Exploration of group II metabotropic glutamate receptor modulation in mouse models of Rett syndrome and *MECP2* Duplication syndrome

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Abstract

Rett syndrome (RTT) and *MECP2* Duplication syndrome (MDS) have opposing molecular origins in relation to expression and function of the transcriptional regulator Methyl-CpG-binding protein 2 (MeCP2). Several clinical and preclinical phenotypes, however, are shared between these disorders. Modulation of MeCP2 levels has recently emerged as a potential treatment option for both of these diseases. However, toxicity concerns remain with these approaches. Here, we focus on pharmacologically modulating the group II metabotropic glutamate receptors (mGlu), $mGlu_2$ and $mGlu_3$, which are two downstream targets of MeCP2 that are bidirectionally affected in expression in RTT patients and mice ($Mecp2^{Null/+}$) versus an MDS mouse model ($MECP2^{Tg1/o}$). $Mecp2^{Null/+}$ and $MECP2^{Tg1/o}$ animals also exhibit contrasting phenotypes in trace fear acquisition,

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a form of temporal associative learning and memory, with trace fear deficiency observed in *Mecp2^{Null/+}* mice and abnormally enhanced trace fear acquisition in *MECP2^{Tg1/o}* animals. In *Mecp2^{Null/+}* mice, treatment with the mGlu$_{2/3}$ agonist LY379268 reverses the deficit in trace fear acquisition, and mGlu$_{2/3}$ antagonism with LY341495 normalizes the abnormal trace fear learning and memory phenotype in *MECP2^{Tg1/o}* mice. Altogether, these data highlight the role of group II mGlu receptors in RTT and MDS and demonstrate that both mGlu$_2$ and mGlu$_3$ may be potential therapeutic targets for these disorders.

Graphical Abstract
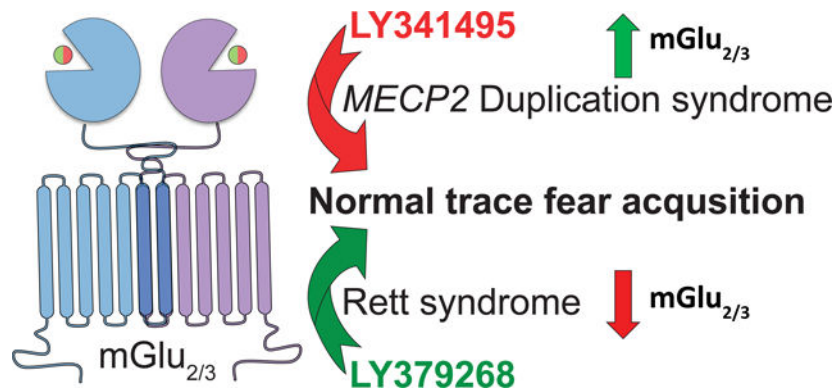
Keywords

mGlu$_2$; mGlu$_3$; Rett syndrome; *MECP2* Duplication syndrome; MECP2; trace fear

1. Introduction

MECP2-associated disorders are X-linked monogenic neurodevelopmental diseases that are caused by abnormal expression and/or function of the protein Methyl-CpG-binding protein 2 (MeCP2), which is encoded on the X chromosome. Rett syndrome (RTT) is caused by loss-of-function (LOF) mutations in *MECP2*, and is observed most often in females (Amir et al., 1999; Lombardi et al., 2015). The multi-domain clinical symptoms of RTT, including motor dysfunction, impaired social skills, cognitive decline, and breathing abnormalities, overlap with another *MECP2*-associated disorder, *MECP2* Duplication syndrome (MDS), which is caused by multiple copies of the *MECP2* gene. Due to random X chromosome inactivation in females, patients diagnosed with MDS are predominantly male (Ramocki et al., 2009; Van Esch et al., 2005). The symptoms observed in RTT patients are recapitulated in mice that ubiquitously or cell type-specifically lack *Mecp2* or express functionally mutated *Mecp2* (Adachi et al., 2009; Ballinger et al., 2019; Brown et al., 2016; Chen et al., 2001; Collins et al., 2021; Fyffe et al., 2008; Gemelli et al., 2006; Goffin et al., 2011; Guy et al., 2001; Heckman et al., 2014; Jentarra et al., 2010; Kerr et al., 2008; Lamonica et al., 2017; Lawson-Yuen et al., 2007; Luikenhuis et al., 2004; Moretti et al., 2006, 2005; Pelka et al., 2006; Pitcher et al., 2015; Samaco et al., 2013, 2009, 2008; Schaevitz et al., 2013; Shahbazian et al., 2002; Vermudez et al., 2021). Similarly, clinical characteristics of MDS are observed in mouse models that either have global or neuronal-specific *MECP2*

overexpression (Collins et al., 2004; Na et al., 2014). Interestingly, many phenotypes in RTT mice are antiparallel to those observed in MDS animal models, including anxiety, motor abnormalities, and cognitive function. For example, RTT mice display deficits in associative learning and memory in a fear conditioning task; in contrast, MDS mice exhibit abnormally enhanced learning in this task in addition to impairments in extinction of fear-learned behavior (Collins et al., 2004; Moretti et al., 2006; Na et al., 2012; Stansley et al., 2018; Stearns et al., 2007).

Previous studies in RTT models have shown that genetic normalization of *MECP2,* even after disease onset, can rescue many phenotypes in *Mecp2* mutant mice, supporting the feasibility of treating the disorder (Gadalla et al., 2017, 2013; Garg et al., 2013; Guy et al., 2007; Luoni et al., 2020; Matagne et al., 2021, 2017; Powers et al., 2019; Sinnett et al., 2021, 2017; Tillotson et al., 2017). Promisingly, recent studies have also demonstrated that genetic manipulations after symptom onset, including normalization of MeCP2 dosage with antisense oligonucleotides, can improve abnormal phenotypes in MDS mice (Koerner et al., 2018; Sztainberg et al., 2015). Pharmacological approaches targeting genes/pathways downstream of MeCP2 could also have potential in treating symptom domains of RTT and MDS. For example, in mice with MeCP2 over-expression in neurons (*Tau-Mecp2*), pharmacological antagonism of $GABA_A$ receptors can alleviate symptoms (Na et al., 2014). Comparably, studies have determined that modulation of receptors involved in neurotransmission and that are sensitive to MeCP2 dosage can improve phenotypes in RTT mice (Bittolo et al., 2016; Degano et al., 2014; Gogliotti et al., 2018, 2017, 2016; Li et al., 2017; Ogier et al., 2007; Roux et al., 2007; Scaramuzza et al., 2021; Zanella et al., 2008). These include the Trkb, glutamatergic AMPA, metabotropic glutamate (mGlu), and muscarinic acetylcholine receptors, which were initially identified in expression studies in RTT patient and mouse samples (Bedogni et al., 2016; Ben-Shachar et al., 2009; Chahrour et al., 2008; Gogliotti et al., 2018, 2017, 2016; Lin et al., 2016; Pacheco et al., 2017). The association of these receptors with neurological and neurodevelopmental disorders, as well as the advent of selective modulators, make these receptors potential therapeutic targets for new drug candidates.

The expression of the group II mGlu receptors, $mGlu_2$ and $mGlu_3$, has been consistently demonstrated to be affected by MeCP2 dosage in patient and preclinical samples (Bedogni et al., 2016; Ben-Shachar et al., 2009; Chahrour et al., 2008; Gogliotti et al., 2018; Lin et al., 2016; Pacheco et al., 2017). Additionally, these dimeric receptors have been implicated in neuropsychiatric disorders such as schizophrenia and depression (Chaki, 2017; Egan et al., 2004; Harrison et al., 2008; Maksymetz et al., 2017; Saini et al., 2017). Studies using subtype-selective modulators, preclinical knockout mouse models, and clinical genetic associations have shown that $mGlu_3$ has a vital role in cognition, specifically in hippocampal and prefrontal cortical function (Dogra et al., 2021; Egan et al., 2004; Fujioka et al., 2014; Joffe et al., 2020, 2019; Lainiola et al., 2014; Pöschel et al., 2005; Rosenberg et al., 2016; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Walker et al., 2017, 2015). $mGlu_2$ has also been shown to be involved in cognition, as $mGlu_2$-selective positive allosteric modulators (PAMs) improve learning and memory in rodent models of schizophrenia (Griebel et al., 2016; Nikiforuk et al., 2010). Given the molecular studies demonstrating the relationship of MeCP2 and the group II mGlu receptors, as well as the

association of these receptors with cognition, we posited that mGlu$_2$ and mGlu$_3$ may play critical roles in the etiology or treatment of the cognitive phenotypes observed in RTT and MDS.

In this study, we show that mGlu$_2$ and mGlu$_3$ receptor levels are decreased in temporal cortex samples from RTT patient autopsies. Expression of these receptors is also reciprocally decreased and increased in the hippocampus of $Mecp2^{Null/+}$ and $MECP2^{Tg1/o}$ mice, respectively. Based on these findings, we tested the hypothesis that an mGlu$_{2/3}$ agonist or antagonist would positively affect behavior in $Mecp2^{Null/+}$ and $MECP2^{Tg1/o}$ mice in a hippocampal-dependent behavioral cognitive assay. We show here that activation of mGlu$_{2/3}$ receptors reverses deficient trace fear acquisition in $Mecp2^{Null/+}$ animals, whereas mGlu$_{2/3}$ antagonism normalizes the abnormal enhanced trace fear acquisition phenotype in $MECP2^{Tg1/o}$ mice. Collectively, these data demonstrate that both mGlu$_2$ and mGlu$_3$ receptors are implicated in the cognitive function of RTT and MDS model mice, and that modulation of mGlu$_{2/3}$ activity may be beneficial in alleviating cognitive symptoms of these two disorders.

2.   Materials and Methods

2.1   Animals

All animals used in the present study were group housed with food and water given ad libitum and maintained on a 12hr light/dark cycle. Animals were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All studies were approved by the Vanderbilt Institutional Animal Care and Use Committee and took place during the light phase. $MECP2^{Tg1/o}$ mice (FVB-Tg(MECP2)1Hzo/J, stock no. 008679) were cryorecovered and $Mecp2^{Null/+}$ (B6.129P2(C)-$Mecp2^{tm1.1Bird}$/J, stock no. 003890) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Given that $MECP2^{Tg1/o}$ mice on the FVB/N background are prone to retinal degeneration, we utilized the F1 generation or hybrid mice (FVB/N x C57BL/6) for our studies as described previously (Samaco et al., 2012; Zhang et al., 2017). These F1 hybrid mice were generated by crossing $MECP2^{Tg1/o}$ mice (FVB/N background) with wild-type (WT) C57BL/6J mice (The Jackson Laboratory, stock no. 000664). To reflect the predominantly male clinical population in MDS (Ramocki et al., 2009), male $MECP2^{Tg1/o}$ mice and WT littermates were used for all experiments, and at 8–12 weeks old, an age range at which $MECP2^{Tg1/o}$ mice were previously observed to exhibit abnormal phenotypes (Samaco et al., 2012; Zhang et al., 2017). Similarly, as a reflection of the predominantly female RTT patient population, female $Mecp2^{Null/+}$ mice and WT littermates ($Mecp2^{+/+}$) were utilized and aged to at least 20 weeks of age prior to experiments to reflect the symptomatic age of female $Mecp2^{Null/+}$ animals (Guy et al., 2007, 2001). $Mecp2^{Null/+}$ animals were maintained on a C57BL/6J background by breeding $Mecp2^{Null/+}$ with WT C57BL/6J mice (The Jackson Laboratory, stock no. 000664).

2.2   Total Protein Preparation

The cortex and hippocampus were microdissected from 8–9-week-old male WT littermates and $MECP2^{Tg1/o}$ mice, and 20–25-week-old female littermates, $Mecp2^{+/+}$ and $Mecp2^{Null/+}$

animals. Total protein was prepared as previously described in (Fisher et al., 2018). Briefly, tissue samples were homogenized using a hand-held motorized mortar and pestle in radioimmunoprecipitation assay buffer (RIPA) containing 10mM Tris-HCl, 150mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and 1% deoxycholate. After homogenization, samples were centrifuged and the supernatant was collected. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce).

2.3 SDS-Page and Western Blotting

As previously described in (Fisher et al., 2018), 50μg of total protein was electrophoretically separated using a 4–20% SDS polyacrylamide gel and transferred onto a nitrocellulose membrane (iBlot2, ThermoFisher (for $mGlu_2$ and $mGlu_3$); Criterion™ Blotter, Bio-Rad (for MeCP2)). Membranes were blocked in TBS Odyssey blocking buffer (LI-COR) for 1hr at room temperature. Membranes were probed with primary antibodies overnight at 4°C: mouse anti-$mGlu_2$ (1:1000 Abcam, cat. no. ab15672), rabbit anti-$mGlu_3$ (1:1000, Alomone, cat. no. AGC_012), rabbit anti-MeCP2 (1:1000, Millipore, cat. no. 07–013), rabbit anti-vGlut2 (1:1000, Cell Signaling Technology, cat. no. 71555) and mouse anti-Gapdh (1:1000, ThermoFisher, cat. no. MA5–15738), followed by the fluorescent secondary antibodies: goat anti-rabbit (800nm, 1:5000, LI-COR, cat. no. 926–32211) and goat anti-mouse (680nm, 1:10,000, LI-COR, cat. no. 926–68020). Fluorescence was detected using the Odyssey (LI-COR) imaging system at the Vanderbilt University Medical Center Molecular Cell Biology Resource (MCBR) Core and then quantified using the Image Studio Lite software (LI-COR). Values were normalized to Gapdh and compared relative to littermate controls (WT littermates or $Mecp2^{+/+}$).

2.4 Total RNA Extraction and cDNA Synthesis

For total RNA extraction of human temporal cortex samples, frozen samples were obtained from the University of Maryland Brain and Tissue Bank and the Harvard Brain Tissue Resource Center, which are Brain and Tissue Repositories of the National Institutes of Health NeuroBioBank (neurobiobank.nih.gov). For mouse total RNA extraction, the cortex and hippocampus were microdissected from 8–9-week-old male WT littermates and $MECP2^{Tg1/o}$ mice, and 20–25-week-old female littermates, $Mecp2^{+/+}$ and $Mecp2^{Null/+}$ animals. Total RNA was prepared from tissue samples using TRIzol Reagent (ThermoFisher) and isolated using a RNeasy Mini Kit (Qiagen) in accordance with manufacturer's instructions. Total RNA was DNase-treated with RNase-Free DNase Set (Qiagen), and cDNA from 2μg of total RNA was synthesized using a SuperScript™ VILO™ cDNA Synthesis Kit (ThermoFisher, cat. no. 11754050).

2.5 Quantitative Real-Time PCR (qRT-PCR)

qRT-PCR (CFX96, Bio-Rad, Vanderbilt University Medical Center MCBR Core) on 50ng/9μL cDNA was run in duplicate using TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG (Life Technologies, cat. no. 4352042) and Life Technologies gene expression assays for human *GRM2* (Hs00968358_m1), *GRM3* (Hs00932301_m1) and *G6PD* (Hs00166169_m1), and mouse *Grm2* (Mm01235831_m1), *Grm3* (Mm00725298_m1) and *Gapdh* (Mm99999915_g1). Similar qRT-PCR was performed

using PowerUp™ SYBR™ Green Master Mix (ThermoFisher, cat. no. A25742) with the following primers (5' to 3'): *Mecp2* (exon 4, forward: ATGAGACTGTGCTCCCCATC, reverse: TTTTCTCACCAAGGGTGGAC) and *Gapdh* (exon 6, forward: CGACTTCAACAGCAACTCCC, reverse: GCCGTATTCATTGTCATACCAGG). All primers used for SYBR qRT-PCR were designed using Primer3 and constructed by Sigma through the Vanderbilt University Medical Center MCBR Core. Ct values for each sample were normalized to G6PD/Gapdh expression and analyzed using the delta–delta Ct method as described in (Gogliotti et al., 2017). Values exceeding two times the standard deviation were classified as outliers. Each value was compared to the average delta-Ct value acquired for control human samples or wild-type littermate control mice (WT littermate or *Mecp2$^{+/+}$*) and calculated as percent-relative to the average control delta-Ct.

2.6 Drugs

LY379268 (mGlu$_{2/3}$ agonist) and LY341495 (mGlu$_{2/3}$ antagonist) were purchased from Tocris (Minneapolis, MN). All drugs used for behavioral experiments were diluted in 10% Tween-80.

2.7 Behavioral Assays

All behavioral experiments were conducted at predicted symptomatic ages (8–12-week-old *MECP2$^{Tg1/o}$* and 20–25-week-old *Mecp2$^{Null/+}$* mice) and using sex- and age-matched littermate controls (WT littermates or *Mecp2$^{+/+}$* mice) at the Vanderbilt Mouse Neurobehavioral Lab (MNL) Core. All experiments were preceded by intraperitoneal (i.p.) injections of the following drugs (T$_{max}$ in parentheses): vehicle (10% Tween-80), LY379268 (1mg/kg, 30 min) or LY341495 (3mg/kg, 20 min pre-LY379268 administration or 30 min). Quantification was performed either by a researcher blinded to the genotype and treatment and/or by automated software.

2.7.1   Open Field—Mice were placed in the activity chamber for 30 min and locomotor activity was quantified as beam breaks in the X, Y and Z axis using Activity Monitor software (Med Associates Inc).

2.7.2   Trace Fear Conditioning—Mice were habituated to the room for 1 hour before all tests. On acquisition or conditioning day, mice were treated with compounds or vehicle prior to being placed into an operant chamber with a shock grid (Med Associates Inc.) in the presence of a 10% vanilla odor cue. Modified from previous studies (Dogra et al., 2021; Xu et al., 2014), mice were acclimated for 1 min and exposed to a mild 1 sec foot shock (0.5 mA for WT littermate and *MECP2$^{Tg1/o}$* mice, and 0.7mA for *Mecp2$^{+/+}$* and *Mecp2$^{Null/+}$* animals) that was preceded by a 15 sec tone. A precise 30 sec interval or "trace" separated the tone and shock. Three or four tone-trace-shock pairings were applied, 240 sec apart, for the *MECP2$^{Tg1/o}$* or *Mecp2$^{Null/+}$* mouse lines, respectively. Percentage of time spent freezing during each trace was measured by Video Freeze software (Med Associates Inc.).

2.8 Statistical Analyses

Statistics were carried out using Prism 9 (GraphPad) and Excel (Microsoft). All data shown represent mean ± SEM. Statistical significance between genotypes was determined using

Student's t-test, or 2-way ANOVA with Sidak's or Tukey's post-hoc. Sample size (denoted as "n"), statistical test and results of statistical analyses are specified in each figure legend.

3. Results

3.1 Group II mGlu receptor expression is decreased in clinical and preclinical RTT samples

We first investigated the expression of $mGlu_2$ and $mGlu_3$ in post-mortem brain tissue from female patients clinically diagnosed with RTT. Complementing our previous studies in the motor cortex and cerebellum (Gogliotti et al., 2018, 2017, 2016), we obtained fourteen temporal cortex samples (Brodmann area 20 or 38, BA 20 or BA 38) from female RTT patients characterized as having truncation mutations in *MECP2* (*R168X*, *R255X*, and *R270X*) and fourteen age-, sex- and post-mortem interval (PMI)-matched controls (Supplementary Table 1). qRT-PCR analyses revealed decreased levels of *GRM2* and *GRM3* mRNA in the temporal cortex of RTT patients (Figure 1A), which is in agreement with previous transcriptomic analyses of other brain regions, including the cerebellum and frontal, motor and temporal cortices (Ben-Shachar et al., 2009; Gogliotti et al., 2018; Lin et al., 2016).

Decreased expression of $mGlu_2$ and $mGlu_3$ mRNA has also been observed in preclinical RTT mouse models, particularly in the cortex of male *Mecp2 null* mice (Bedogni et al., 2016; Chahrour et al., 2008; Pacheco et al., 2017). Consistent with these transcriptomic studies, we found significantly reduced *Grm2* mRNA expression in the cortex of naïve 20–25-week-old female *Mecp2$^{Null/+}$* animals compared to the wild-type (WT) littermate controls, *Mecp2$^{+/+}$* (Figure 1B). Interestingly, cortical *Grm3* mRNA was not statistically different between the mutant and control mice. Given that $mGlu_2$ and $mGlu_3$ have both been linked to cognitive function, specifically hippocampal-dependent cognition (De Filippis et al., 2015; Lyon et al., 2011), we assessed *Grm2* and *Grm3* transcript levels in the hippocampus, and found that hippocampal *Grm2* and *Grm3* mRNA levels were also decreased in *Mecp2$^{Null/+}$* animals (Figure 1C).

Next, we determined whether altered expression of $mGlu_2$ and $mGlu_3$ in *Mecp2$^{Null/+}$* mice, which express ~50% less MeCP2 than littermate controls *Mecp2$^{+/+}$* (Supplementary Figure 1), was maintained at the protein level. As illustrated in the representative immunoblots, only $mGlu_2$ was significantly decreased in the cortex of *Mecp2$^{Null/+}$* animals compared to their WT counterparts, which is consistent with the transcript expression (Figure 1D-E, antibody validation in Supplementary Figure 2; $mGlu_3$ global knockout animals have been previously characterized in (Dogra et al., 2021); monomer level in the cortex of both $mGlu_2$ and $mGlu_3$ was too low to be accurately quantified). Also in agreement with the transcript expression data was the observed decreased expression of $mGlu_2$ and $mGlu_3$ protein in the hippocampus of *Mecp2$^{Null/+}$* mice (Figure 1F-G). Notably, for both receptors, the dimer and total (sum of monomer and dimer) protein levels were significantly reduced in *Mecp2$^{Null/+}$* animals without differences in the control synaptic protein vGlut2 (Figure 1G). These clinical and preclinical data provided rationale for investigating the role and therapeutic potential of group II mGlu receptors in RTT and related disorders.

3.2   Group II mGlu receptor expression is increased in *MECP2$^{Tg1/o}$* mice

We further explored the molecular relationship of mGlu$_2$ and mGlu$_3$ with MeCP2 using a rodent MDS model, *MECP2$^{Tg1/o}$*, and tested the hypothesis that increased MeCP2 expression in the *MECP2$^{Tg1/o}$* context also enhances mGlu$_2$ and mGlu$_3$ expression. Similar to the use of symptomatic female *Mecp2$^{Null/+}$* animals to reflect the clinical population, we utilized 8–9-week-old male *MECP2$^{Tg1/o}$* mice and WT littermates to assess protein and mRNA expression in the hippocampus. In agreement with previous reports (Collins et al., 2004; Fisher et al., 2018; Na et al., 2012; Sztainberg et al., 2015), MeCP2 mRNA and protein expression were increased in *MECP2$^{Tg1/o}$* mice compared to WT littermates (Figure 2A-C). Comparably, *Grm2* and *Grm3* mRNA levels were also significantly increased in *MECP2$^{Tg1/o}$* animals (Figure 2A). To determine if this effect was also observed at the protein level, we evaluated mGlu$_2$ and mGlu$_3$ protein expression. As illustrated in the representative immunoblots, expression of both receptors was increased in *MECP2$^{Tg1/o}$* animals (Figure 2B). In Figure 2C, quantification of expression showed that statistical significance was observed for the expression of the monomer, dimer and total (sum of monomer and dimer) protein for mGlu$_2$, and the level of the dimer and total mGlu$_3$ protein was significantly different between *MECP2$^{Tg1/o}$* mice and WT littermates. These overexpression data of group II mGlu receptors suggested that these proteins could potentially contribute to the abnormal phenotypes in MDS mice, which could be amenable to receptor modulation.

3.3   RTT and MDS mice exhibit abnormal and bidirectional phenotypes in trace fear acquisition

Numerous studies have characterized RTT model mice as exhibiting abnormal phenotypes in cognition, specifically in associative learning and memory, which is often assessed using a cued or contextual fear conditioning assay (Collins et al., 2021; Gogliotti et al., 2017, 2016; Lamonica et al., 2017; Merritt et al., 2020; Moretti et al., 2006; Pelka et al., 2006; Pitcher et al., 2015; Samaco et al., 2013; Schaevitz et al., 2013; Stearns et al., 2007; Vermudez et al., 2021). Interestingly, a recent study highlighted the role of mGlu$_3$ in mediating a temporal- and hippocampal-dependent associative learning and memory phenotype known as trace fear acquisition or conditioning (Dogra et al., 2021). Prior to investigating the relationship of mGlu$_{2/3}$ receptors and trace fear conditioning in a RTT mouse model, we first characterized the trace fear behavior of *Mecp2$^{Null/+}$* animals. In this task, the mice were trained to associate their environment and presented tone with an aversive stimulus in the form of a mild foot shock (0.7 mA); the "trace" period that separates the tone and shock presentations is thought to enhance learning (Figure 3A). As shown in Figure 3B, increases in percent freezing in *Mecp2$^{+/+}$* animals paralleled the increase in tone-trace-shock pairings (black symbols), reflecting learning behavior. However, we demonstrate here that *Mecp2$^{Null/+}$* mice exhibit abnormal trace fear acquisition, as illustrated by the attenuated percent freezing compared to their *Mecp2$^{+/+}$* counterparts. In particular, freezing behavior was significantly different between *Mecp2$^{+/+}$* and *Mecp2$^{Null/+}$* animals at the last trace period (T4), suggesting a deficit in trace fear acquisition.

Similar to RTT model mice, MDS mice have been shown to exhibit abnormal phenotypes in cued or contextual fear associative learning and memory (Collins et al., 2004; Fisher

et al., 2018; Na et al., 2014, 2012; Stansley et al., 2018). However, as in RTT model animals, phenotypes in trace fear conditioning have not been assessed in mice modeling MDS. WT littermate and $MECP2^{Tg1/o}$ animals were subjected to a similar trace fear acquisition paradigm as RTT mice, with the notable differences of a milder foot shock intensity (0.5 mA) and reduced number of tone-trace-shock pairings (Figure 3C). These changes were performed to account for the high percent freezing phenotype that MDS mice exhibit in fear conditioning assays (Collins et al., 2004; Fisher et al., 2018; Na et al., 2014, 2012; Stansley et al., 2018). Compared to WT littermates, $MECP2^{Tg1/o}$ mice displayed enhanced trace fear acquisition during the last trace period measured (T3, Figure 3D). This abnormal enhancement in trace fear acquisition was in contrast to the deficient phenotype in $Mecp2^{Null/+}$ animals; coupled with the increases in expression of mGlu$_2$ and mGlu$_3$ in the hippocampus of $MECP2^{Tg1/o}$ mice, we hypothesized that these changes in trace fear behavior might be sensitive to mGlu$_{2/3}$ receptor modulation.

3.4   mGlu$_{2/3}$ activation reverses deficits in trace fear acquisition in $Mecp2^{Null/+}$ mice

Detection and quantitation of a trace fear acquisition deficit in RTT mice allowed us to then test the hypothesis that administration of a nonselective mGlu$_{2/3}$ agonist, LY379268, to increase receptor activity would improve this abnormal cognitive phenotype in $Mecp2^{Null/+}$ animals. Animals were intraperitoneally administered with either vehicle (10% Tween-80) or 1mg/kg LY379268 30 minutes prior to trace fear conditioning (Figure 4A). LY379268 treatment reversed the trace fear acquisition deficit in vehicle-treated $Mecp2^{Null/+}$ mice with statistical significance observed between vehicle- and LY379268-treated $Mecp2^{Null/+}$ mice at trace periods 2 (T2) and 4 (T4) (Figure 4B-D). The increased freezing behavior at period T2 was only observed in $Mecp2^{Null/+}$ animals (Figure 4C), suggesting an increased sensitivity to LY379268 in the mutant mice compared to their WT counterparts. In contrast, $Mecp2^{+/+}$ animals did not exhibit a significant change in response to LY379268 at any of the trace periods measured. To eliminate the possibility that the increased percent freezing in $Mecp2^{Null/+}$ animals was due to hypolocomotive effects of LY379268, as has previously been shown in WT animals (Imre, 2007; Imre et al., 2006; Woolley et al., 2008), we placed the mice in an open field chamber and monitored spontaneous locomotor activity. As illustrated in Supplementary Figure 2, 1mg/kg LY379268 did not change the distance traveled of $Mecp2^{+/+}$ or $Mecp2^{Null/+}$ animals, which already have reduced locomotion at baseline.

To further validate that the reversal effects in trace fear conditioning were due to mGlu$_{2/3}$ modulation by LY379268 and not an off-target result, we administered the mGlu$_{2/3}$ orthosteric antagonist LY341495 (3mg/kg) prior to LY379268 treatment (Figure 4E). LY341495 blocked the effect of LY379268 in $Mecp2^{Null/+}$ mice, specifically at trace periods T2-T4 (Figure 4F-I). In these trace periods, the freezing behavior of vehicle-treated $Mecp2^{Null/+}$ animals was indistinguishable from $Mecp2^{Null/+}$ mice that received both LY341495 and LY379268. Importantly, the ability of LY341495 to block LY379268's increased freezing effect at period T2 in $Mecp2^{Null/+}$ animals suggests that the left-shifted response of the mutant animals to LY379268 is a consequence of mGlu$_{2/3}$ modulation (Figure 4G). Interestingly, co-administration of LY379268 and LY341495 to $Mecp2^{+/+}$ animals decreased percent freezing at trace periods T3-T4 (Figure 4H-I). Again, this could

potentially be due to alterations in locomotor activity; however, assessment of distance traveled in an open field assay revealed no locomotor effects of LY341495 and LY379268 co-administration in either the $Mecp2^{+/+}$ and $Mecp2^{+/-}$ mice (Supplementary Figure 3). Overall, these data support the ability of mGlu$_{2/3}$ activation to enhance the trace fear acquisition response in $Mecp2^{Null/+}$ animals.

3.5 mGlu$_{2/3}$ antagonism normalizes the abnormal trace fear acquisition phenotype in $MECP2^{Tg1/o}$ mice

Given the beneficial effects of activating mGlu$_{2/3}$ in RTT mice, which display opposite trace fear conditioning phenotypes from MDS mice, we then posited the converse hypothesis: that inhibiting mGlu$_{2/3}$ could alleviate the abnormal increase in trace fear behavior in $MECP2^{Tg/o}$ animals. Prior to trace fear conditioning, animals were intraperitoneally administered either vehicle (10% Tween-80) or the nonselective orthosteric mGlu$_{2/3}$ antagonist LY341495 (3mg/kg) (Figure 5A). LY341495 treatment did not affect trace fear behavior in WT littermates (Figure 5B-C). However, $MECP2^{Tg1/o}$ animals administered LY341495 exhibited significantly reduced percent freezing at the last tone-trace-shock pairing (T3) compared to vehicle-treated $MECP2^{Tg1/o}$ mice (Figure 5C). Notably, LY341495-treated $MECP2^{Tg1/o}$ mice displayed comparable freezing behavior to vehicle-treated WT counterparts. To rule out potential locomotor effects of mGlu$_{2/3}$ inhibition with the nonselective antagonist, we placed vehicle- and LY341495-treated animals in an open field chamber. At baseline, $MECP2^{Tg1/o}$ mice presented with hypolocomotor activity compared to WT littermates (Supplementary Figure 4). Importantly, regardless of genotype, LY341495 administration did not affect distance traveled in the open field task, which is in agreement with previous studies using systemic administration of LY341495 (Fukumoto et al., 2016; Gleason et al., 2013). Overall, these data suggest that mGlu$_{2/3}$ antagonism has positive effects in normalizing enhanced trace fear behavior in $MECP2^{Tg1/o}$ animals.

4. Discussion

Our understanding of *MECP2*-associated disorders has developed tremendously in the past 20 years. However, despite the monogenicity of these disorders and the link of MeCP2 protein expression and/or function to each disease, no effective treatment is currently available for RTT or MDS. Promisingly, recent studies have demonstrated that genetic manipulations of *MECP2* and pharmacological modulations of downstream targets of the protein improve phenotypes in preclinical rodent models of RTT (Bittolo et al., 2016; Degano et al., 2014; Gadalla et al., 2017, 2013; Garg et al., 2013; Gogliotti et al., 2018, 2017, 2016; Li et al., 2017; Luoni et al., 2020; Matagne et al., 2021, 2017; Ogier et al., 2007; Powers et al., 2019; Roux et al., 2007; Scaramuzza et al., 2021; Sinnett et al., 2017; Tillotson et al., 2017; Zanella et al., 2008) and MDS (Koerner et al., 2018; Na et al., 2014; Sztainberg et al., 2015). In this study, we aimed to capitalize on previous reports by investigating two potential therapeutic targets, mGlu$_2$ and mGlu$_3$, and employing pharmacological modulators of these receptors to evaluate effects in RTT ($Mecp2^{Null/+}$) and MDS ($MECP2^{Tg1/o}$) mouse models.

mGlu$_2$ and mGlu$_3$ are group II mGlu receptors that have been robustly implicated in neuropsychiatric disorders (Chaki, 2017; Joffe and Conn, 2019; Maksymetz et al., 2017; Swanson et al., 2005; Walker and Conn, 2015). Their role in neurodevelopmental disorders is limited; however, mGlu$_3$ has been shown to be involved in the cognitive phenotypes of Fragile X syndrome (FXS) (Choi et al. 2011) and mGlu$_{2/3}$ receptors have been implicated in autism-like phenotypes in a rat model of autism (Chen et al. 2014). Moreover, expression studies demonstrate that both mGlu$_2$ and mGlu$_3$ are decreased in male *Mecp2*$^{Null/y}$ mice and patients diagnosed with RTT (Bedogni et al. 2016; Chahrour et al. 2008; Pacheco et al. 2017; Ben-Shachar et al. 2009; Gogliotti et al. 2018; Lin et al. 2016). Here, we further support these data by showing attenuated mGlu$_2$ and mGlu$_3$ expression in the temporal cortex of RTT autopsy samples from patients with truncating mutations in MeCP2. Altered levels of these receptors are conserved in a female RTT mouse model, *Mecp2*$^{Null/+}$, specifically in the cortex and hippocampus. Since RTT and MDS are due to loss-of-function mutations and multiple copies of *MECP2*, respectively, and given the role of MeCP2 as a transcriptional activator (Chahrour et al. 2008), we posited that mGlu$_2$ and mGlu$_3$ expression would be increased in MDS. In the hippocampus of *MECP2*$^{Tg1/o}$ mice, we confirmed previous studies showing increased MeCP2 expression (Collins et al. 2004; Fisher et al. 2018; Na et al. 2012) and also observed increased mGlu$_2$ and mGlu$_3$ mRNA and protein expression. These findings suggest that group II mGlu receptors may be targets of MeCP2 transcriptional regulation, whether through direct or indirect mechanisms.

These molecular data provided our rationale to further explore mGlu$_2$ and mGlu$_3$ in the behavioral phenotypes of RTT and MDS, focusing on the learning and memory phenotypes given the role of group II mGlu receptors in cognition. Numerous studies have established that RTT and MDS mice have contrasting characteristics in contextual fear conditioning, a hippocampal-dependent learning and memory paradigm (Na et al. 2012; Na et al. 2014; Collins et al. 2004; Fisher et al. 2018; Gogliotti et al. 2017; Stansley et al. 2018; Moretti et al. 2006; Samaco et al. 2012; Stearns et al. 2007). In particular, RTT mice exhibit deficits in contextual fear conditioning, whereas MDS mice display an abnormal enhancement of this behavior. Several studies have described a similar hippocampal-dependent cognitive task that relies on both the spatial and temporal factors of learning and memory called trace fear conditioning (Huerta et al. 2000; Xu et al. 2014; Zhao et al. 2005; McEchron et al. 1998). Excitingly, our group has demonstrated a specific role for mGlu$_3$ in trace fear acquisition (Dogra et al. 2021). Before exploring the relationship of mGlu$_{2/3}$ receptors and trace fear conditioning in mouse models of *MECP2*-associated disorders, we first characterized the behavior of both *Mecp2*$^{Null/+}$ and *MECP2*$^{Tg1/o}$ animals in this cognitive task, which has not yet been investigated. Here, we extend previous studies in cognitive domains to show that *Mecp2*$^{Null/+}$ mice exhibit a deficit in trace fear acquisition. Conversely, *MECP2*$^{Tg1/o}$ animals have an abnormal enhanced trace fear acquisition phenotype, which supports the bidirectional behavioral phenotypes of cognition in mouse models of these two disorders.

These data prompted us to test our hypothesis that mGlu$_{2/3}$ modulation could improve abnormal cognitive phenotypes in *Mecp2*$^{Null/+}$ and *MECP2*$^{Tg1/o}$ mice using the trace fear paradigm. Previous preclinical studies have shown that mGlu$_3$ activation and mGlu$_2$ positive allosteric modulators improve deficits in cognitive tasks (Griebel et al. 2016; Nikiforuk et al. 2010; Walker et al. 2017). Given that both receptors are decreased in *Mecp2*$^{Null/+}$

mice, it is likely that the function of both $mGlu_2$ and $mGlu_3$ is also disrupted. Therefore, we activated both receptors using a nonselective $mGlu_{2/3}$ agonist, LY379268. Excitingly, in $Mecp2^{Null/+}$ animals, LY379268 rescued deficiencies in trace fear acquisition. Interestingly, we observed a left-shifted response to LY379268 that was specific to $Mecp2^{Null/+}$ mice. This could be attributed to a ceiling effect of the trace fear paradigm, for example the strong shock intensity, on the baseline trace fear behavior of $Mecp2^{+/+}$ animals, which has also been previously observed in WT animals (Dogra et al. 2021), or to a potential sensitivity of the receptors to agonist in the $Mecp2^{Null/+}$ context. Importantly, LY379268's effect in augmenting freezing behavior in $Mecp2^{Null/+}$ was completely blocked by the $mGlu_{2/3}$ antagonist, LY341495, suggesting that LY379268 mediates its effects via $mGlu_2$ and $mGlu_3$ in the trace fear acquisition paradigm in RTT mice.

In contrast to the effects seen in $Mecp2^{Null/+}$ animals, inhibition of $mGlu_{2/3}$ receptors with LY341495 reversed the abnormally enhanced trace fear acquisition of $MECP2^{Tg1/o}$ mice. The lack of LY341495's effect in WT animals suggests that the heightened trace fear phenotype in $MECP2^{Tg1/o}$ mice is driven, in part, by the increased expression of the $mGlu_2$ and/or $mGlu_3$ receptors. The bidirectionality in $mGlu_{2/3}$ modulation to alleviate the abnormal behavioral cognition in $Mecp2^{Null/+}$ and $MECP2^{Tg1/o}$ animals parallels the contrasting trace fear phenotypes. These together may indicate that, in both the RTT and MDS contexts where both receptors are affected, one or both of the group II mGlu receptors are needed to mediate this form of associative learning and memory.

In $MECP2^{Tg1/o}$ and $Mecp2^{Null/+}$ mice, the functional consequences of altered $mGlu_2$ and $mGlu_3$ receptor expression, such as in impacting long-term synaptic plasticity in the hippocampal Schaffer collateral-CA1 (SC-CA1) synapse, remains to be elucidated. Previous reports have demonstrated that SC-CA1 long-term potentiation (LTP) is bidirectionally altered in $Mecp2$ mutant and $MECP2^{Tg1/o}$ animals (Asaka et al. 2006; Collins et al. 2004; Guy et al. 2007; Moretti et al. 2006; Sztainberg et al. 2015). Additionally, in $Mecp2$ mutant mice, NMDA antagonism has been shown to reverse LTP deficits (Weng et al. 2011). Although no studies have yet shown whether NMDA function is implicated in MDS, NMDA dysregulation has been associated with the enhanced LTP phenotype of mice modeling a related disorder, Pitt-Hopkins syndrome (Kennedy et al. 2016; Thaxton et al. 2018). Coupling these data with previous evidence illustrating that $mGlu_{2/3}$ receptors play a role in NMDA-mediated LTP at the SC-CA1 synapses (Rosenberg et al. 2016), it is therefore possible that $mGlu_{2/3}$ modulation may correct abnormal SC-CA1 LTP in RTT and MDS animals. However, empirical studies are needed to test this theory.

In conclusion, we have demonstrated that the expression of $mGlu_2$ and $mGlu_3$ is altered in RTT clinical samples and bidirectionally affected in $Mecp2^{Null/+}$ and $MECP2^{Tg1/o}$ animals, indicating a potential role of group II mGlu receptors in RTT and MDS phenotypes. Correspondingly, we establish that $Mecp2^{Null/+}$ and $MECP2^{Tg1/o}$ mice present abnormal and contrasting phenotypes in trace fear conditioning, a temporal-dependent form of associative learning and memory. Pharmacological $mGlu_{2/3}$ activation or antagonism exerted efficacy in modulating trace fear behavior of $Mecp2^{Null/+}$ and $MECP2^{Tg1/o}$ mice, respectively. Altogether, our data provide novel evidence that $mGlu_2$ and $mGlu_3$ are implicated in neurodevelopmental disorders, particularly in RTT and MDS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- mGlu$_2$/mGlu$_3$ receptors are decreased in Rett syndrome patients and mice, *Mecp2$^{Null/+}$*

- mGlu$_2$/mGlu$_3$ receptors are increased in *MECP2* Duplication syndrome mice, *MECP2$^{Tg1/o}$*

- mGlu$_{2/3}$ activation rescues trace fear acquisition deficits in *Mecp2$^{Null/+}$* mice

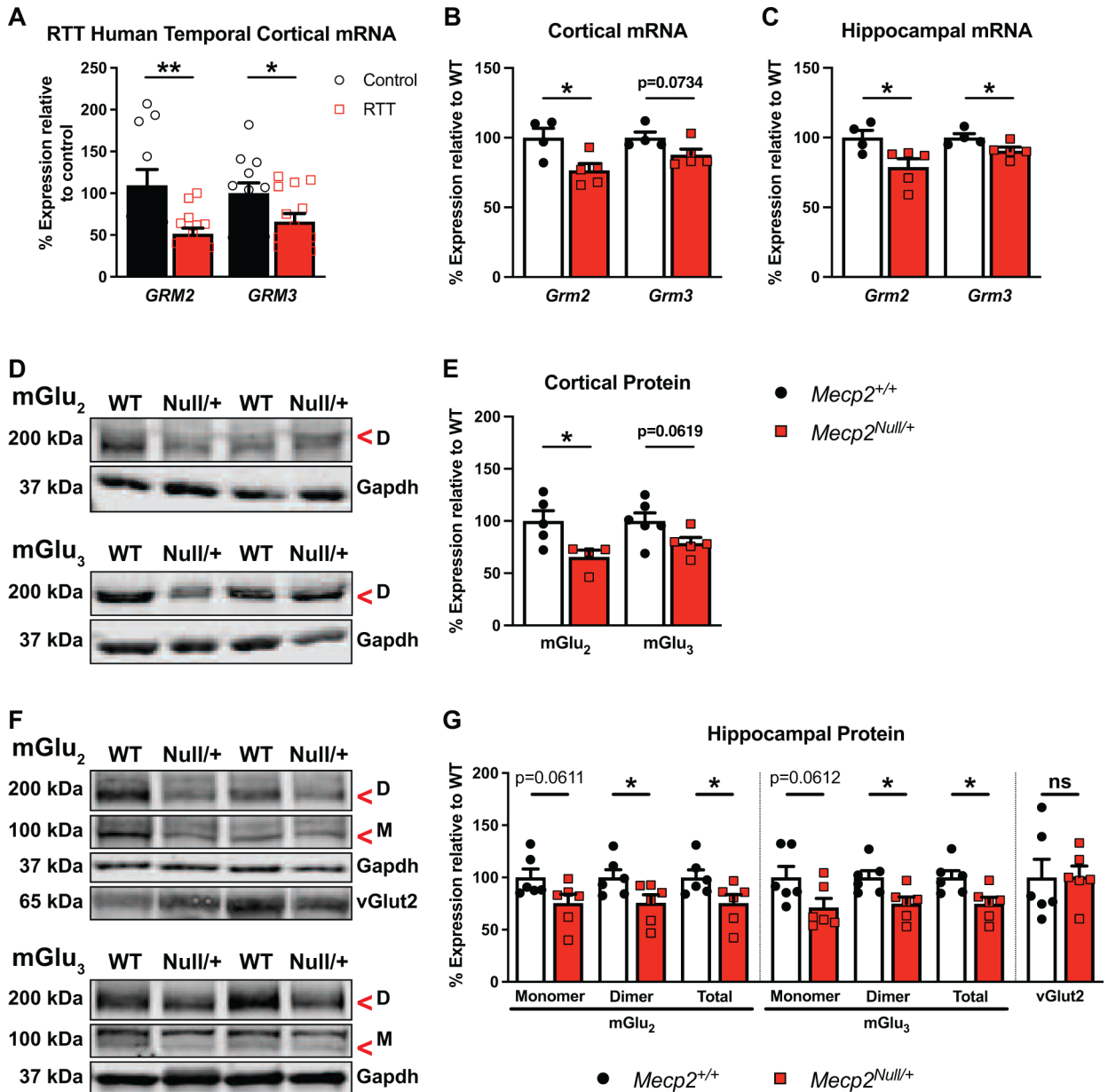- mGlu$_{2/3}$ inhibition reverses heightened trace fear acquisition in *MECP2$^{Tg1/o}$* mice

Figure 1: mGlu₂ and mGlu₃ expression is decreased in RTT patients and mice.
(**A**) Compared to controls (black bars / white circles, n=11–12), *GRM2* and *GRM3* mRNA are both decreased in RTT patients in temporal cortex autopsy samples (red bars / white squares, n=14) (*GRM2*: t(23)=3.144, p=0.0045; *GRM3*: t(24)=2.196, p=0.0380). RTT patient samples used in this study have truncated MeCP2 mutations, specifically R168X, R255X and R270X (see Supplementary Table 1). (**B-C**) Compared to littermate controls, *Mecp2⁺/⁺* (white bars / black circles, n=4–5), *Grm2* and/or *Grm3* mRNA levels are decreased in 20–25-week-old *Mecp2^Null/+* mice (red bars / squares, n=4–5) (cortex: *Grm2*: t(7)=2.897, p=0.0231; *Grm3*: t(7)=2.104, p=0.0734; hippocampus: *Grm2*: t(7)=2.599, p=0.0355; *Grm3*: t(7)=2.460, p=0.0435). (**D**) Representative immunoblots illustrating cortical dimeric ("D", 200 kDa) form of mGlu₂ or mGlu₃ and Gapdh (37 kDa) loading

control in $Mecp2^{+/+}$ ("WT") and $Mecp2^{Null/+}$ ("Null/+") animals. **(E)** Cortical mGlu$_2$ protein expression is significantly decreased in $Mecp2^{Null/+}$ mice (n=4–5) relative to $Mecp2^{+/+}$ animals (n=5–6) (mGlu$_2$: t(7)=2.715, p=0.0300; mGlu$_3$: t(9)=2.131, p=0.0619). **(F)** Representative immunoblots for hippocampal proteins as in **(D)** with the addition of the mGlu$_2$ or mGlu$_3$ monomeric protein ("M", 100 kDa) and vGlut$_2$ (65 kDa). **(G)** Compared to $Mecp2^{+/+}$ animals (n=5–6), mGlu$_2$ and mGlu$_3$ proteins (total = monomer + dimer) are reduced in the hippocampus of $Mecp2^{Null/+}$ mice (n=5–6) (mGlu$_2$: Monomer: t(10)=2.109, p=0.0611; Dimer: t(10)=2.285, p=0.0454; Total: t(10)=2.250, p=0.0482; mGlu$_3$: Monomer: t(10)=2.108, p=0.0612; Dimer: t(10)=2.798, p=0.0188; Total: t(10)=2.811, p=0.0185). vGlut$_2$ is unchanged between genotypes (t(10)=0.04986, p=0.9612). Student's t-test. ns (not significant), *p<0.05, **p<0.01.
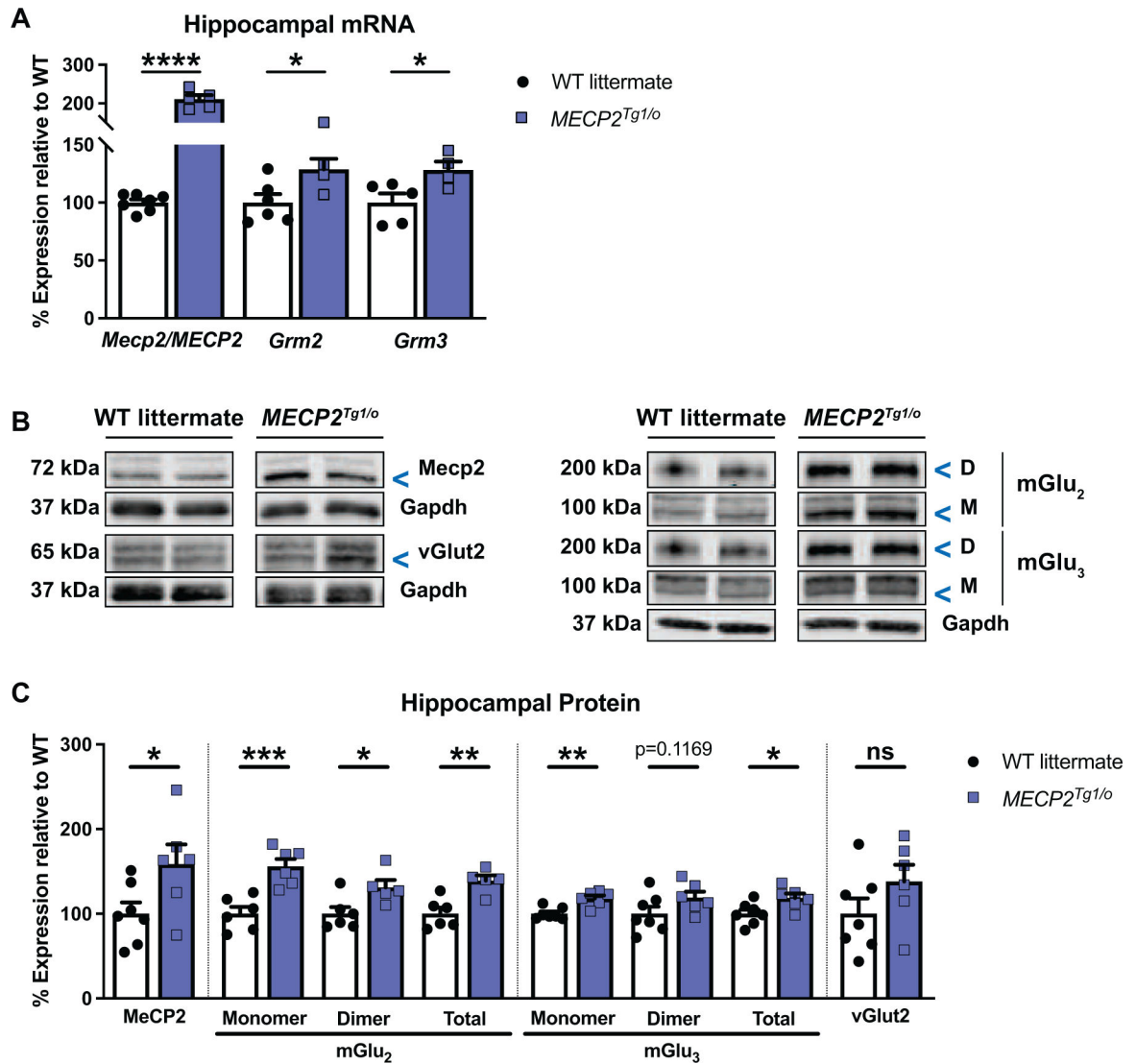
Figure 2: Hippocampal mGlu$_2$ and mGlu$_3$ expression is increased in MDS mice.
(A) Compared to WT littermates (white bars / black circles, n=5–7), *Mecp2/MECP2, Grm2* and *Grm3* transcripts are increased in the hippocampus of 8–9-week-old *MECP2*$^{Tg1/o}$ mice (blue bars / squares, n=4–5) (*Mecp2/MECP2*: t(10)=11.89, p<0.0001; *Grm2*: t(8)=2.476, p=0.0384; *Grm3*: t(7)=2.586, p=0.0362). **(B)** Representative immunoblots illustrating MeCP2 (72 kDa), mGlu$_2$ or mGlu$_3$ dimer ("D", 200 kDa) and monomer ("M", 100 kDa), vGlut2 (65 kDa), and Gapdh (37 kDa) loading control in hippocampal samples of WT littermates and *MECP2*$^{Tg1/o}$ mice. **(C)** Protein expression of MeCP2, mGlu$_2$, and mGlu$_3$ (total = monomer + dimer) is increased in *MECP2*$^{Tg1/o}$ mice (n=5–6) relative to WT littermates (n=6–7) (MeCP2: t(11)=2.269, p=0.0444; mGlu$_2$: Monomer: t(10)=4.669, p=0.0009; Dimer: t(9)=2.642, p=0.0268; Total: t(9)=4.028, p=0.0030; mGlu$_3$: Monomer: t(11)=4.140, p=0.0016; Dimer: t(11)=1.701, p=0.1169; Total: t(11)=2.597, p=0.0248). vGlut$_2$ is unchanged between genotypes (t(11)=1.433, p=0.1797). Student's t-test. ns (not significant), *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
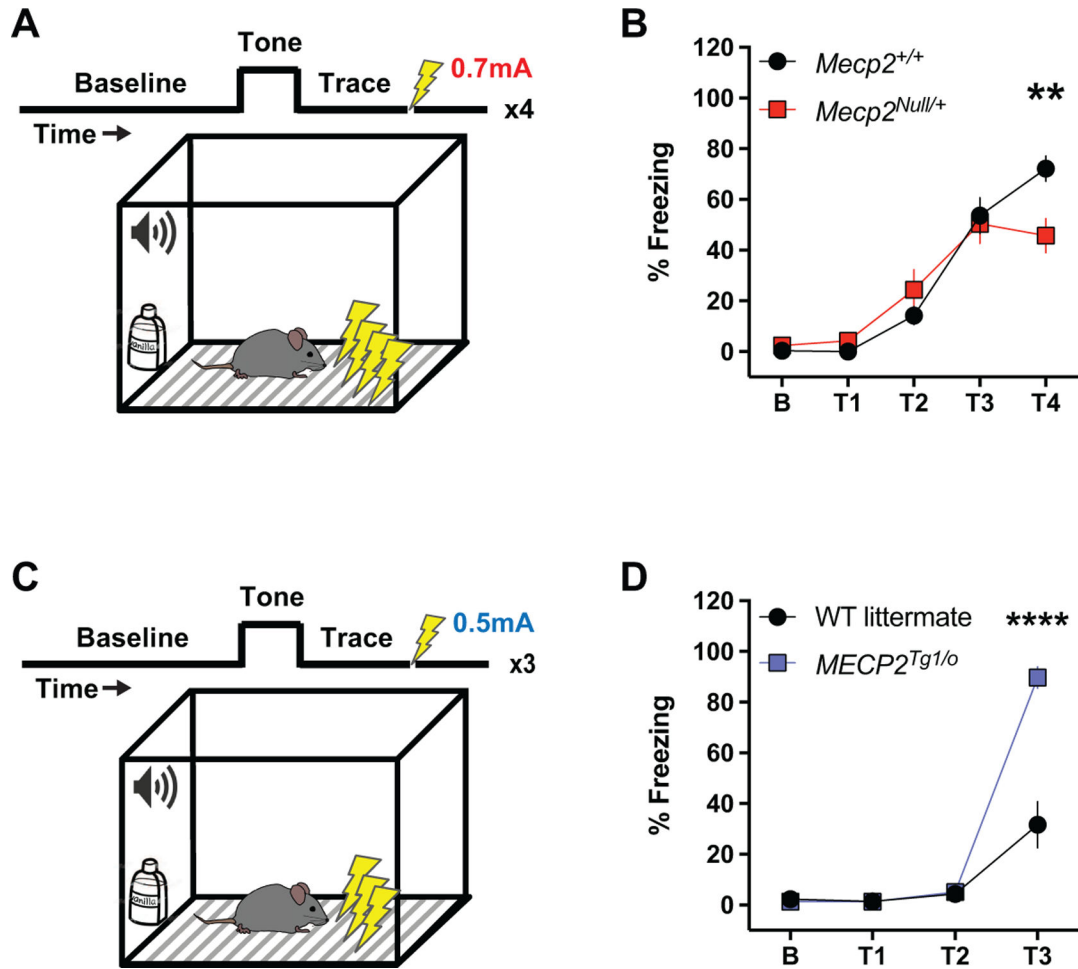
Figure 3: Contrasting phenotypes in trace fear conditioning between *Mecp2^{Null/+}* and *MECP2^{Tg1/o}* animals.

(**A**) Diagram illustrating the trace fear conditioning paradigm. Mice are trained to associate their environment (context and tone) with an aversive stimulus in the form of a mild foot shock (0.7 mA), with a temporal component (trace) separating the tone and shock. Four tone-trace-shock pairings are presented and percent freezing during the trace period is measured as a proxy for fear learning behavior. (**B**) Compared to littermate controls, *Mecp2^{+/+}* (black circles, n=17), 20–25-week-old *Mecp2^{Null/+}* animals (red squares, n=12) display attenuated percent freezing during the fourth trace period (T4) ($F_{(4,108)}=4.276$; p=0.0016). (**C**) Diagram illustrating the trace fear conditioning paradigm in WT littermates and *MECP2^{Tg1/o}* animals. The protocol mentioned above in RTT mice is followed with notable differences in the foot shock intensity (0.5 mA) and the number of tone-trace-shock pairings (three). (**D**) Compared to WT littermates (black circles, n=11), 8–12-week-old *MECP2^{Tg1/o}* animals (blue squares, n=10) exhibit increased percent freezing during the third trace period (T3) ($F_{(3,57)}=35.14$; p<0.0001). 2-way ANOVA with Sidak's post-hoc test. **p<0.01, ****p<0.0001.

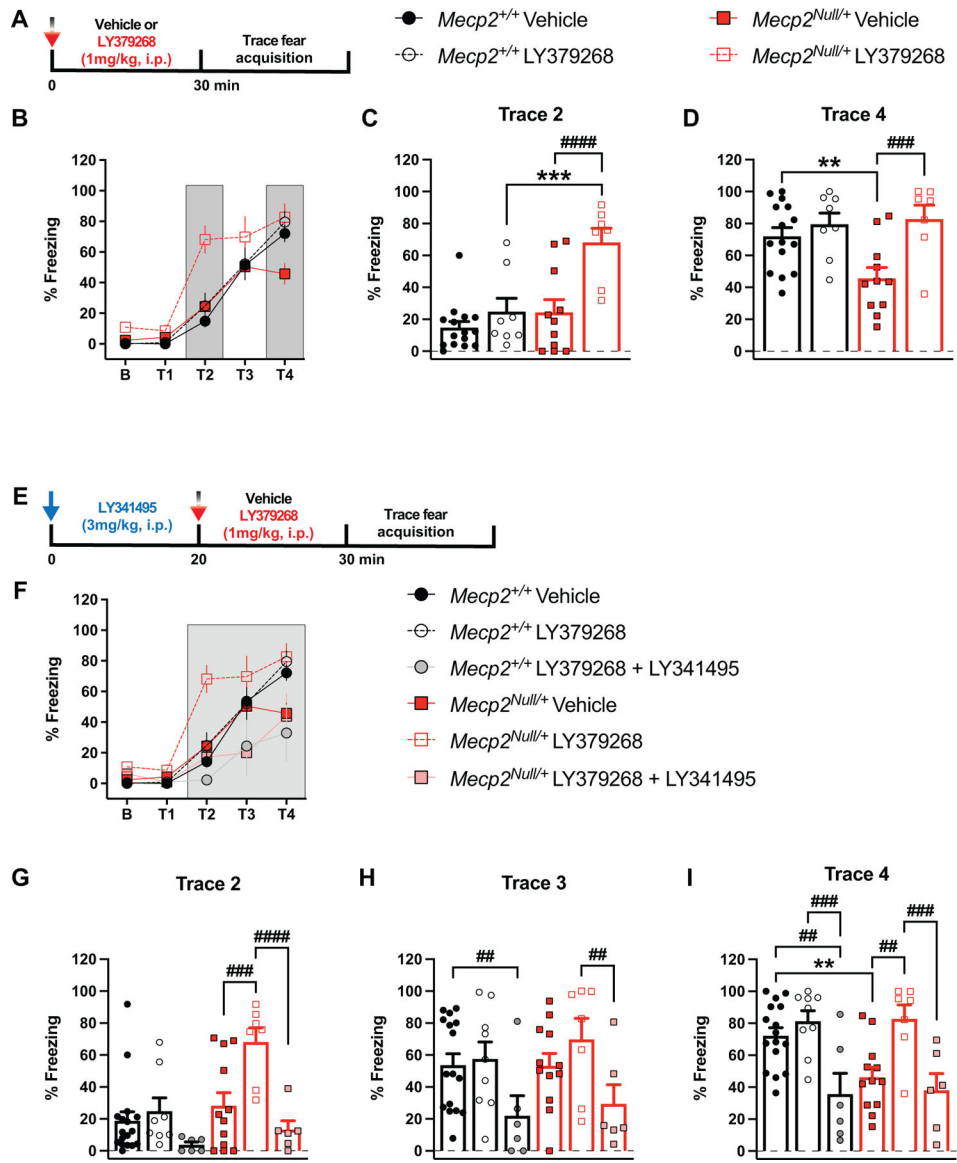**Figure 4: Attenuated trace fear acquisition in *Mecp2$^{Null/+}$* mice is improved with group II mGlu receptor activation.**

(A) Diagram illustrating timeline of drug administration prior to trace fear conditioning. Mice are treated intraperitoneally (i.p.) with vehicle (10% Tween-80) or 1mg/kg LY379268 (mGlu$_{2/3}$ agonist) 30 minutes prior to placement in the fear conditioning box. **(B-D)** Treatment with LY379268 increases trace fear acquisition in *Mecp2$^{Null/+}$* mice (red bars, vehicle: closed red squares, n=15; LY379268: open red squares, n=7). Freezing behavior in littermate controls, *Mecp2$^{+/+}$*(black bars) is not different between the treatment groups, vehicle (closed black circles, n=15) and LY379268 (open black circles, n=8). Analysis of trace periods T2 and T4 (dark grey bars in **B**) are shown in **(C)** and **(D)**, respectively. (F(12,148)=3.314; ***p=0.0001 (T2, LY379268: *Mecp2$^{+/+}$* vs *Mecp2$^{Null/+}$*), ####p<0.0001 (T2, *Mecp2$^{Null/+}$*: Vehicle vs LY379268), **p=0.0034 (T4, Vehicle: *Mecp2$^{+/+}$* vs *Mecp2$^{Null/+}$*), ###p=0.0005 (T4, *Mecp2$^{Null/+}$*: Vehicle vs LY379268)). **(E)** Diagram illustrating timeline of administration of LY341495 (mGlu$_{2/3}$ antagonist) and LY379268

prior to trace fear conditioning. LY341495 (3mg/kg, i.p.) is administered 20 minutes prior to LY379268 (1mg/kg, i.p.). **(F-I)** Co-administration of LY341495 and LY379268 blocked the reversal effect of LY379268 in $Mecp2^{Null/+}$ animals (red bars, vehicle: closed red squares, n=12; LY341495 + LY379268: pink squares, n=6) at trace periods T2-T4. Percent freezing in $Mecp2^{+/+}$ mice (black bars) is decreased with co-administration of LY341495 and LY379268, particularly at trace periods T3-T4 (vehicle: closed black circles, n=17; LY341495 + LY379268: grey circles, n=6). Analysis of trace periods T2-T4 (dark grey) is shown in **(G-I)**. (F(20,200)=3.423; ###p=0.0005 (T2, $Mecp2^{Null/+}$: Vehicle vs LY379268), ####p<0.0001 (T2, $Mecp2^{Null/+}$: LY37968 vs LY379268 + LY341495), ##p=0.0044 (T3, $Mecp2^{+/+}$: Vehicle vs LY379268 + LY341495), ##p=0.0043 (T3, $Mecp2^{Null/+}$: LY379268 vs LY379268 + LY341495), **p=0.0042 (T4, Vehicle: $Mecp2^{+/+}$ vs $Mecp2^{Null/+}$), ##p=0.0011 (T4, $Mecp2^{+/+}$: Vehicle vs LY379268 + LY341495), ###p=0.0008 (T4, $Mecp2^{+/+}$: LY379268 vs LY379268 + LY341495), ##p=0.0020 (T4, $Mecp2^{Null/+}$: Vehicle vs LY379268), ###p=0.0010 (T4, $Mecp2^{Null/+}$: LY37968 vs LY379268 + LY341495)). 20–25-week-old mice. 2-way ANOVA with Tukey's post-hoc test. *between-genotypes. #within-genotypes. **p<0.01, ***p<0.001, ##p<0.01, ###p<0.001, ####p<0.0001.
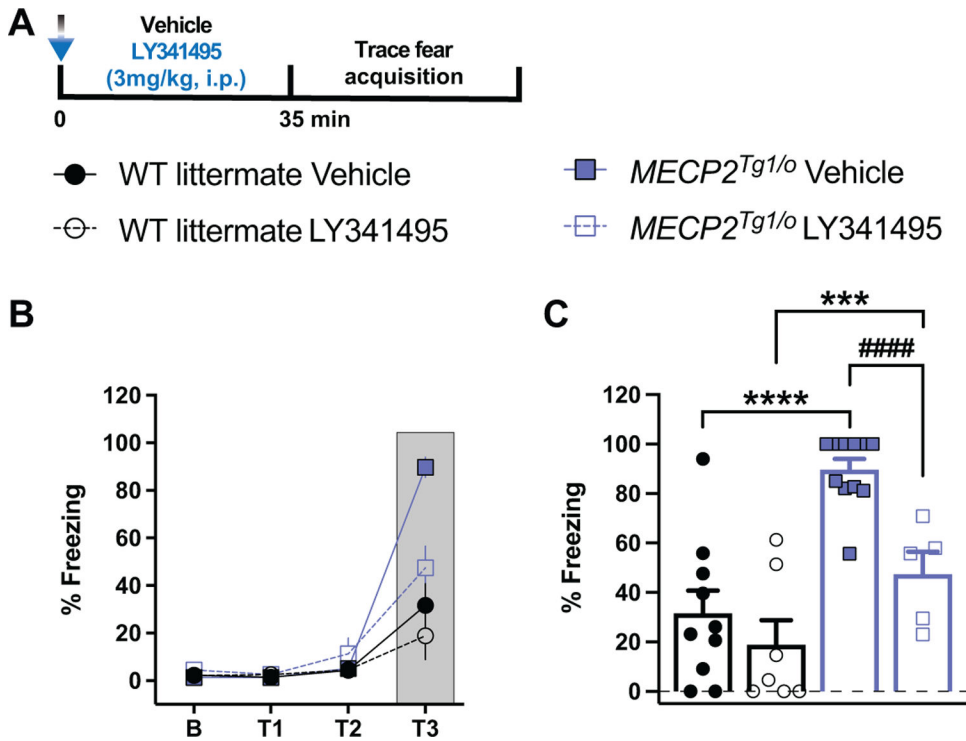
Figure 5: Group II mGlu receptor antagonism normalizes enhanced trace fear acquisition in $MECP2^{Tg1/o}$ mice.

(A) Diagram illustrating the timeline of LY341495 administration prior to trace fear conditioning. Mice are treated intraperitoneally (i.p.) with vehicle (10% Tween-80) or 3mg/kg LY341495 ($mGlu_{2/3}$ antagonist) 35 minutes prior to placement in the fear conditioning box. **(B-C)** LY341495 treatment significantly decreases the enhanced trace fear acquisition phenotype in $MECP2^{Tg1/o}$ mice (blue bars, vehicle: closed blue squares, n=11; LY341495: open blue squares, n=5) but not in WT littermates (white bars, vehicle: closed black circles, n=10; LY341495: open black circles, n=7). Analysis of trace period T3 (dark grey) is shown in **(C)**. ($F(9,87)=16.87$; ****$p<0.0001$ (Vehicle: WT vs $MECP2^{Tg1/o}$), ***$p=0.0008$ (LY341495: WT vs $MECP2^{Tg1/o}$), ####$p<0.0001$ ($MECP2^{Tg1/o}$: Vehicle vs LY341495)). 8–12-week-old mice. 2-way ANOVA with Tukey's post-hoc test. *between-genotypes. #within-genotypes. ***$p<0.001$, ****$p<0.0001$, ####$p<0.0001$.