ABSTRACT

The Role of the mTOR Pathway in Learning and Memory

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Research implicates the mTOR pathway, now known to be a regulator of protein synthesis, as a major player in both neuronal development and cognition. This study demonstrates, both through experimentation and a review of the literature, how disrupting the function of the mTOR pathway has adverse effects on learning and memory. Through delayed fear conditioning procedures, trace fear conditioning procedures, and a novel object test, this study demonstrates marked deficits in learning and memory in homozygous PTEN knockout mice, specifically in the Gfap-Cre^{loxP} induced knockout. This opens the door for further research on treatments with the potential to alleviate the symptoms of the knockout that may then be implemented in the treatment of hyperactive mTOR related disease.

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THE ROLE OF THE MTOR PATHWAY IN LEARNING AND MEMORY

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CHAPTER 1

Introduction to Learning and Memory

An organism's capacity for learning and memory is essential to survival. These processes are adaptive, allowing the organism to analyze properties of the environment and to modify its behavior accordingly. Through direct interaction with the environment or through observation, the organism forms associations between stimuli and learns to avoid those with aversive properties and seek out those with reinforcing properties. This allows it to avoid danger and drives it to pursue elements necessary to survival, like food and water, ultimately promoting its ability to thrive in the environment in which it is placed.

There are two primary types of learning: non-associative and associative. The first, non-associative learning, works by more primitive mechanisms and is common to nearly every organism. This includes behaviors such as habituation; the process by which an organism adapts to a stimulus and gradually grows unresponsive to its presence. Habituation requires little higher order functioning and occurs reflexively. Associative learning, on the other hand, involves the formation of associations between stimuli. This type of learning includes classical conditioning, which allows an organism to associate previously unrelated stimuli by a mechanism that allows a reflexive response to be elicited in a novel situation. Operant conditioning, on the other hand, is the mechanism by which an organism's behavior is influenced by its consequences, namely if a certain

behavior leads to a punishing experience, it will be avoided, and if a behavior leads to a reinforcing experience it will be repeated (Powell, Honey & Symbaluk, 2013).[1]

Abnormalities in the biological machinery controlling learning and memory result in deficits in the ability to form proper associations. This inhibits the organism's capacity to learn from environmental consequences in order to adapt to its surroundings as well as to habituate to insignificant stimuli. These deficits in learning and memory can be observed in a variety of cognitive disorders in humans including autism spectrum disorders, neurofibromatosis, Lhermitte Duclos disease, and cortical dysplasia. Each of these disorders is characterized by symptoms indicating a failure of proper association formation, such as mental retardation, language deficits, and in the case of autism spectrum disorders, the persistence of stereotyped behaviors (Gipson & Johnston, 2012).

The prevalence of such disorders in the human population makes the study of learning and memory and the elucidation of the pathways behind it of the utmost importance. Understanding the underlying mechanisms of these processes will ultimately allow us to better understand and treat human disease and to minimize deficits in learning and memory in these individuals.

One likely player in the physiology of learning and memory is the mammalian target of rapamycin pathway (mTOR). Recent studies implicate the pathway's involvement in synaptic plasticity, a molecular process that many believe to be the underlying mechanism of learning and memory. This study hopes to demonstrate that the manipulation of this pathway, which can lead to aberrant protein synthesis and is a key feature of synaptic plasticity, can influence learning and memory. It also attempts to

[1] Associative learning is employed most heavily in the present study and will be

provide experimental evidence of mTOR involvement by evaluating the cognitive capacity of mice with a knockout of phosphatase and tensin homolog (PTEN), a gene encoding for a major regulator of the pathway.

CHAPTER TWO

Review of the Literature: A Discussion of mTOR Mechanism and Function

The primary role of the mTOR[2] pathway is in the regulation of mRNA translation and cellular protein synthesis (Wong, 2009). Because its influence is exercised over some of the most basic functions of the cell, the mTOR pathway ultimately holds great significance in the developing nervous system. Proper regulation of the pathway is pertinent to appropriate cell growth and differentiation as well as the formation of proper synaptic connections throughout the nervous system. Deficits in the control of this pathway have drastic effects and may result in aberrant cell growth, improper cellular migration, and unregulated formation of synaptic contacts (Ljungberg, 2006 & Ljungberg, 2009).

Current research implicates the mTOR pathway and the cellular abnormalities that often result from deficits in its regulation in many cognitive disorders. This indicates that the pathway may not only be important to cellular control, but possibly to learning and memory on a more macro level as well. Over-activation of the pathway can result in overexpression of genes due to increased translation of mRNA into proteins (Wong, 2009). This may lead to a variety of consequences, including, but not limited to, various forms of cognitive deficit associated epilepsy such as cortical dysplasia and Tuberous Sclerosis (de Vries & Howe, 2007). Pathway dysfunction in the immature brain has also been implicated in the development of various cognitive disorders such as autism, mental retardation, macrocephaly, and Lhermitte Duclos disease (Zhou et. al., 2009 & Kwon

[2] Mammalian target of Rapamycin

2001). In a pathway comprised of numerous kinases, complexes, and regulatory proteins, there are many opportunities for mutations to affect pathway control and disrupt mTOR function.

Pathway Physiology

The mTOR pathway is extraordinarily complex and involves a wide variety protein kinases involved with several different phosphorylation cascades. There are two mTOR protein complexes employed in the pathway: mTORC1 and mTORC2 (reviewed in Ehninger, 2011). However, because mTORC1 is more directly associated with observed results of pathway manipulation, it will be the focus of the following simplified description of pathway physiology.

The mTOR pathway is initiated in several ways. Among these are stress or the binding of growth factors, neurotransmitters, or insulin to extracellular postsynaptic receptors.[3] These receptors are G-protein linked and activate the phosphatidylinositol 3-kinase (PI3K) which then goes on to activate the phosphoinositide dependent protein kinase-1 (PDK1). This is done through the conversion of phosphoinositol diphosphate (PI2) to phosphoinositol triphosphate (PI3). The rate of this particular step is highly regulated by the antagonistic effects of the PTEN protein[4]. The PDK1 protein then phosphorylates Akt into its active form, enabling it to act as an inhibitor to the tuberin protein,[5] also known as TSC2 (Ljungburg, 2009).

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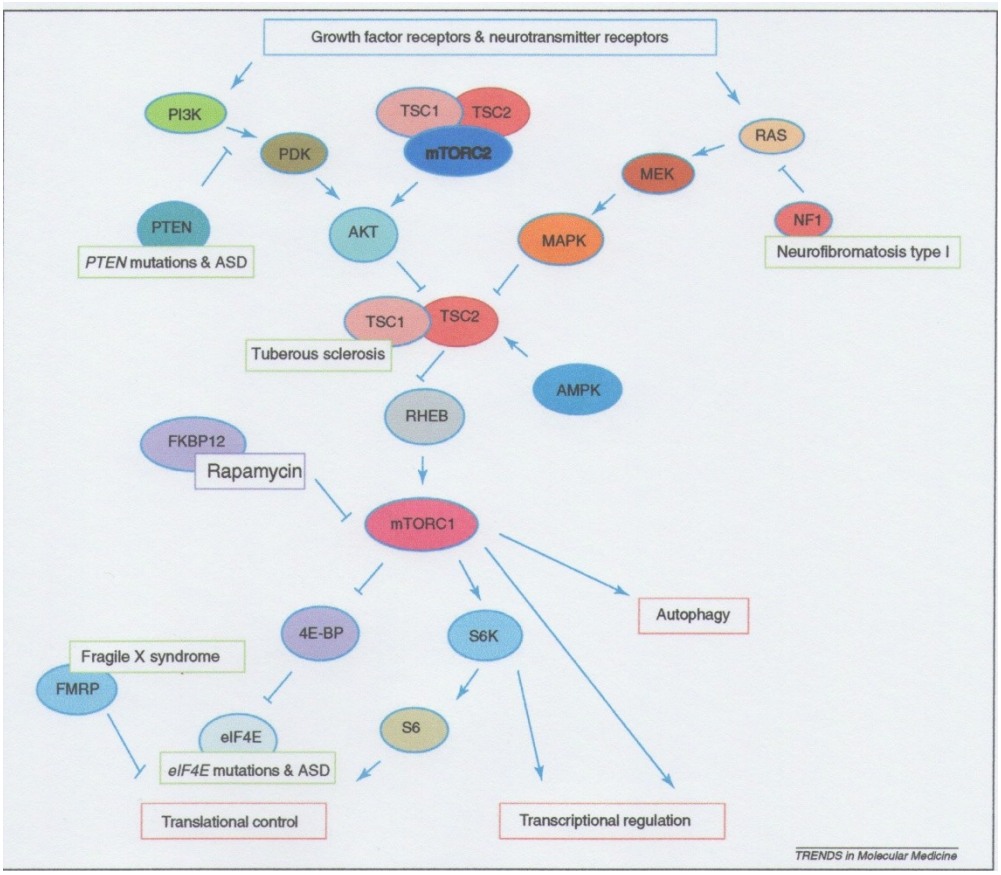
[3] There is also evidence that BDNF and glutamate may also affect mTOR signaling. However, it is still unclear where exactly some of these chemicals stimulate the pathway.

[4] Phosphatase and tensin homolog

[5] This is also known as Tuberous Sclerosis complex 2 due to its association with Tuberous Sclerosis, a form of epilepsy.

The mTORC1 complex is very large and compounds the mTOR protein with the raptor[6], MLST8, PRAS40, and Deptor proteins (Wong, 2009; Ehninger & Silva, 2011). Due to the inhibition of the tuberin protein by Akt, this complex is indirectly activated in the normally functioning pathway (See Figure 1).

Figure 1: From Ehninger & Silva, 2011, p. 4



Translational Control Exercised by mTOR Functioning

The mTORC1 complex controls translation in two ways. One way is through 4E-BPs[7], eukaryotic initiation factors. These are small proteins that bind to the eIF4E

[6] Regulatory associated protein of mTOR
[7] eIF4E-binding protein 1

protein and inhibit translation. When mTORC1 is activated, it phosphorylates the 4E-BP proteins and causes them to dissociate from the complex, increasing the rate of transcription by promoting the formation of the eIF4F complex (Gkogkas, Sonenberg & Costa-Mattiolio, 2010). Conversely, if the complex is in its inactive form the 4E-BP1 proteins are allowed to remain bound, suppressing translation (Wong, 2009 & Ljungberg et. al. 2009).

The second way that the mTORC1 complex affects translation is through the phosphorylation of S6 kinases. The downstream effector for these proteins is the ribosomal protein S6. This directs transcription of mRNAs specifically utilized in the translation of proteins involved in the construction of the ribosome and other translation machinery (Ehninger & Silva, 2011). It also increases the recruitment of ribosomes to the site of translation (Wong, 2009). Thus, when the mTORC1 complex is activated, an increase in ribosome activity will increase overall translational capacity, resulting in the protein synthesis required for changes in synaptic connectivity to occur.

The PTEN model

This particular study utilizes a PTEN knockout to study the effects of mTOR dysfunction. PTEN, or phosphatase and tensin homolog, is a protein that plays an inhibitory role in the mTOR pathway and is so named because the genetic code for the protein sequence is found on chromosome ten (Zhou et. al., 2009). These mice, because they lack inhibitory control over PDK1 activation of Akt, have over-activity of the mTOR pathway (Wong, 2009). Through genetic knockout techniques, the PTEN gene can be disrupted, producing mutant mice with either a heterozygous or homozygous

deletion, each yielding different levels of mTOR over activation available for analysis in a variety of studies.[8]

These mice, as will be demonstrated in the following review of the literature and then in the experiment, often suffer from a variety of physiological abnormalities and cognitive deficits, lending further support for the notion of mTOR involvement in learning and memory.

[8] The PTEN deletion is limited to cells in the central nervous system. Global homozygous PTEN deletion leads to embryonic death of the animal.

CHAPTER THREE

Review of the Literature: Connecting mTOR function with Synaptic Plasticity

A major factor implicating the role of the mTOR pathway in learning and memory is its connection to synaptic plasticity. Synaptic plasticity is considered to be the molecular basis behind learning and memory. Best understood as the strengthening or weakening of a neuronal connection as a result of experience, synaptic plasticity produces alterations in neuronal signaling that lead to long-term changes in mRNA translation and protein synthesis (Purves et. al., 2012). Such changes gradually lead to the modification of dendritic processes and, ultimately, to the overall connectivity of the brain, making their regulation essential to proper development. As noted in the previous chapter, such modification of mRNA translation and protein synthesis is a distinct characteristic of the mTOR pathway, highlighting it as a potential regulator of synaptic plasticity and thus of learning and memory.

Evidence of mTOR Connection to Synaptic Plasticity

Synaptic plasticity depends on strict regulation of the opposing processes of long-term potentiation, in which synapses are strengthened, and long-term depression, in which synapses are weakened. The balance of these two mechanisms results in a precisely organized neuronal network that maximizes learning and memory in the mature brain by allowing those connections relevant to the organism to flourish, and irrelevant connections to be eliminated (Purves et. al., 2012). Successful formation of these

connections can also be observed on a behavioral level by determining the presence normal cognitive capacity.

Because such processes are so basic to the development of an organism, disruptions in proper functioning are easily observable. Because synaptic plasticity is dependent on proper control of mRNA translation and protein synthesis, misregulation of the pathway can result in impaired regulation of cell growth, differentiation, proliferation, or migration, ultimately resulting in abnormality of brain structure. In addition, organisms found to have such misregulation also suffer from a variety of cognitive consequences, such as a reduction in capacity for learning and memory.

Pten Mutant Models

Recent studies analyzing the effects of mTOR pathway dysfunction reflect these consequences of impaired synaptic plasticity. Though the mTOR pathway can be manipulated at multiple steps in the cascade, much recent research has focused on the role of PTEN and how its inactivation affects the pathway as a whole. There are a variety of knockout techniques available to experimenters. In mice, a global, homozygous knockout of PTEN causes death in embryogenesis, while a heterozygous knockout may cause tumors of the endometrium, prostate, or lymphoid system (Kwon et. al., 2006). However, there are also selective knockouts of certain tissue. While deletion in certain types of cells of the nervous system causes hypertrophy, or a cellular enlargement, deletion in other cells may cause hyperplasia, or enhanced proliferation (Kwon et. al., 2006). Thus, while all PTEN manipulations negatively impact synaptic plasticity, the method of PTEN deletion creates variety of cellular abnormalities, some of which are assessed in the following studies (Kwon et. al., 2006).

PTEN Deletion and Effects on Cell Growth and Neuronal Signaling

In 2009, Ljungberg and colleagues created a mouse model of mTOR over-activation for analysis. This study was performed with knockout mice with neuronal subset-specific PTEN mutations. These mutations were created using Cre-*lox*P technology. Mutants were found to have macrocephaly, and especially enlargement of the cortex, cerebellum, and hippocampus that was largely correlated with a loss of PTEN gene expression (Ljungberg et. al., 2009). In addition, independent staining of PTEN negative neurons revealed that these cells are significantly enlarged compared to PTEN positive cells derived from either mutant or wild-type strains. Staining of these cells with phospho-eIF4G and phospho-S6 confirmed that abnormalities of the PTEN negative cells did, in fact, correlate with mTOR pathway over-activation. Finally, mutants were also found to have abnormal EEG activity and spontaneous seizures reflecting the presence of abnormal neuronal signaling. All of these observations by Ljungberg and colleagues (2009) demonstrate the existence of aberrant cell growth and abnormal signaling in association with mTOR over-activation, ultimately suggesting a connection of this pathway with synaptic plasticity.

mTOR Dysfunction Causes Dendritic Abnormalities and Behavioral Deficits

In 2006, Kwon and colleagues performed a study that utilized neuron-specific enolase promotor driven *cre* transgenic mice. This technique limits the PTEN mutation to certain differentiated neuronal populations of the cerebral cortex and hippocampus, specifically in cortical layer III and V and in the granular and polymorphic layer in the dentate gyrus of CA3 respectively.

In this study, progressive macrocephaly was found in these regions of PTEN deletion, as well as soma hypertrophy, or enlargement of the cell body, of PTEN negative cells. They also found a gradual disorganization of the granule layer of the dentate gyrus. Microscopy revealed that mutant cells had a greater number of axonal projections and that these projections spanned to a larger area than those neurons of the dentate gyrus in controls, forming a greater number of synapses. Golgi staining further revealed thickening and elongation of dendritic processes in the cortex of three-month old mutant mice as well as dendritic hypertrophy in the hippocampus. Staining of neurons with phospho-Akt and phospho-S6 revealed that the existence of dendritic ectopia in a cell did, in fact, correlate with mTOR pathway over-activation, again suggesting a connection of the pathway with synaptic plasticity.

This deficit in plasticity also manifested behaviorally in the mutant mice. Social tests were performed at the age of six weeks, before any major morphological changes had occurred. Even at day one, mutant mice had reduced social interaction with another juvenile in comparison to controls. Mutants also had marked deficits in nest formation, a form of social learning. In a social preference task, where the subject had the option to interact with a social target or an inanimate object, mutants spent less time than controls with the social target. Kwon and colleagues also found that while controls preferred a novel social target, mutants showed no preference.

Tests in the six-week old mice also revealed increased anxiety in mutant mice compared to the control mice. Locomotor activity was increased in an open field test, as was the startle response following a repeatedly presented tone of constant intensity. Kwon and colleagues (2006) also found that in comparison to controls, mutants had

reduced sensorimotor gating in a pre-pulse inhibition test, an increased latency to enter the light side of the light/dark box test, and an increased time spent along the sides of the open field apparatus and the Morris water maze. These behavioral abnormalities indicate a deficit in cognitive capacity for learning and memory and further implicate the mTOR pathway in its underlying processes.

mTOR Function and Abnormalities in Cell Growth and Organization

An earlier study done on PTEN pathology by Kwon and colleagues in 2001 revealed further deficits in mice with a PTEN knockout. Using Cre-*lox*P technology, the experimenters eliminated PTEN expression in the brain. This deletion, created by the breeding of *Gfap-Cre* mice with $PTEN^{lox\ P}$ mice, resulted in macrocephaly in comparison to wild-type littermates, and especially an enlargement of the cerebellum. There was an enlargement of the internal granule layer and the molecular layer, as well as poor organization of the Purkinje cell layer. In addition to having fewer Purkinje cells, there was also progressive atrophy of those cells that remained. Misplacement of granule cells was also common, as they failed to migrate properly from the external germinal layer inward to form the internal granule layer.

The concurrence of these phenomena with mTOR overexpression was confirmed again through the use of phospho-Akt. Cre expression confirmed a successful knockout of PTEN in the dentate gyrus and internal granule layer of the cerebellum. Significant phospho-Akt staining was found in the same cell population expressing Cre, indicating that those cells that had PTEN deletion had elevated mTOR activity and contributed to the abnormal molecular anatomy witnessed in mutant mice. The Cre expressing granule cells of the cerebellum and dentate gyrus were also found to have an enlarged soma size

when compared to wild-type littermates at week four. These deficits in cell growth and cellular organization in the cerebellum and hippocampus reveal, yet again, an association between mTOR pathway activity and synaptic plasticity (Kwon et. al., 2001).

Role of mTOR in Disease

Recent research also implicates mTOR dysfunction in a variety of diseases associated with cognitive deficits, further supporting the potential role of the pathway in synaptic plasticity. Not only do the symptoms of many of these diseases include some of the aberrant cell properties observed in the previously mentioned mouse models of mTOR over-activity but they also demonstrate how they manifest behaviorally.

mTOR Dysfunction and Cortical Dysplasia.

In 2006, Ljungberg and colleagues found that over-activation of the mTOR pathway is highly associated with neuroanatomical symptoms of cortical dysplasia in human brain tissue, a disease associated with neuronal signaling abnormalities and cognitive deficits.

Focal cortical dysplasia is a clinical condition in humans comprised of developmental brain abnormalities that are highly associated with intractable childhood epilepsy and a variety of cognitive and behavioral deficits. These abnormalities may include dyslamination as well as enlarged or misoriented neurons and neuroglial cells. There are several dysplastic cell types associated with cortical dysplasia: cytomegalic neurons with a pyramidal cell body, abnormal dendritic and axonal projections, non-pyramidal balloon cells, and immature neurons.

Through the identification and analysis of cytomegalic cells, Ljungberg and colleagues found that an increase in mTOR activity was a common characteristic of cortical dysplasia. Because only cytomegalic cells seemed to be hyperactive, and thus are most likely to be associated with the epilepsy seen in CD patients, these were the cells chosen for the study. Using the tissue from five different FCD patients, these cells were collected via laser capture microdissection and their mRNA submitted to microarray analysis. After determining that the neurofilament heavy-chain gene was overexpressed in cytomegalic cells, markers were used to further distinguish cytomegalic neurons from balloon cells. Incubating tissue with phospho-S6, phospho-eIF4G, and phospho-Akt revealed stronger labeling of NFH positive cells than for others. Because these labels target downstream effectors of the mTOR pathway, these results indicate mTOR pathway over-activation in cytomegalic cells, indicating that the pathway could play a role in the formation of abnormal cell growth and other cellular abnormalities in FCD.

The association of this pathway with deficits in synaptic plasticity is further supported by the behavioral symptoms of cortical dysplasia. In cortical dysplasia, the failure of cells to properly migrate during development and to regulate cell growth results in a variety of symptoms including seizures, a regression in language development upon the onset of such seizures, psychomotor problems, behavioral issues, and mental retardation (Ljungberg et. al., 2006). The undeniable shortcomings in behavioral development in these individuals suggest a connection the observed over-activity of the mTOR pathway in these patients and synaptic plasticity.

mTOR Overactivity and ASD

The Kwon 2006 study suggested a connection between mTOR over-activation and the presence of autistic symptoms in mice. The reduced capacity for social learning in these mice and the increase in anxiety they experience due to an inability to properly habituate stimuli closely parallel symptoms observed in human Autism Spectrum disorder patients. ASD is a disorder characterized by deficits in social communication, delayed language development, and stereotyped behaviors (Bourgeron, 2010). Patients may also experience intellectual deficits, sensitivity to stimuli, and anxiety. The potential of the PTEN knockout model used by Kwon et. al to model a disorder characterized by recognizable deficits in plasticity lend evidence the connection of the mTOR pathway itself with plasticity.

In fact, there are many incidences of autism that are linked with mutations of genes for various effectors in the mTOR pathway other than PTEN. Tuberous Sclerosis Complex is a condition often characterized by autism, intellectual disability, and anatomically, subependymal giant cell astrocytomas and cortical tubers (Gipson & Johnston, 2012). Tuberous Sclerosis Complex is caused by mutations in the genes TSC1 or TSC2, which encode for proteins also known to inhibit the mTOR pathway. Mutation of these genes leads to rapid cell growth, overactive mRNA translation, and ultimately impaired synaptic plasticity (Gipson & Johnston, 2012).

Neurofibromatosis is another disorder associated with autistic behaviors, reduced intelligence, and cognitive impairment due to a mutation in the NF-1 gene. NF-1 encodes a protein known to inhibit Rheb, an activator of the mTOR pathway. NFT patients suffer

from neurofibromas, Lisch nodules, pilocytic astrocytomas, and megalencephaly, all of which also suggest synaptic plasticity impairment (Gipson & Johnston, 2012).

Fragile X Syndrome, the most common form of autism is caused by a mutation in the FMR-1 gene which encodes for FMRP, a protein that when missing, again leads to mTOR overactivation. These patients suffer from anxiety, ADHD, and a variety of unique physical characteristics. This particular mutation is known to reduce the insertion of AMPA receptors into the membrane, ultimately leading to LTD and impaired synaptic plasticity (Gipson & Johnston, 2012)

mTOR Over-activation and Lhermitte Duclos Disease

As mentioned previously, PTEN mutations are often also associated with various cancer types including glioblastoma, endometrial carcinoma, and prostate carcinoma. Another type of unregulated cell growth that may occur due to mTOR misregulation is dysplastic gangliocytoma of the cerebellum, a condition known as Lhermitte Duclos disease in humans.

The abnormalities seen in this mouse model, Kwon and colleagues found in their 2001 study, closely mirrored those of Lhermitte Duclos disease in humans. This disease is characterized anatomically by the dysplastic neurons in the internal granule layer and molecular layer of the cerebellum and the loss of Purkinje cells seen in these mutant mice. Similarly, patients commonly also experience the seizures, hydrocephalus, increased intracranial pressure observed in the mutants. The presence of PTEN mutations has also been confirmed in the dysplastic cells of human patients themselves, revealing the mTOR pathway to be a common physiology in both humans and mouse models of the disease.

CHAPTER FOUR

Methods and Materials

The experimental portion of this study assessed potential cognitive deficits in PTEN knockout mice through the implementation of three experimental procedures: a delayed fear conditioning test, a trace fear conditioning test, and a novel object recognition test. The three tests were designed to gauge memory over the course of different time periods. The two fear conditioning procedures assessed memory over a couple of days, while the novel object recognition test assessed shorter-term memory over the duration of only an hour. This variability in implemented procedures is intended to make results more generalizable to various types of memory.

Each experiment also included subjects of homozygous PTEN knockout genotype, heterozygous knockout genotype, and wild-type control mice in random order. The experimenter was blind to subject genotype, thus eliminating unwanted bias. The technology used to create the knockout models was the Gfap-CreloxP technology (Kwon, 2001). This affected promoters of the PTEN gene only in the hippocampus, cerebral cortex, and cerebellum early in development, resulting in an over-activation of the mTOR pathway in these regions. Different cohorts of mice were used in each experiment and all mice were handled often prior to each experiment to reduce stress due to handling during testing.

Background on Conditioning Principles

The first two procedures used in this study applied the principles of classical conditioning[9]. Classical conditioning is the production of a reflexive behavior in response to a previously neutral stimulus. Conditioning procedures require an unconditioned stimulus (US) that elicits the target behavior reflexively. When the US is paired repeatedly with a neutral stimulus (NS) over an acquisition period, the NS becomes the conditioned stimulus (CS), and is capable of eliciting the behavior on its own. The following classical conditioning procedures aim to determine the successful acquisition of a conditioned response (CR). Such determination will yield insight into the proper regulation of protein synthesis and synaptic plasticity.

Delayed Fear Conditioning Procedure

This procedure is a test of associative learning involving three phases: an acquisition phase (or training phase), a contextual memory test, and a cued fear test.

Materials

Measurements taken during these phases were recorded by the FreezeFrame Monitoring System (Coulbourn; Ohio). For each phase, the mice were placed individually into a 26x22x18 cm chamber with two acrylic walls, two metal walls, and a metal, grid floor that was used to administer a pulsing shock to the subject. To control for extraneous variables, this chamber was then isolated within another sound attenuated chamber that prevented the entrance of outside light. The internal chamber was lit by a background light near the ceiling of one wall. Using the intensity of this light as a

[9] Also called Pavlovian conditioning

baseline, the FreezeFrame system recorded information about the subject's level of activity by monitoring alterations in light intensity. This information was presented as a measurement of freezing throughout indicated intervals. This freezing, also known as conditioned suppression of activity, is used a measurement of fear throughout the experiment.

Acquisition Phase

On training day, one mouse at a time was placed in the chamber and permitted to explore for two minutes. This allowed time for the subject to habituate to the new environment before stimuli presentation. After two minutes, a white noise sound, the neutral stimulus (NS), was presented at an intensity of 80 dB for 30 seconds. This sound was immediately followed by a mild 0.7 mA foot shock, the unconditioned stimulus (US), delivered by the FreezeFrame system. This acquisition phase included two pairings of the NS and US with an inter-trial interval of two minutes. Experimenter verification that the shock was indeed administered required running, jumping, or vocalization of the subject. The chamber was wiped down with 30% isopropyl alcohol between subjects.

In the absence of cognitive deficit, this period of acquisition should result in the subject's formation of a strong association between the sound and the shock, converting the NS to a conditioned stimulus (CS)

Contextual Memory Test

The contextual memory phase was conducted approximately twenty-four hours later. The subject was placed within the chamber and the FreezeFrame system monitored its levels of freezing throughout the duration of the 5 minute period. No stimuli were

administered. The chamber was again wiped down with 30% isopropyl after the subject was returned to its home cage.

This phase was designed to test the subject's memory specifically for the context of the testing chamber.

Cued Fear Test

This phase was conducted two hours after the onset of the contextual memory test. Prior to initiation of this phase, the context of the chamber was altered. The grid floor of the chamber was covered with a clear acrylic insert to alter the texture, color, and shape of the testing chamber. Vanilla extract was placed beneath the acrylic flooring to alter the scent of the chamber. The bedding from the transport cages in which the mice were brought to and from the testing chamber was also replaced with shredded paper. Finally, in this last phase, the chamber was also wiped down with 70% ethanol rather than 30% isopropanol, again to impart a different scent to the chamber. The subjects were exposed to this new environment for three minutes before the onset of the CS. At this point, the white noise was played for another three minutes and freezing was again recorded by the FreezeFrame system.

Alterations in chamber context were made in an effort to isolate and measure memory for the CS alone. The novel context was intended to eliminate the effects of any conditioning that may have occurred to the chamber itself, thus allowing conditioning due to context and conditioning due to the tone to be measured separately.

Data provided by the FreezeFrame system from the three phases was then compared and analyzed to assess the potential presence of cognitive deficits in the PTEN mice for this given test.

Trace Fear Conditioning Procedure

The trace fear conditioning procedure, while similar to the delayed fear conditioning procedure, is designed to identify more subtle deficits in memory through its unique construction of stimulus presentation (Wiltgen et. al., 2005). This procedure also employed the FreezeFrame system to measure the freezing levels of subjects. Three similar phases were used, although of slightly different structure and order.

Acquisition Phase

Subjects were placed in the chamber and allowed to explore for three minutes. After this period an 80 dB, 2700 Hz tone was presented for 20s, serving as a neutral stimulus (NS). Then, after a 20s delay (this is known as the trace interval), the unconditioned stimulus (US) was presented in the form of a 0.5mA shock administered for 2s. Four more consecutive conditioning trials were then presented with a 200s inter-trial period between them. Because of the less salient shock used in this conditioning procedure and because of the specific presentation of the stimuli, more acquisition trials were implemented to maximize associative learning. At the end of the experiment, the mice were returned to their home cage and the chamber was wiped down with 30% isopropyl alcohol before the next subject was tested.

Cued Fear Test

In order to eliminate conditioning to the context, the testing chamber was altered. The grid flooring was again covered with an acrylic insert to alter the texture and look of the chamber, vanilla extract and 70% ethanol were again used to alter its scent, and

shredded paper rather than bedding were used in transfer cages to again modify entry context.

The cued fear test was initiated twenty-four hours after the onset of the training phase. Mice were placed in the chamber and permitted to explore the novel context for two minutes, during which a baseline level of freezing was recorded. This period was followed by three presentations of the tone, or CS, that lasted 20s each. The interval between stimuli, or inter-trial interval, was 220 s each. The FreezeFrame system recorded levels of freezing during the periods of tone presentation, subtracting the baseline amount of freezing found in the periods in which no stimulus was presented. The subjects were then returned to their home cage.

Contextual Memory Test

In the trace fear conditioning procedure, the third phase did not take place until another twenty-four hours after the initiation of the second phase. The original context of the chamber was restored; with the acrylic insert and vanilla extract removed, bedding replaced, and use of 30% isopropanol to clean the chamber resumed. The subjects were placed in the chamber and their activity was recorded for 8 minutes in the absence of any stimuli. The FreezeFrame system recorded freezing levels throughout this period. Subjects were returned to their home cage at the conclusion of the study.

Like in the delayed fear conditioning procedure, the contextual memory test was used to gauge how much freezing occurred as a result of memory just for the context of the conditioning trials, while the cued fear test was used to assess memory for the tone itself.

Novel Object Recognition Test

The novel object recognition test is used in this study to assess cognitive deficits in PTEN knockout mice over the course of one hour. The premise of this experiment is largely based on the discovery that a mouse will investigate a novel object more intensely than a familiar one (Bevins & Besheer, 2006). In this test, a mouse is permitted to inspect both a familiar object and an unfamiliar one. In the absence of cognitive deficits, a mouse should spend more time examining a novel object than one seen in earlier in the experiment. This difference in investigation time reflects memory for the first object. The novel object recognition test assesses the difference in novel object preference between wild type and PTEN knockout mice. Experimenter bias in this procedure was eliminated by randomly ordering mice so that the genotype of the subject was a mystery to the experimenter.

Materials

This test took place in a 25x25x30 cm clear, acrylic chamber. Two objects to be investigated were placed in each of the two back corners on the side of the chamber against the wall. The two sides of the chamber adjacent to the wall were covered with white boards to prevent the subject from forming a bias for one side of the chamber based on stimuli in the external environment. The wall of the chamber facing the room and opposite the objects was left exposed. The experiment was recorded by a video camera suspended from the ceiling above the chamber. Footage of the experiment was then analyzed using the ETHOM scoring system (Event-Recording Computer Software For the Study of Animal Behavior). The experiment was contained in an empty, sound attenuated room with a constant level of lighting and background noise. This constancy

was intended to eliminate any stimuli that may have distracted the subject's attention from the task at hand.

Experimental procedure

The novel object recognition test included a habituation period followed by two phases. The first was exposure of the subject to two identical objects while the second phase introduced the novel object.

On Day 1, the mice were each placed within the apparatus for 20 minutes in the absence of any objects. This was intended to habituate the mice to the testing chamber before the initiation of the experiment.

The first phase was initiated on Day 2, approximately 24 hours after habituation. The objects used in this phase were identical. Two bottles of equal size, shape, and color (clear with a purple cap) were placed into the back corners of the chamber against the wall prior to introducing the subject to the experimental setting. The phase began when the video camera was set to record and stream video to a computer where it was saved for later viewing. A card labeling the identification number of the subject was visible in the recording field. The subject was then removed from the transfer cage and placed at the midline of the chamber on the opposite wall of the objects. His nose was pointed away from the objects with his body parallel to the walls. This placement guaranteed that the mouse was not oriented in a way that biased his approach to the objects. The experimenter then left the room for ten minutes to allow the mouse to explore the chamber and the objects without distraction. After a timer indicated the end of the experimental period, the experimenter reentered the room, stopped the recording, and

removed the subject from the chamber. The mouse was then transferred back to the home cage and the chamber was cleaned with 30% isopropyl alcohol.

The second phase of the experiment began exactly one hour after the initiation of the first phase. This procedure was identical to the previous phase, except that one of the familiar objects was replaced with a novel object. The novel object was an opaque, grey bottle that was taller than the original bottle, and had a black rather than a purple cap. Again, these bottles were placed in the back corners of the chamber. They were also switched to opposite corners between each subject to eliminate the possibility of the confounding variable of a side preference manifesting itself in the appearance of an object preference. The video was then set to record and the mouse moved from the transfer cage to the inside of the chamber and oriented in the manner described in phase one. The experimenter then left the room, returning after ten minutes to stop the video, remove the subject from the chamber, and place the subject in the home cage, or a holding cage if further subjects were to be tested. The chamber was cleaned with 30% isopropyl alcohol.

Video Scoring

To record results from the two phases, the videos of the experiment were later viewed and the behavior of the subjects scored. Scoring was done with the ETHOM program. After the subject was placed in the chamber in the recording, the program was started. Two keys on the keyboard were designated to represent each of the objects. Each time the subject made intentional contact with one of the objects, the designated key was pressed. Two presses were required, one for the onset and one for the offset of contact. Accidental brushes of the object were not recorded. This recording method allowed the

ETHOM system to calculate the frequency with which each of the two objects was contacted as well as the total duration of time spent investigating each object. This was done for both phases of the experiment. Scoring of the first phase was to eliminate the possibility of a side bias. Scoring in the second phase was used to determine whether the animal had a preference, as was expected in a cognitively sound individual, for the novel object. The magnitude of the preference for the novel object was compared between homozygous PTEN knockout mice, heterozygous PTEN knockout mice, and wild type mice.

CHAPTER FIVE

Results and Discussion

Results and Data Analysis

Delayed Fear Conditioning Test

In the delayed fear conditioning test, the percent of time that each subject spent freezing was recorded in each of three conditions: the contextual condition, the tone condition, and the novel context condition. In each condition, the mean percent of time freezing between the PTEN knockout mice, the heterozygous knockout mice, and the wild-type mice were compared using an ANOVA test. The results are presented in Figure 1.

In the contextual conditioning test, the freezing behavior of the subjects was monitored on Day 2 when the mice were returned to the conditioning chamber for a 5 minute period. It was found that the homozygous PTEN knockout mice spent less time freezing than the heterozygous or the wild-type mice ($F_{(2,28)} = 3.44$, $p < 0.05$). This result indicates that the PTEN mice had reduced memory for the context in which the conditioning took place. Unlike the heterozygous and wild-type mice, they did not associate the testing chamber with the delivery of aversive stimuli. Following up with a separate, unpaired samples t-test between the PTEN and wild-type mice confirmed a significant difference in percent time freezing between these two groups ($t_{(1,19)} = 2.6$, $p < 0.05$).

On the other hand, no significant differences between groups were found during the three minute presentation of the CS tone in the new context in the tone condition ($F_{(2,28)}$=0.72, p = 0.49). This indicates that while there was no memory in the knockouts for the context, they had maintained some memory for the tone. This also indicates that the knockout had a greater effect on hippocampal and prefrontal cortex mediated memory than it did on amygdala-mediated memory, as hippocampal learning is often spatial while the amygdala is often more involved in the memory of specific stimuli (Fanselow and LeDoux, 1999).

Finally, there was no significant difference ($F_{(2,28)}$=0.80, p= 0.46) found between the percent freezing in the groups when placed in the new context in the absence of the tone. This was expected, as the new context should not have been associated with the aversive stimulation presented in the conditioning trials, so no memory for the new context should have been detected in any group.

Trace fear conditioning

The results were analyzed similarly for the trace fear conditioning procedure. Again, the percent freezing for each of the three groups was measured under different conditions. Four different conditions were measured for this procedure. The data for these conditions are represented in Figure 2.

The first measurement was taken as a baseline measurement. The percent freezing was compared between the three groups on Day 2 in the two minutes when the mice were introduced to the new context but had not yet undergone conditioning trials. As expected, the baseline condition revealed no significant difference between groups

($F_{(2,60)}$=0.21,p>.8). There should have been no difference in the amount of basal movement in the chamber prior to the conditioning trials.

However, there was a significant difference in percent freezing found between the PTEN mice and the other two groups in the tone condition ($F_{(2,60)}$=5.67,p<0.01). When the tone played for 20 s in the new context, the PTEN mice had reduced freezing compared to the wild-type and the heterozygous mice. This reflects an impaired memory for the tone in this experiment.

There was also a significant difference in percent freezing during the trace-time condition ($F_{(2,60)}$=4.06, p<0.05). This indicates that during the 20 s period between when the tone was played in the new context and when the shock was expected to occur, the PTEN mice froze less than the other two groups, thus suggesting reduced memory for the tone's association with the aversive stimulus.

Finally, there was also a significant difference between groups in the inter-trace interval condition ($F_{(2,60)}$=3.36, p<0.05). The ITI indicates the period of 200s between when the shock was expected to occur in the new context and when the next tone was played. Again, there was reduced freezing in the PTEN mice compared to the other two groups. This again reflects a lack of memory for the conditions of the experiment in the homozygous knockout subjects.

On Day 3, the percent freezing of the three groups was compared when they were placed in the original context in which they were conditioned. Because of a lack of homogeneity of variance, and thus a violation of assumptions, a Kruskal Wallace test was used instead of an ANOVA. Again, it was found that PTEN mice froze less than both heterozygous and wild-type mice (K = 6.136, P < 0.05). Once again, this indicates

reduced learning and memory in the PTEN knockout mice for the stimuli associated with the presentation of aversive stimulation.

Interestingly, the heterozygous knockout mice seemed to freeze less than the wild-type in both the ITI condition and the contextual condition of the trace fear conditioning procedure (see Figure 2). This would indicate that the heterozygous PTEN knockout somehow enhanced learning and memory in comparison to wild-type mice in these particular tests. This is a surprising result and should be further investigated.

Trace fear conditioning is designed to test memory of the hippocampus and the medial prefrontal cortex (Fanselow and LeDoux, 1999). The presence of deficits in the trace fear conditioning procedure, compounded with the failed learning of context in the delayed fear conditioning procedure confirms, as expected, that deletion of PTEN results in deficits in hippocampal and prefrontal cortex learning and memory.

Novel object recognition test

In phase A of the novel object recognition test, mice are placed in the chamber with two of the same object set in opposing corners. This test is designed to familiarize the subject with the repeating object in order to habituate it to its presence prior to the presentation of a novel stimulus. Data was taken for each of the three groups of mice: wild-type, heterozygotes, and PTEN knockouts. In each of the three cases, there was no significant difference found in the time spent exploring each of the two objects (See Figure 3A). This was expected, as the objects were identical.

In phase B of the novel object recognition test, the subjects were placed in the chamber with one of the original objects and one novel object. A subject who spent more time examining the novel object in comparison to the object that had already been

investigated in a previous study was considered to have memory for the original object. This was found to be true of the wild-type group. A paired samples t-test revealed that the mean time spent inspecting the novel object was significantly greater in these subjects than was the mean time inspecting the original object ($t_{(1,15)}$=2.7, p<0.05). However, in the heterozygous knockout mice, there was found to be no significant difference in the time spent exploring the two objects ($t_{(1,7)}$=0.02, p=0.98). There was a similar lack of difference in inspection of the two objects for the PTEN knockout mice ($t_{(1,11)}$=0.93, p=0.36). These results indicate that while wild-type mice demonstrated familiarity with the object presented in phase A of the experiment by spending more time examining the novel object presented in phase B, both heterozygous and homozygous PTEN knockouts did not. This suggests reduced learning and memory in mice with this deletion.

Data

Figure 1A: Delayed fear conditioning test results for contextual condition, tone condition, and novel context condition. The percent freezing in the homozygous PTEN knockout was significantly lower than the other two groups ($F_{(2,28)}$ =3.44, p < 0.05).

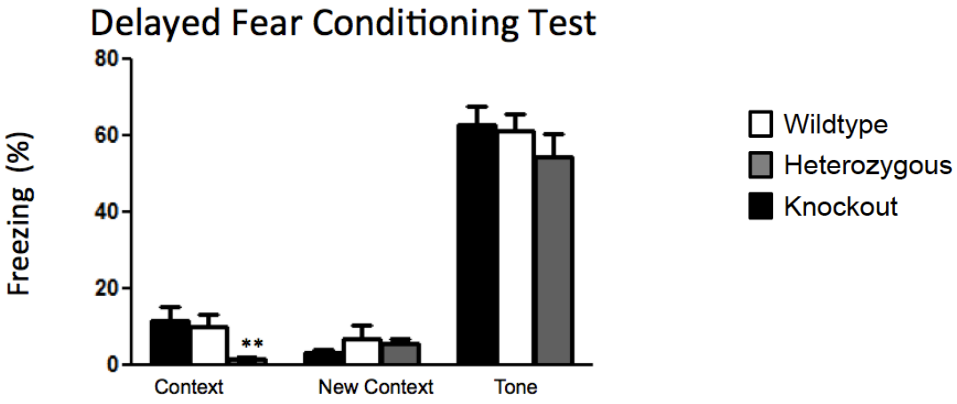
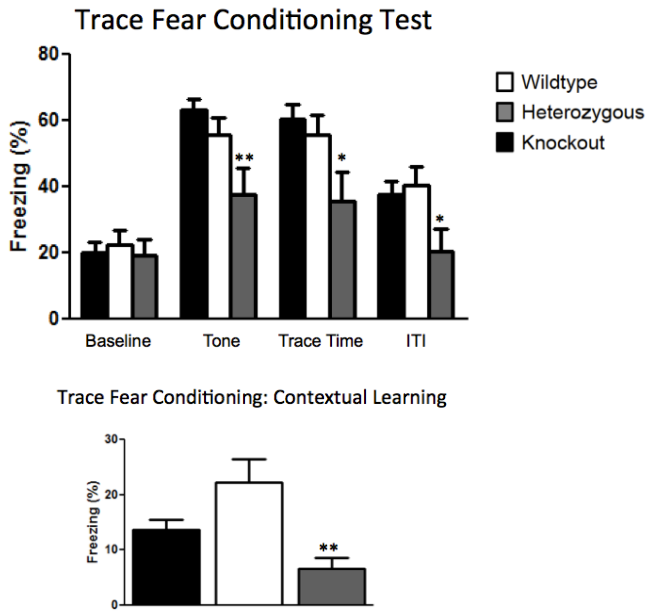


Figure 2: A. Trace fear conditioning results for the baseline, tone, trace time and inter-trace interval conditions (Day 2). Significant reductions in freezing were found for the tone ($F_{(2,60)}$=5.67,p<0.01), the trace time ($F_{(2,60)}$=4.06, p<0.05), and the inter-stimulus interval conditions ($F_{(2,60)}$=4.06, p<0.05). B. Trace fear-conditioning results for the contextual condition (Day 3). Again, there was a significant reduction in freezing for homozygous PTEN knockout mice (K = 6.136, P < 0.05).

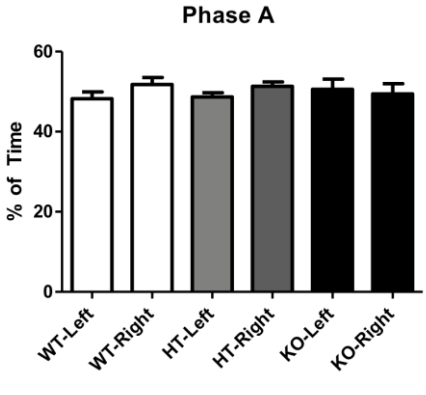
A.



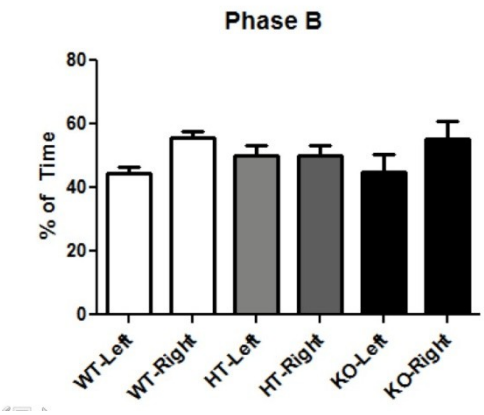
B.

Figure 3: Novel Object Recognition data. A. Phase A. B. Phase B. Note that the "right" side is meant to represent whichever side housed the novel object. While there was a significant difference in the time spent between the two objects for the wild-type mice ($t_{(1,15)}$=2.7, p<0.05), there was no significant difference in time spent between objects for heterozygous ($t_{(1,7)}$=0.02, p=0.98) and homozygous mice ($t_{(1,11)}$=0.93, p=0.36).

A.



B.



Implications

This research indicates a connection of the mTOR pathway with learning and memory. The disruption of the PTEN gene using Gfap-CreloxP technology resulting in a hippocampal localized deficit in memory that was indicated by a reduced ability in the PTEN knockout to recognize the context associated with the delivery of an aversive stimulus. This hippocampal-based learning deficit was also detected in the trace fear conditioning procedure by reduced freezing during the presentation of the tone in the new context, as well as during the trace time and inter-trace interval. Because these procedures take place of over two days in the case of delayed fear conditioning and three days in the case of trace fear conditioning, these deficits are related to impairments in long-term memory.

The deficits found in the novel object recognition test indicated memory impairment in both the PTEN knockout mice and the heterozygous knockouts. This test, which was performed over the course of one hour, reflects impairment in a shorter-term form of memory. It also demonstrates that non-spatial deficits in learning and memory occurred in addition to the spatial deficits determined by the fear conditioning tasks. The fact that different types of memory were affected by the PTEN deletion in this study suggests that manipulation of the mTOR pathway can have widespread behavioral effects on learning and memory, some of which could model effects seen in diseases associated with mTOR dysfunction.

Questions for Further Research

The results from this study, and those found in the studies mentioned in the review of the literature, connect the mTOR pathway both with synaptic plasticity and

learning and memory. However, expanding the battery of tests in this study could improve results by determining more specifically the extent of cognitive deficits caused by the Gfap-CreloxP over-activation of the mTOR pathway in the hippocampus, pre-frontal cortex, and cerebellum. This would be especially useful since few behavioral tests have actually been conducted on this particular knockout. Many types of learning and memory could potentially be tested by avoidance tests, such as the light-dark box test, by a social transmission of food preference tasks[10], by spatial tests such as the radial arm maze, and a variety of others. Once a more comprehensive understanding of the behavioral deficits in learning and memory is formed, these mice may be used to model certain aspects of diseases associated with mTOR over-activation. This would allow for clinically relevant research to be conducted in which the Gfap-CreloxP knockout could be combined with treatment conditions to assess a potential reduction in deficits. Treatments shown to be efficacious in reducing symptoms in mice could serve as potential candidates for clinical research in the treatment of mTOR related disease.

Potential Use of Rapamycin in Future Studies

Rapamycin is one drug known to have direct inhibitory activity on the mTOR pathway. In fact, analysis of the activity of this drug is what gave the pathway its name, "mammalian target of rapamycin". Rapamycin is known to inhibit the mTOR pathway by binding to FK506 binding protein 12 (FKBP12) which then binds to mTORC1 as part of the complex and directly inhibits its effects (Buckmaster, 2009). This, incidentally,

[10] The social transmission of food preference test tests learning of food preferences conveyed from mouse to mouse via interaction after consumption of a novel food source.

reduces the cell's capacity for transcription and protein synthesis by reducing the effectiveness of the mTOR pathway.

Evidence of Symptom Reduction in PTEN Knockout Mice with Rapamycin Treatment

Because rapamycin is an inhibitor of the mTOR pathway, its effects are nearly opposite that of the PTEN knockout, which causes pathway over-activation due to removal of an inhibitory regulation protein. Because of the oppositional effects of rapamycin application, many PTEN knockout studies have used it in comparison of a treatment group to a cohort of control PTEN mice. Not surprisingly, several studies have observed a reduction in both structural and behavioral abnormalities in PTEN knockout mice following treatment of such a drug.

Ljungberg and colleagues, in their previously discussed 2009 study, report that the symptoms caused by mTOR over-activation in NS-PTEN knockout mice can be largely reduced upon treatment with rapamycin. After two weeks of rapamycin treatment, the hypertrophic neurons typical of the PTENs were found to have reduced expression of S6, indicating an overall reduction in mTOR pathway activity. This decrease in activity was even sustained long after the cessation of rapamycin treatment, with hypertrophic neurons maintaining reduced expression of S6 three weeks after the end of treatment. Ljungberg and colleagues also found that rapamycin rescued, to some extent, hypertrophic cells, resulting in a modest decrease in cell body diameter. This reduction in mTOR activity was also expressed on a behavioral level. Rapamycin treatment was found to reduce the frequency and intensity of epileptic seizures in these mice, as well as all forms of epileptiform activity. It also restored a near normal awake

baseline EEG activity that was sustained at least three weeks following the cessation of treatment (Ljungberg et. al., 2009).

The studies conducted by Kwon and colleagues (2001, 2006) demonstrate similar promise in the use of rapamycin to restore overactive mTOR activity to basal levels and to partially reverse aversive effects. In 2003, they demonstrated that PTEN knockouts created from breeding Gfap-cre mice with PtenloxP mice, could be treated efficaciously with a rapamycin analog CCI-779[11]. After daily treatment of the mutant mice with CCI-779 throughout the development of the dentate gyrus, hypertrophy of the soma in the area was prevented and S6 levels reduced almost completely, suggesting near complete inhibition of the mTOR pathway. These changes in physiology were manifested behaviorally as a reduction in seizure occurrence and in the mortality rate of the mice. Kwon and colleagues also found that administration of CCI-779 in adulthood was capable of partially reversing hypertrophy in the dentate gyrus, and with higher doses, in the cerebellum (Kwon, 2003).

The previous studies represent only a portion of the research suggesting the potential of rapamycin to treat mTOR pathway overactivation. The Gfap-CreloxP knockout could represent a model of mTOR related dysfunction with a novel profile of behavioral manifestations. It is important to assess the potential of rapamycin to treat the deficits in learning and behavior present in this particular brand of PTEN knockout. Rapamycin is already being considered as a potential treatment for a wide variety of mTOR related disease such as Tuberous Sclerosis, Fragile X Syndrome, Rhett Syndrome, and autism spectrum disorders. Preliminary evidence based on studies involving

[11] Rapamycin ester

knockouts of various mTOR components suggest that the drug could be used to treat the symptoms of mTOR over-activity present in these diseases. Further study into the behavioral deficits of the Gfap-CreloxP PTEN knockout model could highlight it as a mouse model of yet another mTOR related disease that, if experimental treatment were successful, could benefit from the administration of rapamycin.

Conclusion

The mTOR pathway is heavily involved in mRNA translation and protein synthesis, making its proper regulation imperative to synaptic plasticity. The cognitive deficits found in the Gfap-CreloxP PTEN knockout demonstrate that when vital regulatory proteins such as PTEN fail to function properly, over-activation of the mTOR pathway can occur, ultimately leading to a reduced capacity for learning and memory in an organism. The study of such models is imperative to the molecular understanding of mTOR related disease in humans. Because the behavioral and anatomical deficits observed in these mutant mice are often paralleled in human subjects with known mTOR dysfunction, the models may prove vital to treatment exploration. Future research in this field will allow for a better understanding, not only of the physiology behind learning and memory, but also of how to treat its deficits in the human population.

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