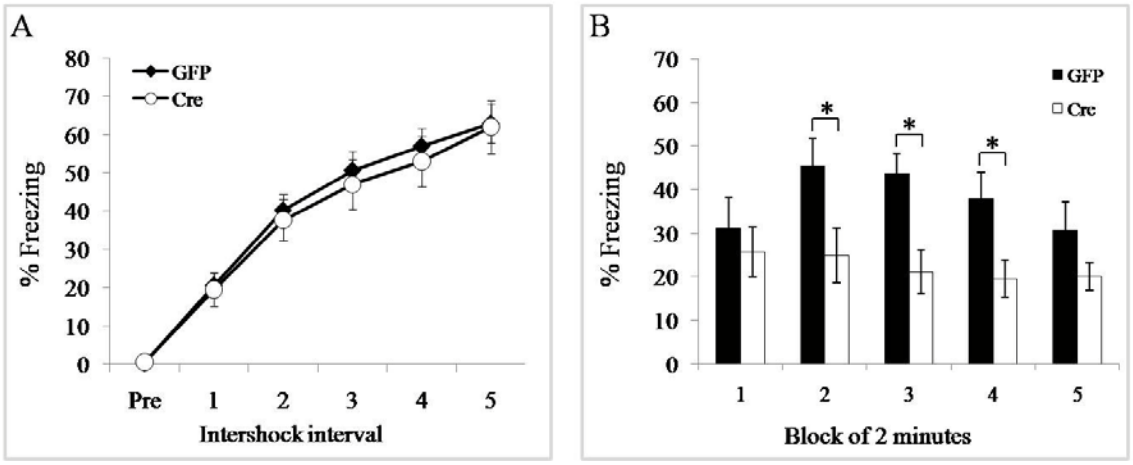

Figure 4.3 – Hippocampal-specific β-catenin deletion prevents the consolidation, but not acquisition of context-dependent fear memories

(A) Acquisition curve for LV-GFP and LV-Cre mice during training. (B) Percentage time spent freezing during the 48 h post-training test in LV-GFP and LV-Cre mice when placed in context for which they were trained. n = 10 for LV-GFP, n = 8 for LV-Cre. Error bars, s.e.m. * $P < 0.05$.

To confirm that deletion of *Ctnnb1* within the hippocampus did not affect other forms of fear memory, we trained the mice to fear a cue, which is amygdala-dependent, but hippocampal-independent. After three days of habituation to a novel set of conditioning chambers, mice received five tone-shock pairings. During the acquisition of the fear memory, we found a significant main effect of time across all mice ($F_{5,80} = 20.836$, $P < 0.01$); however, there was no main effect of virus ($F_{1,16} = 0.268$, $P = 0.612$) (Figure 4.4 A). Forty-eight hours after fear conditioning, we placed the animals into a novel context, and presented them with fifteen conditioned-stimulus tones. The mean percent time spent freezing during these tones was recorded and used as a measure of

conditioned fear. Unlike the effect we observed with context-dependent fear conditioning, we did not observe a difference between LV-GFP and LV-Cre infected mice within the testing period ($F_{1,16} = 0.232$, $P = 0.636$, Figure 4.4 B). Therefore, deficits observed following deletion of β-catenin within the hippocampus are specific to hippocampal-dependent, context-dependent fear memory consolidation.

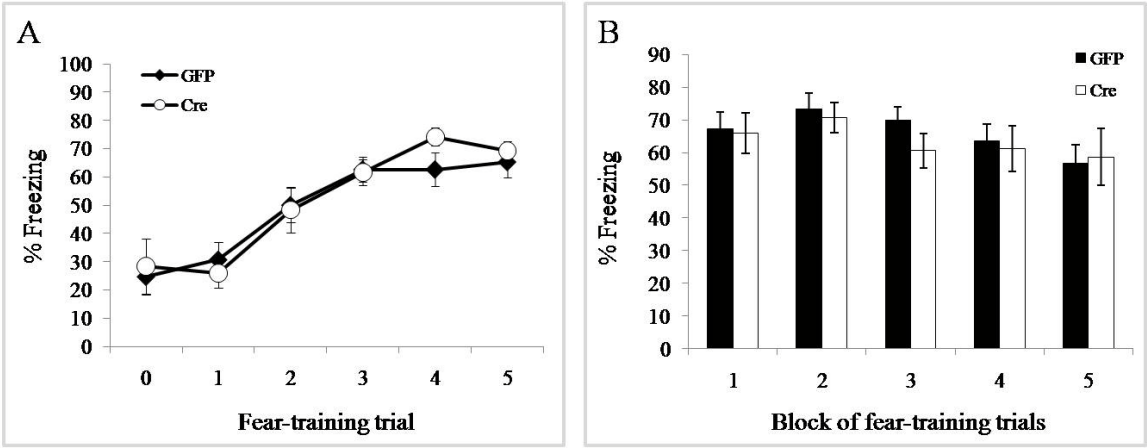

Figure 4.4 - Hippocampal-specific β-catenin deletion does not affect the acquisition of consolidation of cue-dependent fear conditioning

(A)Acquisition curve for LV-GFP and LV-Cre mice during training. (B) Percentage time spent freezing during the 48 h post-training test in LV-GFP and LV-Cre mice in response to the tone presented in a novel context. n = 10 for LV-GFP, n = 8 for LV-Cre. Error bars, s.e.m.

β-Catenin deletion impairs object recognition memory

We then examined whether hippocampal-specific deletion of *Ctnnb1* affects object recognition memory. β-Catenin floxed mice that had received injections of either

LV-GFP or LV-Cre were tested in a novel object recognition task. For two consecutive days, mice were placed in an arena and exposed to two identical objects. Twenty four hours later, they were placed back into the arena with one of the original objects (familiar) and one novel object. The amount of time spent exploring the familiar object versus the novel object was measured, with mice that remember the familiar object spending more time exploring the novel one. In our studies, mice that had been injected with LV-GFP explored the novel object significantly more than the familiar object (t_9 = -3.112, P < 0.05). However mice that had been injected with LV-Cre did not discriminate between the two objects (t_6 = -0.572, P = 0.588, Figure 4.5), suggesting that the LV-Cre infected mice did not form normal object recognition memory, a hippocampal-dependent process.

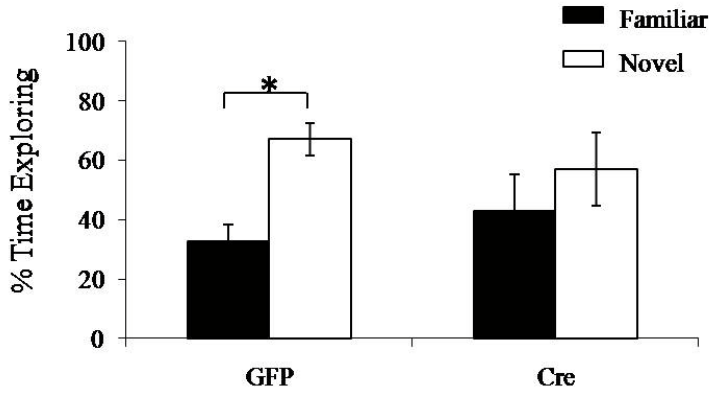

Figure 4.5 – Hippocampal-specific β-catenin deletion impairs object memory

Mice that have been injected with LV-GFP explore the novel object significantly more than the familiar object while mice injected with LV-Cre do not. n = 10 for LV-GFP, n = 7 for LV-Cre. Error bars, s.e.m. * P < 0.05.

β-Catenin deletion impairs consolidation, but not acquisition, of spatial memory

The same mice from above were tested for their spatial learning ability using the Morris Water Maze (MWM). Mice received four training trials per day, for five days, and the average latency to reach the platform was calculated for each day. We found a significant main effect for training day ($F_{4,60}$= 3.6273 , P ≤ 0.01) but no effect of virus ($F_{1,15}$ = 2.441 , P = 0.139, Figure 4.6).

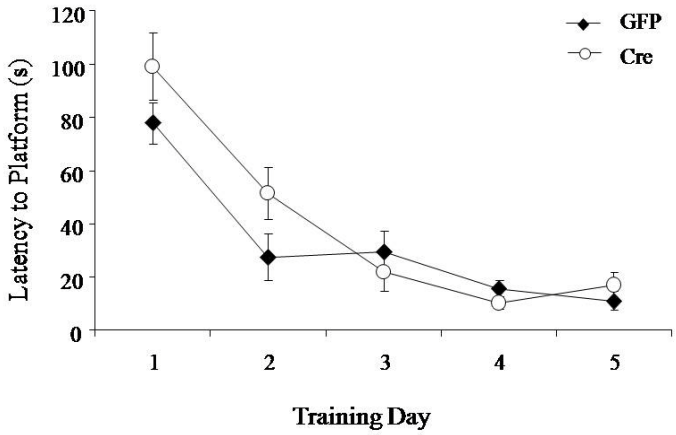

Figure 4.6 – Hippocampal-specific β-catenin deletion does not affect the acquisition of spatial memory

Acquisition of the location of the hidden platform, measured as the average latency to find the platform across 4 trials on 5 separate days. n = 10 for LV-GFP, n = 7 for LV-Cre. Error bars, s.e.m.

Forty eight hours following the last training day, mice were subjected to the probe phase of the MWM. Representative path tracings are shown in Figure 4.7 A. During the probe test, mice injected with LV-GFP spent significantly more time in the target

quadrant than the adjacent (t_9 = 3.827, P < 0.01) or opposite (t_9 = 4.448, P < 0.01) quadrants, indicating their memory of the target quadrant. In contrast, mice injected with LV-Cre did not show a quadrant preference (P > 0.1) (Figure 4.7 B). In addition to the time spent in each quadrant, we also analyzed the distance traveled. Once again, mice injected with LV-GFP traveled significantly more within the target quadrant compared to the adjacent (t_9 = 3.827, P < 0.01) or opposite (t_9 = 4.448, P < 0.01) quadrants, while the mice injected with LV-Cre did not discriminate between the quadrants (P > 0.1) (Figure 4.7 B). Thus, hippocampal-specific β-catenin deletions prevent normal consolidation of spatial learning as indicated by their impaired MWM performance 48hrs after training.

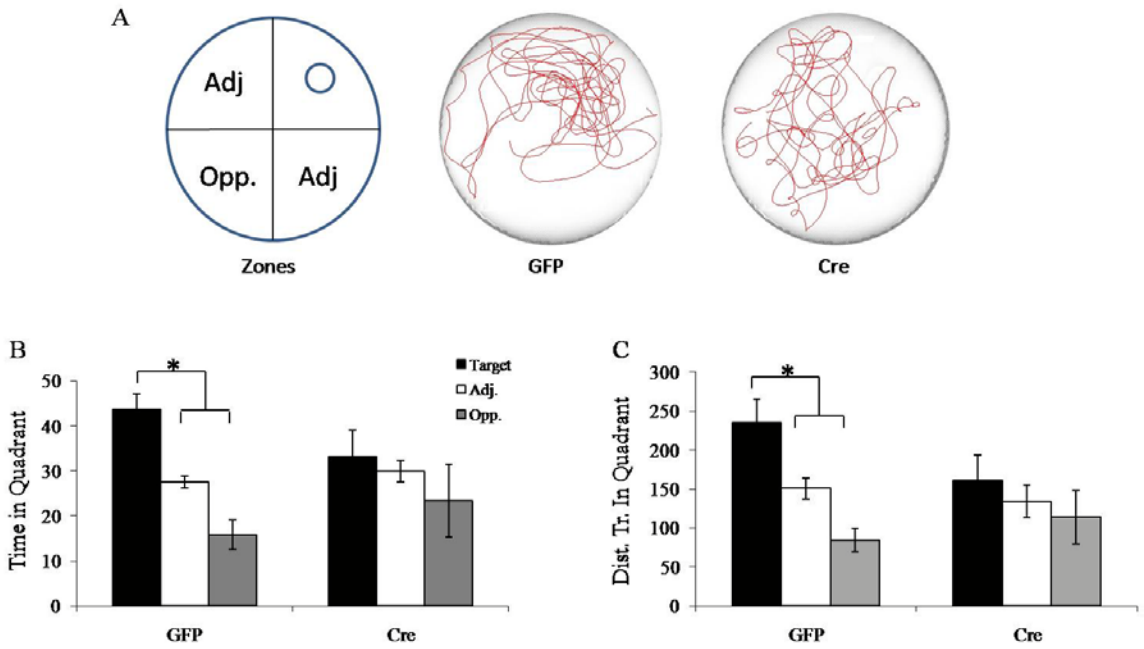

Figure 4.7 - Hippocampal-specific β-catenin deletion does impair consolidated spatial memories.

Forty-eight hours following training, mice were tested on a probe trial in the absence of the platform. (A) Path analysis of animals injected with LV-GFP and LV-Cre. (B) Time spent in the target compared to the adjacent and opposite quadrants. (C) Distance traveled (inch) in the target compared to the adjacent and opposite quadrants. n = 10 for LV-GFP, n = 7 for LV-Cre. Error bars, s.e.m. * $P < 0.05$.

DISCUSSION

Although there is considerable evidence suggesting that β-catenin is important for synapse regulation and plasticity, there have been no studies looking at the role of this protein in hippocampal learning and memory assays in animals. We used lentiviral-

mediated expression of Cre recombinase with floxed β-catenin transgenic mice to directly examine the behavioral effects of hippocampal-dependent β-catenin deletion in adults. We showed that hippocampal-specific β-catenin deletions do not affect baseline anxiety, locomotor activity, or cue-dependent fear learning. However, we found that deletion did produce deficits in context-dependent fear, object recognition, and spatial memories.

The hippocampus is critical for contextual memory. Lesions of this area pre- and post-training have been shown to abolish context-dependent fear learning[138-140]. In our studies we found that β-catenin in the hippocampus is required for the consolidation, but not acquisition, of context-dependent fear memory. However, we did not find an effect on cue-dependent fear learning. Cue-dependent fear learning requires the amygdala, but does not appear to require the hippocampus, as shown by a variety of lesion and inactivation studies[141]. Therefore, the learning deficit that we observed appeared to be specific to hippocampal-dependent fear learning.

In addition to its role in contextual memory, the hippocampus also plays a role in object recognition memory. Inactivation of the hippocampus with lidocaine prior to training has been shown to impair object recognition memory when measured 24 h after training[142]. We found a similar effect with our mice that had received inducible gene deletions. Hippocampal-specific β-catenin did not affect the ability of the mice to explore the objects during training, but when tested 24 h later, the animals did not show a significant preference for the novel object over the familiar one. This suggests that deletion of β-catenin in the hippocampus prevented the mice from recalling the object they had previously seen during training.

The hippocampus is also known to play a critical role in spatial learning. One well-accepted measure of hippocampal-dependent spatial learning is the hidden platform water maze[143]. Damage to the hippocampus has been shown to produce impairments in the ability of mice to perform this task[144-146]. We found that β-catenin deletion within the hippocampus did not affect water maze acquisition, but did produce impairments in the probe test when given 48 h after training. This result is consistent with our other findings showing that β-catenin is required for the consolidation, but not acquisition, of memory.

A variety of tasks that have been shown to be dependent on hippocampal function involve the remodeling of existing synaptic connections or the growth of new connections. For example, some forms of hippocampal-dependent learning have been shown to change spine number and morphology[16, 130-134]. Others, including trace eyeblink conditioning[147] and spatial learning[148-150], have been shown to increase the number of newborn cells, which can then grow axons, dendrites, and synapses. These post-natal born neurons are also capable of receiving synaptic inputs[151, 152]. Therefore, structural plasticity is important for hippocampal-dependent memory formation. Since β-catenin has been shown to be required for synapse formation and function[153], deletion of β-catenin may interfere with the remodeling of synapses during learning and memory.

Interestingly, there is some evidence suggesting that lithium chloride, which is widely accepted as a mediator of β-catenin, has been shown to enhance memory. Lithium acts by inhibiting GSK-3β, preventing it from phosphorylating β-catenin, thus increasing β-catenin stability[36, 91, 92]. Treatment with lithium chloride has been shown to enhance spatial working memory in rats[154]. In addition, there is also data suggesting that bipolar patients who respond well to lithium treatment perform better on neuropsychological

tests measuring spatial memory and sustained attention than patients who do not respond well to lithium[155].

Lithium has also been shown to rescue cognitive deficits associated with Alzheimer's disease (AD). Alzheimer's disease is a neurodegenerative disease associated with progressive memory loss and cognitive impairment. At the molecular level, the disease is characterized by the presence of neurofibrillary tangles (NFTs) and senile plaques, which are extracellular deposits comprised of the β-amyloid peptide (Aβ)[62, 63]. Injection of Aβ fibrils into the dorsal hippocampus of rats has been used as an *in vivo* model of the disease. These rats show a destabilization of endogenous levels of β-catenin, along with impairments in the Morris water maze paradigm. However, chronic lithium treatment can restore β-catenin to control levels and improve the spatial learning deficit[77]. A similar reduction in spatial memory impairments by lithium has been shown in other animal models of the disease[79]. Therefore, understanding how β-catenin may function to regulate memory formation may potentially provide insight into treatment for AD, and other disorders of memory impairment.

METHODS

Animals

Adult male homozygous β-catenin floxed mice (B6.129-Ctnnb1tm2Kem/KnwJ; Jackson Labs) were used for all behavior experiments. Mice were housed four per cage in a temperature-controlled (24 ^{o}C) animal colony, with *ad libitum* access to food and water, on a 12-h light-dark cycle, with all behavioral procedures done during the light cycle.

In situ hybridization

In situ hybridization was carried out as previously described. After behavioral procedures, brains were blocked rapidly and kept frozen at -80 ^{o}C. The full-length clone for β-catenin (GI accession no. 31419847) was used to subclone the regions between exons 2 and 6 of β-catenin, the genomic region flanked by *loxP* sites in the mutant mouse. This subclone targeting the loxP-flanked region was then linearized, and both antisense and sense ^{35}S-riboprobes were generated using the appropriate RNA polymerase and ^{35}S-UTP in the reaction.

Lentiviral vectors and virus infection

Viral vectors were produced and concentrated as previously described. Briefly, a Cre recombinase-expressing vector (LV-Cre) or a GFP-expressing control vector (LV-GFP) with a final titer of 10^{9} infectious units per ml was used for stereotaxic injections into the hippocampus. Mice were anesthetized, and small holes were drilled into the skull above the injection site. Hippocampal coordinates were as follows: anteroposterior, -1.8; dorsoventral, -1.8; mediolateral, ± 1.0 relative to bregma. A 10-μl Hamilton microsyringe pre-coated with bovine serum albumin was used to deliver bilateral injections of lentiviral vectors in the dorsal hippocampus (0.2 μl of virus per side, injected at a rate of 0.025 μl min^{-1}). The needle was left in place for 15 min after the injection, and mice were allowed to recover for 10-14 d before testing. After the behavioral studies described here, we confirmed that Cre recombinase was expressed in dorsal hippocampus, and β-catenin was deleted in the area of infection, similar to our prior work with β-catenin deletion in

amygdala[118] and BDNF deletion in hippocampus[101]. Similarly, GFP was expressed in the hippocampus of animals infected with LV-GFP.

Behavioral Studies

Open field

The open field consisted of a box (27.9 cm x 27.9 cm) made of Plexiglass. The mice were placed in the periphery of the arena at the start of the 10-min test period. At the end of the test, the animal was returned to its home cage. All testing was conducted under standard room lighting. Activity data was obtained and analyzed using the Open Field Activity Software (Med Associates Inc., St. Albans, VT).

Elevated plus maze

Mice were moved to holding area just prior to testing. The animal was then placed quickly onto the center square between the plus maze arms. The mouse was left to explore the plus maze for 5 min, and then returned to its cage. Total time spent in closed verses open arms was recorded.

Context-dependent fear conditioning

Mice were fear conditioned in four identical rodent modular test chambers (ENV-008-MP; Med Associates Inc. St. Albans, VT) with an area of 30.5 cm x 24.1 cm x 21.0 cm. The conditioned stimulus was a scrambled footshock delivered to a removable grid floor that consisted of 36 stainless-steel rods (3.2 mm) placed 7.9 mm apart. Five 0.5 mA footshocks were delivered with an intershock interval of 1.5 min. Percent time spent

freezing during the shock presentations was calculated for each mouse using FreezeFrame (Coulbourn Instruments, #ACT-100, Allentown, PA).

Cue-dependent fear conditioning

Mice were fear-conditioned in eight identical startle-response systems. After 3 d of exposure to the conditioning chambers, mice were placed in the conditioning chamber and after 5 min presented with five tone-shock pairings at an intertrial interval of 5 min. Each pairing consisted of a 30-s tone (12 kHz, 75 db; conditioned stimulus) that co-terminated with a 0.5-s footshock (1.0 mA; unconditioned stimulus). Freezing in startle-response chambers during fear acquisition was assessed as described previously[118]. Forty-eight hours after training, mice were tested for freezing in the Med Associates test chambers. Three minutes later, 15 conditioned stimulus tones (12 kHz, 75 db) with an intertrial interval of 1.5 min were delivered through a high-frequency speaker attached to the side of each chamber. Percentage time spent freezing during the conditioned stimulus presentations was calculated for each mouse as above.

Novel object recognition

The object recognition apparatus consisted of an open box (44 x 44 x 8 cm) made of white polyvinyl chloride (PVC). The apparatus was placed in a room illuminated by normal housing lights. Three different objects made of a combination of plastic, metal, and rubber, were employed in this task. Each object was similar in size, and the weight of the objects ensured that they could not be displaced by mice. These objects were selected on the basis of previous observations that demonstrated a lack of preferential exploration

of one object over the other. The animals' training and testing behaviors were filmed by a video camera mounted over the training area.

For two days prior to training, each animal was placed into the open box and allowed to explore for five minutes in the absence of objects to allow for habituation to the open box environment. For two days following habituation, animals were given a sample session in which two identical objects were placed in the box, 10 cm from the side wall, and diagonal from one another. Each session began with the mouse being placed in the box facing the wall and continued for 5 min, at which point the mouse was promptly removed and returned to its home cage. Forty-eight hours after the training session, mice were returned to the room and tested. The testing session was identical to the training session except that one of the two objects was replaced with a novel object. The objects chosen to be novel as well as the location of the novel object during the testing sessions were counterbalanced between mice. The open box and objects were cleaned with a solution of 50% alcohol and allowed to dry thoroughly between each animal.

Exploration of an object was defined strictly on the basis of active exploration in which mice had to be touching the object with at least their nose. Videotape analyses of total time spent exploring each of the objects were obtained using video recordings and an observer blind to treatment conditions. Measurement of the time spent exploring each object during the testing session was expressed as a percentage of the total object exploration time in seconds.

Morris Water Maze

Morris Water Maze (MWM) consisted of a circular pool made of white PVC plastic with 45-cm-high walls and a diameter of 200 cm. The maze was filled to a depth of 26 cm with 25 ^{o}C water-rendered opaque with nontoxic latex paint. A small clear Plexiglas escape platform was placed at a fixed position in the center of one quadrant and was hidden 1 cm beneath the water surface. The MWM was placed in a room illuminated by normal housing lights and surrounded by a number of fixed visuospatial cues.

The acquisition phase consisted of five consecutive training days with four trials per day, starting at four different positions in a semirandom order. On each trial, mice were placed in a starting location facing the pool wall and allowed to swim until finding a submerged platform or a maximum of 120 s was reached. Mice remained on the platform for 30 s before removal to a retaining cage. If an animal did not reach the platform within 120 s, it was placed on the platform where it had to remain for 30 s. Mice remained in the retaining cage for 60 s before the start of the next trial. At the end of the 4 trial training session, mice were returned to their home cage. For each acquisition session, a mean latency was calculated for each mouse by averaging the latency to reach the platform across all four session trials.

Two days after the acquisition phase, each animal was given a probe session. During this session, the platform was removed from the maze, and animals were allowed to swim freely for 120 s. For the probe session, the percent time spent and distance traveled in each quadrant was analyzed. Trials were filmed by a video camera mounted over the training area, and all measurements were recorded by EthoVision 3.0 (Noldus Information Technology, The Netherlands).

Data analysis

Statistically significant differences were determined by Student's *t*-test or ANOVA, with *post hoc* least-squares difference tests for multiple comparisons. The results are presented as means ± s.e.m.

CHAPTER 5

Concluding remarks

SUMMARY OF FINDINGS

Although it was initially identified for its role in development, β-catenin has also been shown to play a role in neuronal synapse regulation and plasticity. Since alterations in synapse regulation and plasticity are thought to underpin long-term memory formation, β-catenin may play a critical role in this process. However, there has been a scarcity of data exploring this possibility. In my thesis, I aimed to fill this knowledge gap, by examining the role of β-catenin in mammalian learning and memory.

In Chapter 1, I briefly reviewed the basic molecular and structural mechanisms of synaptic plasticity. I then described the structure and function of β-catenin and provided an overview of the literature suggesting that it may function in mediating the structural changes associated with memory formation. Finally, I discussed how perturbations in β-catenin function may lead to pathological states such as Alzheimer's disease.

In Chapter 2, I examined the role of β-catenin in amygdala-dependent fear memory. I began by showing that β-catenin is highly expressed in the adult mouse amygdala and is dynamically regulated at both the transcriptional and post-translational levels with fear learning. I then showed that pharmacological stabilization of β-catenin with lithium chloride resulted in enhanced learning, while genetic deletion of the gene that encodes β-catenin, *Ctnnb1*, in the amygdala resulted in impaired learning. In both cases, the manipulation affected the consolidation, but not acquisition, of the fear memory. Notably, *Ctnnb1* deletion did not affect a number of other behaviors, including locomotor, anxiety-related behavior, or hippocampal-dependent memory.

Memory formation is thought to involve the weakening and strengthening of synapses, and this process can be modulated by the adhesion between pre- and

postsynaptic neurons. Since β-catenin is important for cell adhesion, and deletion of *Ctnnb1* in the amygdala produces deficits in the consolidation of memory, I proposed the following model of how β-catenin may function in memory formation.

β-Catenin can be found in a complex with cadherin at the plasma membrane of dendritic spines. Immediately following a learning event, β-catenin becomes phosphorylated at site Y654. Similar to previous findings, I showed that the increase in phosphorylated β-catenin coincided with a decrease in the interaction between β-catenin and cadherin. This decrease may be required to weaken the bond between the pre- and postsynaptic neurons, allowing for synaptic remodeling to take place. Following a period of β-catenin-cadherin destabilization, β-catenin relocates to the spine and once again forms a complex with cadherin, thereby stabilizing the synapse, and strengthening the memory. In conditions where β-catenin function may be impaired, as in our deletion studies, the initial labile phase may remain unaffected; however, the stable phase may be compromised. If β-catenin is not present, it will not be able to bind to the cadherin and stabilize the synapse. This proposed model suggests that dynamic regulation of β-catenin may be involved in the structural conversion of short-term labile to long-term stable memory traces (Figure 5.1).

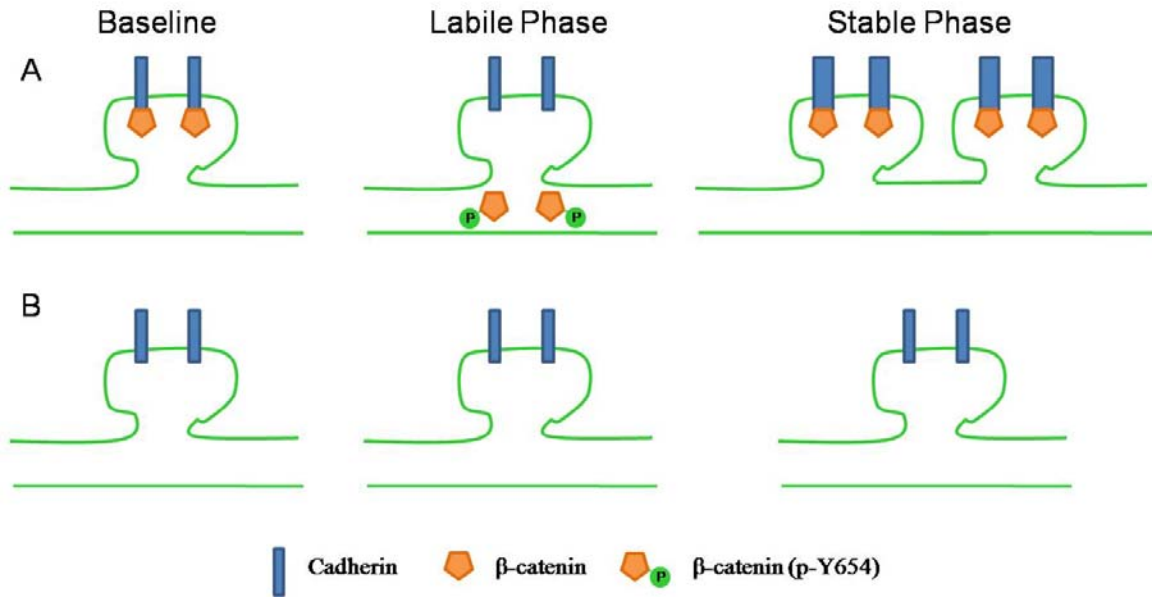

Figure 5.1 - Schematic representation of the role of β-catenin in producing the labile and stable phases of memory formation.

(A) β-Catenin is located in a complex with cadherin. Following a learning event, β-catenin becomes phosphorylated and shifts to the dendritic shaft, allowing for synaptic remodeling to take place (labile phase). At some point later, β-catenin redistributes to the dendritic spine, and re-associates with cadherin to strengthen the memory (stable phase). (B) In the absence of β-catenin, the labile phase of memory can still exist, but the stable phase is impaired.

Although the data in this chapter suggested that the role of β-catenin in cell-cell adhesion may be contributing to memory formation, the Wnt signaling may also play a role. There is emerging data suggesting that Wnt/β-catenin signaling may be involved in structural and functional plasticity of synapses; however, there is no evidence for the role of Wnt signaling *in vivo* underlying learning and memory formation in adults. In Chapter

3, I explored this possibility by examining the role of Wnt signaling in mediating β-catenin-dependent memory formation. I found that disruption of Wnt-mediated signaling with direct infusion of Dickkopf-1 into the amygdala produced a deficit in consolidation, but not acquisition, of fear memory. Injection of this peptide also prevented the re-stabilization of the β-catenin-cadherin complex following memory formation. These findings suggest that Wnt signaling may be an important pathway regulating adult fear learning and memory.

Thus, the next step was to determine how Wnt signaling may be involved in memory formation, I used real-time PCR arrays to analyze the expression of a number of genes related to Wnt-mediated signaling, and found that several genes were dynamically regulated in a time-dependent manner following fear conditioning. I then altered the pattern of Wnt signaling via direct infusion of Wnt1 into the amygdala before or after fear conditioning. The injection of this peptide prior to fear conditioning produced deficits in the consolidation of the memory, without affecting acquisition. Following co-immunoprecipitation studies, I concluded that Wnt1 may impair memory formation by prematurely stabilizing β-catenin cadherin interactions during the early consolidation period, thus preventing the normal transient β-catenin-cadherin disassociation immediately following the learning event.

The finding that infusion of a Wnt signaling antagonist and a Wnt signaling agonist both produce impairments in memory formation suggest that any large perturbation of this pathway can have detrimental effects on neuronal functioning. In addition, both manipulations had an effect on the interaction between β-catenin and cadherin at the synapse, suggesting that Wnt signaling may affect β-catenin-mediated cell

adhesion. These findings agree with other, *in vitro*, lines of evidence suggesting that Wnts may directly and indirectly regulate β-catenin interactions with cadherin[110]. I propose that in conditions where Wnt signaling is inhibited, the interaction between β-catenin and cadherin is decreased, and the re-stabilization of the synapse is prevented (Figure 5.2 B). In contrast, when Wnt signaling is over-activated, the β-catenin-cadherin interaction is increased, and the labile phase is inhibited (Figure 5.2 C). Therefore, both manipulations may interfere with the dynamic and transient destabilization and re-stabilization of synapses during memory consolidation.

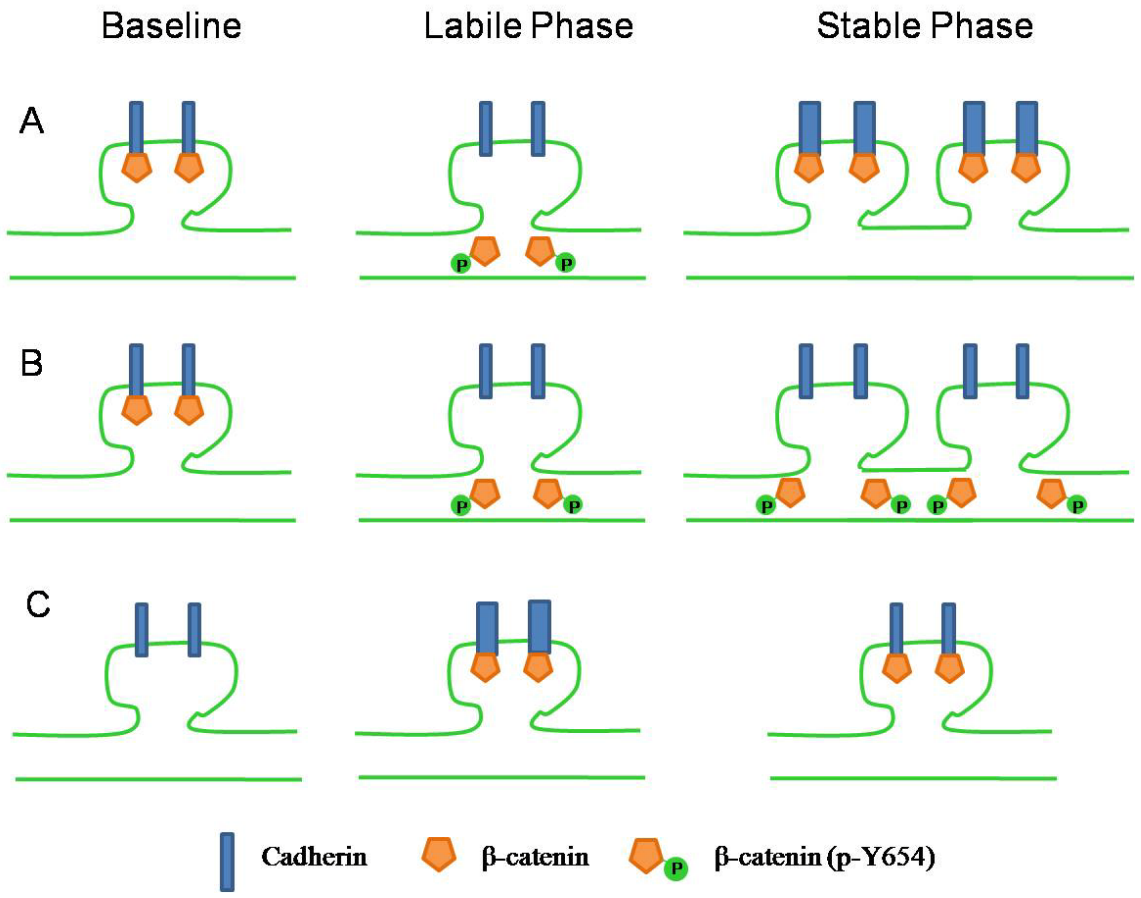

Figure 5.2 - Schematic representation of the effect of a Wnt antagonist and Wnt agonist on the labile and stable phases of memory formation

(A) β-Catenin is located in a complex with cadherin. Following a learning event, β-catenin becomes phosphorylated and shifts to the dendritic shaft, allowing for synaptic remodeling to take place (labile phase). At some point later, β-catenin redistributes to the dendritic spine, and re-associates with cadherin to strengthen the memory (stable phase). (B) In the presence of Dkk-1, a Wnt signaling antagonist, the interaction between β-catenin and cadherin is decreased, thus preventing the stable phase of memory. (C) In the presence of exogenous Wnt1, the association between β-catenin and cadherin is

increased, thus preventing the labile phase of memory. In both B and C, memory formation is impaired.

Thus far, I have provided evidence that β-catenin plays a role in amygdala-dependent learning and memory. In Chapter 4, I explored the possibility that β-catenin may also play a role in hippocampal-dependent learning. First, I examined the effect of hippocampal-specific, time- and spatially restricted β-catenin deletions on contextual fear memory in adults. I found that β-catenin in the hippocampus is required for the consolidation, but not acquisition of fear memory. To confirm that the deletion of *Ctnnb1* within the hippocampus did not affect other forms of fear memory, I tested the ability of these same mice to form cue-dependent fear memory, which is not reliant on the hippocampus. I found no effect of *Ctnnb1* deletion on the acquisition or consolidation of cue-dependent memory, suggesting that the impairments observed are specific to hippocampal-dependent fear memory.

Next, I wanted to determine if β-catenin was required for other forms of memory in addition to fear-related learning and memory. I assessed the performance of mice in a novel object recognition task, and the Morris Water Maze test, and found that deletion of *Ctnnb1* in the hippocampus impaired learning in both hippocampal-dependent tests. These results suggest that β-catenin in the hippocampus also plays a role in the consolidation of object and spatial memories, respectively. Additional studies are needed to determine the specific molecular and cellular roles that β-catenin may play in producing the observed effects on hippocampal-dependent learning and memory.

Dynamic regulation of cadherin/β-catenin interaction

The findings reported for both the amygdala and hippocampal studies show that β-catenin is required for normal consolidation, but not acquisition, of memory. The evidence presented thus far suggests that the decrease and subsequent increase in the interaction between β-catenin and cadherin may be critical for the labile and stable phases of memory formation. Such dynamic regulation has been proposed previously when examining its cellular regulation in hippocampal cultures[43, 156], but it has never been demonstrated *in vivo* or in behavioral learning and memory paradigms.

Neural activity increases the synthesis and secretion of brain-derived neurotrophic factor (BDNF), which has been shown to play a critical role in synaptic plasticity[157]. Treatment with BDNF induces synaptic vesicle dispersion, which is associated with an increase in β-catenin tyrosine phosphorylation and a decrease in β-catenin-cadherin interaction[43]. Within 30 minutes after the dispersion, phosphorylation decreases, and the β-catenin-cadherin interaction is restored[43]. This finding suggests that the disruption and re-stabilization of β-catenin-cadherin complexes may be required for new synapse formation. Our lab and others have previously demonstrated that BDNF is important in both amygdala[97] and hippocampal dependent memory formation[101]. These new findings that β-catenin regulation is also involved in these processes raise the question of whether the specific effects of BDNF on memory consolidation are, in part, via the β-catenin pathway.

Similarly, NMDAR-dependent neural activity has also been shown to induce changes in the interaction between β-catenin and cadherin[156]. NMDAR activation decreases the rate of cadherin endocytosis, increasing the accumulation of cadherin in the

plasma membrane. In addition the level of tyrosine phosphorylated β-catenin is decreased, leading to an increase in the interaction between β-catenin and cadherin in dendritic spines[156]. Furthermore, prolonged stability of the cadherin at the plasma membrane blocks NMDAR-dependent synaptic plasticity, suggesting that the dynamic behavior of β-catenin and cadherin is important for this process.

There have been previous studies showing that BDNF may modulate NMDAR activity. Upon BDNF binding to and activating the tyrosine receptor kinase TrkB, there is an enhancement in glutamatergic synaptic transmission[158] and an increase in the phosphorylation of the NMDAR[159]. *In vitro* studies have shown that the phosphorylation of NMDAR occurs within 5 min of exposure to BDNF[159]. Since BDNF activation of TrkB receptors functions to transiently dissociate the β-catenin from the cadherin, while glutamatergic activation of NMDA receptors functions to increase the association, it is possible that the two systems may interact to produce the transient destabilization and re-stabilization of synapses.

Interestingly, both BDNF and NMDA are required for the consolidation of memories. Therefore, I propose that similar mechanisms are taking place *in vitro* and *in vivo* to stabilize and strengthen synapses (Figure 5.3). BDNF is released following a learning event, which phosphorylates β-catenin, decreasing the β-catenin-cadherin interaction. This decrease in the β-catenin-cadherin interaction increases synaptic vesicle mobility, allowing for synaptic plasticity. At about the same time, BDNF may increase the phosphorylation of NMDAR. The activation of the NMDAR then helps to re-stabilize the synapse, by decreasing the rate of cadherin endocytosis and redistributing β-catenin

into spines. Once the synapse is stabilized, the memory becomes strengthened. Future studies directly examining this interaction, *in vivo*, would be important and interesting.

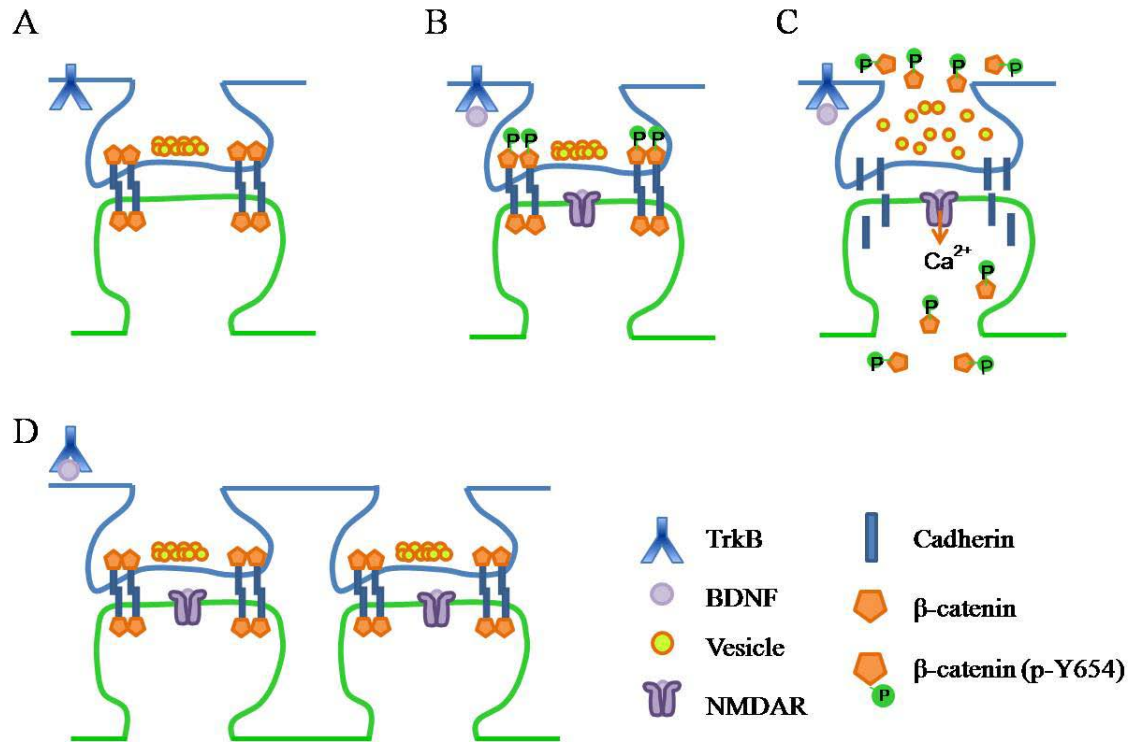

Figure 5.3 - Schematic representation showing a possible interaction between BDNF signaling and NMDAR activation

(A) β-Catenin is located in a complex with cadherin at the plasma membrane of the synapse. (B) Activation of TrkB receptor by BDNF results in the phosphorylation of β-catenin at Y654, thus causing a dissociation of β-catenin from cadherin. (C) BDNF activation also results in the phosphorylation of NMDARs. During this time, phosphorylated β-catenin shifts to the dendritic shaft, and synaptic vesicles disperse. (D) The activation of NMDARs, along with the internalization of TrkB re-stabilizes the synapse by bringing β-catenin and cadherin together at the membrane.

Subcellular distribution of β-catenin

Thus far, the evidence suggesting that β-catenin translocates into dendritic spines following neural activity is based on co-immunoprecipitation and fluorescence microscopy studies. Co-immunoprecipitation studies are a standard method to assess protein-protein interactions, but do not provide data on the localization of these proteins. Fluorescence microscopy can provide information on the localization of the protein, but the resolution is not optimal. In addition, most of the studies showing a shift in β-catenin utilized a green fluorescent protein (GFP)-tagged β-catenin to measure the redistribution of the protein. However, in these conditions, β-catenin is being over-expressed, and it may be possible that endogenous β-catenin would act differently. Therefore, additional studies are needed to confirm the redistribution of endogenous β-catenin following neural activity.

In future studies, one alternative method that could be used to measure changes in β-catenin distribution would be immunogold electron microscopy. Electron microscopy has a greater resolution than optical microscopy, and can be used to examine protein expression at much higher magnifications. The immunogold method produces very specific labeling of the protein, and results can be used to measure qualitative and quantitative changes in β-catenin expression following activity. For example, tissue sections can be prepared from untrained and trained animals sacrificed at different time points following context exposure or conditioning. Then, β-catenin can be labeled with immunogold and analyzed with an electron microscope. The presence of β-catenin

particles in dendritic shafts verses spines can be compared amongst the groups. In addition, the distance of the gold particles to the plasma membrane of the spines can also be measured and compared. The results obtained from these studies would provide more detailed information on the subcellular localization of β-catenin following learning.

The stabilization and de-stabilization of memory

The initial phase of memory formation is thought to be very labile, and can be disrupted by events such as trauma or drug administration. The conversion of short-term memory to long-term memory has been shown to be dependent on protein synthesis. Administration of protein synthesis inhibitors immediately after learning has been shown to prevent long-term memory formation[160]. The greater the interval between training and drug administration, the more resistant memories become to disruption[160]. Thus, this initial protein synthesis-dependent phase of memory appears to be a relatively short period. After this critical period, memories become stabilized through consolidation.

It was previously thought that once a memory is formed, it does not become labile again. However, this classical view of memory stabilization has been argued against by recent discoveries showing that memories can become labile again when recalled or reactivated[161]. This reactivation requires another phase of protein synthesis-dependent consolidation. If protein synthesis inhibitors are administered during the memory recall, the previously stored memory can be disrupted[162]. This protein synthesis-dependent process that is initiated after reactivation is referred to as reconsolidation.

There is much debate on the functional roles of reconsolidation. Some believe that reconsolidation can strengthen memories by preventing the decrease in response due to forgetting or extinction[163]. Others believe that it may function to update previously stored memories[164]. However, there is evidence suggesting that although not identical, some overlap does exist between reconsolidation and consolidation, both in its storage function and underlying mechanisms[165]. Given that my studies have shown a role for β-catenin in the consolidation of memories, it may be interesting to examine the role of β-catenin in reconsolidation.

In order to address this question in future studies, mice could be trained as described in my previous experiments. Then 24 to 48 h later, they would receive a single presentation of the conditioned stimulus, such as a tone, in a novel context. This session is referred to as the retrieval session. Immediately following the retrieval session, mice would receive their manipulation, such as an injection of Dkk-1 or lithium. At least 24 to 48 hours after the retrieval session, the animals would then be placed back in the retrieval context and presented with several tone presentations. Freezing during each of these tones would be measured and analyzed.

Ideally, the above experiment would be performed using the β-catenin floxed mice to examine the effect of *Ctnnb1* deletion on reconsolidation. However, a minimum of 7-10 days is required for optimal lentiviral gene expression. Thus, I would only be able to inject the lentivirus expressing GFP or Cre into the amygdala prior to the retrieval session. Since I would have to wait additional days between the manipulation and retrieval session, I would want to include another group that does not undergo the

retrieval session, to show that the memory remains unmodified in the absence of the reactivation.

In all of the above proposed reconsolidation experiments, it would be interesting to see if β-catenin is required for the re-stabilization of memory following retrieval. Based on my previous findings, I would predict that animals receiving a manipulation that disrupts β-catenin signaling would show decreased freezing during the testing session, due to the inability to re-stabilize the memory. If β-catenin does act to disrupt memory once it has been reactivated, pharmacological agents aimed at manipulating β-catenin levels may be useful in treating psychiatric disorders, including post-traumatic stress disorder (PTSD) and phobias.

Implications for Alzheimer's disease

I have presented evidence showing that β-catenin is critical for memory formation in adult mice. There has been increasing evidence suggesting that β-catenin is also involved in Alzheimer's disease (AD), a neurodegenerative disease associated with progressive memory loss and cognitive impairment. AD is also characterized by the presence of neurofibrillary tangles and amyloid plaques[62].

Studies have shown that β-catenin levels are reduced in AD patients carrying presenilin-1 (PS1) mutations[67], a mutation that accounts for the majority of cases of familial AD. Presenilins are crucial components of the multiprotein γ-secretase complex, which cleaves the amyloid precursor protein (APP), producing Aβ peptides[166, 167].

Mutations of PS1 are associated with the overproduction and aggregation of Aβ peptide[64], which then leads to the formation of amyloid plaques.

Furthermore, it has been shown that PS proteins form complexes with β-catenin[68, 71] and that lower levels of cytoplasmic β-catenin are associated with Aβ-induced neurotoxicity[77]. Aβ-induced neurotoxicity also induces the activation of GSK-3β, which increases the hyperphosphorylation of tau proteins, the main component of neurofibrillary tangles[77]. Interestingly, lithium, which inhibits GSK-3β and enhances β-catenin stability, has been shown to protect rat neurons from Aβ-induced damage[78, 168]. Lithium has also been shown to decrease tau phosphorylation in tau transgenic models with advanced neurofibrillary pathology[169]. Thus, aberrant β-catenin signaling may play a critical role in functional memory decline and the pathogenesis of AD.

Notably, studies have suggested that changes in cognitive functioning can be detected ten years or more prior to the clinical diagnosis of probable AD[170]. Therefore, understanding how abnormalities in β-catenin function affect learning may provide insight into the functional deficits underlying the cognitive impairments associated with AD. If the initial learning and memory deficits, which predate gross neuropathology, can be detected and treated at the earliest stages, the vulnerability of neurons to the formation of neurofibrillary tangles and amyloid plaques may be decreased. It is possible that future drugs which stabilize β-catenin could be helpful both with early memory impairment and in decreasing later neuropathology.

Role of β-catenin in development to disease

β-Catenin is essential for normal embryonic development of the central nervous system as well as normal neuronal functioning in adulthood. Alterations in β-catenin signaling lead to detrimental effects throughout the lifespan. As mentioned earlier, β-catenin knockouts are embryonic lethal[82]. In contrast, embryonic transgenic mice that express stabilized β-catenin in neural precursors develop gross enlargements of the cerebral cortex, hippocampus, and amygdala[171, 172]. Thus, both down- and up-regulation of β-catenin can alter developmental processes. Such tight regulation of β-catenin function is also important for synapse assembly. Perturbations in β-catenin regulation produce deficits in both pre- and postsynaptic structure and function. Understanding how deregulated β-catenin function interferes with homeostasis of the healthy adult vertebrate brain may provide insight into the etiology of neurodegenerative conditions.

In summary, β-catenin is present from development into adulthood. It plays critical roles in many of the cellular and molecular functions that take place during all aspects of life. Understanding how β-catenin may function during development, but also during synapse remodeling in adulthood, may help to understand how alterations in its normal regulation can lead to disease. Furthermore, manipulations of β-catenin in adulthood may alter memory formation and may serve as novel therapeutics in learning and memory related disorders.

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