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The Effects of PTEN Deletion on Cell Size and Plasticity in the Hippocampus

Abstract

The tumor suppressor phosphatase and tensin homolog (PTEN) is the central negative regulator of the phosphatidylinositol 3-kinase (PI3K) -signaling pathway, which mediates diverse processes in various tissues. In the nervous system, the PI3K pathway modulates proliferation, migration, cellular size and synaptic transmission and plasticity. Neurologic abnormalities such as autism, seizures, and ataxia are associated with inherited PTEN mutations. Yet, how PTEN loss contributes to neurologic dysfunction remains unknown. PTEN loss during early development is associated with extensive deficits in neuronal migration and substantial hypotrophy of neurons and synaptic densities. However, whether its effect on synaptic transmission and plasticity is direct or mediated by structural abnormalities remains unknown. Here we analyzed neuronal and synaptic structures and function in Pten-conditional knockout mice in which the gene was deleted from excitatory neurons during late development. Using 2-photon imaging, Golgi staining, immunohistochemistry, electron microscopy, and electrophysiologic tools, we determined that late Pten loss does not affect hippocampal development, neuronal or synaptic structure or basic synaptic transmission. However, it does cause deficits in both major forms of synaptic plasticity, long-term potentiation and long-term depression. Deletion of Pdk1, the positive regulator of the PI3K pathway, rescued Pten-mediated deficits in synaptic plasticity. These results suggest that Pten independently modulates functional and structural properties of hippocampal neurons and is directly involved in mechanisms of synaptic plasticity.

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**THE EFFECTS OF *PTEN* DELETION ON CELL SIZE AND PLASTICITY IN
THE HIPPOCAMPUS**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Margaret Sperow
December 2011

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DEDICATION

I would like to dedicate my dissertation to the memory of my father, James W. Sperow. As a career scientist he exemplified what I hope to achieve. After working many years toward his PhD, he completed his schooling with a Master of Science in Biostatistics. As I did not have an opportunity to know him on an intellectual level, I would like to think that through the completion of my PhD I can continue his legacy and become the first Dr. Sperow in our family.

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ABSTRACT

The tumor suppressor phosphatase and tensin homolog (PTEN) is the central negative regulator of the phosphatidylinositol 3-kinase (PI3K) -signaling pathway, which mediates diverse processes in various tissues. In the nervous system, the PI3K pathway modulates proliferation, migration, cellular size and synaptic transmission and plasticity. Neurologic abnormalities such as autism, seizures, and ataxia are associated with inherited *PTEN* mutations. Yet, how *PTEN* loss contributes to neurologic dysfunction remains unknown. *PTEN* loss during early development is associated with extensive deficits in neuronal migration and substantial hypotrophy of neurons and synaptic densities. However, whether its effect on synaptic transmission and plasticity is direct or mediated by structural abnormalities remains unknown. Here we analyzed neuronal and synaptic structures and function in *Pten*-conditional knockout mice in which the gene was deleted from excitatory neurons during late development. Using 2-photon imaging, Golgi staining, immunohistochemistry, electron microscopy, and electrophysiologic tools, we determined that late *Pten* loss does not affect hippocampal development, neuronal or synaptic structure or basic synaptic transmission. However, it does cause deficits in both major forms of synaptic plasticity, long-term potentiation and long-term depression. Deletion of *Pdk1*, the positive regulator of the PI3K pathway, rescued *Pten*-mediated deficits in synaptic plasticity. These results suggest that *Pten* independently modulates functional and structural properties of hippocampal neurons and is directly involved in mechanisms of synaptic plasticity.

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LIST OF ABBREVIATIONS

<i>αCamKII</i>	Alpha isoform of Ca ²⁺ /calmodulin-dependent protein kinase II
ACSF	Artificial cerebral spinal fluid
ADP	After-depolarization
AHP	After-hyperpolarization
AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid
BDNF	Brain derived neurotrophic factor
BK	Big potassium channels
CA	Cornu ammonis
<i>Cre</i>	<i>Cre</i> -recombinase
DKO	Double knockout
eIF4E	Eukaryotic translation initiation factor 4E
EPSP	Excitatory postsynaptic potential
ERK	Extracellular signal related kinase
fEPSP	Field excitatory postsynaptic potentials
FOXO	Forkhead box protein, class O
Gab1	GRB2-associated-binding protein 1
GABA	γ-aminobutyric acid
GF	Growth factors
<i>GFAP</i>	Glial fibrillary acidic protein
GluR1	Glutamate receptor type 1
GPCR	G-Protein-coupled receptors
H&E	Hematoxylin and eosin stain
HFS	High frequency stimulation
HICAP	Hilar commissural associational pathway-related cells
HIPP	Hilar perforant path-associated cells
I _C	Calcium channel current
I _M	Muscarinic receptor activation
LFS	Low frequency stimulation
LTD	Long-term depression
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinase
mEPSCs	Miniature excitatory postsynaptic currents
mGluR	Metabotropic glutamate receptors
MOPP	Molecular layer perforant path-associated cells
mTOR	Mammalian target of rapamycin
NMDA	N-Methyl-D-aspartic acid
NSE	Neuron-specific enolase

p70S6K	p70 ribosomal S6 kinase
PDK1	3- Phosphoinositide-dependent kinase 1
PDZ	Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (DlgA), and Zonula occludens-1 protein
PFA	Paraformaldehyde
PH	Pleckstrin homology
PIP ₂	Phosphatidylinositol 4,5-bisphosphate
PIP ₃	Phosphatidylinositol 3,4,5-triphosphate
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKM	Constitutively activated form of PKC
PKM ζ	Atypical PKC isoform zeta
PPF	Paired-pulse facilitation
PSD	Postsynaptic density
PTEN	Phosphatase and tensin homolog
Rheb	Ras homologue enriched in brain
RTK	Receptor tyrosine kinase
Shc	“Sarcoma” is a proto-oncogenic tyrosine kinase
SiRNA	Small interfering RNA
SNARE	Soluble NSF attachment protein receptor
TRA	Training quadrant
TSC	Tuberous sclerosis complex
VGCC	Voltage-gated calcium channels
YFP	Yellow fluorescent protein
ZIP	Myristoylated ζ -pseudosubstrate inhibitory peptide
4EBP	eIF4E-binding proteins
5-HT	5-hydroxytryptamine

CHAPTER 1. INTRODUCTION

OVERVIEW

The goal of this study was to examine the interaction and subsequent functional relationship between the phosphoinositide 3-kinase (PI3K) pathway and synaptic function. Previous literature has yet to establish a clear and direct correlation between deletion of *Pten*, a negative regulator of the PI3K pathway, in the hippocampus and changes in synaptic plasticity. I would like to examine the role of *Pten* deletion in CA1 and CA3 synaptic connections and its effect on the two major form of synaptic plasticity: long-term potentiation (LTP) and long-term depression (LTD). Previous studies containing a *Pten* deletion that occurred in multiple cell types showed a gross deficit in synaptic transmission. These results were confounded by this *Pten* deletion causing disruption in cellular migration during development. This disruption in developmental cellular migration prevented previous work from being able to determine the straightforward functional outcome of *Pten* removal and any resulting synaptic defects. The method used to address this question focuses on a novel mouse model deleting *Pten*. This specific mouse model allows the direct study of *Pten*'s role in plasticity since the *cre-loxP* system performs cell specific and temporally precise deletion of *Pten*. This allows us to avoid the lethality associated with the homozygous knockout of *Pten* and focus my studies on a specific cell type. I show a direct correlation between *Pten* deletion in pyramidal neurons and subsequent effects on synaptic function or plasticity in the hippocampus. I also show co-deletion of a second enzymatic protein in this pathway, 3-phosphoinositide-dependent kinase (PDK1), rescues the functional effect of the *Pten* deletion in the same conditional mouse model. The novel results of our findings were that these functional changes occur without the typical neuronal hypertrophy seen in *Pten* deletion models.

BACKGROUND

The PI3 Kinase Pathway

The lipid kinase activity of phosphoinositide 3-kinase (PI3K) is evolutionarily conserved from yeast to mammals (Auger et al, 1989). This family of intracellular lipid kinases functions by phosphorylating the 3'-hydroxyl group of phosphatidylinositol and phosphoinositides (Whitman et al, 1988). Briefly, the primary action of PI3K is phosphorylation and subsequent conversion of membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-triphosphate (PIP₃) (**Figure 1-1**). This process is reversed by the tumor suppressor *PTEN* (phosphatase and tensin homologue on chromosome 10), which is the gene that is the focus of my studies. The primary target of PIP₃ is Akt, which is a serine/threonine protein kinase that translocates to the membrane and binds PIP₃ via its pleckstrin homology (PH) domain. PDK1 (3-phosphoinositide-dependent kinase) is also recruited to the intracellular membrane by PIP₃ via its PH

Figure 1-1 The PI3 kinase pathway.

A schematic demonstrating key components of the phosphoinositide 3-kinase (PI3K) pathway relevant to this study. Included are the activating G-protein coupled receptors (GPCRs), and receptor tyrosine kinases (RTKs). PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-triphosphate (PIP₃) which is reversed by phosphatase and tensin homologue on chromosome 10 (PTEN). Through their pleckstrin homology domains 3-phosphoinositide-dependent kinase (PDK1) and AKT are recruited to the membrane, and activate downstream components such as γ -aminobutyric acid (GABA) receptor, mammalian target of rapamycin (mTOR), tuberous sclerosis complex 1 and 2 (TSC1/2), eukaryotic translation initiation factor 4E (eIF4E), eIF4E-binding protein (4EBP1), and p70 ribosomal S6 kinase (p70S6K).

domain and subsequently phosphorylates the Akt T-loop at threonine (T) 308. (Alessi et al, 1996; Stephens et al, 1998) (**Figure 1-1**). Downstream, Akt phosphorylates the negative signaling regulator tuberous sclerosis complex 2 (TSC2), a negative regulator of mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is then able to phosphorylate eukaryotic translation initiation factor 4E-binding protein (4EBP1) and p70 ribosomal S6 kinase (p70S6K), which promotes mRNA translation and protein synthesis (Huang & Manning, 2008).

The PI3K kinase family is divided into three different functional classes, which play various roles in cellular signaling. The class I PI3Ks are heterodimers which consist of a regulatory and catalytic subunit. The class I PI3Ks are activated either by growth factor receptor tyrosine kinases (RTKs) (Class IA) or G-protein-coupled receptors (GPCRs) (Class IB) (Cantley, 2002). Class II PI3Ks consist of a single catalytic subunit. Class II PI3Ks are able to bind clathrin, and localize to coated pits (involved in membrane trafficking and receptor internalization) (Engelman et al, 2006). Class II PI3Ks can also be activated by RTKs (e.g., epidermal growth factor receptors) as well as cytokine receptors and integrins. Over all, much less is known about class II and class III PI3Ks; therefore my studies are focused on Class I PI3Ks, as they are the most relevant to this study.

An important difference between class IA PI3Ks and class IB PI3Ks is regulatory subunit regulation by RTKs and GPCRs, respectively (Engelman et al, 2006). While the class IB of PI3Ks is important in mammalian cells, the majority of in vivo work has focused on the class IA. PI3K is expressed in both the developing and adult nervous system, and its catalytic subunit is highly expressed throughout the hippocampus, as well in the olfactory bulb and cerebellum, but weakly seen throughout the remainder of the brain (Ito et al, 1995). PI3K also functions in neuronal survival as well as neurite outgrowth (Rodgers & Theibert, 2002).

Functionally, the PI3K signaling pathway has been shown to affect regulation of metabolism downstream of insulin receptors and is therefore relevant to type-2 diabetes (Engelman et al, 2006). Regulation of cellular growth and proliferation has also been an important focus for cancer studies. There are two major regulatory functions of the PI3K signaling pathway that are most relevant to my project. The first is PI3K signaling of cell growth in post-mitotic cells (Backman et al, 2002). The second is the downstream effect on protein synthesis that most likely occurs through the mammalian target of rapamycin (mTOR) (Holz et al, 2005). A hallmark of various PI3K mouse models is post-mitotic cell growth. An example is found in transgenic mice that contain a constitutively active catalytic PI3K subunit: they have increased heart size that results from enlarged myocytes (Shioi et al, 2000). Specifically *Pten* deletion causes increases in post-mitotic cell size. (Backman et al, 2001; Kwon et al, 2001). Protein synthesis is also important as an essential element in long-term synaptic plasticity, which is the neuronal process that is the focus of my studies. There is debate about whether neuronal size changes affect the function of cells. Thus, by examining key proteins in the PI3K pathway, specifically *Pten*, I hope to determine more information on how they affect neuronal plasticity.

I looked at the PI3K pathway in the central nervous system (CNS), specifically in the hippocampus, which is the brain region associated with spatial memory. In the hippocampus, the dentate gyrus granule cells project down the mossy fiber pathway to the CA3, while the pyramidal neurons of the CA3 form the Schaffer collaterals to the CA1 pyramidal neurons. Experimentally, it is possible to study learning and memory by observing the neuronal firing of the CA3 cells through recordings of impulses in postsynaptic CA1 neurons. Depending on the stimulation protocol along this pathway it is possible to elicit an increase in the response of the postsynaptic cells, which is known as long-term potentiation (LTP) or a decrease in the postsynaptic response, which is known as long-term depression (LTD). Studies have been performed showing there are effects of PI3K on the function of these neurons in the brain (Hou & Klann, 2004; Howlett et al, 2008). A cursory explanation of neuronal firing in the hippocampus and a few of the critical receptors involved in synaptic plasticity will help to put previous works in context of the PI3K pathway. In the hippocampus, activation of the CA3 region neurons release glutamate onto the CA1 region neurons and this activates two primary types of ionotropic glutamate receptors among others: *N*-Methyl-D-aspartic acid (NMDA) and 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA). AMPA receptors initially depolarize the membrane upon activation from glutamate, while NMDA receptors initiate synaptic plasticity. In synaptic plasticity, one of the major cellular processes that converts short-term changes to long-term effects is protein synthesis. The PI3K pathway is involved in this regulation of protein synthesis. Studies have shown this correlation, such as those performed by Lingfei Hou and Eric Klann (2004). This group showed that there are increased levels of phosphorylation of Akt at S473, phosphorylation of PDK1 on serine 241, and phosphorylation of mTOR on serine 2448 in the CA1 region of the hippocampus after induction of GPCR metabotropic glutamate receptor (mGluR) a major receptor involved in one form of LTD initiation. Since Akt, PDK1 and mTOR are all activated downstream of PI3K, this shows a relationship between the pathway and LTD. This study experimentally strengthened that relationship by showing that blocking PI3K with two blockers; LY294002, which can act reversibly on the ATP binding site, and wortmannin, which can block substrate binding and the catalytic activity of PI3K, prevented the increased phosphorylation of AKT and mTOR, as well as blocking mGluR-LTD (Hou & Klann, 2004). In whole-cell recordings of CA1 pyramidal neurons, Daw et al, (2002) determined that blocking PI3K did not directly prevent induction of LTD, but did remove synapse specificity in an NMDA and mGluR dependent manner. While these two different conclusions regarding the same relationship between synaptic plasticity and the PI3K pathway seem conflicting they still exhibit a functional relationship between the two albeit via different methods. These types of discrepancies because they are unresolved just further highlight the research still needing to be done in these topics.

In the other major form of synaptic plasticity, LTP, blocking PI3K with wortmannin in perforant path-granule cell synapses of the hippocampal dentate gyrus, prevented the maintenance of LTP (Kelly & Lynch, 2000). Kelly and Lynch (2000) also showed a decrease of glutamate release in the presence of wortmannin. This work strengthened the correlation between PI3K activation and synaptic plasticity. In addition observation that LTP induced at the CA3-CA1 synapses in the hippocampus caused

increased phosphorylation of Akt and p70S6K immediately following potentiation and 30 minutes later (Sanna et al, 2002). This increase in phosphorylation was blocked by LY294002, and if the drug was added 30 minutes after the induction protocol it reduced the established LTP. The novel finding was that wash-out of the drug returned LTP to potentiated levels, suggesting PI3K is required for expression and not maintenance of LTP (Sanna et al, 2002). Importantly if LY294002 was present during the high frequency train of rapidly repeated stimuli or tetanization protocol, but removed 5 minutes after, LTP could be visualized, indicating PI3K activity is not required for LTP induction (Sanna et al, 2002). Lastly Sanna et al, (2002) examined the mechanism of PI3K's role in LTP by blocking the NMDAR component of induction, and determined that LY294002 could also block the L-type voltage-dependent calcium channel component of LTP. These studies show activation of NMDA receptors are a major component of LTP, and PI3K blockers inhibit LTP (Kelly & Lynch, 2000; Sanna et al, 2002). The mechanism of action to look at, is the effect of PI3K on NMDA receptors. In hippocampal slices exposed to NMDA, an increase in phospho-Akt at T308 was completely prevented by treatment with wortmannin (Opazo et al, 2003). This shows a functional correlation between blocking PI3K and affecting LTP, specifically through NMDA receptors. Raymond et al, (2002) concurs that PI3K is involved in synaptic plasticity theorizing that morphological changes could explain the changes in synaptic capabilities. LTP induced in slices decayed back to baseline levels after the addition of LY294002 to block PI3K. This also occurred using rapamycin, the blocker of mTOR, presumably due to downstream effects on p70S6 kinase which plays a role in actin polymerization, and actin dynamics are important for stable LTP (Krucker et al, 2000; Raymond et al, 2002). To summarize, PI3K is not important in LTP induction but is required for maintenance of LTP and it plays a role in both L-type calcium channels and NMDAR.

The other major receptor involved in synaptic plasticity is AMPA and studies have shown that PI3K affects AMPA receptor insertion, as well as its ability to cluster (Arendt et al, 2010; Man et al, 2003). In cultured hippocampal neurons using whole cell recordings, Man et al, (2003) recorded miniature excitatory postsynaptic currents (mEPSCs) that with the pharmacological addition of glycine can produce LTP, or with the application of NMDA can produce LTD. Wortmannin blocked the LTP induced by glycine application, and the addition of LY294002, or wortmannin prevented the increase in cell surface expression of the AMPA receptors that mediated this form of LTP (Man et al, 2003). Also using whole cell recordings in organotypic slice cultures, AMPA synaptic responses were depressed after the addition of a dominant negative protein that bound PIP₃, the end result of PI3K phosphorylation (Arendt et al, 2010).

Long-term potentiation and depression are invoked using different experimental protocols and are used to gain information about neuronal processes in vivo, such as learning or memory. Work has been done to show that PI3K is also important to memory in animals. Retrieval of memories related to specific episodes or contexts requires the hippocampus, and mice trained in contextual fear paradigms had increased levels of phospho-AKT at S473 after being returned to the learning context 24hrs after their last training period (Chen et al, 2005). Bilateral infusion of LY294002 into the CA1 of the hippocampus 15 minutes before the memory retrieval testing significantly decreased the

fear response of these mice (Chen et al, 2005). In object recognition tasks rats, after injection of LY294002, showed a deficit in memory, through reduced novel object and novel location exploration after a 24 hour delay. The injection did not affect exploration after a 10 minute delay (Horwood et al, 2006). This shows that interrupting the PI3K pathway affects consolidation or long-term expression of memories, not the short-term expression of memory, which is in parallel with the studies done in hippocampal slices by Sanna et al, (2002).

This previous work clearly shows the importance of PI3K signaling in synaptic plasticity and memory. Importantly, previous work has shown a variety of results from blocking PI3K itself, and since the phosphorylation from PIP₂ to PIP₃ is the primary action in a series of cellular processes, it would be important to see if other downstream molecules play a role in synaptic plasticity. The next major molecule to examine would be the PI3K effector Akt, since it is activated through binding with PIP₃, the phosphorylated product of PI3K.

Akt

AKT, which is also known as protein kinase B, is a ubiquitously expressed serine/threonine kinase of the AGC superfamily. It has a pleckstrin homology (PH) domain, which is a protein domain of approximately 120 amino acids. This region allows binding to phosphatidylinositol lipids in membranes, for example the PIP₃ which is produced through phosphorylation of PIP₂ by PI3K. There are three isoforms of mammalian Akt; Akt1/PKB α , Akt2/PKB β and Akt3/PKB γ and while there are physiologically different localizations of each isoform they are functionally redundant (Manning & Cantley, 2007; Peifer & Alessi, 2008). Akt1 and Akt2 are ubiquitously expressed throughout the body, and all three isoforms are expressed in the nervous system but Akt3 is the predominate isozyme in tissues of neuroendocrine origin (Li et al, 2009). Akt3 is predominantly expressed in the brain, and mice with this knockout show a normal body size but a smaller brain, due to a reduction in mean cell size (Easton et al, 2005). Akt is known as a proto-oncogene due to its prevention of cytochrome c release and inactivating the transcription factors that lead to apoptosis (Perandones et al, 2004).

AKT is activated by RTKs, and for AKT to be fully activated it must first be translocated to the membrane where it can be phosphorylated. For full activation AKT is phosphorylated on two sites: T308 and S473 (Vasudevan & Garraway, 2010). The T308 site is phosphorylated by PDK1, which stabilizes the activation loop, since this site is located in the protein kinase domain. The S473 site resides in the hydrophobic C-terminal domain, and is phosphorylated by the mTOR complex 2 which consists of the rapamycin insensitive companion of TOR (Rictor) and mLST8 (Dunlop & Tee, 2009; Sarbassov et al, 2005). Phosphorylation at both sites is required for activation of AKT, and its activation phosphorylates the downstream mTOR complex 1, which contains the rapamycin sensitive component. The activation of Akt by PI3K is directly inactivated by Pten and the inactivation is due to a reduction of PIP₃, as Pten reverses the phosphorylation back to PIP₂. *Akt* is also involved in the regulation of cell proliferation,

survival, glucose homeostasis, metabolism, cell migration and invasion, cell growth and protein translation, immunity and angiogenesis, with the majority of these processes occurring through the PI3K pathway (Vasudevan & Garraway, 2010). AKT's role in cell growth and protein translation occurs through the TSC-2 and mTORC1 pathway, since AKT directly phosphorylates TSC2 at multiple sites, which prevents TSC1/TSC2 complex from inhibiting Rheb and therefore activating mTORC1 (Manning & Cantley, 2007). mTORC1 then stimulates protein synthesis by phosphorylating p70S6 kinase and eIF4E binding proteins, increasing translation of mRNA's and promoting cap-dependent translation of mRNA's (Bjornsti & Houghton, 2004). AKT activation is one of the primary methods to detect activation of the PI3K pathway, and deletion of *Pten* results in strong activation of AKT. AKT mutations are also commonly found in tumor models, but models over-expressing AKT are not sufficient in themselves to produce tumors (Vasudevan & Garraway, 2010).

AKT in the hippocampus has been clearly implicated in cell death and aging. Activation of AKT has been shown to protect neurons from ischemia (Fukunaga & Kawano, 2003), as well as neuronal excitotoxicity caused by overactivation of ionotropic glutamate receptors (Kim et al, 2002). Jackson et al, (2009) showed that AKT activity is greater in the CA3 over CA1 region which might partially explain why the CA1 region of the hippocampus is more susceptible to ischemic insult (Alessi et al, 1996; Schmidt-Kastner & Freund, 1991). As mentioned previously, phosphorylation of AKT during induction of LTP occurs on the serine site instead of the threonine. This transient activation causes prolonged activation on target sites of mTOR and the transcription factor Forkhead (FKHR), which is also a AKT target protein (Horwood et al, 2006). Downstream activation during the LTP induction protocol appears to be through AKT related mechanisms, as shown through blocking with the PI3K blocker LY294002 affects LTP expression. This does not affect the phosphorylation of ERK1/2 in the MAPK/ERK pathway which is a pathway not involving AKT that has also been shown to be activated by LTP (Davis et al, 2000; Lin et al, 2001). AKT is phosphorylated by both PDK1 and mTOR so it would follow that changes involving these would also affect synaptic plasticity.

Pdk1

Since PDK1 is one of the two necessary activators of AKT through phosphorylation at T308, I would like to address its role in this pathway. PDK1 is a serine/threonine kinase, belonging to the super-family of AGC kinases which were named due to their conserved homology among protein kinase A (PKA), cGMP-dependent protein kinase (PKG) and protein kinase C (PKC), which have conserved catalytic kinase domain (Bayascas, 2008; Pearce et al, 2010). PDK1 has only one isoform, which is an important benefit to its study. In addition, it is a “master regulator” of approximately 23 protein kinases (PK) in this same AGC super-family including AKT, S6K and PKC (Peifer & Alessi, 2008). PDK1 catalyses the activation loop and activates the major T-loop kinase of the PI3K-regulated members of the AGC family (Mora et al, 2004). It is a ubiquitously expressed enzyme that is constitutively active, but can be

induced to a higher level of activation through extracellular stimulation, and is mainly cytosolic in unstimulated cells and translocated to the plasma membrane upon stimulation (Vanhaesebroeck & Alessi, 2000). Among those protein kinases, PDK1 regulates by activation, is AKT, which is phosphorylated on the T-loop at the T308 that becomes exposed as AKT binds to PIP₃ (Alessi et al, 1996; Stephens et al, 1998). PDK1 also phosphorylates or activates p70S6K, p90 ribosomal S6 kinase, serum and glucocorticoid-induced protein kinase, and several PKC isoforms (Bayascas, 2008). Like AKT, PDK1 has a PIP₃ binding PH domain, and also colocalizes to the membrane with AKT and binds to PIP₃. Phosphorylation of its hydrophobic motif is required for PDK1 to phosphorylate any non-AKT AGC kinases, while the AGC kinases interact with a binding groove located in the catalytic domain (Bayascas, 2008).

PDK1 knockout mice are embryonic lethal, but mice containing a point mutation in the PH domain mice show a similar phenotype to AKT1-null mice as well as AKT2/3 double knockouts. These mice have glucose and insulin intolerance and reduced organ and body size with smaller brain and testis (Bayascas, 2008). The smaller brain size is also true in conditional knockout mice with a *PDK1* deletion in neurons and glia throughout the brain, and almost complete deletion in the hippocampus (Chalhoub et al, 2009). The important role of PDK1 in these studies is in its ability to suppress the tumorigenesis of heterozygous *Pten* mice (Bayascas et al, 2005). *Pten* heterozygous mice are known to develop a range of tumors, including lymphoma, endometrial carcinoma, prostate carcinoma, breast adenocarcinoma and testicular tumors, all of which are markedly reduced by the additional genetic cross with hypomorphic *PDK1* mice (Bayascas et al, 2005).

Protein kinase C is involved in LTP, and has little or no activity unless it is phosphorylated in the activation loop of its catalytic domain by PDK1 (Newton, 2003). In LTP, the atypical protein kinase C isoform protein kinase M ζ is the only protein kinase shown to be critical for the maintenance of LTP and spatial memory in hippocampus (Ling et al, 2002; Sacktor, 2011). Kelly et al, (2007) showed that PDK1 constitutively activates PKM ζ , allowing for maximum phosphorylation and activation and the unique role this protein kinase plays in maintaining memory storage.

Pten

While PI3K is a major initiator of all the aforementioned downstream activations and mechanisms, the major regulator of this pathway is the phosphatase and tensin homolog deleted on chromosome 10, also known as PTEN. PTEN is a lipid phosphatase, most often studied for its role in suppressing tumor formation. The *PTEN* gene encodes a polypeptide with 403 amino acids, and shows homology to protein tyrosine phosphatases. These dephosphorylate tyrosine residues, and tensin, which is a focal adhesion protein (Li & Sun, 1997). Amino acids 1-185 make up the N-terminal phosphatase domain while amino acids 186-403 make-up the C-terminal domain where the C2 domain is found, (the region associated with phospholipid-binding regions) (Lee et al, 1999; Waite & Eng, 2002). The common structural domain PDZ-binding motif (Postsynaptic density protein

(PSD95), Drosophila disc large tumor suppressor (DlgA), and Zonula occludens-1 protein) is also found on the C-terminus, which is significant for protein-protein interactions. Approximately 43% of clinical mutations are found in the C-terminus region, with the majority found in the N-terminal domain. In the last 50-amino acids on the C-terminal tail there are several phosphorylation sites, which are important for protein stability (Waite & Eng, 2002). In vitro, PTEN has protein tyrosine activity by dephosphorylating phosphorylated tyrosine, as well as phosphorylated serine and threonine synthetic substrates, and its phosphatase activity is required for its function as a tumor suppressor (Myers et al, 1998). The primary function of PTEN is to dephosphorylate the plasma membrane lipid, second messenger PIP₃ back to PIP₂, determined by PTEN's preference for highly acidic substrates over tyrosine and serine/threonine phosphoproteins (Maehama & Dixon, 1998). PTEN therefore is predominantly located in the plasma membrane, but also functions in the nucleus (Liu et al, 2005). Investigation into *PTEN*'s role in tumor suppression concluded that out of 123 various brain tumors, 13 out of 42 adult glioblastomas, and 3 out of 13 anaplastic astrocytomas contained mutations in *PTEN* (Rasheed et al, 1997). Out of 32 primary endometrial cancers, half were shown to have mutations in *PTEN* (Tashiro et al, 1997) and in three prostate cancer cell lines as well as 23 out of 80 prostate tumors, *PTEN* mutations were present (Cairns et al, 1997).

The main focus for *PTEN* studies are frequently on the role it plays in cancer, but PTEN is also involved in cell growth (Furnari et al, 1997), extracellular matrix interactions, and cell migration (Tamura et al, 1998). PTEN has been shown to regulate the MAPK pathway through dephosphorylation of Shc, which downstream down-regulates MAPK, or by modulating Gab1's movement to the plasma membrane independent of Shc; both actions indicate a role in insulin signaling (Waite & Eng, 2002). Germline mutations of *PTEN* have also been found in patients with a rare autosomal dominant syndrome, Cowden disease, that causes benign tumors called hamartomas in skin, breast, thyroid and intestinal tissue, with an increased risk of cancer in breast, thyroid and brain (Liaw et al, 1997; Marsh et al, 1998). Bannayan-Riley-Ruvalcaba syndrome, which is a related hamartoma syndrome, is classified by macrocephaly, lipomatosis, hemangiomas and speckled penis, and has also been correlated to germline *PTEN* mutations (Eng, 2003; Marsh et al, 1997). Another hamartoma syndrome that has been associated with germline mutations in *PTEN* is Lhermitte-Duclos disease. It has been associated with Cowden syndrome, and is classified by dysplastic gangliocytoma of the cerebellum, which is attributed to hamartomatous outgrowth of hypertrophic ganglion cells causing ataxia and seizures (Zhou et al, 2003). Germline mutations of *PTEN* are also correlated with proteus and proteus-like syndromes (Eng, 2002). A germline *PTEN* deletion was found in 3 individuals out of 18 autistic subjects in a study by Butler et al, (2005), with another three showing other *PTEN* mutations and all showing macrocephaly. Mouse models manipulating *Pten* would be an important tool to use in figuring out the role *Pten* plays in cellular physiology.

Mice with homozygous deletions for *Pten* are lethal before embryonic day 7.5, but heterozygous mice are viable with multiple features of the human diseases associated with *PTEN* mutation (Di Cristofano et al, 1998). In these mice, intestinal polyps were

observed, as well as hyperplastic changes in cell layers of the epidermis, lymph node hyperplasia, and enlarged areas of the prostate with an increase in proliferating cells and multiple malignant tumors (Di Cristofano et al, 1998; Podsypanina et al, 1999). Heterozygous deletion of the phosphatase domain only caused 14% of mice to develop spontaneous tumors, which was increased to 19% after radiation-induced carcinogenesis (Suzuki et al, 1998). In *Pten* heterozygous mice that formed complex atypical hyperplasia lesions in uteri, lesion sizes could be reduced with rapamycin injections, and the tissue showed a reduction in the typically elevated S6K activity, a downstream translational regulator (Podsypanina et al, 2001). These effects of deletion, however, don't show any neurological changes like those seen in many of these human disorders, so transgenic mice were created with targeted deletion using *Cre-loxP* techniques in the central nervous system (Kwon et al, 2001; Kwon et al, 2006). When the phosphatase motif of *Pten* is deleted in granule neurons of the cerebellum and dentate gyrus, with some deletion throughout the hippocampus and cerebral cortex, these mice developed tonic-clonic seizures, with their brains showing a progressive increase in mass until their death at somewhere between 9 and 48 weeks (Kwon et al, 2001). These mice also exhibited hydrocephaly, and cellular migration during development was disrupted in the cerebellum. Since *Pten* is the negative regulator of PIP₃ creation, an expected upregulation of Akt phosphorylation occurs when it is deleted (Kwon et al, 2001; Kwon et al, 2006). The *Pten* deletion in these mice caused an obvious cell-autonomous enlargement of neurons in the dentate gyrus. Backman et al, (2001) used the same *Gfap-cre* mouse line to activate the deletion of exons 4 and 5 like Kwon et al, (2001), and also reported an approximately twofold increase in neuronal cell size. Additionally there was a disruption of the cell body layer in the dentate gyrus, and a reduction in pyramidal cell density throughout the CA regions. These increases in cell size may be through protein translation as suggested and reviewed by Backman et al, (2002).

Since deletion of *PTEN* in the CNS causes neuronal changes, what role does *PTEN* play in normal brain functions? A fragment of the catalytic domain of *Pten* can be detected in neuronal dendrites of cerebral cortex, cerebellum, hippocampus and olfactory bulb using antibodies to the amino terminal of the protein (Perandones et al, 2004). This fragment was also found in protein fractions isolating the synapse, and a mouse model with impaired synaptogenesis showed reduced expression of the 34 kDa *Pten* fragment, and increased expression of phospho-AKT. This group also used microscopic analysis of immunolabeling and determined that *Pten* was in dendritic shafts and spines (Perandones et al, 2004). Temporal examination of *PTEN* showed variation over the development of these neurons, with the highest variability in the CA3 of the hippocampus and increased labeling in dendritic shafts paralleling synaptic stabilization (Perandones et al, 2004). In dopaminergic neurons, deletion of *PTEN* caused increased neuronal soma size, increased cell proliferation, inhibited apoptosis and thickened axon and dendritic projections in the ventral tegmental area (VTA), and substantia nigra pars compacta (Diaz-Ruiz et al, 2009). Since a hallmark of these neurons is pruning through developmental cell death, and *PTEN* ablation prevents the apoptotic pathway activated by Akt, this would account for increased density. Dopaminergic neurons of the VTA have been implicated in the reward system for addictive drugs, and they are modulated by serotonergic innervations such as the phosphorylation of GPCR 5-HT_{2c}. Maillet et al, (2008) showed that the

protein phosphorylation action of PTEN, but not its lipid phosphatase, regulated 5-HT_{2c}R in vitro. In vivo, interrupting this action suppressed the increase in VTA neuronal firing that was produced by addition of tetrahydrocannabinol, the psychoactive ingredient in marijuana (Maillet et al, 2008). Increased PI3K/Akt pathway signaling leads to increased axon growth, leading to the question of what role PTEN plays in that process. In cultured sensory and dorsal root ganglion cells, PTEN phosphatase inhibitor increased neurite outgrowth, even after preconditioned nerve injury which already shows initiation of robust axon growth (Christie et al, 2010). This effect was also shown to be independent of mTOR by still produced accelerated neurite outgrowth after rapamycin addition. In mice with a nerve trunk transection, pharmacological and small interfering RNAs (siRNA) decreases in PTEN caused the increase in the number and length of outgrowing axons, showing its role in the suppression of regeneration (Christie et al, 2010).

Mice with conditional deletion triggered by the *neuron-specific enolase* (*Nse*) promoter, had deletions in layers III and V of the cerebral cortex and in the hippocampus, the CA3, and the granular and polymorphic layers of the dentate gyrus. These mice after deletion of *Pten* exhibited macrocephaly, and behavioral tests examining social interaction showed the mutant mice to examine a juvenile they had already been exposed to with the same amount of interaction as a novel subject (Kwon et al, 2006). These mice also had reduced nest forming, which indicated abnormal home cage behavior, and they interacted with an inanimate object over a social target, in contrast to control mice that interacted more with the caged adult mouse. Control mice prefer interaction with new over familiar mice, and interact more with the novel mouse, while the mutant mice did not prefer the novel mouse and interacted less with it (Kwon et al, 2006). So not only does the *Pten* deletion cause morphological changes in these mouse models, it also causes functional deficits, and in this case, social interactions that could be correlated to autistic symptoms. Detrimental phenotypes in *Nse-cre* mice such as macrocephaly, granule cell and cortical neuron hypertrophy, dentate gyrus enlargement and impaired social behavior have been reversed through addition of CCI-779 (a rapamycin ester) in vivo (Zhou et al, 2009). The hypertrophy of the dentate gyrus and seizure frequency seen in *Gfap-cre* mice was also rescued by treatment with CCI-779 (Kwon et al, 2003), showing the alteration in growth signaling by PTEN deletion, and is at least partially enacted through downstream mTOR effects.

Another *Gfap-cre* line showed ultrastructural changes, including enlarged mitochondria, increased ribosome density, enlarged presynaptic terminals with increased numbers of vesicles, 60% of which were missing a postsynaptic density (Fraser et al, 2008). The *Pten* knockout mice also had abnormally shaped dendritic spines, and tests of synaptic function showed huge decreases in synaptic transmission across a range of stimulation intensities. Measures of LTP induced with a 200Hz stimulation intensity also showed deficits in this mouse model (Fraser et al, 2008). Wang et al, (2006) didn't see a difference in synaptic input/output curves in heterozygous mice, but they did see a significant reduction in LTP and no LTD could be produced along hippocampal Schaffer collaterals. It has been shown that PTEN plays a role in synaptic plasticity, posing the question of through what mechanisms.

Since NMDA receptors play a major role in synaptic plasticity, it would be of interest to see if PTEN plays a role in regulating them. Ning et al, (2004) found through coimmunoprecipitation studies, that PTEN associated with the NR1 and NR2B subunits of NMDA receptors in hippocampal tissue, but not with the NR2A subunit or the GluR1 subunit of AMPA receptors. In cultured hippocampal neurons, NMDAR-mediated whole cell currents had significantly lower peak amplitude after PTEN siRNA suppression, and, in fact, PTEN suppression reduced the open probability of NMDA channels, as well as the number of functional NMDA channels on the membrane surface (Ning et al, 2004). AMPA receptors are also critical for synaptic plasticity, and have been shown to be regulated by hormones such as leptin, which produces an increase in GluR1 subunit surface expression. NMDA receptor activation also triggers the interaction of PTEN and the postsynaptic density-95 (PSD-95), which is a regulator of synaptic plasticity and function, through the PDZ-domain on PTEN. This recruits and anchors PTEN to the postsynaptic membrane after NMDAR activation in synaptosomes, and PTEN overexpression leads to depression of AMPA responses (Jurado et al, 2010). In fact inhibition of PTEN by the blocker bis-peroxovanadium derivative (bpV(HO)pic) reduced NMDA-dependent, but not mGluR dependent, LTD in hippocampal slices. Further testing indicated the lipid phosphatase action of PTEN was required for LTD (Jurado et al, 2010). This provides ample evidence that deletion of PTEN causes functional and morphological changes to hippocampal neurons, affecting their plasticity. As mentioned above, since the deleterious effects of PTEN deletion can be reversed by the addition of rapamycin, an mTORC1 blocker, it would follow that this downstream part of the pathway plays a role in synaptic plasticity.

mTor

Mammalian target of rapamycin, mTOR, is a 289-kDa serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription, after activation mainly by insulin and growth factors. It was first discovered through studies on the macrolide antibiotic rapamycin, hence its name, which was used as an antifungal and immune suppressor (Bjornsti & Houghton, 2004). In mammalian cells TOR controls translation initiation through two separate mechanisms. The first is activating ribosomal S6K1 to increase translation of mRNAs, which involves the use of four different domains and at least seven sites for activation (Bjornsti & Houghton, 2004). The mRNAs it activates have a 5' terminal oligopyrimidine tract (TOP) and they encode components of the translational apparatus such as ribosomal proteins and elongation factors eEF1A, eEF2 and the poly-A-binding proteins. The second action of TOR is the phosphorylation and inactivation of 4E-BP1 suppressor proteins which then dissociate from RNA cap binding protein eIF4E with the end result of mRNA translation initiation (Bjornsti & Houghton, 2004). mTOR also has significant amino acid homology to PI3 kinase and is found in two cellular complexes; mTORC1 and mTORC2 (Dunlop & Tee, 2009). mTORC1 is a heterotrimeric protein kinase that consists of the mTOR catalytic subunit, mLST8 and the regulatory-associated protein of mTOR (raptor). The mTORC2 replaces raptor with rictor, rapamycin-insensitive companion of mTOR and adds mitogen-activated-protein-kinase-associated protein 1 (mSin1) (Sabatini, 2006).

Work has been done to determine the role of mTOR in synaptic plasticity. Tsokas et al, (2005) found that rapamycin inhibits LTP if applied before the high frequency stimulus used to induce LTP and blocked the phosphorylation of p70S6K. They also saw an increase in eIF1A and phosphor-p70S6K in dendrites after high frequency stimulation (HFS). This same group went on to show that this upregulation of translational machinery was found throughout the apical dendrites, not just the activated synapses. Also they found that this process is regulated by ERK relatively upstream, through action on PDK1, and that PDK1 interacts with 90 kDa ribosomal S6 kinase (RSK) and there is suggested regulation of mTOR by this complex (Tsokas et al, 2007). They found that ERK is in fact required for the HFS induced stimulation of the mTOR pathway in hippocampus. As mentioned above treatment with rapamycin in Pten-null neurons can reverse neuronal hypertrophy, as well as macrocephaly, and can reduce the increased anxiety and impaired social behaviors seen in Pten mutant mice (Kwon et al, 2003; Zhou et al, 2009). Therefore the mTOR complexes play a role in the regulation of the plasticity effects seen by manipulating the PI3K pathway. This concludes the summery of applicable research that lead to our investigation into the specific mechanisms of PTEN deletion and its role in synaptic plasticity. While the basic concepts for this hippocampal synaptic plasticity have been briefly mentioned up till this point, in the next section I would like to elaborate and give the background necessary to fully understand these processes.

Synaptic Plasticity

As addressed in the previous section, multiple molecules in the PI3 kinase pathway have been implicated in synaptic plasticity in the hippocampus. Next I will address the fundamentals of synaptic plasticity and what other mechanisms and molecules are involved. The chemical communication that occurs in the brain involves multiple steps, starting with the release of neurotransmitters from vesicles in the presynaptic neuron. The fusion of these distinct packets allows the interaction of neurotransmitters and postsynaptic receptors. The electrical output produced by these postsynaptic neurons allows us to detect the changes that may occur during this process. Presynaptic changes in neurotransmitter release can also affect the response of the postsynaptic cell, and in concordance, the postsynaptic cell is also modified. This relationship was articulated in 1949 by the psychologist Donald Hebb. The key statement from Hebb's postulate is as follows, "When an axon of Cell A is near enough to excite a Cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased." (Hebb, 1949). While non-Hebbian learning also exists, this theory illustrates one of the basic building blocks behind synaptic plasticity.

Hippocampus

Long-term potentiation (LTP) and long-term depression (LTD) were discovered in the hippocampal formation, and it is the primary brain region used to study synaptic plasticity. In the hippocampus the neuronal pathways contain dense packing of cell

bodies and roughly parallel position of the apical dendrites. This makes for easy synchronous activation, and the highly plastic synapses allow for analysis of the pre and postsynaptic cells. Since the hippocampus is the primary brain region for my experiments, I would like to address some of the intrinsic properties of this area.

The hippocampal formation consists of the dentate gyrus, the hippocampus proper, subiculum, presubiculum, parasubiculum and the entorhinal cortex. Within these collective structures there are two principal cell types; the pyramidal cells and the granule cells. There is a single cell layer that forms organized laminar inputs, with principally unidirectional connections between a series of cortical regions such as the entorhinal cortex receiving inputs from the olfactory bulb. Of the pathways within the hippocampal structure, the layer II neurons of the entorhinal cortex project down the perforant pathway to the dentate gyrus and the CA3 (cornu ammonis) region. The dentate gyrus granule cells project down the mossy fiber pathway to the CA3, while the pyramidal neurons of the CA3 form the Schaffer collaterals to the CA1 pyramidal neurons (Amaral & Witter, 1989). The Schaffer collateral pathway is the region in which I study using our field recordings. This region gives prominent field potentials that can be used as an index of presynaptic activation and the efficacy of postsynaptically translated currents. The CA1 pyramidal neurons project back to the subiculum and the entorhinal cortex, and lastly the subiculum also projects back to the entorhinal cortex along with the CA1 neurons (**Figure 1-2**). While all these regions interact during synaptic activation the two components of the formation that are most relevant are the dentate gyrus and the hippocampus proper (Andersen, 2007).

The dentate gyrus is a V-like structure, with the superior branch between the CA1 and CA3 regions of the hippocampus referred to as the suprapyramidal blade, and the other branch called the infrapyramidal blade. The dentate gyrus is broken down into three layers: the molecular layer, the principal or granule cell layer, and the polymorphic cell layer. The principal cell type of the dentate gyrus, the granule cell, has elliptical cell bodies that are approximately 10 μ m wide and 18 μ m high, and the dendrites are spiny and form cone shaped trees that project to the molecular layer of the dentate (Claiborne et al, 1986). They reside in the granule cell layer, and this is the only cell type that has axons that innervate any other regions of the hippocampal formation. The molecular layer of the dentate gyrus is the closest to the hippocampus, and contains few cells, mostly the dendritic projections from the granule cell layer, basket and polymorphic cells, as well as the terminal axonal arbors of the entorhinal cortex (Andersen, 2007). Lastly the polymorphic cell layer has one prominent cell type, the mossy cells. They have large (25-35 μ m) triangular somas and dendrites that don't enter the CA3, but project to the inner third of the molecular layer (Buckmaster et al, 1992). The proximal dendrites in this region are covered by large complex spines called thorny excrescences, which are immunoreactive for glutamate. The dentate gyrus does not project to any other brain region besides the CA3, and the projections from the mossy fibers arise exclusively from the granule cells (Claiborne et al, 1986). They terminate just above the CA3 pyramidal layer. There are approximately five types of interneurons in the dentate gyrus: pyramidal basket cells, axo-axonic or chandelier cells, HICAP (hilar commissural-associational pathway-related cells), MOPP (molecular layer perforant path-associated cells) and HIP

Figure 1-2 Schematic of the hippocampus.

Abbreviations: CA1 (cornu ammonis); CA3; pcl (pyramidal cell layer); sl-m (stratum lacunosum-moleculare); s-o (stratum oriens); sr (stratum radiatum).

(hilar perforant path-associated cells). The MOPP cells are the only interneurons whose cell bodies are found in the molecular layer, the HIPP, HICAP, and basket cells reside in the polymorphic cell layer while only the axo-axonic cells are in the granule cell layer (Freund & Buzsaki, 1996; Lacaille et al, 1987).

In the hippocampus proper there are five different strata, which are important to our studies because previous work showed Pten deletion disrupted neuronal migration of pyramidal neurons. These different strata, which are created after completion of neuronal mitosis, are important for forming correct cellular connections and to the functioning of this region of the brain. The first stratum is the stratum oriens, which contains the basal dendrites of the pyramidal cells and interneurons. Superior to the stratum oriens is the pyramidal cell layer of the hippocampus, which projects basal dendrites into the stratum oriens, and the apical dendrite tree extends to the hippocampal fissure (Andersen, 2007). The pyramidal cell bodies form the previously mentioned Schaffer collaterals, and the CA3 projects to no other region besides the CA1, once again making it optimal to study pre and postsynaptic stimulations (Amaral & Witter, 1989). Since previous studies using targeted Pten deletions have shown enlargement of neurons (Kwon et al, 2001; Kwon et al, 2006) it is important to record their basic morphology. In the pyramidal layer, the diameters of the CA2/CA3 cell bodies ranges from 20-30 μ m and the total dendritic length can go from 8-18mm. In the CA1 the combined length of all dendritic branches is approximately 13.5 mm and the soma size is 15 μ m in diameter (Ishizuka et al, 1995). This natural variation is particularly important to note as my work is looking for significant changes in neuronal size. The following layer is the stratum lucidum, which is only found in the CA3 region, and not in the CA1/CA2 region, and contains the mossy fibers between the pyramidal cell layer and the stratum radiatum. The s. radiatum is where the CA3-CA3 associational contacts are formed, as well as the CA3-CA1 Schaffer collaterals. The CA1 region is heavily innervated by the CA3 (Amaral & Witter, 1989). Next is the stratum lacunosum-moleculare, which is the layer where the entorhinal cortex fibers terminate. This layer has a lower density of excitatory synapses than the stratum radiatum and oriens. These excitatory neurons are partially regulated by the inhibitory interneurons. Within the hippocampus proper there are six types of interneurons, including basket cells and axo-axonic or chandelier cells, which are found in the pyramidal cell layer along with the bistratified cells and the radial trilaminar cells (Freund & Buzsaki, 1996). The basket, bistratified, and radial trilaminar cells are also found in the stratum radiatum, and the remaining two, horizontal trilaminar cells and the O-LM (oriens lacunosum-moleculare associated cells) are found in the stratum oriens. The basket interneurons are aspiny, and each pyramidal cell only synapses on a basket cell once, while each basket cell innervates 1000-plus pyramidal cells. The axo-axonic or chandelier cells are very similar to those found in the dentate gyrus, and are predominantly GABAergic inhibitory connections (Freund & Buzsaki, 1996).

One of the major connective points between neurons within the hippocampus, and in fact throughout the brain, is formed on dendritic spines. As an important structural and functional component of postsynaptic neurons, whole books have been written on spines (Yuste, 2010). In the mature nervous system it has been found that spines receive most excitatory inputs, and almost all spines have an excitatory synapse on their bulbous end

(Arellano et al, 2007). This follows that each spine corresponds to an excitatory synapse, and CA1 pyramidal neurons have approximately 30,000 dendritic spines. Each spine contains a spine neck that extends off the dendritic shaft and terminates in a spine head that contains a postsynaptic density (PSD). It is also important to record basic spine morphology as the changes in neuronal size after Pten deletion have been shown to affect subcellular morphology (Fraser et al, 2008). Dendritic spines are typically less than 3µm in length, and the head and the diameter are approximately 0.5-1.5 µm in diameter (Yuste, 2010). The most typical classification of spine shapes containing three essential types: thin, stubby, and mushroom. Thin spines are the most common and typically found in adult tissue, as are mushroom spines. While stubby spines, which contain no neck, are more outstanding in early post-natal development, filapodia are long thin spines without a clear head that generally appear after synaptogenesis (Fiala et al, 1998). These important distinctions allowed me to identify changes in the spines after Pten deletion in my studies. The excitatory spines are characterized and identified by the presence of a PSD which can be visualized because it is electron dense, as well as a comparatively wide synaptic cleft. This aligns with a thinner presynaptic density, where vesicles can be seen within an “active zone”, which is the region closest to the synaptic cleft. This set of criteria came from seminal electron microscope studies by E.G. Gray (Gray, 1959a; 1959b), and remains the classification for identifying active synapses that I used in my studies.

The vast majority of spines have a synaptic contact, and only one head, but a subset of spines in dentate gyrus and CA1 pyramidal neurons have branched heads which I also observed under electron microscopy (Harris et al, 1992). Mushroom spines appear to have more complex PSDs. In the larger spines it been found there are a greater the amount of structural components which was something to look for in Pten null neurons. One of the common internal structures is polyribosomes, which are typically found at the base of spines, but also in the head and neck, indicating that spines are capable of local protein synthesis (Yuste, 2010). Another common structure is the smooth endoplasmic reticulum, or SER, and this is found in about half of all hippocampal spines. The SER can contain vesicles, tubules, or a series of cisternae and dense plates that form a “spine apparatus” (Gray, 1959a). Since any functional changes might be due to subcellular differences in these components I also looked for different internal components and changes in these protein synthesizing machineries under electron microscopy.

Synaptic Transmission

The hippocampus has been extensively characterized in terms of its morphology, as mentioned above, but it has also been extensively characterized in terms of its basic neurophysiology. It is important to note the specific properties of these pyramidal neurons since they have been well studied and this information would allow us to compare any differences found in our mouse model. Upon activation, neurons within the brain create an action potential, generated near the cell body that travels down the length of the axon. As this change in membrane potential travels down the cell it opens ion channels, allowing an influx of Ca^{2+} . This triggers the exocytosis of vesicles which are

round organelles approximately 20nm in radius that store neurotransmitters. This release of vesicles does not reliably occur with every action potential and this relationship is modulated by intracellular messengers, extracellular modulators and the activity pattern of the synapse (Sudhof, 2004). There is an ongoing debate about the mechanism of release of the neurotransmitters from the vesicles. Ceccarelli et al, (1973) proposed that the vesicles only briefly touch on the membrane and open a fusion pore releasing neurotransmitter before closing and remaining in the presynaptic terminal, which is called the “kiss and run method”. The other theory considers that the vesicles fuse and dilate completely so as to collapse into the membrane, which is known as the full fusion method (Heuser & Reese, 1973). The recycling pool of vesicles isn’t all the presynaptic vesicles but just those that turn over with activation. It can be divided into the reserve pool which requires higher frequency stimulation to induce release, and the readily releasable pool (RRP), which are vesicles available for immediate release at the introduction of Ca^{2+} , and are therefore docked onto the presynaptic membrane (Schikorski & Stevens, 2001). Vanden Berge and Klingauf showed that these different pools can in fact be activated by different methods of excitation experimentally through loading the vesicles in hippocampal neuronal cultures with FM dyes, and using confocal microscopy to visualize their unloading (Vanden Berghe & Klingauf, 2006). Their study showed low frequency stimulation released those vesicles with faster kinetics, while high frequency stimulation endocytosed a different pool of vesicles that were then placed at the back of the release queue. Two components of vesicle fusion affect plasticity; and the amount of Ca^{2+} allowed into the cell, based on an action potential, and the effect this Ca^{2+} concentration has on the probability of release (Sudhof, 2004). One of the important components I looked at in our studies was the effect of residual Ca^{2+} on repetitive action potentials. If performed in rapid succession this shows a cursory measure of presynaptic function, specifically focusing on the number of release ready vesicles and their sensitivity to Ca^{2+} and is known as paired-pulse facilitation (PPF) (Zucker & Regehr, 2002). These measures could show functional differences created through manipulation of the PI3K pathway.

In the hippocampus the primary excitatory neurotransmitter released is glutamate, which diffuses across the synaptic cleft to bind receptors located on the postsynaptic neurons. There are two classes of glutamate receptors in the hippocampus; ionotropic and metabotropic receptors. Ionotropic glutamate receptors are ligand-gated ion channels, meaning the binding of glutamate allows the channel to open and ions then flow down their chemical and electrical gradients (Andersen, 2007). The flow of ions through these receptors creates the excitatory postsynaptic current (EPSC), which as it depolarizes the postsynaptic membrane it creates an electrical potential or EPSP (Andersen, 2007). If enough of these currents build, it can create depolarize the cell until it reaches threshold and allows an action potential to be produced in the postsynaptic cell. The metabotropic glutamate receptors (mGluRs) actually dissociate G-proteins and trigger intracellular signaling cascades, like the PI3 kinase pathway, after binding with glutamate. There are three groups of mGluRs, with group I receptors (mGluR1 and 5) being located postsynaptically and coupled to phospholipase C, and group II (mGluR2 and 3) and group III (mGluR4,6,7,8) being found presynaptically and negatively coupled to adenylyl cyclase. While the group I receptors may respond to long periods of glutamate exposure

and group II receptors detect extracellular glutamate build-up, the overall mechanism of glutamate activation of mGluRs in hippocampus is less defined but work has shown it plays a role in LTD (Stanton et al, 1991).

Ionotropic glutamate receptors are grouped into three types; 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA), *N*-Methyl-D-aspartic acid (NMDA) and kainite receptors, and are named for the agonists that activate them. These receptors are heteromultimeric and made of four pore-forming subunits. Subunits GluR1-4 form AMPA receptors, GluR5-7 and KA1,2 subunits form kainite receptors and NR1 and NR2A-D subunits form the NMDA receptors (Andersen, 2007). The EPSCs at most hippocampal synapses are mediated by AMPA and NMDA receptors and these have been far more extensively classified in their role of synaptic function and plasticity than the kainite receptors. AMPA receptors are involved in fast glutamatergic transmission and in CA1/CA2 pyramidal neurons expression of GluR1-3 subunits is high in both versions of their splice variants, flip and flop (Wenthold et al, 1996). These receptors can form complexes composed of variations of these subunits, and the insertion, removal and changes in these complexes is a major component of synaptic plasticity. The GluR2 subunit plays a critical role in the ion channel properties of these receptors, such as low Ca^{2+} permeability (Hollmann et al, 1991).

The major difference between the AMPAR and NMDARs is that NMDARs have a Mg^{2+} ion blocking the ion pore at resting membrane potentials and at slight membranes depolarizations (Mayer et al, 1984; Nowak et al, 1984). The depolarization of neuronal membranes due to ions passing through open AMPA receptors allows the release of the Mg^{2+} block in the ion pore. The NMDA receptors are composed of two NR1 subunits, with one or more NR2A-D subunits contributing to the changes in the receptors kinetics. The NR1 subunit is the essential subunit for receptor formation. It doesn't actually bind glutamate, but instead binds a co-agonist such as glycine or D-serine, while the NR2 subunits bind glutamate. The NR2 subunits are the component that modulates open time, conductance and the removal of Mg^{2+} (McBain & Mayer, 1994). Work has been done showing that in striatal neurons, activation of NMDA receptors can activate both the MAP kinase pathway and the Akt pathway through a Ca^{2+} dependent mechanism (Perkinton et al, 2002). This shows a correlation between these major receptors in synaptic plasticity, and the PI3K pathway.

The primary inhibitory neurotransmitter in the hippocampus is GABA, which can activate ionotropic GABA_A receptors or metabotropic GABA_B and GABA_C receptors. The GABA_A receptors are heteropentameric with subunits from seven different families, while the GABA_B are heterodimers with only two types of subunits (Andersen, 2007). The A subtype allow ion conductance of Cl^- and HCO_3^- and are found opposite the release sites of GABA on the postsynaptic neuron. The GABA_B are G-protein-gated and on the presynaptic side are negatively coupled to Ca^{2+} channels, while on the postsynaptic side are inward rectifying K^+ channels (Andersen, 2007). This neurotransmitter plays a modulatory role in the hippocampal system I studied, but the direct role of GABA was not something I looked into.

This gives a very brief overview of the neurotransmitters and their primary receptors in the hippocampus and next I would briefly like to address how they interact to determine the basic properties of the pyramidal neurons. The resting potential of hippocampal pyramidal neurons is from -60 to -70mV, and they have an action potential threshold with a range of -40 to -50 mV, where the resulting action potential is 100mV. Each action potential is followed by four after-potentials: the fast after-hyperpolarization (AHP), the after-depolarization (ADP), the medium AHP and the slow AHP (Storm, 1989). The fast AHP is produced by a fast K^+ conductance, which is voltage-gated, and Ca^{2+} currents such as the BK or I_c . The slow AHP is produced by slow Ca^{2+} -activated K^+ conductance such as the I_{AHP} or SK . The medium AHP is produced through K^+ conductance reduced by muscarinic receptor activation I_M and lastly the ADP is partially produced by R-type Ca^{2+} channels (Andersen, 2007; Storm, 1990). A half width of 1ms is typical for an action potential from a CA1 pyramidal neuron. Using depolarizing currents, multiple action potential firing results in spike-frequency accommodation, where the frequency of firing decreases over a long current injection (Madison & Nicoll, 1984). This phenomenon is due to activation of K^+ conductances such I_M or I_{AHP} , since they do not inactivate and deactivate slowly, and therefore need more time to repolarize back to threshold for the next spike (Lancaster & Nicoll, 1987). These basic firing properties briefly touch on the vast amount of work done on channel conductances and electrophysiological classifications of hippocampal neurons but the predominant interest for my studies are the processes that go into synaptic plasticity.

Long-term Potentiation

Synaptic plasticity, or the effect of persistent activity driven changes in synaptic efficacy, is exemplified by long-term potentiation or LTP. LTP is the ability of repeated firing of a presynaptic cell to cause long-lasting increases in the magnitude of postsynaptic cell output. This creates an overall increase in synaptic communication. This experimental phenomenon was first exhibited in 1970 and 1973 in articles by Timothy Bliss (Bliss & Gardner-Medwin, 1973; Bliss & Lomo, 1970; Bliss & Lomo, 1973), both in the hippocampus of rabbits. One preparation showed an approximately 50% increase in amplitude that lasted for 10 hours and the other showed increases up to 16 weeks. These studies caused interest because of the possibility that these processes underlie learning and memory. Since then, this field of study has exponentially multiplied. One of the questions still studied today is whether the theory still holds that this in vitro process is a model for learning and memory in vivo.

This LTP was reproduced in the CA1 region of hippocampal slices in 1975 (Schwartzkroin & Wester, 1975) and the CA3 and dentate gyrus of hippocampal slices in 1976 (Alger & Teyler, 1976; Teyler et al, 1977). While LTP has been reliably reproduced in many other regions of the brain, in multiple species, for both my studies and the background I will provide, the focus will remain on the hippocampus in *Mus musculus* and *Rattus norvegicus*. Experimentally, LTP can be produced through various methods, and depending on the frequency and intensity of the stimulation, it can produce different durations and forms. A high frequency train of stimulations is referred to as the tetanus,

and immediately following this protocol what is known as the post-tetanic potentiation (PTP) can be observed (Bliss & Lomo, 1973). This increase can be maintained to produce LTP, but can also decline back to baseline. A range of stimulus frequencies from 0.2 Hz to 400 Hz can be used to induce LTP (McNaughton et al, 1978) but the frequencies below 100 Hz can also occasionally produce depression. Also during long trains of stimuli, the postsynaptic cells are hard to monitor and may produce epileptic activity, a problem solved by using multiple trains of fewer stimuli (Douglas, 1977).

During behavioral learning, hippocampal CA1 pyramidal neurons have been recorded to fire in high frequency, short bursts, that oscillate between less than 2 Hz, and more than 15 Hz. These have been classified as theta-rhythms (Buzsaki, 2002). Stimulation using protocols that imitate these naturally occurring patterns can also produce LTP that combines the VGCC and NMDA dependent components, and this protocol produces what is known as compound LTP (Bayazitov et al, 2007; Morgan & Teyler, 2001). There are many protocols used to evoke different kinds of LTP, but in general, the phenomenon can be broken down into two different phases: induction and maintenance.

Induction

The early phase of LTP lasts approximately 60 minutes, while the late phase is protein synthesis dependent after that time point (Davis & Squire, 1984). To induce LTP, a train of high frequency stimuli can be sent along the neuronal pathway of interest. Numerous induction protocols can create NMDA receptor (NMDAR) dependent LTP, and even though NMDARs are required for LTP, the first action of glutamate occurs on the kinetically faster AMPA receptors, due to the voltage gated Mg^{2+} block in the NMDA receptors (Malenka & Nicoll, 1999). The inward flow, through AMPA receptor channels, of the monovalent cations Na^+ and K^+ is the predominant current during low frequency stimulation. When the membrane depolarizes enough to remove the Mg^{2+} block, the NMDAR channels open, leading to an influx of Ca^{2+} into the dendritic spine. This rise in Ca^{2+} is only necessary for approximately 2-3 seconds (Malenka & Bear, 2004). The entrance of Ca^{2+} into CA1 neurons through NMDA receptor channels is a necessary component to induce LTP. This is shown by injection of Ca^{2+} chelators into postsynaptic cells prevents the development of LTP (Grover & Teyler, 1990). There is a diversity of signaling proteins involved in LTP, and multiple intracellular cascades are involved in inducing LTP. One molecule of importance, regardless of induction mechanism, is calcium/calmodulin-dependent protein kinase II (CamKII), due to its role in mediating NMDAR-dependent LTP (Lisman et al, 2002; Malenka and Nicoll, 1999). Another important mediator in LTP is PKA, (protein kinase A), which is a cAMP dependent protein kinase needed during early post-natal development, and later plays an indirect role in enhancing CamKII autophosphorylation through inhibition of protein phosphatase 1 (Huang & Kandel, 1994). Also protein kinase C (PKC) is important for LTP and in particular its atypical isozyme, PKM ζ (Ling et al, 2002). Lastly, as mentioned in the previous section, PI3K is required for LTP through trafficking of AMPARs to synapses in dissociated hippocampal cultures (Man et al, 2003).

One of the most commonly studied pathways for LTP is the Schaffer collaterals of the hippocampus, which are the axon collaterals that project from the CA3 to the CA1 pyramidal neurons. Due to its frequent use and therefore comparative qualities, this region of the brain is the area used for my experiments. The stimulating electrode is placed in the axon pathway of the CA3 cells and the combined electrical activities of the postsynaptic potentials are measured in the dendritic region of the CA1 pyramidal cells. Depending on the stimulation protocol there are different types of LTP that can be induced along this pathway. Using 25 to 50Hz stimulation produces a form of LTP that can be blocked by the NMDA receptor antagonist APV (Grover & Teyler, 1990). If the stimulation protocol is increased to 100-200Hz there is a second form of LTP induced that is only partially blocked by APV (an NMDA receptor antagonist). The remaining component is blocked by the Ca^{2+} channel antagonist nifedipine (Grover & Teyler, 1990). Nifedipine effectively blocks L-type voltage gated calcium channels (L-VGCC), which have been shown to be predominantly located on CA3 neurons (Elliott et al, 1995). Therefore compound LTP, which is induced by the higher frequency train of stimuli, is broken into two types; the faster postsynaptic NMDA dependent component and the slower presynaptic component requiring activation of L-VGCC (Bayazitov et al, 2007). These experimental procedures to produce LTP are physiologically relevant and studies have been performed that reinforce LTP is the current experimental model for memory. Particularly work was done showing LTP stimulation in slice and inhibitory avoidance trials in rats produce the same changes in NMDA phosphorylation at GluR1 receptors, as well as a partial occlusion of in vivo induced LTP following learning trials (Whitlock et al, 2006).

Another mechanism involved in LTP induction is alterations of the AMPA receptor component of the postsynaptic cell (Bliss & Collingridge, 1993; Soderling & Derkach, 2000). Lu et al, (2001) determined that stimulation of postsynaptic NMDA receptors causes an increase in AMPA receptor insertion and clustering via SNARE dependent vesicular trafficking and membrane fusion. Synapses that previously contained no AMPA receptors were deemed “silent” (Isaac et al, 1995). These increases in AMPARs are caused by activity-dependent changes in AMPAR trafficking, specifically the GluR1 subunit, as well as modification in the individual receptors through direct phosphorylation (Bredt and Nicoll, 2003, Malinow and Malenka, 2002).

The division between induction and maintenance has been robustly demonstrated. Studies show that the formation of long-term spatial memory can be prevented by inhibitors that are needed for inducing LTP, such as NMDAR and protein kinases like CamKII, PKA and PKC, while blocking these doesn't reverse already established maintenance phase LTP. There are exceptions for molecules involved in either of these processes, such as brain-specific atypical PKC isoform protein kinase $\text{M}\zeta$, which is both necessary and sufficient for LTP maintenance (Ling et al, 2002). PKM ζ , when applied to slices, potentiates AMPAR responses, while inhibition of it reverses established LTP.

Maintenance

It is well established that late phase LTP requires new protein synthesis and gene transcription (Stanton & Sarvey, 1984). LTP is linked to the nucleus through signaling molecules including PKA, CamKIV, and MAPK, which activate CREB, and then early genes such as *zif268* (Bailey et al, 1996; Malenka & Bear, 2004). A functional aim of this protein synthesis would be to allow permanent changes in neuronal structure. These morphological changes have been experimentally reported, such as transient enlargement of the structure of individual spines as imaged during glutamate activation (Matsuzaki et al, 2004). Cytoskeletal reorganization involvement in maintaining LTP is shown with actin polymerization blockers impairing LTP. Insertion of AMPAR and associated molecules into the postsynaptic density leads to growth of the spine, which is then matched by an increase in the presynaptic active zone and the overall size changes causing the increase in synaptic strength.

This dependence on protein synthesis is shown experimentally through blockers of protein synthesis. In the presence of the protein synthesis blockers anisomycin and emetine, which both block protein synthesis through binding different ribosomal subunits, and cycloheximide, which blocks the translocation of RNA molecules to the ribosome, induction is still possible, but 30 minutes after the repetitive stimulations there is no LTP (Stanton & Sarvey, 1984). Some of the translational initiation factors (4E-BP2 and eIF-4E) that are regulated by mammalian target of rapamycin (mTOR), have been implicated in this protein synthesis through knockout mice (Banko et al, 2005; Costa-Mattioli et al, 2007). Other work has shown that application of rapamycin (mTOR inhibitor) inhibits the late phase of LTP, but leaves the early phase unaffected (Cammalleri et al, 2003). While both *in vivo* memory and LTP exhibit two phases (induction and maintenance), the maintenance phase can also be divided. It starts with a protein independent early phase, lasting a couple hours known as E-LTP (early LTP) and is followed by a protein synthesis dependent late phase or L-LTP. These two phases have been exhibited in slices, anesthetized animals and freely moving animals. Once the process passes into the protein synthesis dependent phase, it is said to have been consolidated (Abraham & Williams, 2008). Mice heterozygous for the translation initiation factor *eIF2 α* show a reduced phosphorylation of this subunit in the hippocampus which leads to a translation of the E-LTP into L-LTP with a brief stimulus train that would normally only induce E-LTP. More specifically Costa-Mattioli et al, (2007) show that a decrease in the phosphorylation of *eIF2 α* is critical component for activation of the gene expression needed for protein translation forming L-LTP. When *eIF2 α* dephosphorylation is blocked by Sal003 there is impairment of induction but not maintenance of L-LTP as well as long-term memory (Costa-Mattioli et al, 2007). This shows that *eIF2 α* dephosphorylation is essential for inducing L-LTP and LTM. During repeated training or titanic stimulation the reduced phosphorylation of *eIF2 α* lowers the transcription factor ATF4's mRNA translation levels, removing inhibitory constraint and increasing protein synthesis (Costa-Mattioli et al, 2007). Field potential recordings in dentate gyrus of freely moving animals have lasted from hours, to weeks, and even up to a year. In hippocampus, CA1 and neocortex LTP lasts weeks and in rodents, memories last a similar time course. The maintenance phase has been correlated between both long-

term memory and LTP because of the shared dependence on protein synthesis (Abraham & Williams, 2008; Grover & Teyler, 1990).

The first hour does not need protein synthesis but does involve posttranslational modifications and glutamate receptor trafficking, while the longer phases are dependent on protein synthesis (Abraham & Williams, 2008). Specifically, AMPA receptors mediate the maintenance phase of LTP while the NMDA receptors mediate the induction (Bayazitov et al, 2007; Soderling & Derkach, 2000). The standing theory in the field is that the protein-synthesis stabilizes the LTP, however it may just create a resistance to the reversal that would return synaptic transmission to baseline (Abraham & Williams, 2008). It is also known that protein kinase M zeta (PKM ζ), a constitutively active independent catalytic domain of protein kinase C, is necessary and sufficient for the maintenance of LTP as shown by the PKM ζ inhibitor chelerythrine reversing established LTP (Ling et al, 2002). Application of a PKM ζ inhibitor cell-permeable synthetic peptide derived from complete PKM ζ isoform called ZIP, (myristoylated ζ -pseudosubstrate inhibitory peptide) 22 hours after induction of stable LTP, rapidly reversed established late LTP in experimentally stimulated perforant path in the subgranular layer of the dentate gyrus (Pastalkova et al, 2006). While V-GCCs are important for the presynaptic component of LTP they are also involved in the maintenance as shown by fluorescence change in the presence of V-GCC blocker nitrendipine (Bayazitov et al, 2007).

Even though LTP implies a permanent change in synaptic efficacy, it is not irreversible. The effects of low frequency stimulation (LFS), and high frequency stimulation (HFS), are sensitive to reversal early after inductions. This is related to activation of adenosine receptors and activation of protein phosphatases. LTP is highly resistant to these for approximately 1-2 hours after induction but is still susceptible in the presence of protein synthesis inhibitors. In the dentate gyrus, stable and consolidated LTP can be reversed by heterosynaptic stimulation protocols (Abraham & Williams, 2008). Lateral path LTP can be reversed by medial path HFS up to 90 days. Medial perforant path LTP can be reversed days later by HFS in lateral path. Novel environments or stimuli can reverse LTP in a time-dependent manner. The reversibility of LTP generalizes across brain regions showing a more flexible and changing entity that is updated expanded or impaired by subsequent experiences. The protein synthesis and consolidation is not a permanent stamping in of synaptic change but possibly just raises the threshold for further change (Abraham & Williams, 2008).

The Pre or Postsynaptic Locus of Expression

An interesting debate ongoing in the field is which side of the synapse plays the biggest role in the changes exhibited. In compound LTP both the presynaptic and postsynaptic sides of the synapse are involved (Bayazitov et al, 2007), but what are the components that perform this change? Since neurotransmitter release is a major component of the synaptic operation, how these neurotransmitters affect synaptic plasticity upon release from vesicles is a critical line of investigation. Originally there were indirect methods of determining changes that occur on the presynaptic side. The

quantal method is a calculation that determines the number of vesicles released, by taking the mean size of the evoked EPSP as a function of the average number of vesicles released, multiplied by the mean amplitude of the postsynaptic potential evoked from a single vesicle released (Katz, 1969). Paired-pulse facilitation, which is defined as a short lasting increase in evoked postsynaptic response within 1 second of a previous postsynaptic response, is also an indirect measure of the presynaptic effect (Schulz et al, 1994). It can be used to give an estimation of any changes that are occurring on the presynaptic side across different synaptic connections. As two EPSCs are stimulated in rapid succession the second pulse has the potential to show a greater evoked response which has been attributed to a transient increase in the probability of transmitter release (McManus et al, 1987).

On the postsynaptic side, AMPA receptors (AMPA) are critical for LTP. The increased response is either due to an upregulation of AMPARs or the recruitment of new receptors to synapses devoid of AMPAR (Kerchner & Nicoll, 2008). The AMPA receptor effect could also be due to the internalization of the AMPAR subunit GluR1 and its replacement with GluR2. This switching of receptor subunits has been shown to occur after the induction of plasticity and relies on protein degradation (Colledge et al, 2003). Postsynaptic structural changes are also important for the maintenance of LTP such as expanding the postsynaptic density and growth of the dendritic spines through actin polymerization (Bramham, 2008).

LTP maintenance without protein synthesis: Application of BDNF can stabilize newly formed LTP for several hours even in the presence of anisomycin. This result suggests that BDNF is one of the key proteins that needs to be created to stabilize LTP possibly by promoting glutamate receptor movement to the cell surface (Abraham & Williams, 2008). BDNF however plays a role in dendritic protein synthesis and gene expression. Work has shown that late-phase LTP is unaffected by anisomycin if no test pulses are delivered during the inhibition of synthesis. If pulses are delivered, then severe inhibition of the LTP occurs and the same goes for early phase LTP. The conclusion is that synaptic activity during LTP maintenance utilizes and turns over the proteins needed for LTP stabilization and expression, which then causes decay of LTP and the machinery is non-functioning to make new proteins (Abraham & Williams, 2008).

Long-term Depression

While the up-regulation of synaptic efficacy is important, neurons form networks that cannot remain in this increased or saturated condition at all times. Mechanisms must also be present to allow weakening of these connections under certain circumstances, which may be where long-term depression or LTD plays a role in synaptic plasticity. This form of synaptic activity was originally termed “depotentiation” in studies showing the potential to reverse LTP. It wasn’t until 1992 that a separate stimulus protocol was established to reliably induce homosynaptic LTD (Dudek & Bear, 1992). Instead of a brief high frequency train of stimuli, the typical induction protocol for LTD is a prolonged low frequency stimulation. For LTD, the collective literature seems to agree

that the postsynaptic cell is the site of plasticity. Since plasticity is a global phenomenon, there are numerous variations across cell type and species so the hippocampus still remains the conventional model for experiments. The excitatory synapses in the hippocampus contain ionotropic glutamate receptors, specifically those responding to NMDA and AMPA. Research led by the Malenka group has thoroughly established that the internalization of AMPA receptors is a critical mechanism behind LTD (Carroll et al, 1999b). There appear to be two major forms of LTD, the NMDARs form and the metabotropic glutamate receptor (mGluRs) form (Nicoll et al, 1998).

Induction

Modest depolarization of postsynaptic neuron reduces the number of 0.5-1 Hz stimuli that are needed to induce LTD, which relieves the Mg^{2+} block of the NMDAR. Inhibiting NMDARs blocks LTD and activating of them induces it, which induces a rise of Ca^{2+} into the postsynaptic neuron that prevents LTD when buffered. NR2A/B and NR2C/D subunits of NMDA receptors contribute differently to LTP and LTD (Malenka & Bear, 2004). Ca^{2+} release from intracellular stores is also proposed to play a role in LTD, particularly when there is sub-optimal activation of NMDARs. LTD is also prevented by the postsynaptic application of protein phosphatase inhibitors that target calcineurin or protein phosphatase 1, and LTD is correlated with dephosphorylation of postsynaptic PKC and PKA. Postsynaptic activation PKA can reverse previously established LTD (Malenka & Bear, 2004).

Induction of LTD in the CA1, that is mGluR dependent, is voltage dependent and the group 1 mGluRs are most likely responsible for this form of LTD (Oliet et al, 1997). While induction of LTD dependent on NMDARs it also requires voltage dependence, and requires an additional rise in postsynaptic Ca^{2+} , at a lower activation level than necessary for LTP (Dudek & Bear, 1992; Nicoll et al, 1998). The effective stimulation train to produce LTD involves 0.5-1.0 Hz stimuli for approximately 900 pulses, but modest depolarization of the postsynaptic neuron, which relieves the Mg^{2+} block of the NMDAR, reduces the number of stimuli needed (Selig et al, 1995). Inhibition of NMDARs blocks LTD, while activation of them induces it, through a rise in Ca^{2+} in the postsynaptic neuron through these channels. Induction of both forms of LTD can be blocked by the addition of the Ca^{2+} chelator BAPTA to the patch pipette, as well as blocking NMDA receptors with APV (Carroll et al, 1999b). While LTP has been related to a phosphorylation of ser-831 on the C-tail of the GluR-1 subunit of AMPA receptors with no change in ser-845, NMDA dependent LTD has been shown to be the opposite with a dephosphorylation ser-845 and no change in ser-831 (Gladding et al, 2009; Lee et al, 2000). More specifically, a PKC inhibitory peptide only blocks the mGluR and not NMDA dependent LTD (Nicoll et al, 1998). The mGluR dependent LTD shows a decrease in frequency but not the size of quantal release, or as demonstrated with FM 1-43 loading and unloading, a change in the method of vesicle release (full fusion to transient opening of a fusion pore, “kiss and run”) (Zakharenko et al, 2002) indicating a presynaptic expression mechanism. The NMDA dependent LTD shows a decrease in quantal size indicating a postsynaptic expression mechanism (Nicoll et al, 1998).

Maintenance

LTD also consolidates into a maintenance phase, requiring an internalization of AMPA receptors. The AMPARs predominate in LTD, however NMDA receptor activation plays a role, as well as the postsynaptic increase in Ca^{2+} . Cultures exposed to glutamate during stimulation showed a decrease in synaptic membrane localized GluR1 subunits. Carroll et al, (1999a) found that the addition of glutamate caused an LTD that was directly correlated to the loss of the surface AMPARs. Consistently AMPARs internalized during LTD inducing protocols, while these protocols have no effect on the distribution of NMDA receptors. The actual endocytosis appears to require clathrin coated pits as shown by co-localization of clathrin antibody AP2 and GluR1 surface labeling (Carroll et al, 1999a). By using hypertonic conditions, which are disadvantageous to clathrin-coated endocytosis since it prevents the internalization of low density lipoprotein (Heuser & Anderson, 1989), there was less internalization of AMPARs (Carroll et al, 1999a). The methods of expression experimentally differ between the two forms of LTD, with mGluR dependent LTD producing altered synaptic vesicle cycling and a reduced release of presynaptic neurotransmitter (Zakharenko et al, 2002) while NMDA dependent LTD expression is due to decreases in the number of surface synaptic AMPARs (Carroll et al, 1999b).

Phosphorylation on the C-tail of the GluR1 subunit at ser-831 is found associated with LTP, which is a substrate of CamKII and PKC without change in ser-845 which is a PKC substrate (Kessels & Malinow, 2009). LTD was found associated with the dephosphorylation of ser-845, likely because it decreases AMPAR open channel probability, without any change in ser-831 (He et al, 2009). AMPARs are rapidly internalized in response to LTD-inducing stimuli and this process occurs through a dynamin- and clathrin-dependent mechanism (Carroll et al, 1999a; Wang & Linden, 2000). The AMPAR GluR2 subunit is a key regulator of AMPAR endocytosis initiation LTD. When NMDAR are activated then the clathrin adaptor protein complex AP2 gets recruited to the region on the membrane close to the GluR2 C-tail initiating the endocytosis (Carroll et al, 1999a). Slot proteins are present to maintain constant transmission in the face of AMPAR turnover on primary candidate is PSD-95. PSD-95 makes a good candidate because of its ability to bind stargazin, which is a known transporter of AMPARs to the surface of the synapse. Therefore PSD-95 might form a "docking site for AMPA receptors" (Colledge et al, 2003). AMPARs are increased during over expression of PSD-95, as well as decreased during LTD and the activation of NMDA receptors. Experiments using NMDA stimulation not only showed a reduction in PSD-95 protein levels, but also immunoprecipitated samples of hippocampal neurons after treatment with NMDA showed an increase in ubiquitination (Colledge et al, 2003). Since PSD-95 is supposedly holding slots for AMPAR insertion, Colledge et al, (2003) looked at the relationship between NMDA induced loss of PSD-95 and surface AMPARs. The same NMDA treatment caused a loss of surface GluR1 and when the ubiquitin tagging and proteasomal degradation of PSD-95 is blocked, the internalization of surface AMPARs is also blocked. NMDA treatment also causes internalization of GluR2 and in cells that are unable to be ubiquitinated, NMDA treatment causes a significantly reduced amount of AMPAR endocytosis (Colledge et al, 2003).

There is clearly a correlation between the PI3K pathway and the protein synthesis dependent transfer of short-term to long-term synaptic changes. Hippocampal synaptic plasticity has also been well studied but my studies should provide insight into the role upstream components of this pathway contribute to controlling the protein synthesis of the PI3K pathway, specifically Pten. I also aimed to determine what role these processes had on both the in vivo and in vitro manifestations of this synaptic plasticity.

CHAPTER 2. RESULTS

PTEN KNOCKOUT MOUSE MODEL

Previous studies had been performed using a cross between mice expressing *Cre*-recombinase (*cre*) under control of the glial fibrillary acidic protein (GFAP) promoter, and mice with exons 4 and 5 floxed in *Pten* (Fraser et al, 2004; Kwon et al, 2003; Kwon et al, 2006). This promoter activated recombination of the *cre* deleting *Pten* in astrocytes throughout the brain, as well as 80-90% of hippocampal neurons and cerebellar granule neurons (Fraser et al, 2004). *GFAP*, which is a marker of astrocytes, as a promoter, activated deletion of *Pten* early in development (Bonni et al, 1997) affecting development in the hippocampus and causing a defect in pyramidal cell migration (Fraser et al, 2004). To avoid the confounding effects of this early *Pten* deletion, the floxed *Pten* mouse was crossed with mice expressing *Cre*-recombinase under control of the alpha subunit of the calmodulin-dependent protein kinase promoter (*αCamKII*) hereafter referred to as *Pten* KO (**Figure 2-1**) (Tsien et al, 1996).

The T29.1 founder strain from the Tonegawa lab was used kindly donated from the Baker lab, with the *αCamKII* promoter driving recombination of the loxP sites. The expression pattern provides activation in the pyramidal neurons of the forebrain which includes the pyramidal layers of the hippocampus and the dentate gyrus. There is also *αCamKII* expression throughout the cortex, with layers 2 and 3 having twice the intensity of expression of layers 4 through 6 (Burgin et al, 1990). *Cre* activation would delete exon 4 and 5 of *Pten* between postnatal days 10-30, allowing the majority of the hippocampus to be developmentally intact before activation of the construct (Yamauchi, 2005). In our hands these mice develop normally with no gross size changes (**Figure 2-2**). However some of the mice develop an unspecified macrocephaly at approximately 8 weeks of an indeterminate cause (**Figure 2-3**). This macrocephaly is expected based on previous *Pten* deletion mouse models (Backman et al, 2001; Kwon et al, 2001).

Basic behavioral observations do not indicate any gross abnormal interactions or movements in these mice from birth through week 8. After week 8 perhaps 50% of the mice develop what could be termed “seizures in absentia”. The mice react to noise or touch with a period of freezing, occasionally accompanied by twitching of a single body part, most frequently the tail. This is followed by death of an unknown nature, with maximum life span to date reaching 80 days, and mortality as early as 58 days (**Figure 2-4**). These seizures have also been reported in previous conditional KO models under the GFAP promoter (Backman et al, 2001; Kwon et al, 2001). Since these seizures appear after 8 weeks of age, I used the 8 week time point for electrophysiological and behavioral studies with these mice.

To determine if I had functional deletion of *Pten*, I started with western blots of *Pten* protein levels. I also looked at the phospho-antibodies for sites on Akt at S473 and T308 to see if any changes occurred in phosphorylation of downstream members of the

Figure 2-1 Schematic representation of the conditional *Pten* KO mouse line.

The top line shows the α CamKII promoter mouse line driving expression of *cre*. The middle line shows the *Pten* gene at the placement of the loxP sites between exons 3 and 4 and between exons 5 and 6. The end result, in line three, is the *Pten* KO mouse where exons 4 and 5 have been deleted.

Figure 2-2 Nine week old mice showing no gross size changes.

Sibling mice sedated and photographed at nine weeks of age. Top mouse *Pten* KO (*Pten*^{fl/fl}; *Cre*+) and bottom mouse WT (*Pten*^{fl/fl}; *Cre*-).

Figure 2-3 Nine week old mouse exhibiting unspecified macrocephaly.

Sibling mice sedated and photographed at nine weeks of age. Left mouse *Pten* KO ($Pten^{fl/fl}; Cre+$) and right mouse WT ($Pten^{fl/fl}; Cre-$).

Figure 2-4 Survival curve for *αCamKII-cre Pten* KO mice versus WT.

Survival curve of 15 *αCamKII-cre Pten* KO mice and 2 WT mice with death in *Pten* KO mice occurring on average at 77 days and the earliest morbidity at 67 and the longest lifespan measuring 98 days. The survival percentage is significantly different (Mann-Whitney Rank Sum, $p=0.030$) with WT mice typically living past 1 year.

pathway. I dissected out randomized unilateral hippocampi and snap froze them. The tissue was lysed using disposable pestles and microtubes in ice-cold RIPA buffer supplemented with protease and phosphatase inhibitions. I quantified protein concentrations and 25µg of protein extract was electrophoresed on 10% SDS-PAGE gels, and protein was transferred to PVDF membrane. The primary antibodies and their dilutions used for chemiluminescent detection were pAkt (T308) (1:1000), pAkt (S473) (1:1000), total Akt (1:1000), p-S6 (Ser235/236) (1:1000), total S6 (1:1000), mouse anti-β-actin (1:5000). Blots were probed with secondary antibody goat anti-rabbit IgG horseradish peroxidase (HRP) (1:5000). A slight decrease was visible in the bands for Pten but the difference is difficult to attribute to the deletion without considering experimental variation in protein concentrations (**Figure 2-5**). The remaining probes however clearly show increased phosphorylation at both the S473 and T308 sites on Akt, which shows that the reduction in Pten is enough to disrupt the pathway, creating greater amounts of PIP₃ causing co-translocation of Akt and PDK1 to the membrane and the phosphorylation of Akt at T308. The phosphorylation of Akt at S473 by mTOR also shows that Pten was sufficiently deleted to disrupt the regulation of downstream mechanisms. I also looked at phosphorylation of S6 at (S235/236) and while some of the samples look upregulated, the difference between the wildtype (WT) and *Pten* KO mice is not as robust. The total S6 levels show variability, which emphasizes the increased phosphorylation levels for example even in the seventh lane where total levels were decreased the phosphorylation was still increased. This variability could be attributed to variations in the cre-recombination which would give different levels of Pten deletion in difference mice and therefore their protein lysates.

Since in the chemiluminescent western blots the reduction in Pten protein levels was difficult to quantify, I performed western blots using the Odyssey infrared imaging system. Using the same protein extraction methods and gel electrophoresis protocols, the blots were probed with primary antibodies Pten (1:1000; #9559, Cell Signaling), and anti-mouse (1:5000) and anti-rabbit (1:5000) secondary antibodies conjugated to IR dye 680 or 800. Blots were imaged using the Odyssey infrared imaging system. The Odyssey software was used to quantify the I.I or K counts, which is the measure of fluorescent intensity. The background was subtracted from the band selection and the Pten fluorescent intensity measures were divided by the intensity of the β-actin bands and normalized to 100% of WT Pten levels. The quantification shows approximately a 40% reduction in levels of Pten (**Figure 2-6**).

To evaluate the cell-autonomous distribution of *cre* activation, I performed immunohistochemistry on paraffin embedded hippocampi. Mice were perfused transcardially with PBS, followed by 4% paraformaldehyde (PFA) in PBS. Tissues were post-fixed in 4% PFA overnight, embedded in paraffin, cut into halves sagittally, and then cut into 5µm thick sections. Littermate controls were all genotypic combinations without *cre* to allow consistency through multiple mouse models, and were processed at the same time as mutant littermates. Using microwave antigen retrieval, sections were probed using primary antibody Pten (1:100). Detection of primary antibodies was performed using biotinylated secondary antibodies (1:200) peroxidase-conjugated avidin. and treatment with DAB substrate and hematoxylin counterstain The *Pten* deletion

Figure 2-5 Western blot of hippocampi from WT and *Pten* KO mice showing *Pten* deletion.

Western blot taken from unilateral hippocampi from 4 WT littermates (first 4 lanes) and 5 *Pten* KO mice (last 5 lanes). 25ug protein was loaded onto an 10% SDS-page gel, transferred to PVDF and probed with primary antibodies against Pten (1:1000), pAkt (T308) (1:1000), pAkt (S473) (1:1000), total Akt (1:1000), p-S6 (Ser235/236) (1:1000), total S6 (1:1000), mouse anti- β -actin (1:5000). Blots were probed with secondary antibody goat anti-rabbit IgG HRP (1:5000).

Figure 2-6 Fluorescently quantified Western blots for Pten in WT and *Pten* KO mice showing *Pten* deletion.

Western blot taken from either left or right hemispheres of hippocampi from 4 WT littermates (first 4 lanes) and 5 *Pten* KO mice (last 5 lanes). 25ug protein was loaded onto an 10% SDS-page gel, transferred to PVDF and probed for primary antibody against Pten (1:1000). Red represents the 680nm wavelength from the secondary conjugated to Pten while green fluorescence emits at 800nm wavelength conjugated to β -actin. The graph shows a 38% decrease of levels of Pten, which is significant ($p=0.040$).

in this mouse model occurs in a mosaic fashion but with approximately 80-90% deletion in the hippocampus by nine weeks (**Figure 2-7**). The authors of the original paper (Tsien et al, 1996) reported a 0.3% expression in the dentate gyrus while qualitative examination of the Pten immunohistochemistry shows an upwards of 70% deletion of Pten in this region. The founder line only showed 1.4% deletion in the CA3 and a 98% deletion in the CA1 (Tsien et al, 1996). Immunohistochemistry for Pten shows a slightly more robust deletion in the CA3, with a comparable deletion in the CA1. Upon cursory examination, the impressive cell size increase that occurs with the *Gfap-cre* line by P14 is substantially reduced, if not absent, in the *α CamKII* knockouts at the same age.

MORPHOLOGICAL CHARACTERISTICS

Since an established phenotype of *Pten* deletion is the increase in cell size, I did extensive work to analyze size changes in this *CamKII-cre Pten* KO mouse model. First, I attempted to quantify the cell size in pyramidal neurons. The simplest method was to use hematoxylin and eosin (H&E) stained paraffin sectioned tissue to measure nuclear diameter. For every embedded brain, 100 slides worth of paraffin sections were produced, and every tenth slide was selected for staining. Those slides were imaged producing representative hippocampi in approximately 150 μ m increments. 20x images were taken of the CA1, CA3 and dentate gyrus cell bodies and ImageJ was used to analyze nuclear diameter and cell density. The nuclear diameter was determined at the widest horizontal point of each nucleus and the density was determined by the number of cell bodies divided by the area of the image, with the pyramidal cell layer centered in the image.

In **Figure 2-8**, the graph shows measurements of 1169 CA1 nuclei in one WT mouse averaged across 8 slides compared with 891 measurements from one *Pten* KO mouse averaged across 6 slides. In the CA3, 723 *Pten* KO nuclei were measured across 6 slides with 1040 WT neurons measured, averaged over 9 slides. There was a significant difference between the nuclear diameters (T- test, $p=0.002$) but in the opposite direction than the literature would suggest. The *Pten* null cells actually had smaller nuclear diameters. In the dentate there were 597 *Pten* KO nuclear diameters measured across 6 slides, and 1352 WT diameters averaged across 8 slides. There was no significant difference in the nuclear diameters in the dentate gyrus. Distinct from this particular mouse model it is important to note that within a brain there is a variation in nuclear diameter that may not seem large, but is enough to be statistically different along the rostral-caudal plane (**Figure A-1; Figure A-2**). So between mice, choosing a slide that is not of the same section of hippocampus will show a statistically significant difference ($p<0.001$) in cell sizes. The actual nuclear diameters only differ by 1, so while the statistics show significance, it's hard to place high experimental value on this difference. The important thing to acknowledge is that the previously reported doubling in size when *Pten* is removed from a neuron (Backman et al, 2002; Kwon et al, 2001) is not seen in this mouse model.

Since the percentage of cells null for *Pten* was significantly less in the CA3 and

Figure 2-7 Classification of the *αCamKII-cre Pten* KO model with immunohistochemistry.

Immunohistochemistry of 4, 6, and 9 week old *Pten* KO mice with age matched littermate controls. Brains were fixed in PFA and paraffin embedded followed by microwave antigen retrieval. Probed for Pten (1:100) and peroxidase-conjugated secondary (1:200) antibodies. Top images showing the deletion progression at 4, 6 and 9 wks, bottom images show an increased magnification of the CA1 pyramidal cell layer at 9 weeks.

Figure 2-8 Hematoxylin and eosin staining showing *Pten* KO nuclear diameters are slightly smaller than WT at 8 weeks.

Eight week old brains were paraffin embedded and sectioned and stained for hematoxylin and eosin. Top images are of the hippocampal pyramidal cell layer in the CA1 region of the WT littermates (WT) and *Pten* KO mice. Bar graphs are shown of the nuclear diameters in CA1, CA3 and dentate gyrus. The *Pten* KO cells are significantly smaller (P=0.002) in the CA1 and CA3 regions with no significant difference in the dentate gyrus (DG).

dentate gyrus compared to the CA1 area, I used the sections labeled for Pten immunohistochemistry to measure nuclear diameter, so that I could identify the Pten positive and negative cells (**Figure 2-9**). I did not see significant difference between CA3 Pten positive and Pten negative neurons (T-test, $p=0.338$). 227 Pten positive nuclei from the CA3 were measured and averaged across 9 slides, while 53 negative neurons were measured and averaged across 12 slides.

As there is significantly less *cre* activation in the CA3 region, it was more difficult to gather data on these cells. Within the dentate gyrus I actually saw the expected increase in Pten negative cell's nuclear size. The Pten null nuclei were significantly larger (T-test, $p<0.001$) with 224 Pten null nuclei across 4 slides and 595 Pten positive nuclei measured, averaged across 6 slides, but this increase constituted only approximately $0.8\mu\text{m}$. While this increase is convincing it is substantially smaller than the previously reported approximately 70% increase of cerebral granule cells in a mouse model with a *Gfap-cre* activated deletion of Pten (Kwon et al, 2001).

The *Pten* deletion could be causing a change in the number of cells and their proliferation. To look at this I measured density in different regions of the hippocampus (**Figure 2-10**). I actually saw an increase in the cellular density of the CA1 region in *Pten* KOs (Mann-Whitney rank sum, $p=0.002$) but while significant, there was only a difference between 1.2 cells per $20\mu\text{m}^2$ and 1.4 cells per $20\mu\text{m}^2$, which appears small and may not be significant. There was no difference in the CA3 region (Mann-Whitney rank sum, $p=0.322$) or the entire dentate gyrus region (T-test, $p=0.223$ and Mann-Whitney rank sum $p=0.212$).

Since it was difficult to determine the soma membrane in the H&E preparation I used Golgi stain to label the entire cell body. Golgi stain was performed using the Golgi-Cox impregnation method, from the FD Rapid Golgi Stain Kit. I impregnated whole brains for the 2 week incubation period, embedded them in tissue freezing medium, and sectioned $200\mu\text{m}$ sections before developing. Images were taken of cell bodies to measure soma area and I used Image J to analyze the area of the cell bodies. I determined that there was no difference in soma area using this technique with samples of 102 WT cells from 3 different mice and 94 neurons from 3 *Pten* KO mice (**Figure 2-11**).

The majority of research in the field has shown that *Pten* deletion causes an increase in cell size and because of this I wanted to thoroughly verify that there was no change in the cell size using multiple experimental methods. I crossed our *Pten* KO mice with a reporter mouse with a stop codon flanked by loxP sites, so that *cre* activation deleted *Pten* and expressed YFP in the same subset of cells. Since *cre* activation can be irregular, and our mouse model shows mosaic deletion of *Pten*, I did dual labeling with immunohistochemistry for Pten and YFP (**Figure 2-12**). Procedures were as mentioned above for Pten immunolabeling, with the addition of primary antibodies for GFP (ab6556) which labels YFP, due to an overlap in sequence homology and fluorescent secondary antibodies. Quantification of representative images from one mouse each showed that in the CA3, 64% of the neurons still contained Pten while 29% were both

Figure 2-9 Nuclear diameters in CA3 and dentate gyrus as measured from *Pten* immunohistochemistry.

Eight week old *Pten* KO and WT mice brains were fixed in PFA and paraffin embedded followed by microwave antigen retrieval. Probed for *Pten* (1:100) and peroxidase-conjugated secondary (1:200) antibodies. Nuclei were measured in identified *Pten* positive and negative cells.

Figure 2-10 Cellular density of CA1, CA3 and dentate gyrus.

Eight week old brains were paraffin embedded and sectioned and stained for hematoxylin and eosin. Images were taken with a 20x objective centered over the pyramidal layer. Cellular density was measured as number of cell bodies per image. Graphs showing the cellular density with an increase in the cellular density of the CA1 region ($p=0.002$). There was no difference in the CA3 region ($p=0.322$) or the dentate gyrus (DG) region ($p=0.223$).

Figure 2-11 There is no difference in soma area as measured in Golgi stain.

Golgi stain (FD Rapid Golgi Stain Kit) performed on 8 week old α CamKII-cre *Pten* KO mice and WT littermates. The top images showing representative golgi stained soma. The graph shows no difference in soma area for 102 neurons from two WT mice and 94 neurons from two α CamKII-cre *Pten* KO mice ($p=0.911$).

Figure 2-12 Dual labeling immunofluorescence of YFP and Pten.

Eight week old *Pten* KO and WT mice brains were fixed overnight in PFA, followed by submersion in 25% sucrose and frozen. Representative sections were probed for Pten (1:100) and GFP (1:1000) and fluorescent secondaries (1:400) mounted with Vectashild with DAPI.

YFP and Pten positive. However in the CA3 region there were only 7% of neurons that had the correct cre recombination of the YFP in addition to recombination in the Pten construct and could be used to correctly identify Pten null cells. The odds of correct reporting greatly increased in the CA1 region which had 76% of the neurons both YFP positive and Pten negative allowing for visualization of Pten null cells with florescence. Ten percent of cells were still positive for Pten, while 14% of cell showed as YFP positive but still contained Pten. This shows that the YFP is an accurate identifier of *Pten* deletion in CA1.

Using this reporter mouse we further tested morphological properties of CA1 pyramidal neurons of *Pten* null neurons in CA1. Acute transverse hippocampal slices (400 μ m) were prepared as described in Bayazitov et al, (2007). Briefly, mouse brains were quickly removed and placed in cold (4 °C) dissecting artificial cerebrospinal fluid (ACSF) containing 125 mM choline-Cl, 2.5 mM KCl, 0.4 mM CaCl₂, 6 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 20 mM glucose (285-295 mOsm), under 95% O₂/5% CO₂. After dissection, slices were incubated for 1h in ACSF containing 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 10 mM glucose (285-295 mOsm), under 95% O₂/5% CO₂ at room temperature and then transferred into the submerged recording chamber and superfused (2-3 ml/min) with warm (30-32 °C) ACSF. Two-photon laser experiments were performed by Ildar Bayazitov using an Ultima imaging system, a Ti:sapphire Chameleon Ultra femtosecond-pulsed laser, and 60 $\times$ (0.9 NA) water-immersion, IR objective. Alexa Fluor 594 was included in the internal solution and was excited at 820 nm. YFP positive neurons in the CA1 were identified and patched with glass pipettes (open pipette resistance, 3-5 M Ω that were filled with an internal solution containing 140 mM KMeSO₄, 8 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, 5 mM MgATP, 0.4 mM Na₂ GTP, and 10 to 25 μ M Alexa 594 (pH 7.3). While the YFP neurons were easy to identify (**Figure A-3, Figure A-4**) filling them with Alexa dye allowed for easier visualization of the dendrite branches, dendrite spines and gave a clear outline of the soma. Both YFP positive, Pten negative and YFP negative, Pten positive cells were patched and filled and images taken of their soma's. Using Image J the area of the soma was measured, and once again I report no significant difference in soma size in 15 WT neurons from two mice and 13 *Pten* null neurons from two Pten KO mice (Mann-Whitney Rank Sum, p=0.790) (**Figure 2-13**).

Since I detected no significant differences in the soma or nuclear size I wanted to look at the dendritic branching in these neurons to determine if there were other structural changes that were occurring through the deletion of *Pten*. Using the same YFP neurons as were labeled for measurement of soma area, the Alexa dye was allowed to diffuse for longer until the whole neuron and all its projections were filled with dye. These were imaged in multiple sections and reconstructed using Adobe photoshop. The first measurement taken was total length of the the apical dendrite. This was measured using Image J, starting at the base of the soma where the dendrite thickness became uniform and was no longer tapering from the soma. The trace was followed along the path that leads to the longest total measurement. I saw no differences in total apical dendrite length (Mann-Whitney Rank Sum, p=0.247) from 6 YFP positive neurons and 5 YFP negative neurons (**Figure 2-14**). Since the number of branches could also be affected I performed

Figure 2-13 2-Photon imaging of pyramidal neurons in the CA1 area and quantified area in Pten positive and negative soma in the CA1 showing no difference.

YFP positive Pten null neurons and YFP negative WT neurons were identified and patched with micropipettes using 2-photon microscopy and filled with Alexa594. Areas were measured and showed no significant difference in size ($p=0.790$).

Figure 2-14 Neuronal reconstructions under 2-photon microscopy.

YFP positive Pten null neurons and YFP negative WT neurons were identified and patched with micropipettes using 2-photon microscopy and filled with Alexa594. Multiple images were taken and the dendritic tree was reconstructed. The apical dendrite length was measured from the base of the soma and traced down the longest path with no difference ($p=0.247$).

Scholl analysis on the neurons that had been filled for reconstruction. The results were inconclusive because too few neurons were traced for complete reconstruction which would be easy to resolve with a greater number of neurons patched and filled for reconstruction.

I also looked at the dendritic spines on *Pten* positive and negative neurons to see if there were differences between them. Spine length and width were measured in 1135 spines in *Pten* negative neurons, and 1227 spines in YFP, *Pten* null neurons (**Figure 2-15**). There were no significant differences with the width, averaged per 8 *Pten* positive neurons and 5 YFP, *Pten* negative neurons (T-test, $p=0.640$) or with the length (T-test, $p=0.597$). Spine density was measured as spines per μm , taken from 20 YFP negative sections from 7 neurons and 59 sections taken from 7 neurons. There was no significant difference (Mann-Whitney Rank Sum, $p=0.101$) in the density of spines in YFP positive and negative cells.

There were no differences in morphology determined with any of these techniques at the cellular level, but for complete verification I also looked at the sub-cellular components of these neurons. To analyze the ultrastructural composition of the *Pten* KO mice, I imaged sections of CA1 and CA3 of the hippocampus using electron microscopy. Hippocampal slices taken from 8 week old mice were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, rinsed in the same buffer three times, and dehydrated in a graded series of alcohol and then propylene oxide washes. The tissue was infiltrated and embedded in epon resin and polymerized overnight at 70 °C. I cut 70-nm sections on a Leica UC6 ultramicrotome fitted with a Diatome diamond knife and stained with 2% lead citrate and 2% uranyl acetate. Thick sections were trimmed to a region containing the CA1 or CA3 cell body layer, and several hundred synapses were imaged. The sections were imaged on a JEOL 1200EX11 transmission electron microscope with an AMT XR111 11 megapixel digital camera. Using the criteria as mentioned in the introduction from seminal electron microscope work by E.D. Gray (Gray, 1959a; 1959b), synapses were identified by the presence of a PSD, which can be visualized because it is electron dense, as well as a comparatively wide synaptic cleft. This aligns with a thinner presynaptic density, where vesicles can be seen within an “active zone” which is the region closest to the synaptic cleft. The images were transferred to ImageJ where the embedded scale bar was used to accurately measure postsynaptic density length, area of presynaptic synapse (as a single cross section), total vesicle counts, counts of vesicles within 150nm from the synaptic cleft and vesicle diameter (**Figure 2-16**).

The presynaptic area measurements show no significant difference (Mann-Whitney rank sum, $p=0.818$) with 44 areas from *Pten* KO mice and 46 areas from WT mice. There was also no difference between the total vesicle counts (Mann-Whitney rank sum, $p=0.381$) and the number of vesicles within 150nm from the synaptic cleft (Mann-Whitney rank sum, $p=0.590$) from the same number of presynaptic areas. There was a difference in the diameter of the vesicles, with the *Pten* KO mice having smaller vesicles (Mann-Whitney rank sum, $p=0.020$). The smaller vesicle diameter could indicate a reduction in the amount of neurotransmitter release from the presynaptic side upon

Figure 2-15 Representative 2-photon images of dendritic spines and graphs showing no difference in spine width, length and density.

YFP positive Pten null neurons and YFP negative WT neurons were identified and patched with micropipettes using 2-photon microscopy and filled with Alexa594. Spine length ($p=0.597$) width ($p=0.640$) and density as a function of spines per μm ($p=0.101$) showed no difference.

Figure 2-16 Electron microscopy measurements of presynaptic area, total vesicles counts, postsynaptic density length, vesicle counts in the active zone and vesicle diameter.

Hippocampal slices from 8 week old mice, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, rinsed three times, and dehydrated in a graded series of alcohol and then propylene oxide washes. Tissue was infiltrated and embedded in epon resin and polymerized overnight. 70-nm sections were cut and stained with 2% lead citrate and 2% uranyl acetate. Thick sections were trimmed to contain the CA1 or CA3 cell body layer. Postsynaptic density length (PSD), area of presynaptic synapse (as a single cross section), total vesicle counts, counts of vesicles within 150nm of synaptic cleft and vesicle diameter were measured. The presynaptic area measurements ($p=0.818$), the total vesicle counts ($p=0.381$) and the number of vesicles within 150nm from the synaptic cleft ($p=0.590$), show no significant difference. The diameter of the vesicles, ($p=0.020$) and the length of the PSDs ($p<0.001$) showed a significant difference.

activation of an action potential. There was a difference between the KO and WT mice vesicle diameter of 2.63nm which produces an approximately 20% change in the volume. These diameters were taken from cross-sections of vesicles which may not be widest point of the vesicle so it is hard to determine how accurate these volume extrapolations would be. This decrease in the *Pten* KO mouse model has the potential to greatly change the function of these neurons and this would need to be tested using electrophysiology to determine relevance. Within a presynaptic area fluctuations in vesicle diameter can be 10% which causes a 30% variation in vesicle volume within the same bouton or the entire presynaptic area (Bekkers et al, 1990). Assuming a constant concentration of neurotransmitter within the vesicle and since the vesicle release method can vary this can bring the quantal release variation up to 40% (Liu, 2003). Therefore the variation of 20% I observe between the average vesicle diameters could be entirely within normal variation. There was also a difference found in the length of the postsynaptic densities (PSDs) (Mann-Whitney rank sum, $p < 0.001$). Since the PSD is representative of the density of postsynaptic receptors these differences might cause changes in the functioning of the neurons in the *Pten* KO mouse model. An increase in postsynaptic receptors could produce a greater neuronal response from smaller neurotransmitter release. The electron microscope images however give no indication about what kind of receptors form the postsynaptic densities in these neurons. Therefore this significant increase in the PSDs in the *Pten* KO mouse model could be comprised of inactive receptor types and a functional analysis would need to be performed to examine this. This data shows that in *αCamKII-cre Pten* KO mice there are none of the extreme morphological changes seen in previous studies. The gross enlargements of neuronal cell size typical of *Pten* deletions is not present, nor are the changes in dendritic spines, density of neurons and subcellular morphology at 8 weeks of age. Next I wanted to know does this conditional knockout cause any changes to the functioning of neurons in these mice.

SYNAPTIC CHARACTERISTICS

The next step in evaluating this *αCamKII-cre Pten* KO mouse model was to determine functional changes at the 8 week time point. The first thing I wanted to test was basic synaptic transmission. I used acute hippocampal slices prepared as mentioned above to record along Schaffer collaterals. The field recordings were performed using a setup with 8 submerged recording chambers. I recorded field excitatory postsynaptic potentials (fEPSPs) from the CA1 stratum radiatum by using an extracellular glass pipette (3-5 MΩ) filled with ACSF. Schaffer collateral/commissural fibers in the stratum radiatum were stimulated with a bipolar tungsten electrode placed 200 to 300 μm away from the recording pipette. Stimulation intensities were chosen to produce a fEPSP with a 1 V/s slope based on an input/output curve from stimulations of 0, 25, 50, 100, 150, 200, 250, and 300 μA. I determined no differences in basic synaptic transmission at any of the stimulation intensities between the slices from *Pten* KO mice and their WT littermates (**Figure 2-17**). This suggests that these mice have normal connectivity in the CA3 to CA1 area. Next I wanted to see if a cursory measure of presynaptic function, paired-pulse facilitation, showed any differences. Paired-pulse facilitation (PPF) experiments were

Figure 2-17 Input /output curve in Pten KO mice and WT littermate hippocampal slices.

In acute hippocampal slices field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum with a extracellular glass pipette (3-5 M Ω) filled with ACSF. Schaffer collateral/commissural fibers in the stratum radiatum were stimulated with a bipolar tungsten electrode placed 200 to 300 μ m. Stimulation intensities of 0, 25, 50, 100, 150, 200, 250, and 300 μ A were applied. Red =Pten KO, black = WT.

performed using a pair of stimuli of the same intensity delivered 20, 50, 100, 200, and 1000 ms apart (**Figure 2-18**). This is a measure of presynaptic function since it shows a functional increase in postsynaptic response as a function of the interval between pulses. Castillo and Katz (1954) performed the first quantal analysis showing that the number of failures of release was decreased. This was a function of the increased release of quanta and therefore an increased probability of release, all measures of presynaptic function. Once again I didn't see any differences between the WT littermates and the Pten KO mice. Both of these measures are short-term components of synaptic transmission. Disruption in the PI3K pathway causes changes in protein translation mechanisms, which is a function of long-term changes in plasticity, so it is not unexpected.

The next functional measure to look at is LTP. LTP was induced by 3 periods of 200-Hz tetanization, delivered every 5 min. The stimulation intensity was the same as that used to produce the PPF which produced a fEPSP with a 1 V/s slope based on the input/output curve. Every period of tetanization consisted of 10 trains of 200-Hz stimulation, delivered at the same intensity for 200 ms (40 stimulations), every 5 s. A similar protocol has previously been used to induce compound (presynaptic and postsynaptic) LTP at CA3-CA1 synapses in the hippocampus (Cavus and Teyler, 1996; Zakharenko et al, 2001; Zakharenko et al, 2003). I measured the rise/ slope (mV/ms) of these traces, between 30 and 90 percent. **Figure 2-19** shows representative traces of individual slices, taken as the average of the last 20 traces before the 20 minute baseline measurements prior to inducing the stimulus protocol, and average of 20 sweeps at the 3 hour time point. The fEPSP's were collected every 30 seconds during the baseline and after the induction protocol, and the data was normalized to the mean rise/slope of the 20 minute baseline period. This normalized data was averaged at each 30 second interval from 22 Pten KO slices from 13 mice, and 42 WT littermate slices from 13 mice. There were a potential of 8 slices per mouse, based on the two, 4-chamber recording apparatuses.

Slices were eliminated if their baseline was unstable, or the average baseline was not between .75 and 1.3 mV. After the induction protocol, slices were eliminated if the normalized rise/slope (mV/ms) dropped suddenly (approximately a 10 min period) by more than 20-50 mV/ms, there was more than one oscillation greater than 20 mV/ms or the span of the rise/slope was greater than 30mV/ms. Measures of LTP were taken up to three hours after the induction protocol and there were significant deficits at the post-tetanic potentiation (PTP) (Mann-Whitney rank sum, $p=0.017$) and at the 3-hr time point LTP (Mann-Whitney rank sum, $p=0.013$). PTP and LTP measurements were averages of 10 stimuli at minute 20 to 24.5 for PTP and minute 187 to 191.5 for LTP. The difference seen at the 3-hour LTP was not unexpected and reinforces the previous literature that there is an effect on synaptic plasticity when manipulating the PI3K pathway and more specifically Pten. It is unexpected that there is a difference between the Pten KO mice and the WT littermates immediately following the tetanus since at this point protein translation wouldn't be playing a role in the synaptic plasticity.

I next looked for differences between the Pten KO mouse and their WT littermates in regards to another form of plasticity, LTD. Experimentally I induced

Figure 2-18 Paired-pulse ratios in Pten KO mice and WT littermate hippocampal slices.

In acute hippocampal slices field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum with a extracellular glass pipette (3-5 M Ω) filled with ACSF. Schaffer collateral/commissural fibers in the stratum radiatum were stimulated with a bipolar tungsten electrode placed 200 to 300 μ m. Stimulus intensities were chosen to produce a fEPSP with a 1 V/s slope based on the input/output curves. A pair of stimuli of the same intensity were delivered 20, 50, 100, 200, and 1000 ms apart. Red =Pten KO, black = WT.

Figure 2-19 Long-term potentiation in Pten KO versus WT littermates.

LTP was induced by 3 periods of 200-Hz tetanization, every 5 min with each period of tetanization consisted of 10 trains of 200-Hz stimulation, for 200 ms (40 stimulations), every 5 s. The rise/ slope (mV/ms) of these traces was measured between 30 and 90 percent. Representative traces of individual slices are inset. Graphs show significant differences in averages of 10 stimuli at the post-tetanic potentiation (PTP) ($p=0.017$) and of LTP taken three hours after the induction protocol ($p=0.013$).

LTD with a protocol using one train of 900 stimulations at 1Hz which produces NMDA dependent LTD as previously reported (Dudek & Bear, 1993). Using the same selection criteria for appropriate slices, there were 24 slices from 5 WT littermate mice and 33 slices from 9 *Pten* KO mice. Once again I saw a significant (Mann-Whitney rank sum, $p=0.010$) deficit at the 3 hour time point (**Figure 2-20**). Interestingly there was no significant difference immediately following the tetanization protocol (Mann-Whitney Rank sum, $p=0.072$). These results also mimic previous results seen in heterozygous mice (Wang et al, 2006) showing deletion of *Pten* causes deficits in LTD. This is in agreement with the hypothesis that *Pten* deletion affects protein synthesis through downstream mechanisms in the PI3K pathway.

Next I wanted to determine if there were any differences in the individual cellular properties of neurons null for *Pten* compared to WT. To experimentally examine this I used the previously mentioned YFP reporter mouse strain. This allowed us to identify those cells that were null for *Pten* through identification of the YFP marker. Under 2-photon microscopy those cells were identified and patched in the CA1 region since this is where we had the most confidence in our cellular reporter. Whole-cell recordings were obtained from the cell bodies of CA1 neurons. For current-clamp recordings, patch pipettes (open pipette resistance, 3-5 M Ω) were filled with an internal solution containing 140 mM KMeSO₄, 8 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, 5 mM MgATP, 0.4 mM Na₂ GTP, 300 μ M Fluo 5F, and 10 to 25 μ M Alexa 594 (pH 7.3). For voltage-clamp recordings, cesium-based internal solution replaced the potassium-based solution. Whole-cell recordings were registered using a Multiclamp 700B, digitized, and recorded using pCLAMP 9.0 software.

The first thing I wanted to look at was the ability of these neurons to release vesicles containing neurotransmitter. Since the electron microscopy studies showed that there was a difference in the vesicle size and this could have an impact on the neurotransmitter release I wanted to determine if there was a functional effect of this difference. The *Pten* KO neuron vesicles had a reduced volume and this could produce reduced amount of neurotransmitter release explaining the reduction in the postsynaptically recorded LTP. Spontaneous miniature excitatory postsynaptic currents (mEPSCs) were recorded at -70 mV holding potential in the presence of picrotoxin (100 μ M) and tetrodotoxin (1 μ M) in the extracellular solution for at least 1 h. The addition of picrotoxin blocked the GABA_A receptors, which are inhibitory receptors, and prevent the flow of Cl⁻ ions. Tetrodotoxin blocks voltage gated fast Na²⁺ channels. This cocktail prevents the neurons from firing, so experimentally we can identify the individual quanta of spontaneous release from excitatory synapses. Based on the spikes measured in the postsynaptic cell below threshold I looked at four different measures on each spike; amplitude, 10% to 90% rise time, 90-10% decay time, and inter-event intervals of mEPSCs were analyzed off-line using the Mini-Analysis Program. All software detected events were verified visually. Examples of representative recordings are shown in **Figure 2-21**. There were 57,598 events recorded from 12 *Pten* positive neurons and 14,503 events recorded from 25 YFP positive *Pten* null neurons. I saw no difference as averaged per neuron in the amplitude in picoamps (pA) ($p=0.304$), the decay time in milliseconds (ms) ($p=0.194$), the interval between events in ms ($p=0.907$)

Figure 2-20 Long-term depression in Pten KO versus WT littermates.

LTD was induced using one train of 900 stimulations at 1Hz. The rise/ slope (mV/ms) of these traces was measured between 30 and 90 percent. Representative traces of individual slices are inset. Graphs show averages of 10 stimuli at the post-tetanic depression (PTD) ($p=0.072$) and of LTD which is significantly different taken three hours after the induction protocol ($p=0.010$). release and components of vesicle fusion on the presynaptic side are not altered by Pten deletion.

Figure 2-21 Representative miniature excitatory postsynaptic currents for YFP; Pten negative and Pten positive neurons.

Raw traces showing spontaneous miniature excitatory postsynaptic currents (mEPSCs) recorded at -70 mV holding potential with picrotoxin ($100\mu\text{M}$) and tetrodotoxin ($1\mu\text{M}$). Left shows recordings from YFP positive Pten negative neurons and the right Pten positive neurons.

and the rise time in ms as taken between 10-90% ($p=0.878$) (**Figure 2-22**). This data indicates that while there was a significant difference in the vesicle diameter as seen using electron microscopy, there is no functional effect in the spontaneous release of vesicles. To produce a functional response significant enough to overcome the innate variability in the vesicle neurotransmitter release as reported previously (Liu, 2003) I would expect to see an amplitude change along the lines of this variability. The 7% change in amplitude doesn't parallel the magnitude of volume change or the presumed neurotransmitter release variability. Diffusion of the neurotransmitters as they cross the synaptic cleft is another factor affecting the relationship of presynaptic release to postsynaptic response. Diffusion may prevent the released neurotransmitters from activating the postsynaptic receptors. In addition since the functional response postsynaptically is not reflecting the presynaptic vesicle size changes, these vesicles may not be those that are released in a spontaneous fusion. Studies have shown that different populations of vesicles release for evoked versus spontaneous fusion (Sara et al, 2005). The volume decrease we see might be attributed to changes in evoked and not spontaneously released vesicles. These spontaneously released vesicles also indicate that the mechanisms for release and components of vesicle fusion on the presynaptic side are not altered by *Pten* deletion.

The postsynaptic receptors could also reflect any changes in the mEPSC responses I observed. Based on the number of receptors that are present and active it would be possible to visualize postsynaptic changes. To evaluate the postsynaptic receptors I looked at the two main ionotropic glutamate receptors that are activated in synaptic plasticity; AMPAR and NMDAR. To test AMPAR function I looked at the current ratio of AMPA receptors to NMDA receptors (AMPA/NMDAR). Evoked EPSCs were recorded in the presence of QX-314 (lidocaine *N*-ethyl bromide) (5 mM) in the intracellular solution to block the generation of backpropagating action potentials (APs) and picrotoxin in the extracellular solution to block inhibitory transmission. The AMPA/NMDA ratio was calculated from the EPSC traces recorded at +40 mV (**Figure 2-23**). The amplitude used for stimulation was adjusted to evoke 50 pA EPSCs at -70 mV. The AMPAR current was determined at the time points when EPSCs, recorded at -70 mV, reached their peaks, and the NMDAR currents were determined 100 ms after the peaks. EPSCs were analyzed off-line using Clampfit 10.1 software. Seven neurons were measured from two mice each and 14 events recorded from *Pten* positive neurons and 13 YFP; *Pten* negative events. There was no difference detected in the current ratios between *Pten* positive and negative neurons ($p=0.790$). This gives indication that the base levels of AMPA receptors are not changed by deletion of *Pten*. These currents are measures of receptors at basic firing, not the changes in receptors after induction of plasticity.

CA3 SPECIFIC MOUSE MODEL OF *PTEN* DELETION

In the *αCamKII-cre Pten* KO mice, cursory measures of presynaptic function as measured through PPFs showed no difference, nor did the mEPSCs. From this I concluded *Pten* deletion doesn't affect neurotransmitter release on the presynaptic side.

Figure 2-22 Graphs showing no difference in mEPSC amplitudes, decays, intervals, and rise times.

Spontaneous miniature excitatory postsynaptic currents (mEPSCs) recorded at -70 mV holding potential with picrotoxin ($100\mu\text{M}$) and tetrodotoxin ($1\mu\text{M}$). 57,598 events were recorded from 12 Pten positive neurons and 14,503 events from 25 YFP; Pten null neurons. There was no significant difference for amplitude ($p=0.304$) decay from 90-10% ($p=0.194$), interevent interval ($p=0.907$) and rise time from 10-90% ($p=0.878$).

Figure 2-23 AMPA/NMDA current ratios.

Evoked EPSCs were recorded in the presence of QX-314 (lidocaine *N*-ethyl bromide) (5 mM) in intracellular solution and picrotoxin in the extracellular solution. The AMPA/NMDA ratio was calculated from the EPSC traces recorded at +40 mV. The amplitude used for stimulation was adjusted to evoke 50 pA EPSCs at -70 mV. The AMPAR current was determined at the time points when EPSCs, recorded at -70 mV, reached their peak, and NMDAR currents at 100 ms after the peaks. No difference was detected ($p=0.790$).

However, postsynaptic measures of the ratio between the two major ionotropic glutamate receptors involved in synaptic plasticity, AMPA and NMDA, also remain unchanged. These conflicting results make it difficult to determine which side of the synapse is affected by the removal of *Pten* causing the deficit in the *αCamKII-cre Pten* mice. The *Pten* deletion is also more robust in the CA1 postsynaptic cells in *αCamKII-cre Pten* KO mice so it is hard to completely evaluate the effects of the deletion in the presynaptic cells since fewer CA3 neurons are *Pten* negative. To address this issue I used another conditional mouse model that expresses *cre* using the *Grik4* promoter developed in the Tonegawa lab (Nakazawa et al, 2002). This mouse was crossed with the floxed *Pten* mouse to create a CA3 specific *Pten* deletion (**Figure 2-24**).

These mice displayed no premature mortality as seen in the *αCamKII-cre Pten* KO mice, nor did they exhibit any seizure phenotype. Since this was a new promoter, the time course of *Pten* deletion needed to be evaluated to determine the age of maximum deletion and the effectiveness of the *cre* recombination in the CA3 region. Immunohistochemistry for *Pten* showed that the optimal deletion of *Pten* from CA3 neurons was at 10 weeks (**Figure 2-25**). To perform field recordings the maximum deletion at the earliest time point was needed, and based on the immunohistochemistry this time point was 10 weeks of age.

These 10 week old *Grik4 Pten* KO mice were used for electrophysiology studies. LTP was induced with the same protocol as the original *αCamKII-cre Pten* KO mice to produce compound LTP. Data was averaged at each 30 second interval from 32 *Grik4 Pten* KO slices from 7 mice, and 27 WT littermate slices from 5 mice. There was no difference at the PTP (Mann-Whitney rank sum, $p=0.382$) or at the three hour time point of LTP (Mann-Whitney rank sum, $p=0.102$) (**Figure 2-26**). This mouse model focused the *Pten* deletion to the CA3 region of the hippocampus and I saw no difference in LTP. This result shows *Pten* deletion in the presynaptic neurons has no effect on LTP. By using this promoter the deficit in LTP and that was seen in the original *αCamKII* activated conditional knockout is not due to a presynaptic effect of *Pten* deletion.

PDK1 LOSS RESCUES THE SYNAPTIC EFFECTS PRESENT IN THE *αCAMKII-CRE PTEN* KNOCKOUT MOUSE MODEL

***PDK1* Knockout Mouse Model**

While these studies have provided some information there remains information still to be determined about this deletion. Since previous studies had shown that the effects of *Pten* deletion can be reversed by the additional deletion of *Pdk1* (Bayascas et al, 2005; Chalhoub et al, 2009) this molecule was the first place to look to gather information about the *αCamKII-cre Pten* KO mouse model. Mice produced in the lab of Dario Alessi and gifted to Suzanne Baker were originally created using a neomycin cassette inserted between exon 2 and 3 of the *PDK1* gene. The third loxP site was inserted between exon 4 and 5 with the final strain containing loxP sites flanking exon 3

Figure 2-24 *Grik4-cre Pten* KO construct.

The top line shows the *Grik4* promoter mouse line driving expression of *cre*. The middle line shows the *Pten* gene at the placement of the loxP sites between exons 3 and 4 and between exons 5 and 6. The end result, in line three, is the *Grik4 Pten* KO mouse where exons 4 and 5 have been deleted.

Figure 2-25 Grik4 *Pten* KO immunohistochemistry.

Immunohistochemistry of 8, 10 and 12 week old *Pten* KO mice. Brains were fixed in PFA and paraffin embedded followed by microwave antigen retrieval. Slices were probed for Pten (1:100) and peroxidase-conjugated secondary (1:200) antibodies.

Figure 2-26 Grik4-*Pten* KO versus WT LTP.

LTP was performed on Grik4 *-cre Pten* KO mice at 10 weeks and induced by 3 periods of 200-Hz tetanization, every 5 min with each period of tetanization consisted of 10 trains of 200-Hz stimulation, for 200 ms (40 stimulations), every 5 s. The rise/ slope (mV/ms) of these traces was measured between 30 and 90 percent. Graphs show no significant differences in averages of 10 stimuli at the post-tetanic potentiation (PTP) (p=0.382) and of LTP taken three hours after the induction protocol (p=0.102).

and 4 and crossed with the *αCamKII-cre* mouse line (**Figure 2-27**). These mice didn't show any abnormalities or seizures and had a normal life span. I determined there were no differences in basic synaptic transmission at any of the stimulation intensities between the slices from *Pdk1* KO mice and their WT littermates (**Figure 2-28**). Next I wanted to see if paired-pulse facilitation, showed any difference. Paired-pulse facilitation (PPF) experiments were performed using a pair of stimuli of the same intensity delivered 20, 50, 100, 200, and 1000 ms apart (**Figure 2-29**). There were no differences between *Pdk1* KO mice and WT littermates.

Next I looked at LTP in the *Pdk1* KO mice and their WT littermates. LTP was induced using the same compound LTP protocol as mentioned previously. Data was averaged at each 30 second interval from 29 *Pdk1* KO slices from 8 mice, and 28 WT littermate slices from 8 mice. Measures of LTP were taken up to three hours after the induction protocol and there were no significant deficits at the post-tetanic potentiation (PTP) (Mann-Whitney rank sum, $p=0.314$) or at the 3-hr time point LTP (Mann-Whitney rank sum, $p=0.381$) (**Figure 2-30**). PTP and LTP measurements were averages of 10 stimuli at minute 20 to 24.5 for PTP and minute 187 to 191.5 for LTP. This shows that deletion of *Pdk1* alone had no effects on either basic synaptic transmission or long or short-term plasticity. To verify this I also looked at LTD in *Pdk1* KO mice to see if there were any differences. *Pdk1* KO mice and their WT littermates were compared using the same LTD protocol as mentioned previously. Using the same selection criteria for appropriate slices, there were 28 slices from 5 WT littermate mice and 35 slices from 5 *Pdk1* KO mice. There was no significant difference at either the PTD (Mann-Whitney rank sum, $p=0.965$) or a deficit at the 3 hour time point (Mann-Whitney rank sum, $p=0.812$) (**Figure 2-31**). The *αCamKII-cre Pdk1* KO mice showed no differences from wildtype littermates in LTP, LTD or measures of synaptic firing or short term plasticity, which is an unexpected result since *Pten* deletion caused a disruption of the system enough to change long-term synaptic plasticity.

***αCamKII-cre Pten* and *Pdk1* Knockout Mouse Model**

Since previous studies had shown that the effects of *Pten* deletion can be reversed by the additional deletion of *Pdk1* (Bayascas et al, 2005; Chalhoub et al, 2009) I then crossed the *Pdk1* mouse line with the *Pten* KO mouse line which allowed activation of *cre*-recombinase in both *Pten* and *Pdk1* genes under the *αCamKII* promoter (*αCamKII-cre;Pten^{fl/fl};Pdk1^{fl/fl}* and referred to as DKO (**Figure 2-32**).

To evaluate the DKO mouse line I first looked at immunohistochemistry of *Pten* since there isn't a reliable antibody to visualize *Pdk1*. Immunohistochemistry was performed on paraffin embedded hippocampi from mice transcardially perfused with PBS, followed by 4% paraformaldehyde (PFA) in PBS as mentioned previously. Using microwave antigen retrieval, sections were probed using primary antibody to *Pten* (1:100). Detection of primary antibodies used biotinylated secondary antibodies (1:200) peroxidase-conjugated avidin and treatment with DAB substrate and hematoxylin counterstain. Representative images show that there is still *Pten* deleted cells in the DKO mice and that

Figure 2-27 The *Pdk1* KO construct.

The top line shows the α *CamKII* promoter mouse line driving expression of *cre*. The middle line shows the *Pdk1* gene and the placement of the loxP sites between exons 2 and 3 and between exons 4 and 5. The end result, in line three, is the *Pdk1* KO mouse where exons 3 and 4 have been deleted.

Figure 2-28 Input/output curves for *Pdk1* KO mice versus WT littermates.

In acute hippocampal slices field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum with an extracellular glass pipette (3-5 $\text{M}\Omega$) filled with ACSF. Schaffer collateral/commissural fibers in the stratum radiatum were stimulated with a bipolar tungsten electrode placed 200 to 300 μm . Stimulation intensities of 0, 25, 50, 100, 150, 200, 250, and 300 μA were applied.

Figure 2-29 Paired pulse ratios of *Pdk1* KO mice versus WT littermates.

In acute hippocampal slices field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum with an extracellular glass pipette (3-5 M Ω) filled with ACSF. Schaffer collateral/commissural fibers in the stratum radiatum were stimulated with a bipolar tungsten electrode placed 200 to 300 μ m. Stimulus intensities were chosen to produce a fEPSP with a 1 V/s slope based on the input/output curves. A pair of stimuli of the same intensity were delivered 20, 50, 100, 200, and 1000 ms apart.

Figure 2-30 LTP for *Pdk1* KO mice versus WT littermates.

LTP was performed on *Pdk1* KO mice at 8 weeks and induced by 3 periods of 200-Hz tetanization, every 5 min with each period of tetanization consisted of 10 trains of 200-Hz stimulation, for 200 ms (40 stimulations), every 5 s. Normalized graphs produced from 29 slices from 8 *Pdk1* mice and 28 slices from 8 WT mice. The rise/ slope (mV/ms) of these traces was measured between 30 and 90 percent. Graphs show no significant differences in averages of 10 stimuli at the post-tetanic potentiation (PTP) ($p=0.314$) and of LTP taken three hours after the induction protocol ($p=0.381$).

Figure 2-31 LTD for *αCamKII-cre Pdk1* KO mice versus WT littermates.

LTD was induced using one train of 900 stimulations at 1Hz. The rise/ slope (mV/ms) of these traces were measured between 30 and 90 percent. Representative traces of individual slices are inset. Normalized graphs produced from 35 slices from 5 *Pdk1* mice and 28 slices from 5 WT mice. Graphs show averages of 10 stimuli at the post-tetanic depression (PTD) (p=0.965) and of LTD which is significantly different taken three hours after the induction protocol (p=0.812).

Figure 2-32 *Pten/Pdk1* DKO mouse construct.

The top line shows the α CamKII promoter mouse line driving expression of *cre*. The middle line shows the *Pten* gene after recombination. The third line shows the *Pdk1* gene after recombination.

the additional deletion of *Pdk1* does not reduce the numbers of neurons (**Figure 2-33**). Basic synaptic transmission was normal in the DKO mice as shown in the input/ output curve (**Figure 2-34**) and the pair-pulse ratios (**Figure 2-35**). Next I looked at LTP in the DKO KO mice and their WT littermates. LTP was induced using the same compound LTP protocol as mentioned previously; 3 periods of 200-Hz tetanization.

The rise/ slope (mV/ms) of these traces was measured, between 30 and 90 percent and this normalized data was averaged from 32 DKO slices from 9 mice, and 21 WT littermate slices from 9 mice. Measures of LTP were taken up to three hours after the induction protocol and there were no significant deficits at the post-tetanic potentiation (PTP) (Mann-Whitney rank sum, $p=0.659$) or at the 3-hr time point LTP (Mann-Whitney rank sum, $p=0.938$) (**Figure 2-36**). This finding is novel that the co-deletion of *Pdk1* in the same population of cells, reversed the deficit in LTP we observed in the *Pten* KO mice. Since the mechanisms of LTP and LTD are different next I looked at LTD to see if the DKO mouse also rescued this measure of synaptic plasticity. I induced LTD using the same protocol as mentioned previously in 22 slices from 4 WT littermate mice and 24 slices from 6 DKO mice. There was no significant difference at either the PTD (Mann-Whitney rank sum, $p=0.555$) or a deficit at the 3 hour time point (Mann-Whitney rank sum, $p=0.615$) (**Figure 2-37**). PTD and LTD measurements were averages of 10 stimuli at minute 20 to 24.5 for PTD and minute 187 to 191.5 for LTD.

Previous studies had shown that the additional deletion of *Pdk1* in a heterozygous *Pten* KO mouse model suppressed tumorigenesis (Bayascas et al, 2005) there was no work showing any co-effect in synaptic plasticity. These finding are novel and significant that the effects of *Pten* deletion on synaptic plasticity can be reversed by the additional deletion of *Pdk1*. The deletion of *Pten* would disrupt the PI3K pathway by preventing the dephosphorylation of PIP_3 back to PIP_2 and causing an increase in downstream activation starting with co-localization of *Pdk1* and AKT to the membrane. I would hypothesize this is a rescue of LTP and LTD deficit because of the deletion of *Pdk1* inhibiting the downstream activation caused by the increased amount of membrane bound PIP_3 produced by the *Pten* deletion. Follow up experiments would be required to determine if this really a rescue or if the co-deletion produces normal LTP and LTD through another unknown cellular mechanism in these neurons.

Figure 2-33 Immunohistochemistry for Pten in *Pten/Pdk1* DKO mice.

Immunohistochemistry of 8 week old DKO mice showing continued deletion of *Pten* in this *α CamKII-cre *Pten/Pdk1** DKO mouse model. Brains were fixed in PFA and paraffin embedded followed by microwave antigen retrieval. Probed for Pten (1:100) and peroxidase-conjugated secondary (1:200). Top image 5x image, middle image 20x image of the CA1 and the bottom image CA3.

Figure 2-34 Input/output curve for *Pten/Pdk1* DKO mice.

In acute hippocampal slices field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum with an extracellular glass pipette (3-5 M Ω) filled with ACSF. Schaffer collateral/commissural fibers in the stratum radiatum were stimulated with a bipolar tungsten electrode placed 200 to 300 μ m. Stimulation intensities of 0, 25, 50, 100, 150, 200, 250, and 300 μ A were applied.

Figure 2-35 Pair-pulse ratios of DKO versus WT littermates.

In acute hippocampal slices field excitatory postsynaptic potentials (fEPSPs) were recorded from the CA1 stratum radiatum with an extracellular glass pipette (3-5 M Ω) filled with ACSF. Schaffer collateral/commissural fibers in the stratum radiatum were stimulated with a bipolar tungsten electrode placed 200 to 300 μ m. Stimulus intensities were chosen to produce a fEPSP with a 1 V/s slope based on the input/output curves. Normalized graphs produced from 30 slices from 9 DKO mice and 18 slices from 9 WT mice. Pairs of stimuli of the same intensity were delivered 20 (p=0.75), 50 (p=0.113), 100 (p=0.090), 200 (p=0.026), and 1000 (p=0.029) ms apart and showed significant differences at 200ms and 1000ms interpulse intervals.

Figure 2-36 LTP in *Pten/Pdk1* DKO mice versus WT littermates.

LTP was performed on DKO and WT littermates mice at 8 weeks and induced by 3 periods of 200-Hz tetanization, every 5 min with each period of tetanization consisted of 10 trains of 200-Hz stimulation, for 200 ms (40 stimulations), every 5 s. Normalized graphs produced from 32 slices from 9 DKO mice and 21 slices from 9 WT mice. The rise/ slope (mV/ms) of these traces were measured between 30 and 90 percent. Graphs show no significant differences in averages of 10 stimuli at the post-tetanic potentiation (PTP) ($p=0.659$) and of LTP taken three hours after the induction protocol ($p=0.938$).

Figure 2-37 LTD in *Pten/Pdk1* DKO mice versus WT littermates.

Long-term depression (LTD) was induced using one train of 900 stimulations at 1Hz. The rise/ slope (mV/ms) of these traces were measured between 30 and 90 percent. Normalized graphs produced from 24 slices from 6 DKO mice and 22 slices from 4 WT mice. Graphs show averages of 10 stimuli at the post-tetanic depression (PTD) ($p=0.555$) and of LTD which are not significantly different taken three hours after the induction protocol ($p=0.615$).

CHAPTER 3. DISCUSSION

There is a well-established correlation between the PI3K pathway and synaptic plasticity as presented in the background and introduction material. Since the lipid phosphatase *Pten* is the primary negative regulator of this pathway, I would expect its deletion or disruption to affect multiple cellular processes. *Pten* is predominantly a tumor suppressor and germline mutations of *PTEN* have been shown in human diseases such as Cowden disease, Bannayan-Riley-Ruvalcaba and Lhermitte-Duclos disease, and *PTEN* mutations present an increased risk of cancer (Eng, 2002; Eng, 2003; Liaw et al, 1997; Marsh et al, 1997; Marsh et al, 1998). Once this gene was discovered to play a role in these human disorders it was important to create experimental models to study these diseases. The homozygous deletion of *Pten* proved to be embryonic lethal (Di Cristofano et al, 1998) so work was performed to reveal effects produced by heterozygous deletion. Research showed that this heterozygous *Pten* deletion mouse model produced symptoms similar to the human conditions, with incidences of cancer, intestinal polyps and extreme proliferating cells (Di Cristofano et al, 1998; Podsypanina et al, 1999; Suzuki et al, 1998). These mouse models explored the role *Pten* plays in cell growth regulation, cell migration and insulin signaling (Furnari et al, 1997; Tamura et al, 1998; Waite & Eng, 2002). These human disorders also produce neurological symptoms such as ataxia, seizures and *PTEN* was recently found correlated with autism (Butler et al, 2005). The logical follow-up would involve replicating these neurological effects in a mouse models with *Pten* deletion in neurons.

Since germline deletions didn't produce these neurological effects, work was done to refocus the sub-set of cells in which *Pten* was deleted. Conditionally activated transgenic mice were created using *cre-loxP* recombination technology. This system allows the bacterial enzyme *Cre* recombinase to be expressed under activation of genes that are turned on in a specific subset of cells. Using the gene promoters of *glial fibrillary acidic protein* (*Gfap*) or *neuron-specific enolase* (*nse*) to regulate the expression of *cre*, allows the deletions to be restricted to neuronal cells. These *cre* expressing mice were crossed with mice containing loxP sites, flanking exons 4 and 5 of the *Pten* gene, which encode the phosphatase motif (Suzuki et al, 2001). The *Gfap-cre* transgenic mouse deleted *Pten* in the majority of granule neurons of the cerebellum and dentate gyrus, as well as expression in the CA2 and 3 of the hippocampus, and layer 5 pyramidal neurons. The GFAP promoter is activated at embryonic days 13-14 and deletion was essentially complete by postnatal day 5. These mice showed a progressive increase in brain mass, hydrocephaly, and tonic-clonic seizures around week 4 (Kwon et al, 2001). Even though these mice died between 9 and 48 weeks, they show neuronal cell size increases, with cells 110% of baseline at 2 weeks, 130% at 4 weeks and 155% at more than 20 weeks of age (Kwon et al, 2001). While not as significant, I did see a 117% increase above baseline in the dentate gyrus in the *αCamKII-cre Pten* mouse model at 8 weeks. The *Nse-cre* conditional mouse lines showed a similar effect, with the deletion occurring in layer V of the cerebral cortex, CA3, the dentate gyrus granular layer, and the polymorphic layer of the hippocampal formation. The *nse* promoter is activated in development, and 30-60% of the neurons had recombination by 4 weeks. When measured at 2-4 months,

the brain weight due to macrocephaly was approximately 120% of controls and about 130% at greater than 4 months (Kwon et al, 2006). Since the recombination is focused in the hippocampus in this model, the dentate gyrus was examined. It showed progressive enlargement, which disrupted the pyramidal cell layer, as well as compressing or removing of the CA1 pyramidal cell layer.

The significance of these findings is that previous literature is in direct contrast with my studies. Firstly, the deletion of *Pten* under the *αCamKII* promoter produced *cre*-recombination starting at postnatal day 10 and continuing to day 30 (Yamauchi, 2005). Thus activation in this model doesn't occur until the *Pten* deletion had been completed in the *Gfap-cre* and *Nse-cre* conditional knockout models. This later onset of deletion would allow the migration of the pyramidal cells to be more complete prior to *Pten* deletion. While the hippocampus begins development embryonically, the development continues for approximately one month postnatally (Bayer, 1980). This later onset of deletion could explain why hippocampi of 8-9 week old *αCamKII-cre Pten* KO mice have no changes in the cytoarchitecture (**Figure 2-7**). Immunohistochemistry and western blots performed in these mice show that *Pten* deletion has occurred (a 40% reduction of Pten in total hippocampal lysates) and this deletion is affecting downstream phosphorylation, as seen in increased phosphorylation of Akt at both S473 and T308 (**Figure 2-5**). These mice exhibit macrocephaly and seizures similar to previous models, with an unknown cause of mortality. The maximum life span is 98 days with the mean mortality at 77 days and death occurring as early as 67 days. Mice were selected at 8-9 weeks of age for examination of the *Pten* deletion effects, to avoid confounding seizure development toward the end of life. Therefore *cre*-activation had been operational for approximately 53 days (9 weeks minus the 10 day postnatal onset of *αCamKII* expression) prior to experimentation. In comparison the *Gfap-cre* conditional knockout exhibited disruption in hippocampal morphology at 310 days (10 months plus an additional 10 days during prenatally) (Kwon et al, 2006). While Pten may affect neuronal migration, hippocampal structure in the *Gfap-cre* mice at 42 days shows no changes (Kwon et al, 2006). At 42 days the *cre* recombination has been well established and Pten deletion completed, yet the hippocampal structure remains unchanged. So the laminar organization of the hippocampus appears to change over time, even after the *Pten* deletion is complete. This time course could explain why I don't observe disruption of hippocampal structure, since this mouse line cannot be evaluated at a comparable age due to the mortality of these mice. It is possible that functional Pten must be absent from the neurons for greater than 53 days or must be removed at a specific time during development before morphological changes will occur. This could explain the dichotomy between previously published work and my findings.

To further address the absence of gross morphological effects in a *αCamKII-cre Pten* KO mouse, soma size was observed. Soma diameters were significantly enlarged in both *Gfap-cre* and *Nse-cre* conditional knockout models. In the *Gfap-cre* mouse model the soma diameter increased to 130% of WT littermates by 4 weeks, and the average surface area of the dentate gyrus granule cells was approximately two-fold greater at 4 weeks (Kwon et al, 2001). In the *Gfap-cre* mouse model there was a cell size increase produced after only a three week period of *Pten* deletion, as measured after the majority

of cells expressed *cre* in the dentate gyrus. This time course allowed me the ability to compare soma size in the two mouse models. In the *Nse-cre* conditional knockout models the relative soma diameter increased to 120% above controls between 4-6 months of age. Removal of *Pten* is in effect for about 20 weeks at that age (Kwon et al, 2006).

In the *αCamKII-cre Pten* KO model, I did not see any obvious size increases using H&E staining or *Pten* immunohistochemistry as measures of soma diameter in the CA1 and CA3 regions. This is a novel characteristic of this specific transgenic mouse. Since this goes against the standard in the field, it was critical to thoroughly prove a lack of cell size increase in the CA1 and CA3 neurons. For statistical purposes to show that these cells don't increase, paraffin tissue sections were stained with H&E as a starting point, which allowed a greater *n*. Since it was difficult to determine the clear outline of the soma in these preparations, nuclear diameter was used as a representation of soma size, as had previously been shown (Fraser et al, 2008). To insure compensation for the natural variation within neuronal cell sizes, images of the CA1, CA3 and dentate gyrus were taken from 6 to 9 slides and no less than 500 nuclear diameters measured per slide. The measures were averaged with-in slide before the means per slide were also averaged. Based on the nuclear diameter as a representation of soma area there were significant differences in CA1 and CA3, actually showing a smaller cell size in the *Pten* KO mice. There wasn't significance in the dentate gyrus using the H&E stained sections but *Pten* immunohistochemistry displayed the expected enlargement. This indicated that the cellular enlargement may not have occurred in the CA1 and CA3 areas of the hippocampus but it could manifest if we looked at these mice at a future time point. This enlargement in the dentate gyrus reassured us that while the neurons involved in LTP and LTD in the hippocampus did not enlarge like previous models, the *αCamKII-cre* KO *Pten* KO mice were crossed with a YFP reporter mouse, also utilizing *cre*-recombinase technology. This mouse allowed me to determine that the CA1 region was the only area that had robust enough deletion of *Pten* to use the H&E stained tissue to measure size, with 76% of neurons *Pten* negative, versus the 7% in the CA3 region. Since the deletion is mosaic and many cells in the *Pten* KO CA3 and dentate gyrus were still positive for *Pten*, nuclear diameter was re-measured in the CA3 and dentate gyrus using slides stained for *Pten* IHC. There was no difference in the CA3 region but there was a significant increase in the nuclear diameters in the dentate gyrus *Pten* KO cells. This is the first sign that the *αCamKII* driven *Pten* KO deletion in this mouse may cause the same neuronal hypertrophy if it were possible to allow these mice to age past 9 weeks.

To ensure the deletion of *Pten* wasn't inducing cell death or that cellular proliferation was limiting potential for expansion, density was also experimentally determined. There was no difference at the CA3 or dentate gyrus but there was a significant increase in the number of cells in the CA1 region. However this increase was from 1.3 neurons per 20 μm^2 to 1.5 neurons per 20 μm^2 . This minute difference could be attributed to innate variation in pyramidal cell size. These measures were nuclear diameter and while indicative of soma size, this measure does not represent complete soma area. For more accurate measures of soma area Golgi stain was performed on *Pten* KO and WT mice, which allows a subset of cells to be filled completely through still

unknown selection criteria. Once again at 8 weeks old there was no difference in the area of the soma taken from a filled silhouette. Even though the area of these somata were available to measure there was still potential variation produced by the fixation and staining protocol that could obscure differences in soma size.

To address the limitations produced with fixed tissue the previously mentioned YFP reporter mouse was crossed with the *α CamKII-cre Pten* KO mice to allow visualization of neurons containing Pten in live tissue. The 2-photon imaging system allowed easy identification of those cells expressing YFP. Dual labeling florescent IHC showed us that the CA3 region is unreliable in its *cre*-recombination, so all YFP cells were selected from the CA1 region. Once the selected cell was patched, a brighter flourophore could be infused to allow the cellular morphology to be reconstructed and analyzed. Once again, using a third experimental method there were still no differences in *Pten*^{-/-} neurons and *Pten*^{+/+} neurons. These measures clearly establish that there are no differences in the soma size between the *Pten* KO and WT littermate mice.

The soma area was not the only morphological change that had been previously reported in *Pten* conditional KO mice. The *Gfap-cre* mice exhibited changes in the size of dendritic branches as well as a greater density of spines, with the shape of the spine lacking the rounded terminal head (Fraser et al, 2008). Spine density was examined based on these previously reported changes, as well as spine length and width using the 2-photon microscopy imaging of YFP and non-YFP neurons. None of these measures showed significant differences nor did the total length of the apical dendrite. This confirms that at even closer examination the previously reported increases were not present in these mice.

The *Gfap-cre Pten* KO mice also showed effects of the deletion at sub-cellular levels as measured with electron microscopy. These mice showed abnormalities in myelination and the presynaptic terminals were enlarged, and increased numbers synaptic vesicles compared to controls. Normal synaptic connections were hard to identify, particularly since the majority of the synapses were missing the postsynaptic density, while in those synapses that had a postsynaptic density it was enlarged. To complete the morphological analysis of these mice, electron microscopy was performed to visualize any subcellular differences. Five criteria were selected to quantify synaptic changes under electron microscopy and there were no differences in three measures: the total presynaptic area, the total number of vesicles counted in the presynaptic area and the number of vesicles that were docked into the active zone (within 150nm from the synaptic cleft). These are also in direct contrast with the previous work showing enlarged presynaptic areas (Fraser et al, 2008). There were no problems identifying synapses and no differences were found in the presynaptic area. The extreme error bars on **Figure 2-16** were indicative of the range of sizes in presynaptic areas. Since the electron microscopy images were taken as a cross section approximately 70nm thick, this provides an incomplete picture of the total 3-dimensional area of the presynaptic side. It would have been more informative to complete serial reconstructions of the presynaptic area to compare the volumes between the KO mice and their littermate controls. However, this process is challenging to perform, as well as time consuming, and a smaller statistical

sampling would have been obtained. The information is still relevant, as an equal cross sectional presynaptic areas between the two specimens was compared. The issue also arose that the mosaic deletion of *Pten* left *Pten* present in a population of neurons in *Pten* KO sections. I attempted to address this using an electron dense producing reporter mouse for *Pten* that could be visualized with electron microscopy. Using the same principal of the YFP reporter mouse I crossed the *Pten* KO mouse with a Z/AP mouse (Lobe et al, 1999). The Z/AP mice contain a *cre* activated construct that upon *cre*-recombination converts the gene expression from production of lacZ to expression of the human alkaline phosphatase gene. After production, alkaline phosphatase gets transported to the membrane of the cell and can be stained for visualization using electron microscopy (Hugon & Borgers, 1966). Using this reporter mouse it was possible to visualize under electron microscopy the thickened cell membranes that marked *cre*-positive; *Pten* null cells. This method however had a flaw, rendering it impossible to distinguish between alkaline positive and negative cells. The majority of the cells contained the electron dense product and those that didn't shared membranes obscuring the difference. The aim was to identify alkaline phosphatase negative cells interspersed between those expressing alkaline phosphatase for a control within section, but negative cells could not be identified. While this reporter was unhelpful for its intended use, it did produce confidence that the majority of the postsynaptic synapses in the *Pten* KO tissue samples measured were negative for *Pten* and an effective representation of the deletion.

Two measures of electron microscopy produced a difference; the vesicle diameter and the postsynaptic density lengths. The vesicle diameters were significantly smaller in the *Pten* KO mice. This measure could give a functional effect through a smaller volume of neurotransmitter release from the presynaptic side. The electron microscopy work was performed with the intention of examining the CA1 cells, as they have more robust deletion; however the synaptic regions examined are most likely formed from CA3 axonal projections that would be less likely *Pten* null. If the presynaptic neurons did have a change in neurotransmitter release this would be reflected in the mEPSCs recorded from the postsynaptic cells. The amount of neurotransmitter release would be visualized in the amplitude of the currents, or in the rise time of the peaks. Since there were no differences in either of these two functional measures, it's difficult to attribute significance to this change in vesicle diameter. The other measure that revealed a change was the postsynaptic density length, which is visualized as a thickening on the postsynaptic membrane where the synaptic cleft has formed. This density is due to the greater number of membrane receptors focused at that point. An enlargement of the density could indicate a greater number of postsynaptic receptors and also produce a functional change. To look at the two major receptors in this system whole-cell patch clamp was performed to visualize the ion currents through these receptors. Specifically I looked for changes in the ratio of AMPA to NMDA receptors. Since the AMPA receptors activate and open their ion channel first in response to glutamate release, their current is distinguishable from the NMDA current with its delayed response due to the voltage-gated Mg^{2+} block. There were no changes in the ratios of these receptor types postsynaptically. This doesn't exclude the possibility of an equal up-regulation of both AMPA and NMDA in response to *Pten* deletion, which could be examined with immunohistochemistry for AMPA and NMDA receptors or western blots performed on

protein fractions from synaptosomes. Other receptors or membrane bound molecules could also be increased in these *Pten* KO mice causing the enlarged postsynaptic density.

So the novel conclusion from the first part of my studies is that unlike previously reported conditional deletion of *Pten* in neurons, there are no significant morphological changes in the CA1 and CA3 regions of the hippocampus in this *αCamKII-cre Pten* KO mouse. The second part I examined was whether this *Pten* deletion that lacked morphological changes produced any functional effects.

Previous work had shown a *Pten* heterozygous deletion caused a reduction in LTP and the inability to produce LTD (Wang et al, 2006). The *Gfap-cre Pten* KO mice also showed large deficits in 200Hz induced LTP in addition to defects in basic synaptic firing as shown in input/output curves (Fraser et al, 2008). Acute hippocampal slices were produced and fEPSPs were measured to give an indication of general population changes between the CA3 and CA1 hippocampal cells. The first experimental measure performed was basic synaptic transmission using input/output curves and no difference between the *Pten* KO slices and their WT littermates was observed. This finding was not unexpected since the structure of the hippocampus, due to the later onset of *cre*-recombination, is intact, and therefore the synaptic connections are probably intact as well. Next paired-pulse facilitation in these slices was recorded, which also proved unchanged. As a cursory measure of presynaptic function this finding was unsurprising due to a greater percentage of CA3 cells still containing *Pten*. These findings were not unexpected since both of these are measures of short-term firing properties and the PI3K pathway's effect would be on the cellular changes that would occur after long-term changes.

The next thing to look at was long-term changes in synaptic efficacy that might require translation mechanisms through the PI3K pathway. Using a protocol to induce compound LTP, I looked at the long-term effects of *Pten* deletion on hippocampal synaptic firing. There is a significant difference in LTP after 3 hours as well as immediately following the stimulus protocol (PTP). This is striking because it is the first measure of *Pten*'s role in long-term changes in a KO mouse model that has intact cellular migration and cellular connections, and a focused deletion to critical neurons. LTD was also examined in this preparation and there was a significant decrease in the amount of depression induced at the 3 hour time point. These findings are consistent with previous work (Fraser et al, 2008; Wang et al, 2006), but are essential because they show a more direct correlation between *Pten* and long-term synaptic changes.

While these functional changes occurred in the system as a whole, I wanted to determine how *Pten* was causing these changes and then where intercellularly *Pten* was affecting the plasticity. There was no LTP phenotype in the *Grik4-cre Pten* KO mice and this conditional knockout was designed to produce *Pten* deletion in only the presynaptic CA3 cells. *Pten* removal from the presynaptic cell produces no effect on compound LTP and since there are no changes in the spontaneous release from presynaptic neurons as measured in the mEPSCs, it is possible to conclude *Pten*'s effect on LTP is not due to presynaptic neurons.

While the mechanism of action remains undetermined I still wanted to see if a rescue some of the effects produced by the *Pten* deletion was possible. Previous studies have shown that the downstream pathway molecule Pdk1 can rescue some of the effects caused by *Pten* deletion (Bayascas et al, 2005; Chalhoub et al, 2009). Mice with exons 3 and 4 of the *Pdk1* gene were flanked by loxP sites and crossed with the *αCamKII* promoter *cre* line. First the effects of *Pdk1* deletion alone were examined and no differences in synaptic transmission or paired-pulse facilitation were observed. LTP and LTD were also experimentally produced in the *αCamKII-cre Pdk1* KO mice and there were no differences in these measures of long-term synaptic plasticity. This was unexpected since removal of Pdk1 would reduce the ability to activate the downstream molecules such as Akt. I would hypothesize this to change the pathway in the opposite direction of the *Pten* deletion and cause an increase in the long-term synaptic plasticity. However when these mice were crossed with the *αCamKII-cre Pten* KO mice to form a double KO (DKO) this completely abolished the deficit seen in LTP and LTD in the *αCamKII-cre Pten* KO mice. Immunohistochemistry verified that these mice were still missing *Pten* in the same population of neurons as the previous strain (**Figure 2-33**). The DKO mice also showed no differences in synaptic firing through the input/output curves or in pair-pulse facilitation. This finding, while in agreement with results in previous rescues, is impressive due to its complete rescue of the *αCamKII-cre Pten* KO deficit by the additional deletion of a gene that alone produces no electrophysiological phenotype.

Since *Pten* is the negative regulator of the PI3K pathway it would be expected that its removal would upset the pathway. Deletion causes an up-regulation of the downstream molecules as exhibited by the increased phosphorylation of Akt and S6. Since Akt is phosphorylated by Pdk1 at the T308 site, causing the downstream phosphorylation of TSC2 Akt needs to be phosphorylated on two sites to be activated. So removal of Pdk1 would prevent downstream protein synthesis by preventing the TSC1/2 complex from inhibiting Rheb and allowing activation of mTORC1. Interestingly deletion of *Pten* would allow more PIP₃ into the system by not dephosphorylating it back to PIP₂. This would cause more Akt and Pdk1 to translocate to the membrane to bind their PH domains and creating a down-stream up-regulation of protein synthesis. So in the *Pten* KO model, which shows a deficit in LTP and LTD, the deficit could be dependent on protein synthesis that I hypothesize is actually an up-regulation of protein production. In the *αCamKII-cre Pdk1* KO mouse there would be an expected decrease in protein production by limiting the downstream activation of Akt, but this mouse model shows no deficits in LTP or LTD. The deletions of these genes are causing changes in the PI3K pathway at its resting state. It is possible that the mechanism for the deficit is actually an overabundance of protein production to the extent that the system is close to saturation and the increase needed to create plasticity changes cannot occur. The LTP and LTD deficits are only a reduction, not a complete abolishment, so there is still an ability to maintain baseline protein synthesis. In instances where the production would need to be increased, the cellular machinery is already at its maximum and can't meet the full requirements.

Since these experimental protocols and preparations were developed with the hope of studying learning and memory, it would be important to also directly test this in

vivo. So the *αCamKII-cre Pten* KO mice were provided to Raymond Berry, PhD to study their behavior in the Morris water maze. While the data provides an additional story about the application of my research, Dr. Berry performed these experiments without assistance and therefore the data is included as an appendix. Since LTP is the model for learning and memory and we observe a deficit in LTP in the *Pten* KO mice, it would be expected to observe a deficit in their behavior. These mice decreased the distance to find the submerged platform over the 10 day training period (**Figure B-1, Figure B-2**). When tested for their ability to recall the location of the platform on the day following completion of their training, the *αCamKII-cre Pten* KO mice were significantly within the target quadrant, but the number of times they passed directly over the platform location was about half as often as their WT littermates (**Figure B-3, Figure B-4**). This indicates that the reduction seen in the LTP studies is physiologically relevant to the animals' formation of memories. The ability of the *Pten* KO mice to learn effectively over the spatial training period may indicate a role for Pten in the retrieval of memory but not necessarily the formation of memories.

These studies show that this conditional deletion of *Pten* in the hippocampus causes a deficit in the formation of long-term synaptic changes, such as LTP and LTD. A novel finding from this work is that these changes occur without the morphological disruption and size increases typically seen in *Pten* KO mouse models. In fact the functional changes caused by the deletion could be the catalyst that induces the cell size growth and changes in these neurons. The Pten deletion might be disrupting the pathway and the neuronal compensation is enlargement. This is indicated by previous work showing that the disruption of the dentate gyrus actually occurred well after the *Pten* deletion was activated, at day 42 (Kwon et al, 2006). While the exact mechanism for the action of Pten and specifically the pre or postsynaptic side of the synapse remains undetermined I believe I have demonstrated an important role of Pten in long-term synaptic plasticity and learning and memory.

CHAPTER 4. MATERIALS AND METHODS

The following is a list of the materials and methods that were used to complete the aforementioned experiments.

Animal breeding and analysis. The previously described *Pten*^{+/loxP}; conditional knockout mice (Backman et al, 2001; Kwon et al, 2001) were crossed with the *αCamKII cre-loxP*, T29-1 founder line as developed by the Tonegawa lab (Tsien et al, 1996). Both these strains were also crossed with floxed *Pdk1* mice (Lawlor et al, 2002) to create the three lines of mice used in this study: *Cre;Pten*^{fl/fl}; *Pdk1*^{+/+}, *Cre;Pten*^{fl/fl}; *Pdk1*^{fl/fl}, and *Cre;Pten*^{+/+}; *Pdk1*^{fl/fl}. The *Pten*^{+/loxP} mice were also crossed with Grik4 *cre-loxP* mice developed by the Tonegawa lab (Nakazawa et al, 2002). For genotype combinations, *cre* negative *Pten* or *Pdk1* mice were used as WT littermates.

Animal housing and use were in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the local institutional animal care committee at St. Jude Children's Research Hospital.

Brain slice preparation. Acute transverse hippocampal slices (400 μm) were prepared as previously described (Bayazitov et al, 2007). Briefly, mouse brains were quickly removed and placed in cold (4 °C) dissecting artificial cerebrospinal fluid (ACSF) containing 125 mM choline-Cl, 2.5 mM KCl, 0.4 mM CaCl₂, 6 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 20 mM glucose (285-295 mOsm), under 95% O₂/5% CO₂. After dissection, slices were incubated for 1 h in ACSF containing 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, and 10 mM glucose (285-295 mOsm), under 95% O₂/5% CO₂ at room temperature and then transferred into the submerged recording chamber and superfused (2-3 ml/min) with warm (30-32 °C) ACSF.

Field potential recordings. The field recordings were performed using a setup with 8 submerged recording chambers (Campden Instruments, Lafayette, IN). I recorded field excitatory postsynaptic potentials (fEPSPs) from the CA1 stratum radiatum by using an extracellular glass pipette (3-5 M Ω) filled with ACSF. Schaffer collateral/commissural fibers in the s. radiatum were stimulated with a bipolar tungsten electrode placed 200 to 300 μm away from the recording pipette. Stimulation intensities were chosen to produce an fEPSP with a 1 V/s slope based on an input/output curve from stimulations of 0, 25, 50, 100, 150, 200, 250, and 300 μA. Paired-pulse facilitation (PPF) experiments were performed using a pair of stimuli of the same intensity delivered 20, 50, 100, 200, and 1000 ms apart.

LTP was induced by 3 periods of 200-Hz tetanization delivered every 5 min using the stimulation intensities that were chosen to produce an fEPSP with a 1 V/s slope. Every period of tetanization consisted of 10 trains of 200-Hz stimulation delivered at the same intensity for 200 ms (40 stimulations) every 5 s. A similar protocol has previously been used to induce compound (presynaptic and postsynaptic) LTP at CA3-CA1

synapses in the hippocampus (Cavus & Teyler, 1996; Zakharenko et al, 2003; Zakharenko et al, 2001). 50Hz LTP was induced by 3 trains of 50 stimulations separated by 5 minute intervals. LTD was induced with one train of 900 stimulations at 1Hz stimulation.

Whole-cell electrophysiology. Whole-cell recordings were obtained from the cell bodies of CA1 neurons. For current-clamp recordings, patch pipettes (open pipette resistance, 3-5 M Ω) were filled with an internal solution containing 140 mM KMeSO₄, 8 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, 5 mM MgATP, 0.4 mM Na₂ GTP, and 10 to 25 μ M Alexa 594 (pH 7.3). For voltage-clamp recordings, I replaced the potassium-based solution with a cesium-based internal solution. Whole-cell recordings were registered using a Multiclamp 700B (Molecular Devices, Sunnyvale, Ca), digitized (10 kHz; DigiData 1322A, Molecular Devices), and recorded using pCLAMP 9.0 software (Molecular Devices). Spontaneous miniature excitatory postsynaptic currents (mEPSCs) were recorded at -70 mV holding potential in the presence of picrotoxin (100 μ M) and tetrodotoxin (1 μ M) in the extracellular solution for at least 1 h. Amplitude, 10% to 90% rise time, decay time constant, and inter-event intervals of mEPSCs were analyzed off-line using the Mini-Analysis Program (Synaptosoft Inc., Leonia, NJ). All software detected events were verified visually. The current ratio of AMPA receptors to NMDA receptors (AMPA/NMDAR) was calculated from the EPSC traces recorded at +40 mV. The amplitude of stimulation was adjusted to evoke 50 pA EPSCs at -70 mV. The AMPAR current was determined at time points when EPSCs recorded at -70 mV reached their peaks, and the NMDAR currents were determined 100 ms after the peaks. EPSCs were analyzed off-line using Clampfit 10.1 software (Molecular Devices).

Two-photon imaging. Two-photon laser-scanning microscopy (TPLSM) was performed using an Ultima imaging system (Prairie Technologies, Middleton, WI), a Ti:sapphire Chameleon Ultra femtosecond-pulsed laser (Coherent Inc., Santa Clara, CA), and 60 $\times$ (0.9 NA) water-immersion IR objectives (Olympus, Center Valley, PA). Alexa Fluor 594 was included in the internal solution (see above) and was excited at 820 nm. Alexa Fluor 594 fluorescence (red channel) was used to image and reconstruct dendritic and axonal morphology of CA1 neurons with the additional YFP reporter construct allowing for selection of Pten^{fl/fl}; α CamKII-cre neurons with 76% accuracy. ImageJ was used to analyze dendritic branching and morphology of dendritic spines.

Spatial memory testing. I tested spatial memory in the Morris water maze. I used a circular steel water maze (diameter, 4 ft; depth, 2 ft) filled with water (room temperature) clouded with white, nontoxic, water-based paint. Compass points labeled along the rim served as trial starting positions. For the spatial tasks, water levels were raised 0.25 in above the clear, Plexiglass escape platform. For the nonspatial task, the water level was lowered so that the escape platform was visible 0.25 in above the water's surface. The water maze environment was full of visual cues whose locations remained fixed throughout the learning protocol. Mouse movements in the maze were recorded using a video camera tracking system (HVS Image, Co., Buckingham, UK) mounted above the pool, and path length was measured. Animals learned to find a hidden platform in the training (TRA) quadrant using the standard spatial version of the Morris water maze task

for 10 successive days. Each day, animals were given four 1-min trials from each starting position with an inter-trial latency of at least 60 s. The order of starting locations was counterbalanced each day using a Latin square design.

A spatial memory (probe) trial was administered on the day following the completion of spatial learning. With the platform removed, animals received a single 1-min trial in which the animal tried to find the escape platform in the TRA quadrant. This trial started from the point that was the farthest from the platform's location on the previous training day. The overall path length was measured for each mouse, and the relative path length for each quadrant was calculated.

Mice started nonspatial learning tasks at least 8 days after completion of the spatial protocol. In this task, the platform was visible above the water's surface. Animals were trained using the standard nonspatial version of the Morris water maze task for 5 successive days. During training day 1, they saw the escape platform located in the same position used during spatial training. Each day thereafter, the escape platform was rotated, in a clockwise manner, to the next quadrant. Each day, animals were given four 1-min trials in the same manner that occurred during spatial training.

Electron microscopy. Hippocampal slices were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, thrice rinsed in the same buffer, and dehydrated in a graded series of alcohol and then propylene oxide washes. The tissue was infiltrated and embedded in epon araldite and polymerized overnight at 70 °C. 70-nm sections were cut on a Leica UC6 ultramicrotome fitted with a Diatome diamond knife and stained with lead citrate and 8% uranyl acetate. Thick sections were trimmed to a region containing the CA1 cell body layer where synapses were identified. The sections were imaged on a JEOL 1200EX11 transmission electron microscope with an AMT XR111 11 megapixel digital camera. Synapses were counted as regions of membrane enclosing synaptic vesicles in close proximity to a postsynaptic density. Vesicle size and number and postsynaptic density length were measured in ImageJ by tracing with the line or elliptical selection tools followed by measurement. Active zone vesicles were determined as vesicles 150nm from the synaptic cleft.

Immunohistochemistry. Mice were perfused transcardially with PBS, followed by 4% paraformaldehyde (PFA) in PBS. Tissues were post-fixed in 4% PFA overnight, cut into two halves sagittally embedded in paraffin and then cut into 5- μ m thick sections. Littermate controls were of all genotypic combinations without *cre* and were processed at the same time as mutant littermates. Using microwave antigen retrieval, sections were probed using primary antibody Pten (1:100; #9559, Cell Signaling). Detection of primary antibodies was performed using biotinylated secondary antibodies (1:200, Vector labs) peroxidase-conjugated avidin (Elite ABC, Vector Labs) and treatment with DAB substrate and hematoxylin counterstain (Vector Labs). ImageJ was used to analyze nuclear diameter.

For dual labeling fluorescent immunohistochemistry for Pten and YFP mice were perfused transcardially with PBS, followed by 4% paraformaldehyde (PFA) in PBS.

Tissues were post-fixed in 4% PFA overnight and then submerged overnight in 25% sucrose in 1XPBS. Tissue was frozen and sectioned. Probed for Pten (1:100 # 9559, Cell signaling) and GFP (1:1000) (ab5449, Abcam) with FTIC conjugated secondaries (1:400) (ab97050, and ab97099 respectively Abcam). Sections were mounted with vectashield with DAPI.

Nuclear size measurement and cell counting. All mice were selected at 8 weeks of age and perfused with and then submerged in 4% paraformaldehyde overnight. Brains were embedded in paraffin and bilaterally divided and 5µm sections of the whole brain. Tissue sections were stained for hematoxylin and eosin, and 20x images were taken of the CA1, CA3 and dentate gyrus cell bodies from sections in approximately 150µm increments. Nuclear diameter was measured using ImageJ at the widest point of each nuclei in all cells in each of the three areas in the hippocampus. The cell density was measured counting all cell bodies in each image. To compensate for the natural variation within neuronal cell sizes, images of the CA1, CA3 and dentate gyrus were taken from between 6 and 9 slides and no less than 500 nuclei diameters measured per slide. The measures were averaged within slide before the means per slide were also averaged.

Western blotting. Unilateral hippocampi were dissected, snap frozen and the tissue was lysed using disposable pestles and microtubes (Fisher Scientific) in ice-cold RIPA buffer (50 mM Tris-HCl [pH 7.5], 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, and 0.1% SDS and supplemented with protease and phosphatase inhibitions [Roche, Indianapolis, IN]). 25-µg of protein extract was electrophoresed on 10% SDS-PAGE gels (Invitrogen), and protein was transferred to PVDF (Invitrogen). The primary antibodies used for chemiluminescent detection were Pten (1:1000; #9559, Cell Signaling), pAkt (T308) (1:1000, Cell Signaling, #9275), pAkt (S473) (1:1000, Cell Signaling # 9271), total Akt (1:1000, Cell Signaling # 4691), p-S6 (Ser235/236) (1:1000, Cell Signaling # 2211), total S6 (1:1000, Cell Signaling # 2217), mouse anti-β-actin (1:5000, #A5441, Sigma). Blots were probed with secondary antibody goat anti-rabbit IgG HRP (1:5000, Elite ABC, Vector Labs). For quantifiable fluorescent blots the primary antibodies used were Pten (1:1000; #9559, Cell Signaling), GluR1 (AMPA subtype) (1:500, ab31232, Abcam Inc.), NMDAR1 (1:500, ab28669, Abcam Inc.), Anti-GluR 2&3 (1:1000, #AB1506, Millipore Corp) and mouse anti-β-actin (1:10000, A5316, Sigma). Western blots were probed with anti-mouse (1:5000) and anti-rabbit (1:5000) secondary antibodies conjugated to IR dye 680 or 800 (LI-COR Biosciences, Lincoln, NE). Blots were imaged and quantified using the Odyssey infrared imaging system (LI-COR Biosciences).

Statistics. All data are represented as mean ± standard error of the means. Statistics for all experiments, except behavioral testing studies, were computed using nonparametric Mann-Whitney rank sum and Wilcoxon signed rank tests or t-test measured in Sigma Stat (Systat Software, Inc., Point Richmond, CA). Statistics for behavioral testing was computed using repeated measures ANOVA measured in SPSS Statistics (SPSS Inc., Chicago, IL).

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APPENDIX A. SUPPLEMENTAL FIGURES TO CHAPTER 2

Figure A-1 Variability of nuclear diameters in CA1 neurons with-in mouse.

Imaging of CA1 nuclear diameters in H&E stained sections taken from slides sectioned 150 μ m apart. Between 86 and 193 nuclei were measured from each slide. The ** signifies p-values of ≤ 0.001 with * < 0.05 .

Figure A-2 Variability of nuclear diameter in CA3 neurons with-in mouse.

Imaging of CA3 nuclear diameters in H&E stained sections taken from slides sectioned 150 μ m apart. Between 78 and 194 nuclei were measured from each slide. The ** signifies p-values of ≤ 0.001 with * < 0.05 .

Figure A-3 Composite z-stack of 2-photon image showing an abundance of YFP labeled cells in CA1.

2-photon microscopy imaging of z-stack images of YFP neurons in the CA1 region demonstrating the abundance of labeled neurons.

Figure A-4 2-photon image of YFP positive and negative cells before filling with Alexa dye.

2-photon imaging of CA1 region in YFP- reporter mice with the bottom right imaging demonstrating the ability to visualize and patch a non-YFP labeled cell and fill with Alexa 594 dye through the displayed electrode and excited at 820nm. All images are the same field of view with the bottom right image exhibiting all neurons.

APPENDIX B. BEHAVIORAL DATA PERFORMED BY RAYMOND BERRY

Spatial memory was tested in the Morris water maze, which was performed and analyzed by Raymond Berry. He used a circular steel water maze (with a diameter of 4 feet and a depth of 2 feet) filled with water (room temperature) clouded with white, nontoxic, water-based paint. Compass points labeled along the rim served as trial starting positions. For the spatial tasks, water levels were raised 0.25 in above the clear, Plexiglass escape platform. For the nonspatial task, the water level was lowered so that the escape platform was visible 0.25 in above the water's surface. The water maze environment was full of visual cues whose locations remained fixed throughout the learning protocol. Mouse movements in the maze were recorded using a video camera tracking system mounted above the pool, and path length was measured. Animals learned to find a hidden platform in the training (TRA) quadrant using the standard spatial version of the Morris water maze task for 10 successive days. Each day, animals were given four 1-min trials from each starting position with an intertrial latency of at least 60 s. The order of the starting locations was counterbalanced each day using a Latin square design. The Latin square design is a row-column system that allows the each variable to be listed once only in each row and each column.

Pten KO mice and their WT littermates effectively mastered the spatial learning over the 10 day training period as measured by the mean path length in meters (**Figure B-1**). The learning over the trial day showed significance (ANOVA, $p < 0.001$) however there was no significant difference at day 10 using this method of measurement. Another method to interpret the swimming pattern of these mice is to use the Gallagher proximity measure, which is the distance to the platform from the mouse at any given time point (Gallagher et al, 1993). The Gallagher proximity for these mice also showed their ability to find the platform was unimpaired with the distance on training day 1 and training day 10 significantly different ($p < 0.001$) for both groups. Using this measure a difference could be detected, with significant differences between the WT and *Pten* KO mice at the final training day, with the WT mice significantly closer to the platform ($p = 0.003$) (**Figure B-2**). This shows a slight deficit in the learning for the KO mice.

A spatial memory (probe) trial was administered on the day following the completion of spatial learning. With the platform removed, animals received a single 1-min trial in which the animal tried to find the escape platform in the TRA quadrant. This trial started from the point that was the farthest from the platform's location on the previous training day. The overall path length was measured for each mouse, and the relative path length for each quadrant was calculated. Both groups of mice showed a significant portion of their path length in the target quadrant (**Figure B-3**). This showed the mice were able to recall the correct quadrant where the platform had previously been. A more specific measure of the ability to recall the location of the platform was the number of times the mice passed over the location where the platform had been in the training trials (**Figure B-4**). In the probe trial, broken down into 10 second intervals, there was a significant difference of the number of passes the WT mice made versus the

Figure B-1 Pten KO versus WT mean path length during watermaze training.

Distance in path length (meters) of the Pten KO and WT mice during the 10 day training period. Mice showed significant differences between day 1 and day 10 (ANOVA $p < 0.001$) but no significant difference at day 10 between the groups of mice ($p = 0.268$).

Figure B-2 Gallagher proximity during spatial training.

Distance from the mouse to the platform as measured in Gallagher proximity across the 10 day training period. A significance difference ($p=0.003$) of the proximity of the mouse to the platform was seen between the WT and Pten KO mice at day 10.

Figure B-3 Pten KO versus WT mice percent of path spent in 4 quadrants of the water maze during the probe trial.

Mice tested for a 60 second interval 24 hrs after the end of the training period. WT and *Pten* KO mice both showed significant more path distance (meters) in the target quadrant over the other 3 quadrants.

Figure B-4 Pten KO versus WT mice counter passes over the platform during the probe trial.

Number of counter passes over the platform during the probe trial as broken down in 10 second intervals. Passes were significant between WT and Pten KO mice at each of the 10 second time points (the * denotes $p < 0.001$).

Pten KO mice. This shows that the *Pten* KO mice had an impairment in their ability to recall the location of the platform.

Mice started nonspatial learning tasks at least 8 days after completion of the spatial protocol. In this task, the platform was visible above the water's surface. Animals were trained using the standard nonspatial version of the Morris water maze task for 5 successive days. During training day 1, they saw the escape platform located in the same position used during spatial training. Each day thereafter, the escape platform was rotated, in a clockwise manner, to the next quadrant. Each day, animals were given four 1-min trials in the same manner that occurred during spatial training. This was to insure that the mice's ability to visually identify the cues that were placed as well as the ability to swim effectively. There were no differences in the non-spatial tasks between either mouse groups.

The previous tests of long-term synaptic plasticity in acute hippocampal slices were to test experimental measures of learning and memory. However the experimental conditions are artificial so it is advantageous to test these same measures in a behaving animal. The measure of the Morris water maze is to test spatial learning and the differences that were displayed correlate directly to the deficits I saw in LTP. Therefore I can be thoroughly sure that the *Pten* deletion caused deficits in hippocampal dependent memory as demonstrated through in vivo and in vitro experimental preparations.

VITA

Margaret Sperow was born in Philadelphia, Pennsylvania in 1982. She graduated from Springside School for girls in Chestnut Hill, PA in 2001. The following fall she started college at Ursinus College in Collegeville, PA graduated in May 2005 with a Bachelor's of Science Degree in Biology/Neuroscience. In August 2005 she started in the University of Tennessee Health Science Center's Integrated Program in Biomedical Sciences and selected the Neuroscience Track. In May 2006 Margaret joined the lab of Stanislav S. Zakharenko at St. Jude Children's Research Hospital and completed her doctorate degree in December 2011.