HIPPOCAMPAL SYNAPTIC PLASTICITY-ASSOCIATED PROTEIN EXPRESSION IN A RAT MODEL OF RETT SYNDROME

by

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Dedicated to my parents and brother:

Late Viswanathan Mahadevan, Uma Mahadevan and Anand Mahadevan

Thank you for your unconditional love and support

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by

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December 2017

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Rett syndrome is a rare X-linked neurodevelopmental disorder. Mutations in the methyl CpG binding protein 2 (MeCP2) gene is the leading cause for most cases of Rett syndrome, generating severe cognitive impairment in females and some males at a very young age (Zhao et al., 2015). Research across the world from MecP2 null mice models of Rett syndrome reveals dysfunction in the hippocampal region of the brain that is associated with significant alterations in behavior, memory and locomotion. Already existing data from electrophysiology and western blot analysis show severe changes in protein expression, synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD). In our study, we assessed the role of specific synaptic proteins in a MeCP2 KO rat model, to compare the data with the existing mouse models and validate the use of rats as an animal model for Rett syndrome research. Immunoblot analysis revealed decreased expression of calcium calmodulin protein kinase II α (CaMKIIα) and NMDA receptor subunit-GluN2A in both dorsal and ventral hippocampus. No significant change was observed in the NMDA receptor subunit- GluN2B. However, the ratio of GluN2B/GluN2A was significantly increased in the dorsal hippocampus. No significant change was observed in the levels of protein

kinase A (PKA), PSD95 and actin. Protein level values obtained in the rat model of MeCP2 knockout gene were comparable (with slight variations for GluN2B and PSD95) to the already published mouse models across the globe in different laboratories with similar experimental conditions. These data suggest that changes in protein expression could affect synaptic plasticity and alter the ratio of long-term potentiation/long-term depression, all of which could promote the clinical manifestations observed in Rett syndrome. Further, these findings indicate that the rat model can be used to study synaptic dysfunction in Rett syndrome related conditions.

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CHAPTER 1

INTRODUCTION

Rett syndrome is a rare, genetic and X-linked neuro-developmental disorder. It causes impaired brain development in females at a very young age, affecting 1 in every 9,000 girls under the age of 12 and 1 in 30,000 in the general population (Kozinetz et al., 1993).

Andreas Rett, an Australian neurologist, was the first to describe Rett syndrome. Patients with Rett syndrome are born healthy with normal growth up to 6-12 months followed by a slowing in development and other symptoms. Patients are diagnosed with neurological and behavioral deterioration in different stages of the disease (Hagberg and Engerstrom, 1986). The most common characteristic feature of this syndrome includes slower head and brain development, reduced muscle tone, distinctive hand movements, seizures, dyspraxia, inability to speak, irregular breathing pattern, loss of motor skills and some other autistic features. An overall cognitive impairment is witnessed in the patients suffering from Rett syndrome.

Although the etiology of this disease is not well known, mutations in the methyl CpG binding protein 2 (MeCP2) gene causes loss of function or under expression of the MeCP2 transcript in Rett syndrome (Guy J et al., 2011). Since the mutation is on the X chromosome, there is a higher prevalence in females. MeCP2 functions by binding to methylated cytosine on a DNA strand. The primary function of MeCP2 is transcriptional repression by recruiting certain co factors which can alter the structure and function of the gene (Zhao et al., 2015). MeCP2 can also act as a transcriptional activator, suggesting its overall role as a transcriptional regulator of the gene.

Mutation in any part of this gene can hinder normal synapse formation and alter the structure and function of neurons in brain (Gao et al., 2015; Rietveld et al., 2015). Researchers have shown that specific regions of the brain such as the hippocampus, substantia nigra, basal ganglia, cortex, cerebellum and spinal cord are significantly affected in Rett syndrome. In addition, a mutation in MeCP2 also causes significant changes in several neurotransmitter systems like the glutaminergic, GABAergic, and dopaminergic systems (Smeets et al., 2012).

Genetically engineered mutations in mice that either lack the MeCP2 gene (null) or have a truncated form of the MeCP2 gene (KO) or over expressed MeCP2 gene (duplication) closely mimic some frequent mutations observed in humans suffering from Rett syndrome. Such models display many essential clinical features observed in Rett syndrome phenotype (Chen at al., 2001). They can also provide valuable insights into the pathophysiological mechanisms involved in this disorder.

Comparing the data between different murine models can help provide a better understanding of the disease at a phenotypic, genotypic, behavioral and molecular level. Assessing the extent of cognitive impairment may be a challenge in animal models. Some of the existing animal models of Rett syndrome from different laboratories have provided significant data concerning commencement and progression of the disease, neurological and behavior function and synaptic plasticity (Moretti et al., 2006).

Most of the animal research on Rett syndrome has been performed using a mouse model. This is because the mouse genome was the first to be characterized and analyzed. Also, the physiological and genetic similarity between mice and humans is significantly high. Mice have an added advantage of being small in size and they have a short breeding, reproductive and development cycle. They are also easy to maintain compared to other animal models and have similar phenotypic deficiencies that can be related to humans (Smeets et al., 2012). But, over the last few years, the focus has shifted to using rat models for studying specific disorders. Rat genome was characterized later and new genetic tools allow for these kinds of investigations in rats. Also rats offer an increased size, which is advantageous during neurosurgery. Electrophysiology and direct drug delivery to specific brain regions are easier to carry out in rat models, especially during early developmental stages (Patterson et al., 2016). Rat models also demonstrate a wider variety of cognitive and behavioral abilities. Furthermore, use of both mice and rat models would increase the translatability of bench work research into clinical trials because effects that are conserved across species are more likely to be seen in humans with the syndrome.

In spite of the fact that Rett syndrome mostly affects females, there are rare cases where males have also been affected. In humans, the chances of survival of such men are close to zero as the mutation in the gene is lethal causing death at a very early age. Statistics reveal that females with Rett syndrome can survive up to the age of 25-40 (Dimensions of dental hygiene, 2009); however, deterioration of motor skills and behavioral regression starts as early as age 1-3 and this gets worse and more complicated with increasing age. In males, the symptoms are more

intense. Animal studies of MeCP2 have used both males and females. In early stages of development, phenotypic changes and clinical manifestations are more prominently visible in male animal models of Rett syndrome compared to females. So, it would be interesting to use a male rat model with a mutation in MeCP2 that can provide useful information about the defects in cognitive impairments, motor skills and behavior changes in Rett syndrome.

Behavior changes observed in Rett syndrome

Many studies have demonstrated that mouse models with MeCP2 deficiency show Rett like characteristics and dramatic changes in physical and behavioral manifestations. According to a paper published by Patrizi et al., (2015), a mutation of the MeCP2 gene significantly affects several physical traits in mice like body weight, respiration pattern (checked using plethysmography), locomotion activity (using rotor rod test). According to Patterson et al., (2016), male and female rats with a mutated MeCP2 gene showed reduced brain size, brain weight, and body weight. They also developed malocclusions of the teeth due to feeding difficulties with increasing age. According to Na and colleagues (2014), other symptoms of the MeCP2 KO rat included the development of strange hand movements, arrhythmic breathing pattern, increased seizures with age, and social aggressiveness or anxiety-like signs. Along with this, Rett MeCP2 KO rats became lethargic (at the end of 1-2 years) and displayed diminished survival rate and an overall decrease in wellness. Also, locomotion activity of these rats appeared altered in open field tests, and the rotor rod test revealed abnormalities in motor coordination.

Based on the behavior changes that are observed in these animals, scientists have used electrophysiology tools to see plausible changes in neurotransmitters and synaptic activity of neurons that could underlie the behavior changes caused in this syndrome (Na et al., 2014).

Electrophysiology study to assess synaptic activity in Rett syndrome

Researchers have used electrophysiology studies to identify changes in neurotransmitters and synaptic activity in the brain. Electrophysiology involves using electric conductance to measure the electrical activity of cells and tissues in biological samples. Specifically, in neuronal cells, it helps in the assessment of action potential, long-term potentiation (LTP) and long-term depression (LTD). Long-term potentiation refers to the steady increase in synaptic activity between two neurons which causes persistent strengthening of synaptic activity. Since memory formation is mainly dependent on synaptic strength, LTP seems to play an essential role in memory formation. Contrary to that, long-term depression causes a reduction in synaptic activity between two neurons, causing a decrease in synaptic activity. The patch clamp technique is one of the widely used electrophysiology studies that incorporates the use of a microelectrode with a micropipette that allows sampling from the cell membrane to study the activity of ion channels. This is one of the intracellular methods of examining electrophysiology, although there are several other extracellular methods available (Wu et al., 2016).

According to Asaka et al., (2006) long-term potentiation is impaired in the MeCP2 null mice with no improvement even upon induction of theta burst stimulation. Researchers also investigated an alteration in the NMDA receptor in the symptomatic MeCP2 null mice. In

another study, Balakrishnan et al., (2016) used patch clamp electrophysiology in a MeCP2 null mouse hippocampal region to reveal altered expression of the NMDA receptor that also affects LTP levels. Electrophysiology results also reveal impairments in adenylyl cyclase, cyclic-AMP and PKA function in MeCP2 null mice leading to a reduction in LTP activity. All of these findings suggest a possible alteration in synaptic activity in animals lacking the MeCP2 gene.

Since synaptic activity is profoundly affected in patients suffering from Rett syndrome, it is essential to understand how it is influenced (Massey et al., 2004). Synaptic plasticity refers to the capability of the synapse to change its activity over a period in response to external stimuli. It can be measured in the form of long-term potentiation (LTP) and long-term depression (LTD).

Synaptic plasticity also causes alterations in neurotransmitters and their receptors, whose mechanisms are not fully understood, but plasticity proves to be a potential target for treatment. Hence, LTP and LTD are essential for normal development of the brain and a balance in the ratio of LTP/LTD is required for homeostasis. The levels and activity of LTP and LTD are dependent on calcium levels, calcium-calmodulin dependent kinase (CaMKII), N-methyl-D-aspartate receptors (NMDARs) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPARs). In general, changes in calcium level, CaMKII and other neurotransmitter receptors like NMDA and AMPA, could alter the expression of LTP and LTD. Such changes could also stimulate several downstream events that could either be beneficial or harmful to brain activity and development (Bayer et al., 2006; Balakrishnan et al., 2016). Any change in the synaptic/extra synaptic form of these receptors and the influx of calcium levels will significantly affect the LTP/LTD ratio.

Molecular basis for phenotypic/behavior changes in Rett syndrome

The next step would be to examine changes at the molecular level to understand the link between the mutation and the cellular dysfunction observed in the disease. Western blotting can be used to measure differences in protein expression in knockout animals. The experimental setup has three necessary parts: separation of proteins based on charge and mass, transferring the gel to a solid support like polyvinylidene fluoride (PVDF) or nitrocellulose membrane, and probing the target protein using labeled primary and secondary antibodies. Since the characteristic feature of Rett syndrome involves abnormal neuronal development and defects in synaptic plasticity, it is possible to see changes in synaptic proteins. Previous findings indicate reductions in synaptic proteins of specific NMDA receptors (GluN2B, GluN2A, and GluN1), AMPA receptors (GluR1, GluR2), CaMKII, protein kinase A/C, cAMP response element binding protein (CREB), signaling molecules of mitogen-activated protein kinase (MAPK), extracellular receptor kinase (ERK) and protein kinase B (Nguyen et al., 2012).

The NMDA receptor is one of the crucial glutamate receptors present in the nerve cell. It is activated when glutamate or glycine binds to it. The NMDA receptor is well known for its function in synaptic plasticity and memory. Its activity is highly dependent on calcium influx. It is a heterotrimeric receptor with three different subunits NR1, NR2 and NR3. Each subunit has several other subunits, each of them having a unique function: NR1 has 8, NR2 has 4 (NR2A, NR2B, NR2C, NR2D) and NR3 has 2 (NR3A, NR3B). Out of all the subunits, NR2A and NR2B have been studied the most. NR2A, also known as GluN2A, is believed to be involved in cell death pathways whereas NR2B, also known as GluN2B, is believed to be involved in cell

survival cascades (Bayer et al., 2006). Interestingly, GluN2B and GluN2A have differing roles, and both can affect either long-term potentiation (LTP) or long-term depression (LTD) depending on whether they are present in synaptic or extra synaptic locations. In case of Rett syndrome, NMDA receptor subunits- GluN2A and GluN2B seems to be altered.

The AMPA receptor is another glutamate receptor. Like the NMDA receptor, the AMPA receptor is activated when glutamate binds to it. It is primarily involved in synaptic plasticity and transmission across the neurons. The AMPA receptor is a tetramer consisting of 4 subunits: GluR1, GluR2, GluR3 and GluR4. GluR1 and GluR2 subunits play a major role in promoting long-term potentiation (LTP) through phosphorylation of several protein kinases at specific amino acid residues. CaMKII is one of the kinases that interact with both AMPA and NMDA receptors (Lisman et al., 2012). It is one of the many kinases that have the capability of inducing long term-potentiation (Lisman et al., 2012). Protein kinase A is another class of enzymes whose activity depends on the AMP and ATP. It further phosphorylates CREB and several other transcription factors that are important for neurons. Another serine-threonine kinase is MAPK which is involved in several cellular responses in the body like differentiation, proliferation, apoptosis and many more (Zhang et al., 2002). ERK and Akt are the primary transcription factors which regulate normal homeostasis in the cells (Castro et al., 2013).

The proteins mentioned above play a pivotal role in the normal development of the brain. Interestingly, levels of these proteins are also essential in the maintenance of LTP/LTD ratio and activity of several neurotransmitters (Wu et al., 2016). Any variation in the quantity of these

proteins could hinder the healthy development and function of the brain and promote severe neurodevelopment/neurodegenerative/neuropsychiatric disorders such as Rett syndrome. Therefore, it is imperative to understand the interaction of MeCP2 and these proteins in the brain. The majority of these proteins have been studied in MeCP2 null or KO mice model. This study aims to validate a rat model of Rett syndrome by looking into molecular alterations of specific proteins in the dorsal and ventral hippocampal regions of the brain because this region supports the cognitive processes that are affected in this syndrome.

CHAPTER 2

MATERIALS AND METHODS

Rats were obtained from a collaborating laboratory of the Biomedical Engineering department at The University of Texas at Dallas. Female rats with a mutation in MeCP2 gene were bred with male rats. Progenies were obtained and genotyped. Male rats having knockout and wild type MeCP2 genes were euthanized at the age of 36 days and the brains were obtained (from the collaborating lab) which was used for studying different protein expression.

Sample Size: A total of 33 rat brains were used for studying protein expression in the dorsal hippocampus and 30 for the ventral hippocampus. However, for two experiments, samples did not yield measurable protein levels, so those were not included in the data analysis. Thus, each experiment has different sample size.

Tissue Preparation: Rats were euthanized at the age of 32-38 days. They were deeply anesthetized using isoflurane and brains were removed within 2-2:30 minutes after administration of isoflurane. Brains were flash-frozen in ice-cold 2-methyl butane. Brains were stored in -80°C until tissues from dorsal and ventral hippocampus were dissected using a tissue punch kit. 4-5 tissue punches were obtained using a needle from both dorsal and ventral hippocampus in the range of 0.5-0.9mm diameter. The samples were stored in -80°C for running western blots in future.

Sonication of Samples: Tissue samples were sonicated using a sonication buffer. A stock solution of sonication buffer was prepared using phosphate buffer (To 1L water, added 5.52g sodium phosphate (monobasic) and 22.7g sodium phosphate (dibasic), pH set to 7.4) and EDTA (5mM or 1.46g/L). To this stock solution, 400uL glycerol, 80uL protease inhibitor and 40uL phosphatase inhibitor was added and brought to 4mL. Each tissue sample was mixed with 400uL of this solution.

Protein Assay: The working solution was prepared using 1uL of the quant reagent for each sample and 199μL of the quant buffer. 2μL of each sample was mixed with 198μL of working solution, vortexed for few seconds and incubated in room temperature for 15 minutes. Qubit protein assay kit was calibrated using the standards and samples were placed in the apparatus. Protein value for each sample was recorded and all the values were standardized to the smallest amount of protein.

SDS-PAGE: Samples were heated in a hot water bath at 70°C for 10 minutes. Running buffer was prepared using 50mL MOPS SDS running buffer (20X) + 950mL DI water and 435μL NuPage antioxidant was added to each inner chamber. Equal amounts of protein were loaded onto 4–12% gradient Bis-Tris MIDI gels and the gels were run at ~220V for 1 hour. Gel consisted of loading controls, ladder and wild type & knockout samples loaded in order.

Transfer: The gel was transferred onto a nitrocellulose membrane by electroblotting (iBlot system, Life Technologies). The transfer was run for 7 minutes.

Immunostaining: Non specific proteins were blocked with 2.5g milk in tris buffered saline with tween (TBS-T) for one hour at room temperature. The membranes were probed against the following antibodies and incubated overnight at 4.C : GluN2B (Rabbit, 1:300, Millipore), GluN2A (Rabbit, 1:300, Millipore), Actin (Rabbit, 1:1000, Cell Signaling), PSD95 (Rabbit, 1:1000, Cell Signaling), PCaMKIIα (Rabbit, 1:300, Cell Signaling), TCaMKII (Rabbit, 1:500, Cell Signaling), PPKA (Rabbit, 1:300, Cell Signaling) and TPKA (Rabbit, 1:500, Cell Signaling). On the next day, membranes were washed in TBS-T 3X (5mins, 5mins, and then 15mins), probed with a secondary antibody (goat anti-rabbit; Cell Signaling) and incubated for one hour on a shaker at room temperature.

Visualization: Membranes were again washed with TBS-T 3X (5mins, 5mins, and then 15mins) and then visualized using ECL western blotting substrate kit (Pierce). Compression of the membrane against the film for 2-5 minutes transferred a chemiluminescent signal to the film which was then exposed to developer, water and fixer to produce clear bands.

Stripping and Re-Probing: Membranes were first washed with TBS-T 3X (5mins, 5mins, then 15mins) and then with stripping buffer for 7 minutes. Excess stripping buffer was removed and rewashed with TBS-T 3X (5mins, 5mins, and later 15mins). Non specific proteins were blocked with 2.5g (in TBS-T) milk for one hour at room temperature and immunostaining and visualization procedures were repeated.

Data Analysis: Films were scanned to a computer and the free NIH program Image J was used to quantify the density of individual bands. Bands were normalized to a loading control by taking the ratio of the density of the protein of interest/density of the loading protein. For phosphorylated proteins, the total protein was used as the loading control. Other proteins were compared to actin or PSD95. However, we also compared the density of actin and PSD95 across samples to see if there was a difference in expression of these proteins in the knockout rats. We did not normalize the densitometry reading of actin or PSD95 to a loading control. The mean normalized densities of bands from wild-type and the knockout rats were compared using one way ANOVA test in Excel. A P value of 0.05 or less was considered to be statistically significant.

CHAPTER 3

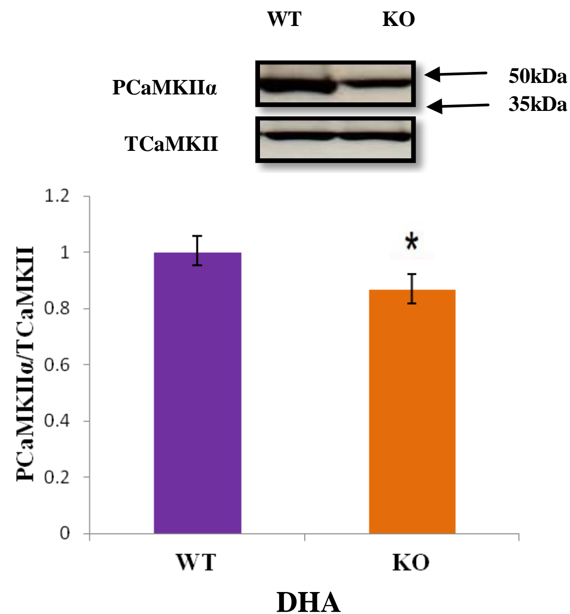
RESULTS

Previous studies across the globe from different mouse models of Rett syndrome suggested significant alterations in the expression of certain proteins in the brain. In our study, we evaluated the state of specific proteins in a MeCP2 knockout rat model specifically in the dorsal and ventral hippocampus by performing a western blotting protocol. Male MeCP2 knockout rats, as well as wild-type rats, were euthanized at the age of 32-38 days. Brains were isolated and tissues were collected from both the hippocampal regions. Synaptic plasticity-associated protein expression in the dorsal and ventral hippocampus was analyzed.

Rats with a knockout in the MeCP2 gene displayed a significant reduction in the phosphorylation of CaMKIIα at Thr286 compared to wild-type in both regions of the hippocampus. [Figure 1(A); $t(33) = 1.69$, $p = 0.045$] and [Figure 1(B); $t(30) = 1.70$, $p = 0.029$].

Protein kinase A (PKA) is another class of enzymes whose activity is important for the normal functioning of neurons. According to our data, there was no significant change in the phosphorylation of PKA between the knockout and wild-type MeCP2 rat model. This was observed for both dorsal and ventral hippocampus. [Figure 2(A); $t(33) = 1.69$, $p = 0.16$] and [Figure 2(B); $t(30) = 1.70$, $p = 0.16$].

A)



B)

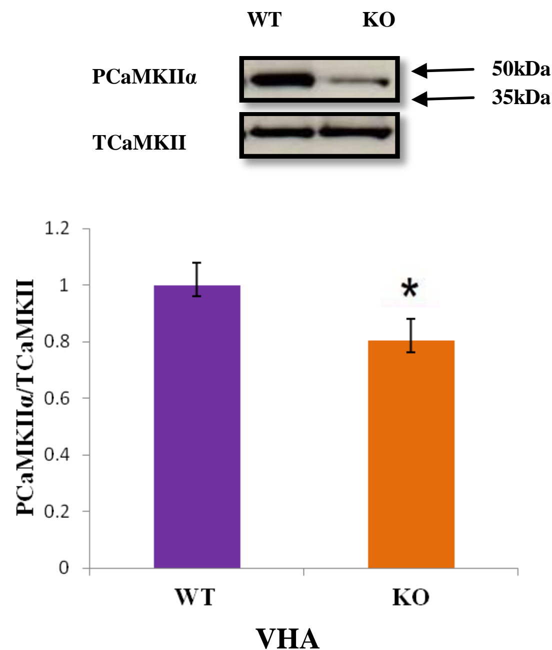
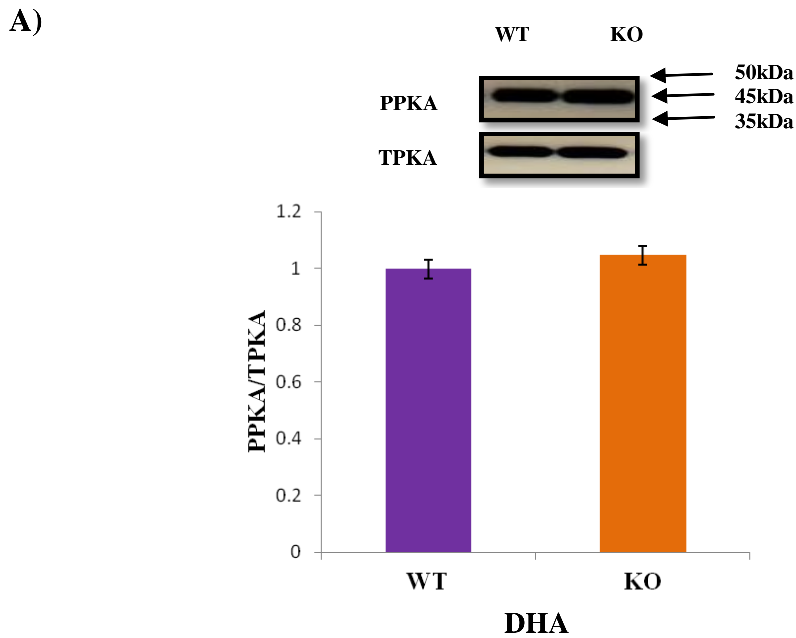


Figure 1. The calcium calmodulin protein-dependent kinase α (CaMKIIα) is significantly altered in the hippocampus of MeCP2 KO male rats. (A) Western blot analysis revealed decreased expression of phosphorylated CaMKIIα to total CaMKII in the dorsal hippocampus [$P<0.05$, n=21 (WT), 12 (KO)]. (B) Western blot analysis showed reduced expression of phosphorylated CaMKIIα to total CaMKII in the ventral hippocampus [$P<0.05$, n=18 (WT), 12 (KO)].



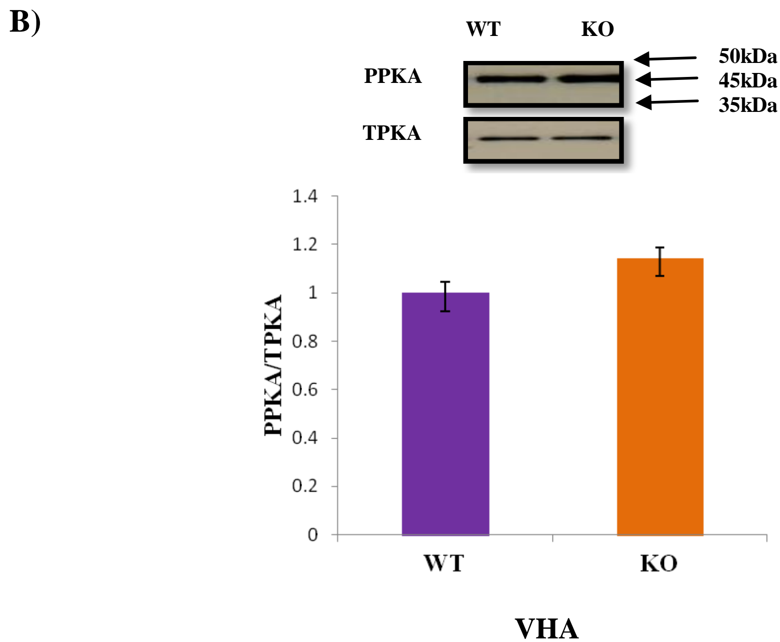
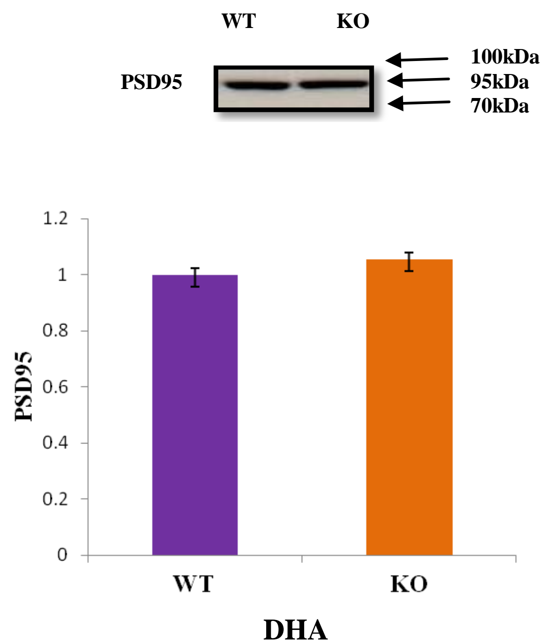


Figure 2. No significant change in protein kinase A (PKA) in the hippocampus of MeCP2 KO male rats. (A) Western blot data suggest no difference in PKA levels between the wild-type and MeCP2 knockout in the dorsal hippocampus [n=21 (WT), 12 (KO)]. (B) Western blot data indicates no change in PKA levels between the wild-type and MeCP2 knockout in the ventral hippocampus [n=18 (WT), 12 (KO)].

A)



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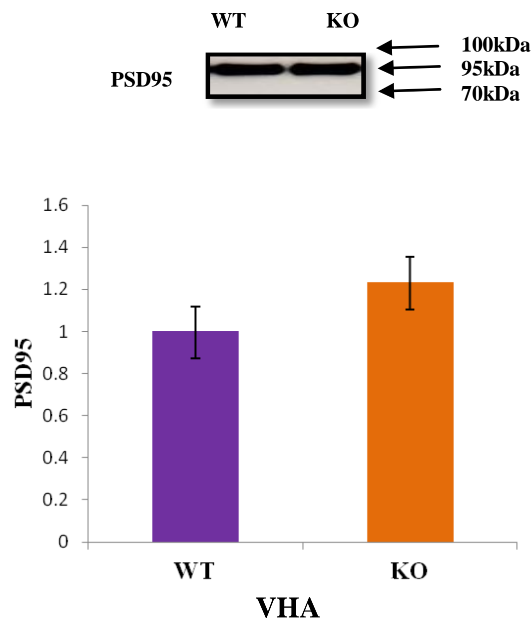
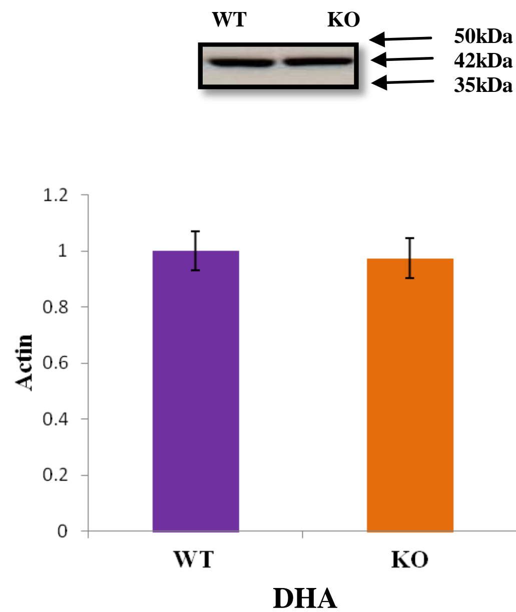


Figure 3. No significant difference in PSD95 in the hippocampus of MeCP2 KO male rats. (A) Western blot results signified no change in the protein expression of PSD95 between the wild-type and MeCP2 knockout in the dorsal hippocampus [n=21 (WT), 12 (KO)] .(B) Western blot meant no change in the protein expression of PSD95 between the wild-type and MeCP2 knockout in the ventral hippocampus [n=18 (WT), 12 (KO)].

A)



B)

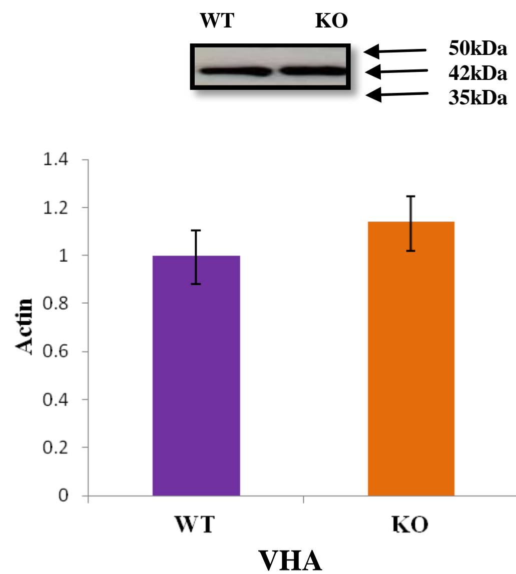
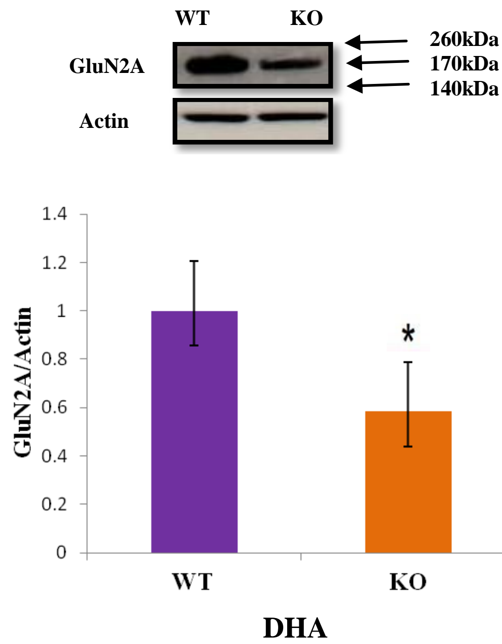


Figure 4. No significant change in actin in the hippocampus of MeCP2 KO male rats.
(A) Western blot results signified no change in the protein expression of actin between the wild-type and MeCP2 knockout in the dorsal hippocampus [n=21 (WT), 12 (KO)]. (B) Western blot results indicated no change in the protein expression of actin between the wild-type and MeCP2 knockout in the ventral hippocampus [n=21 (WT), 12 (KO)].

Although no difference was observed in phosphorylation of protein kinase A, significant reductions in the phosphorylated state of CaMKIIα, led us to elucidate further the molecular effects that could have caused the change. To do so, it was necessary to choose a proper loading control to which the levels of some synaptic protein expression could be quantified. We targeted two loading controls- PSD95 & actin and looked for knockout effects on these two proteins. There was no difference in PSD95 between knockout and wild-type groups in both dorsal and ventral hippocampus. [Figure 3(A); $t(33) = 1.69$, $p = 0.11$] and [Figure 3(B); $t(30) = 1.70$, $p = 0.1$]. Similarly, there was no change in the protein expression of actin between knockout and wild-type groups in both dorsal and ventral hippocampus. [Figure 4(A); $t(33) = 1.69$, $p = 0.45$] and [Figure 4(B); $t(30) = 1.70$, $p = 0.39$].

Since calcium calmodulin protein kinase is a crucial protein kinase involved in maintaining LTP/LTD ratio, we further explored the downstream molecular targets of CaMKII that plays a vital role in synaptic activity. Phosphorylated CaMKII is one of the many kinases which has the capability of inducing LTP and alter the ratio of LTP/LTD through direct and indirect phosphorylation of several signaling targets like ERK, NMDA, and AMPA receptors. In our study, we analyzed the protein extracts from the dorsal and ventral hippocampus to identify any protein level changes in the NMDA receptor subunits – GluN2A and GluN2B. Phosphorylated CaMKII interacts with NMDA receptor subunits and allows its trafficking into the membrane. This is important in strengthening the synapse and maintaining LTP. So, it would be exciting to note any protein change in NMDAR subunits as significant decrease is observed in the phosphorylation of CaMKIIα.

A)



B)

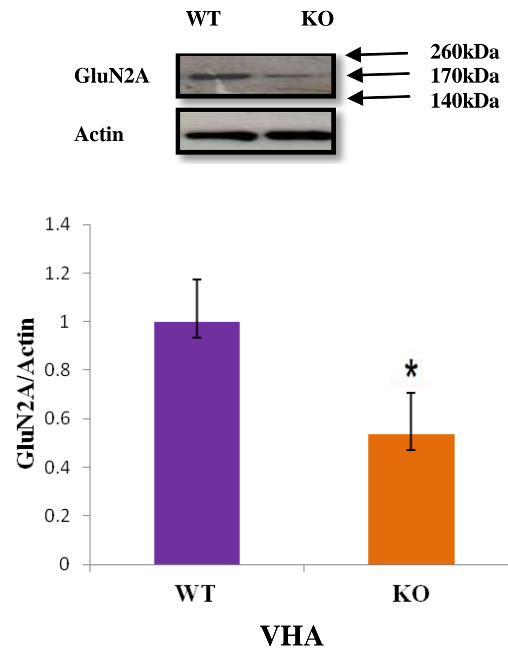
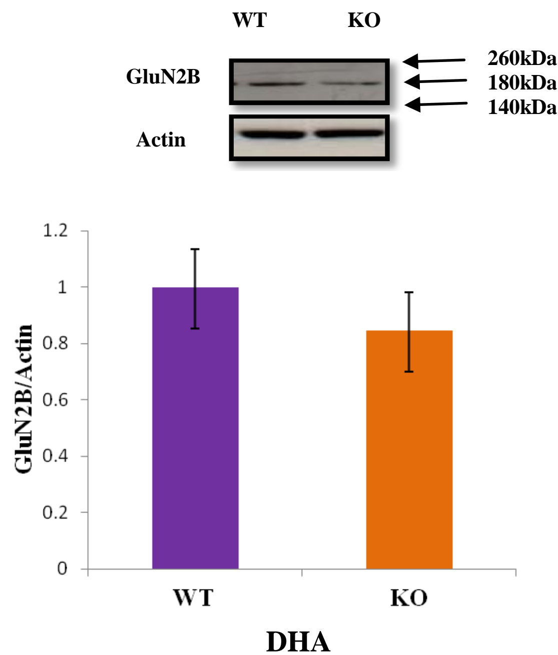


Figure 5. NMDA receptor subunit GluN2A (NR2A) is significantly altered in the hippocampus of MeCP2 KO male rats. (A) Western blot data suggest a decrease in GluN2A protein levels in the dorsal hippocampus [P<0.05, n=20 (WT), 11 (KO)]. (B) Western blot data revealed a significant reduction in the appearance of GluN2A in the ventral hippocampus [P<0.05, n=14 (WT), 11 (KO)]. Actin was used as the loading control.

A)



B)

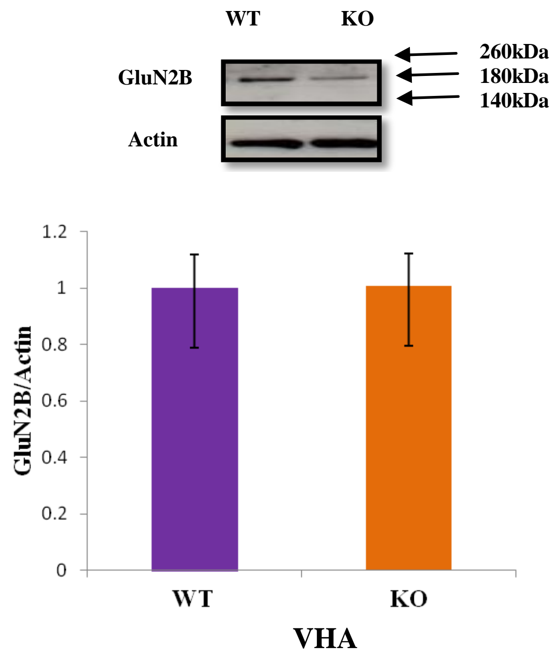
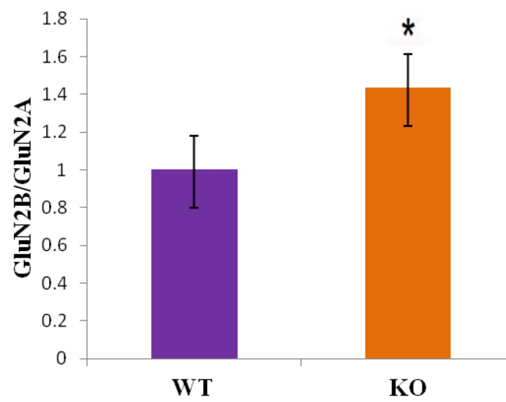


Figure 6. NMDA receptor subunit GluN2B (NR2B) is not altered in the hippocampus regions of MeCP2 KO male rats. (A) Western blot revealed no change in the levels of GluN2B in the dorsal hippocampus [n=21 (WT), 12 (KO)]. (B) Western blot analysis did not show any change in the appearance of GluN2B in the ventral hippocampus [n=18 (WT), 12 (KO)]. Actin was used as the loading control.

According to a t-test analysis of NMDA receptor subunits, GluN2A expression was significantly decreased in knockout and wild-type groups in both dorsal and ventral hippocampus. [Figure 5(A); t (31) = 1.69, p = 0.08] and [Figure 5(B); t (25) = 1.71, p = 0.015]. Contrary to that, there was no change in the expression of GluN2B between the two groups in both dorsal and ventral hippocampus. [Figure 6(A); t (33) = 1.68, p = 0.37] and [Figure 6(B); t (30) = 1.66, p = 0.48].

A)



B)

DHA

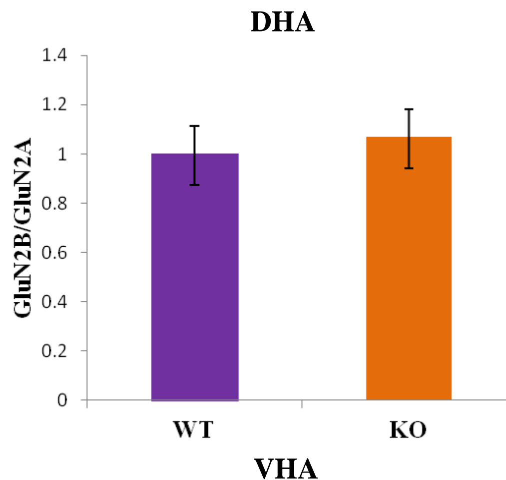


Figure 7. NMDA receptor is significantly altered in the hippocampus regions of MeCP2 KO male rats. (A) GluN2B/GluN2A ratio is significantly increased in the dorsal hippocampus, quantified by western blot [P<0.05, n=20 (WT), 11 (KO)]. (B) Western blot revealed no significant change in the ratio of GluN2B/GluN2A in the ventral hippocampus [n=14 (WT), 11 (KO)].

We also checked the ratio of GluN2B/GluN2A. Western blot data showed decrease in the ratio in dorsal hippocampus. However there was no difference in ventral hippocampus.

Overall, from our study, we observed the following:

1. Significant reductions of NMDA receptor subunit GluN2A (NR2A) in both dorsal and ventral hippocampus.
2. The ratio of GluN2B/GluN2A through western blot data revealed increase in dorsal hippocampus with no change in ventral hippocampus
3. Calcium calmodulin protein kinase II α (CaMKIIα) was significantly decreased in both dorsal and ventral hippocampus.
4. No change was observed in the expression levels of NMDA receptor subunit GluN2B (NR2B), protein kinase A, PSD95 & actin.

CHAPTER 4

DISCUSSION

Over the years, several animal models have been constructed for research in Rett syndrome. Cre-Lox recombinase system has been utilized in editing the MeCP2 gene, providing various mutated/knockout/duplicated models for Rett syndrome. Previous studies in several laboratories on MeCP2 null or knockout models have been performed using mice. As stated earlier, this is because the physiological and genetic similarity between mice and humans is high. Also, mice have advantages of being small in size, short breeding, reproductive and development cycles, and easy maintenance compared to other animal models. Furthermore, they have similar phenotypic effects that can be related to humans. In our study, we utilized a male rat model of the MeCP2 gene knockout to look for changes in protein expression in the dorsal and ventral hippocampus regions of the brain. This is because the mouse genome was the first to be characterized and analyzed. Also, the physiological and genetic similarity between mice and humans is significantly high. Rats are larger than mice, which can be advantageous during brain surgeries. Apart from this, electrophysiology studies which are essential to assess the neural deficits associated with behavior and cognitive impairments are relatively easy to study in rat models. All these factors increase the potential for understanding the molecular mechanisms of RTT pathophysiology and translatability to clinical trials to ameliorate the progression of the disease (Veeraraghavan et al., 2016).

Since the mutation in Rett syndrome is on the X chromosome, it is lethal in males. Use of a male model for studying Rett syndrome presents certain advantages. Because the mutation is so rare and lethal, if diagnosed in young boys early it may improve survival rates. Further, phenotypic changes and clinical manifestations are expected to be more prominently visible in male animal models of Rett syndrome as compared to females. Therefore, males are phenotypically more symptomatic than females, displaying symptoms and regression at an early age.

Patterson et al., (2016), summarized and compared some of the physical, behavioral and motor deficits between male and female animal models (both rats and mice) of Rett syndrome. These results include work of several researchers on behavior phenotypes observed in Rett syndrome. The work provides significant insights into the clinical manifestations seen in Rett syndrome and is the basis for choosing the male rat model for our Rett syndrome research. From the Patterson study, it is clear that males tend to display abnormal symptoms at a very young age compared to the females. This helps in understanding the physiology of the disease at an early stage, followed by the progression of the disease which allows looking into the molecular aspects causing the cognitive changes. Previous papers have established that the Rett mouse model demonstrates various behavioral, motor and synaptic plasticity deficits.

In our study, we assessed the expression of some proteins that are important for synaptic plasticity and memory in a male MeCP2 gene knockout rat to understand changes in molecular mechanisms which could be involved in the behavioral and neurological changes observed in Rett syndrome patients (Moretti et al., 2006). We specifically targeted hippocampus region of the

brain as it plays an essential role in memory and plasticity. We separated the dorsal hippocampus and ventral hippocampus, as they both play distinct roles. Dorsal hippocampus is majorly involved in information processing, memory function and cognitive roles whereas the ventral hippocampus responds to stress and emotions (Fanselow et al., 2014).

Our results display significant alterations in the NMDA receptor subunit GluN2A and calcium calmodulin protein kinase II α (CaMKIIα) in both dorsal and ventral hippocampus. GluN2A expression was significantly decreased between the wild-type and knockout groups. However, there was no significant change in GluN2B expression in both dorsal and ventral hippocampus. This causes an imbalance in the ratio of GluN2B/GluN2A, disturbing homeostasis and affecting the normal development of brain at various levels.

Table 1. Molecular and Behavior Changes in Rett syndrome

MeCP2 model	Mice/Rat (Age)	Behavior Changes	Molecular Changes	Possible Treatments	Author
MeCP2 null mice	Male-28 days, female-42 days	Decreased lifespan, locomotion activity, heart rate, respiration pattern, social and anxiety behavior	Decreased IGF-1, Akt, ERK1/2 and PSD95 levels (whole cell)	Recombinant hIGF-1	Castro et al., (2013)
MeCP2 duplication mice	Male (10-16 weeks)	Loss of episodic memory, motor learning deficits, short-term plasticity	Decrease levels of GABA receptor (hippocampus)	Picrotoxin	Na et al., (2014)
MeCP2 KO mice	Male (6-8 weeks)	Decreased life span, locomotion activity, brain size	Decreased levels of BDNF (cortex, cerebellum, hippocampus)	Over expression of BDNF	Chang et al., (2006)
MeCP2 KO mice	Male (4-6 weeks), female (20 weeks)	Behavior deficits, brain shrinkage, decreased dendritic and astrocytes complexity	Reductions in CaMKII, AMPAR(GluR2/3), NMDAR (GluN2B/GluN2A) (cerebellum, hippocampus, brain stem, cortex)	Over expression of MeCP2 gene	Nguyen et al., (2012)
MeCP2-308 mice	Female (1 Year)	Defects in motor activity, different forms of memory and synaptic plasticity	Changes in phosphorylated rpS6 and Ser 235 and 240 (hippocampus)	Serotonin receptor 7	Filippis et al., (2015)
MeCP2 KO mice	Male (5 weeks)	Long-term potentiation impaired	Increase in the level of regulatory subunit of PKA, cyclic AMP (hippocampus)	Rolipram	Balakrishnan et al., (2016)

Since Rett syndrome is associated with progressive deterioration of cognition, it is essential to look for changes in neurotransmitters and their receptors. In a study by Nguyen et al., in 2012, male mice (4-6 weeks) and female mice (20 weeks) with mutations in the MeCP2 gene displayed marked reductions in the levels of NMDA receptors (NMDAR2A by 50%) and CaMKII (by 50%). This is consistent with our study where MeCP2 KO male rats around the age of 5 weeks showed significant reductions in NMDA receptor subunit GluN2A and the phosphorylated form of calcium calmodulin protein kinase IIα at Thr286. Calcium calmodulin protein kinase II is a critical protein kinase independently capable of inducing long-term potential in brain cells. NMDA receptors also play a significant role in supporting synaptic plasticity and homeostasis in neuronal cells. The phosphorylated form of CaMKII interacts with NMDA receptor subunits and helps in the initiation and preservation of long-term potentiation (LTP). Changes in calcium influx could also trigger further downstream changes in CaMKII. Specifically in Rett syndrome, from the western blot analysis, it is possible that a decrease in phosphorylated form of CaMKII could affect its interaction with NMDA receptor. The significant reduction observed in the GluN2A subunit of the NMDA receptor indicates a possible disruption in the interaction between CaMKII/NMDAR that could alter the ratios of LTP/LTD. Studies suggest that CaMKII alone has the potential to induce LTP (Pi et al., 2010). A decrease in CaMKII activity could decrease the levels of LTP in the brain and affect synaptic plasticity. A reduction in both CaMKIIα and the NMDA receptor subunit GluN2A could further impact synaptic strengthening and LTP/LTD ratios.

According to Asaka et al., (2006), alterations in the hippocampal NMDA receptors- GluN2B and GluN2A significantly affect the ratios between them and, consequently, affect LTP which is a measure of synaptic plasticity. NMDA receptors are important glutamate receptors involved in maintaining synaptic plasticity in neurons. The NMDA receptor subunit- GluN2A is believed to be involved in cell death pathways whereas GluN2B is considered to be involved in cell survival cascades. GluN2B and GluN2A have differing roles and both can affect either long-term potentiation (LTP) or long-term differentiation (LTD) depending on whether they are present in synaptic or extra synaptic locations. Based on our MeCP2 KO male rat model of Rett syndrome, we can predict that a reduction in GluN2A and slight changes in GluN2B will alter the ratios of GluN2B/GluN2A and could affect the levels of long-term potentiation and long-term depression in the brain.

In a study performed by Balakrishnan et al., (2016) at the University of Gottingen, Germany; MeCP2 wild-type and knockout male mice at the age of 5 weeks revealed a two-fold increase in the regulatory subunit of protein kinase A (PKA), while there was no change in the catalytic subunit. Apart from this, use of multiple tetanus also showed significant reductions in cyclic AMP that could affect LTP level. In our study, we utilized male rat models of the MeCP2 gene KO and found similar results with no major alterations in the protein levels of the catalytic subunit of protein kinase A. From this finding, we can probably confer that the catalytic part of PKA is not a cause of the physiological symptoms observed in Rett syndrome. However, from the mouse model study, one can predict to see changes in the regulatory subunit of PKA that could affect various other transcription factors and hinder the process of synaptic strengthening

in Rett syndrome patients. For instance, protein kinase A can interact with cyclic AMP, an important second messenger for signaling transduction. Reductions in PKA and cAMP levels (Balakrishnan et al., 2016) could affect its interactions with other signaling molecules that may disrupt synaptic activity in Rett syndrome patient. For instance, cAMP is involved in regulating some ion channels in brain. Any change in its level could trigger an imbalance in the ion channels and cause severe cognitive deficits (Ali et al., 2016).

Postsynaptic density protein 95 also known as PSD95 is a protein found in the postsynaptic density of neurons. It can interact with several ion channels and receptors like NMDA and AMPA to facilitate synaptic plasticity, stability of various signaling proteins, and synaptic changes during long-term potentiation. In a study done by Castro et al., (2013), insulin-like growth factors levels (IGF-1) are considerably reduced in MeCP2 null male mice at the age of 28 days and female mice at the age of 42 days. There was a further decrease in synaptic PSD95 and phosphorylated Akt and ERK ½ levels. Contrary to this, a study conducted by Nguyen et al., (2012), on male mice (4-6 weeks) and female mice (20 weeks) with mutations in MeCP2 did not reveal any alterations in the levels of PSD93 and PSD95. In our study, male rats with mutations in MeCP2 did not show any difference in the levels of PSD95 compared to the wild-type MeCP2 group. This suggests that PSD95 is relatively preserved at least in the tested brain regions in the MeCP2 null male mice at the experimental ages and there are no changes in the expression of PSD95 in the Rett syndrome rat model.

Actin belongs to the family of globular proteins. It plays an essential role in many cellular processes. In our study, we checked for the expression of actin in dorsal & ventral hippocampus and did not find any difference between the wild-type and knockout groups. No control was used to examine the differences in actin. Actin was initially used as a loading control in the experiments. However, according to some investigators like Cortelazzo and colleagues (2014), differences in actin expression was observed in the KO. Therefore, we compared actin levels between the wildtype and knockout groups. We did not observe a difference in actin expression between the two groups so we used actin as the loading control to look for changes in NMDA receptor subunits GluN2A and GluN2B. This is consistent with the results found by Filippis and colleagues in 2015, where no change was observed in the levels of Actin in a mouse model of Rett syndrome. However, further analysis should compare the levels of actin expression with other loading controls such as GAPDH.

Overall, striking reductions were observed in NMDA receptor subunit GluN2A in the hippocampus. There was not much change in GluN2B expression in the dorsal and ventral hippocampus. This can lead to alterations in the ratio of GluN2B/GluN2A in brain cells. Also, a considerable decrease is seen in the phosphorylated form of CaMKIIα at Thr286 in both dorsal and ventral hippocampus. It is evident that phosphorylated form of CaMKII interacts with NMDA receptor subunits either directly or indirectly. It mainly does so through influx of calcium ions and phosphorylation of CaMKIIα at Thr286 when it interacts with NMDA receptor subunits (Sanhueza et al., 2011). Alterations in the levels of NMDA receptor subunits and phosphorylated CaMKIIα would remarkably affect its interactions with each other and other

molecular targets. It may also greatly impact the levels of LTP and LTD, thereby affecting the process of synaptic plasticity that supports learning and memory and may cause an overall cognitive decline as seen in Rett syndrome patients. Interestingly, there was no change in the PKA catalytic subunit between the wild-type and knockout groups. Assessing the expression of synaptic protein PSD95 and globular protein actin provided a basis for choosing the most appropriate loading, in this case, actin- to check for changes in NMDA receptor subunits protein levels.

On the whole, this study used male rat model of Rett syndrome to look for changes in protein expression of specific synaptic proteins and receptors in the dorsal and ventral hippocampus regions of the brain. Data analyzed by western blot revealed reductions in the levels of GluN2A and phosphorylated (activated) CaMKIIα. There was no significant change in the levels of PKA, GluN2B, PSD95 and actin. All the proteins were studied in rat MeCP2 KO model at the age of around five weeks and these data are mostly consistent with protein expression changes observed in the mouse model of MeCP2 KO during the same age period (4-6 weeks). This suggests that the rat can be used to model changes in protein expression in the hippocampus region in Rett syndrome. Changes in these proteins could affect synaptic plasticity and may be the cause of the cognitive impairments associated with Rett syndrome.

CHAPTER 5

CONCLUSIONS

We have utilized a male rat model of Rett syndrome to understand the molecular mechanisms behind the physiological and behavioral changes observed in Rett syndrome. We tried to validate the rat model by comparing changes in protein expression in established mouse models of Rett syndrome across the world in different laboratories. From our study, we conclude that rats can be utilized as a model for studying Rett syndrome because they have similar protein level changes for specific receptors, signaling molecules and protein kinases as those observed in the mouse model. Western blotting data revealed particular alterations in phosphorylated CaMKIIα and NMDA receptors in the hippocampus region of the brain that may affect synaptic plasticity and lead to the cognitive impairments observed in Rett syndrome patients. The interaction between NMDA receptor subunits and phosphorylated CaMKII is essential for maintaining LTP and LTD levels. Hence any change in these kinases and receptors would significantly affect synaptic plasticity and several other processes in the brain.

It is evident that CaMKII and NMDAR play an important role in understanding the clinical presentations in Rett syndrome, however we did not observe any changes in the levels of regulatory subunit of protein kinase A (PKA), post synaptic density protein (PSD95) and actin.

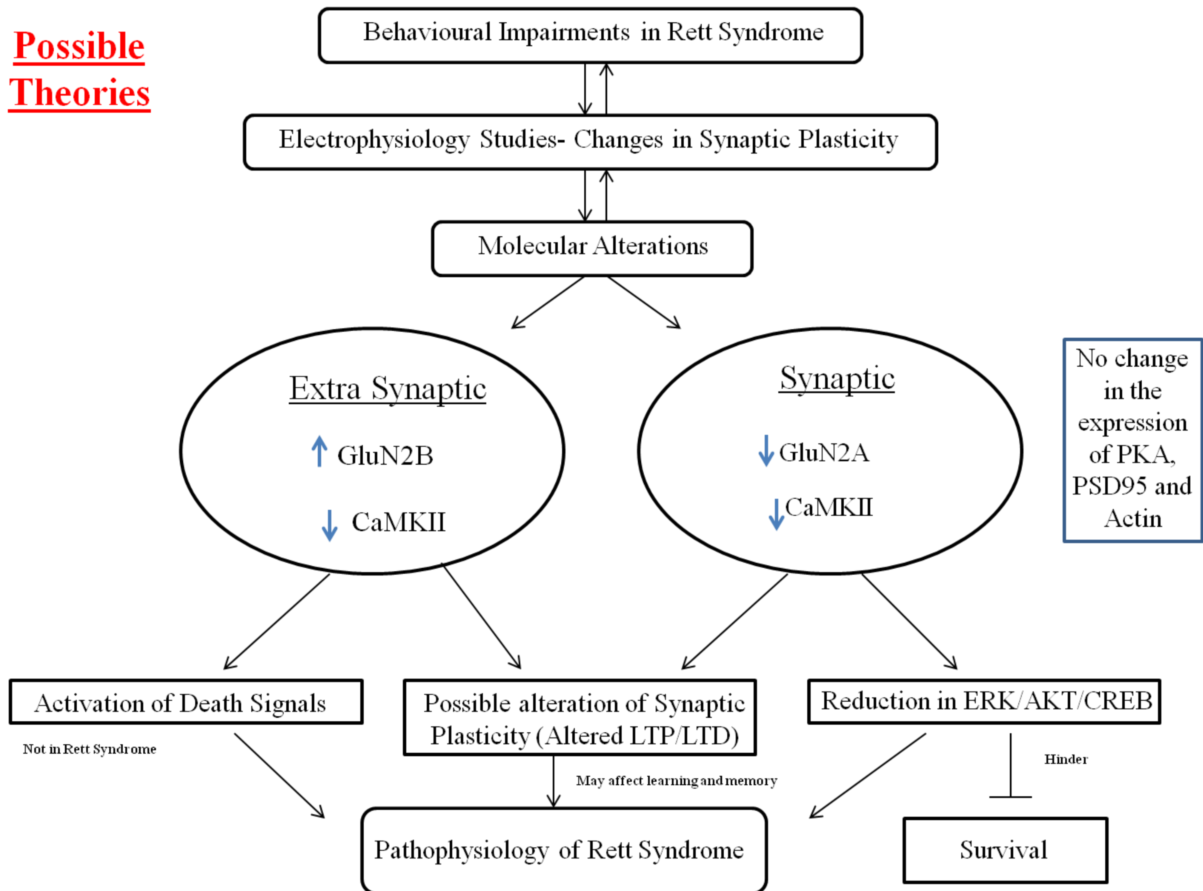


Figure 8. A possible theory of pathophysiology observed in Rett syndrome.
Several behavior impairments are observed in Rett syndrome – brain shrinkage, loss of motor skills, reduced locomotion activity, reduced dendritic complexity, irregular breathing habits, anxiety disorders and so on. These behavior changes led to electrophysiology studies with prominent changes in synaptic plasticity, majorly affecting long-term potentiation and long-term depression. Molecular alterations with changes in protein expression in NMDAR subunit GluN2A and phosphorylated form of CaMKIIα may further lead to changes in synaptic plasticity, affecting the behavior in Rett syndrome patients. This may also affect the process of learning and memory. Several other molecular targets could be varied in Rett syndrome that may cause possible behavior and physiological changes.

Table 2. Comparisons between mice and rat models for variables studied for Rett syndrome

Variable	Mice Model (WT vs KO)* MeCP2	Rat Model (WT vs KO)** MeCP2	
	Hippocampus	Dorsal Hippocampus	Ventral Hippocampus
GluN2A	Decrease $P<0.05$ [1]	Decrease $P<0.05$	Decrease $P<0.05$
GluN2B	Decrease $P<0.05$ [1]	Decrease (Not significant)	No Significant change
GluN2B/GluN2A	-------------	Increase $P<0.05$	No Significant change
PCaMKIIα/TCaMKII	Decrease $P<0.05$ [1]	Decrease $P<0.05$	Decrease $P<0.05$
PPKA/TPKA	No Change (Catalytic subunit) [2]	No Change (Catalytic subunit)	No Change (Catalytic subunit)
Actin	No Change [3]	No Change	No Change
PSD95	No Change [4] Decrease $P<0.05$ [5]	No Change	No Change

*observations from other research papers/lab (ref)
** present study
WT(wild type), KO (Knockout)
[1] Nguyen et al., (2012), Asaka et al., (2006)
[2] Balakrishnan et al., (2016)
[3] Filippis et al., (2015)
[4] Nguyen et al., (2012)
[5] Castro et al., (2016)

In summary, our study tries to validate rat model of Rett syndrome by looking into changes in protein expression that may affect hippocampus dependent behavioral impairments. We used a male rat model of the MeCP2 KO to study molecular changes in Rett syndrome and got similar results to the existing mice models published in different laboratories across the globe with similar experimental conditions (Table 2). Changes in the NMDAR subunit and the phosphorylated form of CaMKIIα indicate alterations in synaptic plasticity. Modifications of synaptic plasticity along with molecular changes could be one of the many reasons for the clinical manifestations observed in Rett syndrome patients. The exact molecular mechanisms are not fully understood yet, however, targeting these synaptic proteins in future research could pave the way for better understanding the pathophysiology of this disease.

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BIOGRAPHICAL SKETCH

Archana Mahadevan Iyer was born to Viswanathan Mahadevan and Uma Mahadevan in Vadodara, Gujarat, India. She attended Bhartiya Vidya Bhavan's V.M. Public School from kindergarden through high school. She received a scholarship for scoring the highest marks in Biology and Chemistry in high school in 2011. She pursued her undergraduate courses at SRM University, Chennai, India and received her degree of Bachelor's of Technology in Biotechnology with honors in 2015. During the final year of her undergraduate studies, she worked on her thesis project. Her thesis topic was "Studies on the therapeutic intervention of angiogenesis: Implications of endothelial gelatinases" at the Central Leather Research Institute (CSIR-CLRI), Chennai. The main aim of the project was to study the effect of thalidomide on expression of MMP (Gelatinases) and the angiogenesis process. She is now pursuing a Master of Science in Molecular and Cellular Biology at The University of Texas at Dallas. She is working as a volunteer research student in Dr. Christa K. Mcintyre's lab. She assisted a PhD student in understanding molecular mechanisms of vagus nerve stimulation. She is now working on a thesis project to study changes in protein expression in Rett syndrome by performing the western blotting technique.

CURRICULUM VITAE

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EDUCATIONAL QUALIFICATIONS:

The University of Texas at Dallas, Richardson, TX
December 2017
M.S., Molecular and Cellular Biology

SRM University, Chennai, India
May 2015
B.Tech, Biotechnology

RESEARCH EXPERIENCE:

Dr Christa McIntyre Rodriguez Lab (Neurobiology of Memory Lab), School of Behavioral and Brain Sciences, The University of Texas at Dallas, August 2016-Present

- Assisted PhD student in the study on effect of fear learning and memory via vagus nerve stimulation in rats
- Acquired skills in several molecular and behavioral studies like Western blotting, Tissue punching, Fear conditioning and extinction, Social interaction, Elevated plus maze, Marble Burying etc.

Molecular Neuropathology Lab, The University of Texas at Dallas, August 2016-April-2017

- Analyzed the mutation and Amyloid Beta plaque present in Alzheimer's disease transgenic mice using Polymerase Chain Reaction (PCR) and Western Blotting respectively
- Compared and distinguished healthy and diseased model of Alzheimer's using Immunofluorescence (IFC)

ACADEMIC PROJECTS:

Central Leather Research Institute (CLRI), Chennai, December 2014-April 2015

- Researched on various angiogenesis and cancer inhibiting drugs
- Tested the effect of thalidomide on a normal, angiogenic and cancer cell line both *in vitro* and *in vivo*
- Acquired skills in Cell culture, ELISA, Zymography, Tube formation assay and CAM assay
- Organized all data and represented a thesis

TRAINING EXPERIENCE:

1. **Department of Biochemistry, Molecular Endocrinology and Stem Cell Research Laboratory, Maharaja Sayajirao University of Baroda, Vadodara, June 2014**
   - Experimented on tissue samples using immunocytochemistry and western blotting
   - Trained to handle, feed and inject (Intraperitoneal, Intramuscular and subcutaneous) rats
2. **Pharmacy Lab, Maharaja Sayajirao University of Baroda, Vadodara, December, 2013**
   - Grasped knowledge on different types of drug dosage and its formulatory aspect
3. **Indo-American Hybrid Seeds Pvt. Ltd., Bangalore, June 2013**
   - Learned recombinant DNA technology by conducting experiments on infected tomato leaf curl virus

TEACHING EXPERIENCE:

- Teaching assistant for Biochemistry course for undergraduate students at The University of Texas at Dallas, 2017

CO-CURRICULAR ACTIVITIES:

- Assisted M. Tech students at their ongoing project on Diabetes in the animal biotech lab at SRM University
- Presented a poster on “Gamma Interferon Response in calves to DNA vaccines against Bovine Tuberculosis”

COMMUNITY SERVICE:

- Active volunteer of a student run organization called “The Green Nest” wherein every weekend; children from various orphanages in Chennai are taught English
- Secured Third place of excellence as a volunteer and recruited as the Deputy HR Director

LANGUAGES: Proficient in English, Hindi, Gujarati and Tamil, Beginner-German