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Vitamin D Modulates Rett Syndrome Phenotypes and Underlying Cellular Pathways in an Mecp2-mutant Mouse Model

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Abstract

Rett syndrome (RTT) is a progressive and severe X-linked neurodevelopmental disorder caused by mutations in the transcriptional regulator MECP2. There is currently no effective treatment for RTT. Female RTT patients develop relatively normally during the first 6-18 months of life, after which they undergo a period of rapid regression, losing the ability to talk, walk and purposefully use their hands, in addition to suffering from deceleration of head growth, and onset of repetitive, autistic behaviors. RTT symptoms can be partially reversed by the re-expression of Mecp2 in adult mice, suggesting the potential for post-symptomatic therapeutic intervention. Among the many dysfunctions caused by the loss of Mecp2 is aberrant NF-κB signaling. Importantly, genetically attenuating NF-κB rescues some characteristic neuronal phenotypes of RTT. Given that vitamin D (VitD) inhibits NF-κB activity and VitD deficiency is prevalent in RTT patients, the focus of this dissertation was to investigate whether VitD supplementation would ameliorate the many Mecp2-null phenotypes in RTT mouse models. Using an in vitro approach, I determined that adding the activated form of vitamin D to Mecp2-knockdown cortical neurons reduces the aberrant NF-κB activation and promotes neurite outgrowth. Further, VitD dietary supplementation moderately extends the lifespan of Mecp2-null mice and rescues dendritic complexity and soma area of 8-week-old Mecp2-null and 5-month-old heterozygous female mice in a dose dependent manner. RNA-sequencing analyses indicate that VitD supplementation normalizes the expression of many differentially expressed genes associated with neuronal morphology in the cortex of Mecp2 heterozygous female mice at 7 months of age. Moreover, I demonstrate that VitD supplementation improves motor deficits and anxiety-like behavior of RTT female mice, in an age dependent manner. Interestingly, I have found that insufficient serum 25(OH)D concentration, the major circulating form of vitamin D, only
disrupts the behavior of *Mecp2* deficient mice, not altering the performance of their wild-type littermates. Additionally, exposure to VitD deficient chow does not exacerbate behavioral outcomes of *Mecp2* heterozygous female mice broadly, even though it leads to extensive transcriptome alterations. Both VitD supplementation and restriction diets result in altered expression of genes involved in the metabolism of VitD. This is observed exclusively in *Mecp2* mutant mice, suggesting that the loss of *Mecp2* increases susceptibility to VitD homeostasis disruptions in mice. Overall, my data demonstrate that VitD supplementation ameliorates phenotypes of *Mecp2* mutant mice, and its modulation could underlie RTT pathology. Moreover, my transcriptome data provides novel insight and opens up new exciting avenues for investigation.
Vitamin D modulates Rett syndrome phenotypes and underlying cellular pathways in an MeCP2-mutant mouse model

by

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Dissertation

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<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>5hmC</td>
<td>5-hydroxymethylcytosine</td>
</tr>
<tr>
<td>5mC</td>
<td>5-methylcytosine</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ACM</td>
<td>Astrocytic conditioned media</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>apoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>ASDs</td>
<td>Autism spectrum disorders</td>
</tr>
<tr>
<td>BACE1</td>
<td>β-site APP cleaving enzyme</td>
</tr>
<tr>
<td>BAFFR</td>
<td>B cell activating factor receptor</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>C/EBPb</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>CaMK IV</td>
<td>Calcium/calmodulin-dependent protein kinase type IV</td>
</tr>
<tr>
<td>CC</td>
<td>Corpus callosum</td>
</tr>
<tr>
<td>Cdk5</td>
<td>Cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>CDKL5</td>
<td>Cyclin-dependent kinase-like 5</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPN</td>
<td>Callosal projection neurons</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CREB1</td>
<td>Cyclic AMP-responsive element-binding protein 1</td>
</tr>
<tr>
<td>CYPs</td>
<td>Cytochrome P450 mixed-function oxidases</td>
</tr>
<tr>
<td>DEG</td>
<td>Differentially expressed genes</td>
</tr>
<tr>
<td>DGCR8</td>
<td>DiGeorge syndrome critical region 8</td>
</tr>
</tbody>
</table>
DIV  Days in vitro
DM   Dissociation medium
EtOH Ethanol
FOXG1 Forkhead box G1
GGTase-I Geranylgeranyl transferase-I
GO   Gene ontology
GSA  Gene specific analysis
Gsk3β Glycogen synthase kinase-3β
Het  Heterozygous
HFD  High-fat diet
HIPK2 Homeodomain-interacting protein kinase 2
hVDR Human VDR
IGF1 Insulin-like growth factor 1
IκB  Inhibitor of κB
IKK  IκB kinase
iPSC Induced pluripotent stem cell
IRAK1 Interleukin 1 receptor-associated kinase 1
IRAK4 Interleukin 1 receptor-associated kinase 4
MBD Methyl-CpG-binding domain
MECP2 Methyl-CpG-binding protein 2
mGlu7 Metabotropic glutamate receptor 7
MIA  Maternal immune activation
MRE miRNA recognition element
MyD88 Myeloid differentiation primary response gene 88
nAChR Nicotinic acetylcholine receptor
NCoR Nuclear repressor co-repressor
NES Nuclear export sequence
NF-κB Nuclear factor-kappa B
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB-RE</td>
<td>NF-κB-response elements</td>
</tr>
<tr>
<td>NGF</td>
<td>Neuronal growth factor</td>
</tr>
<tr>
<td>NID</td>
<td>NCoR/SMRT interacting domain</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB-inducing kinase</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NRF-2/HO-1</td>
<td>Nuclear factor erythroid 2-related factor 2/heme oxygenase 1</td>
</tr>
<tr>
<td>NSCs</td>
<td>Neural stem cells</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>Pdia3</td>
<td>Protein disulfide isomerase family A member 3</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PLAA</td>
<td>Phospholipase A2 activating protein</td>
</tr>
<tr>
<td>poly(I:C)</td>
<td>Polyriboinosinic-polyribocytidylic acid</td>
</tr>
<tr>
<td>Ptk2β</td>
<td>Protein tyrosine kinase 2 β</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylenetetrazol</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator for NF-κB</td>
</tr>
<tr>
<td>RHD</td>
<td>Rel homology domain</td>
</tr>
<tr>
<td>rhIGF1</td>
<td>Recombinant human IGF1</td>
</tr>
<tr>
<td>RIPs</td>
<td>Receptor-interacting proteins</td>
</tr>
<tr>
<td>RTT</td>
<td>Rett syndrome</td>
</tr>
<tr>
<td>RUV</td>
<td>Remove unwanted variation</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid-X-receptor</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage-activating protein</td>
</tr>
<tr>
<td>sGTPases</td>
<td>Small guanosine triphosphate-binding proteins</td>
</tr>
<tr>
<td>SIRT-1</td>
<td>Sirtuin-1</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator of retinoic acid and thyroid hormone receptor</td>
</tr>
<tr>
<td>SRC-1</td>
<td>Steroid receptor coactivator 1</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>Sterol regulatory element binding protein 2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>TADs</td>
<td>Transcription transactivation domains</td>
</tr>
<tr>
<td>TAK1</td>
<td>TGF-β-activated kinase I</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNFR-associated death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNFR associated factor</td>
</tr>
<tr>
<td>TRD</td>
<td>Transcriptional repression domain</td>
</tr>
<tr>
<td>Trk</td>
<td>Tropomyosin-related kinase</td>
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<tr>
<td>USVs</td>
<td>Ultrasonic vocalizations</td>
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<tr>
<td>VBD</td>
<td>Vitamin D binding protein</td>
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<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
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<td>VDR/RXR</td>
<td>VDR/retinoid X receptor</td>
</tr>
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<td>VDRE</td>
<td>VDR response elements</td>
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<td>VitD</td>
<td>Vitamin D</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
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<tr>
<td>XCI</td>
<td>X-chromosome inactivation</td>
</tr>
<tr>
<td>Xi</td>
<td>Inactivated chromosome</td>
</tr>
<tr>
<td>YB-1</td>
<td>Y box-binding protein 1</td>
</tr>
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</table>
Chapter 1: Introduction

This chapter contains the published manuscript:


Sections 1.1.1 and 1.1.2 have been expanded upon from the published manuscript; Sections 1.1.3 to 1.1.8 appear as published, along with Figures 1.1 and 1.2, and Table 1.
1.1. Rett syndrome is a severe neurodevelopmental disorder

Rett syndrome (RTT) was first described in 1966 by the Austrian pediatrician Andreas Rett, after he observed two females who exhibited identical hand-wringer stereotypies in the waiting room of his clinic (Rett, 1966). However, Rett shared his findings in a German publication that was not widely distributed and RTT remained unknown in the medical community for another 17 years. In 1983, Hagberg at al. described 35 girls displaying similar symptoms after a neurotypical early infancy (Hagberg et al., 1983). Today, RTT is known as a progressive neurodevelopmental disorder that occurs almost exclusively in girls with an incidence of 1:10,000 – 15,000 live female births (Burd et al., 1991).

Following a period of apparently normal child development, lasting from 6-18 months of age, RTT progresses in four clinical stages. Stage I (stagnation period): occurs from 6-18 months and it is characterized by a delay in development, including postural delays, hypotonia, and the beginning of microcephaly. This period can last from weeks to months. Stage II (rapid regression): occurs around 1-4 years of age and it is characterized by the loss of acquired communication and motor skills, and the development of repetitive hand movements, breathing irregularities, microcephaly, and onset of seizures in some girls. This period can last from weeks to months. Stage III (pseudo stationary or plateau period): starts around 2 years of age and can last throughout the patient’s life. It is characterized by hand apraxia and loss of motor coordination leaving children to communicate through eye-pointing. Seizures are common during this stage, which can last for decades. Stage IV (motor deterioration): occurs at around 10 years of age until the end of life and it is characterized by scoliosis, dystonia, and bradykinesia. This phase corresponds to severe physical disability; however, not all RTT patients progress to this stage (Hagberg, 2002). In summary, females afflicted with this condition lose their speech,
motor skills, and purposeful use of their hands. Other symptoms of RTT include intellectual disability, early-onset osteoporosis, and gastrointestinal problems (Hagberg, 2002).

Patients also exhibit decreased brain volume largely due to global hypoplasia and cortical grey matter reduction (Armstrong et al., 1999; Casanova et al., 1991; Collins et al., 2004; Murakami et al., 1992; Reiss et al., 1993; Subramaniam et al., 1997). Cortical neurons from layers III and V have smaller cell body, less complex dendrites and are generally more densely packed (Armstrong et al., 1995, 1998; Armstrong, 2002). Smaller neurons with simpler dendritic arborization were also seen in the thalamus, substantia nigra, basal ganglia, amygdala, cerebellum and hippocampus of patients (Armstrong, 2002; Jellinger et al., 1988). Moreover, synaptic function is impaired, evidenced by the dysregulation of neurotransmitters and neuromodulators (Zoghbi et al., 1989). Outside the brain, metabolic dysfunction has also been reported in RTT patients. These include abnormal concentration of lipids in the blood, increased plasma leptin and lactate, abnormally structured mitochondria, and elevated oxidative stress (Acampa et al., 2008; De Felice et al., 2009; Eeg-Olofsson et al., 1988; Justice et al., 2013; Leoncini et al., 2011; Segatto et al., 2014; Wakai et al., 1990). Although RTT patients have a myriad of problems, many survive to middle age and older, usually succumbing to respiratory tract infection, respiratory failure, seizure related illness or cardiac abnormalities (Anderson et al., 2014). There is currently no cure for RTT, and treatment is limited to symptom management.

### 1.1.1. Rett syndrome is caused by mutations in the \textit{MECP2} gene

In 1998, a multipoint linkage analysis performed in a Brazilian family with 3 daughters diagnosed with RTT and 3 unaffected daughters pointed to a gene in the region Xq28 being the cause of RTT (Sirianni et al., 1998). Next, Amir et al. (1999) analyzed close to 100 genes in that
region identifying missense, nonsense, and frameshift mutations in the coding region of the gene methyl-CpG-binding protein 2 (MECP2) of several RTT patients (Amir et al., 1999). *De novo* mutations in MECP2 accounts for 95% of RTT cases (Amir et al., 1999; Neul et al., 2008; Wan et al., 1999). Atypical RTT cases are usually caused by mutations in the genes cyclin-dependent kinase-like 5 (CDKL5), classified as early-onset seizure type, or Forkhead box G1 (FOXG1), classified as congenital type, although some causes remain unknown. A child is deemed an atypical RTT patient when they develop RTT symptoms, but do not meet all clinical requirements for diagnosis (Ariani et al., 2008; Philippe et al., 2010; Sartori et al., 2009; Tao et al., 2004; Weaving et al., 2004; Zappella, 1992).

The discovery in 1999 that the majority of RTT cases are caused by mutations in the gene MECP2 (Amir et al., 1999) has led to an expansive field of research on the role of the transcriptional regulator MECP2 in brain function. Further, the monogenic nature of this disorder has led to the development of a number of mouse models of RTT. Mecp2 mutant mice exhibit a range of neurological abnormalities that recapitulate human RTT, and this model system has already provided crucial insight into the pathology of RTT. For example, selectively re-expressing Mecp2 in postmitotic neurons, either in the whole brain or neocortex and hippocampus, of adult mice has shown that RTT symptoms can be partially reversed (Giacometti et al., 2007; Guy et al., 2007; Luikenhuis et al., 2004), indicating that MeCP2 is necessary for both the development and maintenance of functional mature neurons (McGraw et al., 2011; Nguyen et al., 2012). These discoveries have uncovered the exciting potential of post-symptomatic reversal of RTT symptoms.

Although RTT is an X-linked disorder and human males rarely survive past birth, Mecp2 loss-of-function is less severe in mice and male hemizygous null mice (Mecp2−/y) not only survive until
adulthood, they have been the most commonly studied model system to date and they have provided extensive insight into the molecular pathophysiology of MeCP2 loss-of-function. Heterozygous (Het; MeCP2+/-) female mice have not been as thoroughly characterized, likely because of the added experimental challenges that they present, including delayed and more variable phenotypic progression, and cellular mosaicism due to X-inactivation. However, they are a more clinically relevant RTT model and it is imperative that female heterozygotes are included in any studies of potential therapeutics (Katz et al., 2012). Further, because they have a more delayed phenotypic progression and longer lifespan than males, there is greater potential to investigate clinically relevant alterations in phenotypic development.

1.1.2. MeCP2 is a transcriptional regulator with many functions

Although the genetic cause of RTT, mutations in the methyl CpG binding Protein 2 gene (MECP2; Mecp2 in mouse) was identified 20 years ago (Amir et al., 1999), the complex functions of the MeCP2 protein and the regulation of MeCP2 expression, have hindered the elucidation of molecular disruptions underpinning RTT phenotypes. MECP2/Mecp2 regulation is dependent on the methylation of its promoter with reduced MECP2 expression correlating with promoter hypermethylation in the brains of male autistic subjects (Nagarajan et al., 2008, 2006). Moreover, the brain-enriched microRNA, miR132, interacts with its miRNA recognition element (MRE) in the 3’ UTR region of MeCP2 preventing its translation (Klein et al., 2007). miR132 is induced by the transcriptional factor cAMP response element-binding protein (CREB) and BDNF, and its expression induces neurite outgrowth in cortical neurons (Klein et al., 2007; Vo et al., 2005). Interestingly, post-translation modifications alter MeCP2 function. For example, upon membrane depolarization, protein kinase A (PKA) and calcium/calmodulin-dependent protein
kinase type IV (CaMK IV) phosphorylate threonine residues (T308) of MECP2, inhibiting its binding to the nuclear repressor co-repressor (NCoR)/silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) complex (Ebert et al., 2013). However, in resting neurons, the homeodomain-interacting protein kinase 2 (HIPK2) phosphorylates the serine residues (S80) of MeCP2, leading to a reduction in the protein’s association with chromatin (Bracaglia et al., 2009).

The MeCP2 protein contains multiple domains, including the methyl-CpG-binding domain (MBD), transcriptional repression domain (TRD) and the NCoR/SMRT interacting domain (NID), located within the TRD (Lyst et al., 2013; Nan et al., 1997, 1993). MeCP2 binds to DNA through its MBD domain and functions as a repressor of gene transcription by recruiting SIN3A, histone deacetylases and NCoR/SMRT co-repressors (Guo et al., 2014; Jones et al., 1998; Kokura et al., 2001; Lewis et al., 1992; Nan et al., 1998). In addition to interacting with methylated CpG dinucleotides, Mecp2 binds to methylated Cph (Guo et al., 2014). In fact, MeCP2 recruitment to methylated CpA plays a key role in the downregulation of long genes (Gabel et al., 2015). Additionally, because many of the dysregulated genes in MeCP2-mutant mice acquire high levels of methylated Cph as they mature, it has been proposed that the lack of MeCP2 binding to mCH as neurons mature could underpin the delayed onset of RTT symptoms (Chen et al., 2015). Surprisingly, MeCP2 also acts as a transcriptional activator when it recruits cyclic AMP-responsive element-binding protein 1 (CREB1), with data suggesting that in the absence of Mecp2, a greater number of genes are downregulated than upregulated (Chahrour et al., 2008). Whether these transcriptional changes all represent direct targets of MeCP2 or secondary effects is not clear, however. It has been proposed that MeCP2 acts as a transcriptional repressor when it interacts with 5- methylcytosine (5mC) and as an activator when it binds to 5-
hydroxymethylcytosine (5hmC) (Li et al., 2013; Mellén et al., 2012). However, more recent studies suggest that MeCP2 only has high affinity to 5hmCA and its affinity to 5hmCG is low, similarly to unmethylated regions (Kinde et al., 2015; Mellén et al., 2017); therefore, the low affinity of MeCP2 to 5hmCG would result in its diminished binding and consequently reduction in transcriptional repression by MeCP2 (Ip et al., 2018; Mellén et al., 2017). Further, there is evidence that MeCP2 contributes to chromatin structural organization by displacing histone H1. Importantly, in the absence of MeCP2, there is an increase in histone deacetylation and histone H1, leading to alterations in chromatin structure (Nan et al., 1997; Skene et al., 2010). In addition, MeCP2 is involved in post-transcriptional regulation, evidenced by its enriched binding to alternatively spliced exons and by its interaction with Y box-binding protein 1 (YB-1), a protein that regulates RNA splicing events. Loss of MeCP2 leads to alterations in alternative splicing, in both mice and human cell lines (Maunakea et al., 2013; Young et al., 2005). MeCP2 also binds to DiGeorge syndrome critical region 8 (DGCR8), playing a role in miRNA processing by preventing the formation of the DGCR8-Drosha complex (Cheng et al., 2014). With so many mechanisms of action and functions for MeCP2, it is not surprising that mutations in MECP2 lead to such a complex syndrome with several distinct symptoms. It should be noted that the majority of the mechanistic studies involving RTT model mice have been conducted in MeCP2-null mice, demonstrating their importance to RTT research. Even though the null model does not recapitulate the cellular mosaicism of human RTT, it has been invaluable for accelerating our understanding of MeCP2 and the underlying pathophysiology of RTT. However, due to the complete absence of MeCP2 in these mice, it is difficult to tease apart the direct effects of MeCP2 loss and the indirect outcomes of their severely compromised
development, and further studies are needed to understand the mechanisms of action of MeCP2 in the context of the cellular mosaicism of the female Het brain.

1.1.3 MeCP2 mutant mouse models

1.1.3.1 Female heterozygous mutant mice display milder phenotypes and delayed phenotypic progression

A number of MeCP2-mutant mouse models have been generated that recapitulate certain phenotypes of human RTT. MeCP2\textsuperscript{tm1.1Bird} and MeCP2\textsuperscript{tm1.1Jae} are the most common null allele models used, while MeCP2\textsuperscript{tm1Hzo} (also known as MeCP2\textsuperscript{308}) is one of the most common nonsense mutation models. The MeCP2\textsuperscript{tm1.1Bird} model lacks exons 3 and 4, resulting in a complete loss of expression of MeCP2 mRNA and protein. Male null and female Het MeCP2\textsuperscript{tm1.1Bird} mice display similar physical deficits, but with very distinct phenotypic progression timelines. These phenotypes include irregular gait and reduced mobility, breathing apneas, hindlimb clasping, reduced neuronal size and brain weight. MeCP2\textsuperscript{tm1.1Bird} null males develop overt phenotypes as early as 3 weeks of age, while female Hets might not start showing obvious symptoms until 3 months. Further, female Het mice do not exhibit the rapid phenotypic progression seen in null males, which results in their early death at around 6 to 12 weeks of age (Guy et al., 2001). The MeCP2\textsuperscript{tm1.1Jae} model, on the other hand, lacks only exon 3 and, although mice carrying this allele do not express the full MeCP2 protein, they do exhibit peptides of smaller size. Similar physical deficits are seen in MeCP2\textsuperscript{tm1.1Jae} null males, which display overt symptoms by 5 weeks of age. Het females, on the other hand, are apparently asymptomatic for the first four months of life, later developing aberrant gait, body tremors, and reduced activity as seen with null males and the
The \textit{Mecp2} \textit{tm1.1Bird} model (Chen et al., 2001; Guy et al., 2001). The \textit{Mecp2} \textit{tm1Hzo} model produces a truncated protein, recapitulating a common mutation found in RTT. A premature stop codon was engineered after codon 308 of \textit{Mecp2}; thus, the allele maintains the MBD and TRD domains and the nuclear localization signal (NLS) but lacks the C-terminal third of the sequence. Similar to the null allele models, the heterozygous female mice exhibit milder phenotypes with a delayed symptom onset relative to males. In female Hets, overt symptoms appear after 1 year of age, while males start showing symptoms after 4 months of age, with signs of mild tremor appearing at 6 weeks (Shahbazian et al., 2002b). Due to the fact that the testes of \textit{Mecp2}-null mice remain internal, and they are not able to breed, generating \textit{Mecp2}−/− female mice is uncommon. However, Guy et al (2001) generated \textit{Mecp2}-null females by crossing female mice heterozygous for \textit{Mecp2} and for a Cre transgene on the X chromosome to males carrying an allele flanking exons 3 and 4 of the \textit{Mecp2} gene. The resulting \textit{Mecp2}-null female mice displayed similar phenotypic progression as \textit{Mecp2}-null males (Guy et al., 2001), suggesting that the delayed and highly variable phenotypic progression seen in Het females is due to their cellular mosaicism rather than an overall sex difference. Additional studies on \textit{Mecp2}−/− female mice would be necessary, however, to determine if there are sex-based differences in the RTT model mice that are independent of the cellular mosaicism inherent in the commonly employed Het model.

1.1.3.2. Phenotypes of both male and female \textit{Mecp2} mutant mice depend on genetic background

Importantly, genetic background greatly alters phenotypic presentation in \textit{Mecp2} mutant mice (Table 1). For example, \textit{Mecp2} \textit{tm1.1Bird} male mice on a C57BL/6 background undergo rapid weight loss before death, while males with the same allele on a 129 strain are significantly
heavier than wild-type (Guy et al., 2001). Similarly, $\textit{Mecp2}^{tm1.1\text{Jae}}$ male mice maintained on a mixed background (129,C57BL/6 and BALB/c) display increased body weight (Chen et al., 2001), as do heterozygous $\textit{Mecp2}^{tm1.1\text{Bird}}$ females on an FVB.129F1 (FVB/N × 129S6/SvEv) background. The latter are overweight starting at 8 weeks of age, while heterozygous females on a 129.B6F1 (129S6/SvEv × C57BL/6) background only appear to be heavier at 52 weeks.

Weight gain in mice is altered by the neuropeptide somatostatin in the hypothalamus, which increases as the expression level of MeCP2 rises. Remarkably, the negative correlation between $\textit{Mecp2}$ expression and weight gain is only observed in females on the FVB.129F1 background, but not the 129.B6F1 strain, even though both strains show weight gain with the progression of RTT (Samaco et al., 2013). Although $\textit{Mecp2}^{tm1.1\text{Bird}}$ mutant mice on a CD-1 background display similar overall phenotypes to those on a C57BL/6 background, considerations should be made to metabolic alterations, such as cholesterol levels, which appear to depend more on the genetic background than neurological phenotypes. Genetic background also modifies litter size and maternal care, with $\textit{Mecp2}$ heterozygous mice on a CD-1 background having larger litters with greater survival than C57BL/6 dams (Gigli et al., 2016). Maternal care is particularly important for symptom onset and severity in $\textit{Mecp2}$-mutant mice. This is highlighted by cross-fostering pups between $\textit{Mecp2}^{+/−}$ and wild-type dams, which results in differences in the onset of adult phenotypes and easier detection of behavioral deficits of heterozygous female mice when compared to their wild-type littermates (Vogel Ciernia et al., 2017). The sociability of $\textit{Mecp2}$ mutant mice is also variable and dependent on the allele and strain studied. For males, the $\textit{Mecp2}^{tm1.1\text{Jae}}$ and $\textit{Mecp2}^{tm1.1\text{Bird}}$ alleles are associated with hypersociability (Kerr et al., 2008; Schaevitz et al., 2010); however, $\textit{Mecp2}^{tm1\text{Hzo}}$ males only display increased sociability on a C57BL/6 background (Pearson et al., 2012), and not on the 129/SvEv background (Moretti et al.,
female mice on either a FVB.129F1 or 129.B6F1 background spend more time investigating a novel mouse than a novel object, but less time investigating the novel mouse than their wild-type littermates, suggesting behavioral impairments in social approach (Samaco et al., 2013).

Table 1. Phenotypic variation is dependent on both Mecp2 mutant allele and genetic background

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allele</td>
<td>Background</td>
</tr>
<tr>
<td>Weight</td>
<td>Loss</td>
<td>Mecp2^{m1.1Bird}</td>
</tr>
<tr>
<td></td>
<td>Gain</td>
<td>Mecp2^{m1.1Bird}</td>
</tr>
<tr>
<td>Social behavior</td>
<td>Increased</td>
<td>Mecp2^{m1.1Bird}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mecp2^{m1.1ae}</td>
</tr>
<tr>
<td>Anxiety behavior</td>
<td>Increased</td>
<td>Mecp2^{m1.1ae}</td>
</tr>
<tr>
<td>Light/dark box</td>
<td></td>
<td>Mecp2^{m1.1ae}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mecp2^{m1.1ae}</td>
</tr>
<tr>
<td>Open field</td>
<td>Increased</td>
<td>Mecp2^{m1.1ae}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mecp2^{m1.1ae}</td>
</tr>
<tr>
<td>Elevated Plus Maze</td>
<td>Increased</td>
<td>Mecp2^{m1.1ae}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mecp2^{m1.1ae}</td>
</tr>
<tr>
<td></td>
<td>Decreased</td>
<td>Mecp2^{m1.1ae}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mecp2^{m1.1ae}</td>
</tr>
<tr>
<td>Zero Maze</td>
<td>Increased</td>
<td>Mecp2^{m1.1ae}</td>
</tr>
<tr>
<td></td>
<td>Decreased</td>
<td>Mecp2^{m1.1ae}</td>
</tr>
</tbody>
</table>

Dash (–) indicates absence of phenotype or phenotype not investigated.

Mecp2^{St}: Mecp2 gene floxed by a stop codon.

* Increased time spent with novel mouse when compared to object, but decreased time spent with novel mouse when compared to wildtype littermate.

1.1.3.3. Mecp2 mutant male and female mice share a subset of phenotypes

Although phenotypic progression and severity are highly divergent between the two sexes, a number of neurological phenotypes are consistently comparable between male hemizygous null
and female Het mice. This includes a reduction in the volume of cortical and subcortical regions, with only minor sex differences in the \( \text{Mecp2}^{\text{tm1Hzo}} \) model, mainly in the inferior and superior colliculi of the brainstem (Allemang-Grand et al., 2017). Further, both \( \text{Mecp2} \)-null and Het females display reduced dendritic complexity, spine density and soma area of cortical neurons (Belichenko et al., 2009; Fukuda et al., 2005; Kishi and Macklis, 2004; Rietveld et al., 2015; Stuss et al., 2012; Tropea et al., 2009; Wang et al., 2013). Disrupted contextual fear learning (Pelka et al., 2006; Samaco et al., 2013) and inability to complete hippocampal dependent tasks (Stearns et al., 2007) are also consistently observed in both male and female \( \text{Mecp2} \) mutant mice. Other robust phenotypes observed in mice of both sexes, as well as RTT patients, are respiratory (Mancini et al., 2018; Roux et al., 2007) and cardiac dysfunction (McCauley et al., 2011). A study using Sarizotan, a 5-HT1a and dopamine D2-like receptor agonist, showed reduction in apneas and breathing irregularities in both male and female mice, with only respiratory frequency increased in females and not altered in male mice (Abdala et al., 2014). This drug is currently in clinical trial to assess its efficacy in treating breathing abnormalities of RTT patients (NCT02790034). Reduction in apnea frequency was also observed in \( \text{Mecp2} \) Het female mice exposed to metabotropic glutamate receptor allosteric modulators. Metabotropic glutamate receptor 7 (mGlu7) is downregulated in the cortex and hippocampus of male and female \( \text{Mecp2} \) mutant mice, in addition to RTT patients, and its modulation also improves synaptic transmission between Schaffer collaterals and CA1 in the hippocampus, regardless of sex (Gogliotti et al., 2017).

Additionally, male mice generated with a conditional \( \text{Mecp2} \) allele that exhibit a ~50% reduction in the expression of \( \text{Mecp2} \) showed intact pain sensitivity, which is dependent on spinal cord reflex, but impaired pain recognition, which is dependent on the communication between the
spinal cord and the brain (Samaco et al., 2008). The same phenotype is observed in female mice and RTT patients (Downs et al., 2010; Samaco et al., 2013). This indicates that either pain response is a common result of the loss of MeCP2 independent of sex, or that the similarity in phenotype is due to the roughly 50% reduction of MeCP2 in the brain.

Anxiety-like behavioral phenotypes are even more complex, with results dependent on the paradigm used to test the behavior. The elevated plus and zero mazes consistently show lower anxiety-like behavior in both male and female mutant mice (Meng et al., 2016; Penny et al., 1996; Samaco et al., 2013; Stearns et al., 2007; Ure et al., 2016; Vogel Ciernia et al., 2017), with the exception of the MeCP2tm1Hzo model (De Filippis et al., 2010; McGill et al., 2006). However, the open field test usually evidences increased anxiety-like behavior in both sexes (McGill et al., 2006; Shahbazian et al., 2002a; Vogel Ciernia et al., 2017), while the light/dark box paradigm shows variable results, indicating anxiogenic effect in some cases (McGill et al., 2006; Meng et al., 2016; Ure et al., 2016) and anxiolytic effect in others (Samaco et al., 2013).

Taken together, these data suggest that a subset of RTT phenotypes are consistent between male and female MeCP2-mutant mice and might respond similarly to therapeutic intervention. However, the preponderance of evidence indicates that male null and female Het mice have sex-specific responses to possible therapeutic interventions that must be considered.

1.1.4. MeCP2 has distinct functions in different circuits and cell types across the nervous system

The mammalian brain is comprised of a complex network of neuronal and glial subtypes, each with a distinct transcriptome and epigenome. MeCP2 is expressed in most (if not all) of these
cells, but the molecular pathways regulated by MeCP2 are tissue- and cell-type specific and loss of MeCP2 function in defined central nervous system (CNS) circuits results in distinct RTT phenotypes. To tease apart the contributions of different circuitries and brain regions to RTT phenotypes, cell type- and brain region-specific deletion and re-expression of Mecp2 have been employed. However, few studies have included both male and female mice in their experimental design, which is problematic when the severity of the phenotypes can be idiosyncratic to each sex, greatly impacting the potential for phenotypic rescue. Further, studies that have analyzed both males and female Mecp2 mutant mice have identified some distinct differences in phenotypes, highlighting the need to employ both sexes in such analyses.

1.1.4.1. Mecp2 mutant mice display sex-specific phenotypes in distinct neuronal subpopulations

An example of molecular phenotypic differences between males and females is found in the serotonergic system, which is more highly dysregulated in Mecp2-null mice than Het females (Vogelgesang et al., 2017). Behaviorally, loss of Mecp2 specifically in 5-hydroxytryptamine (5-HT) neurons leads to reduced depression-like and increased anxiety-like phenotypes in the novelty suppression feeding test in male Mecp2-null mice. In contrast, female Het mice display reduced anxiety-like behavior in the elevated plus maze test (Philippe et al., 2018). The authors suggest that the changes in behavior could be a result of increased expression of 5-HT1A autoreceptors due to the loss of MeCP2 enhancement of Deaf1-mediated repression in 5-HT neurons (Philippe et al., 2018).

Restoration of Mecp2 expression specifically in glutamatergic or inhibitory neurons also highlights the differences in male and female phenotypes, and possible limitations of preclinical
studies that focus only on *Mecp2*-null mice. Female mice in which *Mecp2* expression is maintained exclusively in glutamatergic neurons display more extensive phenotypic amelioration than male mice; for example, normalization of ataxia is only seen in Het females (Meng et al., 2016). In contrast, Het female mice expressing *Mecp2* only in inhibitory neurons display less extensive phenotypic improvement than male null mice (Ure et al., 2016). The authors suggest that this phenotypic divergence is indicative of *Mecp2* re-expression being less effective in inhibitory than glutamatergic neurons in *Mecp2* mosaic Het brains (Meng et al., 2016; Ure et al., 2016).

*Mecp2* mutant mice also display sex-specific differences relating to the cholinergic system. *Mecp2* deficient mice express lower levels of choline acetyltransferase in a number of brain regions, such as basal forebrain and striatum (Ricceri et al., 2011; Zhou et al., 2017). With the injection of nicotine or nicotinic acetylcholine receptor (nAChR) agonist, male *Mecp2*-null mice displayed enhanced locomotion, contrary to the suppressive effect these agonists have on wild-type mice. Het female mice, on the other hand, show distinctive phenotypes after nicotine exposure, such as Straub tail, a dorsiflexion of the tail used to measure nicotine sensitivity in mice. Similar phenotypic heterogeneity was found in the expression levels of nAChR subtypes in the midbrain of RTT mice, with males having a significant reduction in α4 and α6 subtype mRNA levels, while females only showed differential expression of α6 (Leung et al., 2017). This could contribute to the sex difference seen in the behavioral response to nicotine exposure. Additionally, the loss of *Mecp2* in the cholinergic system impairs memory recognition of *Mecp2*-null mice, a phenotype that can be rescued with the chronic administration of the acetylcholinesterase inhibitor donepezil (Ballinger et al., 2019).
The hippocampi of *Mecp2*-null and female heterozygous mice, on the other hand, share similar properties. Increased hippocampal activation (Calfa et al., 2011; Li et al., 2017), and a reduction in soma size of hippocampal neurons is observed in both males and females. The same is true for soma size of neurons in the locus coeruleus, which display smaller and hyperexcitable neurons in both male and female mutant mice. However, there are features in the locus coeruleus that distinguish males and females; the decrease in tyrosine hydroxylase levels between MeCP2-neurons in the Het brain and wild-type females is greater than difference seen between *Mecp2*-null and wild-type male mice (Taneja et al., 2009). Neuronal phenotypes in the neocortex are also consistent between male nulls and female Hets. In both, cortical neurons demonstrate reduced dendritic complexity, spine density, and soma size (Belichenko et al., 2009; Fukuda et al., 2005; Kishi and Macklis, 2004; Rietveld et al., 2015; Stuss et al., 2012; Tropea et al., 2009; Wang et al., 2013) as well as spontaneous excitatory input (Asgharhafshejani et al., 2019). Physical and morphological phenotypes of dopaminergic neurons in the substantia nigra are comparable between males and females as well. Both sexes of *Mecp2* mutant mice have reduced cell capacitance and dopamine current density, and increased resistance, although male mice show more severe reduction in capacitance (Gantz et al., 2011). Thus, some neuronal characteristics might provide a consistent measure of potential phenotypic rescue between mutant mice of both sexes, but distinct responses can be expected in other neuronal populations.

1.1.4.2. *Mecp2* loss-of-function in glia disrupts their function and alters neuronal circuitry
Initially, RTT was thought to be predominantly caused by the loss of *Mecp2* in neurons since the protein is expressed up to ten times higher in neuronal cells than other cell types in the brain (Chen et al., 2001; Skene et al., 2010). However, more recently, *Mecp2* loss-of-function in glial
cells has been shown to centrally contribute to RTT pathogenesis, although sex differences have yet to be characterized in these important cellular populations. Expressing MeCP2 exclusively in astrocytes improves several RTT phenotypes, including locomotion and anxiety-like behavior, in addition to prolonging lifespan (Lioy et al., 2011). MeCP2-null astrocytes grow slower in vitro and do not mediate immune response as effectively as wild-type astrocytes. Further, MeCP2-null astrocytes alter wild-type neuronal phenotypes and fail to provide essential support for neuronal health. The negative impact of MeCP2-null astrocytes might become more pronounced with age; hippocampal astrocytes of Het female mice display lower MeCP2 expression at 7 months than at 1 month of age. This MeCP2 deficiency appears to spread through gap junctions, highlighting the negative impact that MeCP2+ cells have on MeCP2- cells (Maezawa et al., 2009).

Microglia also contribute to circuit disruption in RTT, although their role in the pathogenesis of the disorder has been controversial. Wang et al (2015) found that restoration of MeCP2 expression in microglia or the introduction of wild-type microglia in the brain via bone marrow transplantation does not rescue RTT phenotypes in mice, as had been previously reported (Noël C Derecki et al., 2012; Wang et al., 2015). Nevertheless, microglia are a known physiological mediator of synaptic pruning by eliminating unnecessary synaptic connections in the retinogeniculate system of MeCP2-null mice at postnatal day 5 (Schafer et al., 2012) and between postnatal days 30 and 60 (Hong et al., 2014; Schafer et al., 2012). Interestingly, MeCP2-null and wild-type microglia behave similarly in early postnatal and juvenile timepoints; however, after postnatal day 56, microglia lacking MeCP2 excessively prune synaptic connections. This period coincides with late symptomatic stages of MeCP2-null mice, indicating that microglia could enable late-stage circuit defects in males (Schafer et al., 2012). Because female heterozygous
mice become highly symptomatic at a later timepoint than male mice, it would be interesting to see if the same microglia defects would be found in this population, at a later timepoint.

1.1.5. Rett syndrome phenotypes are influenced by patterns of X-chromosome inactivation

Our ability to distinguish differences between male Mecp2-null and female Mecp2 Het mice is further compounded by phenotypic variability caused by skewed X-inactivation. X-chromosome inactivation (XCI) is an event that occurs very early in development, around the time of implantation, in both mice and humans. It allows for dosage compensation between females and males, who only possess one X-chromosome. The process randomly silences either the maternal or paternal X-chromosome in all somatic cells. The inactivated chromosome (Xi) expresses a long non-coding RNA called XIST, which is upregulated prior to Xi undergoing extensive epigenetic modulation, such as DNA methylation and histone modification, responsible for its silencing (Clemson et al., 1996; Escamilla-Del-Arenal et al., 2011; Kohlmaier et al., 2004; Penny et al., 1996; Shahbazian et al., 2002a; Sharp et al., 2011).

Although XCI is a random process and it is expected that Het female mice are mosaic for Mecp2 expression, with roughly 50% of cells expressing the wild-type allele while the other half express the mutated allele, the transcript level of either allele can vary widely from 40 to 85% (Braunschweig et al., 2004; Samaco et al., 2013; Young and Zoghbi, 2004). Whilst the pattern of XCI is mostly skewed towards the wild-type allele in mice, it is interesting to note that MeCP2 protein levels in wild-type-expressing cells within the brain inversely correlates with the number of cells expressing the mutated Mecp2 allele (Braunschweig et al., 2004). Therefore, even with a favorable XCI ratio, overall MeCP2 protein level is decreased due to this non-cell-autonomous impact on the wild-type cells. Hence, it is problematic to base pre-clinical research only on male
mice lacking any MeCP2 expression, since these animals will not show the impact the mutated allele could have on wild-type cells, which could greatly alter phenotypic presentation.

In mice, multiple phenotypes are influenced by the XCI ratio, with body tremor and stereotypic forepaw movements being highly susceptible to changes in the ratio (Young and Zoghbi, 2004). However, how mouse behavior is influenced by levels of MeCP2 is dependent on the region of the brain; protein expression in the cortex inversely correlates with overall phenotype severity, while MeCP2 levels in the cerebellum, hippocampus and spinal cord are not significantly correlated with overall phenotype severity. Specific behaviors, on the other hand, are correlated with MeCP2 expression levels in these regions. For example, open field activity increases as Het mice display higher MeCP2 expression in the hippocampus, but no correlation is found in the cortex, cerebellum, or spinal cord. The same is true for anxiety-like behavior (Wither et al., 2013). This disparity is not only restricted to physical or behavioral phenotypes as it has been shown that neuronal morphology is also impacted by the ratio of cells expressing wild-type or mutant Mecp2; the nuclear area of cells expressing MeCP2 rises with the increase in the number of cells expressing the wild-type Mecp2 allele, while the nuclear area of the cells expressing the null allele decreases (Rietveld et al., 2015). This shows that neurons expressing the mutant Mecp2 allele will have a more severe phenotype when surrounded by skewed XCI favoring the wild-type allele (Fig. 1.1), perhaps indicating the inability of the null neurons to compete with the wild-type expressing cells. In addition, the soma size of MeCP2+ neurons is also reduced in mosaic brains when compared to neurons in wild-type brains, demonstrating a non-cell autonomous effect impacted by variable XCI (Rietveld et al., 2015; Wither et al., 2013).

In contrast to Mecp2 mutant mouse models of the disorder, only a few cases of skewed XCI pattern have been documented in RTT patients, with balanced XCI predominantly found in the
population (LaSalle et al., 2001; Shahbazian et al., 2002a). However, the small number of variable XCI cases seen in patients could be explained by undiagnosed cases in which a skewed ratio towards wild-type MECP2 expression could be neurologically protective, preventing or lessening RTT symptoms (Amir et al., 2000; Knudsen et al., 2006; Young and Zoghbi, 2004; X. Zhang et al., 2012). The paternal X-chromosome is the most commonly inactivated in instances where there is skewed XCI in humans (Nielsen et al., 2001); because de novo mutations in MECP2 usually have a paternal origin (Girard et al., 2001; Trappe et al., 2001; X. Zhang et al., 2012), this could enhance the protective effect of skewed XCI.

In humans (and in mice), different mutations in MECP2 underpin phenotypic variability. For example, missense mutations are more likely to result in scoliosis while truncating mutations frequently cause breathing abnormalities (Amir et al., 2000). Despite this phenotypic variability, the major determinant of RTT phenotypes is still the XCI ratio, which will most likely determine if the patient will or will not meet diagnostic criteria for RTT. This further highlights the importance of employing female mice, which are also subject to XCI, in RTT research.
Figure 1. Skewed X-chromosome inactivation alters RTT phenotypes in *Mecp2* heterozygous female brain.

The female Het brain is a mosaic of cells expressing the wild-type allele (*MeCP2*+) and those expressing the mutant or null allele (*MeCP2*−). Although a 1:1 ratio of *MeCP2*+ and *MeCP2*− cells is the norm (top), skewed X-chromosome inactivation (XCI) can lead to an increase in the relative percentage of either the *MeCP2*+ cells (bottom left) or the *MeCP2*− cells (bottom right). This change in the overall cellular environment alters specific phenotypes of both *MeCP2*+ and *MeCP2*− neurons, through non-cell-autonomous mechanisms. For both *MeCP2*+ and *MeCP2*− neurons, the direction of phenotypic change (arrows) is depicted relative to the same cell type under balanced (1:1) XCI. Magenta indicates expression of wild-type allele and cyan indicates expression of null allele.
1.1.6. MeCP2 exerts both cell autonomous and non-cell-autonomous control

The impact that skewed XCI ratios have not only on cells expressing the mutated allele of *Mecp2*, but also on the cells expressing the wild-type allele reinforces that *Mecp2* mutant phenotypes result from both cell-autonomous and non-cell-autonomous disruptions. For example, *Mecp2*-null neocortical projection neurons exhibit reduced dendritic arborization even when transplanted into the cortex of wild-type mice; the reduction is not worsened when *Mecp2*-null neurons are transplanted in *Mecp2*-null cortices, indicating that the loss of *Mecp2* is the main contributor to this phenotype. In contrast, wild-type neurons transplanted into the *Mecp2*-null cortex demonstrate reduced soma area, similar to null-neurons, indicating a non-cell-autonomous impact on soma size (Kishi and Macklis, 2010).

1.1.6.1. MeCP2+ and MeCP2− cells in the Het brain are distinct from wild-type and null

Similar observations of both cell autonomous and non-cell-autonomous effects of *Mecp2* loss-of-function can be made when comparing wild-type (MeCP2+) with null (MeCP2−) layer V-VI pyramidal neurons in the motor cortex of Het female mice (Fig. 1.2). In addition to altered dendritic spine density and dendritic width in MeCP2− neurons, disruptions were seen between MeCP2+ cells in Het female brain and wild-type littermates. However, the number of dendritic spines on MeCP2+ neurons was reduced when compared to MeCP2− neurons, which was similar to the number of spines found in the brains of *Mecp2*-null male mice, indicating the influence of both cell autonomous and non-cell-autonomous mechanisms in this phenotype. In contrast, the number of dendrites with irregularities, such as narrowing and swelling, appears to be influenced only by cell autonomous processes, since MeCP2+ cells are not different from wild-type neurons, while MeCP2− and Mecp2-null neurons show a significant increase in frequency of
those dendrites when compared to wild-type controls (Belichenko et al., 2009). Soma and nuclear size of MeCP2+ cortical neurons in a Het brain are also significantly reduced relative to those in a wild-type brain, although they are not quite as small as MeCP2− neurons. Overall dendritic length and branching of MeCP2+ neurons is not different from wild-type, on the other hand, but is significantly reduced in MeCP2− neurons (Rietveld et al., 2015). Further, electrophysiological parameters of cortical neurons are under the influence of cell autonomous (reduction in excitatory tone) and non-cell-autonomous (increased IPSCs frequency) mechanisms as well (Asgarihafshejani et al., 2019). Thus, RTT neuronal morphology phenotypes are comprised of both cell-autonomous and non-cell-autonomous disruptions.

Soma size and synaptic connectivity have been shown to be influenced by cell-autonomous and non-cell-autonomous mechanisms in the hippocampus as well. In the absence of MeCP2, there is a decline in Brain-derived neurotrophic factor (BDNF) synthesis and release, which results in a reduction of soma area, nuclear size, and dendritic length of hippocampal neurons, in addition to diminished glutamatergic synaptic outputs in vitro. The same morphological and presynaptic defects are observed in wild-type neurons in which the BDNF pathway is blocked (Sampathkumar et al., 2016). Importantly, increasing the expression of BDNF in vitro, either by normalizing its production in the MeCP2-null neurons or by exogenous application to cell culture medium, rescued both the synaptic and morphological defects of MeCP2-null hippocampal neurons. Overexpressing BDNF in wild-type neurons co-cultured with MeCP2-null neurons did not rescues their phenotypes, however, indicating that BDNF regulates dendritic complexity in a cell-autonomous and autocrine manner (Sampathkumar et al., 2016). The BDNF pathway could play a similar role in this phenotype within cortical neurons, which also express lower levels of BDNF (Chang et al., 2006).
*Mecp2* loss-of-function also leads to both cell-autonomous and non-cell-autonomous disruptions in gene expression. In the brain of Het female mice, MeCP2− and MeCP2+ cortical pyramidal neurons display distinct patterns of gene expression, demonstrating cell autonomous disruptions in the regulation of gene expression by the loss of MeCP2 (Johnson et al., 2017). However, a large number of genes are also differentially expressed in MeCP2+ neurons from Het female mice when compared to wild-type neurons from a control mouse brain. Importantly, these non-cell-autonomous disruptions in transcriptional regulation are more prevalent in mice carrying the R106W human mutation, which results in severe RTT phenotype, than in those with the T158M mutation, which has a less severe phenotype, suggesting that disease progression drives these secondary changes in gene expression. Interestingly, the non-cell-autonomous differentially expressed genes are predominantly associated with cell-to-cell signaling and protein phosphorylation while the cell-autonomous differentially expressed genes are associated with transcriptional regulation (Johnson et al., 2017). Another study found that the genes that are differentially expressed between MeCP2+ neurons in wild-type and Het mouse brains are associated with neuronal activity-dependent gene expression and neurotrophin signaling. In agreement with the previous study, the non-cell-autonomous differentially expressed genes do not seem to be directly regulated by MeCP2, mostly likely resulting from indirect effects of the mosaic RTT brain environment (Renthal et al., 2018).
Neurons display both cell-autonomous and cell-non-autonomous disruptions in the female heterozygous brain. *Mecp2*-mutant neurons in the brains of both female Hets and male nulls display aberrant size and morphology phenotypes. MeCP2− neurons in a Het brain display distinct morphologies from MeCP2+ neurons; however, they are not as severely disrupted as MeCP2− neurons in a null brain. In addition, MeCP2+ neurons in a Het brain are distinct from MeCP2+ neurons in a wild-type (WT) brain, for example, displaying decreased soma size and dendritic spine density. Other phenotypes, such as dendritic branching and morphology, are dependent only on MeCP2 expression within that neuron. Thus, specific phenotypes respond differently to the cellular environment, indicating that they are controlled by distinct cell-autonomous and cell-non-autonomous mechanisms. Arrows denote phenotypic change relative to MeCP2+ in a WT brain, dash indicates no change.

**Figure 1.** Neurons display both cell-autonomous and cell-non-autonomous disruptions in the female heterozygous brain.
1.1.6.2. MeCP2+ and MeCP2− glia alter neuronal morphology and function via non-cell-autonomous mechanisms

Wild-type hippocampal neurons co-cultured with astrocytes derived from MeCP2-null mice or with astrocytic conditioned media (ACM) from MeCP2-null and wild-type astrocytes, mimicking MeCP2 mosaicism in heterozygous brains, fail to thrive, displaying a reduction in neuronal processes and neuronal density (Ballas et al., 2009). Wild-type mouse hippocampal neurons co-cultured with astrocytes differentiated from induced pluripotent stem cell (iPSC) from RTT patients carrying 3 distinct MECP2 mutations similarly show reduced neuronal outgrowth, when compared to wild-type neurons cultured on isogenic control iPSC-derived astrocytes (Williams et al., 2014). Additionally, culturing MeCP2-null hippocampal neurons with ACM derived from wild-type astrocytes ameliorates disrupted neuronal growth (Ballas et al., 2009), providing further evidence of the non-cell-autonomous influence of glia in RTT pathology. Loss of MeCP2 specifically in astrocytes also impairs synaptic transmission. Proper neuronal synaptic response to astrocyte stimulation only occurs in the presence of an astrocyte that expresses MeCP2; an MeCP2+ neuron within a Het brain will demonstrate impaired synaptic transmission if coupled with an MeCP2− astrocyte (Rakela et al., 2018).

These findings that MeCP2 loss-of-function exerts both cell-autonomous and non-cell-autonomous effects highlights the complexity of RTT. Further, it reinforces the need to study the molecular underpinnings of RTT and to investigate potential therapeutics within female Het mice. MeCP2+ and MeCP2− neurons likely respond differently to any intervention and could modify the response of neighboring cells accordingly. Although male hemizygous null mice remove this variability and allow for the study of underlying mechanisms in a more straightforward context, they do not recapitulate the complexity of the human disorder.
1.1.7. Translation of pre-clinical studies performed in mice to human RTT

Although behavioral and physical deficits of MeCP2 mutant mice are dependent on the allele, genetic background, and sex of the animal being studied, the most confounding aspect of RTT research is, perhaps, the fact that phenotypic rescue experiments in mice that show very promising results are not recapitulated in clinical trials. One such example is the drug desipramine, an antidepressant that inhibits norepinephrine reuptake, which was evaluated for breathing disorders in RTT patients. Preclinical experiments showed robust rescue in the number of apneas and tyrosine hydroxylase-expressing neurons in the brainstem, and increased lifespan with treatment of MeCP2-null mice with desipramine (Roux et al., 2007; Zanella et al., 2008). However, the clinical trial was unsuccessful, showing no improvement in the patients treated with desipramine when compared to the placebo group (Mancini et al., 2018). An underlying cause of such dichotomy could be that the pre-clinical research focused only on male mice.

Many of the insulin-like growth factor 1 (IGF1) preclinical studies were also done in male mice. Recombinant human IGF1 (rhIGF1) extends MeCP2-null lifespan, and improves apnea, bradycardia and locomotion in these animals (Castro et al., 2014; Mellios et al., 2014; Tropea et al., 2009). Females were only used to study visual plasticity due to the severe phenotypes of male mice in adulthood; in this study, rhIGF1 treatment curbed the enhanced critical period of heterozygous females (Castro et al., 2014). Although, a few studies with human female RTT patients found IGF1 treatment to be well tolerated and to improve some features of the disorder (Glaze et al., 2017; Khwaja et al., 2014; Pini et al., 2012), another clinical trial did not see significant improvement in patients, with some parameters worsening (O’Leary et al., 2018).

It will be interesting to observe the outcome of other clinical trials currently underway that were also based on preclinical studies focused on MeCP2-null mice, and whether the expanding use of
female Het mouse models will improve our understanding of therapeutic targets. Any therapeutic approach will need to consider the immense challenges posed by the cell-type specific transcriptional targets and functions of MeCP2 and the unique stoichiometry of its expression between neurons and glia, in addition to the cellular mosaicism of the Het brain.

1.1.8. Summary of Mecp2 dysfunction in RTT mice model

It would be a gross understatement to say that RTT syndrome is a complex disorder. MeCP2 has multiple functions, and the pathways that it regulates are cell-type and tissue-type specific. It is the combination of MeCP2 loss-of-function across these distinct cell types and circuits that leads to the amalgam of symptoms that characterize RTT. Teasing these apart and identifying the disruptions that are paramount for targeting in therapeutic intervention is a monumental task. In so doing, however, it is imperative to recognize the cellular mosaicism of the female Het brain as a critical factor in the pathophysiology of RTT. Therapeutic strategies for MeCP2 re-expression, such as via adeno-associated virus (AAV) MeCP2 transgenes, must consider the cellular mosaicism of the female Het brain in designing a targeting strategy. For example, exogenously expressing MeCP2 within the MeCP2+ cells as well as the MeCP2− cells would be highly detrimental as MeCP2 overexpression leads to severe neurological disruptions (Collins et al., 2004). Similarly, driving expression of MeCP2 at the same levels in glia as in neurons would likely lead to an over-expression phenotype as neurons express MeCP2 at a 10-fold higher level than other cell types in the brain. Further, MeCP2 loss-of-function causes both cell-autonomous and non-cell-autonomous disruptions in the brain. Thus, we must consider not only the phenotypes and molecular underpinnings of MeCP2− neurons and glia, but also the impact of these cells on MeCP2+ cells. MeCP2+ cells in a Het brain are distinct from MeCP2+ cells in a
wild-type brain, and MeCP2− cells in a Het brain are distinct from MeCP2− cells in a male hemizygous null brain. These variations contribute to the distinct phenotypes observed in MeCP2 mutant female mice in comparison to male null mice, as well as their increased variability in phenotypic progression.

1.2. The NF-κB pathway as a therapeutic target for Rett syndrome

NF-κB signaling has been extensively studied in the immune system due to its role in the regulation of genes involved in cell survival, cell proliferation, and inflammatory and immune responses (Vallabhapurapu and Karin, 2009). In the brain, NF-κB signaling is first observed at E12.5 (Schmidt-Ullrich et al., 1996), and it can be activated by inflammatory stimuli, neuronal growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotransmitters (Dresselhaus and Meffert, 2019; Snow and Albensi, 2016). NF-κB signaling has roles in neurogenesis and neuroprotection, in addition to modulating synaptic plasticity and cognitive behavior (Dresselhaus and Meffert, 2019; Zhang and Hu, 2012).

Due to its role in many essential aspects of CNS physiology, NF-κB dysregulation is involved in the pathology of several neurological disorders (Jha et al., 2019; Mattson and Camandola, 2001; Sivandzade et al., 2019), including autism spectrum disorder (ASD) (Chiara Manzini et al., 2014; Naik et al., 2011; Young et al., 2011) and epilepsy (Blondeau et al., 2001; Lubin et al., 2007; Teocchi et al., 2013; Yu et al., 2011). In RTT, NF-κB signaling is aberrantly increased in both the brain and in peripheral systems (Colak et al., 2011; Kishi et al., 2016; O’Driscoll et al., 2015; Pecorelli et al., 2020). Transcriptome alterations in the NF-κB pathway have been reported in whole blood of human patients (Colak et al., 2011). Elevated signaling and pro-inflammatory
cytokines have also been found in MECP2-deficient human peripheral blood mononuclear cells (PBMCs), in the human monocyte line THP1 (O’Driscoll et al., 2015), and in skin biopsies from RTT subjects (Pecorelli et al., 2020). Moreover, proteomic analysis found significant enrichment of the gene ontology (GO) term ‘positive regulation of NF-κB transcription factor activity’ among the dysregulated biological processes in primary dermal fibroblasts from RTT patients (Cicaloni et al., 2020). In addition, the administration of an inhibitor of glycogen synthase kinase-3b (Gsk3b) lowered NF-κB signaling and increased dendritic arborization and spine density in MeCP2-null mice (Jorge-Torres et al., 2018). Similar morphological rescue was seen with NF-κB genetic attenuation, which also resulted in phenotypic amelioration and increase in the lifespan of MeCP2-null mice (Kishi et al., 2016). Taken together, these data suggest that NF-κB attenuation could provide a path towards RTT therapeutics.

1.2.1. NF-κB signaling occurs through canonical and non-canonical mechanisms

The mammalian nuclear factor-kappa B (NF-κB) transcription factor family is ubiquitously expressed and functions as a hetero- or homodimer comprised of five possible protein subunits. The subunits Rel A (p65), c-Rel and Rel B contain transcription transactivation domains (TADs), while the subunits p50 and p52 do not. In addition, p50 and p52 are cleaved post-translationally from their precursors proteins p105 (NFKB1) and p100 (NFKB2), respectively (Hayden and Ghosh, 2012; Sen and Baltimore, 1986). All five subunits bind to κB sites within regulatory regions of target genes via their shared N-terminal Rel homology domain (RHD). Once bound to κB response elements, NF-κB dimers recruit coactivators or corepressors. TAD is necessary for
activation of genes; therefore, the subunits p50 and p52 can only repress gene transcription, unless coupled with a subunit containing TAD (Hayden and Ghosh, 2008; Zhang et al., 2017).

The NF-κB pathway is inactive under unstimulated conditions as its dimers remain bound by a protein from the inhibitor of κB (IκB) family, which is typically comprised of IκBα (NFKBIA), IκBβ (NFKBIB) or IκBε (NFKBIE) (Cheng et al., 1998; Hayden and Ghosh, 2008). The most studied IκB is IκBα, which has as its primary target the p65/p50 heterodimer. The IκBα/NF-κB complex appears to be sequestered in the cytoplasm, when in reality, it constantly shuffles between the nucleus and cytoplasm due to the nuclear export sequence (NES) in the IκBα protein, and the fact that IκBα masks the nuclear localization sequence (NLS) of p65, but not p50 (Ghosh and Karin, 2002).

Following cellular stimulation, the NF-κB pathway is activated by the phosphorylation of IκB serine residues leading to its ubiquitination and proteasomal degradation. IκB phosphorylation is mediated by the IκB kinase (IKK) complex, which consist of a kinase subunit, IKKα/IKK1 or IKKβ/IKK2, and a regulatory unit NEMO/IKKγ (Didonato et al., 1997; Häcker and Karin, 2006; Krappmann et al., 2000). IKKβ is the main participant of canonical activation mediating the phosphorylation of IκBα and IκBβ while the non-canonical pathway is exclusively dependent on the IKKα subunit (Ghosh and Karin, 2002; Kwak et al., 2000).

The canonical pathway can be activated by a variety of stimuli ranging from physical and chemical stresses to endogenous and exogenous ligands. Toll-like receptors (TLRs), tumor necrosis factor receptor (TNFR) and proinflammatory cytokine receptors are among the many receptors involved in NF-κB canonical activation (Hui Yu et al., 2020). Interestingly, NF-κB
canonical signaling induces the expression of cytokines such as TNF-α and IL-1β while TNFR1 and IL-1RI receptors are also involved in the activation of the pathway itself. Both TLRs and IL-1R receptors are involved in the recruitment of myeloid differentiation primary response gene 88 (MyD88), which activates interleukin 1 receptor-associated kinase 1 (IRA1) and 4 (IRA4) (Fig. 1.3). TNFR associated factor (TRAF) family members are subsequently recruited to activate TGFβ-activated kinase I (TAK1) (Bowie and O’Neill, 2000; Cao et al., 1996; Deng et al., 2000; Didonato et al., 1997; Jiang et al., 2002; Verstrepen et al., 2008; Wesche et al., 1997). The pathway activation through TNFR, on the other hand, depends on the activity of the TNFR-associated death domain (TRADD) and receptor-interacting proteins (RIPs) (Hsu et al., 1996, 1995). The latter recruit the IKK complex through interactions with NEMO, forming a TAK1-IKK complex and leading to IκB degradation and release of p65, p50 or c-Rel (Ea et al., 2006; Rahighi et al., 2009). Canonical signaling is rapid and short-lived while non-canonical signaling is slow and persistent (Sun, 2017).

The non-canonical pathway is facilitated by NF-κB-inducing kinase (NIK), which is responsible for the activation of IKKα, resulting in p100 processing into p52. p100 is an IκB-like protein that regulates Rel B, preventing it from nuclear translocation until it is cleaved to p52 (Senftleben et al., 2001; Xiao et al., 2001). The main activators of the non-canonical pathway are ligands of the TNFR superfamily, including B cell activating factor receptor (BAFFR) and receptor activator for NF-κB (RANK) (Claudio et al., 2002; Kayagaki et al., 2002; Novack et al., 2003). These TNFR receptors bind to TRAF family members, which function upstream of NIK, with TRAF2 and TRAF3 acting as negative regulators of non-canonical signaling by mediating the ubiquitylation of NIK (Liao et al., 2004; Vallabhapurapu et al., 2008).
Figure 1.3 NF-κB acts in a canonical and non-canonical manner.

In the canonical pathway (left), TNFR activation depends on TRADD and RIPs. RIP acts through TAK1 to activate IKK, while TRADD depends on TRAF2. TLRs and IL-1R, however, recruit MyD88, which activates IRAK1 and IRAK4. This leads to the recruitment of TRAF and subsequent activation of TAK1 and IKK. Ultimately, IκB is degraded and the subunits p65/p50 translocate to the nucleus, where it binds to response elements in the DNA. In the non-canonical
pathway (right), NIK activates IKKα, which processes p100 into p52. Then RelB/p52 translocate to nucleus to bind DNA.

1.2.2. The NF-κB pathway has many functions in the CNS

Various components of the NF-κB pathway are expressed in areas of active neurogenesis in the early postnatal mouse brain, more specifically in radial glial cells, migrating neuronal precursors and in cells of astrocytic lineage (Denis-Donini et al., 2005). It has also been shown that the p65/p50 dimers are necessary for the growth and proliferation of neurosphere cultures (Young et al., 2006). Moreover, NF-κB signaling is necessary for the control of asymmetric division of neural stem cells (NSCs) and it participates in the initiation of NSCs differentiation by inhibiting CCAAT/enhancer binding protein (C/EBPβ) (Y. Zhang et al., 2012). In adult hippocampal neurogenesis, TLRs regulate neuronal proliferation and differentiation via NF-κB pathway activation (Rolls et al., 2007). Interestingly, stress-induced impairment in adult neurogenesis is mediated by IL-1β/NF-κB signaling and it results in decreased proliferation of NSCs (Koo et al., 2010). However, studies conducted in p50 knockout mice showed no alteration in the rate of proliferation of progenitor cells within the dentate gyrus. Yet, the number of surviving newborn neurons was significantly decreased in the absence of p50 (Denis-Donini et al., 2008).

NF-κB signaling is also involved in neuronal morphology. Axonal development, and subsequent neuronal polarity, are disrupted with the inhibition of IκBα phosphorylation in the axon initial segment of hippocampal neurons (Sanchez-Ponce et al., 2008). NF-κB activation in glutamatergic neurons promotes the formation of dendritic spines and excitatory synapses.
Moreover, preventing NF-κB activation in layer 2 somatosensory pyramidal neurons leads to reduced dendritic arborization (Gutierrez et al., 2005) while dampening aberrant NF-κB signaling in a mouse model of RTT increases dendritic complexity and soma area (Kishi et al., 2016), suggesting that the activity of the pathway needs to be tightly regulated.

Disruption in NF-κB pathway activation has been associated with behavioral deficits as well. p65 deficient (Meffert et al., 2003) and c-Rel knockout (Ahn et al., 2008) mice have spatial learning deficits and hypomobility, respectively; while p50 knockout mice display learning and memory impairment and reduced anxiety-like behavior (Denis-Donini et al., 2008; Kassed and Herkenham, 2004). Additionally, synaptic plasticity is disrupted with a reduction in NF-κB activity; long-term potentiation and long-term depression are both decreased after the pathway is inhibited (Ahn et al., 2008; O’Mahony et al., 2006; Oikawa et al., 2012). Thus, dysregulation of the NF-κB pathway could be at the core of several RTT phenotypes.

1.3. Vitamin D plays an essential role in the brain

Vitamin D is one of the many inhibitors of NF-κB signaling (Al-Rasheed et al., 2015; Y. Chen et al., 2013a; D’Ambrosio et al., 1998; Giarratana et al., 2004; J. Li et al., 2016; Penna et al., 2009; Sun et al., 2006). Intriguingly, RTT patients are known to be vitamin D deficient (Motil et al., 2011; Sarajlija et al., 2013) and this prevalence is higher in RTT than in patients with other neurological disorders (Sarajlija et al., 2013). The mechanism responsible for this deficiency is not known, although it is possible that the impaired cholesterol metabolism associated with RTT (Buchovecky et al., 2014) contributes to the phenotype, seeing as cholesterol is needed for
vitamin D synthesis (Prabhu et al., 2016). Male MeCP2-null mice exhibit lower sterol synthesis in the brain and increased serum cholesterol and triglycerides (Buchovecky et al., 2013), while a subset of RTT patients show higher levels of serum cholesterol and triglycerides (Justice et al., 2013; Segatto et al., 2014). Therefore, understanding vitamin D metabolism and its role in the pathology of RTT could prove crucial to the development of therapeutics for the disorder.

1.3.1 Vitamin D synthesis involves several metabolic steps

Vitamin D is obtained via metabolic synthesis or from dietary intake (Fig. 1.4). For vitamin D to be synthesized, UV light (spectrum 280-320 UVB) is needed for the conversion of 7-dehydrocholesterol, found in the skin, into pre-vitamin D3, which isomerizes to vitamin D3 (cholecalciferol or calcifol). Only fatty fish contain vitamin D3, while most foods are fortified with vitamin D2 or ergocalciferol. Vitamin D2 can also be produced from ergosterol, which is found in plants and fungi (Holick et al., 1980; Jeon and Shin, 2018). Vitamin D2, however, has a weaker affinity for the vitamin D binding protein (VBD) and it is cleared from circulation more rapidly than vitamin D3 (Houghton and Vieth, 2006).

The first step in the metabolism of vitamin D is 25-hydroxylation, which takes place in the liver with the help of cytochrome P450 mixed-function oxidases (CYPs). CYP27A1 hydroxylates vitamin D3 exclusively while CYP2R1 processes both vitamin D3 and vitamin D2, being the main 25-hydroxylase enzyme in the liver (Zhu et al., 2013). Calcidiol, also known as 25-hydroxyvitamin D3 or 25(OH)D3, is biologically inactive and the most abundant form of circulating vitamin D. Calcidiol is then hydroxylated into 1,25(OH)2D3, also known as calcitriol, by the CYP27B1 enzyme (Takeyama et al., 1997). Calcitriol is the active form of vitamin D. The levels of Calcidiol and calcitriol are regulated by the enzyme CYP24A1, which catabolizes
vitamin D into calcitroic acid and lactone (Jeon and Shin, 2018; Jones et al., 2014; Schuster, 2011). Calcitriol synthesis is regulated transcriptionally by a negative feedback loop, in which the complex of 1,25(OH)_{2}D_{3} and its receptor (VDR) regulates the expression of Cyp24a1, preventing accumulation of the active form of vitamin D (Makin et al., 1989).

The biologically active metabolite calcitriol is synthesized mainly in the kidneys; however, multiple tissues express CYP27B1, including the lungs, intestine, and cells of the immune system, among others (Bikle, 2009). The presence of enzymes involved in the synthesis of vitamin D in the brain of rodents (Gezen-Ak et al., 2013; Landel et al., 2018) and humans (Eyles et al., 2005; Fu et al., 1997) suggests that calcitriol can be synthesized in the CNS as well. Both calcidiol and calcitriol are capable of crossing the blood brain barrier (Pardridge et al., 1985), however, CNS levels of calcitriol better correlate with plasma levels of calcidiol than calcitriol in mice (Spach and Hayes, 2005), indicating that calcitriol is generated within the CNS.
Figure 1.4 Schematic of vitamin D metabolism.

Vitamin D3 is synthesized from 7-dehydrocholesterol found in the skin or obtained through diet, along with vitamin D2. Vitamin D is transported by vitamin D binding protein to the liver where it is converted to calcidiol with the help of the enzymes CYP27A1 and CYP2R1. Calcidiol is then transported to the kidneys, and the brain, where it is converted to calcitriol, the activated form of vitamin D, by the enzyme CYP27B1. Calcitriol is transported to the body and brain, where it can exert its function. The brain also contains several of the enzymes involved in the metabolism of vitamin D. Finally, CYP24A1 is involved in the catabolism of calcitriol into calcitroic acid and lactone.
1.3.2. Vitamin D has two models of action

Vitamin D can function in both a direct gene regulatory manner (genomic) and an indirect signaling pathway (non-genomic manner). The genomic mechanism is dependent on its nuclear receptor, VDR, which is a transcription factor containing three domains, an N-terminal DNA binding domain, a C-terminal ligand binding domain, and a hinge region that connects both domains (Rochel et al., 2000). Upon ligand binding, VDR forms a heterodimer with the Retinoid-X-receptor (RXR) and binds to response elements in the DNA (VDRE). The classic VDRE sequence, known as DR3, consists of hexanucleotides repeats, 5’-(A/G) G(G/T)TCA-3’, separated in its half-site by 3 nucleotides (Carlberg et al., 1993). Once the VDR/RXR complex binds to the VDRE consensus sequence in the genome, coregulatory partners are recruited leading to gene expression changes. The recruited complexes can be specific to genes and cell types. One such example is a histone acetyltransferase belonging to the SRC family, SRC1, which is recruited upon the displacement of the corepressor complex NcoR2/SMART after the VDR/RXR binds to regulatory regions (Haussler et al., 2013). There are thousands of VDR binding sites throughout the genome; thus, vitamin D can cause widespread changes in the transcriptome, impacting a multitude of biological processes.

Vitamin D’s mode of action is not restricted to its transcriptional role, however, and it can also induce rapid signal transduction responses via the activation of kinases, phosphatases, and ion channels in what is referred to as the non-genomic mechanism. The receptors involved in the non-genomic response include a different configuration of VDR (VDRm) and protein disulfide isomerase family A member 3 (Pdia3), also known as 1,25-MAARS, ERp57, ERp60 or Grp58 (Chen et al., 2010; Mizwicki and Norman, 2009). Both receptors are found in the caveolae of the plasma membrane and interact with the scaffolding protein caveolin-1; however, VDRm acts
through c-Src whereas Pdia3 interacts with phospholipase A2 activating protein (PLAA) to activate phospholipase A2 (PLA2) (J. Chen et al., 2013).

*Pdia3* is expressed in many brain cells types, including astrocytes and neurons; although it is more highly expressed in brain endothelial cells (Landel et al., 2018). VDR is widely expressed in the brain as well, as early as embryonic day 12 (Cui et al., 2013), and its pattern of expression includes different population of cells, such as neurons, astrocytes and oligodendrocytes, in addition to multiple brain regions (Brown et al., 2003; Cui et al., 2013; Eyles et al., 2005; Landel et al., 2018; Prüfer et al., 1999), highlighting the importance of vitamin D signaling in the CNS.

### 1.3.3. Vitamin D has many roles in the brain

For decades, vitamin D has been studied for its classical functions, such as bone and calcium homeostasis (Holmlund-Suila et al., 2012; Lieben and Carmeliet, 2013) and for its role in osteoporosis (Boonen et al., 2006; Nishii, 2003) and inflammation (Vuillermot et al., 2017). However, more recently, vitamin D has been implicated in brain development (Cui et al., 2007; Ko et al., 2004), oligodendrocyte differentiation (De La Fuente et al., 2015; Shirazi et al., 2015), neurotransmitter release (Cass et al., 2014, 2012; Jiang et al., 2014), and neuroprotection (Landel et al., 2016), among others, rendering further studies of the role of vitamin D in the brain essential.

A common method of investigating the importance of vitamin D in the developing brain is studying its deficiency. In a model of gestational vitamin D deficiency, there was a decrease in apoptosis and an increase in cell proliferation in several developmental stages of the rat brain due to the altered expression of genes involved in the progression of the cell cycle (Ko et al., 2004).
Vitamin D has also been shown to regulate cell proliferation in the subventricular zone of the developing cortex. In the absence of vitamin D, there was an increase in the number of subventricular zone neurospheres formed in culture whereas added vitamin D led to neurospheres reduction. VDR also seems to be highly concentrated in the subventricular zone (Cui et al., 2007).

Changes brought on by vitamin D deficiency in utero are not restricted to developmental phenotypes and can persist in adulthood. Impaired learning was observed in 30-week-old mice subjected to gestational vitamin D deficiency (Fernandes de Abreu et al., 2010). At 10 weeks of age, rats displayed a reduction in NGF protein expression and downregulation of genes involved in neurofilament, like MAP-2, and neurotransmission, like GABA-Aα4 (Féron et al., 2005). The timing of in utero deficiency is also important; rats subjected to late and full gestational deficiency exhibited hyperlocomotion induced by the NMDA receptor antagonist MK-801, used in models of schizophrenia. However, vitamin D deficiency only in early gestation is not sufficient to alter adult behavior under MK-801 exposure (O’Loan et al., 2007).

Adult deficiency has been shown to impact hippocampal-dependent learning and memory and reduce perineuronal net positive cells in the hippocampus of mice (Al-Amin et al., 2019). However, adult vitamin D deficiency did not reduce proliferation or survival of adult born neurons in the hippocampus of mice (Groves et al., 2016) even though maternal deficiency reduced neurogenesis in the adult hippocampus of rats (Keilhoff et al., 2010). This indicates that vitamin D may play distinct roles in the brain during its development and adulthood and its effect could also be species specific. The latter is further supported by alteration in lateral ventricles of rodents subjected to developmental vitamin D deficiency. Rats displayed enlarged lateral
ventricles (Eyles et al., 2003; Féron et al., 2005) whereas mice showed the opposite effect (Fernandes de Abreu et al., 2010; Harms et al., 2012; Hawes et al., 2015).

Meta-analysis and epidemiological studies indicate that vitamin D deficiency is a risk factor for Alzheimer’s disease (AD) and dementia (Chai et al., 2019; Feart et al., 2017; Ouma et al., 2018). Vitamin D supplementation in animal models of AD improves memory, hippocampal neurogenesis and reduces amyloid-β accumulation in the brain, while its deficiency exacerbates cognitive decline and amyloid plaque deposition (Durk et al., 2014; Ito et al., 2011; Morello et al., 2018; Taghizadeh et al., 2011). Vitamin D deficiency has been reported in patients suffering from schizophrenia (Akinlade et al., 2017; O’Loan et al., 2007; Viani-Walsh et al., 2020; Zhu et al., 2020) and multiple sclerosis (Mowry et al., 2010; Simpson et al., 2010; Van Der Mei et al., 2007) as well, with similar beneficial effects observed in vitamin D supplementation studies, demonstrating the broad impact that vitamin D can have on brain health (Burton et al., 2010; Ghaderi et al., 2019; Krivoy et al., 2017; Soilu-Hänninen et al., 2012).

The modulation of brain development by vitamin D is not exclusive to its direct genomic function. In a non-genomic manner, VDRm mediates the rapid uptake of extracellular calcium via L-type voltage-dependent calcium channels in the cortex of early postnatal rats, an effect dependent on intra and extracellular calcium concentrations. VDRm calcium regulation is also dependent on the activity of several protein kinases (PKA, PKCaMII, PI3K and p38MAPK) and potassium and chloride ion channels (Zanatta et al., 2012). Vitamin D has also been shown to modulate synaptic transmission in a non-genomic manner. In the hypothalamus, juvenile gonadotrophin-releasing neurons displayed rapid decrease in inward currents mediated by the excitatory receptors NMDA and kainite, after vitamin D application. Vitamin D was able to lower the frequency of GABAergic postsynaptic currents as well (Bhattarai et al., 2016).
addition, the non-genomic receptor Pdia3 is expressed by neurons, glia and brain endothelial cells (Landel et al., 2018), indicating that vitamin D plays an important role in the CNS through indirect signaling pathways.

1.3.3.1. Vitamin D is involved in the pathology of ASD and epilepsy

Although the phenotypic alterations caused by vitamin D deficiency have not been fully investigated in RTT, studies have been conducted in disorders with shared pathology, such as epilepsy and ASD. Epidemiological studies have found that lower levels of 25-hydroxyvitamin D during pregnancy and early infancy is associated with increased odds of a child developing ASD (Chen et al., 2016; Fernell et al., 2015; Lee et al., 2019; Vinkhuyzen et al., 2018; Whitehouse et al., 2013). Additionally, a study with a population of Chinese Han children found an association between polymorphisms of enzymes involved in vitamin D synthesis, CYP27B1 and CYP2R1, and an increase risk of ASD and its severity (Hong Yu et al., 2020).

Viral infection during pregnancy is another risk factor for ASD (Lee et al., 2015; Patterson, 2011; Vuillermot et al., 2017), and according to data obtained with animal research this risk is mediated by maternal immune activation (MIA) (Choi et al., 2016). Vuillermot et al. (2017) investigated vitamin D neuroprotection role in juvenile mice exposed to the viral mimic polyriboinosinic-polyribocytidylic acid (poly(I:C)), a common model of MIA. They found that the administration of vitamin D to dams starting on gestation day 9, which is equivalent to the first trimester in humans, prevented male offspring from developing ASD phenotypes, such as social approach deficits, altered stereotyped behavior and reduced conditioned fear response. However, no changes were seen in the expression of pro-inflammatory cytokines in the brains of dams and fetuses (Vuillermot et al., 2017). In rats, vitamin D deficiency during gestation lowers
pup grooming and licking behavior by dams and alters pup ultrasonic vocalization. Adult male offspring exposed to vitamin D deficiency *in utero* exhibit increased grooming, decreased social interaction and impaired learning and memory. Additionally, there was an increase in the volume of the lateral ventricle of the adult offspring, accompanied by reduced expression of FOXP2, a protein associated with language development, and dysregulation of genes involved in dopamine and glucocorticoid pathways (Yates et al., 2018).

Thus, vitamin D acts in a neuroprotective manner in ASD, by preventing impairment in social interaction, diminishing compulsive behavior, and improving learning and memory.

Furthermore, ASD is only one example of a disorder ameliorated by vitamin D. Epilepsy research also indicates that vitamin D can be a potential option for a partial therapeutic. Even though studies investigating the effects of vitamin D in human epilepsy are limited (Christiansen et al., 1974; Holló et al., 2012), compelling evidence suggests that vitamin D supplementation could have a beneficial impact in this disorder (Pendo and Degiorgio, 2016).

The prevalence of vitamin D deficiency in patients suffering from epilepsy is well known, as is their propensity for developing osteoporosis (Sheth et al., 2006; Teagarden et al., 2014). Studies have also associated hypocalcemia-induced seizures in newborns with gestational vitamin D deficiency (Camadoo et al., 2007; Oki et al., 1991). Interestingly, certain antiepileptic drugs, such as carbamazepine and phenytoin, are known for inducing vitamin D deficiency by promoting the conversion of the active metabolite into its inactivated form (Ali et al., 2004; Hahn, 1980).

A seminal paper describing the study of vitamin D supplementation in epilepsy using animals was published in 1984 (Siegel et al., 1984). The study showed that intravenous administration of calcitriol significantly raises seizure threshold in the hippocampi of rats for 30 minutes post
injection (Siegel et al., 1984). Another study found similar results, showing that mice that underwent subcutaneous injection of vitamin D 40 minutes prior to pentylentetrazol (PTZ) administration displayed reduced mortality, duration of tonic-clonic seizures and longer mean latency to seizure onset. The neuroprotective effect did not occur if vitamin D was injected 3, 6, 12 or 24h before PTZ dosing. This rapid and short-lived anticonvulsant effect might indicate that vitamin D acts in a non-genomic manner in epilepsy (Kalueff et al., 2005). However, there is also evidence that VDR is involved in seizure threshold. VDR-knockout mice injected with PTZ exhibited higher Racine seizure scores and mortality, in addition to lower latency to seizure onset, when compared to wild-type animals (Kalueff et al., 2006). In addition, it appears that calcium metabolism is not involved in vitamin D anticonvulsant effect given that vitamin D injected and control mice had normal calcium levels (Kalueff et al., 2006, 2005). Calcitriol is not the only form of vitamin D that has anticonvulsive potential; cholecalciferol has also been shown to alter seizure threshold. Mice injected with higher doses of cholecalciferol had increased electroconvulsive threshold. Additionally, cholecalciferol augmented the efficacy of the antiepileptic drugs valproate, phenytoin, oxcarbazepine and lamotrigine at a lower dose and carbamazepine, phenobarbital and topiramate at a higher dose (Borowicz et al., 2015, 2007). Therefore, vitamin D has the potential to improve the quality of life of patients suffering from seizures, a common comorbidity of RTT.

1.3.3.2. The role of vitamin D as an NF-κB pathway inhibitor

As mentioned previously, vitamin D can alter inflammatory response. This is due, in part, to the modulation of the NF-κB pathway. Although the brain has not been the focus of most studies,
vitamin D has been shown to inhibit NF-κB signaling in several contexts. This inhibition has been found to be dependent on the VDR. Embryonic fibroblasts originated from VDR-null mice displayed reduced IκBα protein expression and higher NF-κB transcriptional activity, which was significantly lowered after the transfection of human VDR (hVDR) into the cells. In addition, fibroblasts lacking VDR show high pathway activation without the need for pro-inflammatory cytokine stimulation, indicated by a similar distribution of p65 in the cytoplasm (inactive) and nucleus (active) of the cells; in comparison, cells from VDR+/− mice display a pattern of p65 distribution nearly exclusive to the cytoplasm. These data demonstrate that VDR is directly involved in the inhibition of NF-κB signaling by vitamin D (Sun et al., 2006). Interestingly, NF-κB p65 does not bind directly to VDRE or to the VDR/retinoid X receptor (VDR/RXR) dimer (Farmer et al., 2000; Lu et al., 2004); however, it has been shown that TNFα-activated p65 can become part of the VDR transcriptional complex by binding around the VDRE found in the promoter region of the gene osteocalcin, preventing the steroid receptor coactivator 1 (SRC-1) from binding. The binding of p65 does not occur unless VDR is activated by its ligand, vitamin D, and as a result, p65 limits the transcriptional effect of vitamin D stimulation in bone cells (Lu et al., 2004).

Vitamin D inhibition of the NF-κB pathway has also been observed in cancer research. Treatment with the activated form of vitamin D in vitro restricted NF-κB p65 to the cytoplasm of breast tumor cells and prevented the growth and proliferation of cancer cells; however, in tumor cells that underwent CYP27B1 ablation, the inactive prohormone 25(OH)D did not alter p65 nuclear distribution, indicating that only the active metabolite of vitamin D has antiproliferative tumor activity and inhibits NF-κB signaling (J. Li et al., 2016). Moreover, the addition of 1α,25-dihydroxyvitamin D3 in the growth medium of tamoxifen-resistant breast cancer cell line
stimulated IκBα expression. Vitamin D treatment also reduced the phosphorylation of p65 and its translocation to the nucleus of breast cancer cell lines after TNFα induction (Lundqvist et al., 2014). A vitamin D receptor agonist, elocalcitol, was shown to arrest NF-κB p65 nuclear translocation in human prostate tissue in vitro as well (Penna et al., 2009).

A similar mechanism was observed in the left ventricle tissue of the heart of adult male Wistar albino rats exposed to isoproterenol, which induces cardiac hypertrophy in rodents. In the absence of vitamin D treatment, the expression of NF-κB p65 was significantly elevated in the group exposed to isoproterenol, in contrast with IκBα expression, which was reduced; however, with vitamin D supplementation, p65 expression was significantly reduced while IκBα expression increased (Al-Rasheed et al., 2015). Increased expression of IκBα was also seen in human keratinocytes after vitamin D treatment, leading to the downregulation of NF-κB downstream targets. This demonstrates that vitamin D dampens NF-κB signaling in multiple tissues by inducing the expression of its inhibitor, IκBα.

Vitamin D supplementation has been studied as a therapeutic for autoimmune disorders as well, based on its role in the inhibition of IL-12, an interleukin involved in the development of T helper 1 (Th1) cells associated with the pathogenesis of inflammatory autoimmune disorders. The interaction between IL-12 and NF-κB, along with NF-κB activation, is diminished in the presence of vitamin D and an intact VDR/RXR complex, resulting in the downregulation of IL-12 in monocytic cells (D’Ambrosio et al., 1998). In addition, BXL-219, a vitamin D analog, up-regulates IκBα expression in islet cells of non-obese diabetic mouse model, preventing p65 nuclear translocation, which results in the decrease of proinflammatory chemokine production in vitro and in vivo (Giarratana et al., 2004).
NF-κB inhibition by vitamin D is not only accomplished through IκBα upregulation; vitamin D can physically interact with IKKβ via its receptor, VDR. GST pull-down and co-immunoprecipitation assays done in human embryonic kidney cell line showed that 1,25(OH)2D3 augments the interaction between VDR and IKKβ, which form a complex that prevents the physiological activity of IKKβ. Without IKKβ phosphorylation, IκBα maintains the p65/p50 dimer in the cytoplasm, diminishing NF-κB signaling (Y. Chen et al., 2013a).

Evidence of vitamin D inhibition of NF-κB activity in the brain is sparse, since not much research has been conducted in the CNS. However, with the increased interest in the role of vitamin D in neuroinflammation (Calvello et al., 2017; de Oliveira et al., 2020), more studies are now focusing on the mechanisms responsible for vitamin D inhibition of NF-κB signaling in the brain. For example, Hajiluian et al. (2017) studied the effects of vitamin D supplementation in the brain in a high-fat diet (HFD) context, exposing male Wistar rats to HFD for 4 months before treating them with vitamin D for 5 weeks, while still being exposed to the HFD. HFD-treated rats displayed cognitive impairment, assessed with the Morris water maze test, increased blood brain barrier (BBB) permeability, reduced BDNF concentration and elevated NF-κB protein expression. However, after vitamin D supplementation, both cognitive deficits and BBB permeability were normalized, in addition to a reduction in NF-κB concentration and an increase in BDNF expression in the hippocampus (Hajiluian et al., 2017).

Furthermore, it has been shown that vitamin D, together with progesterone, was more effective in conserving the spatial memory of Sprague-Dawley rats subjected to bilateral medial frontal cortical impact, which mimics traumatic brain injury (TBI) (Hua et al., 2012). Importantly, the combined treatment lowered phosphorylated NF-κB p65 and TNFα protein expression. Reduced
NF-κB signaling was a result of decreased IκBα phosphorylation, which was significantly lower in TBI rats receiving the progesterone and vitamin D treatment when compared to TBI rats only being treated with progesterone (Tang et al., 2015).

In a D-galactose oxidative stress mouse model, vitamin D administration prevented the reduction of synaptic proteins, such as PSD-95 and synaptophysin, in addition, to preventing memory impairment in the Morris water maze and the Y-maze tests. Ali et al (2021) argued that vitamin D’s therapeutic effect is due to the activation of NAD-dependent deacetylase sirtuin-1 (SIRT-1), which in turn activates the nuclear factor erythroid 2-related factor 2/heme oxygenase 1 (NRF-2/HO-1) signaling axis, limiting oxidative stress burden. Moreover, vitamin D was also beneficial for its inhibition of NF-κB signaling (Ali et al., 2021); D-galactose treatment is known to activate the NF-κB pathway and exacerbate neuroinflammation (Nonn et al., 2006); however, D-galactose administration in conjunction with vitamin D significantly lowered NF-κB signaling and the expression of its downstream targets, TNF-α and IL-1β, in the brains of adult male mice (Ali et al., 2021). Furthermore, under hypoxia conditions, p65 nuclear translocation is elevated in brain endothelial cells; however, vitamin D treatment inhibits p65 translocation to the nucleus of those cells (Won et al., 2015). Therefore, even though most of the research investigating the mechanism of NF-κB pathway inhibition by vitamin D has utilized tissues involved in inflammation or tumorigenesis, there is compelling evidence to argue that vitamin D plays a similar role in the CNS.
1.4. Vitamin D modulation of NF-κB signaling as a therapeutic avenue for RTT

As previously mentioned in detail, vitamin D is an inhibitor of NF-κB activity (Y. Chen et al., 2013a; D’Ambrosio et al., 1998; Giarratana et al., 2004), and aberrant NF-κB signaling has been observed in MeCP2-null mice (Kishi et al., 2016). Importantly, genetically attenuating NF-κB activity by crossing MeCP2 mice with the Nfκb1 strain, which lacks the p50 subunit, has been shown to rescue the morphology of cortical neurons and extend the lifespan of the mice (Kishi et al., 2016). Thus, we hypothesized that vitamin D supplementation of RTT model mice would lower NF-κB signaling and similarly ameliorate the phenotypes of the disorder.
Chapter 2: Vitamin D supplementation rescues aberrant NF-κB pathway activation and partially ameliorates Rett syndrome phenotypes in MeCP2 mutant mice

This chapter is a duplicated version of the published manuscript:


Author contributions: M.C.R., N.K., J.L.M, and J.D.M. conceptualized and designed the study. M.C.R. and S.M.M. performed and analyzed the in vitro assays (p65 localization - M.C.R.; neurite outgrowth - S.M.M.). M.C.R. and J.L.M. performed and analyzed the in vivo phenotyping and morphology analyses, with assistance from S.M.M. (male phenotypic score, lifespan, dendritic complexity, soma area - J.L.M.; male spine density - M.C.R.; female morphology - M.C.R. and S.M.M.). M.C.R. performed the transcriptional analyses. M.C.R. and J.L.M. wrote the manuscript with input from J.D.M.
2.1. Abstract

Rett syndrome (RTT) is a severe, progressive X-linked neurodevelopmental disorder caused by mutations in the transcriptional regulator MECP2. We previously identified aberrant NF-κB pathway up-regulation in brains of MeCP2-null mice and demonstrated that genetically attenuating NF-κB rescues some characteristic neuronal RTT phenotypes. These results raised the intriguing question of whether NF-κB pathway inhibitors might provide a therapeutic avenue in RTT. Here, we investigate whether the known NF-κB pathway inhibitor vitamin D ameliorates neuronal phenotypes in MeCP2-mutant mice. Vitamin D deficiency is prevalent among RTT patients, and we find that MeCP2-null mice similarly have significantly reduced 25(OH)D serum levels compared to wild-type littermates. We identify that vitamin D rescues aberrant NF-κB pathway activation and reduced neurite outgrowth of MeCP2 knockdown cortical neurons in vitro. Further, dietary supplementation with vitamin D in early symptomatic male MeCP2 hemizygous null and female MeCP2 heterozygous mice ameliorates reduced neocortical dendritic morphology and soma size phenotypes, and modestly improves reduced lifespan of MeCP2-nulls. These results elucidate fundamental neurobiology of RTT and provide foundation that NF-κB pathway inhibition might be a therapeutic target for RTT.

2.2. Introduction

There is currently no effective treatment for Rett syndrome (RTT), a severe X-linked progressive neurodevelopmental disorder caused by mutations in the transcriptional regulator MECP2 (Amir et al., 1999). Girls with this devastating disorder develop relatively normally for 6-18 months, after which they undergo a period of rapid regression, with loss of motor skills, including
purposeful hand movement, deceleration of head growth, and onset of repetitive, autistic behaviors (Chahrour and Zoghbi, 2007). Importantly, selectively re-expressing MeCP2 in adult mice has shown that RTT symptoms can be partially reversed (Giacometti et al., 2007; Guy et al., 2007; Luikenhuis et al., 2004), indicating that MeCP2 is necessary for both the development and maintenance of mature neurons (McGraw et al., 2011; Nguyen et al., 2012). These results suggest the potential for post-symptomatic therapeutic intervention, and open up the exciting prospect to at least partially stall or reverse phenotypic progression by restoring homeostasis of downstream targets of MeCP2.

One such potential downstream target is the NF-κB pathway. The NF-κB pathway regulates many cellular processes, including neural process development, structural plasticity, and learning and memory (Gutierrez and Davies, 2011). Mutations in components of the NF-κB pathway cause a spectrum of cognitive phenotypes in humans, including intellectual disability and autism spectrum disorders (ASDs) (Chiara Manzini et al., 2014; Philippe et al., 2010). Previously, we identified aberrant up-regulation of Irak1, encoding a signaling kinase and scaffold protein within the NF-κB pathway, in purified cortical callosal projection neurons (CPN) from male MeCP2-null mice (Kishi et al., 2016). Up-regulation of Irak1 has also been observed in different regions of the brain across RTT mouse models, correlating with phenotype severity (Gabel et al., 2015), further supporting our results. We found that Irak1 over-expression recapitulates the reduced dendritic complexity phenotype of MeCP2-null CPN, and that NF-κB pathway signaling is aberrantly up-regulated in cortical neurons with MeCP2 loss-of-function. We genetically attenuated the aberrant NF-κB signaling in MeCP2-null mice by crossing them with mice heterozygous for Nfkb1. Strikingly this genetic attenuation partially rescues the reduced cortical
dendritic complexity in MeCP2-null mice – a hallmark of RTT that is recapitulated in these animals, and it substantially extends their normally shortened lifespan.

There are many known inhibitors of the NF-κB pathway. The known ability of vitamin D to inhibit NF-κB signaling (Chen and Chen, 2013; Lundqvist et al., 2014) is particularly compelling given the high prevalence of vitamin D deficiency in RTT patients (Motil et al., 2011; Sarajlija et al., 2013). Developmental vitamin D deficiency leads to severe neurodevelopmental disruptions and behavioral abnormalities in rodents (Cui et al., 2017; Eyles et al., 2013), and there is growing evidence of a correlation between vitamin D deficiency and neurodevelopmental disorders, including ASD (Fernell et al., 2015), epilepsy (Holló et al., 2012), and cognitive function (Mayne and Burne, 2019). Vitamin D supplements can improve behavioral measures in some children with ASD (Jia et al., 2015), and phenotypes in rodent models of ASD-like characteristics (Vuillermot et al., 2017). The precise mechanisms by which vitamin D regulates neurodevelopment are not known and might include modulation of NF-κB as well as parallel pathways.

Here, we investigated whether vitamin D supplementation can inhibit the aberrant NF-κB signaling in cortical neurons that occurs with MeCP2 loss-of-function, and whether such supplementation can ameliorate RTT phenotypes in male and female MeCP2 mutant mice. We determined that addition of the activated form of vitamin D rescues the increased NF-κB-dependent transcription that occurs with MeCP2 knockdown and increases neurite outgrowth in vitro. Further, we employed custom chow to discover that dietary vitamin D supplementation in vivo rescues the neuronal morphology of both male MeCP2-null and female heterozygous mice, and modestly extends the lifespan of male MeCP2-nulls. These results provide proof-of-concept
that NF-κB pathway inhibition, including via vitamin D supplementation, could provide a novel therapeutic target for some RTT phenotypes.

2.3. Materials and Methods

2.3.1. Experimental Design and Statistical Analyses

Animals were placed on custom chow in a rotating order based on date of birth. In rare instances when a litter contained 3 or more nulls or heterozygous females, the mice were randomly divided into two cages by an investigator blinded to experimental conditions and were treated as sequential litters to avoid over-representation of littermates in one treatment group. Mice were weighed weekly, and assessed with a phenotypic score following criteria established by Guy et al. (Guy et al., 2007) by an investigator blinded to genotype and chow concentration. In brief, the mice were evaluated for abnormal gait, hind limb clasping, irregular breathing, tremor, impaired mobility and poor general body condition. Each symptom was scored as 0 (absent), 1 (present) or 2 (severe), and the score for each symptom was summed to provide an overall phenotype score with a maximum possible score of 12. Any mouse scoring a 2 (highly symptomatic) for general body condition, tremor, or breathing, or that lost greater than 20% of pre-symptomatic body weight was euthanized and the day of euthanasia was considered day of death for lifespan analysis. The selection of sample size was based on standards in the field, and on criteria established by the RTT research community (Katz et al., 2012). All morphological and phenotypic analyses were performed by investigators blinded to experimental conditions (genotype and treatment group).
GraphPad Prism 8.0 (GraphPad Software, San Diego, CA) was used to carry out the statistical analyses. No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those generally employed in the field. Our statistical tests consisted of two-tailed t-test, one-way ANOVA with Tukey’s multiple comparison, or two-way ANOVA with Bonferroni post-test analyses to determine statistical significance between groups. Data distribution was handled as if normal, but this was not formally tested (since potential differences in results would be minor). Variance between groups was analyzed using the f test procedure. For the survival curve analysis, we used the log-rank test, since this method is commonly used to compare the survival distributions of two groups. All data shown represent means ± SEM. Sample size and statistical test are specified in each figure legends.

2.3.2. Animals

All animal experimental protocols were approved by the Harvard University and/or Syracuse University Institutional Animal Care and Use Committee and adhere to NIH guidelines. Mice were group housed at a maximum of 5 mice per cage on a 12:12 h light/dark cycle and were given food and water *ad libitum*. CD-1 timed pregnant female mice were purchased from Charles River. Female *Mecp2* heterozygous mice were purchased from Jackson Labs (B6.129P2(C)-*Mecp2*<sup>tm1.1Bird/J</sup>; RRID:IMSR_JAX:003890), and were maintained on a C57BL/6 background. Genotypes were determined by PCR on genomic DNA as follows:

*Mecp2* mutant mice - forward primer oIMR1436 5’- GGT AAA GAC CCA TGT GAC CC -3’; reverse primer oIMR1437 5’- TCC ACC TAG CCT GCC TGT AC -3’; reverse primer oIMR1438 5’- GGC TTG CCA CAT GAC AA-3’.
2.3.3. Constructs

To knock down Mecp2 expression, a construct consisting of a bicistronic cassette encoding an shRNA sequence targeted against Mecp2 driven by a U6 promoter, and GFP driven by a ubiquitin promoter, was used. In control experiments, a scrambled sequence replaced the Mecp2 shRNA (both constructs were a generous gift of Dr. Z. Zhou, University of Pennsylvania (Zhou et al., 2006). To measure NF-κB activation, a plasmid containing 5 copies of an NF-κB response element driving expression of the luciferase reporter gene luc2P was purchased from Promega (Cat# E8491). Relative luminescence was normalized to a co-transfected Renilla luciferase construct, derived from the psiCHECK-2 vector (Promega, Cat# C8021) with the HSV-TK promoter and Firefly luciferase cut out by digestion with NotI and Xba1.

2.3.4. Embryonic Cortical Neuron Culture

E15.5 embryos were collected from timed pregnant CD1 mice, and the cortex was dissected out in dissociation medium (DM) containing, MgKyn (Sigma-Aldrich), glucose, AP-V (Sigma-Aldrich), Penicillin-Streptomycin (Invitrogen) and B27 supplement (Invitrogen). The cells were dissociated using cysteine (Sigma-Aldrich, St. Louis, MO), papain, and OptiMem media. Glass coverslips were precoated with Poly-D-lysine hydrobromide (Sigma-Aldrich P-6407). For neurite outgrowth experiments, 5 million cells were electroporated with 20µg of either shScram or shMecp2 plasmid (BTX ECM 830 Square Wave Electroporation system, following the parameters: 700V, 1 unipolar pulse at 100µs pulse length in a 100ms interval). After a recovery period of 5 minutes, 50,000 cells per coverslip were plated in neurobasal based medium containing Glutamax (Invitrogen, Carlsbad, CA), fetal bovine serum (Invitrogen), and Penicillin-Streptomycin (Invitrogen). After 4 hours, the plating medium was removed, and growth medium
was added, which contained neurobasal, Glutamax (Invitrogen), Penicillin-Streptomycin (Invitrogen), N2 and B27 supplements (Invitrogen). Calcitriol treatment started on DIV2 and continued until the cells were fixed on DIV7. For p65 nuclear quantification experiments, 50,000 cells were plated per coverslip immersed in plating medium for 4 hours before being replaced by growth media. After 3 days, cells were transfected via lipofectamine 2000 (Invitrogen) with 1ug/ul of either shScram or shMecp2 plasmid, following manufacturer guidelines. Calcitriol treatment started on DIV4 and lasted until the cells were fixed on DIV14. 10µM calcitriol stock solution was prepared by dissolving 1α,25-Dihydroxyvitamin D₃ (Sigma-Aldrich) in ethanol. For the no treatment group, only growth medium was added; for the vehicle group, only ethanol was added; for the treatment group, 100nM of calcitriol stock solution was added. The final ethanol concentration for both the vehicle and calcitriol groups was 1%. Growth medium was changed every other day.

2.3.5. Immunocytochemistry

Coverslips containing DIV 7 or DIV 14 cells were fixed with 4% paraformaldehyde in PBS for 15 minutes, followed by three PBS washes. The cells were blocked with 8% goat serum, 0.33% Triton X, 0.3% bovine serum albumin (Sigma-Aldrich) in PBS for 20 min. The coverslips were then incubated in primary antibodies diluted in blocking solution for 1 hour. Coverslips were rinsed three times with PBS for 5 minutes each and incubated in secondary antibodies diluted in blocking solution for 1 hour. The coverslips were washed three times with PBS, rinsed with 1/3 PB, and mounted on a slide in Fluoromount (SouthernBiotech, Birmingham, AL) prior to imaging. Antibody dilutions were as follows: rabbit α-MeCP2 (1:500, Cell Signaling Technology Cat# 3456, RRID:AB_2143849); chicken α-GFP IgY (1:1,000, Thermo Fisher Scientific Cat#
A10262, RRID:AB_2534023); rabbit α-NF-κB P65 (1:500, Cell Signaling Technology Cat# 8242, RRID:AB_10859369); mouse α-MAP2 (1:1,000, Sigma-Aldrich Cat# M1406, RRID:AB_477171). Secondary antibodies from Molecular Probes Alexa Series were used based on the primary antibody dilution (1:500 or 1:1,000, Invitrogen).

2.3.6. p65 Nuclear Quantification

Cortical neurons positive for both GFP and MAP2 were imaged with a Nikon Ni-U upright fluorescence microscope with a Zyla CMOS digital camera. Three independent experiments were performed, and 6-10 neurons per condition were imaged from each experiment. DAPI was used to identify the nucleus, and p65 translocation was quantified using ImageJ as corrected total cell fluorescence \[\text{CTCF} = \text{Integrated density} - (\text{area of selected cell} \times \text{mean fluorescence of background readings})\] (Burgess et al., 2010; McCloy et al., 2014). Images were assembled using Photoshop CC 2017 (Adobe, San Jose, CA).

2.3.7. NF-κB Luciferase Reporter Assays

P1 C57Bl/6 wild-type brains were dissected and dissociated as described for embryonic cultures. Dissociated cells were nucleofected with the NF-κB reporter construct and control Renilla luciferase construct, along with either scrambled shRNA or Mecp2 shRNA constructs, using an Amaxa Mouse Neuron Nucleofector kit (Lonza, Basel, Switzerland), and the Amaxa Nucleofector II Device (Lonza). Cells were cultured for 48 hours at high density in 96 well plates coated with poly-D-lysine (Sigma-Aldrich), in growth medium composed of 50% DMEM-F12 and 50% Neurobasal (Gibco, Gaithersburg, MD), with N2, B27, and GlutaMax supplements (Invitrogen). Calcitriol or vehicle control was added at 24 hours. At 48 hours, Firefly and Renilla
luciferase activities were measured using the Dual-Glo Luciferase Assay system (Promega, Madison, WI) and a GloMax 96 microplate luminometer (Promega). The luminescence of each well was normalized individually, and triplicate wells were averaged within each experiment. Relative luminescence was normalized to the control, shScram experimental condition, and data represent four independent biological replicates.

2.3.8. Quantitative Real-Time PCR (qPCR)

RNA was extracted using TRIzol (Invitrogen), and cDNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) or qScript™ cDNA SuperMix (Quanta Biosciences, Beverly, MA). qPCR was performed on a CFX Connect™ Real-Time System (Bio-Rad Laboratories) according to the manufacturer's instructions. Primer pairs for Irak1, Gapdh, and S16 were as follows; each primer of each primer pair was designed in different exons, so as not to amplify genomic DNA:

Irak1: Forward 5′- GCTGTGGACACCGATACCTT -3′
       Reverse 5′- GGTCACTCCAGCCTCTTCAG -3′

Gapdh: Forward 5′- GGCATTGCTCTCAATGACAA -3′
       Reverse 5′- TGTGAGGGAGATGCTCAGTG -3′

S16: Forward 5′- CACTGCAAAACGGGAATGG -3′
     Reverse 5′- TGAGATGGACTGTCGGATGG -3′

Mecp2: Forward 5′- TATTTGATCAATCCCCAGGG -3′
      Reverse 5′- CTCCCCCTCTCCAGTATCCGT -3′

For the PCR reactions, we used PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences) Master mix, and each PCR reaction consisted of 1X LightCycler FastStart DNA Master SYBR
Green I mixture, 0.2 μM primers, and cDNA. We used the mean of *Gapdh* and *S16* expressions as the reference gene. Each sample was run in triplicate and averaged. The relative quantification analysis was performed as follow: \( \Delta \text{Cq} = \text{Cq of gene of interest} - \text{geometric mean of Cq of reference genes} \); \( \Delta \Delta \text{Cq} = \Delta \text{Cq} - \text{Mean of } \Delta \text{Cq of wild-type samples} \); Fold change = \( 2^{\Delta \Delta \text{Cq}} \).

We also performed melt curve analysis to verify the specificity of the amplicons.

**2.3.9. Vitamin D Serum Measurements**

Serum was collected from 4 pairs of *Mecp2+/y* and *Mecp2-/y* littermates at 8 weeks of age, following standard protocols. Total serum 25(OH)D levels were measured by radioimmunoassay by Heartland Assays (Ames, IA). For measurement of the vitamin D supplemented animals, 3-4 serum samples of *Mecp2+/y* and *Mecp2-/y* littermates at 8 weeks of age and 3-4 serum samples of *Mecp2+/+* and *Mecp2 +/+* littermates at 5 months of age on the different concentrations of vitamin D were analyzed via Mass Spectrometry by ZRT Laboratories (Beaverton, OR).

**2.3.10. Vitamin D Supplementation**

Custom chow obtained from Bio-Serv (Flemington, NJ) was based on the AIN-93G Rodent Diet, varying only in Vitamin D₃ concentration. Male *Mecp2+/y* and *Mecp2-/y* littermates, and female *Mecp2+/+* and *Mecp2 +/+* littermates were each weaned together at 4 weeks of age, and placed on chow containing 1 IU/g vitamin D (standard chow), 10 IU/g, or 50 IU/g in rotating order based on date of birth. As per established standards for preclinical studies in *Mecp2*-null mice (Katz et al., 2012), 15-18 *Mecp2-/y* mice were analyzed for lifespan and phenotypic progression for each vitamin D concentration. Mice were weighed weekly, and assessed with a phenotypic score following criteria established by Guy et al. (Guy et al., 2007) by an investigator blinded to
genotype and chow concentration. Any mouse scoring a 2 (highly symptomatic) for general body condition, tremor, or breathing, or that lost greater than 20% of pre-symptomatic body weight was euthanized and the day of euthanasia was considered day of death for lifespan analysis.

2.3.11. Golgi Staining, Dendrite and Soma measurements

For dendrite and soma size analyses, 4-5 mice of each sex and genotype were analyzed per condition, as per established standards. Mice were euthanized with avertin overdose (at 8 weeks of age for males and 5 months of age for females), and brains were immersed in freshly prepared Golgi impregnation solution (FD Rapid GolgiStain kit; FD Neurosciences, Columbia, MD). Brains were processed according to the protocol provided by the company. Neurons were systematically selected for analysis and imaged by an investigator blinded to genotype and experimental condition with the following a priori selection criteria: 1) overall cellular morphology of superficial layer cortical pyramidal neurons; 2) dendritic trees well impregnated, and not obscured by stain precipitate, blood vessels, or astrocytes; and 3) the entire dendritic tree appearing intact and visible within the 150 μm thickness of the section. Neurons were imaged on a Nikon Ni-U upright microscope with a Zyla CMOS digital camera under a 20x objective, equipped with an Optiscan XYZ motorized stage to allow for Z stacks. NIS-Elements software was used for Simple Deconvolution and Extended Depth of Focus, after which the neurons were traced using Adobe Illustrator CS5 (Adobe, San Jose, CA). Dendritic complexity was quantified using Sholl analysis (Sholl, 1953), employing ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland) with the Sholl Analysis Plugin (v1.0) (Ghosh Lab, www.ghoshlab.org/software/index.html). The following parameters were used for dendrite analysis: step = 10 μm, beginning radius = 20 μm, final radius = 200 μm.
2.3.12. Dendritic Spines Measurements

For apical dendritic spine density quantification, 3 Mecp2-/-y and Mecp2+/- littermates were analyzed per condition. Neurons were selected and imaged by an investigator blinded to genotype and experimental condition, following the criteria: 1) morphology of superficial layer cortical pyramidal neurons; 2) well impregnated dendritic trees; and 3) the entire apical dendritic tree appearing intact. Neurons were imaged under a 60x-oil objective using with a Nikon Ni-U upright microscope with a Zyla CMOS digital camera, and Optiscan XYZ motorized stage, enabling Z-stacks. For the quantification, we used the software RECONSTRUCT following the directions described (Risher et al., 2014). Images were reconstructed using Photoshop CC 2017 (Adobe).

2.4. Results

2.4.1. Vitamin D serum levels are reduced in Mecp2-/-y mice

The high prevalence of vitamin D deficiency in RTT patients (Motil et al., 2011; Sarajlija et al., 2013), and the known ability of vitamin D to inhibit the NF-κB pathway (Y. Chen et al., 2013b; Lundqvist et al., 2014), which is up-regulated in brains of hemizygous null (Mecp2-/-y) male mice (Kishi et al., 2016), raises the intriguing questions of whether this simple, cost-effective dietary supplement might rescue the aberrant NF-κB pathway activation in these mice, and whether it can contribute to phenotypic improvement. To investigate this and to further test the mechanistic motivation for this approach, we first analyzed vitamin D levels in the serum of 8-week-old Mecp2-null mice and wild-type littermates (Mecp2+/-) by radioimmunoassay. Previous studies employing dietary vitamin D supplementation in mice have demonstrated that
1,25(OH)₂D₃ levels in the brain correlate with plasma 25(OH)D₃ levels (Spach and Hayes, 2005); thus, we measured plasma 25(OH)D₃ levels. We found that, similar to RTT patients, MeCP2-null mice have significantly reduced (~50%) total serum 25(OH)D levels compared to wild-type littermates (Fig. 2.1A), further suggesting that vitamin D supplementation might have therapeutic benefit.
Figure 2. 1 Vitamin D rescues aberrant NF-κB activation in MeCP2 knockdown cortical neurons. (A) MeCP2-null mice have reduced serum vitamin D levels (25(OH)D) compared to the wild-type littermates at 8 weeks of age (N = 4 mice / genotype). (B-C) E15.5 cortical neurons were nucleofected with a construct expressing GFP as reporter and either a control shRNA (shScram)
or an shRNA targeting Mecp2 (shMecp2). shMecp2 nucleofection visibly reduced the expression of MeCP2 protein at 7 days in vitro (B) and downregulated the overall expression of Mecp2 approximately 50% after 14 days in vitro, in cultures in which the transfection efficiency was about 60% (C). Arrowheads indicate nucleofected GFP-positive neurons; arrows indicate neighboring non-nucleofected GFP negative neurons. N = 4 experimental replicates. (D) Dissociated P1 cortical neurons were nucleofected with shScram or shMecp2, then were cultured for 2 days. Addition of calcitriol, the activated form of vitamin D (VitD), to culture medium for 24 hours rescues the ~1.75-fold increase in NF-κB-dependent transcription that occurs with knockdown of Mecp2 in cortical neurons in vitro. However, calcitriol has no effect on shScram control nucleofected neurons (N = 4 biological replicates). (E-F) Mecp2 knockdown results in increased nuclear p65 localization in cortical neurons, which is indicative of NF-κB activation. Addition of calcitriol to the culture medium reduces p65 protein expression in the nucleus of Mecp2 knockdown cortical neurons, but not control (shScram) neurons. C-D: N = shScram no treatment: 33 neurons, vehicle: 30 neurons, 100 nM VitD: 33 neurons; shMecp2 no treatment: 23 neurons, vehicle: 22 neurons, 100 nM VitD: 22 neurons from 3 independent experiments.

Expression of GFP was employed to identify transfected neurons. AU = relative luminescence units. A, B, D: two-tailed t-test. E: one-way ANOVA with Tukey’s Multiple Comparison. * P < 0.05, ** P < 0.01, NS = not significant. Scale bar = 20 μm. Error bar: ± SEM.
2.4.2. Vitamin D supplementation rescues aberrant NF-κB activation in cortical neurons in vitro

Vitamin D and its analogues have been found to inhibit the NF-κB pathway, but this has not been well-studied in neurons. In the inactive state, the NF-κB dimer is tethered in the cytoplasm by Inhibitor of κB (IκB). When the pathway is activated, IκB is phosphorylated, targeting it for proteasomal degradation. The NF-κB dimer is thus released, and translocates to the nucleus, where it binds to consensus NF-κB response elements in the DNA to activate transcription of target genes. The predominant form of NF-κB in the nervous system is a p65/p50 heterodimer (Gutierrez and Davies, 2011), and NF-κB subunits are expressed throughout the CNS, by neurons as well as by glia.

To investigate whether vitamin D supplementation can rescue aberrant NF-κB activation resulting from MeCP2 knockdown in cortical neurons, we first employed an in vitro NF-κB response element luciferase assay. We previously employed this reporter construct with tandem NF-κB -response elements (NF-κB-RE) and a minimal reporter driving luciferase to assay NF-κB transcriptional activity in cortical neurons following Irak1 over-expression or MeCP2 knockdown, and identified significant up-regulation of NF-κB dependent transcriptional activity (Kishi et al., 2016). We employed an shRNA-mediated MeCP2 knockdown approach in wild-type neurons, allowing for an efficient, higher-throughput in vitro system; the high transfection efficiency obtained with the nucleofection approach (approx. 60% of surviving cells) recapitulates the heterogeneous MeCP2 expression of a MeCP2+/- cortices. The MeCP2 knockdown and control shRNA constructs employed have been previously validated and published (Kishi et al., 2016; Zhou et al., 2006). Both constructs contain eGFP driven by an independent promoter. This shRNA-mediated knockdown of MeCP2 is sufficient to visibly
reduce, but not eliminate, protein detection by immunocytochemistry in cortical neurons (Fig. 2.1B-C). In the vehicle control, MeCP2 knockdown results in an approximate 1.75-fold increase in NF-κB dependent transcriptional activity relative to shScram, which is similar to the previously published ~2-fold increase observed with MeCP2 knockdown without treatment (Kishi et al., 2016). We treated neurons with the bioactive form of vitamin D (1α,25-Dihydroxyvitamin D3; calcitriol) for 24 hours prior to performing NF-κB-RE luciferase assays. We find that addition of calcitriol has no effect on relative NF-κB activation in control neurons, but significantly reduces the elevated NF-κB signaling in MeCP2 knockdown neurons, bringing the level back down to that of control neurons (Fig. 2.1D).

Next, we investigated whether calcitriol might also reduce nuclear translocation of the p65 subunit of NF-κB, which is indicative of NF-κB pathway activation. For these experiments, E15.5 cortical cells were dissociated and cultured for 14 days. They were transfected with either shScram or shMeCP2 at 3 days in vitro (DIV), and treated with either vehicle (ethanol) or 100 nM of calcitriol starting at 4 DIV. Transfected neurons were identified by co-expression of GFP and the neuronal marker MAP2. shMeCP2-transfected neurons express higher levels of p65 protein in their nucleus compared to control (shScram) transfected cells (Fig. 2.1E, left). Interestingly, the addition of the vehicle (EtOH) increases p65 nuclear translocation in control transfected neurons, but not in MeCP2 knockdown neurons, perhaps indicating that pathway activation is already maximal in these neurons (Fig. 2.1E, middle). Ethanol is known to alter NF-κB signaling via ROS-dependent pathways, increasing p65 phosphorylation and its nuclear translocation in neurons and glia (Davis and Syapin, 2004; Lippai et al., 2013; Okabe et al., 2016; Vetreno and Crews, 2018). However, addition of 100 nM calcitriol reduces p65 nuclear localization in MeCP2 knockdown neurons without affecting the control neurons (Fig. 2.1E-F), indicating that vitamin
D supplementation can reduce aberrant NF-κB signaling in *MeCP2*-deficient cortical neurons *in vitro*.

### 2.4.3. Vitamin D rescues reduced neurite outgrowth of *MeCP2* knockdown cortical neurons *in vitro*

We next investigated whether addition of calcitriol might also rescue the reduced neurite outgrowth of *MeCP2* knockdown neurons *in vitro*. E15.5 cortical neurons transfected with *shMeCP2* demonstrate a significant reduction in total neurite outgrowth by 7 DIV, in comparison to control *shScram* transfected neurons (Fig. 2.2A-C). Transfected neurons were again identified by GFP and MAP2 expression, with GFP used to trace total neurites. Calcitriol or ethanol (vehicle) was added to the culture medium from 2 to 7 days *in vitro* (Fig. 2.2A-B). The vehicle marginally reduced the total neurite outgrowth of both *shScram* and *shMeCP2* neurons; however, *MeCP2* knockdown neurons have significantly decreased neurite length compared to *shScram* control, both in untreated and vehicle-treated cultures. Ethanol has also been shown to negatively alter neuronal dendritic complexity and neurite development, as discussed above for p65 localization. However, there is no difference in total neurite outgrowth between vehicle and calcitriol treated *shScram* neurons, while there is a significant increase in total neurite outgrowth of *shMeCP2* neurons treated with calcitriol compared to vehicle. (Fig. 2.2C). Together, these data indicate that vitamin D is able to act on neurons to modify NF-κB signaling and *MeCP2* knockdown cortical neuronal phenotypes *in vitro*, thus motivating investigation of how vitamin D modifies *MeCP2*-mutant neuronal phenotypes *in vivo*. 
A

Neurite Outgrowth of Cortical Neurons at 7 DIV

<table>
<thead>
<tr>
<th>No Treatment</th>
<th>Vehicle</th>
<th>100 nM VitD</th>
</tr>
</thead>
<tbody>
<tr>
<td>shScram</td>
<td>GFP</td>
<td>GFP</td>
</tr>
<tr>
<td>shMecp2</td>
<td>GFP</td>
<td>GFP</td>
</tr>
</tbody>
</table>

B

No Treatment  
shScram  shMecp2

Vehicle  
shScram  shMecp2

100 nM VitD  
shScram  shMecp2

C

Total Neurite Outgrowth (µm)

- shScram
- shMecp2

* ns
**Figure 2.** Vitamin D rescues reduced neurite outgrowth of *Mecp2* knockdown cortical neurons. (A-C) Dissociated E15.5 cortical neurons were nucleofected with a construct expressing a GFP reporter and either a control shRNA (shScram) or an shRNA targeting *Mecp2* (sh*Mecp2*), then were plated and cultured for 7 days. Neurons were either maintained in standard culture medium, or were supplemented with vehicle (EtOH) or 100nM calcitriol (VitD) from 2-7 DIV. (A) Representative images of GFP+ cortical neurons at 7 days *in vitro* under each condition. (B) Representative traces of GFP+ cortical neurons under each condition. (C) Total neurite outgrowth of GFP+ neurons was quantified from randomly selected neurons, from each of 3 independent experiments (N = shScram no treatment: 30 neurons, vehicle: 26 neurons, 100 nM VitD: 27 neurons; shMecp2 no treatment: 28 neurons, vehicle: 26 neurons, 100 nM VitD: 27 neurons). Supplementation with calcitriol rescues the reduced neurite outgrowth of *Mecp2* knockdown neurons relative to EtOH vehicle control, but does not have a significant effect on control cortical neurons. Thus, *shMecp2* neurons with calcitriol are not significantly different from *shScram*. C: one-way ANOVA with Tukey’s Multiple Comparison. * P < 0.05, NS = not significant. Scale bar = 50 μm. Error bar: ± SEM.
2.4.4. Dietary vitamin D supplementation moderately extends the reduced lifespan of

*Mecp2*-null mice

To investigate whether vitamin D supplementation might also improve specific *Mecp2*-null phenotypes *in vivo*, we treated *Mecp2*-null (*Mecp*2<sup>tm1.1Bird</sup>) and wild-type littermates with vitamin D supplemented chow, and analyzed complexity and soma size of cortical neurons in one cohort of mice, and overall phenotypic progression and (morbidity-limited) lifespan in a second cohort (Fig. 2.3A). *Mecp2*-null and wild-type littermates were placed on chow containing one of three vitamin D concentrations in a strict rotation based on date of birth: 1 IU/g (standard chow concentration, serving as control), 10 IU/g, or 50 IU/g vitamin D. Chow with 10 IU/g and 50 IU/g are well tolerated and can alter neuronal pathology (Gianforcaro et al., 2013; Gianforcaro and Hamadeh, 2012; Latimer et al., 2014). A pilot study included chow with 200 IU/g vitamin D, which is well below the published toxic range; however, we found that it led to reduced lifespan in *Mecp2*-<sup>y</sup> mice, and thus this dosage was halted. Mice on a diet supplemented with 50 IU/g of vitamin D have more than a 2-fold increase in total 25(OH)D serum concentration compared to the mice on 10 IU/g of vitamin D, regardless of genotype (Fig. 2.3B), indicating that the dietary supplementation is effective at increasing circulating vitamin D in *Mecp2*-<sup>y</sup> mice, beyond that observed in *Mecp2*<sup>+/y</sup> mice under control conditions (Fig. 2.1A).

At 4 weeks of age, *Mecp2*-<sup>y</sup> mice are mildly symptomatic, already demonstrating reduced body weight relative to wild-type littermates, and a small, but significant increase in phenotypic score (data not shown). Dendritic complexity and soma size of layer II/III CPN are not significantly disrupted in *Mecp2*-null mice at 4 weeks of age, but are significantly reduced compared to wild-type by 8 weeks of age (Kishi and Macklis, 2004). *Mecp2*-nulls display an overall rapid
phenotypic progression between 4 and 8 weeks of age, and a median survival between 10 and 11 weeks (Guy et al., 2001); we thus treated the mice with vitamin D during this critical window.

The mice were weighed weekly, and assessed with a phenotypic score (Guy et al., 2007) by an investigator blinded to genotype and chow concentration. Briefly, the mice were assigned a score of 0 (absent), 1 (present) or 2 (severe) for each of the following six phenotypes: abnormal gait, hind limb clasping, irregular breathing, tremor, impaired mobility, and poor general body condition. The score for each symptom was summed to provide an overall phenotype score, with a maximum possible score of 12.

While Vitamin D supplementation does not significantly alter the reduced weight of Mecp2-/y mice, Mecp2-/y mice on 50 IU/g vitamin D demonstrate a small, but significant, reduction in total phenotypic score by 8 weeks of age (Fig. 2.3C). Thus, to investigate whether vitamin D supplementation can, indeed, slow broad phenotypic progression, we analyzed lifespan as an indicator of overall phenotypic progression and health. Following established standards for preclinical trials in Mecp2 mutant mice (Katz et al., 2012), 14-17 Mecp2-null mice and wild-type littermates were maintained on each vitamin D concentration from 4 weeks of age until death. Supplementation with 50 IU/g significantly increased the median lifespan of Mecp2-null mice (83 days, log-rank test P = 0.04), while supplementation with 10 IU/g vitamin D produced a trend to increased median lifespan (from 68.5 days on control chow to 81 days) (Fig. 2.3D). The mean age at death is significantly increased for Mecp2-/y mice on 50 IU/g vitamin D, relative to those on the control chow (Fig. 2.3E). While this ~20% increase in survival is not as extensive as that obtained with genetic attenuation of NF-κB signaling (Kishi et al., 2016), it is similar to results seen with other treatments currently under investigation, such as human recombinant IGF1 (Castro et al., 2014). Taken together, these results provide highly intriguing evidence that
dietary supplementation with vitamin D might provide a partial improvement of some RTT phenotypes.
**Figure 2.** Vitamin D supplementation modestly improves *Mecp2*-null phenotypes and increases their reduced lifespan.

(A) Experimental plan for *in vivo* vitamin D treatment of *Mecp2*-*/y* and *Mecp2*+*/y* littermates.

(B) Supplementing the diet of the mice with vitamin D (VitD) significantly increases their total serum levels of 25(OH)D, regardless of genotype, which is most apparent with 50 IU/g supplemented chow. (C) *Mecp2*-/*/y* on 50 IU/g VitD have a small, but significant, reduction in total phenotypic score at 8 weeks of age compared to *Mecp2*-/*/y* on control 1 IU/g VitD. (D) Kaplan-Meier survival curves. *Mecp2*-/*/y* mice on 50 IU/g VitD chow survive significantly longer than *Mecp2*-/*/y* mice on control chow, while *Mecp2*-/*/y* mice on 10 IU/g VitD display a trend toward increased median lifespan (P = 0.04; log-rank test). The median lifespan of *Mecp2*-/*/y* on 1 IU/g is 68.5 days, 81 days on 10 IU/g and 83 days on 50 IU/g. (E) The mean age of death of *Mecp2*-/*/y* mice on the control chow is significantly lower than the animals on 50 IU/g VitD. B, C, E: one-way ANOVA with Tukey’s Multiple Comparison. * P < 0.05, ** P < 0.01, *** P < 0.001, NS = not significant. B: N = 4 mice per condition. C-E: N = 16 *Mecp2*+*/y* 1 IU, 17 *Mecp2*-/*/y* 1 IU, 15 *Mecp2*-/*/y* 10 IU, 14 *Mecp2*-/*/y* 50 IU. Error bar: ± SEM.
2.4.5. Dietary vitamin D supplementation rescues projection neuron dendritic complexity and soma size phenotypes in MeCP2-/y neocortex

To investigate a specific RTT neuronal phenotype that is recapitulated in MeCP2 mutant mice, we analyzed the complexity and soma size of layer II/III callosal projection neurons (CPN). CPN, the broad population of commissural neurons whose axons connect the two cerebral hemispheres via the corpus callosum (CC), are excitatory pyramidal projection neurons whose cell bodies reside in neocortical layers II/III (~80% in mouse), V (~20%), and a few % in VI (Fame et al., 2011). Layer II/III CPN increasingly express MeCP2 as they mature, and loss of MeCP2 function reduces their dendritic complexity in a largely cell-autonomous manner (Kishi and Macklis, 2010, 2004). Reduced dendritic complexity of neocortical layer II/III CPN has also been observed in post-mortem brains of RTT patients (Armstrong et al., 1995; Armstrong, 2002), with synaptic circuit abnormalities identified in this population in mouse (Wood et al., 2009). In fact, perturbed dendritic complexity of layer II/III CPN is observed in multiple neurodevelopmental disorders, including ASD (Brian Egaas et al., 1995; Herbert and Kenet, 2007; Srivastava et al., 2012), ADHD (Hynd et al., 1991; Roessner et al., 2004; Seidman et al., 2005), and schizophrenia (Swayze et al., 1990; Tibbo et al., 1998). Further, genetic attenuation of the NF-κB pathway improves the reduced complexity of CPN in MeCP2-/y mice (Kishi et al., 2016). We thus focused on this important neuronal population as a window into the broader pathophysiology of RTT.

Supplementing with vitamin D between 4 and 8 weeks of age has no significant effect on dendritic complexity or soma size of CPN in wild-type mice (Fig. 2.4A-D), nor does it affect overall health (measured by total phenotypic score) or weight of wild-type mice (Fig. 2.4E-F). Thus, for clarity and rigor, we compare MeCP2-/y mice on all vitamin D concentrations to wild-
type (Mecp2+/y) mice on 1 IU/g (control) chow in subsequent analyses. Strikingly, we find that supplementation with 50 IU/g vitamin D fully rescues the reduced dendritic complexity of Mecp2-null layer II/III CPN, as measured by Golgi staining and Sholl analysis (Fig. 2.5A-B). This rescue appears to result from both an increase in the number of branch points, relative to Mecp2-/y on control chow (Fig. 5C), and total dendritic length (Fig. 5D).
Figure 2.4 Dietary vitamin D supplementation does not significantly alter neuronal morphology or health in wild-type (Mecp2+/y) mice.

Treatment of Mecp2+/y mice with vitamin D supplemented chow between 4 and 8 weeks of age does not alter (A) soma size (P = 0.67, one-way ANOVA; 1 IU/g n = 76, 10 IU/g n = 103, 50 IU/g n = 84) or (B-D) dendritic complexity of layer II/III pyramidal neurons, as measured by Golgi staining and (B) Sholl analysis, (C) quantification of the number of dendritic branches, or
(D) quantification of total dendritic length (1 IU/g n = 20 neurons, 10 IU/g n = 29, 50 IU/g n = 18). In addition, vitamin D supplementation does not alter the (E) total phenotypic score (P = 0.34, one-way ANOVA) or (F) weight of Mecp2+/y mice (P = 0.66, one-way ANOVA). B, E, F: Two-way ANOVA with Bonferroni post-tests, A, C, D: One-way ANOVA with Tukey posttests. Error bar: ± SEM.
Further evaluation of the data reveals that the total dendritic length reduction in *Mecp2-*y CPN is not due to primary dendrites, but, rather, to reduced secondary and tertiary+ dendrites. Strikingly, these secondary and tertiary dendrites in *Mecp2-*y mice receiving 50 IU/g of vitamin D supplementation are not significantly different from wild-type (Fig. 2.5E). However, this rescue is limited to basal dendrites; the total basal dendritic length of *Mecp2-*y CPN on vitamin D supplementation is not significantly different from wild-type, while the apical dendritic branches continue to show significant reduction in length (Fig. 2.5F). Further, 50 IU/g vitamin D, but not 10 IU/g, rescues the reduced soma size of *Mecp2-*y layer II/III CPN (Fig. 2.5G). It is likely that the morphological abnormalities observed in this neuronal population underlie at least some aspects of the cognitive, behavioral phenotypes observed in RTT, suggesting that amelioration of these phenotypes via vitamin D supplementation might potentially alleviate some RTT symptoms.
**Figure 2.** Vitamin D supplementation rescues reduced cortical dendritic complexity and soma size phenotypes in *Mecp2*-null mice.

(A) Representative traces of layer II/III cortical callosal projection neurons (CPN) following Golgi staining. (B-F) Dendritic complexity of CPN, as measured by (B) Sholl analysis, (C) number of branch points, and (D) total dendritic length, is significantly reduced in *Mecp2-*y mice on both control 1 IU/g and 10 IU/g VitD chow, compared to *Mecp2*+y on control 1 IU/g chow. Dendritic complexity of *Mecp2-*y mice on 50 IU/g VitD, however, is essentially indistinguishable from wild-type (*Mecp2*+/y). (E) *Mecp2-*y mice on both control 1 IU/g and 10 IU/g VitD chow have reduced secondary and tertiary dendrite lengths, which are rescued in *Mecp2-*y mice on 50 IU/g VitD. (F) The length of apical dendrites is also significantly lower in *Mecp2*-nulls on all chows, compared to wild-type mice. However, the length of basal dendrites of *Mecp2-*y on 10IU/g VitD and 50IU/g VitD chow is rescued, and it is not significantly different from *Mecp2*+/y mice. (G) Soma area of layer II/III CPN is significantly reduced in *Mecp2-*y cortex on both control chow and 10 IU/g VitD chow, relative to *Mecp2*+/y on control chow, but is rescued with 50 IU/g VitD. B: two-way ANOVA, Bonferroni post-test. C-F: one-way ANOVA with Tukey’s Multiple Comparison. * P < 0.05, ** P < 0.01, *** P < 0.001, NS = not significant. * Compared to *Mecp2*+/y. # Compared to *Mecp2-*y 1 IU/g VitD. B-F: N: *Mecp2*+/y IU = 21 neurons from 3 brains, *Mecp2-*y 1 IU = 28 neurons from 4 brains, 10 IU = 19 neurons from 3 brains, 50 IU = 35 neurons from 5 brains. G: N = *Mecp2*+/y IU = 228 neurons from 3 brains, *Mecp2-*y 1 IU = 263 neurons from 4 brains, 10 IU = 193 neurons from 3 brains, 50 IU = 204 neurons from 5 brains. Error bar: ± SEM.
2.4.6. Dietary vitamin D supplementation rescues dendritic spine density of Mecp2-/- CPN

In addition to alterations in dendritic complexity and soma area of cortical neurons, RTT patients and Mecp2 mutant mice are known to have reduced dendritic spine density (Armstrong et al., 1995; Belichenko et al., 2009; Fukuda et al., 2005). To investigate whether vitamin D might rescue this phenotype, we analyzed apical dendrites of layer II/III cortical projection neurons in the neocortex of 8-week-old Mecp2-null and wild-type mice on control (1 IU/g) and 50 IU/g vitamin D chow (Fig. 2.6A-D). We focused our analyses on 50 IU/g vitamin D because this concentration rescues both dendritic complexity and soma size of the neurons. The data reveal significant reduction in spine density in the apical dendrites of Mecp2-/- mice on control chow, when compared to wild-type littermates. Vitamin D supplementation, however, fully rescues the decreased dendritic spine density of Mecp2-/- CPN, while not significantly altering the number of dendritic spines in wild-type littermates (Fig. 2.6E). Together, these results indicate that dietary vitamin D supplementation is able to rescue reduced neuronal size and complexity of Mecp2-null neurons but does not modify morphology of wild-type neurons.
Figure 2.6 Vitamin D supplementation rescues reduced dendritic spine density in Mecp2-/-y layer II/III CPN.
(A-D) Representative images of apical dendrites of layer II/III CPN in somatosensory cortex following Golgi staining. Boxes indicate areas displayed at higher magnification in (A’-D’). (E) Spine density is significantly decreased in MeCP2-null neurons compared to wild-type littermates. This decrease is rescued with 50 IU/g vitamin D supplementation. * P < 0.05, one-way ANOVA with Tukey’s Multiple Comparison. N = MeCP2+/y 1IU: 43 dendrites from 3 brains, MeCP2-/y 1IU: 54 dendrites from 3 brains, MeCP2+/y 50IU: 33 dendrites from 3 brains, MeCP2-/y 50IU: 64 dendrites from 4 brains. Scale bar = 200 μm (A-D), 5 μm (A’-D’). Error bar ± SEM.
2.4.7. Female *Mecp2* heterozygous mice also display aberrant NF-κB pathway activation

Although RTT is an X-linked disorder, and human males with a mutation in *MECP2* rarely survive past birth, *Mecp2* loss-of-function is less severe in mice. Male hemizygous null mice not only survive until adulthood, they have been the most commonly studied model system. Heterozygous female mice (*Mecp2*+/−) have not been as thoroughly characterized, likely because of the added experimental challenges that they present, including delayed and more variable phenotypic progression, and cellular mosaicism due to X-inactivation (Guy et al., 2001; Ribeiro and Macdonald, 2020; Samaco et al., 2013; Vogel Ciernia et al., 2017). However, they are a more clinically relevant RTT model, and it has become a consensus opinion that it is imperative to include female *Mecp2*+/− for optimal information in studies of potential therapeutics (Katz et al., 2012).

We first investigated whether female *Mecp2*+/− also display aberrant NF-κB pathway activation. Over-expression of *Irk1*, encoding a signaling kinase and scaffold protein within the NF-κB pathway, is highly prevalent in male *Mecp2*−/y mice, identified in a transcriptome study from CPN (Kishi et al., 2016), as well as in studies from other brain regions and different strains (Gabel et al., 2015). Over-expression of *Irk1* leads to aberrant NF-κB pathway activation and NF-κB pathway attenuation can rescue the reduced dendritic complexity of *Mecp2*-null neurons and extend the usually shortened lifespan of male *Mecp2*-null mice (Kishi et al., 2016). We find that *Irk1* is significantly up-regulated in the cortex of *Mecp2*+/− mice as well (Fig. 2.7A). Additionally, *CamkIIId*, a downstream target of the NF-κB pathway, is up-regulated in the cortex of *Mecp2*+/− mice when compared to their wild-type littermates (Fig. 2.7B), as previously reported in *Mecp2*−/y animals (Kishi et al., 2016). These results support the conclusion that
aberrant NF-κB pathway activation is also prevalent within the female \textit{Mecp2}+/− neocortex and contributes to their neuronal phenotypes.

2.4.8. Vitamin D supplementation partially rescues reduced CPN dendritic complexity in female heterozygous \textit{Mecp2}+/− mice

To investigate whether \textit{Mecp2}+/− mice also display improvement of neuronal morphology phenotypes with vitamin D supplementation, \textit{Mecp2}+/− and wild-type littersmates (\textit{Mecp2}+/+) were placed on custom chow at 4 weeks of age, as outlined for males. Vitamin D serum levels and dendritic complexity were analyzed at 5 months, an age at which cortical dendritic complexity and soma size phenotypes are already apparent (Rietveld et al., 2015) and \textit{Mecp2}+/− mice consistently display motor impairments (Samaco et al., 2013). Unlike \textit{Mecp2}-null mice, \textit{Mecp2}+/− females on control chow do not display significantly reduced levels of 25(OH)D under control conditions. However, 10 IU/g vitamin D dietary supplementation significantly increases 25(OH)D serum levels for both \textit{Mecp2}+/+ and \textit{Mecp2}+/− mice (Fig. 2.7C). Supplementation with both 10 and 50 IU/g vitamin D significantly increases layer II/III CPN dendritic complexity in \textit{Mecp2}+/− cortex, compared to \textit{Mecp2}+/− on control chow, although it does not fully rescue to wild-type complexity (Fig. 2.7D-E). Similar to \textit{Mecp2}-ly males, \textit{Mecp2}+/− females exhibit a reduced number of branch points and total dendritic length compared to their wild-type littersmates. Although vitamin D supplementation does not fully rescue these phenotypes, there is a trend toward increased total dendritic length with vitamin D supplementation, particularly 10 IU/g vitamin D (Fig. 2.7F-G). \textit{Mecp2}+/− mice on 10 IU/g vitamin D demonstrate a significant increase in secondary dendrite length, relative to \textit{Mecp2}+/− on 1 IU/g vitamin D, with \textit{Mecp2}+/− on both 10 and 50 IU/g vitamin D supplemented diets showing primary dendrite length that is
not significantly different from wild-type (Fig. 7H). Intriguingly, MeCP2+/- females on 10 IU/g vitamin D chow show a rescue in apical dendritic length (Fig. 7I). This differs from the MeCP2-null male mice, which demonstrated rescue of the length of their basal dendrites, but not of their apical dendrites. Additionally, supplementation of 10 IU/g vitamin D appears to have the most beneficial effect by also rescuing the reduced soma size of MeCP2+/- layer II/III CPN (Fig. 2.7J). Together, these results demonstrate that vitamin D supplementation in the 10-50 IU/g range ameliorates neuronal size and complexity phenotypes in female heterozygous as well as male hemizygous null mice, and further suggests that there might be sex-specific differences in optimal dose, so the treatment paradigm should be optimized independently for each sex. These results have implications more broadly regarding other potential pharmacologic routes to NF-κB inhibition, perhaps contributing to RTT therapy.
Figure 2.7 Mecp2+/- female cortex has increased Irak1 expression, and displays partial rescue of reduced dendritic complexity and soma size phenotypes with vitamin D supplementation.

(A) Female Mecp2+/- cortex also displays up-regulation of Irak1 expression at 5 months, as previously determined in male Mecp2-/-y cortex at 8 weeks (two-tailed t-test, $P = 0.009$; N:
Mecp2+/+ = 8, Mecp2+- = 7). (B) 5-month-old Mecp2+- mice show increased expression of the NF-κB downstream target CamkIlδ (two-tailed t-test, P = 0.015; N: Mecp2+/+ = 8, Mecp2+/– = 7). (C) Mecp2+- females on control chow (1IU) do not display lower levels of VitD at 5 months of age; however, supplementing the diet of the mice with 10 IU/g VitD from 4 weeks of age significantly increases total serum levels of 25(OH)D, independent of genotype. (D) Representative traces of layer II/III cortical callosal projection neurons. (E) At 5 months of age, Mecp2+- mice on both 10 IU/g and 50 IU/g vitamin D have increased dendritic complexity compared to Mecp2+- on control 1 IU/g chow, as measured by Golgi staining and Sholl analysis, although it is not fully rescued to wild-type (Mecp2+/+) levels. Asterisks denote significant difference for Mecp2+- on 1 IU (blue), 10 IU (red), and 50 IU/g VitD (green) compared to Mecp2+/+ on control chow. (F-G) Mecp2+- on all VitD chows show reduced number of branch points (F) and total dendritic length (G) compared to wild-type, although there is a trend toward increased branch points and dendrite length with VitD supplementation. (H-I) Mecp2+- mice on 10 IU/g VitD demonstrate a significant increase in secondary dendrite length relative to control chow (H), and apical dendritic length that is not significantly different from wild-type (I). (J) Mecp2+- mice on 10 IU/g vitamin D chow also show increased soma area, which is not significantly different from Mecp2+/+ mice on control chow. C: One-way ANOVA with Tukey’s multiple comparisons test. E: Two-way ANOVA with Bonferroni post-test. F-I: One-way ANOVA with Tukey post-test. C: N: Mecp2+/+ 1 IU, Mecp2+- 1 IU and Mecp2+/– 10 IU = 4 animals, Mecp2+/+ 10 IU = 3 animals. E-I: N: Mecp2+/+ 1 IU = 46 neurons from 5 brains, Mecp2+- 1 IU = 68 neurons from 6 brains, 10 IU = 62 neurons from 6 brains, 50 IU = 47 neurons from 5 brains. J: N = Mecp2+/+ 1 IU = 192 neurons from 5 brains, Mecp2+/– 1 IU = 366
neurons from 6 brains, 10 IU = 323 neurons from 6 brains, 50 IU = 234 neurons from 5 brains. * P < 0.05 ** P < 0.01, *** P < 0.001. Error bar: ± SEM.

2.5. Discussion

In this study, we tested the ability of vitamin D – a simple, cost-effective inhibitor of NF-κB signaling – to rescue the aberrant NF-κB pathway activation in MeCP2-mutant neurons, and to improve specific RTT phenotypes. We identified a surprisingly efficacious, dose-dependent amelioration of both layer II/III CPN dendritic complexity and soma size phenotypes, in addition to moderate improvements to overall health and longevity. Our multi-stage experiments show efficacy in both female MeCP2+/- mice that most closely model the human disease, and in male MeCP2-/-y mice, which have been more widely used in earlier analyses due to their rapid progression. Our results have broader relevance for the potential of NF-κB pathway inhibition to contribute to therapeutic approaches for RTT, with a range of increasingly specific, controllable, and potentially targetable inhibitors of this pathway in existence or under development. That said, vitamin D provides more than simply a proof-of-concept, since it is already known to be safe, has no or little toxicity at the dosage ranges in question, and also directly addresses known vitamin D deficiency in RTT patients.

The NF-κB pathway regulates many cellular processes, including immune response, and MeCP2 knockdown has also been found to lead to enhanced NF-κB signaling in myeloid lineage cells (O’Driscoll et al., 2013, 2015). NF-κB subunits are also expressed throughout the CNS, and there is an extensive literature implicating the NF-κB pathway in regulation of neural process development and structural plasticity, in addition to learning and memory (Gutierrez and Davies, 2011). Further, previous results demonstrate that genetic attenuation of this pathway in MeCP2-/-y
mice rescues RTT phenotypes (Kishi et al., 2016), and it has been shown that inhibition of the Gsk3b pathway improves neuronal morphology in Mecp2-null neurons by reducing NF-κB signaling (Jorge-Torres et al., 2018). Together, these results indicate that abnormal activation of NF-κB signaling contributes to the pathogenesis of Mecp2-null mice, and likely RTT. The broad neurological phenotypes of RTT overlap with multiple other neurological disorders, both neurodevelopmental (e.g., ASD, some forms of cerebral palsy and epilepsy) and acquired (e.g. traumatic brain injury), raising interesting questions regarding converging underlying mechanisms and possible involvement of NF-κB signaling, either causal or potentially permissive for enhanced recovery. Thus, NF-κB pathway inhibition might provide a novel therapeutic target not only for the devastating disorder RTT, but also potentially to treat elements of neurological disorders with overlapping pathology.

Previous studies have identified other compounds, such as rhIGF1, ketamine, and Cannabidivarin that appear to also significantly improve behavioral and morphological phenotypes of Mecp2 mutant mice (Castro et al., 2014; Patrizi et al., 2016; Zamberletti et al., 2019). Further, genetic attenuation of the NF-κB pathway (Kishi et al., 2016), is more effective at rescuing Mecp2-null lifespan than our early-symptomatic vitamin D supplementation (Fig. 2.3), suggesting that either earlier onset of NF-κB pathway inhibition and/or other, more specific NF-κB inhibitors might be more efficacious. That said, our results reported here offer a straightforward, readily implementable, and immediately available option: vitamin D, which is cost-effective and of easy access. For this reason, our work strongly motivates that vitamin D supplementation be more thoroughly investigated as a simple, partial therapeutic avenue for RTT, likely in combination with other approaches.
Although the vitamin D deficiency repeatedly observed in RTT patients has been largely attributed to poor nutrition and/or lack of exposure to sunlight, our results that MeCP2-null mice that are maintained in a controlled environment on chow considered to be vitamin D-sufficient also have reduced vitamin D serum levels (Fig. 2.1) suggests an underlying deficiency. One potential mechanism contributing to this vitamin D deficiency could be the disrupted cholesterol homeostasis reported in MeCP2-null mice (Buchovecky et al., 2013), since the primary natural source of vitamin D is dermal synthesis from cholesterol. The findings that heterozygous female mice maintained in the controlled environment do not display reduced vitamin D serum levels might indicate that their roughly 50% mosaic of MeCP2+ cells is sufficient to maintain the synthesis of vitamin D. However, increased vitamin D still partially rescues the neuronal morphology phenotypes. Thus, it is interesting to speculate that MeCP2+/− maintained on a vitamin D deficient diet might likely have more severe phenotypes, perhaps more closely resembling the male MeCP2−/− mice.

In addition to the vitamin D receptor (VDR), which is mainly localized in the nucleus of cells within the brain, protein disulfide isomerase family member 3 (PDIA3) is a known vitamin D receptor localized in the cellular membrane (Eyles et al., 2014; Nemere et al., 2004). PDIA3 is associated with rapid nongenomic response to vitamin D, although both receptors are thought to work in conjunction (Boyan et al., 2012; J. Chen et al., 2013). While the expression of Vdr is very low in the brain compared to kidney and liver of rodents, Pdia3 displays greater abundance in brain than in other organs (Landel et al., 2018). We found no difference in Pdia3 expression in the cortex of either MeCP2 mutant male (P = 0.56) or female mice (P = 0.84), suggesting that they do not have a disruption in their ability to respond to vitamin D.
Our data suggest that vitamin D can act directly on cortical neurons to rescue their reduced dendritic complexity \textit{in vitro}, with complementary work by us and by others indicating primarily direct action with regard to dendritic complexity. \textit{MeCP2} mutant cortical phenotypes result from both cell-autonomous and cell non-autonomous disruptions (Ribeiro and MacDonald, 2020). For example, reciprocal cross-transplantation studies demonstrate that \textit{MeCP2}-/y CPN display reduced dendritic complexity even in the context of a wild-type cortex, but that soma size is dependent on the recipient cortical \textit{MeCP2} genotype (Kishi and Macklis, 2010). Further, in heterozygous females, dendritic complexity of layer V cortical neurons correlates with MeCP2 cell-autonomous expression, while soma size is reduced even in wild-type neurons (Rietveld et al., 2015). In addition, the molecular pathways regulated by MeCP2 are tissue- and cell-type specific (Chao et al., 2010; Noël C. Derecki et al., 2012; Lioy et al., 2011; Samaco et al., 2009), and loss of MeCP2 function in defined CNS circuits results in distinct RTT phenotypes (Adachi et al., 2009; Fyffe et al., 2008; He et al., 2014; Kerr et al., 2008; Nguyen et al., 2012; Wither et al., 2013). NF-κB signaling is prevalent in glia, and the vitamin D receptor (VDR) is expressed by both neurons and astrocytes (Eyles et al., 2005). Thus, vitamin D might act on distinct cellular targets to differentially improve specific RTT phenotypes.

Interestingly, we also observe a sex difference in how \textit{MeCP2} mutant mice respond to our supplementation paradigm, though both sexes display increased circulating 25(OH)D after vitamin D dietary supplementation. Male \textit{MeCP2}-null mice demonstrate CPN morphological rescue when treated with 50 IU/g of vitamin D (Fig. 5, and Fig. 6) while heterozygous females respond better to 10 IU/g of vitamin D supplementation (Fig. 2.7). Furthermore, vitamin D supplementation rescues basal dendrite length in \textit{MeCP2}-/y cortex (Fig. 2.5), and apical dendrite length in \textit{MeCP2}+- females (Fig. 2.7). Different genes selectively control basal or apical
Therefore, it is tempting to speculate that the distinct treatment responses we see in males and females might be a result of different genes responding to Mecp2 mosaic expression in Mecp2+/- mice, and/or to non-cell autonomous effects regulating dendritic branching. Another consideration is the duration of the treatment: while mice of both sexes were placed on custom chow when weaned at P28, Mecp2-null male mice treatment lasted only 4 weeks, due to their shortened lifespan, while heterozygous female mice were on the custom diet for 4 months until reaching a typical symptomatic age.

In summary, we identify that dietary vitamin D supplementation, within a widely acceptable and nontoxic dosage range, rescues aberrant NF-κB pathway activation and partially ameliorates downstream neuropathological effects of NF-κB signaling in Mecp2 mutant mice. These results further solidify the NF-κB pathway as a potential novel therapeutic target for RTT. We demonstrate that vitamin D inhibits this pathway in Mecp2 knockdown neurons in vitro, ameliorates reduced neocortical dendritic morphology and soma size phenotypes in a dose-dependent manner in vivo in both male and female RTT model mice, and modestly improves the reduced lifespan of male Mecp2-null mice. While it is known that neuronal morphological rescue can lead to behavioral improvements of Mecp2-null mice (Bu et al., 2017; Chin et al., 2018), it will be important for future studies to assess both complex mouse behavior and electrophysiological properties of Mecp2-null neurons in mice with vitamin D supplementation, to further investigate the breadth of therapeutic potential of vitamin D supplementation and the specific phenotypes that are or are not improved. Together, our results both provide new insight into the fundamental neurobiology of RTT, and motivate consideration of NF-κB pathway
inhibition, including via vitamin D dietary supplementation, as a potential partial therapeutic intervention for RTT.
Chapter 3: Dietary vitamin D supplementation ameliorates motor and anxiety-like behavior of MeCP2 heterozygous mice and partially normalizes transcriptome alterations
3.1. Abstract

Rett syndrome (RTT) is an X-linked, severe neurodevelopmental disorder caused by mutations in the transcriptional regulator MECP2. Previous research found that in the absence of Mecp2, NF-κB signaling is increased in the CNS of mice. Genetically attenuating the aberrant NF-κB activity ameliorates several hallmark phenotypes of RTT, including neuronal morphology. Among the known inhibitors of NF-κB signaling is vitamin D. Interestingly, vitamin D deficiency is prevalent in RTT patients, and male Mecp2-null mice have significantly lower serum levels of 25(OH)D. Importantly, vitamin D supplementation lowers NF-κB activity and promotes neurite outgrowth of Mecp2-knockdown cortical neurons in vitro. Additionally, we have found that Mecp2-null males and heterozygous females with dietary vitamin D supplementation display increased dendritic complexity and soma area when compared to Mecp2 mutant mice on the control chow at 4 and 20 weeks of age, respectively. In this study, we have investigated whether vitamin D supplementation improves behavioral outcomes of RTT female mice. Our results show improved motor coordination and decreased anxiety-like behavior in Mecp2+/− mice on 10IU/g vitamin D supplementation at 5 and 7 months of age, respectively. Excitingly, RNA-seq analyses on total cortex of a subset of female mice that underwent behavior assays reveal that over 200 differentially expressed genes found in Mecp2+/− mice on 1IU/g vitamin D are no longer significantly different in Mecp2+/− mice on 10IU/g vitamin D, when compared to wild-type littermates on the control or supplemented chows. According to gene ontology analysis, these differentially expressed rescued genes are involved, among other things, in neuronal morphology, which is rescued in our dietary vitamin D supplementation model. Interestingly, restricting dietary vitamin D does not exacerbate behavioral deficits of Mecp2+/− mice; however, vitamin D deficiency leads to extensive transcriptome changes. Importantly,
vitamin D modulation, either through supplementation or restriction, results in alterations in its own metabolism. Thus, our data indicate that vitamin D contributes to RTT pathology and its supplementation could be a simple and cost-effective therapeutic avenue for RTT.

3.2. Introduction

Rett syndrome (RTT) is a severe X-linked neurodevelopmental disorder caused by mutations in the transcriptional regulator MECP2 (Amir et al., 1999). Female RTT patients develop normally during the first 6-18 months of life, after which they go through a period of regression, losing the ability to talk, walk and purposefully use their hands. Additional symptoms include microcephaly, breathing abnormalities and the onset of seizures and autistic behaviors (Chahrour and Zoghbi, 2007). Mouse models have been widely utilized to investigate the role of MeCP2 in the CNS and the progression of RTT phenotypes (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002b; Tudor et al., 2002). Common mouse phenotypes include motor deficits (Samaco et al., 2013; Vogel Ciernia et al., 2018, 2017), impaired cognition (Pelka et al., 2006), social dysfunction (Kerr et al., 2008; Moretti et al., 2005; Pearson et al., 2012; Samaco et al., 2013; Schaevitz et al., 2010), and altered anxiety-like behavior (Samaco et al., 2013; Vogel Ciernia et al., 2017). While RTT patients are usually female, male MeCP2-null mice have been used extensively in RTT research due to more severe and early-onset phenotypes, and rapid phenotypic progression. Although MeCP2-null mice provide valuable information regarding the outcome of complete loss of MeCP2 expression, heterozygous female mice better mimic the human condition and can display distinct phenotypes, likely due to MeCP2 mosaicism (Ribeiro and MacDonald, 2020).
Among the many dysfunctions observed in MeCP2-null mice is aberrant NF-κB signaling (Kishi et al., 2016). MeCP2 loss leads to the overexpression of Irak1, which encodes a kinase within the NF-κB pathway, and subsequent upregulation of NF-κB activity in the cortex of mice. More importantly, it has been shown that the genetic attenuation of the NF-κB pathway, a result of crossing MeCP2 deficient mice with the Nfkb1 line, is sufficient to extend lifespan in male MeCP2-null mice and rescue dendritic complexity of callosal projection neurons (CPN) (Kishi et al., 2016). Vitamin D is a known inhibitor of NF-κB activity (Al-Rasheed et al., 2015; Y. Chen et al., 2013a; D’Ambrosio et al., 1998; Giarratana et al., 2004; Penna et al., 2009; Sun et al., 2006), and its deficiency during CNS development is associated with neurological disorders such as autism spectrum disorder (ASD) (Chen et al., 2016; Fernell et al., 2015; Lee et al., 2019; Vinkhuyzen et al., 2018; Whitehouse et al., 2013) and epilepsy (Camadoo et al., 2007; Teagarden et al., 2014). Vitamin D deficiency is also prevalent in RTT patients (Motil et al., 2011; Sarajlija et al., 2013) and MeCP2-null mice (Ribeiro et al., 2020). Unsurprisingly, vitamin D supplementation prevented the development of ASD phenotypes in mice (Vuillermot et al., 2017), augmented the efficacy of antiepileptic drugs in mice (Borowicz et al., 2015, 2007; Kalueff et al., 2005), and improved behavioral deficits of a vitamin D deficient child with ASD (Jia et al., 2015).

Previously, we have shown that adding the activated form of vitamin D to MeCP2-knockdown cortical neurons reduces NF-κB activation and increases neurite outgrowth in vitro. We have also demonstrated that vitamin D dietary supplementation moderately extends the lifespan of MeCP2-null mice and rescues dendritic complexity and soma area of 8-week-old MeCP2-null and 5-month-old heterozygous female mice (Ribeiro et al., 2020), providing evidence that vitamin D supplementation could be a partial therapeutic option for RTT.
In this study, we investigated whether dietary vitamin D supplementation can ameliorate behavioral outcomes of heterozygous female mice, similar to the morphological benefits already established. We have also sought to understand the molecular mechanism involved in the phenotypic rescue seen with the increased exposure to vitamin D. We determined that vitamin D supplementation improves motor deficits and anxiety-like behavior of RTT female mice, with no effect in sociability. Interestingly, we have found that low 25(OH)D serum concentration only disrupts the behavior of MeCP2 deficient mice, not altering the performance of their wild-type littermates. Additionally, RNA-sequencing analyses indicate that vitamin D supplementation normalizes the expression of many differentially expressed genes associated with neuronal morphology. Thus, these data show further evidence that vitamin D supplementation could offer a simple and cost-effective therapeutic avenue for RTT.

3.3. Materials and Methods

3.3.1. Animals

All animal experimental protocols were approved by the Syracuse University Institutional Animal Care and Use Committee and adhere to NIH guidelines. Mice were group housed at a maximum of five mice per cage on a 12/12 h light/dark cycle and were given food and water ad libitum. Female MeCP2 heterozygous mice were purchased from The Jackson Laboratory (B6.129P2(C)-Mecp2tm1.1Bird/J; RRID:IMSR_JAX:003890), and were maintained on a C57BL/6 background. Genotypes were determined by PCR on genomic DNA as follow: MeCP2 mutant mice, forward primer oIMR1436 5’ - GGT AAA GAC CCA TGT GAC CC - 3’; reverse primer oIMR1437 5’ - TCC ACC TAG CCT GCC TGT AC - 3’; reverse primer oIMR1438 5’ -
GGC TTGCCACATGACAA - 3’. Mice were weighed weekly by an investigator blinded to genotype and chow concentration.

3.3.2. Vitamin D supplementation and serum measurements

Custom chow obtained from Bio-Serv was based on the AIN-93G Rodent Diet, varying only in Vitamin D3 concentration. Female *Mecp2*+/+ and *Mecp2*+- littermates were each weaned together at 4 weeks of age and placed on chow containing 1 IU/g (standard chow), 10 IU/g (supplemented) or 0.1 IU/g (deficient) vitamin D in rotating order based on date of birth.

Serum was collected from *Mecp2*+/+ and *Mecp2*+- females on all chows at 7 months of age, after the completion of the behavioral assays. Total serum 25(OH)D levels were measured via mass spectrometry by ZRT Laboratories (Beaverton, OR).

3.3.3. Behavioral analysis

Behavior was assessed at 3, 5 and 7 months of age. All behavioral tests (described in detail below) were run during the light cycle. The mice were acclimated to the behavior room for at least 30 min prior to testing.

*Accelerating Rotarod.* The rotarod apparatus (Ugo Basile) was set to accelerate from 5 to 50rpm in an interval of 5 min. Each mouse was tested three times per day, with one hour between trials, for three consecutive days. Each trial entailed the placement of the mouse on the stationary apparatus and given 5 sec to acclimate to its movement before it was accelerated. In cases where the mouse fell from the beam before 30 sec had elapsed, the test was reset, and the mouse was
placed on the apparatus; this was repeated for a total of three times. The latency to trial end, which indicates the fall of the mouse to the floor of the rotarod, was recorded for each mouse. The trial was manually ended if the mouse clutched the rotating rod without walking for five consecutive turns (Vogel Ciernia et al., 2017).

*Open field exploration.* Each mouse was placed in the center of a 40 cm x 40 cm open field maze with a photo-beam recording system (SD Instrument) for 30 min. Total distance travelled and time/distance spent in the center of the maze (10 cm x 10 cm, equivalent to ¼ the area of the apparatus) was quantified with San Diego’s PAS Reporter software.

*Elevated plus maze.* Each mouse was placed in the center of a plus-shaped maze (arms: 35 cm x 5 cm; wall: 20 cm in height) elevated 63.5 cm off the ground for a total period of 5 min. Travel distance, time spent in each arm, number of entries in each arm and time spent with the nose over the edge of the open arms were quantified with the Ethovision XT (Noldus) software.

*Social approach.* The social approach apparatus consisted of three chambers (40 cm x 20 cm) connected by sliding doors. Each mouse was placed in the closed center chamber for 5 min. After, the doors were removed, and the mouse was free to explore all three chambers for another 10 min to acclimate to the apparatus. After acclimation, the mouse was returned to the enclosed center chamber and one wire pencil cup was placed in the right chamber and another identical cup was placed in the left chamber. One of the cups contained an age and sex matched mouse (novel mouse) while the other cup remained empty (novel object) (Vogel Ciernia et al., 2017). After the apparatus was set, the doors were open once again and the mouse was free to explore the chambers for 10 min. The Ethovision XT (Noldus) software was used to calculate total distance travelled, time spent in each chamber, number of entries to each chamber, and time sniffing the novel mouse/object.
3.3.4. Quantitative reverse transcription PCR (RT-qPCR)

RNA was extracted using TRIzol (Invitrogen), and cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences). RT-qPCR was performed on a CFX Connect Real-Time System (Bio-Rad Laboratories) according to the manufacturer’s instructions. Primer pairs were designed in intron-spanning regions or exon-exon junctions as to not amplify genomic DNA. The primer pairs were as follow:

*CYP2RI*: forward 5’ - TGTATGGCGAGATTTTCAGTTTAG - 3’; reverse 5’ - CAAAGGAAGGCATGGTCTATCT - 3’.


*CYP27B1*: forward 5’ - AGTGGTAGATTGTACCCTGTG - 3’; reverse 5’ - TAGGGAGACTAGCTATCTTGG - 3’.


We used PerfeCTa SYBR Green FastMix (Quanta Biosciences) Master mix, and each PCR consisted of 1X LightCycler FastStart DNA Master SYBR Green I mixture, 0.2 μM primers, and cDNA. We used the mean of Gapdh and S16 expressions as the reference gene. Each sample was run in triplicate and averaged. The relative quantification analysis was performed as follow: ΔCq = Cq of gene of interest - geometric mean of Cq of reference genes; ΔΔCq = ΔCq - mean of ΔCq of wild-type samples; fold change = 2-ΔΔCq. Melt curve analysis was also performed to verify specificity of the amplicons.
3.3.5. RNA-sequencing

Total RNA was extracted from the cortices of 7-month-old mice using TRIzol reagent (Invitrogen) and RNaseasy Mini Kit (Qiagen) (Untergasser, 2008). The samples consisted of 3 Mecp2+/+ and 3 Mecp2+-/- on 1IU/g, 10IU and 0.1IU/g vitamin D. Libraries were made using the kit QuantSeq 3’ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen) according to the manufacturer’s instructions. The libraries were sequenced in an Illumina NextSeq 500 next-generation sequencing instrument using the NextSeq 500/550 High Output kit v2.5 (75 cycles) (Illumina) to generate approximately 22M reads per sample.

**Analysis.** RNA-sequencing analysis was performed with the PartekFlow software. In brief, adapters and bases were trimmed, followed by alignment with STAR 2.7.3a index (mus musculus mm10 assembly, whole genome aligner index) and quantification to model (Gencode M25). Normalization to remove unwanted variation (RUV) was performed with R (Risso et al., 2014). Gene specific analysis (GSA) and heatmaps were done on PartekFlow. Genes were deemed differentially expressed based on their p-value (<0.05) and false discovery rate (FDR<0.1). Volcano plots were made in GraphPad Prism. Upset plots were created with the web-version of UpsetR (Lex et al., 2014), while gene ontology (GO) analysis and transcription factors enrichment were performed on g:Profiler (Raudvere et al., 2019).

3.3.6 Statistical analysis

GraphPad Prism 8.0 (GraphPad Software) was used to carry out the statistical analyses. No statistical method was used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. Our statistical tests consisted of two-tailed t test, two-way ANOVA with Tukey’s multiple comparison, or Pearson correlation of multivariate analysis to
determine statistical significance among groups. Data distribution was handled as if normal, but this was not formally tested (since potential differences in results would be minor). All data shown represent mean ±SEM. Sample size and statistical test are specified in each figure legend.

3.4. Results

3.4.1. Vitamin D dietary supplementation ameliorates disrupted motor coordination of Mecp2+/− mice

To investigate whether vitamin D dietary supplementation has a beneficial impact on the behavior of Mecp2+/− mice, in addition to neuronal morphology, we placed the animals on control (1 IU/g vitamin D) or supplemented chow (10 IU/g vitamin D) starting at 4 weeks of age, when the mice were weaned (Fig. 3.1A). We chose 10 IU/g vitamin D chow as it was the dose that improved neuronal morphology phenotypes of Mecp2+/− mice most effectively (Ribeiro et al., 2020). We performed a longitudinal experiment to evaluate the effect of vitamin D on the phenotypic progression and longitudinal decline displayed by the Mecp2+/− mice, assessing their behavior at early symptomatic (3 months), symptomatic (5 months), and late symptomatic (7 months) stages. At each age, we assessed motor coordination (rotarod), motor activity (open field), anxiety-like behavior (open field and elevated plus maze), and sociability (three chamber social approach).

RTT murine models display motor deficit that are comparable to symptoms observed in RTT patients (Kondo et al., 2008; Santos et al., 2007; Stearns et al., 2007; Vogel Ciernia et al., 2017). To assess motor behavior, we used the accelerating rotarod assay, which correlates deficits in
motor coordination with lower latency to trial end (Deacon, 2013). We find that at 3 months of age, \textit{Mecp2}+/- mice on both control 1 IU/g vitamin D and supplemented 10 IU/g vitamin D chow display lower latency to trial end than their \textit{Mecp2}++/+ littermates, indicating impaired motor coordination (Fig. 3.1B). At 5 months of age, however, the \textit{Mecp2}+/- females on 10 IU/g vitamin D chow display a significant improvement in motor coordination when compared to \textit{Mecp2}+/- mice on 1 IU/g vitamin D. At 7 months of age, both groups of \textit{Mecp2}+/- mice exhibit lower latency to trial end (Fig. 3.1B), indicating that dietary vitamin D supplementation delays the decline in motor coordination but does not fully prevent it. Vitamin D supplementation does not significantly alter the distance travelled or the average speed of locomotion in the open field test at any of the ages examined (Fig. 3.1C), however. Therefore, vitamin D supplemented chow partially ameliorates or delays motor behavior phenotypes of \textit{Mecp2} deficient female mice.
Figure 3.1 Vitamin D supplementation mitigates motor coordination deficits of Mecp2+/- mice. (A) Experimental plan for in vivo vitamin D treatment and behavior testing of Mecp2+/- and Mecp2+/- littermates. (B) In the rotarod test, Mecp2+/- mice on 1 and 10IU/g vitamin D have a
shorter latency to trial end than *Mecp2*+/+ animals at 3 and 7 months of age. However, at 5 months the latency to trial end of *Mecp2*+/- mice on 10IU/g vitamin D diet is not significantly shorter than *Mecp2*+/+ mice on 1IU/g vitamin D and it is significantly higher than *Mecp2*+/- on 1IU/g vitamin D. (C) No differences are seen in the total distanced travelled or average speed of mice in the open field test at 3 and 5 months of age. At 7 months of age, both *Mecp2*+/- animals on 1 and 10IU/g vitamin D travelled less and had a lower average speed than *Mecp2*+/+ on 1IU/g vitamin D. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. NS = not significant. Two-way ANOVA with Tukey’s post hoc analysis. N = 12 – 17 per condition, genotype, and age group, indicated by dots. Error bar: ±SEM.
3.4.2. Vitamin D dietary supplementation improves anxiety-like behavior in the open field test

Next, we measured anxiety-related behavior, a phenotype that has been previously reported in RTT patients (Barnes et al., 2015) and mouse models (Adachi et al., 2009; Meng et al., 2016; Philippe et al., 2018). For this analysis, we used the open field test, which assesses anxiety-like behavior by analyzing how much time the animal spends in the center of the arena compared to its periphery. Mice that spend more time in the center of the maze display lower anxiety-like behavior (Lezak et al., 2017). We find that in early and mid-symptomatic stages, vitamin D supplementation does not improve anxiety-like behavior of Mecp2+/− mice (Fig. 3.2A). At 3 months of age, Mecp2+/− female mice on 1 IU/g vitamin D and 10 IU/g vitamin D chow spend less time in the center of the open field arena than Mecp2+/+ mice on 1 IU/g vitamin D chow.

At 5 months of age, no group displays increased anxiety-like behavior. However, at a late symptomatic stage, Mecp2+/− mice on 1 IU/g vitamin D chow spend less time in the center of the arena than their Mecp2+/+ littermates whereas Mecp2+/− treated with 10 IU/g vitamin D diet do not display increased anxiety-like behavior when compared to Mecp2+/+ mice (Fig. 3.2A).

We also employed the elevated plus maze test to assess anxiety-like behavior. Animals that spend an increased amount of time in the closed arm of the elevated maze relative to the open arm are thought to have higher levels of anxiety-related behavior (Lezak et al., 2017). Yet, RTT model mice act differently in this paradigm; studies have found that Mecp2 deficient mice spend more time in the open arm of the maze, indicating less anxiety-like behavior (Meng et al., 2016; Ribeiro and Macdonald, 2020; Samaco et al., 2013; Ure et al., 2016; Vogel Ciernia et al., 2017).

Recently, a study found that clipping the whiskers of Mecp2 mutant animals eliminates elevated plus maze deficits, suggesting that this test might assess sensory hypersensitivity of RTT mice.
and not anxiety-related behavior (Flores Gutiérrez et al., 2020). Our data show that, at 3 months of age, *Mecp2+/-* mice on control and supplemented chow spend more time in the open arm of the elevated plus maze than *Mecp2+/+* mice, indicating decreased anxiety-like behavior (or increased sensory hypersensitivity) in this paradigm. At 5 months of age, *Mecp2+/-* females on 10 IU/g vitamin D diet spend more time in the open arm of the maze when compared to the *Mecp2+/+* mice. However, at 7 months of age, no statistically significant difference was seen between groups (Fig. 3.2B). Thus, these data indicate that vitamin D supplementation might modestly improve anxiety-like behavior of late symptomatic *Mecp2+/-* mice.
Figure 3. 2 Mecp2+/- mice on the supplemented diet do not display anxiety-like behavior at 7 months of age.

(A) At 3 months of age, Mecp2+/- on 1IU/g, Mecp2+/- and Mecp2+/+ mice on 10IU/g vitamin D have increased anxiety-like behavior when compared to Mecp2+/+ animals on 1IU/g. At 5 months of age, no differences are seen among any of the groups. However, at 7 months of age, only the Mecp2+/- mice on 1IU/g vitamin D have increased anxiety-like behavior when compared to Mecp2+/+ on 1IU/g vitamin D. (B) At 3 months of age, both Mecp2+/- mice on 1 and 10IU/g vitamin D spend more time in the open arm of the elevated plus maze when compared to Mecp2+/+ mice, indicating lower anxiety-like behavior. At 5 months, however, only Mecp2+/- mice on 10IU/g vitamin D spend more time in the open arm of the maze when
compared to \textit{Mecp2+/-} mice. At 7 months of age, no differences are seen among groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$. NS = not significant. Two-way ANOVA with Tukey’s post hoc analysis. N = 12 – 17 per condition, genotype, and age group, indicated by dots. Error bar: ±SEM.

3.4.3. Sociability is not altered in \textit{Mecp2+/-} mice

Alterations in social interaction of \textit{Mecp2} mutant mice is dependent on strain and background, which do not always reveal a deficit in sociability (Ribeiro and Macdonald, 2020; Vogel Ciernia et al., 2017). To examine this behavior in our mice, we used the three-chambered social approach test. This assay consists of allowing the mouse to investigate a novel object or a novel age- and sex- matched mouse. Mice are considered to have impaired social behavior when they prefer to spend more time with the novel object than the novel mouse (Yang et al., 2011). Both \textit{Mecp2+/-} female mice on control and supplemented chow did not exhibit altered sociability when compared to \textit{Mecp2+//} mice (Supplemental Fig. 3.1). Therefore, these data demonstrate that increased dietary vitamin D does not alter social behavior of \textit{Mecp2}-mutant mice.

3.4.4. Sufficient 25(OH)D serum levels correlates with improved motor activity in \textit{Mecp2+/-} mice

We analyzed vitamin D serum concentrations at the end of the experiment (7 months) to determine if the dietary supplementation resulted in higher circulating 25(OH)D serum levels. We measured serum 25(OH)D because it better correlates with 1,25(OH)D brain levels than
serum 1,25(OH)D (Spach and Hayes, 2005). We found that the 10 IU/g vitamin D supplementation does not significantly alter 25(OH)D serum levels of Mecp2+-/- or Mecp2+-/+ mice overall; however, we observed high variability in 25(OH)D levels within groups (Fig. 3.3A). To investigate whether circulating levels of vitamin D correlate with behavioral outcome, we analyzed our data based on the 25(OH)D serum concentration, regardless of the chow they were treated with. We considered insufficient levels to be below 29 ng/ml and sufficient levels to be above 29 ng/ml (Holick et al., 2011; Mallya et al., 2016).

By grouping the data in this manner, we observe that Mecp2+-/- mice with insufficient levels of serum 25(OH)D travel significantly less in the open field arena than Mecp2+-/- mice with sufficient levels, and Mecp2+-/+ mice in both groups (Fig. 3.3B). Importantly, Mecp2+-/+ mice with insufficient 25(OH)D serum concentration are not impacted and display similar exploratory behavior to Mecp2+-/+ mice with sufficient levels. Moreover, Mecp2+-/- female mice with sufficient 25(OH)D serum concentration do not display increased anxiety-like behavior in the open field test when compared to Mecp2+-/+ mice, while Mecp2+-/- mice with insufficient 25(OH)D serum levels exhibit increased anxiety-like behavior (Fig. 3.3B). However, lower levels of serum 25(OH)D do not significantly impact motor coordination (Fig. 3.3C), anxiety-like behavior in the elevated plus maze (Fig. 3.3D) or social approach (Supplemental Fig. 3.1B). Thus, our results suggest that low levels of serum 25(OH)D in Mecp2+-/- mice could contribute to exploratory motor and anxiety-like behavioral deficits.
Figure 3. Sufficient serum 25(OH)D rescues motor activity of Mecp2+/- mice.

(A) Total serum 25(OH)D is not increased with vitamin D supplementation at 7 months of age. N = 11 – 17 per condition and genotype, indicated by dots. (B) For this analysis, animals were grouped based on serum 25(OH)D concentration, and not treatment. Sufficient 25(OH)D vitamin D rescues total distance travelled in the open field assay and ameliorates anxiety-like behavior of Mecp2+/- mice. Mecp2+/+ mice with insufficient 25(OH)D serum concentration behave similarly to Mecp2+/+ animals with sufficient levels. (C) Mecp2+/- mice with insufficient and sufficient serum 25(OH)D display shorter latency to trial end when compared to Mecp2+/+ mice. No differences are seen between Mecp2+/+ in both groups. (D) No differences among groups are seen in the elevated plus maze test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. NS =
not significant. Two-way ANOVA with Tukey’s post hoc analysis. B, C: N = 7 – 24 per condition and genotype, indicated by dots. Error bar: ±SEM.

3.4.5. *Mecp2*+/- mice increased weight correlates with poor behavior performance

One common phenotype seen with *Mecp2*+/- mice is increased body weight as they age (Gigli et al., 2016; Samaco et al., 2013). To investigate if 25(OH)D serum concentration has an effect on body weight or whether the weight of the animals impact their behavioral performance, we ran a multivariate analysis with Pearson correlation. Our data show that for *Mecp2*+/* mice on 10 IU/g vitamin D custom chow, there is no correlation between weight and behavioral performance, while a negative correlation was found for the weight of *Mecp2*+/+ mice on 1 IU/g vitamin D and the amount of time spent in the open arm of the elevated plus maze only (Supplemental Fig. 3.2).

However, for *Mecp2*+/- female mice on 1 IU/g vitamin D, there is a negative correlation between weight, rotarod, and open field performance, with heavier mice spending less time in the center of the open field arena and on the accelerating rotarod. A negative correlation was also observed between 25(OH)D serum levels and the amount of time those mice spent sniffing the novel mouse in the social approach assay. As for the *Mecp2*+/- female mice on 10 IU/g vitamin D, only the latency to trial end in the rotarod assay showed a negative correlation with the weight of the animals. Interestingly, a significant positive correlation was seen between *Mecp2* expression level and distanced travelled in the open field test and latency to trial end in the accelerating rotarod of those mice. However, no correlation was found between *Mecp2* expression and weight of the animals for any of the conditions and genotypes (Supplemental Fig. 3.2). These data indicate that body weight, 25(OH)D serum concentration, and *Mecp2* expression
can impact behavioral performance in specific contexts, particularly affecting *Mecp2*+/- females. More importantly, the combinatorial effect of higher body weight, insufficient 25(OH)D serum levels and lower *Mecp2* expression contribute to a negative outcome.

### 3.4.6. Vitamin D supplementation normalizes the expression of dysregulated genes in *Mecp2*+/- cortex that are associated with neuronal morphology

We next performed bulk RNA-sequencing on a subset of cortical samples collected from *Mecp2*+/+ and *Mecp2*+/- mice on 1IU/g and 10IU/g vitamin D chow at 7 months of age, post-behavior testing. The purpose of the experiment was to investigate whether differentially expressed genes (DEG) in the cortex of *Mecp2*+/- mice on the control chow are rescued in the *Mecp2*+/- mice treated with VitD supplemented diet. We observe 989 DEG (p < 0.05, FDR < 0.1) in the cortex of *Mecp2*+/- animals on the 1 IU/g diet when compared to their wild-type littermates. Of those DEG, 420 genes are downregulated, and 569 genes are upregulated (Fig. 3.4A). In contrast, of the 5,233 DEG between *Mecp2*+/- and *Mecp2*+/+ treated with the supplemented chow, the majority (3,187) are downregulated and not upregulated (2,046) (Fig. 3.4B). *Mecp2* expression is reduced in *Mecp2*+/- cortices, independent of treatment group. Conversely, the expression of *Irak1*, the kinase upstream of the NF-κB pathway, is increased in the cortex of *Mecp2*+/- mice on 1 IU/g and 10 IU/g vitamin D (Fig. 3.4A-B). Further, over 3,000 DEG are found between *Mecp2*+/+ mice on 10IU/g vitamin D chow and *Mecp2*+/+ on the control diet, indicating that vitamin D supplementation can lead to gene expression changes in the cortex, independent of *Mecp2* loss, and those alterations mainly lead to the upregulation of genes (2,680 versus 1,177).
Importantly, we hypothesized that genes that had their expression normalized with vitamin D supplementation underpin the morphological (Ribeiro et al., 2020) and behavioral rescue observed in Mecp2+/- mice. Thus, we considered to be normalized the genes that are differentially expressed in the cortex of Mecp2+/- females on 1 IU/g vitamin D chow when compared to their wild-type littermates, and that are no longer differentially expressed between Mecp2+/- treated with the supplemented diet and Mecp2+/+ on the control diet; 283 genes fit those requirements (Fig. 3.4C). The majority of the 283 genes are downregulated in the Mecp2+/- mice on 1IU/g vitamin D chow when compared to the three groups (Fig. 3.4D). GO analysis of cellular components demonstrate that the rescued DEG are involved in neuronal structure (Fig. 4E). Deficits in neuronal morphology is one of the hallmark phenotypes associated with RTT model mice (Belichenko et al., 2009; Fukuda et al., 2005; Kishi and Macklis, 2004; Rietveld et al., 2015; Stuss et al., 2012; Tropea et al., 2009) and human patients (Armstrong et al., 1995, 1999; Armstrong, 2002; Jellinger et al., 1988). Notably, we have previously published data demonstrating that vitamin D supplementation rescues neurite outgrowth of cortical neurons in vitro and dendritic complexity and soma area in male and female mice in vivo (Ribeiro et al., 2020). Thus, the rescued DEG in this study are likely involved in the phenotypic amelioration of cortical neuronal morphology of Mecp2-mutant mice after vitamin D supplementation.

Importantly, of the 283 rescue DEG, 74 contain the VDR transcription factor binding motif (GGGKNARNRRGWSA), and have the potential to be directly regulated by vitamin D/VDR. Moreover, 165 of the rescued DEGs have been previously found to be altered in RTT (Gabel et al., 2015), with 8 of those genes also appearing in the SFARI database, a curated catalogue of genes associated with ASD (Abrahams et al., 2013). Excitingly, we have identified 118 genes
that have not been previously reported in the compilation of altered RTT genes, with 5 of those being found in the SFARI database (Fig. 3.4F). Therefore, vitamin D supplementation normalizes the expression of many genes that are relevant to not only RTT pathology, but also to other conditions that share its biological basis, and vitamin D should be further evaluated for its potential broad therapeutic benefits.
Figure 3.4 Vitamin D supplementation normalizes differentially expressed genes of Mecp2+/− mouse cortex.

Volcano plots showing the change in gene expression between Mecp2+/+ and Mecp2+/− mice on 1IU/g vitamin D (A) and 10IU/g vitamin D diets (B). Gray dotted line indicates FDR = 0.1. Red indicates upregulated genes and blue indicates downregulated genes. (C) Upset plot illustrating the criteria for selecting differentially expressed gene that are normalized. Purple indicates the groups that were selected. (D) Heat map showing the pattern of expression of the Mecp2+/− mice differentially expressed genes that were normalized with vitamin D supplementation. (E) GO of cellular components analysis of the normalized genes, indicating that they are involved in neuronal morphology. (F) Venn diagram illustrating the intersection between the normalized genes (DE rescue genes), genes involved in the RTT pathology (Gabel et al, 2015) and genes associated with ASD (SFARI database).
3.4.7. Vitamin D deficient diet does not exacerbate behavioral phenotypes

Having demonstrated that vitamin D improves aspects of behavioral performance and rescues DEG in the cortex of Mecp2+/− mice treated with the supplemented diet, we next hypothesized that treating Mecp2+/− mice with a diet deficient in vitamin D (0.1IU/g) would exacerbate their behavioral phenotypes and lead to further disruptions in their cortical transcriptome. To test this hypothesis, we followed the same experimental design used in the supplementation study, placing the Mecp2+/+ and Mecp2+/− mice on 0.1IU/g vitamin D at weaning (1 month of age), and performing the behavioral tests at 3, 5, and 7 months of age.

Data from the accelerating rotarod test show that at all time points, Mecp2+/− females on 1IU/g vitamin D and 0.1IU/g vitamin D diets have a shorter latency to trial end when compared with their Mecp2+/+ littermates; although at 3 months of age Mecp2+/− on 0.1IU/g vitamin D does not display statistically significant motor coordination deficit when compared to Mecp2+/+ on 1IU/g vitamin D (Fig. 3.5A). No significant difference is seen in the elevated plus maze test at 3, 5 (Supplemental Fig. 3.3B) and 7 months of age for all groups (Fig. 3.5B). We observe a similar result in the social approach test (Supplemental Fig. 3.3C).

In the open field paradigm, we observe no difference among groups in the distance travelled at 3 and 5 months of age (Supplemental Fig. 3.3A); however, at 7 months of age, there is a significant reduction in exploratory motor behavior and average speed of Mecp2+/− on 1IU/g and 0.1IU/g vitamin D (Fig. 3.5C). Moreover, at 3 (Supplemental Fig. 3.3A) and at 7 months of age (Fig. 3.5C), Mecp2+/− mice display increased anxiety-like behavior, regardless of their treatment group, when compared to Mecp2+/+ mice on 1IU/g vitamin D chow. At 5 months of age, we see no change in anxiety-related behavior in either treatment groups (Supplemental Fig. 3.3A). Thus, our data show that exposure to a vitamin D deficient diet does not lead to an exacerbation of
Mecp2+/− behavioral outcomes, since the performance of Mecp2+/− mice on the control or deficient chow are not statistically different from each other, while being significantly worse than Mecp2+/+ on the control diet.
Figure 3. 5 Mecp2+- mice treated with vitamin D deficient and control chow display similar behavioral deficits.

(A) In the rotarod test, Mecp2+- mice on 1 and 0.1IU/g vitamin D chow have lower latency to trial end when compared to Mecp2+/+ on 1IU/g vitamin D. At 5 and 7 months of age, Mecp2+- mice on 1 and 10IU/g vitamin D have a shorter latency to trial end than Mecp2+/+ animals. (B) At 7 months of age, no differences are observed between animals treated with 1IU/g or 0.1IU/g vitamin D chow in the elevated plus maze assay. (C) In the open filed test, both Mecp2+- mice on 1IU/g and 0.1IU/g vitamin D demonstrate impaired motor activity and increased anxiety-like behavior at 7 months of age. *p < 0.05, **p < 0.01, ***p < 0.001. Two-way ANOVA with Tukey’s post hoc analysis. N = 11 – 15 per condition and genotype, indicated by dots. Error bar: ±SEM.
3.4.8. Mecp2 expression is positively correlated with 25(OH)D serum levels

As with the animals on the control and supplemented diet, we performed a multivariate analysis with Pearson correlation to investigate whether low 25(OH)D serum levels correlate with behavioral outcomes of mice treated with 0.1 IU/g vitamin D chow. Our data demonstrate that no major correlation is observed between behavioral performance of Mecp2+/+ mice on 0.1IU/g vitamin D and their circulating 25(OH)D, with only a small positive correlation found between 25(OH)D serum concentration and time spent with the novel mouse in the social approach test (Supplemental Fig. 3.4A).

However, for the Mecp2+-/- mice exposed to deficient chow, we found a negative correlation between body weight and total distance travel and rotarod performance. In contrast, Mecp2 expression and latency to trial end share a significant positive correlation, indicating better rotarod performance in animals that express higher levels of Mecp2. Moreover, there is a positive correlation between 25(OH)D serum levels and the amount of time Mecp2+-/- animals spend in the center of the open field arena, demonstrating that low levels of circulating vitamin D could result in increased anxiety-like behavior. Interestingly, 25(OH)D serum concentration also showed a positive correlation with Mecp2 expression (Supplemental Fig. 3.4B) in those animals, suggesting that perhaps higher gene expression, likely due to skewed X-chromosome inactivation, could have a favorable effect in the metabolism of vitamin D.

3.4.9. Vitamin D deficiency leads to extensive transcriptome changes

To investigate whether vitamin D deficiency further exacerbates the transcriptional dysregulation found in the Mecp2+-/- cortex, and/or leads to unique disruptions, we performed RNA-seq on the total cortex of Mecp2+-/- and Mecp2+/+ mice on the 0.1 IU/g chow. These samples were
processed in parallel with the 1 IU/g and 10 IU/g and analyzed in comparison to the same 1 IU/g controls as the 10 IU/g samples. Our data identify 9,671 DEG between Mecp2+/- and Mecp2+/+ on the 0.1IU/g vitamin D diet, with 4,845 being upregulated and 4,826 being downregulated (Fig. 3.6A). The change in gene expression between Mecp2+/- mice and their wild-type littermates is the highest on the deficient diet (compared to 1IU/g and 10IU/g vitamin D).

Further, Mecp2+/+ animals on the vitamin D deficient diet display 10,500 DEG compared to Mecp2+/+ on control diet. Among those, 4,378 are upregulated and 6,122 are downregulated. These data highlight the remarkable impact of vitamin D deficiency on gene expression within the cortex. Interestingly, by following the same requisites we used to select rescue genes resulting from vitamin D supplementation, we find that 192 genes are differentially expressed between Mecp2+/- and Mecp2+/+ on 1IU/g vitamin D and between Mecp2+/- on 0.1IU/g vitamin D and Mecp2+/- 1IU/g vitamin D chow (Fig. 3.6B). However, when analyzed with g:Profiler, none of those genes are enriched for GO cellular components terms. In addition, none of those 0.1IU/g vitamin D genes are enriched for the VDR motif, which is indicative of secondary effects and not direct vitamin D regulation.

Further, when comparing the genes that were rescued with vitamin D supplementation and the genes rescued with its deficiency, we find an overlap of 54 genes (Fig. 3.6C), indicating that the expression of these genes can be normalized with vitamin D modulation, regardless of whether it is supplementation or restriction. Additionally, of those 54 genes, 26 are also known to be associated with RTT (Gabel et al., 2015) and 3 are found in the SFARI database (Fig. 3.6D).
Figure 3.6 Vitamin D deficiency leads to extensive transcriptome changes in *Mecp2*+/- mice. (A) Volcano plot showing the change in gene expression between *Mecp2*+/+ and *Mecp2*+/- mice on 0.1IU/g vitamin D. Gray dotted line indicates FDR = 0.1. Red indicates upregulated genes and blue indicates downregulated genes. (B) Upset plot illustrating the selection criteria for a differentially expressed gene to be considered normalized. Purple indicates the groups that were selected. (C) Heat map showing the pattern of expression of the genes that were normalized with vitamin D supplementation. Most of the genes are still differentially expressed after vitamin D
deficiency. (D) Venn diagram illustrating the intersection between the normalized genes with vitamin D supplementation and with vitamin D deficiency (top). Venn diagram of the 54 normalized genes in common between vitamin D supplementation and deficiency (DE rescue genes), and their intersection with genes involved in the RTT pathology (Gabel et al, 2015) and genes associated with ASD (SFARI database) (Bottom).

3.4.10. Vitamin D homeostasis is disrupted in multiple tissues of Mecp2+/− mice

In view of the moderate effect that vitamin D supplementation and deficiency exerted on Mecp2+/− mice, we investigated the hypothesis that vitamin D homeostasis might be altered with the loss of Mecp2, leading to the activation of compensatory mechanisms. Vitamin D metabolism is complex and involves multiple genes and tissues. Vitamin D3 and D2 are processed into calcidiol in the liver by the cytochrome oxidases CYP27A1 and CYP2R1. Calcidiol is then transported to the kidney and processed into calcitriol, the activated form of vitamin D, by the enzyme CYP27B1 (Zhu et al., 2013). Finally, the enzyme CYP24A1 is responsible for the catabolism of calcitriol (Fig. 3.7A) (Jeon and Shin, 2018; Jones et al., 2014; Schuster, 2011). There is a feedback mechanism in place that adjusts the levels of these enzymes in response to vitamin D, maintaining homeostasis. For example, higher levels of serum 25(OH)D corresponds with lower levels of CYP27B1 and upregulation of CYP24A1 (Fleet et al., 2008). The cytochrome oxidase enzymes are also expressed in the brain (Gezen-Ak et al., 2013; Landel et al., 2018) and both forms of vitamin D, calcidiol and calcitriol, are able to cross the blood brain barrier (Pardridge et al., 1985). Importantly, it has been shown previously that calcidiol concentration in the serum better correlates with brain calcitriol, suggesting that the brain synthesizes vitamin D in situ (Spach and Hayes, 2005).
To investigate if vitamin D homeostasis is disrupted in MeCP2+/− mice, we analyzed the expression of the genes involved in vitamin D metabolism. In the liver, there is no alteration in the expression of CYP2R1, however, MeCP2+/− females display reduced levels of CYP27A1 expression, regardless of treatment group (Fig. 3.7B). Further, only the MeCP2+/− females on the supplemented diet display elevated levels of CYP27B1, and a trend towards lower CYP24A1 expression in the kidney (Fig. 3.7C). Importantly, no change in gene expression was observed in the MeCP2+/+ mice treated with 10IU/g vitamin D chow, indicating that MeCP2 loss could contribute to vitamin D homeostasis disruption, which is not normalized even with vitamin D supplementation.

Interestingly, when analyzing cortical expression of the genes that were altered in the liver and kidney, we observe that the MeCP2+/− female mice on the control diet do not display altered expression of CYP27A1. The same is true for the MeCP2+/− females on the supplemented diet, which do not show dysregulated cortical CYP27A1, CYP27B1, and CYP24A1 (Fig. 3.7D), indicating that the alterations in vitamin D homeostasis might be specific to peripheral tissues.
Figure 3.7 Vitamin supplementation leads to vitamin D homeostasis alterations in Mecp2+- mice. (A) Illustration of vitamin D biosynthesis process. In the liver, the enzymes CYP27A1 and CYP2R1 process vitamin D into 25(OH)D, which is then transported to the kidneys. The enzyme CYP27B1 hydrolyzes 25(OH)D into 1,25(OH)2, which is transported to various organs or broken down by the enzyme CYP24A1. The brain expresses the enzymes involved in the vitamin D metabolism, indicating synthesis in situ. (B) CYP2R1 expression is unaltered with vitamin D supplementation. Mecp2+- on 1 or 10IU/g vitamin D show reduced expression of hepatic CYP27A1. (C) The expression of renal CYP27B1 is elevated in Mecp2+- on 10IU/g vitamin D,
while CYP24A1 expression has a trend towards downregulation. (D) No alteration is observed in cortical gene expression. *p < 0.05, **p < 0.01, ****p < 0.0001. B, C: Two-way ANOVA with Tukey’s post hoc analysis. D: Two-tailed t test. N = 3 – 7 per condition and genotype, indicated by dots. Error bar: ±SEM.
3.4.11. Vitamin D deficient diet leads to homeostasis disruption as well

Unlike the supplemented study, treating mice with vitamin D deficient diet alters their 25(OH)D serum concentration, which is significantly lower in those animals than in the mice exposed to the control chow (Fig. 3.8A). This result is consistent with previous studies (Belenchia et al., 2017; Comer et al., 1993; Fleet et al., 2008; Kasatkina et al., 2020; Rowling et al., 2007), and it is likely due to a reduction in the availability of 25(OH)D precursor. However, most of the alterations in the expression of the genes involved in the metabolism of vitamin D occur exclusively in the Mecp2+/− mice. In the liver, the CYP2R1 remains unaltered in animals of both genotypes treated with 1IU/g vitamin D or 0.1IU/g vitamin D diet; however, CYP27A1 is altered in the liver of Mecp2+/− mice on both diets (Fig. 3.8B). In addition, only the Mecp2+/− on 0.1IU/g vitamin D chow display increased expression of CYP27B1 and a trend towards reduced expression of CYP24A1 in the kidney (Fig. 3.8C). Cortical CYP27A1 and CYP27B1 have a small trend towards downregulation, while CYP24A1 remains unchanged in the Mecp2+/− animals treated with 0.1IU/g vitamin D (Fig. 3.8D).

Overall, our data suggest that even though the serum level of 25(OH)D is altered in animals exposed to 0.1IU/g vitamin D chow, which does not occur with the animals treated with 10IU/g vitamin D, the dysregulation seen in vitamin D homeostasis in peripheral tissues is similar between the Mecp2+/− mice on both treatment groups. These results suggest that vitamin D modulation in either direction could be having the same metabolic effect, although, vitamin D supplementation appears more beneficial to RTT in view of the behavioral performance rescue.
Figure 3.8 Vitamin D deficient diet lowers serum 25(OH)D and alters vitamin D metabolism. (A) Exposure to vitamin D deficient chow leads to a reduction in 25(OH)D serum concentration, independent of genotype. (B) CYP2R1 expression is unaltered with vitamin D restriction. Mecp2+/+ on 1 or 0.1IU/g vitamin D show reduced expression of hepatic CYP27A1. (C) The expression of renal CYP27B1 is elevated in Mecp2+-/- on 0.1IU/g vitamin D, while CYP24A1
expression has a trend towards downregulation. (D) No alteration is observed in cortical gene expression. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. A-C: Two-way ANOVA with Tukey’s post hoc analysis. D: Two-tailed t test. A: N = 11 – 16 per condition and genotype, indicated by dots. B- D: N = 4 – 7 per condition and genotype, indicated by dots. Error bar: ±SEM.
3.5. Discussion

In this study, we examined whether dietary vitamin D supplementation rescues aberrant behavior outcomes of Mecp2+/- mice, after previously demonstrating its beneficial role on cortical neuronal morphology (Ribeiro et al., 2020). Our longitudinal experimental approach demonstrates that vitamin D supplementation ameliorates motor coordination, motor activity, and anxiety-like behavior of Mecp2+/- mice in an age- and vitamin D status-dependent manner. Motor coordination of Mecp2+/- on 10IU/g vitamin D chow, assessed with the accelerating rotarod, significantly improves at 5 months of age, while vitamin D’s benefit on anxiety-like behavior was seen at 7 months. For exploratory motor activity, Mecp2+/- mice with higher 25(OH)D serum concentration travelled significantly more in the open field arena than Mecp2+/- with insufficient 25(OH)D. Interestingly, an insufficient level of serum 25(OH)D negatively impacts only Mecp2 deficient mice since wild-type animals in either group did not behave differently, suggesting that the loss of Mecp2 could leave mice more vulnerable to the effects of disruptions in vitamin D status.

We measured serum 25(OH)D concentration at 7 months of age as it is considered the best indicator of vitamin D status; the activated form of vitamin D (1,25(OH)_{2}D) is very tightly regulated and might not reveal the start of adverse effects of its modulation (Dirks et al., 2018). Additionally, brain 1,25(OH)_{2}D concentration better correlates with serum 25(OH)D concentration than with serum 1,25(OH)_{2}D (Spach and Hayes, 2005). Importantly, even though we see no change in 25(OH)D concentration after supplementation, serum 1,25(OH)_{2}D could still be altered. We have previously reported an increase in serum 25(OH)D levels with 10IU/g vitamin D supplementation at 5 months of age when compared to mice on the control chow, independent of genotype (Ribeiro et al., 2020). Thus, it is possible that after 6 months of
treatment (7 months of age), 25(OH)D serum level is not altered in the mice due to a shift in vitamin D homeostasis, but it could still be altered after 4 months of treatment (5 months of age), which might explain the rescue of motor coordination behavior observed exclusively at that time point.

Previous reports have shown that vitamin D supplementation raises serum 25(OH)D (Fleet et al., 2008; Rowling et al., 2007; Shepard and Deluca, 1980) while another study observed little change in vitamin D status after supplementation (Seldeen et al., 2017), in accordance with our findings at 7 months. Another possible explanation for not seeing an increase in 25(OH)D serum concentration after supplementation could be due to the increase in body weight of Mecp2+/− mice. Studies have shown that obesity can diminish the effects of vitamin D supplementation (Castaneda et al., 2012; Gallagher et al., 2013; Lee et al., 2009; Wortsman et al., 2000). The mechanism responsible for this is still poorly understood, but it might involve volumetric dilution of vitamin D in a large body mass (Drincic et al., 2012) or fat sequestration (Wortsman et al., 2000). Our data show that Mecp2+/− mice are significantly heavier at 7 months of age than at 5 months (Supplemental Fig.3.5); therefore, it is reasonable to assume that Mecp2+/− mice might need a higher dose of vitamin D later in life in order to enhance 25(OH)D serum concentration to compensate for the increase in body weight. However, we do not observe a correlation between 25(OH)D status and body weight at 7 months of age, consistent with previous findings (Seldeen et al., 2017).

Although dietary vitamin D deficiency did not significantly exacerbate behavioral phenotypes of Mecp2+/− mice, when compared to Mecp2+/− on the control chow, a positive correlation between Mecp2 expression level and 25(OH)D concentration was observed for Mecp2+/− mice with very low levels of serum 25(OH)D. This observation suggests that in mice with lower Mecp2
expression, possibly due to skewed X-chromosome inactivation, vitamin D status is at greater risk for disruption. In addition, there is a positive correlation between 25(OH)D serum concentration and anxiety-like behavior for MeCP2+/− mice on 0.1IU/g vitamin D diet, which is not observed for any of the other groups. Thus, it is possible that there is a threshold effect, in which very low serum 25(OH)D could compromise behavior more strongly than moderately insufficient levels.

One possible reason for the influence of low 25(OH)D concentration on behavior lies within vitamin D homeostasis disruptions. We investigated this hypothesis transcriptionally and identified the downregulation of hepatic CYP27A1, a rate-limiting factor in the production of 25(OH)D, in MeCP2+/− mice in all treatment groups when compared to their littermates. The reduced expression of CYP27A1 in the liver in MeCP2+/− mice on 10 IU/g vitamin D could explain why renal CYP27B1 expression is upregulated in response to vitamin D supplementation; greater concentration of 25(OH)D precursor could lead to the ramping up in the expression of the gene required for the production of the active metabolite, in an effort to normalize vitamin D metabolism. There is also a trend toward reduced CYP24A1 in the liver, the enzyme responsible for catabolizing 1,25(OH)2D, which could also help normalize vitamin D metabolism. However, since CYP27A1 is a limiting factor, vitamin D homeostasis remains disrupted in the MeCP2+/− mice.

It is interesting to note that MeCP2+/− on the vitamin D deficient diet also display reduced CYP27A1 expression in the liver, increased CYP27B1 and a trend towards decrease CYP24A1 expression in the kidney, further highlighting the disruption in vitamin D homeostasis in these mice. The reason for low serum 25(OH)D and unaltered expression of the genes involved in vitamin D metabolism in our wild-type mice exposed to vitamin D dietary restriction is not clear.
Previous reports found that those genes change in response to vitamin D modulation (Fleet et al., 2008; Roizen et al., 2018), which we do not observe in our wild-type mice. It is important to note that our supplementation and deficiency experiments had a longitudinal component and lasted for over 26 weeks, while those studies were much shorter and lasted for 4 to 12 weeks.

Although vitamin D supplementation does not raise 25(OH)D serum concentration and normalize its metabolism in Mecp2+/- mice, this paradigm still shows therapeutic promise due to the amelioration of behavioral outcomes and neuronal morphology phenotypes (Ribeiro et al., 2020). In addition, we demonstrate that vitamin D supplementation normalizes the expression of 283 dysregulated genes in the Mecp2+/- cortex, many of which play important roles in neuronal morphology. The major contributor to cholesterol catabolism in the brain, CYP46A1, is among those genes (Lund et al., 1999). Cholesterol is essential for proper brain function and it is synthesized in situ, for it cannot cross the blood-brain-barrier (Turley et al., 1998, 1996). Cholesterol metabolism is significantly altered in a number of conditions affecting the CNS, including RTT (Reviewed in Martín et al., 2014). Downregulation of CYP46A1 is observed in severely symptomatic male Mecp2-null mice, while at early symptomatic stages, its expression is increased (Buchovecky et al., 2014). In our study, CYP46A1 is downregulated in the cortex of Mecp2+/- mice on the control chow; vitamin D supplementation normalizes CYP46A1 expression, while deficiency further downregulates it. Thus, even though dietary supplementation does not rescue aberrant vitamin D homeostasis in Mecp2+/- mice, it is still sufficient to normalize the expression of a critical gene involved in cholesterol turnover within the brain. Moreover, CYP46A1 also plays an important role in other neurological disorders, and it is currently being studied as a target for therapeutics in Huntington’s (Boussicault et al., 2016;
Kacher et al., 2019) and Alzheimer’s diseases (Chen et al., 2020; Djelti et al., 2015; Kölsch et al., 2009).

Surprisingly, vitamin D deficient dietary exposure leads to the highest number of DEG between \textit{Mecp2}+/+ and \textit{Mecp2}+- animals. Among those, vitamin D deficiency exacerbates the dysregulation of 23 of the DEGs that are rescued by supplementation. According to GO analysis, those genes are particularly involved in the regulation of neurotransmitter receptor activity, such as histamine (HRH3) and GABA receptors (GABBR1). Interestingly, 54 of the DEG rescued with vitamin D supplementation are also normalized with its deficiency. A few of those are relevant to neurodevelopmental disorders (Abrahams et al., 2013; Gabel et al., 2015). For example, copy number variants in KANK1 represent a risk factor for ASD (Vanzo et al., 2019), and ANK2 is one of the high-confidence ASD genes (Iossifov et al., 2014; Yang et al., 2019), demonstrating that alterations in vitamin D homeostasis, whether through supplementation or restriction, normalizes gene transcription of a select number of clinically relevant genes in the \textit{Mecp2}+- cortex.

There are additional DEG between \textit{Mecp2}+/+ and \textit{Mecp2}+- mice on control chow that are no longer differentially expressed in \textit{Mecp2}+- animals on the deficient chow. Unlike the 74 DEG normalized after supplementation that are enriched for the VDR motif, none of the normalized genes in the deficiency paradigm appear to be enriched for the motif, suggesting that their transcription is not directly regulated by vitamin D. Vitamin D does not act exclusively in a genomic capacity; PDIA3 is a membrane receptor that mediates vitamin D non-genomic, rapid response (Boyan et al., 2012; Chen et al., 2010; J. Chen et al., 2013). We have shown previously that \textit{Pdia3} expression remains unaltered in the cortices of male and female \textit{Mecp2} mutant mice at 2 and 5 months of age, respectively (Ribeiro et al., 2020). Our RNA-sequencing data confirm
this result on female mice on control chow at 7 months of age. However, with vitamin D supplementation, Pdia3 expression increases in Mecp2+/- females when compared to their wild-type littermates and Mecp2+/- mice on the control chow. Interestingly, Mecp2+/- mice on the deficient chow showed a similar up-regulation of Pdia3; however, Mecp2+/- mice on deficient chow also displayed Pdia3 upregulation, even more so than Mecp2+/- animals. This indicates that non-genomic vitamin D response is altered with supplementation and restriction, with the latter being independent of genotype.

Finally, dietary approaches with the goal of improving disease phenotypic progression can be very complex due to the broad impact they have on a combination of cellular pathways and organ systems. Vitamin D homeostasis is an added challenging factor that still has much to be elucidated. Further studies on cholesterol and vitamin D metabolism in the brain of Mecp2 deficient mice are warranted to fully understand the possible benefits of vitamin D dietary modulation. However, in this study, we provide compelling data suggesting that vitamin D supplementation may provide valuable benefit to the quality of life of RTT patients by ameliorating essential behavior outcomes and gene regulation.
Supplemental Figure 3.1 Vitamin D supplementation does not alter sociability of MeCP2+/- mice.

(A) We observe no difference in social behavior in any of the studied groups at 3, 5 and 7 months of age. (B) Insufficient serum 25(OH)D does not alter sociability of mice at 7 months of age. Two-way ANOVA with Tukey’s post hoc analysis. N = 12 – 17 per condition, genotype and age group, indicated by dots. Error bar: ±SEM.
Supplemental Figure 3. Higher weight of Mecp2+/- mice correlates with poor motor behavior in the open field (1 IU/g) and rotarod (1 and 10 IU/g) tests. Moreover, Mecp2 expression positively correlates with total distance travelled in the open field test and rotarod performance.
of \textit{Mecp2+/-} mice on the supplemented diet. Pearson correlation of multivariate analysis (p < 0.05).

\textbf{Supplemental Figure 3.} Vitamin D deficiency does not exacerbate behavior performance of \textit{Mecp2+/-} mice.

(A) Total distance travelled, and average speed are not altered at 3 and 5 months of age. At 7 months, \textit{Mecp2+/-} mice on 1 and 0.1IU/g vitamin D and \textit{Mecp2+/-} on 0.1IU/g vitamin D exhibit increased anxiety-like behavior when compared to \textit{Mecp2+/-} mice on 1IU/g vitamin D. (B) At 3 months of age, \textit{Mecp2+/-} mice on 1IU/g vitamin D spend more time in the open arm of the elevated plus maze than \textit{Mecp2+/-} on the control chow, indicating lower anxiety-like behavior.
At 5 months, no differences are seen among groups. Social behavior of mice is not altered at 3 and 5 months of age. \(*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.\) Two-way ANOVA with Tukey’s post hoc analysis. \(N = 12 – 15\) per condition and genotype, indicated by dots. Error bar: ±SEM.
Supplemental Figure 3.4 Low serum 25(OH)D correlates with anxiety-like behavior of Mecp2+/− mice.

(A) The weight of Mecp2+/− mice on 0.1IU/g vitamin D negatively correlated with motor behavior in the open field and rotarod tests. Moreover, Mecp2 expression positively correlates with rotarod performance of Mecp2+/− mice on the deficient diet. Importantly, serum 25(OH)D positively correlates with Mecp2 expression and anxiety-like behavior of Mecp2+/− mice.

Pearson correlation of multivariate analysis.
**Supplemental Figure 3.** *Mecp2 +/-* mice are heavier in late symptomatic stage. *Mecp2 +/-* mice exhibit increased weight as they age, regardless of vitamin D treatment. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Two-way ANOVA with Tukey’s post hoc analysis. N = 12 – 15 per condition and genotype, indicated by dots. Error bar: ±SEM.
Chapter 4: Discussion and future directions
4.1 Main findings

The goal of this dissertation was to elucidate whether vitamin D supplementation could lower aberrant NF-κB activation in neurons and rescue RTT phenotypes in MeCP2-mutant mouse models of the disorder. The main findings of this dissertation include: (1) vitamin D lowers the aberrant NF-κB activation that occurs with MeCP2 loss-of-function, and increases neurite outgrowth of cortical neurons in vitro; (2) dietary 50IU/g vitamin D supplementation rescues dendritic complexity, soma area, and dendritic spine density of male MeCP2-null mice, in addition to moderately extending their reduced lifespan. Further, 10IU/g and 50IU/g supplementation partially rescue dendritic complexity of female MeCP2 heterozygous cortical neurons (significantly higher than MeCP2+/- mice on the control chow, but still significantly lower than MeCP2++/+ animals), while the soma area of MeCP2+/- mice on 10IU/g vitamin D is no longer significantly different from MeCP2+/- on 1IU/g vitamin D; (3) motor and anxiety-like behavioral deficits of MeCP2+/- mice are mitigated by 10IU/g vitamin D supplementation, while restricting vitamin D supply to 0.1IU/g does not exacerbate behavior impairments; (4) transcriptome analyses indicate that vitamin D supplementation rescues the expression of over 200 genes in MeCP2+/- cortex, including those involved in neuronal morphology; (5) vitamin D metabolism is disrupted in MeCP2+/- mice and vitamin D modulation does not alter the expression of the gene that encodes the 25(OH)D rate limiting enzyme, CYP27A1, in the liver.

4.2 Vitamin D lowers NF-κB activation in vitro and ameliorates neuronal morphology

Deficits in neuronal morphology and reduced soma size are hallmark phenotypes of RTT, both in model animals and human patients (Armstrong, 2002; Armstrong et al., 1999; Jellinger et al., 1988; Noriyuki Kishi, 2008). Increased activity of the NF-κB pathway contributes to the reduced
dendritic arborization of cortical neurons of Mecp2-null mice. Importantly, genetically attenuating NF-κB signaling by crossing Mecp2-mutant mice with Nfkb1+/- mice, encoding the p50 subunit, normalizes dendritic deficits and extend the lifespan of these animals (Kishi et al., 2016). Aberrant NF-κB activation has been reported in a number of neurological conditions (Dresselhaus and Meffert, 2019; O’Sullivan et al., 2010). For example, in Alzheimer’s disease (AD), neurotoxic amyloid β peptides activate the NF-κB pathway in neurons and glia (Kaitschmidt et al., 1997). Additionally, NF-κB directly regulates the expression of β-site APP cleaving enzyme (BACE1), a central protein in the generation of amyloid plaques in AD brains (Chen et al., 2012). Taken together, these data indicate that research into NF-κB inhibition would be widely applicable to numerous disorders, extending beyond RTT. However, using genetic manipulation as the approach for dampening NF-κB activation might not be feasible, and the use of simpler pathway inhibitors seems the more practical approach.

There are over 700 inhibitors of NF-κB activation (Gilmore, 2006), including vitamin D. Vitamin D’s ability to inhibit the NF-κB pathway has been reported in multiple studies (Y. Chen et al., 2013a; Liu et al., 2013; Sun et al., 2006); however, none of those reports included the CNS. Thus, we set out to investigate whether the addition of calcitriol, the activated form of vitamin D, to the culture media of cortical neurons would lower NF-κB activity. By using two different approaches, we were able to demonstrate that calcitriol lowers relative NF-κB activation, which was up-regulated in cortical neurons transfected with an shRNA targeting Mecp2 (shMecp2), compared to neurons transfected with a control scrambled shRNA. The challenge of finding an antibody that cleanly recognizes phosphorylated p65, which is indicative of its activation, directed us to use cellular localization of the protein instead. As a result, we observed that p65 nuclear localization is increased in shMecp2 cortical neurons, but calcitriol
rescued the p65 nuclear localization, indicating that the pathway activation was diminished. Additionally, calcitriol increased the reduced total neurite outgrowth of shMecp2 cortical neurons.

Further, our data confirm the potential of vitamin D as a therapeutic for RTT by modestly extending the lifespan of Mecp2-null animals and rescuing neuronal morphology of mice in a dose-dependent manner. Mecp2-null mice on 50IU/g vitamin D display increased dendritic complexity, soma area and dendritic spine density, while similar results are observed with Mecp2+/- mice on 10IU/g vitamin D (Fig. 4). It is possible that our females respond differently to vitamin D supplementation due to a combination of cell autonomous and non-cell autonomous effects, the increased length of vitamin D treatment or due to other sex-specific components, such as the estrous cycle. Nevertheless, we established that vitamin D can not only inhibit NF-κB activation in vitro, but it can also mitigate neuronal deficits in vitro and in vivo. Another study confirmed the importance of reducing NF-κB activation in Mecp2-null mice, which also led to neuronal morphology and synaptic markers rescue (Jorge-Torres et al., 2018), demonstrating that the inhibition of the pathway by vitamin D could be a simple and cost-effective therapeutic option for RTT.
Figure 4.1 Vitamin D supplementation rescues neuronal morphology and behavioral outcomes of *Mecp2* mutant mice.

The addition of vitamin D to culture media lowers NF-κB signaling and promotes neurite outgrowth of *Mecp2*-knockdown cortical neurons *in vitro*. Further, dietary vitamin D supplementation rescues cortical neuronal morphology, such as reduced dendritic complexity, of *Mecp2* mutant mice in a dose dependent manner (Top). At 5 months of age, *Mecp2+/-* mice
supplemented with vitamin D exhibit a higher latency to trial end than $MeCP2^{+/−}$ animals on the control chow. At 7 months of age, $MeCP2^{+/−}$ mice on the control chow display higher anxiety-like behavior when compared to their wild-type littermates. $MeCP2^{+/−}$ on the supplemented chow spend similar amount of time in the center of the open field arena when compared to wild-type mice (Bottom).
4.3 Vitamin D supplementation mitigates behavioral deficits of MeCP2 heterozygous mice

Studies have shown that there is an association between neuronal morphology rescue and behavioral amelioration in mice (Heun-Johnson and Levitt, 2018; Karadurmus et al., 2019; Medendorp et al., 2018; Wu et al., 2016). Therefore, in view of the positive effect of vitamin D on cortical structural changes, we assessed possible behavioral benefits as well. In this dissertation, I have shown that vitamin D dietary supplementation improves certain behavioral outcomes of MeCP2+/- in an age specific manner, such as motor coordination at 5 months of age and anxiety-like behavior at 7 months (Fig. 4.2). Interestingly, analyzing animals based on their 25(OH)D serum concentration, rather than treatment group, reveals that vitamin status is more important for exploratory motor behavior of MeCP2+/- mice than MeCP2+/, and that higher serum 25(OH)D leads to better performance. We also observed a relationship between very low serum 25(OH)D concentration, found in MeCP2+/- mice treated with vitamin D deficient chow, and anxiety-like behavior, a phenotype rescued with supplementation. Nevertheless, exposure to vitamin D deficient diet was not sufficient to significantly exacerbate behavioral phenotypes of MeCP2+/- mice overall.

An aspect of vitamin D metabolism to note is its relationship with parathyroid hormone (PTH). The release of PTH is regulated by 1,25(OH)2D (Levin et al., 2007; Tang et al., 2019). Lower calcium concentration results in an increase in PTH levels, leading to the upregulation of CYP27B1, which encodes the enzyme responsible for the final step in the synthesis of 1,25(OH)2D (Rowling et al., 2007). PTH plasma concentration shares a negative correlation with 25(OH)D in RTT patients (Bowden et al., 2008; Sarajlija et al., 2013), while their fractional calcium absorption is increased (Motil et al., 2006). Notably, alterations in calcium concentration can modify behavior; rats supplemented with calcium displayed higher ambulation and reduced
anxiety-like behavior (Godinho et al., 2002). Therefore, we cannot discount the contributions that PTH and calcium levels might have in our paradigm.

4.4 Vitamin D supplementation normalizes the expression of genes involved in neuronal morphology

After the behavior experiments were completed, we used a subset of mice for RNA-sequencing analysis of whole cortex. We observed that hundreds of genes are differentially expressed between Mecp2+/- mice, and their wild-type littermates supplemented with vitamin D, more so than the genes found in the comparison between Mecp2+/- and Mecp2+/- on the control chow. This is likely due to vitamin D’s ability to act as a transcriptional factor when it is bound to its receptor, VDR. Still, Mecp2+/- mice treated with vitamin D deficient chow had an even greater number of differentially expressed genes when compared to their wild-type littermates, suggesting that vitamin D restriction can further dysregulate the cortical transcriptome of Mecp2+/- mice.

Among the differentially expressed genes in Mecp2+/- cortex that were normalized with vitamin D supplementation is cyclin-dependent kinase 5 (Cdk5), which is essential for neural development; Cdk5 is downregulated in Mecp2+/- mice on control chow but not in those on vitamin D supplementation. Cdk5 in association with p39, one of its two activators, is necessary for dendritic development. The loss of p39 or Cdk5 results in shorter and less complex dendritic arborization (Ouyang et al., 2020). An additional example of a gene that is no longer downregulated in Mecp2+/- mice after supplementation is protein tyrosine kinase 2 beta (Ptk2b), also known as Pyk2. Ptk2b is a non-receptor tyrosine kinase that is enriched in the forebrain and that is activated by Ca^{2+} (Ouyang et al., 2020). Interestingly, Ptk2b is also associated with the
regulation of lipid metabolism (Ni et al., 2018), and its modulation improves phenotypes of mouse models of AD (Giralt et al., 2018). More importantly, vitamin D deficiency did not normalize the expression of these genes, illustrating that our supplementation paradigm exclusively rescues the expression of genes involved in neuronal morphology and that are associated with neurological disorders other than RTT.

Another exciting candidate gene that has been normalized with vitamin D supplementation is Cyp46a1, which is downregulated in Mecp2+/- mice on the control chow and further downregulated in Mecp2+/- on the deficient chow. Cyp46a1 is essential for cholesterol turnover and it is predominantly found in the brain, with highest expression in the striatum, followed by the cortex, hippocampus and cerebellum (Popiolek et al., 2020). CYP46A1 plays a role in neuronal development and increases dendritic outgrowth and spine density. It acts through small guanosine triphosphate-binding proteins (sGTPases), resulting in the phosphorylation of tropomyosin-related kinase (Trk), in addition to promoting Trk interaction with geranylgeranyl transferase-I (GGTase-I) (Moutinho et al., 2016). Importantly, the use of TrkB activators restores hippocampal synaptic plasticity and motor learning in Mecp2-mutant mice (Adams1 et al., 2020; Li et al., 2017). Moreover, in the brains of Cyp46a1+/- mice, the phosphorylation of many proteins that are associated with microtubules, neurofilaments, synaptic vesicles and neurotransmission, such as proteins of the MAP, NEF, SLC and SHANK families, is altered (Mast et al., 2017). Thus, it is possible that those proteins are similarly altered in the cortex of Mecp2+/- mice with lower Cyp46a1 expression and that vitamin D supplementation could lead to the increase in these proteins and cellular pathways. The genes mentioned here are only a few examples of the many that were normalized with vitamin D supplementation, demonstrating the likely broad benefits vitamin D can have on RTT pathology. The data provided by this
transcriptome experiment will open up new avenues of study on RTT cortical pathology and have the potential to identify new avenues for therapeutic treatment.

4.5 Vitamin D modulation alters its own metabolism and cholesterol biosynthesis in *Mecp2+/-* mice

In addition to the genes discussed above, we observed dysregulation in the expression of a hepatic gene essential for vitamin D metabolism. The downregulation of *Cyp27a1* is prevalent in all *Mecp2+/-* mice, regardless of diet. In addition to vitamin D regulation, CYP27A1 is associated with cholesterol metabolism and bile acids biosynthesis (Zurkinden et al., 2020). Treating *Cyp27a1* knock out and *Cyp27a1/Apolipoprotein E* double knock out mice with bile acids ameliorates the metabolic dysregulation observed in these animals and alters cholesterol absorption (Repa et al., 2000; Zurkinden et al., 2020). Thus, our vitamin D modulation paradigm might be altering other metabolic pathways, such as bile acids and cholesterol biosynthesis.

Cholesterol metabolism is a precursor to vitamin D synthesis. Thus, it was expected that our dietary vitamin D manipulation paradigm could alter components of cholesterol metabolism. Cholesterol biosynthesis dysfunction has already been reported in RTT. A study found that along with the reduction in *Cyp46a1* expression, the genes *Sqle* and *Hmgcr* were also downregulated in whole brain lysates of severely affected male *Mecp2*-null mice (Buchovecky et al., 2014). SQLE and HMGCR are rate-limiting enzymes in cholesterol biosynthesis (Gill et al., 2011; Nieweg et al., 2009; Yamamoto and Bloch, 1970). We found no significant difference in *Sqle* expression in the cortex of female animals in all conditions tested. Conversely, cortical *Hmgcr* expression was downregulated in *Mecp2+/-* mice on the supplemented and deficient chow. Of the *Mecp2+/-* mice, only the ones exposed to vitamin D deficient diet show *Hmgcr* alteration, which is further
downregulated when compared to Mecp2+/- mice treated with the same diet. Our contrasting result could be due to the fact that we utilized older female mice in our study and performed RNA-sequencing on cortical tissue, while Buchovecky et al. (2014) used whole brain lysates of younger male mice.

Cholesterol biosynthesis is a tightly regulated process mediated by the sterol regulatory element binding protein 2 (SREBP-2), which is initially sequestered in the endoplasmic reticulum as its inactivated precursor. As sterol levels lower, the cholesterol sensor SREBP cleavage-activating protein (SCAP) guides SREBP-2 to the Golgi apparatus, where it is cleaved and activated (Brown and Goldstein, 2009). Then, SREBP-2 travels to the nucleus and activates genes involved in cholesterol synthesis, including Hmgcr and Sqle (Brown and Goldstein, 2009; Suzuki et al., 2013; Wu et al., 2013). Our data demonstrate that there is a reduction in the expression of Scap, and no alteration in Srebf2 (encodes SREBP-2) levels, in all Mecp2+/- mice, regardless of treatment group. Interestingly, Mecp2+/+ mice on 0.1IU/g vitamin D display an even greater downregulation of Scap. Murine homozygous deletion of Scap in the brain results in the downregulation of Srebf2 and its downstream targets, Hmgcr, Sqle, Fdps, Idi1 and Ldlr (Suzuki et al., 2013). However, our data only points to the downregulation of Scap, not Srebf2 or Sqle. The other downstream genes have variable dysregulation, summarized in Table 1.
Table 2. Differential expression of genes involved in cholesterol biosynthesis and turnover.

<table>
<thead>
<tr>
<th>Genes</th>
<th>1 M+/- vs 1 M+/+</th>
<th>10 M+/- vs 1 M+/+</th>
<th>0.1 M+/- vs 1 M+/+</th>
<th>10 M+/+ vs 1 M+/+</th>
<th>0.1 M+/+ vs 1 M+/+</th>
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<tr>
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<tr>
<td>Scap</td>
<td>Down Down NC NC NC NC</td>
<td>Down Down NC NC NC Up</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Fdps</td>
<td>NC NC NC NC NC NC</td>
<td>NC NC NC NC NC Up</td>
<td></td>
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</tr>
<tr>
<td>Idi1</td>
<td>*NC Down NC Down NC Down NC Down</td>
<td>*NC Down NC Down NC Down</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ldlr</td>
<td>NC Up Up NC Up Up</td>
<td>NC Up Up NC Up Up</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Down = downregulated, Up = upregulated, NC = no change. *Nearly upregulated (p = 0.01, FDR = 0.13).

Overall, previous reports and our data demonstrate that the loss of Mecp2 results in aberrant regulation of genes involved in cholesterol metabolism in the brain. Vitamin D treatment, either supplementation or restriction, alters those genes in different ways. For example, exposure to vitamin D deficiency downregulates Fdps in Mecp2+/- and upregulates it in Mecp2+/- mice while no changes are observed with supplementation. The consequences of altering cholesterol metabolism with vitamin D dietary modulation and its clinical significance to RTT are still unclear. It is also important to note, however, that these findings are based on transcriptomic analysis and they might not correlate with protein status. Nevertheless, the use of pharmacological inhibitors of cholesterol synthesis has been proven beneficial to neurological disorders. Lovastatin treatment of a mouse model of fragile X syndrome prevent epileptogenesis (Osterweil et al., 2013), while Mecp2 deficient mice receiving lovastatin or fluvastatin displayed motor improvements and extended survival (Buchovecky et al., 2014).

Astrocytes are the main contributors to brain cholesterol synthesis, delivering to neurons complexes of apolipoprotein E (apoE)-lipoprotein-bound cholesterol (Mauch et al., 2001; Pfrieger and Ungerer, 2011). Biosynthetic profiles of cholesterol producing enzymes differ
between neurons and astrocytes, with the former expressing very low levels of enzymes capable of processing lanosterol, which is a precursor of cholesterol. Thus, astrocytes synthesize cholesterol more efficiently than neurons and respond more rapidly to the inhibition of its biosynthesis (Nieweg et al., 2009). Disruptions in astrocytic cholesterol production can result in cognitive dysfunction. Knocking out Srebf2 in astrocytes leads to motor, social and memory deficits in mice (Ferris et al., 2017). Astrocytes with expansions in the huntingtin protein produce less apoE-lipoprotein, leading to the reduction in cholesterol synthesis and reduced neurite outgrowth, features of Huntington’s disease (Valenza et al., 2015). In view of the essential contribution of astrocytes to brain cholesterol homeostasis, it is likely that our vitamin D modulation paradigm alters astrocytic function in some capacity. Astrocyte dysfunction contributes to RTT pathophysiology (Dong et al., 2020; Rakela et al., 2018; Williams et al., 2014); re-expressing Mecp2 exclusively in astrocytes helps alleviate locomotor activity deficits (Lioy et al., 2011) and rescues RTT respiratory phenotypes (Garg et al., 2015).

With the myriad of downstream pathways altered by vitamin D, vitamin D supplemented diet can generate beneficial and detrimental impacts at the same time. In high fat diet research, it has been shown that treating mice with higher levels of vitamin D and calcium aggravated their metabolic alterations, resulting in higher glucose and insulin concentrations. Yet, vitamin D and calcium supplementation staved off the increase in inflammatory markers that was observed in animals that did not receive vitamin D (Bastie et al., 2012). It is important to note that our vitamin D supplemented chow did not include higher calcium concentrations.

Overall, in this dissertation, we have clearly demonstrated that vitamin D manipulation can improve certain features of RTT; however, it is essential that further studies closely investigate the relationship between vitamin D and cholesterol metabolism in the brains of RTT animals.
treated with vitamin D supplementation and/or deficiency. This will allow us to better understand whether using vitamin D, as a therapeutic option for RTT, needs to be introduced in a more targeted instead of systemic approach.

4.6 Future directions

My dissertation research offered compelling evidence that vitamin D dietary supplementation could be a potential simple, and cost effective, therapeutic option for RTT patients. Additionally, it evinced important metabolic factors that likely contribute to RTT pathology, such as vitamin D homeostasis and cholesterol metabolism. However, there are several limitations to these studies that should be addressed and expanded upon in future research.

Vitamin D metabolism is a highly complex process that involves multiple genes and takes place across organs systems. In addition, this process is dynamic, and it needs to rapidly adapt to environmental cues. Therefore, investigating 25(OH)D serum concentration only at limited time points could overshadow the pathological contribution of vitamin D homeostasis dysfunction to RTT. For the present study, serum 25(OH)D in the female mice was only measured at 5 (mice that were used for morphological analysis) and 7 (animals that went through behavior experimentation) months of age. Thus, a longitudinal analysis of components of vitamin D metabolism might better reveal how it affects RTT. One possibility would be to collect blood every other week (or every week) from mice exposed to different vitamin D concentrations, starting at weaning age (baseline). A previous study found that the lowering of serum 25(OH)D occurs two to three weeks after mice are treated with vitamin D deficient chow. Additionally, when mice are removed from that diet and placed on control diet, it takes approximately two weeks for serum 25(OH)D to normalize (Belenchia et al., 2017). The proposed experiment could
be expanded and include the testing of metabolites, mainly 1,25(OH)₂D and 24,25(OH)D. In case there is a dynamic regulation of vitamin D as MeCP2+/- mice become more symptomatic, including fluctuations in serum metabolites, repeating behavioral experiments at 3 and 5 months of age would allow us to correlate the mice behavior outcomes to vitamin D metabolites concentration. Behavior experiments could also be expanded to add the light/dark box assay, another measure of anxiety (Lezak et al., 2017), to confirm the results observed with the open field test and that contradict the elevated plus maze data.

It would also be interesting to study whether there are any differences in the expression of genes involved in vitamin D synthesis and/or cholesterol metabolism in MeCP2+/- mice at early symptomatic stages or if the downregulation of CYP27A1, for example, only occurs when mice are symptomatic. According to a previous study, Cyp46a1 expression is increased at P28 in the brains of MeCP2-null mice. At P56, however, Cyp46a1, along with other important genes for cholesterol biosynthesis, Hmgcr and Sqle, are downregulated while total cholesterol concentration is abnormally high, when compared to wild-type mice. In addition, the cholesterol intermediates desmosterol and lanosterol are lower at P70, even though there is no significant difference between MeCP2-null and wild-type mice brain total cholesterol levels at this specific time point (Buchovecky et al., 2014). This illustrates how dynamic cholesterol metabolism is and how it can be altered during phenotypic progression of MeCP2-null mice. Therefore, performing a longitudinal study evaluating genes involved in cholesterol biosynthesis and turnover, in addition to its intermediates, would be helpful in understanding the aberrant interplay between cholesterol and vitamin D that likely contributes to RTT pathology.

A study investigating whether in utero exposure to vitamin D modulation would prevent the dysregulation of vitamin D homeostasis and cholesterol metabolism could be done as well, since
starting treatment at an early symptomatic age leads to a limited impact, ameliorating phenotypes, but not preventing their development. Currently, only a small pilot experiment has been conducted to study the effects of in utero exposure to vitamin D supplementation, in which we found that supplemented pups produce more ultrasonic vocalizations (Appendix). Maternal vitamin D status is essential for offspring wellbeing and neurotypical development. Exposure to lower levels of vitamin D metabolites leads to greater risk of attention-deficit/hyperactivity disorder (Sucksdorff et al., 2021) and ASD in the offspring (Chen et al., 2016; Grant and Soles, 2009; Vinkhuyzen et al., 2018), for example. Thus, evaluating if in utero vitamin D modulation could more fully prevent phenotypic development would be essential to support vitamin D as a therapeutic option for RTT.

Another line of research would be to explore whether other regions of the brain are altered in a similar manner to the cortex. Buchovecky et al. (2014) used whole brain lysates when studying cholesterol disruption in RTT, and in our experiments, we used cortical samples, which could be one of the reasons for the contradictory data between our studies. Moreover, it has been shown that cortical and hippocampal neurons differ in how they respond to cholesterol disruption. For example, removal and addition of cholesterol leads to a reduction in cortical neurite outgrowth; however, cholesterol depletion increases neurite outgrowth of hippocampal neurons while cholesterol supplementation results in no alterations (Ko et al., 2005). Thus, the disruption we see in genes involved in cholesterol synthesis could be brain-region specific. Hippocampal deficits have been implicated in RTT in multiple studies (Balakrishnan and Mironov, 2018; Kee et al., 2018; W. Li et al., 2016; Lozovaya et al., 2019; Lu et al., 2016) and understanding the role of vitamin D in this region would bring us a step further towards efficient treatment for the disorder.
Finally, it has been reported that vitamin D can be a powerful adjuvant in the treatment of epilepsy, one of the many symptoms of RTT. Borowicz et al. (2015, 2007) demonstrated that vitamin D enhances the efficacy of antiepileptic drugs in an electroshock epilepsy model by raising seizure threshold (Borowicz et al., 2015, 2007). Vitamin D’s benefit in epilepsy research has been pursued numerous times. The partial deletion of VDR resulted in shorter latency to seizure onset, higher mortality and scores in the Racine scale of chemically induced seizure in mice (Kalueff et al., 2006). Administering 1,25(OH)\(_2\)D prior to pentylenetetrazol (PTZ) induced seizures improved mice phenotypes, such as longer latency to seizure onset, lower mortality and seizure duration (Kalueff et al., 2005). We did not observe significant improvements in seizure threshold of Mecp2\(^{+/-}\) mice supplemented with vitamin D in a pilot study (Appendix).

Nevertheless, investigating the effects of vitamin D in conjunction with another drug, perhaps even another NF-κB inhibitor, could prove beneficial to this RTT phenotype. NF-κB upregulation during status epilepticus is well known (Blondeau et al., 2001; Lubin et al., 2007; Shin et al., 2014) and administering NF-κB inhibitors dampens its response and ameliorates seizure outcomes (Chen et al., 2017; Yu et al., 2011). Thus, there are many exciting avenues to be pursued in the study of vitamin D role in RTT therapeutics.

Overall, my dissertation provides evidence that vitamin D modulation alters phenotypic presentation, behavioral outcomes, and gene regulation of Mecp2 mutant mice. However, studies investigating the impact of vitamin D deficiency on RTT phenotypic progression are still very limited. Currently, symptom management is the only treatment plan available for RTT patients, which is why finding therapeutic options that are able to improve their quality of life is essential for the community. Therefore, unraveling how vitamin D biosynthesis, and other associated metabolic processes, contribute to RTT pathology will likely be very challenging, but rewarding.
Pilot experiment 1

Seizures commonly occur in RTT patients and mouse models (Calfa et al., 2011; Tarquinio et al., 2017). Reports have shown that vitamin D has a beneficial role in epilepsy and can increase seizure threshold (Borowicz et al., 2015, 2007; Kalueff et al., 2006, 2005). Thus, we investigated whether vitamin D supplementation could prevent seizures in Mecp2+/- mice. However, due to the metabolic alterations observed in Mecp2 deficient mice, which could impact the uptake of drugs that induce chemical seizures, we decided to test an acute corneal electroshock paradigm. For this experiment, 3-4-month-old female mice, on 1 or 10IU/g vitamin D, were acclimated to the room at least one hour prior to testing. An anesthetizing solution was applied to each eye of the mouse, followed by saline solution. The electrodes were placed on the eyes of the mouse and after the release of the current, the mouse was observed for 15 minutes. The experiment ended when the mouse suffered a tonic seizure, did not suffer a tonic seizure after the highest current (4, 8, 12 and 16 mA), or did not survive the electroshock. Survival rate was also recorded for each group. The frequency (60Hz) and duration (0.2s) were maintained for all current stimulations. This pilot experiment did not result in significant differences between Mecp2+/+ and Mecp2+/- mice on the control chow (Appendix Fig.1.1), which led to its termination.
Appendix Figure 1. 1 Mecp2+/- mice do not display lower seizure threshold.
The current in which mice experienced a tonic seizure was not different between groups or
genotypes. Two-way ANOVA with Tukey’s post hoc analysis. N = 3 – 11 per condition and
genotype, indicated by dots. Error bar: ±SEM.

Pilot experiment 2
It is well known that RTT symptoms are delayed and do not appear soon after birth. However,
there has been evidence of patients exhibiting mild deficits earlier in life (Einspieler et al., 2005a,
2005b). Mecp2 mutant mice also display alterations that occur soon after birth (De Filippis et al.,
2010; Santos et al., 2007). Thus, to investigate early postnatal alterations in Mecp2 mutant mice
and whether vitamin D in utero supplementation could prevent phenotypic development, we
tested maternal separation-induced ultrasonic vocalizations (USVs) and latency of righting
reflex. In the Mecp2-308 mutation RTT model, pups deficient in Mecp2 produce less calls at P6
than their wild-type littermates (De Filippis et al., 2010). Moreover, Santos et al (2007)
demonstrated that Mecp2 mutant mice have higher righting reflex time than wild-type pups at P6
(Santos et al., 2007).
For this experiment, the dams were placed on vitamin D supplemented (10IU/g vitamin D) or control (1IU/g vitamin D) chow once they reached sexual maturity (8 weeks of age) for a period of 2 weeks prior to mating. Once mated, both male and female mice remained on the custom chow. The USVs test was conducted at P4, P8, P10 and P14, followed by the righting reflex analysis on each day. Pups were removed one by one from their home cage and individually placed in a sound proofed chamber. USVs were recorded with a microphone (Patterson M500-384) for a period of 5 min. After this, the pup was placed on an empty cage for the righting reflex analysis. In brief, the pup was placed on its back and allowed to right itself for a period of 30 s; the test ended when the pup flipped back, or the 30 s had elapsed. Each pup was tested twice, and the righting reflex time was averaged. After all the pups had been tested for both USVs and righting reflex, they were returned to their home cage. Number of calls was measured using Noldus UltraVox XT software. The criteria for call classification were as followed: frequency between 30 and 125 kHz, duration between 5 and 500 ms with a gap greater or equal to 6 ms, and amplitude greater than 250 a.u. above background.

The number of pups in this experiment was limited. Additionally, most of the pups supplemented with vitamin D in utero were birthed by the same dam. Thus, this experiment is not conclusive; however, it is interesting to note that vitamin D in utero supplementation seems to alter righting reflex and call production, especially in late postnatal ages (Appendix Fig. 1.2). To confirm that early postnatal changes are promoted by vitamin D in utero supplementation, more pups need to be tested.
Appendix Figure 1.2 *In utero* vitamin D supplementation alters early behavior of pups

Female pups displayed no significant difference in righting reflex average time (left), while *Mecp2+/y* pups on the control chow displayed lower average time than *Mecp2+/y* animals on the supplemented chow at early postnatal. However, at P8, *Mecp2-/y* pups on the control chow take longer to right themselves than *Mecp2-/y* and *Mecp2+/y* pups supplemented with vitamin D *in utero* (left). Further, *Mecp2+/y* pups on 10IU/g chow produce more calls than pups on the control chow P4 (right). Similarly, *Mecp2+/y* pups on the supplemented chow call more often than all other pups at P8. At P10, *Mecp2+/y* only produce more calls than *Mecp2-/y* pups on control chow. *p < 0.05, **p < 0.01. Two-way ANOVA with Tukey’s post hoc analysis.

Females: *Mecp2+/+ 1IU/g*: N = 7; *Mecp2+/− 1IU/g*: N = 6; *Mecp2+/+ 10IU/g*: N = 5; *Mecp2+/− 10IU/g*: N = 4. Males: *Mecp2+/+ 1IU/g*: N = 4; *Mecp2−/− 1IU/g*: N = 8; *Mecp2+/y 10IU/g*: N = 3; *Mecp2−/y 10IU/g*: N = 4. Error bar: ±SEM.
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WORK EXPERIENCE

08/2015-present: **Graduate Research Assistant**, Syracuse University, Syracuse, NY
Advisor: Dr. Jessica MacDonald
* Investigated the phenotypic effects of vitamin D dietary supplementation in a mouse model of Rett syndrome.
* Optimized and developed protocols for various lab techniques, and pioneered mice behavior assays and RNA-sequencing in the lab.
* Mentored 4 undergraduate students and taught laboratory techniques to 16 lab members.
* Presented scientific data at national (5) and international (4) conferences and symposiums.

08/2015-05/2019: **Graduate Teaching Assistant**, Syracuse University, Syracuse, NY
* Laboratory section instructor for 3 undergraduate level biology courses (5 semesters).
* Assisted professors of 3 graduate biology courses (3 semesters); held weekly office hours and tutorial sessions.

03/2013-12/2014: **Intern**, Monsanto Company, Campinas, SP, Brazil
* Collaborated with stakeholders to develop a robust onboarding and mentoring program for incoming interns.
* Assisted the transgenic sugarcane production team (1st year) and the molecular diagnostics team (2nd year).
* Developed and implemented an innovative rooting system for seedlings (50% more efficient).
* Implemented a trackable online safety data sheets system for all teams under the molecular assays umbrella.

11/2012-03/2013: **Intern**, Molecular Genetics of Cancer Lab at UNICAMP University, Campinas, SP, Brazil
* Assisted with blood sample collection from patients.
* Assisted with lab organization and experiments.

10/2010-11/2012: **Customer Service Representative**, Mercedes-Benz, Campinas, SP, Brazil
* Assisted customers via phone, mail, chat, and email in Portuguese, English, and Spanish.
* Developed a comprehensive bank of editable email responses for various customers concerns.
* Member of the team tasked with maintaining the brand’s presence on social media.
* Represented the company at expositions, conferences, and award events.

EDUCATION

**Ph.D. in Biology - Neuroscience concentration** (In progress)
Syracuse University (08/2015-present)
Syracuse, NY
GPA: 4.00/4.00

**B.S. in Biological Sciences and Science Teaching**
Pontifícia Universidade Católica de Campinas (01/2011-12/2014)
campinis, sp, brazil
GPA: 9.22/10.00

associate degree in liberal arts - mathematics and science
Norwalk Community College (09/2008-08/2010)
Norwalk, CT
GPA: 3.98/4.00

Publications


Presentations


* Ribeiro, M.C., Moore, S.M. Macklis, J.D., MacDonald, J.L. Vitamin D supplementation rescues aberrant NF-κB pathway activation and partially ameliorates Rett syndrome cortical phenotypes in MeCP2 mutant mice. Talk: Society for Neuroscience (SfN), Chicago, October 2019.


* Fridkis, G.F., Ribeiro, M.C., † MacDonald, J.L. Vitamin D supplementation as a therapeutic agent for Rett syndrome. Poster: Arts & Sciences Undergraduate Research Festival, Syracuse University, April 2019. Poster: Life Science Symposium, Syracuse University, April 2019. †Co-first authors. Best poster award.

* Ribeiro, M.C., Moore, S.M., Kish, N., Macklis, J.D., MacDonald, J.L. Reduction of aberrant NF-κB signaling and vitamin D supplementation ameliorate Rett syndrome cortical phenotypes in MeCP2-null mice. Poster: Society for Neuroscience (SfN), San Diego, November 2018.


**HONORS AND AWARDS**

* Travel fellowship recipient, awarded by the Syracuse University Graduate Student Organization and the Biology Department to attend the 2019 Society for Neuroscience (SfN) meeting in Chicago, IL.

* Research Excellence Doctoral Funding (REDF) fellowship, awarded by the Syracuse University Graduate School for the 2019-2020 academic year.

* Travel fellowship recipient, awarded by the Syracuse University Neuroscience program to attend the 2019 ISN-ASN meeting in Montreal, Canada.

* Travel fellowship recipient, awarded by the Syracuse University Graduate Student Organization and the Biology Department to attend the 2018 Society for Neuroscience (SfN) meeting in San Diego, CA.

* Fellowship to attend the ISN-JNC Flagship School, awarded by the International Society of Neurochemistry and Journal of Neurochemistry in Alpbach, Austria. September 2018.

* Best Graduate Student Poster Presentation, Biology Recruitment Weekend, Syracuse University Biology Department. March 2018.

**DIVERSITY AND INCLUSION**

* Lead Facilitator for First-Year Experience Initiative – September 2018:
  - Led group discussions regarding identity, belonging, diversity, inclusion, health, and wellness with incoming undergraduate students

**MENTORING**

* Teaching Mentor Program – 03/2020 - 03/2021
  - Developed the ‘Effective Assessment’ module for the orientation of incoming Teaching Assistants at Syracuse University.
  - Led a small group discussion regarding the university’s policies and the teaching assistants’ questions and concerns.

* Mentoring of Lab Members – 01/2016 - Present
  - Taught laboratory techniques and mouse colony management to undergraduate and graduate students, lab technicians, and research associates.
  - Assisted undergraduate students with poster design and presentation, literature search, paper reading comprehension, and thesis writing.

* Monsanto Intern Program – 12/2013 - 12/2014
- Mentored various interns from R&D and Marketing areas.
- Collaborated with stakeholders from several areas to develop an onboarding and mentoring program for incoming interns.

OUTREACH

* Biology Graduate Student Organization Outreach Committee – Member. August 2017 – May 2020.

AFFILIATIONS

* Biology Graduate Student Organization:
  President (May 2018 – May 2020): Acted as a liaison between graduate students, faculty, and the chair of the department; responsible for implementing directives from the university-wide graduate student organization; responsible for the budgeting and finances of the organization; led and supported five direct reports and oversaw three committees within the organization; organized annual elections; ensured that all members and activities upheld the organization’s constitution.
  Chair of Invited Speaker Committee (August 2017 – August 2018): Led the planning of the Biology department’s annual Life Science Symposium.
* Society for Neuroscience (SfN). Member, July 2017 – present.
* American Society for Neurochemistry (ASN). Member, February 2018 – present.
* Future Professorial Program. Syracuse University. Member, August 2015 – present.

MISCELLANEOUS

* Experience using ImageJ, SPSS, GraphPad Prism, and Adobe Creative Suite (Photoshop, Illustrator, InDesign).
* Intermediate Spanish.
* Native Portuguese speaker.