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Assessment Of A Bdnf Mimetic To Improve Behavioral Deficits In Female Mecp2 Heterozygous Mice, A Model Of Rett Syndrome

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ASSESSMENT OF A BDNF MIMETIC TO IMPROVE BEHAVIORAL DEFICITS IN FEMALE *Mecp2* HETEROZYGOUS MICE, A MODEL OF RETT SYNDROME

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BIOLOGY PROGRAM

ABSTRACT

Rett syndrome (RTT) is a neurodevelopmental disorder representing the second most common cause of intellectual disability in women. Most RTT cases are caused by mutations in the gene coding for the transcriptional regulator Methyl-CpG-binding Protein 2 (MeCP2). The neurotrophin BDNF (Brain-Derived Neurotrophic Factor) has therapeutic potential for RTT because BDNF levels are lower in RTT autopsy brains and in *Mecp2* deficient mice, while conditional *Bdnf* deletion results in phenotypes similar to those of *Mecp2* knockout (KO) mice. In addition, *Bdnf* overexpression in forebrain excitatory neurons in *Mecp2* KO mice significantly improved their lifespan, locomotor function, and brain weight. However, BDNF has low blood-brain barrier permeability and potential undesired effects by activation of the p75 neurotrophin receptor. Prompted by these limitations, a search for small molecule mimetics of the BDNF loop domain yielded the compound LM22A-4, which selectively activates the BDNF receptor TrkB. LM22A-4 improves respiratory function, object location memory, and hippocampal synaptic plasticity in female *Mecp2* heterozygous (HET) mice. Recently, our lab reported social memory deficits and atypical social behaviors in male *Mecp2* KO mice. To expand on these findings, we tested whether female *Mecp2* HET mice exhibit similar deficits, with the aim of testing improvement by a 4-week treatment with LM22A-4. In this study, all mice were tested in two social interactions tasks: a novel Unrestricted Social Interaction assay and the

standard Three-Chamber social test. We found that female *Mecp2* HET mice display RTT-like disease progression, with four-month-old mice exhibiting transient atypical social behaviors not observed in six-month-old mice, and the worsening of two other behaviors: increased time in repetitive stereotypy-like digging and fear-induced shuffling. LM22A-4 also reduced the atypical repetitive digging behavior in female *Mecp2* HET mice, reaching levels observed in female wild-type mice. However, LM22A-4 did not affect social behaviors in both genotypes. Overall, these results define a set of behavioral deficits that occur transiently during disease progression in female *Mecp2* HET mice, and others that progress with a slower time course. In addition, LM22A-4 decreased atypical repetitive behaviors in female *Mecp2* HET mice, without affecting typical behaviors in female wild-type mice, underscoring its potential therapeutic value.

Keywords:

Rett syndrome, MeCP2, social behavior, BDNF, TrkB

DEDICATION

To my parents, Haydee Baylon and Lenin Mejia, whose love, support and encouragement shaped my life and continue to help me navigate life's many turns.

To my best friend, Hailey Egido-Betancourt, for the never-ending encouragement to live and enjoy life to the fullest and for showing me that anything is possible if you dream it.

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LIST OF ABBREVIATIONS

BDNF	Brain-Derived Neurotrophin Factor
HET	Heterozygous
KO	Knockout
MeCP2	Methyl CpG-binding Protein 2
RTT	Rett Syndrome
TrkB	Tropomyosin-related kinase B
WT	Wild-type
XCI	X-Chromosome Inactivation

INTRODUCTION

Rett Syndrome

Rett syndrome (RTT) is a neurodevelopmental disorder associated with intellectual disability, neurological symptoms, and autistic features that affects 1:10,00 females worldwide (reviewed by Berger-Sweeney, 2011; Chapleau et al., 2013). First noted by Austrian pediatrician Andreas Rett in the 1960's, it became more widely recognized in the 1980's and currently has diagnostic criteria that are continuously updated (reviewed by Banerjee, Castro, & Sur, 2012; Neul et al., 2010; Pozzo-Miller, Pati, & Percy, 2015). This neurodevelopmental disorder is typically caused by loss-of-function mutations in the *MECP2* gene, which encodes methyl-CpG-binding protein-2 (MeCP2), located on chromosome Xq28 (reviewed by Li & Pozzo-Miller, 2014). Due to the X chromosome location of *MECP2*, male individuals with RTT are rare and usually have a 47, XXY karyotype (reviewed by Gonzales & LaSalle, 2010). Afflicted individuals develop typically for the first 6-18 months of age and then enter a regression stage and lose previously-acquired cognitive and motor skills (reviewed by Katz et al., 2012; reviewed by Ricceri, De Filippis, & Laviola, 2013). Although this regression eventually stabilizes, RTT individuals develop prominent afflictions, such as severe intellectual disability, lack of communication skills, repetitive hand movements (stereotypies), gait problems, gastro-intestinal problems, respiratory and cardiac abnormalities, and seizures, with some afflicted individuals entering another stage of motor decline later in life (reviewed by Katz

et al., 2012; reviewed by Ricceri et al., 2013). Currently, therapies are only aimed at alleviating the most severe RTT symptoms, such physical therapy and surgery for scoliosis and anti-epileptic medication for seizures, and RTT individuals commonly survive until middle age, with some living even longer (Chapleau et al., 2013; National Institute of Child Health and Human Development, 2016). Although there is no cure for RTT, therapeutic progress continues to be made, providing hope that a cure could be possible in the near future (reviewed by Katz et al., 2012).

Phenotypic Variance

MECP2 is subject to a X-linked inheritance pattern, with females obtaining a maternal and paternal chromosome and males obtaining only a maternal chromosome (reviewed by Gonzales & LaSalle, 2010). Additionally, *MECP2* is subject to X-Chromosome Inactivation (XCI) in females: a gene silencing process that makes female expression pattern similar to those in males by silencing or inactivating one of the X chromosomes (reviewed by Gonzales & LaSalle, 2010). XCI is a random process occurring in every cell, and it likely accounts for some of phenotypic variability seen in RTT individuals, with higher wild-type *MECP2* gene expression resulting in milder RTT symptoms and higher mutant *MECP2* gene expression resulting in more severe RTT symptoms (reviewed by Gonzales & LaSalle, 2010). As with all X-linked disorders, afflicted RTT males, who express only the mutant *MECP2* gene, are more severely affected and have low survival rates, explaining the female prevalence seen in RTT (reviewed by Gonzales & LaSalle, 2010; reviewed by Na & Monteggia, 2011). Surviving RTT males typically have the 47, XXY karyotype and undergo XCI similarly to females

(reviewed by Gonzales & LaSalle, 2010). Although XCI likely accounts for a good portion of RTT phenotypic variability, other differences arise from the types of *MECP2* mutations. Most mutations arise spontaneously (de novo) in the paternal chromosome and more than 250 different *MECP2* mutations have been identified in RTT individuals, with loss-of-function *MECP2* mutations accounting for a majority of RTT cases (reviewed by Calfa, Percy, & Pozzo-Miller, 2011; reviewed by Katz et al., 2012).

MeCP2

MeCP2, or Methyl-CpG-binding Protein 2, is a transcriptional regulator with genome-wide distribution and ubiquitous expression in mammalian tissues (reviewed by Calfa et al., 2011). Although best known for its transcriptional repressor role of binding to methylated DNA at CpG dinucleotides and to ultimately form tightly-bound chromatin complexes, MeCP2 can also bind to promoter regions and promote gene activation (reviewed by Berger-Sweeney, 2011). MeCP2 is found in remarkably high levels in the brain, with mature neurons having the highest expression; although neurons initially have low protein levels, MeCP2 levels continuously increases during post-natal neural development (reviewed by Gonzales & LaSalle, 2010; Guy, Cheval, Selfridge, & Bird, 2011). This expressional timeline suggests a role in the synaptogenesis and synaptic pruning of differentiating neurons (reviewed by Berger-Sweeney, 2011; Guy et al., 2011). Loss of MeCP2 function in RTT individuals results in smaller neuronal sizes as well as shorter axonal and dendritic processes and lower dendritic spine density (reviewed by Calfa et al., 2011; reviewed by Na & Monteggia, 2011). Moreover, hippocampal MeCP2-deficient neurons have fewer dendritic spines and reduced arborization (reviewed by Na &

Monteggia, 2011). Additionally, long-term synaptic plasticity, a widely-accepted cellular basis for learning and memory, is negatively impacted by changes in MeCP2 expression (reviewed by Na & Monteggia, 2011). These abnormalities likely contribute to the cognitive impairments seen in RTT individuals and further demonstrate MeCP2's role on synaptogenesis and the development of cortical networks and behavior (reviewed by Na & Monteggia, 2011).

It should be noted that MeCP2 is implicated in other neurodevelopmental disorders, such as *MECP2* duplication syndrome, a disease characterized by higher *MECP2* expression levels and the development of severe intellectual disability, progressive spasticity and susceptibility to respiratory infections in males (with milder symptoms in females) (reviewed by Gonzales & LaSalle, 2010). This emphasizes the need for precise regulation of *MECP2* expression for proper neuronal development and the challenges of rescuing MeCP2 dysfunction (reviewed by Gonzales & LaSalle, 2010).

RTT Mouse Models

To assess the role of MeCP2 on brain development and RTT symptomatology and neuropathology, several mouse models have been created, including mice with global mutations and even cell-type-specific mutations (reviewed by Calfa et al., 2011; Guy et al., 2011). Because both human and mouse *MECP2*/*Mecp2* genes contain four exons, the Cre recombinase-loxP system was used to render exons 4 and/or 3 non-functional, creating two of the most commonly used global *Mecp2* mutations (*Mecp2*$^{tm1.1Bird}$ and *Mecp2*$^{tm1.1Jae}$, respectively) (reviewed by Calfa et al., 2011; Guy et al., 2011). These mouse models result in the creation of *Mecp2* hemizygous knockout (KO) males and *Mecp2* heterozygous

(HET) females. Considering that RTT mutations are typically loss-of-function, these global *Mecp2* null mutations have construct validity (reviewed by Katz et al., 2012). Moreover, male *Mecp2* KO mice develop phenotypes consistent with RTT symptomatology, such as atypical gait with splaying hind limbs, hind limb clasping upon tail suspension, disheveled fur and erected whiskers, labored breathing, tremors and seizures (reviewed by Calfa et al., 2011). Additionally, this phenotype progresses with time, becoming evident around the 4th-5th week of life and worsening until death around 10 weeks of life, and involves varied neurological features, such as abnormal social and anxiety-related behavior and learning and memory deficits (reviewed by Calfa et al., 2011).

As male *Mecp2* KO mice show a complete lack of *Mecp2* expression, they are useful in revealing *Mecp2*-related mechanisms without interference from other cofounding factors (Samaco et al., 2013). However, this true knockout or null condition in male mice is rarely seen in RTT individuals, possibly accounting for 10% of RTT individuals at most (reviewed by Katz et al., 2012). Considering that male *Mecp2* KO mice display severe phenotypes and short life spans, and that most afflicted RTT individuals are females displaying genetic mosaicism, female *Mecp2* HET mice are better candidates for clinical studies (reviewed by Katz et al., 2012; Samaco et al., 2013). Similarly to female RTT individuals, female *Mecp2* HET mice undergo XCI and display mosaic *Mecp2* expression also resulting in diverse phenotypes (reviewed by Katz et al., 2012). Nevertheless, phenotypic variation among individual mice in addition to delayed disease progression (e.g., hind limb clasping takes six months or longer to become evident) has made the use of female *Mecp2* HET mice scarce in research studies (Stearns et al., 2007). To fill this

gap, female *Mecp2* HET mice usage in pre-clinical studies has increased in recent years (reviewed by Katz et al., 2012).

Therapeutic Progress

Despite its disease severity, several findings have instilled hope that most RTT symptoms and phenotypes could be reversed with effective therapies. Evidence indicates that RTT is not a neurodegenerative disorder: RTT brain autopsies do not display neuropathological changes, atrophy, demyelination, nor neural degeneration (reviewed by Katz et al., 2012). In addition, reactivation of silent *Mecp2* alleles reversed Rett-like symptoms in a mouse model (reviewed by Berger-Sweeney, 2011; reviewed by Katz et al., 2012). Nevertheless, other transgenic lines have shown that *Mecp2* overexpression also results in the development of RTT-like phenotypes (reviewed by Berger-Sweeney, 2011; reviewed by Ricceri et al., 2013). For this reason, the prospect of *MECP2* gene therapy has proved even more difficult, requiring not only the development of safe delivery vectors but the avoidance of *MECP2* overexpression in wild-type cells of the mosaic RTT brain (reviewed by Pozzo-Miller et al., 2015).

Nevertheless, advances have been made toward treatment avenues other than gene therapy. One therapeutic aim is extending MeCP2's half-life to increase its expression time before its degradation (reviewed by Chapleau et al., 2013). Another therapeutic target focuses in the modulation of neurotransmitters (reviewed by Ricceri et al., 2013). Several RTT mouse models have revealed physiological impairments: hyperexcitability in the hippocampus and brainstem, synaptic hypoconnectivity in the cerebral cortex, and transmitter release dysregulation in hippocampal neurons (reviewed by Ricceri et al.,

2013). Therefore, some treatments are aimed at modulating glutamate levels to reduce hippocampal hyperexcitability through the use of a weak NMDA receptor blockers (reviewed by Ricceri et al., 2013). Similarly, there is also interest in the use of selective GABA uptake inhibitors, such as NO-711, which was shown to reduce breathing irregularity in a mouse model (reviewed by Ricceri et al., 2013).

Other therapeutics bypass MeCP2 and focus on its downstream targets (reviewed by Ricceri et al., 2013). MeCP2 regulates various important genes, such as the gene encoding Brain Derived Neurotrophic Factor (BDNF), a neurotrophic factor playing a role in neuronal survival and differentiation, synaptic plasticity, and cognition (reviewed by Berger-Sweeney, 2011; Schaevitz, Moriuchi, Nag, Mellot, & Berger-Sweeney, 2010). Although their precise regulatory mechanism is not well defined, there is evidence of their relationship: low *BDNF/Bdnf* expression was observed in RTT brain samples and *Mecp2* mutant mouse brains (reviewed by Li & Pozzo-Miller, 2014). Additionally, *Bdnf* overexpression improved RTT-like phenotypes in male *Mecp2* KO mice (longer lifespan, improved locomotion, increased brain weight) and reverse impaired axonal and dendritic complexity, further illustrating BDNF's therapeutic potential (Chang, Khare, Dani, Nelson, & Jaenisch, 2006; reviewed by Li & Pozzo-Miller, 2014).

The major limitations of all BDNF-based therapies are BDNF's low blood-brain permeability, short half-life, and potential for undesired side-effects upon interaction with neurotrophin receptor p75 (NTR), such as coupling with apoptosis-inducing mechanisms (Massa et al., 2010). These difficulties led to the search for BDNF "mimetics." One of them, LM22A-4, selectively targets and activates the BDNF receptor tropomyosin-related kinase B (TrkB) receptor (Massa et al., 2010). This TrkB partial-agonist prevented *in vitro*

cell death in models of Alzheimer, Huntington, and Parkinson diseases, improved motor learning in rats with traumatic brain injury, and improved RTT-like phenotypes in mice such as abnormal respiratory functions and object location memory, showing promise as a therapeutic method (Li et al., 2017; Massa et al., 2010; Schmid et al., 2012).

APPROACH

RTT individuals display cognitive deficits, a trait that is also present in several *Mecp2*-based mouse models such as impaired contextual fear conditioning and object recognition (reviewed by Katz et al., 2012). Recently, the Pozzo-Miller laboratory demonstrated that male *Mecp2* KO mice display atypical general behaviors and social memory deficits through the use of the established Three-Chamber assay and a novel Unrestricted Social Interaction assay (an open field that allows test mice to interact freely with familiar and novel sentinels for unbiased behavioral sorting and scoring) (Phillips, Robinson, & Pozzo-Miller, 2018). Our laboratory also demonstrated that a one-month-long LM22A-4 drug treatment on female *Mecp2* HET mice restored object location memory (Li et al., 2017). Our lab set out to determine whether these atypical behaviors and social memory deficits seen in male *Mecp2* KO mice are also exhibited by female *Mecp2* HET mice, and whether LM22A-4 improves them.

We hypothesized the behavioral differences observed in male *Mecp2* hemizygous (KO) mice are also present in female *Mecp2* heterozygous (HET) mice and that LM22A-4 treatment improves these atypical behaviors. To answer these questions, we treated female HET and wild-type mice for 30 days with either LM22A-4 or saline solution by intraperitoneal injections followed by two behavioral assays (Unrestricted Social Interaction and Three-Chamber Social assays, with the use of two mouse sentinels to detect any social memory deficits). We tested 4-month-old female mice and 6-month-old female mice to explore differences on more severe phenotypes.

MATERIALS AND METHODS

Mice

Female mice lacking exon 3 of *Mecp2* (B6.Cg-*Mecp2*$^{tm1.1Jae}$, Jaenisch strain in a pure C57BL/6 background) were purchased from the Mutant Mouse Regional Resource Center (University of California, Davis) and a colony was generated and maintained at the University of Alabama at Birmingham by mating *Mecp2*$^{tm1.1Jae}$ heterozygous female mice with wild-type C57BL/6 males. All experimental subjects were female *Mecp2*$^{tm1.1Jae}$ heterozygous mice (HET mice) and female wild-type littermates (WT mice) around 4 months of age (130 days ± 7 days) and 6 months of age (190 ± 7 days) at the start of treatment. Additionally, other age-matched female wild-type littermates were used as sentinels for behavioral experiments. Mice were handled and housed according to the Committee on Laboratory Animal Resources of the National Institutes of Health. All experimental protocols were reviewed and approved annually by the Institutional Animals Care and Use Committee of the University of Alabama at Birmingham.

LM22A-4 Treatment

Female *Mecp2*$^{tm1.1Jae}$ HET mice and age-matched WT littermates were given intraperitoneal (i.p.) injections of either sterile LM22A-4 (50mg/kg) or vehicle/saline (0.9% NaCl) solution twice a day for 30 days prior to behavioral assays, following a previously established dosing regimen (Li et al., 2017; Schmid et al., 2012). Mice were randomly allocated to either treatment (LM22A-4 or saline). Researcher was blind to treatment types.

Unrestricted Social Interaction Behavioral Assay

This behavioral assay allowed us to explore mouse behavior without any restrictions, as the test mouse is allowed to freely interact with one or two sentinels for 10 minutes, in a 12 in. x 16 in. plexiglass open box containing about 5 full "scoops" of clean rodent bedding from a 500 mL beaker. For the 4-month-old test mice, the behavioral assay used novel sentinels, which were selected and housed separately. For the 6-month-old test mice, the behavioral assay used familiar and novel sentinels, which were selected and randomly assigned as familiar or novel. Familiar sentinels were housed with test mice at the start of treatments.

Four to 7 days prior to behavioral testing, all test mice and sentinels were brought to the testing location (a room specifically set up to run behavioral testing) for location acclimation. During this time, sentinels underwent a hair dying process (with Born Blonde Maxi, Clairol) to display distinct patterns on their backs for computer video tracking and placed back into their original cages. Three days prior to the Unrestricted Social Interaction Assay, mice were acclimated to the field daily. This 3-day acclimation period started with mouse handling (i.e. mice hand holding in the palm of hand) for 3 minutes per test mouse. Each mouse was then placed in the middle of the field and allowed to interact with it for 10 minutes. Next, test mice were placed into empty cages as they finished acclimation, and the used rodent bedding was discarded and replenished for the next test mouse. Finally, after all test mice were acclimated, they were placed back into their original cages.

On the day of Unrestricted Social Interaction testing, sentinels were placed on an empty cage if needed or applicable. Similarly to the acclimation days, mice handling was performed; then, each test mouse was placed in the middle of the field and filmed for 10

minutes while it freely roamed the open field. Next, one familiar cage-mate sentinel and one novel sentinel (or just one novel sentinel for the 4-month-old test mice) were added to the field and all mice are allowed to freely interact with each other, while being filmed for an additional 10 minutes. Then, the test mouse was removed and placed in an empty cage and the sentinels were returned to their corresponding empty cages. Used bedding was discarded and replaced for the next test mouse. Sentinels were alternated to avoid tiring them out. Sentinels were individually filmed for five minutes each on an additional day, to create identity videos. Finally, once all test mice were assayed, test mice and sentinels were returned to their original cages.

All handling and testing were performed in the dark phase of the 12 h : 12 h light/dark cycle through the use of a red headlamp and video acquisition was obtained through infrared illumination with a Basler acA780-75gm camera with a Edmund Optics 5800 C-mount lens (Phillips et al., 2019). Individual identity and test videos were run through the *Motr* program (Ohayon, Avni, Taylor, Perona, & Roian Egnor, 2013; Phillips et al., 2019) to create track files that were exported to Janelia Automatic Animal Behavior Annotator (*JAABA*) (Kabra, Robie, Rivera-Alba, Branson, & Branson, 2012; Phillips et al., 2019) for unbiased computer identification of behaviors. JAABA classifiers have been previously trained on pilot data sets (Phillips et al., 2019). Nineteen behaviors were scored and analyzed. Locomotive behaviors analyzed were wall climbing, wall jumping, shuffling, and walking. General behaviors analyzed were digging, freezing, front grooming, rear grooming, scratching, and air sniffing. Aggressive social behaviors analyzed were fighting and chasing. Finally, non-aggressive social behaviors analyzed were following, face following, being followed by sentinel, rear sniffing, nose sniffing,

side sniffing and jumping on. Additionally, two other behaviors were analyzed in the 6-month-old female *Mecp2* HET mice: fighting and chasing initiated by a sentinel.

Three-Chamber Social Assay

This assay was adapted from Yang, Silverman, & Crawley (2011) with a 3-minute middle chamber acclimation period. The Three-Chamber apparatus is a plexiglass rectangular open box separated into three connected chambers. These three chambers are connected through "doorways" or openings in the middle chamber to allow the mice to travel back and forth into the other chambers. These openings can be blocked with other plexiglass pieces as needed. Our apparatus is 23 in. x 11 in., with two 10 in. outer chambers and a 3 in. middle chamber. The Three-Chamber test started with a non-filmed 3-minute middle chamber acclimation, with blocked doorways. Then, the doorways were unblocked, and the test mouse was allowed to explore all three chambers, while being filmed for 10 minutes. This time was used to assess any chamber preference; any test mice spending more than 75% of the acclimation time in any chamber was placed into another cage, to await a second and final acclimation attempt after all other test mice had completed testing. Mice showing no chamber preference were allowed to continue to be filmed and assayed for social preference (or sociability) and social memory. Sentinels used for this test were housed separately and trained daily, for at least 1 week prior to testing, to stay inside the Three-Chamber mesh cages/cups without rattling the cups or causing any other disturbances.

For the sociability assay, the mouse was first allowed to return to the middle chamber and, then, enclosed. Next, an empty mesh cage/cup was placed in the center of

one of the chambers, while a mesh cage/cup containing Sentinel 1 (one of the trained Three-Chamber sentinels) was placed in the middle of the opposite chamber. Two flasks were placed on top of the mesh cups to prevent the test mice from climbing and sitting on top of the mesh cups. Then, the doorways were unblocked, again, and the test mouse was allowed to explore the chambers again for 10 minutes, while being filmed. For the social memory assay, the mouse was allowed to return to the middle chamber and, then, enclosed. Next, Sentinel 2 (another trained Three-Chamber sentinel) was placed in the previously-empty mesh cup. Afterward, the doorways were unblocked again, and the test mouse was filmed and allowed to explore for another 10 minutes. The test mouse was then taken out of the chamber and placed in an empty cage, while the sentinels were placed back into the sentinel-housing cage. Finally, the whole apparatus, including the mesh cups, beakers and plexiglass "door" pieces, was wiped clean with isopropyl alcohol in preparation for the next test mouse.

Test mice were acclimated to the testing location for at least 4 days prior to behavioral testing. Mice handling was performed every day prior to acclimation and testing. All handling and testing were performed in the dark phase of the 12 h : 12 h light/dark cycle as described above (Phillips et al., 2019). Sentinels were randomly assigned to starting chamber and alternated to avoid exhausting them. Interaction times with each cup/sentinel were manually recorded with a digital timer when test mice were within close proximity and physically sniffing, touching and/or climbing the mesh cups.

Statistical Analyses

All statistical analyses were performed in R programming, post behavioral testing. Graphs were created using Prism 8 (GraphPad). Unrestricted Social Interaction behavior times were obtained from JAABA in MATLAB (MathWorks) and Three-Chamber times were manually recorded. Based on the use of two genotypes and two treatment types, four groups were created for comparison: Wildtype LM22A-4, Wildtype Saline, Heterozygous LM22A-4, Heterozygous Saline, with LM22A-4 and Saline representing mice who underwent LM22A-4 and vehicle/saline treatments, correspondingly. One-Way ANOVA and post-hoc Tukey's 'Honest Significant Difference' were performed on datasets with normal distribution and homogenous variances. Welch's ANOVA and post-hoc pairwise t-tests comparison with non-pooled S.D. and Holm-Bonferroni corrections were performed on datasets with normal distribution and heterogenous variances. Kruskal Wallis rank sum test and post-hoc pairwise Wilcoxon rank sum tests with Holm-Bonferroni corrections were performed on datasets with non-normal distribution.

Graphs display only statistical group comparisons of interest: WT Saline versus HET Saline, HET Saline versus HET LM22A-4, WT Saline versus WT LM22A-4. WT Saline versus HET Saline comparisons will serve to determine any genotypic differences. HET Saline versus HET LM22A-4 comparisons will serve to assess any treatment impact. Finally, WT Saline versus WT LM22A-4 comparisons will serve to ascertain any negative treatment impact.

Behavioral assays with two dependent variables (such as time spent with familiar sentinel versus time spent with novel sentinel) were represented and analyzed through the use of a discrimination index ($\frac{(T_1 - T_2)}{(T_1 + T_2)}\ x\ 100\%$, with T_1 representing time spent with mouse

cup, sentinel 2 or novel sentinel and T_2 representing time spent with empty cup, sentinel 1, or familiar sentinel, correspondingly) for Social Memory comparisons, or through the use of Total Time doing the behavior (T_1+T_2) for behavioral comparisons. Statistical tests used for experiments were stated in the main text and within its associated figure legend. Significance was defined as $p < 0.05$, with the specific statistical test provided in main text or within associated figure legend. Significance conventions are as follows: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Sample sizes (n) refers to the number of animals. Sample sizes are provided in the main text and within associated figure legend. Behavioral data is represented as mean +/- SD.

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RESULTS

Four-month-old Female *Mecp2* Heterozygous Mice Display Atypical Social Behaviors

To assess behavioral differences between female wild-type (WT) and *Mecp2* heterozygous (HET) mice, we first used the Unrestricted Social Interaction Assay with 4-month-old female mice (130 ± 7 days), an assay previously used by our laboratory to demonstrate social impairments in male *Mecp2* knockout (KO) mice (Phillips et al., 2019). Our laboratory has previously identified and classified a variety of behaviors in *JAABA*, allowing us to compare many behaviors from just one test (Phillips et al., 2019). We analyzed scores for nineteen behaviors.

Female *Mecp2* HET mice spent more time than female WT mice on non-aggressive social behaviors (nose sniffing and face following) and fighting, an aggressive social behavior (Figure 1), demonstrating that female *Mecp2* HET mice show atypical social interactions. In addition, female *Mecp2* HET mice spent less time than female WT mice on general and locomotive behaviors, such as digging, wall climbing, and air sniffing (Figure 2). These results are in line with our previous observations of atypical social behaviors found in male *Mecp2* KO mice (Phillips et al., 2019). However, it should be noted that these studies did not find the same behavioral differences. Male *Mecp2* KO mice spent less time than male WT mice following, rear sniffing, and fighting sentinels, while female *Mecp2* HET mice were more socially active than female WT mice, spending more time face following, nose sniffing and fighting the sentinels (Phillips et al., 2019). Nevertheless, these results further indicate that *Mecp2* deletion results in impaired social interactions. In addition, female *Mecp2* HET and WT mice did not spend statistically different times

jumping on (and holding on) the sentinels, while male *Mecp2* KO mice spent more time than male WT mice jumping on the sentinels (Figure 1.A) (Phillips et al., 2019).

Furthermore, we found female *Mecp2* HET mice spent less time than female WT mice air sniffing and wall climbing, behaviors without any significant differences in male *Mecp2* KO mice, and possibly demonstrating lower environmental exploration behavior (Phillips et al., 2019). There were no time differences in wall jumping or shuffling, which were observed to be higher in male *Mecp2* KO mice (Phillips et al., 2019). Female *Mecp2* HET mice spent less time digging compared to female WT mice, a phenotype also observed in the male *Mecp2* KO mice (Phillips et al., 2019). Although this shorter digging time could be related to the severity of the RTT phenotype as a motor deficit, there were no differences in walking time between female WT mice and *Mecp2* HET mice nor between male WT mice and male *Mecp2* KO mice (Phillips et al., 2019). Together with a shorter digging time, the shorter air sniffing and wall climbing times likely represent lower environmental exploration in female *Mecp2* HET mice.

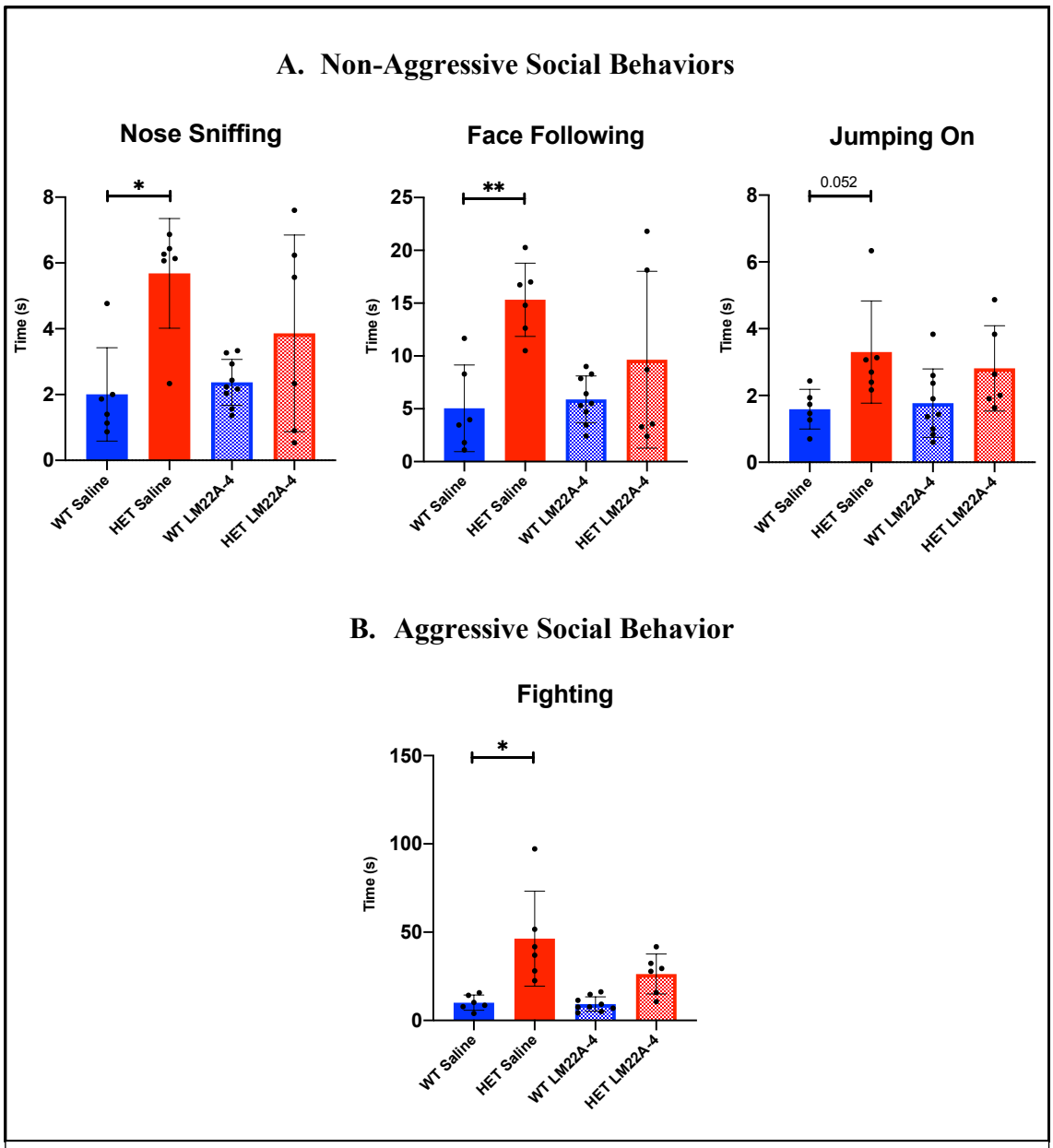

Figure 1. Atypical Social Interactions displayed in 4-month-old female *Mecp2* HET mice compared to female WT mice. A. Non-aggressive social behaviors of interest obtained from the Unrestricted Social Interaction Assay. Nose Sniffing: WT Saline-HET Saline p = 0.013, Welch's ANOVA and pairwise t-test comparisons with Holm corrections. Face Following: WT Saline-HET Saline p = 0.006, One-Way ANOVA and post-hoc TukeyHSD analysis. Jumping On: WT Saline-HET Saline p = 0.052, Kruskal Wallis rank sum test and post-hoc Wilcoxcon rank sum test with Holm corrections. **B.** Aggressive social behaviors of interest obtained from the Unrestricted Social Interaction Assay. Fighting: WT Saline-HET Saline p = 0.011, Kruskal Wallis rank sum test and post-hoc Wilcoxcon rank sum test with Holm corrections. WT Saline n = 6; HET Saline n = 6; WT LM22A-4 n = 9; HET LM22A-4 n = 6.
*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Mean ± S.D.

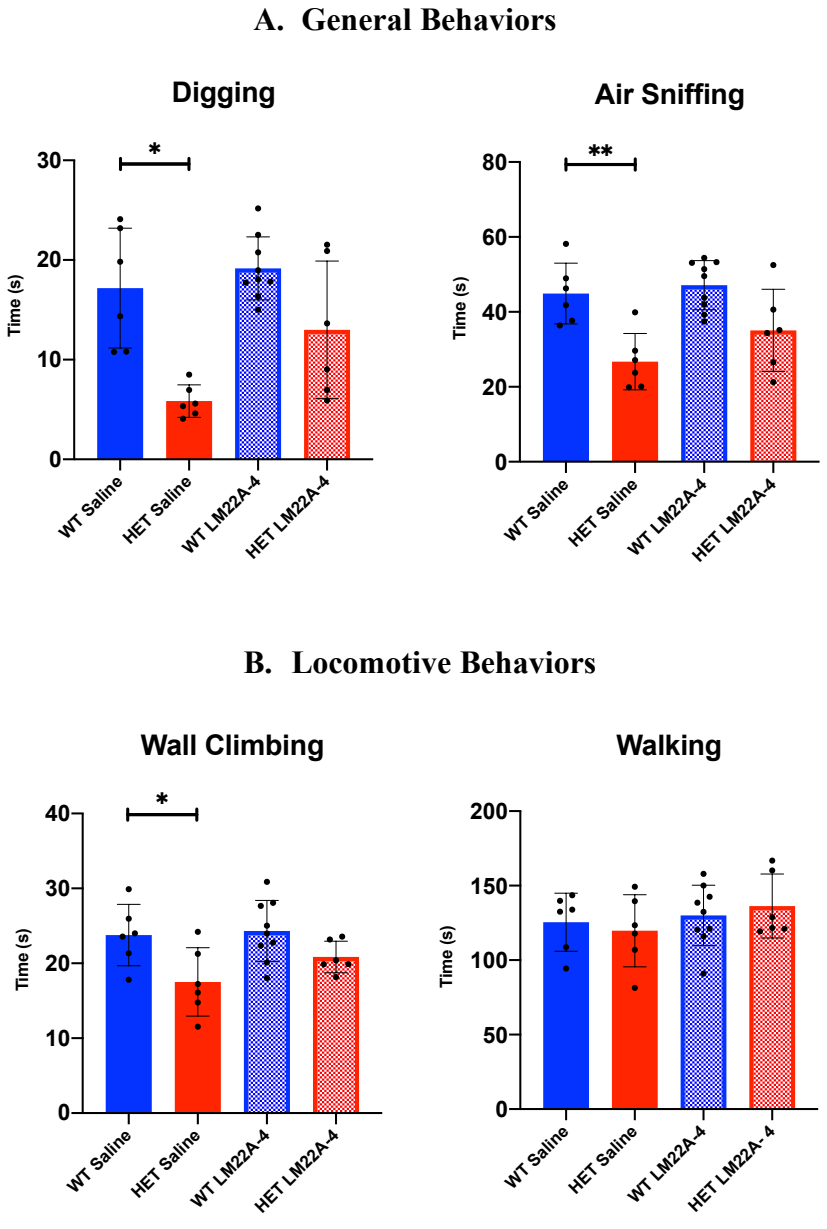

Figure 2. Atypical behaviors displayed in 4-month-old female *Mecp2* HET mice compared to female WT mice. A. General behaviors of interest obtained from the Unrestricted Social Interaction Assay. Digging: WT Saline-HET Saline p = 0.024, Welch's ANOVA and pairwise t-test comparisons with Holm corrections. Air Sniffing: WT Saline-HET Saline p = 0.004, One-Way ANOVA and post-hoc TukeyHSD analysis. **B.** Locomotive behaviors of interest obtained from the Unrestricted Social Interaction Assay. Wall Climbing: WT Saline-HET Saline p = 0.046, One-Way ANOVA and post-hoc TukeyHSD analysis. Walking: p = n.s, One-Way ANOVA. WT Saline n = 6; HET Saline n = 6; WT LM22A-4 n = 9; HET LM22A-4 n = 6. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Mean ± S.D.

Disease Progression in Six-month-old Female *Mecp2* Heterozygous Mice

We used 6-month-old females (190 ± 7 days), that display more severe Rett-like phenotypes, and, to compare sociability and social memory differences between female WT and *Mecp2* HET mice simultaneously, we used the Unrestricted Social Interaction Assay with two age-matched sentinels: one familiar cage-mate sentinel and one novel sentinel. Out of a total 10 min. session, we obtained two measures of interaction time: time spent interacting with the familiar sentinel and time spent interacting with the novel sentinel. We analyzed the scores for 21 behaviors: the same 19 behaviors measured in the 4-month-old test mice, with the addition of fighting and chasing initiated by a sentinel mouse.

We expected to find the same behavioral phenotypes as the 4-month-old female *Mecp2* HET mice, with a progressive worsening due to age. Surprisingly, none of the atypical social behaviors nor the general and locomotive behavioral differences (air sniffing and wall climbing) were present (Figure 3.C). Although there was a difference in digging time, at 6 months of age, female *Mecp2* HET mice spent more time digging than female WT mice (Figure3.A). These results suggest that female *Mecp2* HET mice develop compulsive obsessive-like digging over time, similar to the stereotypies (repetitive hand movements) in RTT individuals. In addition, 6-month-old female *Mecp2* HET mice spent more time than female WT mice shuffling, a running-like avoidance behavior possibly as a result of fear or anxiety (Figure 3.B). These two behavioral differences, atypical digging and shuffling times in 6-month-old female *Mecp2* HET mice, was also described in male *Mecp2* KO mice, further suggesting that these two behavioral differences are a result of

RTT-like disease progression (Phillips et al., 2019). It should be noted, that there were no differences in walking time between 6-month-old HET and WT mice (Figure 3.B).

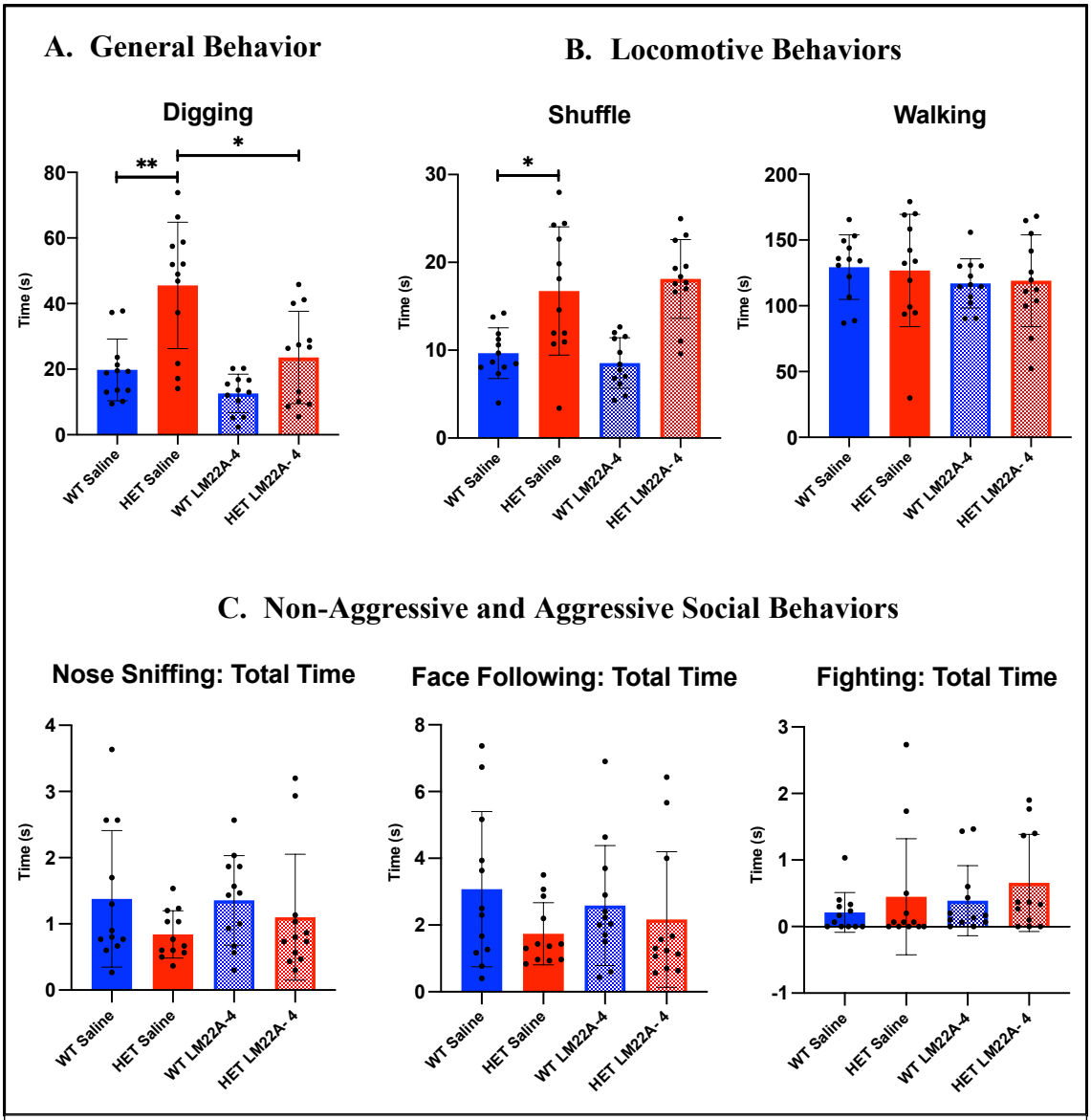

Figure 3: Unrestricted Social Paradigm reveals different set of atypical behaviors with seemingly typical social behaviors in 6-month-old female *Mecp2* HET mice. **A.** General behaviors of interest obtained from the Unrestricted Social Interaction Assay on 6-month-old female mice. Digging: WT Saline-HET Saline p = 0.004, HET Saline-HET LM22A-4 p = 0.018, Welch's ANOVA and pairwise t-test comparisons with Holm corrections. **B.** Locomotive behaviors of interest obtained from the Unrestricted Social Interaction Assay on 6-month-old mice. Shuffle: WT Saline-HET Saline p = 0.022, Welch's ANOVA and pairwise t-test comparisons with Holm corrections. Walking: p = n.s, One-Way ANOVA. **C.** Non-Aggressive and Aggressive Social Behaviors of interest obtained from the Unrestricted social Assay on 6-month-old mice. Nose Sniffing, Face Following and Fighting: p = n.s. One-Way ANOVAs. Total Times obtained by adding times doing behavior on familiar sentinel and novel sentinel. WT Saline n = 12; HET Saline n = 12; WT LM22A-4 n = 12; HET LM22A-4 n = 12. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Mean ± S.D.

Six-month-old Female *Mecp2* Heterozygous Mice Do Not Display Atypical Sociability nor Social Memory Deficit

As an addition to the Unrestricted Social interaction assay, we performed the Three-Chamber social assay in the 6-month-old test mice. The first part of the assay, the Social Preference test, showed that both female WT and female *Mecp2* HET mice spent more time interacting with the inverted mesh cup containing Sentinel 1 than with the empty mesh cup in the opposite chamber (Figure 4.A). The discrimination indexes show no sociability difference between female WT and female *Mecp2* HET mice (Figure 3.A). These results are in line with those of the Unrestricted Social Interaction assay at this age, which also showed typical social interactions (Figure 3.C).

The second part of the Three-Chamber social assay, the Social Memory Test, showed that female *Mecp2* HET mice spent more time interacting with novel Sentinel 2 than with familiar Sentinel 1, in the same way as female WT mice do (Figure 4.B). In addition, the discrimination indexes of time spent with sentinels during the Unrestricted Social Interaction behavioral assay showed that female *Mecp2* HET mice have typical social memory, similar to female WT mice (Figure 4.C).

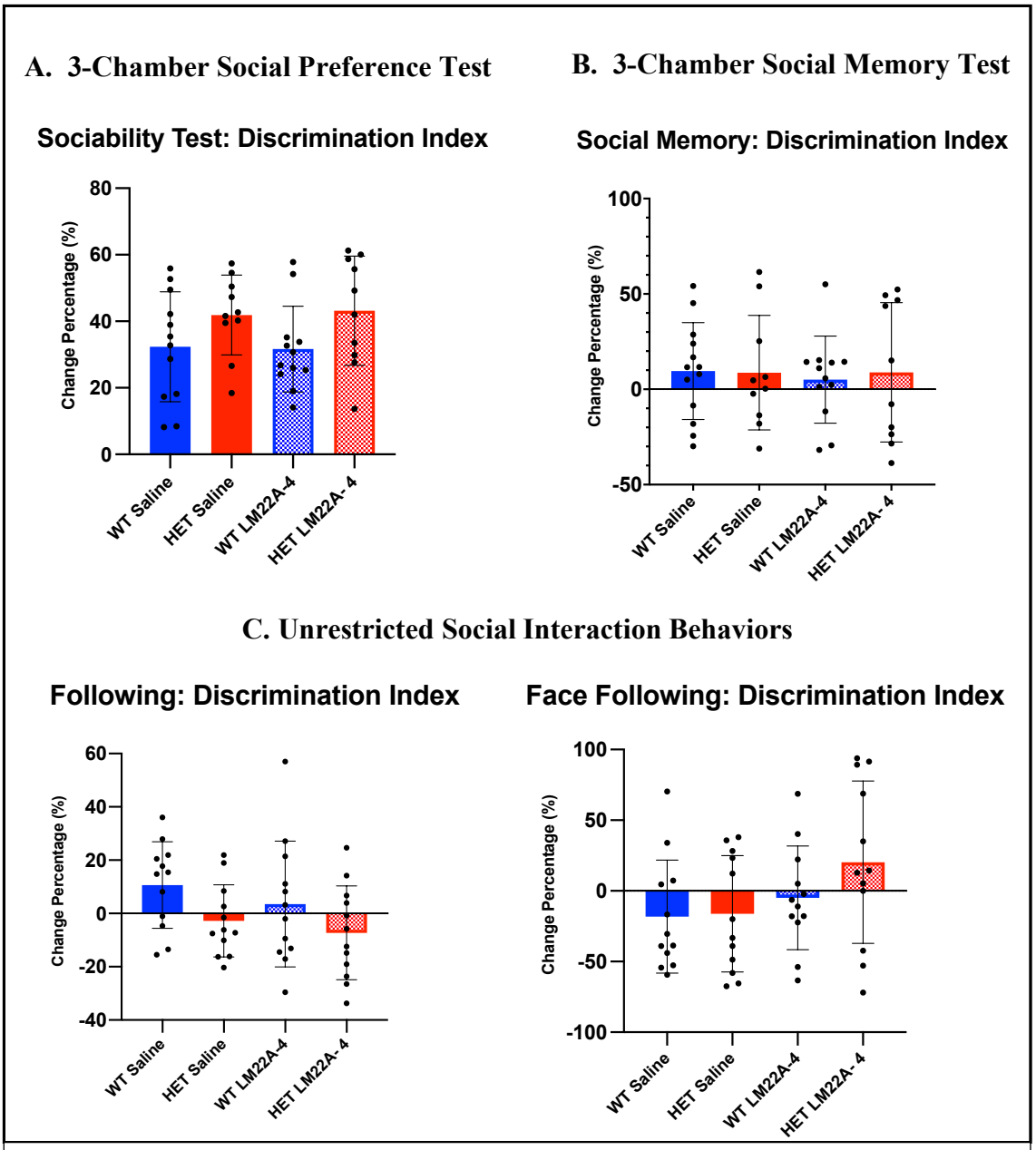

Figure 4: No Sociability or Social Memory differences between 6-month old *Mecp2* HET and WT mice. A. Social Preference Test results from the 3-Chamber Assay. Discrimination Index: p = n.s., One-Way ANOVA. WT Saline n = 12; HET Saline n = 10; WT LM22A-4 n = 12; HET LM22A-4 n = 10. **B.** Social Memory Test results from the 3-Chamber Assay. Discrimination Index: p = n.s., One-Way ANOVA. WT Saline n = 13; HET Saline n = 10; WT LM22A-4 n = 12; HET LM22A-4 n = 10. **C.** Social behaviors of interest obtained from the Unrestricted Social Interaction Assay on 6-month-old mice. Discrimination Index used. Following: p = n.s., One-Way ANOVA. Face Following: p = n.s., One-Way ANOVA. WT Saline n = 12; HET Saline n = 12; WT LM22A-4 n = 12; HET LM22A-4 n = 12.
*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Mean ± S.D.

The TrkB-ligand improves Repetitive Digging Behavior in 6-month-old Female *Mecp2* Heterozygous Mice

A 30-day treatment of twice daily LM22A-4 i.p. injections did not have any significant effects in the behaviors of either female WT or *Mecp2* HET mice at 4 months of age (Figure 1-2). On the other hand, LM22A-4 reduced the digging time spent by 6-month-old female *Mecp2* HET mice to levels similar to those observed in female WT mice (WT Saline - HET LM22A-4 $p = 0.447$, Welch's ANOVA and pairwise t-test comparisons with Holm corrections) (Figure 3.A). Furthermore, LM22A-4 did not affect the digging behavior of female WT mice (WT Saline - WT LM22A-4 $p = 0.076$, Welch's ANOVA and pairwise t-test comparisons with Holm corrections) (Figure 3.A). In addition, LM22A-4 did not have any other effect on either genotype for all other behaviors (Figure 3). Therefore, the LM22A-4 treatment had no effect on sociability nor social memory on either genotype (Figure 4).

DISCUSSION

Rett syndrome (RTT) and autism spectrum disorders share many clinical manifestations during the early stages of RTT, such as the loss of language and social skills (Moretti, Bouwknecht, Teague, Paylor, & Zoghbi, 2004). Indeed, girls with RTT display almost the same autism-related deficits and anti-social behavior as girls with autism, and RTT patients were misdiagnosed with autism prior to awareness of RTT as a distinct disorder (Moretti et al., 2004). Although RTT's autistic features are of interest in finding treatment answers, not a lot of research is focused on behavioral changes. For this reason, when Phillips et al. (2019) elucidated atypical social behaviors in male *Mecp2* KO mice, we considered it was necessary to test if those behavioral differences were also present in female *Mecp2* HET mice, as they provide the best experimental RTT model.

In this study, we used two age groups: 4-month-old and 6-month-old mice; and found that 4-month-old female *Mecp2* HET mice displayed atypical social behaviors that were not present in 6-month-old female *Mecp2* HET mice. We found that the 4-month-old female *Mecp2* HET mice spent more time "Face Following" (peculiar face grabbing and sniffing interaction), nose sniffing, and fighting than female WT mice, hence showing a preference for social interaction. Similarly, an eye-tracking study of RTT girls (mean age: 9.9 ± 6 years S.D.) indicated a preference for socially weighted stimuli, with more eye contact on people versus objects or backgrounds (Djukic & Valicenti McDermott, 2012). "Face Following" is an interesting phenotype as it does not represent social avoidance but rather an atypical social interaction. Even though these results differ from those in male *Mecp2* KO mice (less time following, rear sniffing and fighting, excibiting lower social preference), Phillips et al. (2019) also found an atypical behavior that was performed more

by male *Mecp2* KO mice: jumping on, a peculiar behavior in which the test mouse jumps on the sentinel and holds on as the sentinel moves. Although, this particular behavior was not significantly higher in female *Mecp2* HET mice, it does represent an atypical social behavior not commonly performed by mice. While we were surprised to not find these atypical social behaviors displayed in the 6-month-old female *Mecp2* HET mice, it has been suggested that social behavior deficits in RTT could improve with age (Samaco et al., 2013). Autistic features are linked to the early regression phase of RTT, and likely last through childhood (Kaufmann et al., 2012).

Other differences detected in 4-month-old female *Mecp2* HET mice were shorter digging, air sniffing, and wall climbing times in *Mecp2* heterozygous females, similar to the shorter digging times observed in male *Mecp2* KO mice (Phillips et al., 2019). These three behaviors could represent lower field-exploration drive in female *Mecp2* HET mice, as these are common mouse behaviors present during exposures to new open fields. On the other hand, these lower exploration times could be due to the test mice having spent 10 minutes in the field prior to interaction with the sentinels. In order to ensure that this lower exploration drive is due exclusevely due to genotypic differences, the test needs to be modified to not have the field acclimation prior to the sentinels being added on test day.

Analysis from the Unrestricted Social Interaction assay in 6-month-old mice, using a familiar and a novel sentinel, showed how disease progression normalized the atypical social interactions, as well as worsened other general and locomotive behaviors like digging and shuffling. Although digging could reflect exploratory behavior, it is also used as a measurement of obsessive compulsive/repetitive behaviors in marble burying assays (Angoa-Pérez, Kane, Briggs, Francescutti, & Kuhn, 2013). Considering that RTT

individuals display stereotypies (repetitive hand movements), these increased digging times could reflect sterotypy-like behavior in female *Mecp2* HET mice. In addition, the marble burying assay is sometimes used as an anxiety test, although it was recently deemed unreliable to measure anxiety and obsessive compulsive tendencies by itself and, hence, needs additional behavioral methodology for more accurate conclusions (Wolmarans, Stein, & Harvey, 2016). For this reason, it should be noted that other measurements of anxiety and/or repetitive behaviors in the Unrestricted Social Interaction behavioral assay, such as freeezing, front grooming and rear grooming, were not significantly different between female WT and *Mecp2* HET mice. Therefore, to assess whether this digging time difference is truly a representation of a stereotypy-like phenotype, further testing using other behavioral methods such as the nestlet test (which measures tearing of nest blocks) is needed (Angoa-Pérez et al., 2013).

Due to the severe communication deficits in RTT, accurate assessments of cognitive skills are difficult (reviewed by Katz et al., 2012). RTT mouse models recapitulate RTT neuropatholgy, such as smaller brain and neuronal cell bodies, higher neuronal densities, reduced dendritic and axonal arborizations, and abnormal dendritic complexity (reviewed by Katz et al., 2012). In addition, cognitive deficits have been found through the use of several tests, such as the water maze task, open field, three-chamber test, fear conditioning, and, recently, the unrestricted social interaction assay (reviewed by Katz et al., 2012) (Phillips et al., 2019). However, there are many discrepancies in these findings, even from mice carrying the same *Mecp2* mutation. For example, male *Mecp2* KO mice of the "Jaenisch" line have increased sociability and social memory through a Three-Chamber assay, while our laboratory found that these male *Mecp2* KO mice have intact sociability

and a social memory deficit using the same assay (Phillips et al., 2019; Schaevitz et al., 2010). It could be that these differences arise from how the assay was set up, with the Schaevitz study cleaning the chambers after every step while the our study cleaned after each test mouse, or by the statistical tests used, repeated measures ANOVA for the Schaevitz study versus paired t-tests for ours (Phillips et al., 2019; Schaevitz et al., 2010).

Due to these different results, we assesed whether the social memory deficit observed in male *Mecp2* KO mice was also present in female *Mecp2* HET mice, and we did not find any social memory deficits nor differences between female WT and *Mecp2* HET mice at 6 months of age. Considering that we followed the same protocols as in Phillips et al. (2019), these observations suggest that the deficit in social memory observed in male *Mecp2* KO mice is either transient (i.e. shows temporarily in female *Mecp2* HET mice younger than 4 months of age) or develops later in life (i.e. in female *Mecp2* HET mice older than 6 months of age).

Finally, the TrkB-ligand LM22A-4 did not affect the atypical social behaviors exhibited by in 4-month-old female *Mecp2* HET mice. In addition, LM22A-4 decreased repetitive digging behavior time in female *Mecp2* HET mice to levels shown by female WT mice. Considering that LM22A-4 improved other RTT-like phenotypes, such a breathing irregularity and object location memory, it could be tested as a therapeutic treatment on RTT individuals (Li et al., 2017; Schmid et al., 2012). It should be noted that LM22A-4, being a partial agonist, had no significant effects on female WT mice (Massa et al., 2010).

Overall, this study determined that female *Mecp2* HET mice displayed atypical behaviors, but typical social memory deficits. These atypical behaviors in 4-month-old

female *Mecp2* HET mice are transient and seem to normalize by 6 months of age. On the other hand, other behaviors worsen or appear after 6 months of age. In addition, the TrkB-ligand LM22A-4 rescues repetitive digging behavior in female *Mecp2* HET mice, which provides more evidence of the therapeutic potential of LM22A-4, although more studies are needed.

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