LIMK1 Regulation of Long-Term Memory and Synaptic Plasticity

by

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Department of Physiology

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Abstract

The LIM-Kinase family of proteins (LIMK) plays an important role in actin dynamics through its regulation of ADF/cofilin. A subtype of LIMK, LIMK1, is mostly expressed in neuronal tissues with high levels in the mature synapse. Previous studies from the Jia lab have shown that LIMK1−/− mice exhibit abnormal spine morphology as well as altered hippocampal synaptic plasticity. LIMK1 has been shown to interact with CREB during neuronal development (Yang et al., 2004). We propose that LIMK1 is able to phosphorylate CREB in response to a synaptic activity. We hypothesize that if LIMK1 activates CREB in mature neurons, then LIMK1 knockout mice will have decreased L-LTP and deficits in long-term memory.

My results show that LIMK1 and CREB exist in a complex and are bound to each other in mature neurons. LIMK1−/− mice exhibit deficits in the late phase of long-term potentiation and specific deficits in long-term memory while short-term memory remains unaltered. Pharmacological activation of CREB attenuates the observed deficits in synaptic plasticity and long-term memory. These results show a potentially novel mechanism of CREB activation in response to synaptic activity. Moreover, using peptides to manipulate actin dynamics in LIMK1
lacking animals only has effects on early LTP and is not able to rescue the late phase LTP deficits found in LIMK1 -/- mice. These results indicate a specific role of LIMK1 long-term memory and synaptic plasticity through regulation of CREB and not through regulation of the actin cytoskeleton.
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1 Background

1.1 Learning, Memory and Synaptic Plasticity

1.1.1 Learning and Memory

We acquire new experiences throughout our lives, and we have recollections of those experiences which we can maintain for years or a lifetime. The process behind the acquisition and storage of this information we acquire is called learning and memory. Their storage and acquisition involves the alterations down to the molecular level in our brain. The processes that underlie these molecular changes underlie the biological basis of learning and memory. This can be a complicated task, as these processes are required to be specific, stable, and in some cases permanent; yet they also need to be adaptable and flexible to incorporate new information and alter old information. For example, childhood memories can last the entire length of one’s lifetime. Essentially, long-term memory refers to memories that we keep with us over extended periods. The name of a childhood friend and the name of our first school are both examples of long-term memories. On the other hand, short-term memories need to be encoded for relatively brief periods and may involve somewhat different molecular processes. Memorizing a series of digits, such as in a phone number, for a short time is an example of short-term memory at work. Short-term memories and long-term memories appear to be distinct and therefore, there need to be mechanisms in the brain that distinguish between short-term and long-term memory (Kandel, 2001).

Additionally, the particular type of memory can depend on the how it is acquired and its function. Episodic memory refers to our memories of events, such as a high school graduation.
Semantic memory deals with the defined information about our world, for example, remembering that the sky is blue. Both of these types of memories are declarative in that they require work to form and to recall.

1.1.2 The Hippocampus and Its Importance in Learning and Memory

Learning and memory have been studied in various regions of the brain. However, much of the knowledge we have about the molecular processes that involve learning and memory comes from studies of the hippocampus. The hippocampus is a brain region that is necessary for the early stages of memory formation. It is well known that it sends and receives information from a number of different parts of the neocortex (Lynch, 2004). It is generally proposed that, along with the subiculum and entorhinal cortices, the hippocampus is necessary for transferring short-term memory into long-term memory (Lynch, 2004). A number of studies have revealed the hippocampus’ importance in long-term memory. For example, patients undergoing bilateral hippocampal lesions have been found to experience anterograde amnesia, a condition in which those afflicted are unable to form new long-term memories (Zola-Morgan et al, 1982; Káldy and Sigala, 2004; Lynch et al, 2004). Moreover, the amount of connectivity that exists between the hippocampus and the other regions of the brain suggests that it may be important in associating particular sensory information from various regions of the brain about a certain experience and consolidating it into a single memory of specific place, person or event. Interestingly, the immature development of the hippocampus is responsible for us not remembering our early childhood memories (Káldy and Sigala, 2004). These findings indicate a central and important role for the hippocampus in memory. Accordingly, it has been a viable target for those trying to discern the molecular basis for learning and memory.
1.1.3 Circuitry of the Hippocampus

As stated above, the hippocampus receives input from several regions of the neocortex. Signaling in the hippocampus itself is largely unidirectional. The hippocampus itself is divided in different regions. Axons projected from cells in the entorhinal cortex make connections with the granule cells in the dentate gyrus as well as the Cornu Ammonis (CA) 3 pyramidal neurons. CA3 neurons also receive input from the mossy fibre cells of the dentate gyrus. The CA3 neurons, in turn, send axons toward the CA1 region pyramidal cells via the Schaffer collateral pathway (Figure 1.1).

Importantly, the CA3-CA1 Schaffer collateral pathway of the hippocampus is a well studied region when it comes to synaptic plasticity. A great deal what we understand about long-term potentiation, a specific type of experimentally induced synaptic plasticity, comes from investigations done in this particular region (Andersen et al., 2007, Lynch, 2004).
Figure 1.1
Figure 1.1 Simplified Circuitry of the Hippocampus. The hippocampus forms a generally unidirectional network. Signals from the entorhinal cortex (EC) form connections with cells in the dentate gyrus (DG), shown in yellow, and with cells in CA3 regions, shown in blue, via the perforant pathway (PP). The cells of the dentate gyrus form connections with those of the CA3 via the mossy fibre pathway (MF). CA3 cells send their signals via the Schaffer collateral pathway (SC) to the CA1 neurons. The signal is then sent from the CA1 cells to neurons in the subiculum (Sbc). (Adapted from Daumas et al, 2009)
1.2 Synapses

Communication between the neurons occurs mainly at the synapse (Anderson et al., 2007). Synapses are highly specialized connections that exist between neurons that allow for synaptic plasticity and ultimately, learning and memory. The manner in which the cell manipulates the strength of these synaptic connections is called synaptic plasticity. Accordingly, the synapse has been the focus of intensive studies that are trying to examine the molecular mechanisms of learning and memory.

1.2.1 Structure of the Synapse and Synaptic Transmission

The synapse itself consists of presynaptic and postsynaptic sites along with the space that separates them which is referred to as the synaptic cleft. The presynaptic site is also referred to as the terminal bouton. This is the site where once action potential arrives it is subsequently followed by sufficient calcium ion influx. The calcium ion influx causes vesicles containing neurotransmitters to fuse with the membrane and release their contents. Much of the excitatory neurotransmission in the mammalian brain uses glutamate as a neurotransmitter. The neurotransmitter diffuses across the synaptic cleft and comes in contact with specific neurotransmitter receptors on the postsynaptic membrane of the dendritic spine, a small mushroom shaped protrusion of the dendrite. In glutamatergic neurotransmission, there are several different types of postsynaptic receptors that are present on the postsynaptic cell. These include the AMPA (α-amino-5-hydroxy-3-nethyl-4-isoxazole propionic acid) and NMDA (N-methyl-D-aspartic acid) receptors and mGluRs (metabotropic glutamate receptors). NMDA and AMPA receptors are ligand gated ion channels, in that they require glutamate binding in order for them to allow for the passage of ions (Dingledine et al., 1999). Both types of receptors
consist of multiple subunits that govern the functionality of the channels. For example, AMPA receptors are generally impermeable to calcium ions if they contain the GluR2 subunit (Jia et al, 1996, Hollmann et al, 1991, Bochet et al, 1994). Moreover, NMDA receptors act as coincidence detectors; they require ligand binding and cell depolarization in order for activation and removal of the Mg\(^{2+}\) block respectively (Nowak et al., 1984, Mayer et al., 1984). At basal conditions Mg\(^{2+}\) resides in the pore of the NMDA receptor and depolarization is required for this block to be removed. Once glutamate and glycine are bound and the postsynaptic membrane is depolarized cations are able to flow through the NMDA receptor. It is this property of the NMDA receptors that allows the postsynaptic cell to differentiate between spontaneous random firing from the presynaptic cell and strong correlated activity resulting from action potentials (Cooke and Bliss, 2006). Hippocampal long term potentiation (LTP) is thought to be induced by calcium influx through NMDA receptors and maintained by more complex processes including the insertion of AMPA receptors to the synapse (Lynch, 2004, Malenka, 2004) (Figure 1.2).

1.3 Synaptic Plasticity: A general overview

Synaptic plasticity is thought to be the biological correlate of learning and memory and is usually defined as a long-lasting change in synaptic efficacy (Lynch 2004). Over a hundred years ago, Santiago Ramon y Cajal and other neuroscience pioneers hypothesized that learning and memory involved changes in the way neurons interact with each other. Indeed, much empirical evidence has shown that synaptic plasticity involves altering the strength of the synaptic connections between neurons (Cooke and Bliss, 2006; Lamprecht and LeDoux, 2004; Mullet at al 2002; Sweatt, 2004). This often includes changes that essentially increase the sensitivity of the neurons and in some cases, increasing the number of connections between neurons (Lamprecht and
Taking all this into consideration, synaptic plasticity involves alterations in the manner in which neurons communicate with each other, as well as changes to the neurons themselves in their ability to respond and send signals.

1.3.1 Long-Term Potentiation

A well studied form of synaptic plasticity is long-term potentiation (LTP) (Figure 1.2). LTP can be induced both \textit{in vivo} in intact animals and \textit{in vitro} on brain tissue slices and cell cultures (Cooke and Bliss, 2006). LTP is often viewed as an experimental analogue of learning and memory (Bliss and Collingridge, 1991, Kandel, 2001). Because of the hypothesized relationship between learning and memory and LTP, LTP is a widely and well studied experimental phenomenon. LTP in Schaffer collateral pathway has been extensively correlated with learning and memory, such that proteins found to be important for LTP are also found to be important for learning and memory (Bliss and Collingidge, 1993; Malenka and Nicoll, 1999, Cooke and Bliss, 2006).

LTP in the CA1 region of the hippocampus is characterized by a number of different properties. The first is co-operativity, which means that several different synaptic connections have to be activated to induce LTP. The second is associativity, in that concomitant activity is required in both the presynaptic and postsynaptic neurons. LTP is also marked by input specificity, such that, LTP occurring at a given synapse is not propagated to other synapses in the same neuron. These properties are shared with associative learning and memory, and therefore make CA1 LTP a useful model for studying these processes (Cooke and Bliss, 2006).
CA1 LTP shares another characteristic with memory in that there are two distinct forms of LTP. Memory consists of short-term memory and long term memory. Similarly, there is early LTP (E-LTP) and late LTP (L-LTP). L-LTP depends largely on de novo protein synthesis (Kandel, 2001). This means that L-LTP involves the activation of transcription factors and new gene expression which leads to new protein synthesis. During L-LTP the signal from the dendritic spine undergoing LTP has to reach the nucleus of the neuron. Experimentally, L-LTP lasts at least three hours but can persist for as long as days and weeks in some cases. On the other hand, the mechanisms responsible for E-LTP include, amongst others, phosphorylation of already existing proteins and insertion of neurotransmitter receptors into the synapse. Thus, E-LTP depends largely on the modulation of the activity and location of already existing proteins. In fact, protein synthesis inhibitors have no effect on E-LTP but abolish L-LTP (Kandel 2001). Moreover, this mechanistic difference translates to a shorter overall duration for E-LTP, generally no more than 2 hours.

1.3.2 Synaptic Plasticity and Memory Hypothesis

LTP and memory share many of the same mechanisms, however, whether LTP equals learning and memory is more complicated question. For one it has to be qualified as to what type of learning and what type of memory we are investigating and whether LTP is responsible for the persistence of memory (Stevens, 1998). Indeed, LTP shares many of the same mechanisms as learning and memory. Blockade of GluA1R synaptic expression prevents induction of LTP (Jensen et al., 2003,) and also prevents memory formation (Reisel et al., 2002). However, there are interesting exceptions, for example in some memory GluA1 lacking mice exhibit LTP deficits but no deficits in memory (Zamanillo et al., 1999). Much of the evidence tying LTP to learning and memory is correlative in nature and it has been difficult to provide direct evidence.
However, what we do know is that LTP and memory share many of the same mechanisms (Martin et al, 2000) and further work is being done to strengthen the evidence tying LTP to learning and memory. For example, training rats to retrieve pellets with their preferred paw induced LTP like changes in the corresponding cortex which are absent in the opposite hand cortex (Rioult Pedotti, et al., 1998, Monfils and Teskey, 2004). The most compelling evidence comes from work done in Mark Bear’s lab. Training animals in this study led to in vivo recorded LTP in 27% of the electrode that were arranged in the multielectrode array. Further LTP was also occluded in those 27% of electrodes but was induced in the unpotentiated pathways (Whitlock et al, 2006). These studies go a long way in establishing that LTP and memory are closely related and however as things remain LTP serves as the best experimental analogue which provides us with the insight on the mechanisms that underlie learning and memory.
Figure 1.2
Figure 1.2 Excitatory Synaptic Transmission and Plasticity at CA1 Synapses. The synapse is comprised of the presynaptic terminal bouton and the postsynaptic dendritic spine, and the space between them is termed the synaptic cleft. To initiate synaptic transmission, an action potential travels down the bouton where it triggers calcium influx through voltage gated calcium channels. This causes a vesicle to fuse and release its contents into the synaptic cleft (1). The neurotransmitter binds to neurotransmitter receptors such as NMDA receptors, AMPA receptors and mGluRs (2). Binding to NMDA and AMPA receptors allows for calcium influx (2) into the dendritic spine. Calcium ion influx leads to activation of calmodulin which activates Calmodulin kinase II (CamKII), extracellular signal regulated kinase (ERK) and adenyl cyclase among other signaling molecules (4) which collectively lead to increase the expression of surface AMPA receptors (5). Binding of glutamate to mGluRs leads to activation of G proteins which leads to activation of adenyl cyclase (3) which can also be activated by signalling from calmodulin. Activation of adenyl cyclase leads to an increase in cyclic adenylyl monophosphate (cAMP) and activation of protein kinase A (PKA). In turn, PKA signaling activates cyclic response element binding protein (CREB) (5) which leads to gene transcription. CREB has also been shown to be activated by ERK.
1.3.2.1 Mechanisms of LTP: A general Overview

The key mechanism of LTP induction is an increase in intracellular calcium levels in the postsynaptic neuron (Bliss and Collingridge, 1993, Lynch, 2004). This increase in calcium levels is mediated by, in large part, NMDA receptors (Lynch, 2004). Calcium influx through NMDA receptors then leads to activation of a calmodulin kinase II (CaMKII) (Malenka et al, 1989). CaMKII activation leads to a number of changes that result in the initiation and maintenance of LTP. For example, CaMKII activation is thought to lead to phosphorylation and insertion of AMPA receptors to the synapse (Shi et al, 1999: Shi et al, 2001).

AMPA receptor insertion is a key mechanism that drives the expression of LTP. AMPA receptor insertion itself is a dynamic process that occurs at basal conditions (Luscher and Frerking, 2001, Malinow and Malenka, 2002). However, this cycling can be modulated by activation of NMDA receptors and leads to activity-dependent AMPA receptor insertion (Malinow and Malenka, 2002, Liao et al, 2001, Lu et al, 2001). In addition, LTP studies in mice lacking AMPA receptor subunits (Jia et al., 1996, Asrar et al., 2009) implicate AMPA receptors as important factors in LTP.

CaMKII activation and AMPA receptor insertion modulate the changes that are largely responsible for the early phase of LTP maintenance. Cyclic adenosine monophosphate (cAMP) is another key molecule that is activated during LTP. Much evidence has shown that cAMP and PKA activation is important in the maintenance of L-LTP (Huang and Kandel, 1994, Huang et al, 2000, Otmakhova et al, 2000). Moreover, cAMP/PKA signaling has been found to lead to
activation of transcription factors which result in new gene expression that has been implicated as being critical for the occurrence of L-LTP (Deadwyler et al., 1987, Frey et al., 1988, Huang and Kandel, 2001, Kandel, 2001).

In addition, increases in cAMP levels also activate extracellular response kinase/mitogen activated protein kinase (ERK/MAPK) (Impey et al, 1998). Because of the large number of downstream targets of ERK, it appears to play a key role in both E-LTP and L-LTP (Lynch, 2004). For example, pharmacological inhibition of ERK activation leads to a depression in E-LTP as well as L-LTP (McGahorr et al, 1999). ERK substrates include CREB, MAP-2, c-fos, and Tau, which vary from transcription factors to cytoskeletal proteins (Lynch, 2004). The regulation of such varied proteins can lead to wide ranging implications in terms of the processes activated during LTP. CREB, L-LTP and Long Term Memory

A common target of the above named proteins and their signaling cascades is the transcription factor cAMP response element binding protein (CREB). It is one of the important transcription factors that are activated during L-LTP and accordingly it is one of the best studied transcription factors involved in synaptic plasticity (Kandel, 2001, Huang and Kandel, 2002). Moreover, CREB has been shown to play a role in a number of other key processes such as cell proliferation and cell death. In relation to synaptic plasticity, CREB has been shown to be critically involved in long-term memory in a number of organisms including Aplysia, Drosophila and mice (Kandel, 2001; Silva et al, 1998; Hummler et al, 1994; Impey et al, 1999; Casadio et al, 1999). For example, mice lacking certain CREB isoforms have decreased L-LTP as well as deficits in long-term memory (Silva et al, 1998; Hummler et al, 1994, Bourtchuladze et al...
1994). Prevention of CREB activation also leads to a deficit in L-LTP (Pittenger et al, 2006). Drugs that increase CREB activation can rescue L-LTP deficient phenotypes and can recover deficits in long-term memory (Bailey et al, 2004). It is important to note that these above studies show effects on L-LTP and long-term memory specifically while E-LTP and short-term memory remain largely unaffected by CREB manipulations. This further implicates the role of CREB as an important factor specifically during L-LTP and long-term memory.

1.3.2.2 CREB activation

CREB is expressed in all cells of the brain and is thought to be important in coupling extracellular signals with gene expression (Bailey et al, 2004). Indeed, regulation of CREB can be quite complex as it has numerous phosphorylation sites. However, it is widely accepted that CREB activation requires phosphorylation at the serine-133 position (Kandel, 2001, Bailey et al, 2004). When activated, CREB forms dimers that are then bound to the cAMP Response Element (CRE) of the DNA strand and initiates transcription and expression of a number of immediate early genes. Through this process, CREB mediates the consolidation of long-term memory from short-term memory (Kandel, 2001, Bailey et al, 2004, Silva et al, 1998).
Figure 1.3
**Figure 1.3 CREB signaling.** CREB is activated by a number of different extracellular signals. Surface receptors such as N-Methyl-D-aspartic (NMDA) receptors, and receptor tyrosine kinases (RTK) initiate the signaling cascade. Calcim influx via NMDA receptors activates adenyl cyclases (AC), which in turn use cyclic adenyl monophosphate (cAMP) as a messenger. cAMP activates protein kinase A (PKA) which then activates cyclic response element binding protein (CREB). In addition calcium influx through NMDA receptors activates calmodulin. Calmodulin, in turn, acts on Calmodulin Kinase IV which then activates CREB. Another pathway of CREB activation is through Ras GTPases. Ras GTPases can be activated by calcium influx in addition to activation by RTKs. Upon activation, Ras GTPases trigger extracellular signal-regulated kinases (ERK) signaling which eventually leads to CREB activation.
1.4 The Dendritic Spine and Synaptic Plasticity

1.4.1 Dendritic Spine Shape

As mentioned earlier, the dendritic spine is the major postsynaptic site of excitatory neurotransmission. The unique conformation of the dendritic spine allows for its specialized role. Although dendritic spines can be found in a variety of shapes and can alter their form during development, the majority of mature spines are mushroom shaped with a relatively large head and a thin, constricted neck (Tada and Sheng, 2006). This characteristic shape of the mature dendritic spine is thought to be beneficial for compartmentalizing the chemical changes within the spine itself. The small size of the head is thought to be necessary for achieving a fast diffusional equilibrium, while the length of the spine neck controls the time constant of calcium extrusion from the dendritic spine (Oertner and Matus, 2005). Young, immature dendritic spines are more elongate with a thicker and longer neck and they lack a clear distinction between the head and neck. Immature spines tend to be more plastic than mature spines and are more likely to undergo potentiation (Tada and Sheng, 2006).

1.4.2 Molecular Determinants of Dendritic Spine Structure

Actin governs the shape of dendritic spines as it is the major cytoskeletal component (Tada and Sheng, 2006). Therefore, these changes in how the actin cytoskeleton is regulated are likely to have effects on the shape of the dendritic spine. This is particularly the case during synapse development, and during synaptic activity (Lamprecht and LeDoux, 2004). It is because the actin cytoskeleton is important in spine morphology and the structural plasticity of the spine a
number of proteins that regulate the actin cytoskeleton are thought to be important in synaptic plasticity, learning and memory as well as during development.

Dendritic spines can alter their morphology in response to external signals. For example, evidence has shown that spines increase in size when stimulated or during LTP (Lang et al, 2004; Yuste and Bonhoeffer, 2001, Fischer et al, 1998). The mechanism that drives the changes in size is the differential recomposition and breakdown of actin filaments in the actin cytoskeleton of the dendritic spine. Actin is a good candidate for regulating the shape of synaptic structure because it is quite dynamic. Actin filaments undergo constant cycling and turnover during a process termed “treadmilling” (Dillon and Goda, 2005). By regulating the cycling between actin filaments (f-actin) and globular actin monomers (g-actin), the actin cytoskeleton can change from a quite rigid structural component to a somewhat soft and malleable one and vice versa. For instance, during expansion and growth of the dendritic spine, the cycling can be controlled to favour expansion of the actin filaments and prevent their depolymerization. Conversely, during instances of dendritic spine shrinkage, actin cycling is regulated such that it favours actin depolymerization (Lamprecht and LeDoux, 2004).

1.4.3 The dendritic spine and synaptic plasticity

The majority of the changes that occur during CA1 LTP are thought to occur postsynaptically. The dendritic spine is a vital structure in mediating these changes as it is the major site of excitatory transmission in the postsynaptic neuron. The structural changes that occur during LTP give us a hint as to the importance of the dendritic spine in synaptic plasticity. For example, increase in the size of the head of the dendritic spine leads to an increase in the overall area of synaptic contact. This may allow for an increased number of AMPA receptors at the
postsynaptic site. Indeed, it has been shown that these AMPA receptors originate from the dendritic spine itself and are recruited from endosomes found in dendritic spines (Malinow and Malenka, 2002). It is widely established that it is the AMPA receptors that drive LTP (Malenka and Bear, 2004; Malinow and Malenka, 2002). Previous studies have also suggested LTP induced by high-frequency stimulation leads to an increase in the number of spines (Lamprecht and LeDoux, 2004, Yuste and Bonhoeffer, 2001). The emergence of new dendritic spines is thought to be important for tying structural modifications to an enduring change in synaptic strength (Kandel, 2001, Lamprecht and LeDoux, 2004). Therefore, it seems likely that these observed changes seen in dendritic spines are closely linked to the formation of new memories. Although it may be speculative to suggest dendritic spines are equivalent to memories, the processes that lead to new spine formation are likely to be involved in learning and memory.

The importance of dendritic spines can be further emphasized if we consider the fact that many diseases that result in cognitive deficits or abnormalities such as Down’s syndrome, Fragile X syndrome and epilepsy produce abnormalities in dendritic spine morphology or spine number (Lamprecht and LeDoux, 2004). Therefore, dendritic spines are vital components of the central nervous system and are necessary for its proper function.
Figure 1.4
**Figure 1.4** Dendritic spines during synaptic plasticity. 1) Synaptic transmission induces changes in dendritic spine shape. 2) During long-term potentiation (LTP) dendritic spines are found to increase in size. This allows for insertion of more receptors to the synaptic site and other changes that facilitate an increase in synaptic response. 3) Over time, new spines can form. This has been found to occur during LTP as well as in animals undergoing learning tasks. 4) Spine shrinkage can occur in times of induced synaptic activity. Long-term depression (LTD), an experimentally used form of synaptic plasticity which can be seen as the opposite of LTP, induces a depression in the synaptic response. LTD can cause spine shrinkage. 5) With a prolonged depression in synaptic activity, spine “pruning” can occur. In this case, the spine has been completely removed.
1.5 The Rho family of small GTPases and Synaptic Plasticity

The Rho family of small GTPases (RhoGTP), which includes RhoGTPases, related to PKA and PKC protein (Rac) and cell division cycle 42 protein (cdc42) are known to be potent regulators of the actin cytoskeleton (Jaffe and Hall, 2005). They are activated by increases in intracellular calcium levels in addition to signals from adhesion molecules and extracellular signals from ephrins (Rossman et al, 2005; Lamprecht and LeDoux, 2004). The RhoGTPases function as molecular switches by adopting conformational changes in response to binding to GDP or GTP (Rossman et al, 2005). They are active when bound to GTP and inactive when bound to GDP (Van Aelst and DSouza-Schorey, 1997). The cycling between the GDP and GTP bound states is controlled by guanine nucleotide exchanges factors (GEFs) which exchange GDP for GTP (Hart et al 1991; Schmidt and Hall, 2002).

The majority of the work done on RhoGTPase dependent regulation of the actin cytoskeleton has taken place in fibroblasts and human embryonic kidney (HEK) cells. Studies implicating Rho GTPases in synaptic plasticity have been particularly prevalent in the last 20 years (Tolias et al, 2011; Oh et al, 2010; Nishimura et al, 2006; Newsome et al, 2000)). Indeed, much recent work has implicated the RhoGTPases and their downstream targets in several neurodevelopmental and mental retardation syndromes. For example, oligophrenin1 (OPHN1) an inhibitor of RhoA, and p21 activated kinase 3 (PAK3) a regulator of the actin cytoskeleton and CREB (Meng et al, 2006) are involved in X-linked mental retardation (Ramakers et al, 2002). As well, RhoGTPase signaling is involved in syndromic mental retardation, that is, mental retardation in combination with other medical conditions. Faciogenital dysplasia protein (FGDY) and LIM-kinase 1
(LIMK1) are involved in Aasrkog-Scott and Williams-Beuren syndrome respectively (Lebel et al., 2002; Martindale et al., 2000).

However, in much of these cases, the mechanisms by which these proteins can affect learning and memory and synaptic plasticity are not well characterized. In fact, some of these proteins may not simply act on regulation of the actin cytoskeleton but may additionally affect other pathways that are important in learning and memory and synaptic plasticity. For instance, it has been shown that PAKs are capable of interacting and regulating ERK (Meng et al, 2006), as well it has been shown that LIMK1 interacts with CREB during development in hippocampal neuroprogenitor cells (Yang et al, 2004).
**Figure 1.5 Actin filament regulation is largely controlled by the RhoGTPases.** The RhoGTPases are activated by a number of signaling pathways in the postsynaptic neuron. Signals such as calcium influx from NMDA receptors can activate tyrosine kinases (TK) which then lead to activation of RhoGTPase activating protein (RhoGap) which regulates the RhoGTPases. In addition, activation by ephrins of ephexin inhibits RhoGTPases. When active, RhoGTPases activate Rho-associated kinase (Rock) and p21 associated kinase (PAK). PAK and Rock both activate LIMK which inhibits actin depolymerisation factor (ADF)/cofilin. ADF/cofilin is thus prevented from binding to and depolymerising actin filaments. The Rac/Cdc42 pathway also plays a role in this signaling. Upon activation by Rac/Cdc42 guanine exchange factors GEFs, Rac/Cdc42 activates PAK. In addition, Rac/Cdc42 also activates Arp2/3, which then polymerises or extends actin filaments. (Adapted from Lamprecht and LeDoux, 2004)
1.6 Regulation of Actin Dynamics

In the cell, actin exists in two states, filamentous actin and globular actin. There is a constant turnover of actin filaments, and the equilibrium shifts toward each type of actin in an activity dependent manner. Depolymerisation and polymerisation of the actin cytoskeleton is largely controlled by downstream proteins of the Rho family of GTPases. The barbed end of the actin filament (f-actin) is constantly broken down whereas the opposite, or pointed end, is expanded. The expansion or the addition of g-actin to actin filaments is what generates force and growth in the dendritic spine. This process of constant actin filament turnover is known as “treadmilling” and actin filaments undergo complete turnover in 60 seconds, with only a small fraction remaining stable for longer periods of time (Kuhn and Pollard, 2005). At the dendritic spine, it has been shown that actin filament growth occurs from the spine tip to the spine base, suggesting that the synapse is the major point of polymerization (Kuhn and Pollard, 2005). A number of actin regulators control the size of the actin filaments by increasing or decreasing f-actin polymerization and depolymerization. Actin depolymerization is regulated by a number of proteins, key of which is the actin depolymerizing factor (ADF)/cofilin family of proteins. Actin depolymerizing factor (ADF)/cofilin are a family of proteins that are known to be important regulators of the actin cytoskeleton (Kuhn et al, 2000). ADF/cofilin binds to f-actin and facilitates both severing and depolymerization (Huang et al, 2006)) (Figure 1.6). LIMK1 negatively regulates ADF/cofilin by direct phosphorylation (Bamburg and Wiggan, 2002). LIMK1 is a direct regulator of ADF/cofilin and serves as a potent regulator of actin dynamics and the actin cytoskeleton (Yang et al, 1998). Through its regulation of ADF/cofilin and, thereby actin and the actin cytoskeleton, LIMK1 can be a critical factor in regulation of synaptic plasticity.
Figure 1.6
**Figure 1.6 Actin turnover as regulated by coflin.** Actin turnover occurs constantly in all cells. Several important regulatory proteins have been found that regulate this process. Actin depolymerizing factor (ADF)/cofilin binds to and breaks down actin filaments. Additionally, ADF/Cofilin is itself subject to regulation by LIM-kinase (LIMK) and slingshot phosphatase. Phosphorylation by LIMK inactivates ADF/Cofilin activity whereas dephosphorylation by slingshot phosphatase activates its function.
1.6.1 Actin and Synaptic Regulation

At the presynaptic terminal, actin aggregates at the active zone and nearby synaptic vesicle clusters (Blecker et al, 2012; Shirao and Sekino, 2001). Moreover, it has been now widely reported that actin plays an important role in neurite outgrowth and axon formation (Gordon-Weeks, 1991).

At the post-synaptic dendritic spine, actin interacts with the post synaptic density (PSD). Scaffolding proteins, which make up the PSD, interact with actin filaments (Boeckers, 2006). Indeed, inhibiting actin polymerization by latrunculin results in a loss of important scaffolding proteins from the PSD, (Kuriu et al, 2006) while preventing depolymerization by jasplakinolide disrupts any morphological changes in the PSD (Blanpied et atl, 2008). Moreover, NMDA receptors are attached to actin via intermediate proteins and their trafficking has been shown to require depolymerization of the actin cytoskeleton (Morishita et al, 2006). AMPA receptor stabilization and endocytosis is also regulated by the actin cytoskeleton. AMPA receptor stabilization occurs by direct interaction with PSD-95 and stargazin (Schnell et al 2002). AMPA receptor endocytosis occurs by disruption of the Arp2/3 complex by PICK1 which concurrently binds to the GluA2 subunit of the AMPA receptor (Liu and Cull-Candy, 2005; Rocca et al, 2008). Additionally, our group has also shown that hippocampal long term depression (LTD), a form of synaptic plasticity, depends on an actin based mechanism (Zhou et al, 2011). Indeed, we have demonstrated that mGluR LTD is abolished with inhibition of LIMK1 and cofilin activity (Zhou et al, 2011).
Regulation of actin cytoskeleton turnover has been shown to be important in learning and memory and synaptic plasticity (Kramar et al, 2006, Fukazawa et al, 2003, Matsuzaki et al, 2004, Fifkova et al, 1992). For example, one study has specifically shown that during L-LTP several actin regulating genes have their activity upregulated (Yamazaki et al, 2001), while a recent study has shown that actin remodeling is crucial for the activity induced conversion of silent synapses (Shen et al, 2006).

1.7 LIM-kinase

1.7.1 Importance of LIMK1 in cognition: Williams-Beuren Syndrome

Williams-Beuren syndrome (WBS) is a developmental disorder with distinct neurocognitive profile. Individuals with WBS tend to be overly social with well developed language skills but severe deficits in spatial cognition. Because of these symptoms it is thought that WBS appears to show a distinction between spatial learning and language development. Patients are also characterized with a mean IQ of 60, which falls in the mentally retarded range (Bellugi et al, 1999). Moreover, LIMK1 is one of the genes that is duplicated in autism spectrum disorders (Sanders et al, 2011) and suggesting a strong link between it and autism. Additionally, the behavioural phenotype of Williams-Beuren syndrome can be thought of as a reverse autism phenotype. Both of these findings taken together suggest a possible link between LIMK1 and sociability.

The genetic hallmark of WBS is a deletion in the chromosome band 7q11.23 which includes the limk1 gene (Robinson et al., 1996). Moreover, it appears that LIMK1 can be directly linked to the visuo-spatial abnormalities present in Williams-Beuren syndrome (Frangiskakis et al., 1996).
This deficit in spatial abilities suggests that there may be abnormal hippocampal function (Bellugi et al, 1999, Morris and Mervis, 2000). Moreover, deficits in verbal memory in Williams Syndrome individuals further alludes a role for the hippocampus since it is thought to play a role in verbal memory in humans. In this way, Williams Syndrome underlines the potentially important role that LIMK1 may have in overall cognition, thereby making it an interesting protein to study in order to understand the mechanisms involved in learning and memory.

1.7.2 General Properties of LIM-kinase

LIMK is serine/threonine kinase consisting of two LIM domains, a PDZ domain and a kinase domain (Figure 1.7). The LIM acronym is derived from the three genes that it is a product of: lin-11, isl-1 and mec-3. The LIM domain of LIMK is defined by a cysteine rich sequence, contributing to a double zinc finger motif. The LIM domain is thought to aid in protein-protein interactions as well as cellular localization (Mizuno et al, 1994, Bernard et al, 1994, Okano et al, 1995). The PDZ domain aids in protein binding and interactions, and is often found in scaffolding proteins in protein complexes (Hung and Sheng, 2002; Kim and Sheng, 2004). There are two isoforms of LIM-kinase: LIMK1 and LIMK2. LIMK1 and LIMK2 are both present in all tissues in mammals, with LIMK1 being the most abundant in neuronal tissues, including hippocampal pyramidal neurons (Proschel et al, 1995).
Figure 1.7
Figure 1.7 Structure of LIM-kinase. LIM-kinase consists of LIM, PDZ and protein kinase domains. The LIM domain is a product of three genes (lin-11, isl-1 and mec-3) and it contains a cysteine rich sequence, which contributes to a zinc finger motif. It is followed by a PDZ domain. Both of these domains aid in protein-protein interactions and binding. The kinase domain phosphorylates serine, tyrosine and threonine residues. This domain also contains a nuclear targeting region which helps localize LIMK in the nucleus.
1.7.3 LIM-kinase signaling and effect on actin dynamics

Rho and Rac induced cofilin phosphorylation and regulation of the actin cytoskeleton can be blocked by inhibition of LIMK (Sumi et al., 1999). This suggests that LIMK is a common downstream target of the Rho family of small GTPases and that LIMK is a key regulator of the actin cytoskeleton in both of the above pathways. LIMK1 is known to be functionally regulated by two proteins, p21 activated kinase (PAK) and Rho associated kinase (Rock) (Ohashi et al, 2000; Edwards et al, 1999). In this regard, the Rho family of small GTPases can directly associate with and activate both PAK and Rock.

Previous studies have suggested that LIMK, is a potent regulator of actin dynamics by regulation of ADF/cofilin (Arber et al, 1998; Yang et al, 1998). Mice lacking LIMK1 have decreased levels of phosphorylated cofilin, and the effects of actin depolymerising drugs are altered in electrophysiological experiments (Meng et al, 2002). Also, our group has shown that LIMK1-/− mice have abnormal LTP and memory suggesting an important role for LIMK1 in those processes (Meng et al, 2002; Meng et al, 2004). Moreover, aside from Rock and PAK, LIMK1 has been shown to interact with protein kinase C and neureglins (Kuroda et al, 1996; Wang et al, 1998). These proteins are known to be important in the development and function of the nervous system (Metzger and Kapfhammer, 2003; Corfas et al, 2004). Thus, there is significant evidence to implicate LIMK1 as a potentially important factor in the function and development of the CNS.
1.7.4 LIMK1 in Synaptic Regulation and Cognition in Mice

Because of LIMK1’s important role in cognition, as indicated by human genetic studies of individuals with Williams-Beuren Syndrome, and its potent role in regulation of actin dynamics our lab has generated and analyzed LIMK1/- mice. We examined their phenotype in order to determine the role of LIMK1 in synaptic plasticity, and learning and memory. An interesting and novel finding is the abnormal shape of dendritic spines in LIMK1/- mice (Meng et al, 2002). As previously described, mature wild type spines have a characteristic shape that consists of a thin constricted neck and a large round head. It is thought that this distinct shape helps concentrate and localize chemical signals such as intracellular calcium. LIMK1/- mice exhibited thin, elongated spines with no distinction between head and neck. Dendritic spines found in LIMK1/- mice are more akin to filipodia, that is, immature dendritic spines (Meng et al, 2002). These results suggest that LIMK1 is important in dendritic spine maturation and/or maintenance.

The altered spine shape is likely related to altered cofilin and actin regulation. Our previous results have shown that LIMK/- mice under basal conditions have decreased amounts of phosphorylated ADF/cofilin (pAC) whereas total amounts of cofilin are unaltered (Meng et al, 2002). Furthermore, when hippocampal brain slices are treated with NMDA or glutamate brain slices derived from LIMK1/- mice show no enhancement of pAC at basal conditions. On the other hand, in wild type controls NMDA or glutamate treatment leads to increased levels of pAC. This indicates abnormal ADF/cofilin regulation at basal conditions and during synaptic activity in LIMK1/- mice. Actin staining also revealed an abnormal distribution of f-actin in the dendrites. In normal conditions, very little f-actin is present in the dendritic shaft and most actin
staining occurs at the dendritic spine heads. However, LIMK1-/- mice exhibit a large increase in actin staining at the dendritic shaft and show comparatively less actin at the dendritic spine when compared to wild type controls (Meng et al, 2002). As previously stated, this data strongly suggests altered actin dynamics and distribution in LIMK1-/- mice.

E-LTP, induced by high frequency stimulation, was also found to be enhanced in LIMK1-/- mice when compared to wild type controls. This is represents a robust indication of altered synaptic plasticity in the LIMK1-/- mice. Interestingly, effects of actin depolymerising drugs, such as cytochalasin-D, are also reduced in LIMK1-/- mice. This is consistent with the hypothesis that LIMK1 is critical to ADF/cofilin phosphorylation and F-actin stability.

As indicated by the LTP studies, LIMK1-/- mice were found to exhibit abnormal learning and memory. In the fear conditioning test, mice lacking LIMK1-/- mice exhibited higher freezing than wild type controls. This clearly demonstrates the abnormal cognition found in the LIMK1 lacking mice.

The Morris Water maze is another test that assesses the contextual memory of the subjected animals. During this test, it was found that LIMK1-/- mice and the wild type controls performed equally well in initial learning. However, the LIMK1-/- mice performed poorly on the reversal learning task. The reversal learning task consisted of retraining the previously trained mice in the Morris water maze but with the platform then moved to another location for the second test. The mice are then trained to memorize the second location of the platform. The LIMK1-/- mice did not learn the new location of the platform as proficiently as the wild type controls (Meng et al, 2002). These results indicate abnormal memory function in LIMK1-/- mice.
An interesting recent study found that LIMK1 interacts and phosphorylates CREB in hippocampal neuroprogenitor cells. Moreover, the same study found that LIMK1 phosphorylates CREB at serine-133, which is the site that is widely believed to activate CREB function (Yang et al, 2004). This finding suggests that there are likely additional mechanisms of action for LIMK1 in the regulation of synaptic plasticity and learning and memory. However, it is not known whether LIMK1 and CREB interact and regulate each other in mature neurons and what effects this may have on synaptic plasticity.
2 Hypothesis and Objectives

2.1 Rationale

It is not known whether the LIMK1 and CREB interact in the mature brain and whether this interaction contributes to the regulation of synaptic plasticity by LIMK1. If this interaction exists, I aim to investigate the underlying mechanisms and functional consequences that this interaction encompasses.

LIMK1 is known to play an important role in cognition through its association with WBS. Animal studies from our own lab have indicated that LIMK1 is important for learning and memory, hippocampal LTP, dendritic spines and actin dynamics (Meng et al, 2002, Meng et al, 2004). However, the mechanisms by which LIMK1 is important for these processes have yet to be fully identified. Based on a recent study showing that LIMK1 interacts with CREB in hippocampal neuroprogenitor cells and the fact that CREB is a key regulator of L-LTP and long-term memory (Yang et al, 2004), it is possible that LIMK1 may also interact with CREB in mature hippocampal neurons and can therefore regulate synaptic plasticity and learning and memory (Figure 2.1).
2.2 Hypothesis and Objectives

My hypothesis is that LIMK1 interacts with CREB and this interaction is responsible for normal long-term synaptic plasticity and long-term memory function.

The goal of my project is to investigate whether there are interactions between CREB and LIMK and specifically whether there is an activation of CREB by LIMK1 and whether these interactions contribute to synaptic plasticity. To achieve these objectives, I will perform biochemical, electrophysiological and behavioural tests using various knockout mice including LIMK1, PAK1 and Rock2 knockouts. Specifically, I will determine whether:

1. CREB and LIMK1 interact in the mature brain using immunoprecipitation and Western blots.
2. L-LTP is altered in mice lacking LIMK1 using field recordings at the CA1 synapse.
3. Long-term memory is deficient in mice lacking LIMK1 with the use of fear memory tests.
4. Whether this interaction if it exists could be rescued by enhancing CREB activity.
Collectively, the results from these experiments will provide evidence that LIMK1 is involved in L-LTP and long-term memory via regulation of CREB.
Figure 2.1
Figure 2.1 Illustration of Hypothesis. The main hypothesis that is being investigated examines the role of LIMK1 in synaptic plasticity specifically during late-LTP and long-term memory. In particular, it is proposed that via its phosphorylation of CREB and through its regulation of the actin cytoskeleton LIMK plays a role in long-term memory and L-LTP. As stated earlier, LIMK1 has been shown to play a role in synaptic plasticity (Meng et al., 2002) but the underlying mechanisms remain largely unknown.
3 Methods

3.1 Study animals

For experiments, mature mice aged from 2 to 6 months of age were used. Mice lacking the LIMK1 gene were generated from a CD1 strain (Meng et al, 2002). The mice in this study used those LIMK1-/- CD1 background mice backcrossed with C57/bl6 mice for 6 generations. Animals were reared according to standards of the animal care committee at the Hospital for Sick Children. Animals were housed in a Plexiglas shoe box cages with ad lib access to food and water with 12 hour light/12 hour dark cycles.

3.2 Euthanasia and slice preparation

As per the guidelines of the animal care committee mice were euthanized with a cervical dislocation. The head was then removed to be dissected and in order to remove the brain. Once the brain has been extracted, it was placed in ice cold ice cold oxygenated artificial cerebrospinal fluid (ACSF) (120mM NaCl, 4.0mM KCl, 1.0 mM MgSO4, 1.0 mM NaH2PO4, 26 mM NaHCO3, 2.0 mM CaCl2, and 11 mM D-glucose) for 1-2 minutes. The brain was then removed from the ACSF and the cerebellum is removed. The brain was then cut along the midline and placed in the vibratome chamber which contains ice cold oxygenated ACSF. Using the vibratome, 400 µm thick sagital brain slices were cut and placed in a different holding chamber containing oxygenated ACSF at room temperature. The slices were picked up using an eyedropper and were placed gently in a recovery chamber again containing oxygenated ACSF. While in this chamber, the slices were then allowed to recover for at least 1hr before electrophysiological recordings or preparation of cell lysate.
3.3 Electrophysiology

3.3.1 Background

The principles of field electrophysiology involve generating an extracellular current flowing across the membrane resistance between the recording and ground electrodes. Since the hippocampus is a highly laminated structure these synchronized currents that are generated by synaptic activation of the axons give rise to characteristic response field excitatory postsynaptic potential (fEPSP). This synaptically generated current flows into the dendrites from the activated region. Once inside the cell, the current flows away from the activated dendrites and exits where the membrane area is the greatest, or the membrane resistance is least, at the neuronal cell body. Completing the current loop is the ground electrode which gives rise to fEPSPs that are negative in the region of the current sink.

Using extracellular recordings in the hippocampus allows for an accurate reading of synaptic activity with regards to the amplitude, time and polarity of the response. The apical dendrites are all arranged in a roughly parallel direction which allows them to be easily activated synchronously and thereby generating a field potential of a large magnitude.

The hippocampus has been studied extensively using electrophysiological methods. Since the discovery of LTP by Bliss and Lomo (1973), electrophysiological recordings have served as our basis of molecular investigation of learning and memory. In the hippocampal slice preparation, LTP was discovered soon after (Andersen et al, 1977), in an experiment detailing the input
specificity property of LTP. In this experiment, LTP was induced only in the tetanized pathway whereas the response in the untetanized pathway remained at baseline levels.

Moreover, there exists a significant correlation between LTP and memory. Overwhelming evidence has suggested that at the very least LTP and memory formation share extremely similar mechanisms. For example, upregulation of the AMPA receptor subunit GluA1 occurs both during memory formation and LTP (Lee et al, 2009; Shi et al, 1999; Rumpel et al, 2005; Resel et al., 2002).

3.3.2 General Methods

Previously cut hippocampal slices were placed in a recording chamber continuously perfused with oxygenated ACSF at the rate of 2-2.5 mL/min. Once there, a stimulating electrode was positioned in the hippocampus so as to stimulate multiple axons along the Schaffer collateral pathway. Then, a recording electrode was placed in the CA1 region of the hippocampus (Figure 3.1). The slices were stimulated at 60% of the maximum evoked response. The size of the evoked response was measured by the slope of the field excitatory postsynaptic potential (fEPSP). All experiments were performed at room temperature.

3.3.3 Induction of LTP

The fEPSP was recorded for an initial baseline period where the quality and stability of the evoked response was evaluated. L-LTP was induced after a sufficient baseline period, generally a half hour, when the response was deemed to be stable. For high frequency stimulation (HFS) induced L-LTP, four trains of high frequency stimulations of 100Hz lasting one second each were given at 5 minute intervals. The response was then recorded for 3 hours after the last HFS. For theta burst induced L-LTP, 3 trains of 10 theta bursts with an intertrain interval of 10
minutes were used. As before, the response was then recorded for 3 hours after the last stimulation. For the tetraburst HFS stimulation protocol 4 trains of HFS were given at intervals of 20s.

Early-LTP was induced by one train of HFS at 100Hz. The response was recorded for 40 minutes following the stimulation protocol.

3.3.4 Data collection and analysis

Data was collected using pCLAMP 8 or pCLAMP7 software (Axon Instruments). Field EPSPs were measured by taking the slope of the rising phase between 5% and 60% of the peak response. The size of the response was normalized using the mean baseline evoked response value as 100%. In some cases, averages for comparison purposes were calculated for specific portions of the recording as indicated. All data was statistically evaluated by using Student’s t-test.
Figure 3.1
Figure 3.1 Extracellular Electrophysiology in the Hippocampus. CA1 LTP has been well characterized and well correlated with learning and memory; in order to record an evoked response a stimulating is placed in the Schaffer Collateral pathway (see Figure 1.1) while the recording electrode is placed in the CA1 region of the hippocampus. The stimulating electrode generates a test pulse every 15 seconds. Once the response of the hippocampal slices is determined to be stable a stimulating protocol is used to induce a form LTP and the response is recorded again.
3.3.5 Immunochemistry

3.3.5.1 Background

In order to determine protein-protein interactions, amounts of different proteins, as well as phosphorylation of those proteins, immunochemical methods need to be employed. The basic principle of immunochemistry involves animal derived antibodies interacting with the specific proteins that are being probed for. These antibodies are generated by injecting animals with the molecule in question and isolating the immune response β-cells in a process which will eventually generate antibodies specific to the injected molecule. In turn, these primary antibodies serve as antigens for a secondary set of antibodies that are linked to horseradish peroxidase. A chemiluminescent agent is then added and cleaved by the horseradish peroxidase causing. This process causes luminescence to be produced in proportion to the amount of protein that is present. This can then be visualized using varying methods (Figure 3.2). Moreover, the variations in fluorescence intensity can be used to determine the presence or absence of a protein, its relative amounts, as well as the relative amounts of phosphorylation.

It is possible for primary antibodies to not be entirely specific to the molecule that has generated them. Therefore, it is important to use control samples lacking the probed molecule of interest to ensure the specificity of that particular antibody.

In immunoprecipitation, the basic principle involves the use of microscopic beads that have an affinity for antibodies. The interaction between the beads and the antibody and their subsequent isolation from the rest of the protein sample ensure a high amount of purity. In other words, the isolated sample is likely to contain a largely pure sample of the molecule in question. Moreover,
if a particular protein does interact with certain other proteins; they will also be likely pulled down along with the molecule of interest. In this case, the sample can then be subsequently probed with varying antibodies to determine whether such interactions exist.
Protein Primary Antibody

Secondary Antibody linked with Horseradish Peroxidase

Figure 3.2
Figure 3.2 Illustration of Immunochemistry. Immunochemistry uses antibody/antigen reactions and combines with fluorescence or other detection methods. The principle is that a protein has a specific and unique site that a particular primary antibody will bind. A secondary antibody containing a fluorochrome then binds to an antigen site on the primary antibody. The use of detection agents will cause fluorescence in the fluorochrome. Using this reaction, one can determine the presence or absence, as well as the relative amounts of specific proteins.
3.3.5.2 Preparation of brain lysate for immunochemistry

Hippocampal brain slices were isolated in an identical manner as for electrophysiology. In this case, slices were set to recover for 2 hours in oxygenated ACSF. After recovery, the slices were placed in small centrifuge tubes while on ice, with the ACSF was removed and an appropriate homogenization buffer was added. For Western blots, 140μL of lysis buffer (50mM Tris 8.0, 150mM NaCl, 5mM EDTA, 0.5% NP-40, 1% Triton X-100, 0.5mM PMSF, 1mM Na3VO4) was used. For immunoprecipitation, immunoprecipitation buffer (1% Triton X-100, 137mM NaCl, 5mM EDTA, 5mM EGTA, 20 mM Tris, and 1mM Na3VO4) was used. Both solutions contained 1% protease inhibitor (Canbiochem). The slices were then broken down and allowed to be digested for at least one hour.

Following that, they were then centrifuged at 12,500 rpm for 20 minutes. For Western blots, 70μL of the supernatant containing the protein was added to a separate tube and 20μL of 6X loading buffer along with 10μL of 1M Dithiothreitol (DTT) were added. The samples were then stored at -70°C until further use. For immunoprecipitation, tubes containing 500μl were created by pooling the contents of the centrifuged tubes. The samples were then either used immediately or placed at -70°C for storage.
3.3.6 Immunoprecipitation

Whole brains were extracted and lysed as described above. In 500 μL of extracted protein, 50μL of a primary antibody was added. The sample was then placed and incubated overnight at 4°C with shaking. The next day, 50μL of protein-A agarose beads were added to the sample on ice. It was then left to incubate with shaking for 2 hours at 4°C. Following that, the samples were then centrifuged, the supernatant was discarded and the beads were washed with lysis buffer three times, discarding the supernatant each time. The beads were then resuspended in 70μL of lysis buffer and 20μl of 6X loading buffer along with 10μL of 1M DTT were added. The samples were then probed using Western blot or were stored at -70°C.

3.3.7 Western blot

Protein samples of 15 to 20μl were loaded on 10% polyacrylamide gels. The separated samples were then transferred to nitrocellulose membranes. The blotted proteins were probed with polyclonal anti-CREB (Upstate) anti-pCREB (Upstate), anti-LIMK1 (Santa Cruz), anti-PAK1 (Upstate) respectively. The blots where then probed with the appropriate secondary antibodies linked with horseradish peroxidase and the proteins were visualized with enhanced luminescence (Amersham).

3.3.8 Data acquisition and analysis

To estimate relative protein amounts, a densitometer was used. Exposed films were scanned, and the density was measured in all the lanes used and was normalized to the background of each lane. For measurements of phosphorylated CREB, the density value obtained was then
normalized using the density value of total CREB. The values were further normalized in the same blot against the value obtained from wild type controls. This data was then analyzed using Student’s t-test.

3.4 Behaviour Tests

3.4.1 Background

Assessing the learning and memory of mice involves the use of behaviour tests that are designed to assess specific parts of their memory. A simple and robust test that provides a good assessment of learning and memory in mice is the fear conditioning test. It exploits the freezing response that results from a fearful stimulus in mice. The fear conditioning test involves a basic classical conditioning paradigm. A mouse is placed in a conditioning chamber where the visual context is controlled and then a foot shock is administered which is accompanied by the tonal cue. The principle behind this experiment is that the mouse will then associate the context and/or the tone with the pain received from the foot shock. Accordingly, presenting the context and/or cue to the mouse after the training should elicit a freezing response even though no shock is applied.

When testing contextual memory, the mouse is placed in the cage where it was trained with all the contextual cues preserved. It is hoped that the mouse will likely freeze when it recognizes the context. When testing other aspects of memory with the cued test, the mouse is placed in a different context. In this case, the mouse should only freeze at the time during which the tone that accompanied the initial foot shock is played. Moreover, contextual memory is thought to be largely dependent on the hippocampus while cued memory is largely associated with the
amygdala (Lynch, 2004). Therefore, with the use of the fear conditioning test one can test different brain regions and examine how they are affected by different manipulations.

One of the most widely used tasks in assessing spatial memory in rodents is the Morris water maze. The task involves placing an animal in a circular pool of water and having it learn and memorize the location of an escape platform based on various visual cues placed around the pool. The task itself was developed by Richard Morris and first described in 1981.

This task represents a form of higher spatial learning in mice, as it requires several training trials for the animal to fully acquire the location of the platform. After sufficient training trials, the platform is removed and the animal is placed again inside the apparatus. Once inside, the amount of time that the animal spends searching in the former platform area is seen as evidence of spatial memory. Varying the time between the training trials and probe trial allows for testing of short-term and long-term memories.

The fact that the Morris water maze requires spatial learning suggests that it requires proper hippocampal function. Accordingly, it has been shown that mice with hippocampal lesions have difficulty in retaining the memory of the platform location.

3.4.2 Open field test

To assess the general motor activity and general exploratory behaviour of the mice, an open field test was used. This test consists of placing a mouse in the middle of the testing chamber and its movements are tracked using an electronic laser grid for 5 minutes. Parameters such as distance, movement time and rest time were tracked using the laser grid.
3.4.3 Fear conditioning test

3.4.3.1 Training

A standard fear conditioning paradigm was used. Individual mice were placed in a conditioning chamber with controlled contextual cues and an electrified shock floor (Coulbourn Instruments). The mice were allowed to acclimate to the chamber for 2 minutes. Afterwards, a 30 second tone (85dB, 18,000 Hz) was played. A mild foot shock (0.5mA) was applied during the last two seconds of the tone. The mouse was allowed to remain in the chamber for one minute after the shock. This was done to get an assessment of freezing from both the knockout mice and their wild type littermates. This was important in order to determine whether there was a difference in pain sensitivity between the transgenic mice and their wild type littermates. Freezing is defined as lack of any movement with the exception of respiration.

3.4.3.2 Testing

At the indicated intervals (24hr, 2weeks), the fear memory of the mice was tested. Long-term memory tests using mice often involve testing at 24 hours or longer after the training session (Kandel, 2001). Moreover, extending this time interval was performed to obtain a more complete assessment of long term memory. During the contextual test, the contextual cues that were present during conditioning remained. The mouse was placed in the recording chamber for 5 minutes. During the cued test, the chamber and the contextual cues were changed. Following a two minute acclimation, the same tone was played for 2 minutes.
3.4.4 Data acquisition and analysis

Data acquisition was done using TruScan Linc 2.0 software (Coulbourn Instruments). Mouse behaviour was recorded using the TruScanLinc laser grid. Parameters recorded included: number of movements, movement time, rest time, distance traveled, and movement speed. An adjusted value of rest time was used as an indicator of freezing. To calculate freezing time, the rest time of the mice during the testing phase was used. Rest time prior to the foot shock was calculated as normal, baseline resting time. The increase in rest time post shock was judged to be freezing. For a particular group of mice, values for freezing during the cued and contextual test were then normalized using the average freezing time found post shock during the training phase. The data was analyzed using Student t-test in SigmaPlot 2001. In addition, manual recordings of freezing were also performed to confirm the results from the digitally collected data.

3.4.5 Morris Water Maze

3.4.5.1 Training

The mice were handled for 3 days prior to the beginning of training. The training consisted of placing the mice in the water maze (1.3m in diameter) with the arranged spatial cues. The starting position of the mice was randomized. The mice were then left to swim until the platform (10cm in diameter) was found or until 60s elapsed. In cases where the platform was not found, the mice were directed and placed on the platform for 5s and then removed from the water maze. When the platform was found, the mouse was allowed to stay on the platform for 5s and then removed. The mice were trained for 4 training session per day for three consecutive days. This protocol was used in order to compensate for any ceiling effects and to avoid any effects of
spaced training may have on CREB function as has been shown previously (Kogan et al., 1997).
The position of the mice was tracked and recorded using a camera connected to a video tracking system (Noldus Information Technology).

3.4.5.2 Testing
For the test of Morris water maze memory, the platform was removed and mice were placed in the opposite quadrant from the platform and allowed to swim for 60s. As previously, the movements of the mice were tracked and recorded using a camera connected to a video tracking system (Noldus Information Technology). The platform zone was defined as a zone of 20cm diameter with the centre located at the position of the platform. The amount of time the animals spent searching the platform zone was recorded and compared to similar zones in the other quadrants.

3.4.6 Elevated plus maze
The elevated plus maze consisted of four arms (two open arms without walls and the other two enclosed by 15.25-cm-high walls) 30 cm long and 5 cm wide. Each arm was attached to sturdy legs such that they were elevated 50 cm off the ground surface. To initiate the test, a mouse was placed on the edge of an open arm, and once the mouse reached the maze center, the data recording started and the mouse was given 5 min to explore the maze. The arm entry and the time spent in each arm in the maze center were recorded manually. For all behavioral tests, only male mice between the ages of 2 to 4 months were used, and the experiments were performed during the light phase of the cycle (between 9:00 a.m. and 2:00 p.m.). All animals were handled 3 times daily for 3 days before any behavioral tests.
4 Results

4.1 Electrophysiological deficits in LIMK1-/- mice

4.1.1 Introduction

LTP is a phenomenon associated with learning and memory, and is seen as its \textit{in vitro} analogue. Since its discovery by Bliss and Lomo in the rabbit hippocampus, LTP has been extensively studied. Indeed, it has been discovered to occur in various regions of the brain and spinal cord and in different species. For example, LTP has been found to occur in the amygdala, the cerebral cortex, and the cerebellum, and to occur in many mammalian models along with the occurrence of similar phenomena in invertebrates.

Moreover, there exists a significant correlation between LTP and memory. Overwhelming evidence has suggested that at the very least LTP and memory formation share extremely similar mechanisms. For example, upregulation of the AMPA receptor subunit GluA1 occurs both during memory formation and LTP. Both processes involve activation of CaMKII. LTP and memory also both require activation of CREB. However, as stated earlier, the most convincing evidence comes from occlusion experiments that have been performed. In these experiments, either prior learning or prior LTP in a particular brain region occluded either LTP induction or the formation of new memories respectively. Additionally, processes that inhibit the type of memory with respect to it being long-term and short-term specifically also inhibit the type of LTP that is analogous to it i.e. E-LTP and L-LTP (Martin et al, 2000).

For my studies, I examined the role of CREB in L-LTP. There is a large amount of evidence that implicates CREB in this type of L-LTP. CREB has been shown to be important in the
maintenance of L-LTP in the hippocampus. Its activation leads to the activation of immediate early genes, many of which are themselves transcription factors while some are structural proteins putatively required for the accompanying structural changes during synaptic plasticity. Inhibition of activation by CREB prevents L-LTP but does not affect E-LTP (Kandel, 2001).

LIMK1 is a potent regulator of the actin cytoskeleton and the actin cytoskeleton has been implicated in L-LTP. Prevention of actin depolymerization has been shown to prevent L-LTP, and disturb actin dynamics, that is, the turnover of actin filaments, has also been shown to be important for L-LTP (Fukazawa et al, 2003). Indeed, actin dynamics has been involved in many aspects of synaptic plasticity including mGluR-LTD. The observed structural changes within the post-synaptic dendritic spine that occur during LTP induction are mediated in large by the actin cytoskeleton. Again, disturbing these structural changes also prevents LTP in these neurons. Interestingly, there are LTP induction protocols that can induce actin polymerization. Theta burst stimulation has been shown to induce actin polymerization in dendritic spines. This finding could potentially serve as a mechanism to define the roles of CREB and the actin cytoskeleton play in the LIMK1 lacking mice L-LTP can be discerned.
4.1.2 Methods

4.1.2.1 General Methods

Previously cut hippocampal slices were placed in a recording chamber continuously perfused with oxygenated ACSF at the rate of 2-2.5mL/min. Once there, the hippocampal slice was stimulated along the Schaffer-Collateral pathway. The baseline evoked response was elicited at 60% of the maximum evoked response. The fEPSPs were recorded for an initial baseline period where the quality and stability of the response was evaluated. L-LTP was induced after this baseline period once the response was stabilized. For high frequency stimulation (HFS) induced L-LTP, four trains of high frequency stimulations of 100Hz lasting one second each were given at 5 minute intervals. The response was then recorded for 3 hours after the last HFS. For theta burst induced L-LTP, 3 trains of 10 theta bursts with an intertrain interval of 10 minutes were used. As before, the response was then recorded for 3 hours following the last stimulation.

Data collection was done using pCLAMP 8 software (Axon Instruments). The slope of the Field EPSPs was measured by using the rising phase between 5% and 60% of the peak response. This was then normalized using the mean baseline evoked response value as 100%. All data is statistically evaluated by using Student’s t-test.
4.1.3 Results

4.1.3.1 LIMK1 is Important for L-LTP

In order to determine whether synaptic plasticity in LIMK1-/- mice is altered I used a number of electrophysiology induction protocols. My initial experiments involved using a induction protocol of 4 trains at 5 minutes apart, I found that LIMK1-/- mice exhibit significantly lower L-LTP than control wild type when the amount of potentiation was compared in the last half hour [Wild type: 173.21 ± 1.92 (n=6); LIMK1-/-: 129.55 ± 2.38 (n=7) | p<0.05] (Figure 4.1). This is in an indication that LIMK1 is important for L-LTP maintenance. This data suggests that LIMK1 is specifically important for maintenance because as shown by our previously published studies, LIMK1-/- mice exhibited enhanced early LTP when compared to wild type controls. In my results, LIMK1-/- mice also showed enhanced potentiation at one hour following stimulation [Wild Type 183.21 ± 2.11 (n=6); LIMK1 -/-: 213.22 ± 5.32 (n=7) | p<0.05]. The size of the response then begins to gradually decrease to a point at 3 hours where LIMK1-/- mice demonstrate the deficit in L-LTP.

Additionally, LTP induced by 4 trains spaced 20 seconds apart which has been shown to be protein synthesis dependent (Woo and Nguyen, 2003) is also reduced in LIMK1-/- mice [Wild type: 179.11 ± 1.00 (n=7); LIMK1-/-: 158.41 ± 1.46 (n=6) | p<0.05]. With this protocol no initial increase was observed in the first hour (Figure 4.2).

Theta burst activity has been shown to occur in the hippocampus of animals during exploratory behaviour (Bland et al, 1980). Other evidence also suggests that theta burst activity occurs during memory formation (Klimesch, 1999). This evidence suggests that theta burst activity
may be a more physiologically relevant protocol in which to induce L-LTP. To help elucidate the role of LIMK1 and its mechanism of action in synaptic plasticity I induced L-LTP using three trains of 15 theta bursts 10 minutes apart. It has been shown previously that this method can reliably induce L-LTP for at least 3 hours (Pittenger et al, 2002). Using theta burst induction, I found that LIMK1-/- mice again exhibited a deficit in L-LTP when compared to wild type controls [Wild Type: 165.17±1.36 (n=7); LIMK-/-: 130.63±1.81 (n=6) | p<0.05] (Figure 4.3). Furthermore, it is interesting to note that the enhanced early phase of the L-LTP seen early after induction with HFS in the LIMK1-/- mice is no longer present with TBS induction [Wild Type: 213.74±6.18 (n=7); LIMK1-/-: 211.74±3.23 (n=6) | p<0.05]. This data further implicates LIMK1 as being important to L-LTP specifically.
Figure 4.1
Figure 4.1 LIMK1-/- Mice Exhibit Deficits in L-LTP induced by 4 Trains of HFS Spaced by 5 minutes. Using this protocol LIMK1-/- mice showed a significant deficit in L-LTP [Wild type: 173.21 ± 1.92 (n=6); LIMK1-/-: 129.55 ± 2.38 (n=7)| p<0.05]. Moreover, the depressed response was only observed during the late phase of the recording with LIMK1-/- mice showing an enhancement in the early phase of the recording. Arrows indicate tetanus of high frequency stimulation. Representative traces shown from one experiment, where 1 and 2 indicate the time point at which the traces were taken. Right panel shows the average fEPSP slope for the last 15 minutes of both LIMK1-/- and wild type mice. Error bars represent standard error mean.
Figure 4.2
Figure 4.2 LIMK1-/‐ Exhibit a Deficit in L-LTP Induced by 4 trains spaced 20 seconds apart. This form of L-LTP has been shown to be protein synthesis dependent and blocked with PKA antagonists (Woo et al, 2003). Using this protocol, LIMK1-/‐ mice exhibited a deficit in the late phase of the recording [Wild type: 179.11 ± 1.00 (n=7); LIMK1-/‐: 158.41 ± 1.46 (n=6) | p<0.05]. Arrows indicate tetanus of high frequency stimulation. Representative traces shown from one experiments, 1 and 2 indicate the time point at which the traces were taken. Right panel shows the average fEPSP slope for the last 15 minutes of both LIMK1-/‐ and wild type mice. Error bars represent SEM.
Figure 4.3
Figure 4.3 LIMK1-/- Mice Exhibit Deficit in TBS Induced L-LTP. Utilizing the theta burst protocol (TBS), LIMK1-/- mice also exhibited a deficit when compared to wild type littermate control consistent with a role for LIMK1 in long-term synaptic plasticity [Wild Type: 165.17±1.36 (n=7); LIMK-/-: 130.63±1.81 (n=6) | p<0.05]. Arrows indicate tetanus of high frequency stimulation. Representative traces shown from one experiment, where 1 and 2 indicate the time point at which the traces were taken. Right panel shows the average fEPSP slope for the last 15 minutes of both LIMK1-/- and wild type mice. Error bars represent SEM.
4.1.4 Discussion

LIMK1-/- mice exhibited deficits in all forms of L-LTP that were induced. L-LTP induced by 4 trains at 5 mins apart has been reliably shown to induce protein synthesis and CREB activation (Vickers and Wylie, 2007, Pittinger et al, 2004, Frey et al 1993, Huang &. Kandel, 1994, Nguyen et al 1994). The finding that LIMK1-/- mice exhibit a deficit in this type of induced L-LTP indicates that a protein synthesis or CREB activation pathway is abnormal in LIMK1-/- mice suggesting that LIMK1 may be important for activity dependent signaling. Additionally, saturation experiments previously done by our lab (Meng et al., 2004) where the response was given repeated HFS at 5 min intervals showed that LIMK1-/- mice saturate and potentiate at higher levels than wild type controls. My results (Figure 4.1) are consistent with those findings. The response in the first 30 minutes post HFS in LIMK1-/- mice is indeed higher than wild type controls. Furthermore, there is a drop off in the response in LIMK1-/- mice where we observe a deficit in the late phase of L-LTP. This evidence is consistent with the view that E-LTP and L-LTP are maintained by different underlying mechanisms (Kandel, 2001). It is important to note that the decrease in potentiation we observe in the LIMK1-/- mice induced L-LTP is not likely due to damage or unhealthy hippocampal slices. This is because the potentiation seems to stabilize in the last 30 minutes. This indicates that LIMK1-/- hippocampal slices are capable of achieving and maintaining L-LTP though not at wild type levels, thus indicating a deficit in the magnitude of the maintenance mechanisms.

LTP induced by 4 trains of HFS spaced 20 seconds apart (Figure 4.2) which has been shown to be protein synthesis dependent and requires activation of PKA (Woo and Nguyen, 2003) is also
reduced in LIMK1 knockouts. This induction protocol was used in order to compensate for the potential lack of CREB activation in LIMK1-/- mice. However, LIMK1 lacking mice exhibited a deficit in this type of induced L-LTP suggesting that PKA activation by this type of induction was likely not sufficient to overcome any potential deficit in CREB signaling in LIMK1-/- mice. Moreover, it is not known whether this induction protocol saturates PKA activation or that it solely requires PKA activation. It is known that is heavily PKA dependent but other pathways are also likely to be activated. It is precisely these alternate pathways that seem to be responsible for the LIMK1-/- L-LTP deficit. This is assuming that PKA activation is normal in LIMK1-/- animals, and further experiments will need to be done to assess whether that is the case.

Theta burst stimulation protocol has been shown to polymerize actin (Lin et al, 2005). Certain mutations of CREB are not affected by theta burst L-LTP specifically (Pittenger et al, 2002). With this induction protocol it was surmised that deficits in L-LTP observed in LIMK1 could be rescued by restoring actin polymerization. This was important in order to separate the effects of LIMK1 on the actin cytoskeleton and CREB, respectively. TBS stimulation failed to rescue the deficits observed in LIMK1-/- mice suggesting that the effects of TBS on the actin cytoskeleton were not sufficient to rescue the LTP deficit. This is consistent with my hypothesis that it is the deficit in CREB activation that is responsible for the L-LTP deficit in LIMK1-/- mice.

Moreover, based on these results, we can predict that there will be a deficit in long-term memory in LIMK1-/- mice in hippocampal based tasks. It would be important to investigate whether the deficits in L-LTP translate to deficits in long-term memory in LIMK1-/- mice.
4.2 Memory Deficits in LIMK1-/- Mice

4.2.1 Introduction

Testing memory in rodents often involves incorporating certain innate behaviours of the animal with protocols that require the animal to learn or memorize a certain task. Hippocampal dependent memory in mice often involves memory of their surroundings. Things like spatial maps based on visual cues, certain odours and textures all provide context specific information for the mouse which all contributes to forming a memory.

It has been shown that contextual memory in mice can be comprised of short-term and long-term memory. These processes appear to be related, and formation of a short-term memory is required in order to form a new long-term memory. However, one can inhibit long-term memories without altering short-term memories. Studies have shown that several processes such as inhibition of protein synthesis, or of CREB specifically, and inhibition of structural proteins can block the formation of new long-term memories. Those previous studies also show that this inhibition does not affect short-term memory.

As stated above, CREB is a key modulator in mediating the changes necessary for converting a short-term memory to a long-term memory. CREB’s role in long-term memory has been widely studied, in a large number of organisms. Studies from mammalian models of memory indicate that CREB is required in a number of different types of long-term memories, including spatial, associative, and social learning.
4.2.1.1 Types of memory tests

*Morris Water Maze*

One of the most widely used tasks in assessing spatial memory in rodents is the Morris water maze. The task involves placing an animal in a circular pool of water and having learn and memorize the location of an escape platform based on various visual cues placed around the pool. The task itself was developed by Richard Morris and first described in 1981.

The task represents a form of higher spatial learning in mice, as it requires several training trials for the animal to fully acquire the location of the platform. After sufficient training trials the platform is removed and the animal is placed inside the apparatus. Once inside, the amount of time that the animal spends searching in the former platform area is seen as an evidence of spatial memory. Varying the time between the training trials and probe trial allows for testing of short-term and long-term memories.

The fact that Morris water maze requires spatial learning suggests that it requires proper hippocampal function. Accordingly, it has been shown that mice with hippocampal lesions have difficulty in retaining the memory of the platform location.

*Fear Conditioning*

Fear conditioning requires the pairing of an unconditioned stimulus, in this case a foot shock causing pain, to conditioned stimuli, such as a tone or a particular context.
The actin cytoskeleton is a key structural component in the dendritic spine. Although there is not a large amount of evidence with regards to actin and memory, an emerging story is that actin dynamics are necessary for memory formation (Lamprechet and LeDoux, 2004). Additionally, there is a large number of evidence of actin dynamics being important in the structural changes seen in and required of the dendritic spines undergoing learning. This is important since dendritic spines are postulated to be the units of location for new memories and accordingly, their structural integrity is potentially to the requirement for memory storage in the neuron. It is most important for dendritic spines to be flexible and allow for changes according to external stimuli. These changes underlie the molecular basis of memory.

4.2.2 Methods

For the water maze test, the acquisition phase consisted of 5 training days with four trials every day, and the probe trial was given 1 h and 24 h after the last training session to assess short-term and long-term memory, respectively. The spatial memory for the platform location was evaluated by the analysis of the dwelling time in the platform zone (a 20-cm-diameter circular area in which the platform was centered). The swim path was recorded by a camera connected to a video tracking system (Noldus Information Technology) and analyzed with EthoVision software.

For fear-learning and memory tests, the prehandled animals were placed in a chamber (22.5-cm-wide by 32.5-cm-long by 33.3-cm-high Plexiglas cage with a grid floor, encased in an isolation cubicle) and allowed to explore the cage for the first 120s, after which a 30s white noise tone was delivered followed by a 2s, 0.7-mA foot shock. Three such training sessions were delivered in succession, with 30s intertrain intervals. Following 2 and 24 h after the training, mice were tested for short-term and long-term fear memory, respectively. In the contextual test, mice were returned to the same chambers in which they were trained and their freezing response was
analyzed over the course of 4 min. In cued tests, the mice were placed in a separate chamber with an entirely different context and allowed to explore for 120s, after which the tone was delivered for the next 120s. All data were analyzed using FreezeView2 (Coulbourn Instrument, Whitehall, PA). Freezing was defined as a motion index of 25 or less.

4.2.3 Results

4.2.3.1 Water Maze

Long term memory of LIMK1-/- animals was assessed using the Morris Water maze. The mice were trained over a period of 3 days with 4 training trials per day. During training, LIMK1-/- mice and wild-type controls were able to learn the task with equal proficiency (Latency [Wild Type: 29.5±2.5 (n=14); LIMK-/-: 28.9±2.8 (n=11) | p>0.05] (Figure 4.4Ai). Example traces of routes showing the paths taken by animals of the different genotypes demonstrate similar direct paths taken by LIMK1 lacking mice and wild type littermates in contrast to with general exploratory or thigmotaxic behavior observed during the first trail.

Memory was then assessed by removing the platform and measuring the amount of time the mice spent in an area 200% the size of the platform. Short term memory was evaluated two hours post training. During this test, there was no significant difference between LIMK1 -/- mice and wild type littermates. The amount of time that the mouse spends in the quadrant where the platform is located is indicative of its ability to remember the location of the platform. These results suggest that the short term memory of the LIMK1 knockout mice in this task is intact. Additionally, no significant difference was found in the swim speed of the mice across genotypes.

However, performing the probe trial 48hrs later shows LIMK1 lacking mice having a deficit in the amount of time spent in the quadrant containing the platform [Wild Type: 16.3±2.1 (n=14);
LIMK-/-: 8.9±2.9 (n=11) | p>0.05 (Figure 4.4Bii). Additionally, LIMK1/- mice spend more time searching around the perimeter indicative of a poor searching strategy [Wild Type: 9.3±1.8 (n=14); LIMK-/-: 27.3±2.3 (n=11) | p>0.05] (Figure 4.4Cii). Again, swim speed was similar when comparing genotypes suggesting that the differences observed during this task were not a product of locomotor differences across genotypes.

Taken together, these results are indicative of a deficit in long-term memory in LIMK1/- animals. Furthermore, these results are suggestive of a role for LIMK1 in long-term memory.
Figure 4.4
Figure 4.4. LIMK1/- mice exhibit long-term memory deficit in the Morris Water Maze.

A. Both LIMK/- mice and wild type littermates were able to learn the task equally well after 3 days of training with 4 trials per day. B. i) Two hours post training there was no significant difference between LIMK1 -/- mice and wild type littermates suggesting that short term memory in this task was intact. [Wild Type: 18.4±2.6 (n=14); LIMK-/-:  23.9±2.9 (n=11) | p>0.05]. ii) At 48 hrs post training LIMK1 -/- mice showed a significant deficit when compared to wild type littermate controls [Wild Type: 16.3s±2.1 (n=14); LIMK-/-:  8.9s±2.9 (n=11) | p>0.05]. C. i) When measuring thigmotaxis, the amount of time the mice spend searching around the pool wall did not reveal a difference between LIMK1/- mice and littermate controls at 2 hrs post training. ii) However, at 48 hrs post training LIMK1/- mice spent more time searching around the perimeter of the pool indicating a poor searching strategy [Wild Type: 9.3s±1.8 (n=14); LIMK-/-:  27.3s±2.3 (n=11) | p>0.05]. D. Swim speed was unaltered between LIMK1 lacking mice and wild type littermate controls both at i)2 hrs [Wild Type: 18.5cm/s±2.2 (n=14); LIMK-/-:  19.2cm/s±2.7 (n=11) | p<0.05]. and ii) 48hrs post training [Wild Type: 17.9cm/s±1.8 (n=14); LIMK-/-:  18.5cm/s±2.6 (n=11) | p<0.05] and ii) 48hrs post training.
4.2.3.2 Fear Conditioning

To evaluate cognitive performance and to assess whether the long-term memory of LIMK1-/- has been affected we employed a battery of behavior tests. In the fear conditioning test, during the training phase the fear conditioning protocol, LIMK1 knockout mice exhibited the same amount of freezing as wild type littermate controls [Before shock: LIMK1 -/-: 8.30±4.80 (n=13), WT 15.09±6.53 (n=15); After shock: LIMK1 -/-: 49.65±8.14, WT 55.45±5.31] p>0.05 (Figure 4.5A). In the contextual memory test, LIMK1 knockout mice showed a significant decrease in freezing at 1 week and 2 weeks post training [1 week: LIMK1 -/-: 42.81±6.75 (n=13); WT: 71.49±4.37 (n=15); p=0.002 | 2 weeks LIMK1 -/-: 27.94±6.20 (n=13), WT: 46.58±5.71 (n=15); p<0.05] (Figures 4.5C and 4.5D). This data suggests that LIMK1 knockout mice have impaired contextual long-term memory while short term memory appears to be intact. Indeed, our data indicates that there is no difference in the contextual memory test when done at 2hrs post training suggesting that there is no deficit in short term memory in LIMK1 knockout mice [LIMK1 -/-: 75.53±9.98 (n=5), WT: 85.05±3.49 (n=5); p<0.05] (Figure 4.5B).
Figure 4.5
Figure 4.5 LIMK1/- Mice Exhibit a Deficit in Long Term Contextual Fear Memory. A. Mice underwent contextual fear training and were exposed to 3 shocks at 60s intervals. During training, LIMK1/- mice do not show any significant difference in freezing from wild type mice. [Before shock: LIMK1 -/-: 8.30±4.80 (n=13), WT: 15.09±6.53 (n=15); After shock: LIMK1 -/-: 49.65±8.14, WT: 55.45±5.31 | p<0.05] in freezing behaviour before and after the foot shock. B. When placed in the same chamber where the electric foot shocks were given at 2hr no significant difference in freezing behavior was found between LIMK/- mice and wild type controls [LIMK1 -/-: 75.53±9.98 (n=5), WT 85.05±3.49 (n=5) | p<0.05] C. One week after training LIMK1 knockout mice exhibit significantly decreased freezing [LIMK1 -/-: 42.81±6.75 (n=13); WT: 71.49±4.37 (n=15) | p<0.05]. D. Two weeks post training, LIMK1 knockout continue to exhibit significantly decreased freezing when compared to wild type littermates [LIMK1 -/-: 27.94±6.20 (n=13), WT:46.58±5.71 (n=15); p<0.05].
4.2.4 Discussion

Both in the Morris water maze and the fear conditioning task, LIMK1 lacking mice exhibit deficits specifically in long-term memory. The deficit in long-term memory is consistent with the earlier electrophysiology deficit in L-LTP. Moreover, the contextual fear conditioning test and the Morris water maze tasks are dependent on the hippocampus, which is where the electrophysiological experiments were performed. Taken together these results suggest abnormal function of long-term memory and synaptic plasticity in the hippocampus of LIMK1-/− mice.

Moreover, these results are consistent with a lack of CREB activation during memory formation since CREB activation is highly important in long-term memory function (Kandel, 2001). CREB transgenic mice show deficits in long-term memory and in the Morris water maze specifically (Gass et al., 1998, Porte et al., 2008), as well as the contextual fear conditioning task (Kida et al., 2002).

Previous work by our lab (Meng et al., 2004) has shown that LIMK1-/− mice do not exhibit any locomotor deficits in the open field task. Additionally, I show that there is no difference in swim speed between LIMK1-/− and wild type controls. These results mean that any deficits in freezing and time spent in the target zone cannot be attributed to any differences in locomotion between the genotypes.

However, the observed memory deficits are not inconsistent with actin regulation of memory since actin has been shown to be important for long-term memory as far as regulating the structural changes necessary for memory formation (Fukazawa et al, 2004). Further experiments
in this thesis will attempt to elucidate and separate the mechanisms responsible for the memory
deficits.

Previous data published by our lab showed that LIMK1 lacking mice did not exhibit differences
in freezing 24 hrs post training in the contextual fear test It is has been shown before that spaced
training such as that used in the Morris water maze task is able to overcome deficits in CREB
activation (Kogan et al.,1997). To avoid any such effects I used a weaker training protocol that
would compensate for these effects. I also used a longer time interval to ensure that the memory
I am investigating is largely long-term memory. Indeed, I show that memory at 48 hours is
deficient in LIMK1-/- mice in the same task. These results suggest that the initial data could have
potentially been a result of training the mice at an enhanced level to sufficiently overcome any
deficits of CREB activation.

Moreover, in my experiments I show that short term memory is intact in LIMK1-/- mice. Indeed,
a number of mouse models with deficits in CREB activation show intact short term memory and
only deficits in long term memory. It is this body of evidence that has implicated CREB
activation as necessary for long-term memory (Kandel, 2001).

Additionally, the intact short-term memory provides evidence that there are not any gross
cognitive deficiencies and, more importantly, that LIMK1-/- mice are able to learn each of the
tasks adequately. Taken together, these results indicate a role for LIMK1 in long-term memory
specifically.
4.3 CREB signalling is deficient in LIMK1-/- mice

4.3.1 Introduction

4.3.1.1 CREB Signaling

A common target of the above named proteins and their signaling cascades is the transcription factor CREB. It is one of the most important transcription factors activated during L-LTP and accordingly it is one of the best studied transcription factors that are involved in synaptic plasticity (Kandel, 2001, Huang and Kandel, 2002). Moreover, CREB has been shown to play a role in a number of other key cellular processes such as cell proliferation and cell death. In relation to synaptic plasticity, CREB has been shown to be critically involved in long-term memory in a number of organisms including Aplysia, Drosophila and mice (Kandel, 2001; Silva et al, 1998; Hummler et al, 1994; Impey et al, 1999; Casadio et al, 1999). For example, mice lacking certain CREB isoforms have decreased L-LTP as well as deficits in long-term memory (Silva et al, 1998; Hummler et al, 1994, Bourtchuladze et al 1994). Prevention of CREB activation also leads to a deficit in L-LTP (Pittenger et al, 2002). Drugs that increase CREB activation can rescue L-LTP deficient phenotypes and can rescue deficits in long-term memory (Bailey et al, 2004). It is important to note that these studies show effects on L-LTP and long-term memory specifically while E-LTP and short-term memory remain largely unaffected by CREB manipulations. This further implicates CREB as an important factor specifically in L-LTP and long-term memory.
4.3.1.2 CREB activation

CREB is expressed in all cells in the brain and is thought to be important in coupling extracellular signals with gene expression (Bailey et al, 2004). Indeed, regulation of CREB can be quite complex as it has numerous phosphorylation sites. However, it is widely accepted that in order to activate CREB it needs to be phosphorylated at the serine-133 position (Kandel, 2001, Bailey et al, 2004). When activated, CREB forms dimers that are then bound to the cAMP Response Element (CRE) of the DNA strand and initiate transcription and expression of a number of immediate early genes. Through this process, CREB mediates the consolidation of long-term memory from short-term memory (Kandel, 2001, Bailey et al, 2004, Silva et al, 1998).

4.3.2 CREB localization and LIMK1 localization

Much evidence has shown CREB subcellular localization to be in the nucleus (Hagiwara et al, 1993; Ginty et al, 1993, Waeber and Habener, 1991) . However, other evidence has also suggested that newly translated CREB can be found in the distal regions of the axon of dorsal ganglion neurons as well as subcellular organelles (Cox et al, 2008). LIMK1 has also been shown to be found in the nucleus and the cytoplasm as well as cell organelles (Foletta et al, 2004, McConnel at al., 2011, Rosso et al, 2004). Both proteins’ ability to found in similar regions suggest that there is a possibility of their interaction.
4.3.3 Results

4.3.3.1 LIMK1 interacts with CREB in mature hippocampal neurons

In order to test whether LIMK1 interacts with CREB in mature hippocampal neurons I used a co-immunoprecipitation assay. Using this assay, it was found that LIMK1 and CREB exist in an immunocomplex. This suggests that LIMK1 may directly interact with CREB and help regulate its activity or subcellular localization. No evidence of an interaction was found in LIMK1-/- mice (Figure 4.6). Previous studies have shown that LIMK1 and CREB interact in hippocampal neuroprogenitor cells (Yang et al, 2004). Here, I show the first evidence of this interaction in mature hippocampal neurons.

Moreover, in hippocampal slices, we found that LIMK1 and CREB were expressed in similar subcellular regions in the mouse dentate gyrus and in the CA1 region of the hippocampus. CREB showed localized staining in the neuronal cell body whereas LIMK1 was present in the cell body as well as the dendrites (Figure 4.7).

In hippocampal neuronal cultures at 17-20 days in vitro (DIV) we found that CREB was expressed in similar subcellular regions as LIMK1. Interestingly, LIMK1 was present in the nucleus as well as the dendrites of the neuron, whereas CREB staining was present only in the nucleus. The interaction and subcellular localization results indicate that the deficits may be caused by a lack of CREB activation in LIMK1-/- mice (Figure 4.8).
Figure. 4.6
**Figure. 4.2 LIMK1 interacts with CREB.** Brains from wild type and LIMK1-/- were isolated, homogenized and lysed. They were then incubated with LIMK1 or CREB antibody. Protein A agarose beads were added which pulled down the incubated antibody. Once spun down, the bead pellet was loaded and separated using gel electrophoresis. The samples were then probed with CREB and LIMK1 antibodies. In wild type mice pulldown with LIMK1 and probing with CREB showed that CREB is bound to LIMK1. This is also the case with CREB pulldown and LIMK1 probe. This interaction does not occur in LIMK1-/- mice.
Figure 4.7
Figure 4.7 LIMK1 and CREB expression the mouse dentate gyrus. Hippocampal slices from wild type mice. Were cut at thickness of 10μm and incubated with CREB and LIMK1. CREB, in red, shows localized staining in the neuronal cell body whereas LIMK1, in green, is present in the cell body as well as the dendrites. The merged image shows a similar expression profile in the cell bodies of the soma of the dentate gyrus granule cells for both LIMK1 and CREB.
Figure 4.8
Figure 4.8 LIMK1 and CREB Expression in Hippocampal Neuronal Cultures.

Hippocampal cultures were isolated from wild type mice. They were grown in neuronal media for up to 28 days *in vitro* (DIV). In hippocampal neuronal cultures at 17-20 DIV CREB, in red, is expressed in the cell body and nucleus. LIMK1, in green, is expressed throughout the neuron. Both LIMK1 and CREB are expressed in the nucleus, thus allowing for the possibility of a putative interaction.
4.3.3.2 CREB phosphorylation

In order to assess whether the LIMK1 and CREB interaction has an effect on CREB phosphorylation (pCREB) hippocampal slices were treated with NMDA (30μM) and glycine (3μM). Indeed, it was found that phosphorylation in LIMK1-/- mice to be significantly reduced at later time intervals after treatment. Moreover, we found a different time course in CREB phosphorylation in LIMK1-/- suggesting that CREB signaling is altered in LIMK1 lacking mice. A significant difference was seen at the 5 minutes [LIMK1-/-: 131.7±7.3 n=9, Wild Type: 179.7±4.3 n=8 | p<0.05] and 10 minutes [LIMK1-/-: 99.2±1.1 n=9; Wild Type: 138.1±4.1 n=8 | p<0.05] time points specifically (Figure 4.9). The difference in CREB phosphorylation in LIMK1-/- mice is consistent with the hypothesis that LIMK1 affects CREB activation. Moreover, basal levels of pCREB were unaltered in LIMK1 lacking mice suggesting that the difference in pCREB levels is activity dependent [LIMK1-/-: 104.4±6.2 n=9, Wild Type: 101.1±4.3 n=8 | p>0.05] (Figure 4.9).

Treatment of hippocampal slices with forskolin (50μM), which activates the PKA activation pathway of CREB, did not produce any differences in CREB phosphorylation between LIMK1 lacking hippocampal slices and wild type controls [LIMK1-/-: 101.1±4.2, Wild Type: 103.1±4.8; p>0.05] (Figure 4.11). Hippocampal slices were also treated with rolipram (2μM) for 10 minutes. Rolipram prevents the breakdown of cyclic AMP and acts to prolong activation of PKA. Activation of the PKA signaling pathway by rolipram did not show any difference in CREB phosphorylation levels between LIMK1-/- mice and wild type controls [WT (Rolipram) = 322.7±18.5; LIMK1 -/- (Rolipram) = 242.2±10.6 | p<0.05] (Figure 4.12). These data suggest that CREB activation through the PKA pathway is unaltered in LIMK1-/- mice.
Treatment of slices with 4 trains of HFS at 5 minutes apart appeared to have increased CREB phosphorylations levels in LIMK1/- mice and wild type controls. However, with this particular treatment it was found that pCREB levels in LIMK1/- mice were not raised as highly as in wild type controls [LIMK1/-: 128.2±7.1 n=6, Wild Type: 157.8±10.2 n=7 | p<0.05] (Figure 4.10). The data in this particular experiment shows that CREB phosphorylation is deficient in LIMK1 lacking mice.
Figure 4.9
Figure 4.9 Abnormal CREB Activation in LIMK1-/- Mice. Hippocampal slices were cut and prepared as described for electrophysiology experiments. NMDA (30μM) and glycine NMDA (3μM) treatment of hippocampal slices indicates a deficit in CREB phosphorylation levels at later time intervals. At 5 minutes LIMK1-/- hippocampal slices show a deficit in CREB phosphorylation levels when compared to wild type controls [LIMK1-/-: 131.7±7.3 n=9, Wild Type: 179.7±4.3 n=8 | p<0.05]. This is also the case at 10 minutes where LIMK1-/- continued to show a deficit in CREB phosphorylation [LIMK1-/-: 99.2±1.1 n=9; Wild Type: 138.1±4.1 n=8 | p<0.05].
Figure 4.10
Figure 4.3 Deficit in CREB Phosphorylation in LIMK1-/- Hippocampal Slices Treated With High Frequency Stimulation. Slices were prepared as for electrophysiology experiments. After recovery they were treated with 4 trains of high frequency stimulation (HFS) 5 minutes apart. HFS treatment led to increased levels of pCREB in WT and LIMK1 littermates. However, LIMK1 -/- showed a significant deficit in pCREB relative density levels compared to wild type littermates [LIMK1-/-: 128.2±7.1 n=6, Wild Type: 157.8±10.2 n=7 | p<0.05]. * indicates p<0.05 compared across genotype in same condition. ** indicates p<0.05 compared to from baseline control. Error bars indicate SEM.
Figure 4.4
Figure 4.11 LIMK1 Does Not Affect Forskolin Dependent CREB Phosphorylation.

Hippocampal slices were prepared as for electrophysiology experiments. Hippocampal slices were treated with forskolin (50 μM) for 10 minutes. Forskolin activates the PKA signaling pathway and leads to an increase in CREB phosphorylation. Activation of CREB by forskolin did not show any difference in CREB phosphorylation levels between LIMK1-/- mice and wild type controls [LIMK1: 138.23± 6.78 (n=9), WT: 146.29 ± 4.63 (n=7) | p<0.05] * indicates p<0.05 compared to from baseline control. Error bars indicate SEM.
Figure 4.12
Figure 4.12 LIMK1 Does Not Affect Rolipram Dependent CREB Phosphorylation.

Hippocampal slices were prepared as for electrophysiology experiments. Treatment with Rolipram (2µM) for 10 minutes was able to increase CREB phosphorylation in both WT and LIMK1 lacking mice. Treatment with Rolipram (2mM) for 10 minutes was able to increase CREB phosphorylation in WT and LIMK1−/− mice to similar levels [WT (Rolipram) = 322.7±18.5 n=8; LIMK1−/− (Rolipram) = 242.2±10.6 n=7] p<0.05. Treatment with the vehicle, DMSO, did not show any increase in pCREB levels. * indicates p<0.05 compared to vehicle control for each genotype. Error bars indicate SEM.
4.3.4 Discussion

The existence of the LIMK1 and CREB interaction suggests that this is a possible signaling pathway for activation of CREB during synaptic activity. I have shown evidence that LIMK1 and CREB are located in similar regions in hippocampal sections and dissociated hippocampal neurons in culture. This initial evidence makes it appear likely that CREB and LIMK1 are able to interact thereby allowing for the possibility of a LIMK1 activation of CREB in mature hippocampal neurons. Moreover, I have shown the first evidence of LIMK1 localization in hippocampal slices and mature dissociated neuronal cultures. A previous study has shown that LIMK1 contains a nuclear localization signal in its kinase domain (Yang and Mizuno, 1999) but this study was not done in neurons. The likelihood of a CREB and LIMK1 interaction existing is increased if they are likely to be expressed in similar regions of the neuron as my results demonstrate.

The most convincing evidence that shows LIMK1 and CREB interact is shown in Figure 4.6 with the immunoprecipitation experiment. It appears that LIMK1 and CREB exist in a complex, and it is therefore highly likely that they interact. Moreover, this interaction is consistent with deficits observed in L-LTP and long-term memory seen in LIMK1-/- mice. It can be speculated that the lack of this LIMK1/CREB complex is responsible for the observed deficits.

Since I have shown that LIMK1 and CREB exist in a complex, it was important to examine whether CREB activation via phosphorylation is deficient in LIMK1-/- mice. Indeed, in response to NMDA treatment, LIMK1-/- mice show a deficit in CREB phosphorylation and therefore activation. This is indicative of an activity dependent role of CREB activation by LIMK1.

Moreover, the timing of the CREB deficit in LIMK1-/- mice suggests a temporal role of CREB activation by LIMK1. The deficit in CREB activation in LIMK1-/- mice appears only at 5 and
10 minutes of NMDA treatment. This data is consistent with electrophysiological results where the L-LTP deficit is observed during the late stages of the electrophysiological recording.

Additionally, baseline levels of pCREB appear to be unchanged between LIMK1-/- mice and wild type controls. This suggests that this is indeed an activity dependent phenomenon.

Forskolin and rolipram both activate CREB via the PKA pathway, which is intact in LIMK1-/- mice, accordingly, did not reveal a difference in activated CREB levels with forskolin treatment. These results indicate that that CREB signaling is intact in LIMK1-/- mice. Additionally, these results indicate that there are no gross abnormalities with CREB activation in LIMK1-/- mice. Furthermore, these results suggest a possible means of activating CREB to sufficient levels in LIMK1-/- mice. Indeed, PKA activation of CREB in LIMK1 -/- mice can be used to potentially eliminate the deficit observed in long-term memory or L-LTP. The possible elimination of these deficits by CREB activation suggests that the deficits are indeed caused by insufficient CREB activation.
4.4 CREB activation can rescue synaptic and memory deficits whereas cofillin deactivation does not

4.4.1 Introduction

Because the mechanisms of L-LTP deficits in LIMK1 lacking mice were not discerned and could be caused by insufficient CREB activation or abnormal regulation of actin dynamics, experiments were designed to distinguish between these possibilities. CREB activation was enhanced with the use of forskolin and rolipram whereas cofillin activation was manipulated using the S3 and S3P peptides.

4.4.1.1 Forskolin

Forskolin is a naturally occurring labdane diturpene produced by the Indian Coleus plant (*Coleus forskohlii*). Forskolin is an adenylyl cyclase (AC) activator which leads to increased levels of cAMP. These increased levels of cAMP leads to increased activation of PKA. PKA is a known activator of CREB, and via this pathway forskolin has been used as an activator of CREB. Forskolin was first described in 1980 as a cardioactive drug (Inamdar et al., 1980) but has been used extensively to activate CREB during electrophysiological experiments (Huang et al., 1995, Otmakhov et al., 2004)

4.4.1.2 Rolipram

Rolipram is a phosphodiesterase 4 (PDE4) inhibitor. PDE4 is protein which degrades cAMP thereby the inhibition of its activity leads to an increase in cAMP in the cell. The effects of rolipram on long-term memory have been known for some time. Indeed, rolipram injection prior to training has increased long-term memory while not affecting short term memory (Barad et al,
This is indicative of its ability to enhance CREB activity via preventing cAMP degradation (Monti et al, 2006).

**4.4.1.3 S3 and S3P Peptides**

Peptides containing the serine-3 (S3) and the phosphorylated serine-3 (S3P) sequences of cofilin were used to manipulate cofilin activity and thereby actin regulation. These have been previously used in our laboratory examining the effects of the actin cytoskeleton on LTD. The S3 peptide works in a dominant negative manner in that binds to the active site of LIMK1 and prevents it from binding and deactivating cofilin. The PS3 peptide inhibits slingshot phosphatase which activates cofilin. Both peptides contained a penetratin sequence allowing it to cross the cell membrane (Zhou et al, 2010). For these experiments, hippocampal slices were pretreated with the peptide for one hour and electrophysiology experiments were performed on the pretreated slices.

**4.4.2 Methods**

**4.4.2.1 Electrophysiology**

The fEPSP was recorded for an initial baseline period where the quality and stability of the response was evaluated. LTP was induced after a sufficient baseline period, generally a half hour, where the response was determined to be stable. For high frequency stimulation (HFS) induced L-LTP, four trains of high frequency stimulations of 100Hz lasting one second each were given at 5 minute intervals. For E-LTP, 2 trains of high frequency stimulation were given at 10 seconds intervals. The response was then recorded for 3 hours after the last stimulation for L-LTP and 40 minutes after stimulation for E-LTP. For forskolin treatment, hippocampal slices were perfused with ACFS containing forskolin (50μM) for 20 minutes prior to induction of L-
LTP and 10 minutes after initiation of LTP induction. For the peptide experiments, hippocampal slices were pretreated with the each peptide for 1hr after recovery and before being placed in the recording chamber.

4.4.3 Results

4.4.3.1 CREB Activation Rescues L-LTP

In order to dissect the mechanisms underlying the deficits in L-LTP and memory, pharmacological methods for increasing CREB activity were used. Forskolin was also able to induce LTP at 3 hours post-stimulation in WT hippocampal slices while DMSO controls showed no induced LTP at 3 hours [Wild Type (Forskolin)-/-: 224.9±0.4 n=8, Wild Type (vehicle): 157.0±0.7 n=7; p<0.05] (Figure 4.13). In LIMK1-/- mice, forskolin was unable to induce LTP at 3 hours [LIMK1-/- (forskolin): 143.6±0.4 n=6, LIMK1-/- (vehicle): 134.5±0.7 n=5 | p>0.05] (Figure 4.14). Forskolin has been showed to induce LTP when perfused in hippocampal slices. In this experiment, LTP was induced in LIMK1-/- mice as well as wild type littermates. However, the magnitude of the forskolin induced LTP was decreased in LIMK1-/- mice [Wild Type (forskolin): 190.6±0.5 n=6, LIMK1-/- (forskolin): 157.6±0.2 n=6 | p<0.05] (Figure 4.15). Induction of L-LTP with the use 4 HFS trains spaced 5 minutes apart as well as addition of forskolin is able to rescue the deficit in LIMK1-/- mice [LIMK1 -/- (forskolin and 4 trains): 206.6±0.8 n=6, Wild Type (forskolin and 4 trains): 204.5±0.7 n=7 | p>0.05] (Figure 4.16). These data suggest that both an increase in CREB activation and a strong stimulation are required in order to rescue the observed deficits in LIMK1-/- mice.
Figure 4.13
**Figure 4.13 Induction of LTP With Addition of Forskolin.** Forskolin treatment and weak HFS stimulation has been widely shown to induce L-LTP. Forskolin (50μM) was able to induce L-LTP with a 2 trains of 100Hz in wild type slices [Wild Type (Forskolin)-/-: 224.9±0.4, Wild Type (vehicle): 157.0±0.7; p<0.05]. Forskolin was perfused for 10 minutes during baseline recording and 10 minutes after induction of LTP> Arrows indicate tetanus of high frequency stimulation. Representative traces shown from one experiment, 1 and 2 indicate the time point at which the traces were taken. Error bars represent SEM. The solid black bar represents the duration of forskolin perfusion, in this case 20 minutes.
Figure 4.14

- LIMK1 -/- (DMSO) (n=5)
- LIMK1 -/- (Forskolin) (n=6)

% fEPSP

Time (min)
Figure 4.14 Addition of Forskolin is unable to induce L-LTP in LIMK1-/- mice. Addition of forskolin (50μM) was unable to induce L-LTP in LIMK-/- mice, at 3 hours [LIMK1 -/- (Forskolin): 143.6±0.4, LIMK1-/- (vehicle): 134.5±0.7; p>0.05]. Hippocampal slices perfused with ACSF containing forskolin for 20 minutes showed no difference compared to DMSO controls. Forskolin was perfused for 10 minutes during baseline recording and 10 minutes after induction. Arrows indicate tetanus of high frequency stimulation. Representative traces shown from one experiment, 1 and 2 indicate the time points at which the traces were taken. Error bars represent SEM. The solid black bar represents the duration of forskolin perfusion, in this case 20 minutes.
Figure 4.15
Figure 4.15 LIMK1-/- Mice Exhibit a Deficit in Forskolin Induced LTP. Forskolin treatment alone can elicit LTP in hippocampal slices. Addition of forskolin (50μM) for 20 min did induce LTP in wild type and LIMK1-/- hippocampal slices but to a lower level in LIMK-/- slices. [Wild Type (forskolin): 190.6±0.5, LIMK1 -/- (forskolin): 157.6±0.2; p<0.05]. Representative traces shown from one experiment, 1 and 2 indicate the time points at which the traces were taken. Error bars represent SEM. The black bar represents the duration of forskolin perfusion.
Figure 4.16

WT (Forskolin) (n=7)
LIMK1 -/- (Forskolin) (n=6)
Figure 4.16 Strong Tetanic Stimulation and Addition of Forskolin is Necessary for Inducing L-LTP in LIMK1/- mice. Attempts to overcome the L-LTP deficit in LIMK1/- mice led to the use of strong tetanic stimulation and forskolin perfusion. Four HFS trains spaced 5 minutes apart are able to rescue the deficit in LIMK1/- mice [LIMK1 -/- (Forskolin and 4 trains): 206.6±0.8, Wild Type (forksolin and 4 trains): 204.5±0.7 | p>0.05]. Forskolin was perfused during the last 10 minutes of baseline recording and for the first 10 minutes of the induction protocol. Arrows indicate tetanus of high frequency stimulation. Representative traces shown from one experiment, 1 and 2 indicate the time point at which the traces were taken. Error bars represent SEM. The solid black bar represents the duration of forskolin perfusion, in this case 20 minutes.
4.4.3.2 CREB Activation Rescues Long-Term Memory

CREB activity can be enhanced with the use of the drug rolipram (Monti et al, 2006, Nakagura et al. 2006). Injection of rolipram was able to overcome deficits in LIMK1 -/- mice long-term memory. For the Morris water maze, rolipram was administered 1 hour prior to the first training session of each training day. The mice were allowed to recover and then training was begun. All mice were able to learn the task to similar levels (Figure 4.17A). At two hours after the final training session the platform was removed, the mice were placed in the water maze and their preference for the target zone was recorded. At two hours post training, all mice showed a similar time spent searching in the target zone [Wild Type (Rolipram): 17.0s±2.8 n=8, Wild Type (DMSO): 22.8s±3.4 n=8; LIMK1-/- (Rolipram): 22.9s±3.6 n=7; LIMK1-/- (DMSO): 18.0s±2.6 n=8 | p< 0.05]. At 48 hours, in the water maze task, rolipram administration was able to increase the time spent in the target zone to near wild type levels for LIMK1 lacking animals [LIMK1-/- (Rolipram): 13.9s±2.40 n=7, Wild Type (DMSO): 15.0s±2.1 n=8 | p<0.05] (Figure 4.17Bii). Additionally, rolipram administration also increased the performance of wild type animals [Wild Type (Rolipram): 18.8s±2.80 n=8, Wild Type (DMSO): 15.s0±2.1 n=8 | p<0.05] (Figure 4.17Bii).

In the contextual fear task, rolipram administration was able increase freezing to near wild type levels [WT (DMSO)=79.0±3.2 n=8; LIMK1-/- (Rolipram)=70.9±4.7 n=7 | p>0.05] (Figure 4.18). In this task rolipram was administered one hour prior to training. In this task, however, the increase of performance in wild type animals given rolipram was not significant. This could be a result of ceiling effects as the initial freezing is quite high for wild type animals. Taken
together, these results suggest that increasing CREB activity was able to partially rescue some forms of the LIMK1 -/- memory deficit.
Figure 4.17
Rolipram was able to rescue deficits in LIMK1 -/- in the Morris water maze task. A. All mice were able to acquire the tasks at the same level on the 3rd day of training. Bi) At 2 hours all mice showed a preference for the target zone at similar levels [Wild Type (Rolipram): 17.0s±2.8 n=8, Wild Type (DMSO): 22.8s±3.4 n=8; LIMK1-/- (Rolipram): 22.9±3.6 n=7; LIMK1-/- (DMSO) 18.0s±2.6 n=7]. Bii) At 48 hours, rolipram administration was able to increase the time spent in the target zone to near wild type levels for LIMK1 lacking animals [LIMK1-/- (Rolipram): 13.9s±2.40, Wild Type (DMSO): 15.0s±2.1; p<0.05]. Additionally, rolipram administration also increased the performance of wild type animals [Wild Type (Rolipram): 18.8s±2.80, Wild Type (DMSO): 15.0s±2.1; p<0.05]. Ci) No differences were found in swim speed across the conditions at Di) 2hrs and Dii) 48 hrs.
Figure 4.18
Figure 4.18 Fear Conditioning Test With Administration of Rolipram. A. Mice across all conditions were able to acquire the task at similar levels. B. Rolipram administration at 2hrs appeared to have increased the amount of freezing across all conditions. C. Administration of rolipram was able to increase freezing of LIMK1 lacking mice to near wild type levels at 48 hours [WT (DMSO)=79.0±3.2 n=8; LIMK1-/- (Rolipram)=70.9±4.7n=7]. Moreover, rolipram administration also appeared to increase the performance of wild type mice at 48 hours.
4.4.3.3 Cofilin Activity Enhances LTP in Wild Type Animals but Does Not Rescue LIMK1-/- Mouse L-LTP Deficit.

We have shown previously that LIMK1-/- mice have enhanced E-LTP (Meng et al., 2002). It was proposed then that this enhancement was caused by abnormal actin distribution. I replicated these results by inducing E-LTP by 1 train of 100Hz of HFS [LIMK1-/-: 201.6±5.6 n=4; Wild Type 165.5±4.7 n=4] p<0.05] (Figure 4.19). To further determine that this enhancement was caused by abnormal actin distribution, I pretreated hippocampal slices for 1hr prior to induction of LTP with a cofilin enhancing peptide, S3P, and a cofilin inhibiting peptide S3. Pretreatment with the S3P peptide that inhibits LIMK1 action showed no effects on LIMK1-/- mice on E-LTP induced by HFS of 1 train at 100Hz [LIMK1-/-: 238.2±11.6 n=4; Wild Type 225.7±9.7 n=4 | p>0.05] (Figure 4.20). The S3P peptide reduced E-LTP of both LIMK1 lacking mice and wild type controls [LIMK1-/-: 144.2±7.6 n=4; Wild Type 139.3±6.4 n=4 | p>0.05] (Figure 4.21).

In order to rule out the contribution of the actin cytoskeleton to the L-LTP deficit in LIMK1 lacking mice, I pretreated hippocampal slices with the S3 peptide. When examining L-LTP induced by HFS 4 trains 5 mins apart it was found that pretreatment with the S3 peptide had no effect on L-LTP on either LIMK1 lacking mice or wild type controls [LIMK1-/-: 238.2±11.6 n=4; Wild Type: 225.7±9.7 n=4 | p>0.05] (Figure 4.22). Overall, pretreatment with the both the cofilin enhancing S3P and cofilin inhibiting S3 peptide appeared to only have effects on E-LTP while having little or minimal effects on L-LTP. These results indicate that it is CREB inactivation in LIMK1 lacking mice that is responsible for the observed L-LTP deficit.
Figure 4.19
**Figure 4.19 LIMK1-/- Mice Exhibit Enhanced E-LTP.** E-LTP induced by one train of 100HZ was able to induce LTP in LIMK1-/- mice and wild type littermates. LIMK1 -/- exhibited enhanced LTP using this protocol [LIMK1-/-: 201.6±5.6 n=4; Wild Type: 165.5±4.7 n=4 | p<0.05]. These data replicate previous findings by our lab (Meng et al., 2002). Arrows indicate tetanus of high frequency stimulation. Representative traces shown from one experiment, 1 and 2 indicate the time points at which the traces were taken. Right panel shows the average fEPSP slope for the last 15 minutes of both LIMK1-/- and wild type mice. Error bars represent SEM.
Figure 4.20
Figure 4.20 Enhancement of Cofilin Increases E-LTP in Wild Type Mice. Addition of the cofilin enhancing peptide, S3, increased the induced LTP of the wildtype mice to LIMK1 levels. [LIMK1-/-: 238.2±11.6 n=4; Wild Type 225.7±9.7 n=4 | p>0.05]. Arrows indicate tetanus of high frequency stimulation. Representative traces shown from one experiment, 1 and 2 indicate the time points at which the traces were taken. Right panel shows the average fEPSP slope for the last 15 minutes of both LIMK1-/- and wild type mice. Error bars represent SEM.
Figure 4.21
Figure 4.5 Inhibition of Cofilin Decreases LTP in Wild Type and LIMK1 -/- mice. Addition of the cofilin inhibiting, S3P, peptide accordingly decreased the induced LTP of the wildtype mice to LIMK1 levels. [LIMK1-/-: 144.2±7.6 n=4; Wild Type 139.3±6.4 n=4 | p>0.05]. Arrows indicate tetanus of high frequency stimulation. Representative traces shown from one experiment, 1 and 2 indicate the time points at which the traces were taken. Right panel shows the average fEPSP for the last 15 minutes of both LIMK1-/- and wild type mice. Error bars represent SEM.
Figure 4.6
Figure 4.22 Inhibition of Cofilin Activity Has no Effect on L-LTP. The cofilin inhibiting peptide S3P does not have an effect on L-LTP in wild type and LIMK1-/- mice [Wild type (S3P)=196.1±11.1 n=4, LIMK1 (S3P)=130.0±8.2 n=4 | p<0.05]. Arrows indicate tetanus of high frequency stimulation. Representative traces shown from one experiment, 1 and 2 indicate the time points at which the traces were taken. Right panel shows the average fEPSP slope for the last 15 minutes of both LIMK1-/- and wild type mice. Error bars represent SEM.
4.4.4 Discussion

Forskolin activates CREB via the PKA pathway which is intact in LIMK1 knockout mice. Accordingly, no difference was found between LIMK1-/- mice and wild type controls in activated CREB levels under forskolin treatment. Interestingly, forskolin application with 2 trains of HFS was not able to induce L-LTP in LIMK1-/- mice as it did in wild type controls. While it can be interpreted that other mechanisms are responsible for the L-LTP deficit in LIMK1-/- mice from this result, it can also be argued that CREB activation by forskolin and 2 trains of HFS does not activate CREB levels sufficiently in LIMK1-/- mice. Indeed, further evidence for this latter explanation is that forskolin induced LTP in LIMK1-/- mice is lower than in wild type controls. This finding indicates that CREB signaling is intact in LIMK1-/- mice since they do exhibit LTP with forskolin treatment. However, it also indicates that CREB activation by PKA alone is not sufficient to overcome the electrophysiological deficits observed in LIMK1-/- mice.

In the electrophysiological experiments it is both forskolin activation and a strong tetanic stimulation that were required in order to rescue the deficits in the LIMK1-/- mice. This evidence suggests that that CREB activation by LIMK1 is only required during L-LTP and long term memory formation and potentially weaker stimulations do not require either mobilization of LIMK1 to the nucleus or LIMK1 activation of CREB. Additionally, spaced training has been shown to overcome CREB activation deficits (Kogan et al., 1997). Therefore, it could be argued that the spaced trains (4 trains at intervals of 5 minutes) used to induce L-LTP along with the additional activation of CREB are what is required to overcome the deficit observed in LIMK1-/-
mice. Furthermore, this finding suggests that LIMK1 signaling and CREB activation is a key component in L-LTP maintenance.

CREB activation by rolipram in vivo appeared to abolish the deficits observed in LIMK1/-/ mice. Rolipram administration was able to overcome the long term deficits in the Morris water maze and contextual fear conditioning task. PKA activation along with spaced training in both the contextual and Morris water maze was able to abolish the long-term memory deficits. As stated earlier, spaced training is capable of overcoming CREB activation deficits. This is consistent with the electrophysiological experiments with forskolin. These data suggest that insufficient CREB activation in LIMK1/-/ mice is responsible for the long-term memory deficits observed in LIMK1 lacking mice.

It should be acknowledged that rolipram does increase the performance of wild type mice as well, and it is widely known that rolipram can enhance the memory of rodents via CREB activation. It is important to note that the enhancement observed in LIMK1/-/ mice does not bring them up to levels rolipram demonstrated in the wild type mice. This result can be interpreted that PKA activation is still not sufficient to overcome the absence of LIMK1 activation in LIMK1/-/ mice. Moreover, this is also consistent with the delayed time point deficit in CREB phosphorylation in LIMK1/-/ mice. Indeed, it indicates that LIMK1 signaling becomes more important as time goes by and that LIMK1 activation of CREB is required to maintain the CREB activation necessary for the maintenance of long-term memories.

As shown previously (Meng et., 2002), I also demonstrated that LIMK1/-/ mice have enhanced LTP when induced by 1 train of HFS. Previously, it was speculated that this enhancement is caused by abnormal actin regulation due to enhanced cofilin activity in LIMK1/-/ mice.
Accordingly, pretreatment with the cofilin enhancing peptide had no effect on the LIMK1-/- LTP and enhanced wild type LTP. The cofilin inhibiting peptide did decrease the LTP in wild type and LIMK1-/- mice but to a greater magnitude in LIMK1-/- mice. This is consistent with the hypothesis that increased actin depolymerization plays a role in enhanced E-LTP found LIMK1-/- mice.

To determine the effects of actin depolymerization on L-LTP hippocampal slices were pretreated with the cofilin enhancing peptide. In this experiment pretreatment with the peptide is not sufficient to alter the L-LTP of wild type and LIMK1-/- hippocampal slices. This data suggests that cofilin activity has no effect on L-LTP. Moreover, it shows that LIMK1 works through two different mechanisms when regulating synaptic plasticity. LIMK1 appears to regulate E-LTP via cofilin and actin regulating pathways, and L-LTP via CREB mediated pathways.
4.5 LIMK1-/- Mice as a Model for Williams Syndrome and Autism Spectrum Disorders

4.5.1 Introduction

Williams-Beuren Syndrome is a rare developmental disorder that has a distinct neurocognitive profile. It combines relatively proficient language skills with profound visuospatial deficits. Those afflicted with WBS are also characterized with a mean IQ of 60, which falls in the mentally retarded range (Bellugi et al, 1999).

WBS is associated with a deletion in chromosome band 7q11.23. A number of genes deleted are speculated to be critically involved in cognition, one of which is LIMK1. Moreover, it appears that LIMK1 can be directly linked to the visuo-spatial abnormalities present in WMS. The deficit in spatial abilities suggests that there may be abnormal hippocampal function (Bellugi et al, 1999, Morris and Mervis, 2000). In this manner, WBS underlines the potentially important role that LIMK1 may have in overall cognition.

4.5.1.1 LIM-Kinase1 knockout mice as a potential model for Williams Syndrome

Since LIMK1 is deleted in individuals with WBS it is possible for LIMK1 knockout mice to be used as a potential model for the syndrome. The neurocognitive profile of WS individuals consists of strengths in verbal skills and language but profound deficits in visuospatial cognition (Greer et al 1997, Mervis et al 1998). Additionally, individuals with WBS exhibit unique
personality traits including a high level of gregariousness, an excess in anxiety including specific phobias, sensory defensiveness, and restricted interests (Bellugi et al., 1999). Thus, WBS appears to share a number of characteristics of the autism spectrum disorders (Klein-Tasman et al., 2009). A number of these traits can be tested in animals if we are to propose that LIMK1 mice are to be used as a putative model for WBS. For example, social interaction can be tested in mice to measure “gregariousness”, and the Morris water maze can be used to assess visuo-spatial cognition. Those afflicted with WBS tend to exhibit more anxiety and tend to have and exhibit more fears and phobias which can be assessed in part by the elevated plus maze.

4.5.2 Methods

4.5.2.1 Social Interaction

One of the hallmarks of Williams-Beuren Syndrome is the hypersociability of those affected by the disease. Patients with WBS are described as “unusually sociable, friendly and empathic” (Levine and Wharton, 2000). If I am to propose that LIMK1 knockout mice can serve as a model for WBS than it would be necessary to examine their sociability. Using a social interaction test where a mouse, the exposure mouse, is placed in a clear plexiglass cylinder with numerous holes drilled into the walls to allow for odour cues and then another mouse, the tester mouse, is placed in the arena the time spent investigating can be used as a measure of sociability of the mice. The mice are placed in the arena for 5 minutes for 4 trials. On the 5th trial, the exposure mouse is replaced with a novel mouse and the tester mouse is placed in the same arena.
4.5.2.2 Extinction

Fear extinction involves the decline of the fear response following continuous but not reinforced exposure to a feared conditioned stimulus. The extinguished fear can recover following reexposure to the original context where the initial conditioned stimulus was given (Myers and Davies, 2007). For this protocol, a mouse was placed in a test chamber (chamber A) for 5 minutes and a foot shock was delivered for 2s at 2.5, 3.5, and 4.5 minutes at the end of a 30s tone. The next day, the mouse was placed in a different chamber, chamber B, and was exposed to the 30s tone at 1 minute intervals for 15 minutes.

4.5.2.3 Elevated Plus Maze

The elevated plus maze has been widely used as a behavioral assay in order to investigate the underlying anxiety-related behavior of rodents. In this assay, mice were placed in the centre of a four arm maze consisting of two open and two closed arms. The mice are placed facing an open arm. The number of entries and duration in each arm are recorded for 5 min. A decrease in open arm activity is thought to reflect anxious behavior.
4.5.3 Results

4.5.3.1 LIMK1-/- Mice Exhibit Increased Social Interaction

Mice were placed in a chamber that they were accustomed to during prior sessions and a tester mouse was presented to them. The time that the mouse spent exploring the area around the tester mouse was measured and recorded. The same exposure mouse was removed and replaced at 15 minute intervals. For four trials the same mouse was used and on a new one was placed on the 5th trial. Because of the short time interval between exposures of the tester mouse this aspect of the test removes any role that memory might play in this experiment.

In this test, it was found that LIMK1-/- mice do not habituate to repeated exposures of the same mouse. Moreover, they also spent significantly more time investigating when compared to wild-type controls (Figure 4.23). When the mouse is replaced by an object no differences were found between genotypes. These results suggest that LIMK1-/- mice share the hypersociability trait with those afflicted with WBS (Figure 4.24).
Figure 4.23
Figure 4.23 LIMK1 -/- Mice Exhibit Abnormal Social Interaction. Mice were exposed to the same mouse for four interactions and a new mouse was presented on the 5th trial. The time the mice spent interacting was recorded for each trial. LIMK1 lacking mice do not habituate to repeated exposures of the same mouse, moreover, they also spent significantly more time investigating when compared to wild-type controls.
Figure 4.24
Figure 4.24 LIMK1 -/- Mice Exhibit Normal Object Recognition. Mice were exposed to the same object for four trials and a novel object was presented on the 5th trial. In this behaviour test, no differences were found between genotypes in the time spent interacting with the object. These results suggest that LIMK1 -/- have similar exploratory and object recognition behaviour as wild type controls.
4.5.3.2 LIMK1 -/- Mice Do Not Exhibit Extinction Type of Behaviour

During the fear conditioning test, LIMK1-/- mice and wild type littermate controls showed similar levels of freezing. In the extinction training protocol, the mouse is given repeated exposures to the auditory cue that was accompanied with the foot shock. Normally, the amount of freezing that the mouse undergoes decreases with repeated exposures of the tone. Indeed, wild type mice show this decline in freezing after repeated exposures. However, during extinction, LIMK1-/- mice failed to show a decline in the freezing response (Figure 4.25A). Extinction can be thought of as new learning and when placed in the chamber where extinction occurred, animals tend to not exhibit freezing behaviours. However, in this case, LIMK1-/- mice continued to show enhanced freezing when placed in the chamber 24 hours after the extinction training (Figure 4.25B). When placed in the original chamber where the fear conditioning trial but not the extinction trial took place, all animals exhibited a renewal of freezing, although it took an extended period of time for the LIMK1-/- mice increase their freezing to wild type levels (Figure 4.25C). However, during extinction LIMK1-/- mice failed to show a decline in the freezing response. Additionally LIMK1-/- continued to exhibit freezing behaviour when exposed to the extinction context 24 hours after extinction training [Fig 4.23]. These results suggest a potential rigidity in learning present in LIMK1-/- mice.
Figure 4.25
Figure 4.25 LIMK1 Mice Exhibit Abnormal Extinction Behaviour. A. During extinction training, LIMK1-/− mice fail to show a decrease in freezing. B. Additionally, when placed in the same extinction chamber LIMK1-/− mice exhibit increased when compared to wild-type controls. C. All animals exhibit a renewal of the fear response when placed in the original chamber where fear conditioning was done.
4.5.3.3 LIMK1 -/- Mice Exhibit Increased Anxiety in the Elevated Plus Maze

Since those afflicted with WBS exhibit increased anxiety and phobias, anxious behaviour was examined using the elevated plus maze. Placing animals in the middle of the maze and tracking their movements revealed that LIMK1 -/- mice spent more time in the more arm cages [LIMK1-/-: 132.2±9.7 Wild Type: 117.3±6.4 | p<0.05] (Figure 4.27). During the experiments, it was observed that LIMK1-/- exhibited freezing like behaviours in the open arm cages but this data was not recorded. However, LIMK1 lacking mice did enter the open arms a significantly less than wild type littermates [Open arm: LIMK1-/-: 5.4±0.4 Wild Type: 8.2±0.8 | p>0.05] (Figure 4.26). The finding that LIMK1-/- mice enter the open arms can suggest that they exhibit more anxious behaviour.
Figure 4.26
Figure 4.26  LIMK-/- Exhibit More Anxious Behaviour in Elevated Plus Maze Task. Mice were placed the elevated plus maze and the amount of time the mice spent in the open and closed arms was measured. In this case, LIMK1 -/- mice spent more time in the open arm sections when compared to the wild type littermates [LIMK1 -/-: 132.2s±9.7 Wild Type 117.3s±6.4 | p<0.05].
Figure 4.27
Figure 4.27 LIMK1 -/- Mice Did Not Show Any Differences in Open Arm Entries. In the same task as in Fig 4.24 the number of entries in the open and closed arms of the elevated plus mouse was measured. In this case, LIMK1 -/- mice did enter the open arms significantly less than wild type littermates. [Open arm: LIMK1 -/-: 5.4±0.4 Wild Type 8.2±0.8 | p>0.05].
4.5.3.4 Discussion

LIMK1 lacking mice tend to exhibit a number of phenotypes associated with William-Beuren syndrome. One of the defining characteristics of Williams-Beuren syndrome is hypersociability. In order to propose LIMK1/- mice as a model for Williams-Beuren Syndrome it was key to have that phenotype present. Indeed, LIMK1/- mice exhibit an increase in social interaction. It is important to note that LIMK1/- mice show both enhanced overall social interaction when presented with the same mouse and also spend more time interacting with the novel mouse. This suggests that LIMK1/- mice have a global enhancement of overall social interaction.

We have previously shown that LIMK1/- mice show deficits in relearning tasks in the Morris water maze (Meng et al., 2002). One can hypothesise from that that LIMK1/- mice exhibit a rigidity in learning, that is if extinction is thought to be a form of novel learning as has been proposed as has been thought of previously (Quirk and Mueller, 2008). These results are consistent with my extinction memory results where LIMK1/- exhibit no extinction learning. Moreover, extinction learning has been shown to be actin dependent and destabilizing actin dynamics has been shown to impair extinction learning (Fischer et al., 2004). Since LIMK1/- exhibit abnormal actin regulation it is likely that this impairment in extinction learning is likely due to abnormal actin dynamics.

Additionally, LIMK1/- mice exhibit a decreased duration in the time spent in the open arms of the elevated maze. An important characteristic of Williams-Beuren Syndrome is an increased anxiety and phobias in those afflicted. Open arm entries in the elevated plus maze have been shown to relate to anxious behaviour (Walf and Frye, 2007). These results cannot be interpreted as a locomotor deficit since the number of entries into the open arms between genotypes is
similar. It can also be interpreted that LIMK1-/ show normal exploratory behaviour in the elevated plus maze. Taken together, the data suggest that LIMK1-/ mice exhibit a number of key traits associated with Williams-Beuren Syndrome and accordingly can be used as a putative model for Williams Beuren Syndrome.
4.6 Regulators of LIMK1 and their role in long-term memory

4.6.1 Introduction

As discussed earlier both PAK1 and Rock2 are positive regulators of LIMK1. It would expect that they are impaired in long term memory. This hypothesis also provides a logical manner in which to examine the role of LIMK1 on long-term memory.

Our lab has done previously published work on both of these proteins. We have found PAK1/- mice have normal basal synaptic function. However, they did have deficits in L-LTP and changes in the actin cytoskeleton as well as activation of LIMK1/- downstream target ADF/Cofilin (Asrar et al 2009). Double knockout PAK1/3/- mice had microcephaly, reduced dendritic arborisation, a deficit in E-LTP as well as short-term memory deficits (Huang et al, 2011). Interestingly, dominant negative PAK transgenic mice, in which all the forms of PAK were inhibited, exhibited fewer dendritic spines and enhanced LTP (Hayashi et al., 2004).

Utilizing Rock2/- mice our lab has performed experiments analyzing synaptic plasticity. We found that Rock2/- mice exhibited deficits in basal synaptic transmission as well as L-LTP. We also showed deficits in spine properties, synaptic deficits, along with the deficits in the actin cytoskeleton (Zhou et al, 2009).
4.6.2 Open field test

To assess the general motor activity and general exploratory behaviour of the mice an open field test was used. The test consists of placing a mouse in the middle of the testing chamber and its movements are tracked using an electronic laser grid for 5 minutes. Parameters such as distance, movement time and rest time were tracked using the laser grid.

4.6.3 Fear conditioning test

4.6.3.1 Training

A standard fear conditioning paradigm was used. Individual mice were placed in a conditioning chamber with controlled contextual cues and an electrified shock floor (Coulbourn Instruments). The mice were allowed to acclimate to the chamber for 2 minutes. Afterwards, a 30 second tone (85dB, 18,000 Hz) was played. A mild foot shock (0.5mA) was applied during the last two seconds of the tone. The mouse was allowed to remain in the chamber for one minute after the shock. This was done to get an assessment of freezing from both the knockout mice and their wild type littermates. This was important in order to determine whether there was a difference in pain sensitivity between the transgenic mice and their wild type littermates. Freezing is defined as lack of any movement except for respiration.

4.6.3.2 Testing

At the indicated intervals (24hr, 2weeks) the fear memory of the mice was tested. Long-term memory tests using mice often involve testing at 24 hours or longer after the training session (Kandel, 2001). Moreover, extending this time interval was performed to obtain a more complete assessment of long term memory. In the contextual test the contextual cues that were present during conditioning remained. The mouse was placed in the recording chamber for five
minutes. During the cued test, the chamber and the contextual cues were changed. Following a
two minute acclimation the same tone was played for 2 minutes.

4.6.3.3 Data acquisition and analysis

Data acquisition was done using TruScan Linc 2.0 software (Coulbourn Instruments). Mouse
behaviour was recorded using the TruScanLinc laser grid. Parameters recorded included:
number of movements, movement time, rest time, distance travelled, and movement speed. An
adjusted value of rest time was used as an indicator of freezing. To calculate freezing time the
rest time of the mice during the testing phase was used. Rest time prior to the foot shock was
calculated as normal, baseline resting time. The increase in rest time post shock was judged to
be freezing. For a particular group of mice, values for freezing during the cued and contextual
test were then normalized using the average freezing time found post shock during the training
phase. The data was analyzed using Student t-test in SigmaPlot 2001. In addition, manual
recordings of freezing were also performed to confirm the results from the digitally collected
data.
4.6.4 Results

4.6.4.1 Open Field

Prior to assessing any differences in learning and memory, we had to establish that there were not any gross motor deficiencies in the knockout mice we were using. The open field test is a good indicator of basal locomotor activity and provides a good indication of normal exploratory behaviour of mice. When measuring the average distance traveled by the mice there was no difference between the PAK1 knockouts and the wild type controls [Wild Type: 22.98 ± 2.89; PAK1−/−: 22.69 ± 2.08] (Figure 4.28A). Rock2−/- mice did seem to have a slight increase in locomotor activity according to the distance traveled when compared to wild type controls [Wild Type: 27.17 ± 0.18; Rock: 27.86 ± 0.40 | p>0.05] (Figure 4.28B). The lack of difference between the knockouts and their wild type littermates allows for the difference in the memory test to be attributed to abnormal memory function rather than motor deficits. The small increase in locomotor activity observed with the Rock2 knockouts was deemed to not be significant enough (i.e. p>0.05) to warrant concern about the next round of tests.
Figure 4.28
Figure 4.28 No Differences in LIMK1 Regulators in the Open Field Test. A. When measuring the average distance traveled by the mice there was no difference between the PAK1 knockouts and the wild type controls [Wild Type: 22.98 ± 2.89; PAK1-/- 22.69 ± 2.08]. B. Rock2 knockout mice appear to have a slight increase in locomotor activity according to the distance traveled when compared to wild type controls but it was not found to be significant [Wild Type: 27.17 ± 0.18; Rock2-/- 27.86 ± 0.40 p<0.05].
4.6.4.2 Fear Conditioning

The fear conditioning test is a simple and robust test in assessing the memory function of mice. The mice are able to remember the context and the cue associated with the noxious stimulus quite quickly which allows for rapid testing of a large number of animals. Using this test, we found that PAK1 knockouts exhibited deficits in long-term contextual memory only at two weeks after training [Wild Type: 99.99 ± 11.29; PAK1/-/-: 73.10±13.18; p=0.18]. At 24 hours post training, mice lacking PAK1 performed equally well when compared to wild type littermates in both the cued and contextual tests [Contextual: Wild Type: 69.81±10.99; PAK1/-/-: 76.18±13.32; p>0.05 | Cued: Wild Type: 101.43±15.15; PAK1/-/-: 94.11±7.93; p>0.05]. The only deficit found was at two weeks in the contextual test--although with a p value of 0.18--and no difference was observed in the 2 week cued test (Figure 4.29). In summary, although there was a trend toward decreased freezing for mice lacking PAK1, there were no significant results that were found (Figure 4.8).

The Rock2/-/- mice on the other hand, exhibited increased contextual long-term memory overall. This difference occurred at 24 hours and two weeks after training. Mice lacking Rock2 froze significantly more than the wild type controls in both cued and contextual tests at two days and 2 weeks after training [24 hours Contextual: Wild Type: 78.21±16.1; Rock2/-/-: 129.31±11.4; p<0.05 | 24 hours Cued: Wild Type: 79.57±13.66; Rock2/-/-: 128.24±13.62; p<0.05 | 2 weeks Contextual: Wild Type: 72.96±10.27; Rock2/-/-: 111.98±13.57; p<0.05| 2 weeks Cued: Wild Type: 96.55±11.10; Rock2/-/-: 138.99±8.07; p<0.05] (Figure 4.30). This suggests that the Rock2/-/- mice may have
enhanced memory. Taken together, the results from the behaviour experiments indicate that Rock2 may function differently than PAK1.
Figure 4.29
Figure 4.29 PAK1 -/- Mice Exhibit Deficits in Fear Conditioning Memory. PAK1 knockouts exhibited deficits in long-term contextual memory only at two weeks after training [Wild Type: 99.99 ± 11.29; PAK1: 73.10±13.18 | p>0.05]. At 24 hours post training, mice lacking PAK1 performed equally well when compared to wild type littermates in both the cued and contextual tests [Contextual: Wild Type: 69.81±10.99; PAK1: 76.18±13.32; p>0.05 | Cued: Wild Type: 101.43±15.15; PAK1-/-:94.11±7.93 | p>0.05].
Figure 4.30
Figure 4.30 Rock2 -/- Mice Exhibit Increased Fear Conditioning Memory. The Rock2 knockout mice exhibited increased contextual long-term memory overall. Rock2-/- mice froze significantly more than the wild type controls in both cued and contextual tests at two days and 2 weeks after training [24 hours Contextual: Wild Type: 78.21±16.1; Rock2-/-: 129.31±11.4 | p<0.05; 24 hours Cued: Wild Type: 79.57±13.66; Rock2-/-: 128.24±13.62 | p<0.05; 2 weeks Contextual: Wild Type: 72.96±10.27; Rock2-/-: 111.98±13.57 | p<0.05; 2 weeks Cued: Wild Type: 96.55±11.10; Rock2-/-: 138.99±8.07 | p<0.05].
4.6.5 Discussion

Neither PAK1-/− or Rock2-/− mice exhibited deficits in the open field test. However, there was an interesting difference between the open field experiments in that the PAK1 knockouts and the wild type littermates showed habituation, as in the distance covered decreased over time, whereas the data obtained using the Rock2 knockouts and their controls did not show such a habituation. This difference can be interpreted to be caused by handling differences as well as the relatively low number of mice used in the Rock2 open field test. However, the Rock2-/− data is also consistent in experiments that demonstrate habituation with LIMK1-/− mice. For example, in the extinction and social interaction experiments LIMK1-/− mice do not show as much habituation as wild type littermates. It is possible that this absence of habituation in Rock2-/− mice is caused by irregular LIMK1 regulation in these mice.

Another important finding of is that the mice lacking the regulators of LIMK1 have altered fear memory. This further supports the hypothesis that LIMK1 signaling is critically involved in learning and memory. Interestingly, PAK1-/− mice exhibited a trend toward lower than normal freezing, whereas Rock2-/−mice exhibited higher than normal freezing suggesting that PAK1 and Rock2 may play differential roles in regulating LIMK1 and brain function. It is important to further investigate how these differential effects are achieved.

Furthermore, my results have important implications in terms of the function and regulation of the RhoGTPases. This means that in addition to the regulation of the actin cytoskeleton, there are other pathways important for learning and memory that can be regulated by the RhoGTPases. This finding may be applicable to other cellular processes as well.
5 Summary of Results

Electrophysiological studies showed that LIMK1 -/- mice have specific L-LTP deficits. In the last half hour of recording LIMK1/-/- LTP was consistently deficient using three different induction protocols. Briefly, induction protocols of 4 trains of 100Hz spaced 5 minutes apart, 3 trains of TBS stimulation 10 minutes apart, and 4 trains of 100Hz spaced 20 seconds apart all showed consistent deficits in LIMK1 lacking animals.

The electrophysiology data is consistent with follow up behavioural experiments. In the Morris water maze at 48 hours post training, LIMK1/-/- mice showed deficits in the amount of time they spent searching for the platform in the correct quadrant compared to wild type littermates. LIMK1 lacking mice did not have deficits in acquiring the task and were able to learn it as well as wild type littermates. Moreover, no deficit was observed 2 hours post training on the last day training was performed. These results suggest that short term memory is intact and long-term memory is impaired in LIMK1 -/- mice in the Morris water maze task.

Additionally, fear conditioning experiments are consistent with the Morris water maze data. In this task LIMK1/-/- animals show no deficits 2 hours post training but froze significantly less at 48 hours post training. These results again show that long-term memory in LIMK1/-/- mice is specifically impaired.

In order to examine whether LIMK1 can potentially interact with CREB immunohistochemical and immunocytochemical experiments were conducted. LIMK1 and CREB were found to exist in an immunocomplex, and to localize in similar subcellular areas in the mouse hippocampus, as well as dissociated hippocampal neurons. Additionally, CREB phosphorylation was decreased
in LIMK1 lacking mice in acute hippocampal slices treated with NMDA and glycine but unchanged with forskolin treatment.

Experiments increasing the activity of CREB were conducted and the electrophysiological properties of LIMK1 lacking mice were examined. Forskolin was able to rescue the L-LTP deficit in LIMK1 mice only when accompanied by a strong tetanic stimulation. Forskolin along with a weak tetanic stimulation was not able to induce L-LTP in LIMK1-/- mice and forskolin induced LTP was deficient in LIMK1-/- mice when compared to littermate controls.

CREB activation was also able to rescue the memory deficit in LIMK1-/- mice. Administration of rolipram rescued the Morris water maze deficit at 48 hours after training for LIMK1 -/- mice while also increasing wild type performance. This was also the case in the fear conditioning experiments where rolipram administration increased freezing to wild type levels in LIMK1 lacking mice. Rolipram administration did not appear to have any effects on memory performance at 2 hours post training in either the Morris water maze or fear conditioning task.

Electrophysiological experiments examining the role of cofilin phosphorylation in LIMK1-/- animals investigated the role of the actin cytoskeleton on LTP. Pretreatment with the S3P peptide that inhibits LIMK1 action showed no effects on LIMK1-/- mice during E-LTP and no effect on L-LTP. The S3P peptide did manage to enhance wild type LTP, however, suggesting that cofilin deactivation by LIMK1 plays an important role in E-LTP. The addition of the S3 peptide showed an attenuation of the increased E- LTP in both LIMK1-/- mice and wild type controls and did not appear to have any effect on the late LTP deficit in LIMK1-/- mice. Moreover, the L-LTP in wild type mice was also unchanged.
Experiments were also performed that assessed other aspects of LIMK1-/- mice that could be used to determine the suitability as a model for Williams-Beuren Syndrome. Since WBS patients exhibit rigid types of learning and previous experiments from our lab have shown that LIMK1-/- mice show deficits in the Morris water maze relearning task (Meng et al, 2002), I performed experiments examining extinction learning in these mice. In the extinction task, LIMK1-/- mice showed increased freezing and no habituation in the novel context. Additionally, they showed increased freezing when placed in the original context.

Moreover, in the elevated plus maze, LIMK1-/- mice appeared to show increased anxiety as measured by the decreased amount of entries in the open arms of the elevated plus maze. As WBS patients exhibit more anxiety than the general population, these results are consistent with that data (Bellugi et al, 2002).

Taken together, these results are consistent with the idea of LIMK1 interacting through CREB to regulate long-term memory and synaptic plasticity, as well as providing a basis for the use of LIMK1-/- as a putative model for WBS.
6 Discussion

The molecular processes and properties of learning and long-term memory are still not well understood. A large body of evidence implicates CREB as having a critical role in long-term memory and L-LTP (Nguyen et al., 1994, Yin and Tully, 1996, Josselyn et al., 2001, Kandel, 2001, Brightwell et al. 2007). Moreover, LIMK1 has been shown to be important in the regulation of the actin cytoskeleton (Meng et al, 2002). The actin cytoskeleton itself has been implicated as being important in synaptic plasticity and long-term memory and L-LTP (Fukazawa et al., 2004). The results that show LIMK1 is able to interact with CREB suggest a potential for cross-talk and dual regulation of synaptic plasticity by LIMK. Moreover, my results show that there appears to be a clear distinction between the mechanisms responsible for E-LTP and L-LTP. Generally, most LTP phenotypes show either similar or depressed E-LTP levels leading to deficits in L-LTP. My data demonstrates a L-LTP deficit accompanied in the same phenotype with an enhanced E-LTP. While it has been postulated that the mechanisms for the different phases of LTP are distinct (Kandel, 2001), my current findings demonstrate a distinct separation of these pathways that has not been previously reported thus providing more evidence that E-LTP and L-LTP require completely different processes for maintenance.

6.1.1 Role of LIMK1 in LTP

The actin cytoskeleton plays a major role in LTP both during the early and late phases (Fukazawa et al, 2003; Rex et al, 2010). However, it is during the early phase that actin turnover plays an important role. Interaction with AMPA receptors and actin associating proteins such as PICK1 contributes to the enhancement of LTP (Rocca et al., 2008, Nakamura et al., 2011). Moreover, actin filaments play an important role in the quantal release probability (Lee et al.,
Indeed, our lab has previously shown that the frequency of miniature excitatory postsynaptic currents (mEPSCs) is increased in LIMK1-/- mice. An increased frequency of mEPSCs has been correlated with increase an in LTP (Oliet et al, 1996). Actin filament distribution has been shown to be important for AMPA receptor trafficking (Kerr and Blanpied, 2012; Allison et al, 1998; Arendt et al, 2010), an important contributor to a single train LTP and NMDA receptor dependent LTD (Morishita et al, 2005). Indeed, the post-synaptic density has been shown to be abundant in actin associating proteins (Matus et al, 1982). LIMK1 regulation of the actin cytoskeleton can contribute to the enhancement of single train LTP. Moreover, actin regulation has been shown to be important for the maintenance of L-LTP (Fukazawa et al, 2003). However, according to my results, this does not seem to be the cause of L-LTP deficits observed in LIMK1-/- mice. Treatment with a cofilin inhibiting peptide does not appear to rescue that deficit. Indeed, only enhancement of CREB activation was able to rescue the electrophysiological deficit. Additionally, TBS has been shown to induce actin polymerization in dendritic spines (Lin et al, 2005), thereby potentially rescuing the effects of increased actin depolymerization in LIMK1-/- mice. Taken together, the findings with LIMK1-/- mice suggest a role for actin polymerization in one train induced E-LTP and a role for CREB in L-LTP induced by spaced stimulation.

6.1.2 Consequences of the LIMK1/CREB interaction

An important finding of this thesis is that LIMK1 interacts with CREB in mature hippocampal slices. This is important in a number of ways. Firstly, in addition to actin dynamics, this finding suggests that LIMK1 may also regulate other cellular processes. Secondly, given the importance of CREB in synaptic plasticity and memory formation, LIMK1 regulation of CREB may provide an important mechanism for the regulation of brain function by LIMK1.
However, the nature of this interaction still needs to be further examined. One question that
remains is whether LIMK1 does indeed directly activate CREB in mature neurons. As stated
previously, LIMK1 has only been shown to directly activate CREB in hippocampal
neuroprogenitor cells (Yang et al, 2004). Therefore, answering this question is likely to lead to
revealing the functional significance. However, an activation interaction is not necessarily the
only one that may be significant for regulation of synaptic activity. A binding interaction
between LIMK1 and CREB may also potentially change other properties such as the subcellular
localization of the two proteins.

CREB activation has been shown to induce new protein synthesis required for the induction and
maintenance of long-term memory and synaptic plasticity. Moreover, the deficit in pCREB
levels in LIMK1-/− mice with NMDA stimulation is indicative of an activity dependent role of
CREB activation by LIMK1. I have also shown that basal levels of pCREB are unaltered in
LIMK1-/− mice. Indeed, forskolin activation of CREB via the PKA pathway was still intact in
LIMK1 knockout mice. This last finding suggests that CREB activation aside by LIMK1 is
functional in LIMK1 mice. Additionally, the finding that treatment with forskolin and the use of
a strong stimulation protocol is able to elicit L-LTP in LIMK1-/− mice shows that these mice
have the cellular machinery capable of L-LTP maintenance provide sufficient CREB activation
is provided. Indeed, this finding indicates that it is particularly the insufficient CREB activation
that is responsible for the L-LTP deficit in LIMK1-/− mice.

The presence of a LIMK1 and CREB interaction also poses a few obvious questions as to the
suitability of using LIMK1 knockout mice as a model for studying learning and memory. The
LIMK1 and CREB interaction occurs during development in hippocampal neuroprogenitor cells,
which suggests that there could be developmental deficits in mice lacking LIMK1. Moreover,
this may purport that the changes observed in these mice are due to developmental deficits. However, this is not likely the case since no significant abnormalities were found in the gross structure of the brain, where LIMK1 is highly expressed (Meng et al. 2002). In addition, there was no difference in other proteins that either regulated or are regulated by LIMK1, such as PAK1, PAK3, Rock2, and ADF/Cofilin. As well, total levels of LIMK2 were unaltered (Meng et al, 2002). This evidence suggests that no compensation in the form of upregulated protein levels occurred in those animals and that the differences are not likely due to developmental changes.

6.1.3 Altered L-LTP in LIMK1 knockout mice

Altered L-LTP observed in the LIMK1-/− hippocampal slices indicates that LIMK1 is important in long-term synaptic plasticity. These results are consistent with the finding that LIMK1 and CREB exist in one complex. However, it is also possible that alterations at the level of the actin cytoskeleton could be contributing to the decrease in L-LTP. Actin has been shown to be important for L-LTP in in vivo experiments (Fukazawa et al, 2002). Therefore, it is important to dissect which of these two mechanisms are responsible for the deficits in the L-LTP deficit observed in mice deficient in LIMK1. It is also possible that both actin regulation and CREB signaling are involved. It is then important to determine the relative significance of the two signaling pathways to the occurrence of L-LTP.

The deficits observed in LIMK1 lacking animals were specifically long-term deficits and, moreover, I was able to rescue them by enhancing CREB activity. Rolipram was able to attenuate the memory deficits seen in LIMK1-/− mice in both the Morris water maze and the fear conditioning task. These results are consistent with insufficient CREB activity being the cause of the observed deficit. In contrast, forskolin and rolipram had no effect on cofilin activation.
Previously, we have also shown that PAK3/- animals have a deficit in L-LTP and also exhibit a deficit in CREB signaling (Meng et al., 2006). PAK3 is an important regulator of LIMK1 (Edwards et al., 1998). Taken together with my current findings, these results show an important role for the Rho GTPases in CREB regulation. The Rho GTPases have been previously implicated in mental retardation (MR), and are known to play important roles in cognition (Ramakers, 2004). However, the mechanisms of their action have not been well understood or it has been proposed that they have acted through regulation of the actin cytoskeleton. Here I show that Rho GTPase signaling is involved in new protein synthesis important for the induction and maintenance of long-term memory and synaptic plasticity.

My data concerning the LIMK1 regulation of CREB sheds further light on the manner in which neuronal cells regulate structural and synaptic plasticity. It has been known that dendritic spines alter their shape by increasing or decreasing in size during activity (Lampechrt and Ledoux, 2002, Nimchinsky et al., 2002. Matsuzaki et al., 2004). This activity is regulated by the actin cytoskeleton, which is the major structural component of the dendritic spine. Rho GTPases are potent regulators of the actin cytoskeleton and our previous results have shown LIMK1 is involved in dendritic spine structure (Meng et al, 2002). Since LIMK1 regulates CREB this may indicate that the cell possibly uses Rho GTPase signaling as a link between structural plasticity and new protein synthesis necessary for the maintenance of long-term memory and synaptic plasticity. Indeed, my results together with other previously published work on Rho GTPase signaling provide evidence of signaling pathways converging to produce the required cellular phenotypes observed in the induction of long-term memory.

The actin cytoskeleton is thought to play a role in the structural morphology of the dendritic spine and therefore changes in its morphology may be seen as secondary or a consequence of
processes involved in LTP maintenance or memory formation. However, showing that LIMK1, which is involved in the structural plasticity of the dendritic spine, also plays a role in CREB signaling suggests that structural plasticity and synaptic plasticity are part of the same ongoing process and one is not a result of the other. Furthermore, my data suggest that LIMK1 serves as a link between structural and synaptic plasticity.

6.1.4 LIMK1 and Williams-Beuren Syndrome

WBS patients and those with various forms of MR exhibit deficient memory (Ramakers, 2002). With LIMK1 being one of the genes deleted in those with WBS as well as the downstream target of the Rho GTPases which have been implicated in various forms of MR our results present a possible explanation for that feature of MR. Indeed, my results also indicate that LIMK1-/- mice show increased social interaction and an increase in anxious behaviour. Previous attempts to model WBS in mice have also shown some of these features including increased social interaction (Young et al, 2008; Fujiwara et al., 2006). However, often those mouse models do not show deficits in long-term memory as has been shown to exist in Williams Syndrome patients (Rhodes et al., 2011). Taken together, this may suggests that LIMK1-/- mice can serve as robust model for study of the neurocognitive aspects of WBS. Additionally, future work needs to focus on a haploinsufficiency model of LIMK1 deletion to better mimic the presenting symptoms of LIMK1 deletion found in WBS. More work also needs to be done in determining the underlying cause of the phenotypes observed in human MR. My work represents a possible therapeutic target for future studies. Moreover, my findings aid in our understanding of cellular signaling during long-term memory and synaptic plasticity, and additionally shed light on the pathophysiology and mechanisms of MR.
7 Future Directions

Williams Syndrome is caused by a deletion in one of the 7th chromosomes and my data is based on work on a complete knockout of the gene in animals where the two copies of the gene are deleted. In terms of developing a model for Williams Syndrome, more work needs to be done in LIMK1+/− mice. It is important to investigate whether the long-term synaptic plasticity and long-term memory deficits are present in the heterozygous mice. Providing that data would strengthen the case of LIMK1 as a the determining gene for the neurocognitive profile in Williams Syndrome.

My data has shown that LIMK1 lacking mice exhibit deficits in LTP and long term memory. I have shown that this is likely due to mechanism involving insufficient CREB activation. I have also shown evidence of a complex involving CREB and LIMK1 in hippocampal neurons. However, LIMK1 has been found to exist in a complex with its antagonist protein, Slingshot phosphatase, (SSH) and PAK in fibroblasts (Soosairajah et al, 2005). We have previously shown that PAK3 lacking mice have deficits in L-LTP and CREB phosphorylation (Meng et al, 2005). Potentially, it is the interplay in the proteins in this complex that may be important in CREB regulation and the possibility needs to be investigated. The significance of CREB on synaptic plasticity and learning and memory is well documented (Kandel, 2001) therefore elucidating mechanisms responsible for its activation are highly important. Additionally, it is unknown whether this LIMK1/SSH/PAK3 complex exists in neurons and what effect, if any, do the other proteins in the complex have on the observed effects in LIMK1 lacking mice. Using immunoprecipitation of fluorescence resonance transfer assays we can determine whether this SSH/LIMK1/PAK complex exists in neurons. Pharmacological manipulation during electrophysiological behavioural experiments of the other proteins in this putative complex can also yield answers as to the functional consequences of the proteins involved in this complex.
This is important convergence of CREB signaling may be critical for achieving a threshold of gene activation for maintenance of long term memory (Davis et al., 2002).

Moreover, it is yet to be determined where the proposed LIMK1 and CREB interaction occurs. LIMK1 contains a nuclear targeting region, which allows it to move back and forth between the nucleus and the cytoplasm (Yang and Mizuno, 1999). It is widely known that CREB is localized in the nucleus and perinuclear regions (Waeber and Habener, 1997). As well it has been shown that translation of the CREB mRNA occurs in distal axons of neurons (Cox et al, 2008).

Therefore, LIMK1 and CREB are able to interact in several sites of the neuron. Determining the subcellular site of the interaction can elucidate whether phosphorylation of CREB by LIMK1 is responsible for the translocation of CREB to the nucleus or whether the interaction is a classical activation phosphorylation. To date research has shown CREB activation is thought to occur in the nucleus, however, LIMK1 and CREB have a similar distribution in axons and the neuronal nucleus. The potential for a novel mechanism of CREB activation outside of the nucleus exists. It would be interesting to separate cytosomal and nuclear fractions and perform immunoprecipitation experiments to determine where the interaction occurs. Moreover, high resolution confocal microscopy can also reveal co-localization of LIMK1 and CREB outside of the nucleus. Experiments involving fluorescence resonance transfer (FRET) can provide more accurate results about co-localization of CREB and LIMK1.

Another study suggests that proteasomes are recruited to dendritic spines with synaptic activity and they associate with actin filaments (Bingol and Schuman, 2006). Since f-actin is perturbed in LIMK1 lacking mice this could be a possible mechanism by which LIMK1 affects L-LTP. With the use of proteasome markers, we can visualize whether the basal distribution of the proteasome is altered along with examining whether their recruitment to the dendritic spines is affected in
LIMK1 knockout mice. This would further elucidate the actin contribution to the deficits observed in memory and synaptic plasticity in LIMK1 lacking mice.

My findings that LIMK1 lacking mice exhibit memory deficits can be related pathophysiologically to Alzheimer’s disease (AD). Indeed, recent studies have shown that a high level of pLIMK, activated LIMK1, is found after treatment of cells with filamentous amyloid beta (Aβ), in addition to increased levels of pCofilin (Heredia et al, 2006). Moreover, immunofluorescence of human AD brains shows an increase of pLIMK1 positive neurons in those areas affected by AD pathology (Heredia et al, 2006). In addition, other studies has shown that treatment with Aβ oligomers induced dendritic spine loss via a cofilin mediated pathway (Davis et al, 2011, Maloney and Bamburg 2007).

Moreover, the role of Aβ protein in the regulation of synaptic plasticity and how it contributes to the effects observed in patients with Alzheimer’s disease has not been investigated. As stated previously, one of the pathological signs of Alzheimer’s disease is a glutametergic signaling deficit. Recent evidence suggests that this could be caused by Aβ dependent pruning of dendritic spines (Heredia et al, 2006). A recent study has shown that secreted Aβ oligomers decrease spine density. The mechanism proposed in that study was thought to involve NMDA receptor signaling and cofilin (Shankar et al, 2007). LIMK is activated by calcium influx through NMDA receptor and regulates cofilin activation (Lamprecht and Ledoux, 2004). Aging and testing LIMK1-/- mice in memory tasks could shed light on LIMK1 involvement in AD. In addition, the formation of amyloid plaques and spine density in aged LIMK1-/- mice could be measured and compared to aged controls. Again, background evidence suggests that LIMK1-/- mice could be less susceptible to AD symptoms (Heredia et al, 2006) implying that LIMK1 may play a role
in the decreased cognitive ability of Alzheimer’s patients. A comprehensive study of this role this role may lead to the discovery of novel therapies.
8 References


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