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A protocol for evaluation of Rett Syndrome symptom improvement by metabolic modulators in *Mecp2*-mutant mice

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Abstract

Mouse models recapitulate many symptoms of Rett Syndrome, an X-linked disorder caused by mutations in methyl-CpG-binding protein 2 (MECP2). The study of *Mecp2*-null male mice has provided insight into pathogenesis of the disorder; most recently, dysregulation of cholesterol and lipid metabolism. Perisymptomatic treatment with statin drugs successfully mitigates the effects of this metabolic syndrome, increases longevity and improves motor function. Described here is a metabolic drug screening protocol and timeline for symptom evaluation in *Mecp2*-mutant mice. Specifically, mice are treated twice weekly with a compound of interest alongside subjective health assessments, bi-weekly body composition measurements and blood chemistries. Throughout treatment, behavioral phenotyping tests are carried out at specific time points. This protocol is highly adaptable to other neurological diseases; however, the time for completion depends on the specific mutant model under study. The protocol highlights the use of several different CPMo protocols to carry out testing in a preclinical model.

Keywords

Drug Treatment; Cholesterol; Lipid; Behavioral Assessment; Rett Syndrome; Neurodevelopmental Disorders; Metabolism

INTRODUCTION

Emerging evidence indicates an important role for maintenance of homeostasis between lipid synthesis, storage, and recycling in neurological diseases. Perturbations in biosynthesis and intracellular trafficking of cholesterol are responsible for the onset of Niemann-Pick type C and Smith-Lemli-Opitz syndrome, respectively. Moreover, inheritance of the E4

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AUTHOR CONTRIBUTIONS

MJJ and CMB conceived of the work. CMB performed early statin injections, phenotyping, and necropsies, and designed the protocol. MGH handled later compounds and performed injections and rotarod assessments. JMB performed weekly phenotyping, rotarod, and open field assessments. MGH and JMB together compiled data, performed necropsies and processed tissues. SMK performed metabolic assays. CMB analyzed data and, together with MHG and JMB optimized the protocol. CMB, SMK and MJJ wrote the manuscript.

variant of apolipoprotein E (APOE), a cholesterol-carrying protein, greatly increases risk for development of late-onset Alzheimer disease (Vance, 2012). Statin drugs, which are designed to lower cholesterol, can ameliorate neurological symptoms in mouse models of Fragile X (Osterweil et al., 2013) and are being used in clinical trials to ameliorate cognitive problems in children with neurofibromatosis Type I (Arderin-Holmes and North, 2011). Most recently, dysregulation of cholesterol and lipid metabolism has been shown to play a role in the pathogenesis of Rett Syndrome (RTT: OMIM 312750), and statin drugs improved symptoms in a mouse model (Buchovecky et al., 2013). Cholesterol abnormalities have also become apparent in RTT patients: in one recent paper, girls with RTT were found to have a serum LDL cholesterol level that is 14.7 mg per dL higher on average than a healthy control cohort of a similar age range (Sticozzi et al., 2013).

In a genetic suppressor screen, we identified a novel mutation that mitigates Rett-like symptoms in 129.*Mecp2*^{tm1.1Bird}/Y mice: a premature stop mutation in the gene encoding squalene epoxidase, SQLE, a rate limiting cholesterol biosynthesis enzyme. Consequently, we investigated lipid metabolism in the 129.*Mecp2*^{tm1.1Bird}/Y mouse model and found it to be perturbed, both in the brain and systemically. 129.*Mecp2*^{tm1.1Bird}/Y mice display complex dysregulation of the pathway in the brain: presymptomatic buildup of cholesterol, followed by a sharp post-symptomatic downregulation of cholesterol synthesis. Systemically, mice showed progressive worsening of metabolic symptoms, including increased liver cholesterol synthesis, increased serum cholesterol, and buildup of triglycerides and other neutral lipids in the liver. Under the theory that a pharmacologic inhibitor of cholesterol synthesis would, like the *Sqle* mutation, attenuate symptoms, we treated 129.*Mecp2*^{tm1.1Bird}/Y mice and wildtype littermates with statin drugs. Not only were metabolic symptoms improved; statin treated 129.*Mecp2*^{tm1.1Bird}/Y mice showed improvement on a number of health and behavioral parameters commonly monitored in RTT mouse models, most notably longevity and motor function. Furthermore, we successfully adapted this protocol to 129.*Mecp2*^{tm1.1Bird}/+ female mice in order to evaluate the effects of statin drugs in mice that more closely model female RTT patients, in which the MECP2 mutation is mosaic due to X-inactivation, and saw similar results (Buchovecky et al., 2013).

However, the ability to treat dysregulation of cholesterol and lipid metabolism is not limited to statin drugs. In addition to dyslipidemia, symptomatic *Mecp2*-null mice display hyperinsulinemia, glucose tolerance, and insulin resistance, suggesting their pathology may be confounded by metabolic syndrome (Pitcher et al., 2013). Furthermore, *Mecp2*-null mice develop fatty liver due to a significant buildup of triglycerides and other neutral lipids (Buchovecky et al., 2013). Cholesterol and triglyceride metabolism is tightly regulated by insulin sensitivity and the ability to utilize glucose. These metabolic pathways are complex and interconnected and are naturally regulated at numerous feedback points (Figure 1), which have been co-opted in pharmaceutical design. Therefore, many more possibilities exist for optimizing treatment of metabolic symptoms.

Described herein is a generalized protocol for the evaluation of Rett Syndrome symptom suppression by metabolic modulators in *Mecp2*-mutant mice (Basic Protocol 1), as well as supporting protocols describing accelerating rotarod (Support Protocol 1), open field activity

(Support Protocol 2), prepulse inhibition (Support Protocol 3), plethysmography (Support Protocol 4), and body composition (Support Protocol 5) assays. We have designed this procedure to be highly adaptable to additional *Mecp2*-mutant mouse models, or even to models of other neurological diseases that have a metabolic component or respond to statin treatment. We hope that providing a generalizable procedure and extensive commentary based on our own experiences with treatment regimen design and phenotype evaluation will encourage more researchers working on mouse models of Rett Syndrome and other neurological diseases to consider a role for metabolism in their model. Further investigation into the cross talk between lipid dysregulation and neurological symptom manifestation is warranted for the effective and safe design of treatments for patients. Thoughtful and controlled treatment of mouse models with metabolic modulators represents the first step and can be achieved through careful consideration of the variables discussed here.

BASIC PROTOCOL 1: Treatment of *Mecp2*-mutant mouse models with metabolic modulators

This is a highly adaptable and generalizable protocol that will walk a researcher through the planning, treatment, and evaluation stages of testing metabolic modulators for suppression of symptoms in *Mecp2*-mutant mice. Please see the commentary section and associated references for information to be considered when selecting the mouse model and compound(s) to be tested, as well as for determining the timeline and assessments to be used.

Note: All experiments should be performed in accordance with relevant animal care and use guidelines and regulations.

Note: Follow all manufacturers' instruction and MSDS guidelines with regard to handling and storage of chemical reagents.

Materials

Planning the experiment

Age and sex-matched *Mecp2*-mutant mice and wildtype littermates

Compound of interest

Appropriate vehicle for compound (*e.g.* sterile saline, ethanol and/or DMSO)

Scale for weighing compounds

Glass vials for storing organic compounds

Appropriate protective equipment for handling compounds (follow manufacturers' guidelines)

Appropriate equipment for compound administration (*e.g.* 10cc syringes with ~26-gauge needles for subcutaneous injection)

Waste container for sharp items

Compound Administration and Routine Assessments

Age and sex-matched *Mecp2*-mutant mice and wildtype littermates

Compound of interest

Appropriate vehicle for compound (*e.g.* sterile saline, ethanol and/or DMSO)

Scale for weighing compounds

Glass vials for storing organic compounds

Appropriate protective equipment for handling compounds (follow manufacturers' guidelines)

Appropriate equipment for compound administration (*e.g.* 10cc syringes with ~26-gauge needles for subcutaneous injection)

Waste container for sharp items

Scale for weighing mice

Optional: mouse whole body composition analyzer (*e.g.* Bruker MiniSpec NMR)

Equipment required for bi-weekly blood draws (method TBD by experimenter and institution) performed in accordance with relevant animal care and use guidelines and regulations

Serum separator tubes (*e.g.* BD Vacutainer® Blood Collection tubes)

End of life studies

Treated and control, age and sex-matched *Mecp2*-mutant mice and wildtype littermates

Waste container for sharp items

Scale for weighing mice

~25-gauge needles and syringes for cardiac punctures

Equipment for mouse euthanasia (method TBD by experimenter and institution) performed in accordance with relevant animal care and use guidelines and regulations

Serum separator tubes (*e.g.* BD Vacutainer® Blood Collection tubes)

Cryo-safe tubes for tissue collection

Liquid Nitrogen

Planning the experiment

1. Chose the mouse model you plan to treat with metabolic modulators.

Note: See “Critical Parameters: Selection of Mouse Strain” for discussions concerning this process. Import and start a colony if you have not already done so, keeping in mind import regulations and quarantine times at your institution.

2. Choose the singular behavioral assays you will use for evaluation and determine the ages at which they will be administered.

Note: See “Critical Parameters: Selection of Behavioral and Metabolic Assessments and Timing” for discussions concerning this process.

Note: This is also a good time to get familiarized with these assays and all other techniques used in subsequent steps.

3. Determine the appropriate age at which to begin treatment.

Note: Generally, we have found an effective age to begin treatment is perisymptomatically. The timing of symptom onset and progression of specific symptoms differs depending on the mutant model, genetic background, and sex of the mice. For example, the earliest sign of perturbed cholesterol metabolism in 129.Mecp2^{tm1.1Bird}/Y mice is increased levels of the brain cholesterol turnover enzyme, Cyp46a1 at postnatal day (P) 28, at which time the mice are beginning to display mild behavioral symptoms, such as occasional limb clasping and tremor. Systemically, metabolic symptoms, most notably rising serum cholesterol, worsen between P28 and P56; we chose to begin treatment at P35. In 129.Mecp2^{tm1.1Bird}/+ female mice, however, behavioral symptoms emerge and progress more slowly and early buildup of lipids in the liver is more pronounced than increased serum cholesterol. In this case, we delayed the start of treatment, but only until six weeks of age to account for the changes already occurring in the liver (Buchovecky et al., 2013). Therefore, it is important to consider all possible phenotypic changes when deciding when to begin treatment. In many cases, the existing literature will not be sufficient to determine when metabolic symptoms appear in your model and it will be necessary to perform some preliminary assessments of cholesterol and lipid status over time. To this end, see sample protocols (Brufau and Groen, 2011; Tailleux and Staels, 2011).

Note: The developmental requirements of the mice should also be kept in mind when determining that age at which to begin treatment. In 129.Mecp2^{tm1.1Bird}/Y mice we attempted, unsuccessfully, to begin treatment as early as P14, but this was detrimental to the health of the mice (data not shown), possibly due the brain’s high requirement for cholesterol for neuronal myelination at this early age (Pfriege and Ungerer, 2011).

4. Select and obtain the compound to be tested and determine an appropriate vehicle and route of administration.

Note: See “Critical Parameters: Considerations Regarding Compound of Interest” for discussions concerning this process. Store according to manufacturers’ instructions.

Note: Consider lipophilicity when selecting a compound to test. In our experience, more lipophilic statin drugs, such as lovastatin and simvastatin, were more effective than less lipophilic ones, which may be a result of their

greater ability to cross the blood-brain barrier (Cibicková, 2011). However, treatment with the relatively hydrophilic statin drug, fluvastatin, also altered brain sterol composition in 129.*Mecp2*^{tm1.1Bird/Y} mice. It is not currently understood how much the ability of statin drugs to alter brain cholesterol levels is a direct result of statin activity in the brain and how much is an indirect effect of altered systemic lipid metabolism (Cibicková, 2011; Stranahan et al., 2011).

5. Determine the desired dose of the compound and the frequency at which it should be administered. Prepare a small amount of your chosen compound according to manufacturers' instructions (e.g. certain statin drugs require an activation step). Dilute the compound in chosen vehicle to the concentration at which it will be administered.

Note: See "Critical Parameters: Considerations Regarding Compound of Interest" for discussions concerning this process.

Note: In our experience, the most successful treatment for 129.*Mecp2*^{tm1.1Bird/Y} mice was a twice-weekly 1.5 mg per kg body weight dose of lovastatin. For comparison, human patients taking lovastatin for hypercholesterolemia are commonly prescribed 20-80 mg tablets taken daily, or 0.25-1.00 mg per kg body weight daily, assuming an 80 kg (176 lb.) patient. In general, successful statin doses for treating Rett-like symptoms in mice were not higher than what is commonly prescribed in human patients when averaged over the course of a week to account for the differing regimens. In fact, doses ten times higher were detrimental (Buchovecky et al., 2013).

6. From an existing *Mecp2*-mutant colony, create sex-matched cohorts of at least six *Mecp2*-mutant mice and four wildtype littermates, subsequent to weaning and genotyping, but prior to penetrant symptom onset for the mutant line of choice (e.g. prior to P35 for 129.*Mecp2*^{tm1.1Bird/Y} mice). A vehicle control cohort should be processed alongside treatment cohorts. Cohorts started at the same time should contain mice as close in age as possible (e.g. P35±2)

Note: Do not use newly transferred mice in this protocol. Mecp2-mutant mice do not tolerate stress well and the stress of transport could skew experimental results.

*Note: For Mecp2-mutant mice, timelines will likely vary significantly depending on whether you are working with male or female mice. See "Critical Parameters" for detailed discussions of why this is the case and a basis for designing your timeline. For suggested timelines using male or female 129.*Mecp2*^{tm1.1Bird} mice, see Figure 2 and Table 1.*

7. Have the researcher who will be performing injections code the cohorts such that the researcher performing the phenotype assessments is blinded to genotypes and treatments of the mice.

8. Sample size and statistical parameters should be established prior the start of the experiment.

Note: Estimates of effect sizes for compounds of interest and for the mouse models under study can commonly be ascertained from the literature. For example, for behavioral assessments in Mecp2-mutant mice, 12-16 mice per treatment group should confer statistical significance (Katz et al., 2012).

Compound Administration and Routine Assessments

Note: If a moribund mouse is discovered during the course of performing any phenotyping assay or compound administration, that mouse should be euthanized in accordance with relevant guidelines and regulations.

- 9 The day prior to the initial administration of the compound of interest or vehicle control, fast the mice for 6 hours then collect blood for clinical blood chemistries in serum separator tubes. Send separated serum for clinical blood chemistries, including lipid panels, immediately after collection (Rathkolb et al., 2011a). Repeat this step biweekly (for mutants with rapid symptom progression) or monthly.

Note: The method of collection is at the discretion of the researcher, but we do not suggest retroorbital bleeds for Mecp2-mutant mice. Instead, the saphenous vein is a good source from which blood can be repeatedly obtained (Rathkolb et al., 2011b).

Note: Repeated non-terminal blood draws must be performed in accordance with relevant animal care and use guidelines and regulations. Some methods can be used more frequently than others. Plan accordingly.

- 10 The day of administration, an experimenter blinded to treatment group should perform subjective health assessments according to guidelines proposed for preclinical evaluation in mice (Katz et al., 2012). Repeat subjective health assessments at least once per week on designated days, typically 24 hours after administration of the compound.

Note: Typically, symptoms of each mouse are scored from 0-2 (0 being like wildtype, 2 being severely symptomatic) on mobility, hindlimb clasping, tremor, breathing, and general condition. Some experimenters prefer a 5-point scale for each symptom being assessed in order to make finer distinctions.

- 11 Record body weight for each mouse. Repeat this step prior to each administration of the compound.

- 12 Optional: Perform whole body composition analysis on all mice according to analyzer manufacturer instructions. Repeat this step biweekly (for mutants with rapid symptom progression) or monthly.
- 13 Prepare the working dilution of the compound and begin administration according to the timing determined when planning the experiment. Repeat compound administration according to the predetermined schedule (e.g. twice weekly until P70 for 129.*Mecp2*^{tm1.1Bird}/Y mice)

Note: Because cholesterol and lipid metabolism follows a circadian rhythm, each treatment and assay or assessment should occur at the same time of day.
- 14 Perform chosen singular behavioral assessments (e.g. accelerated rotarod, open field activity, pre-pulse inhibition of startle response) according to the timeline determined when planning the experiment. These assessments are performed within the framework of repeating steps 9-13 of this protocol.

Note: See support protocols to view procedures for author-suggested assessments.

End of life studies

- 15 Once all behavioral assessments are complete, perform the final set of injections, subjective health assessments, and optional body composition analysis.
- 16 The day after the last injection, fast the mice for 6 hours during the light cycle.
- 17 Record body weight for each mouse.
- 18 Euthanize a mouse with an appropriate anesthetic to incorporate cardiac puncture, with the goal of exsanguination and collection of blood for clinical chemistries, including a lipid panel.
- 19 Dissect the mouse, collecting and weighing any tissues of interest, including brain regions of interest and liver.

Note: Handle tissues according to requirements for future experiments (e.g. flash freeze an approximately 50-100 mg portion of the liver in liquid nitrogen for lipid extraction and quantification).

Note: If exsanguination was poor, large amounts of blood present in the tissues of interest may skew cholesterol quantification results.
- 20 Repeat steps 18 and 19 for all mice in the cohorts.
- 21 Dispose of all biological waste in accordance with relevant guidelines and regulations.

- 22 Extract and dry lipids from brain and liver tissue according to established protocols (Tailleux and Staels, 2011).

Note: Some institution cores will perform this function as part of lipid quantification; follow their guidelines.

- 23 Quantify lipids in tissue extracts according to an established protocol (especially of cholesterol and triglycerides) or have this quantification performed by a lab or core trained in gas-liquid chromatography or another validated process (Tailleux and Staels, 2011).

SUPPORT PROTOCOL 1: Accelerating Rotarod – Repeated Measures

Assessment

Much like human patients, *Mecp2*-mutant mice progressively develop gait irregularities and other motor coordination deficits (Katz et al., 2012). Many methods exist to measure motor coordination, and each has its respective pros and cons. Below, we provide a validated protocol for using accelerating rotarod in *Mecp2*-mutant mice. A detailed discussion of considerations specific to assessment of motor coordination, and protocols for additional methods, can be found in Brooks et al. (2011).

Note: The authors feel the task outlined below is the most efficient and discerning test of motor coordination with minimal experimenter bias currently available. However mouse weight can be a potential confounding factor and should be considered in statistical analyses. Arguably, counting grid-walking foot-slips is a viable alternative that minimizes the effect of weight. However, until a validated automated system becomes available, it is not as high-throughput as rotarod and has greater room for experimenter bias.

Materials

Treated and control, age and sex-matched *Mecp2*-mutant mice and wildtype littermates
Isolated room without noise or traffic
Adjustable light source
Light monitor
White noise source
Noise meter
Rotarod for Mice and Rats (Stoelting; Cat# 52790), or equivalent
Ethanol and paper towels

Protocol Steps

1. Set the rotarod to accelerate steadily from four revolutions per minute up to forty revolutions per minute over the course of five minutes.

2. At the predetermined age (See Basic Protocol 1), place up to five (we suggest no more than three for ease of recording) mice on the rotarod at its lowest speed so that they face opposite the direction of rotation. Start the timer for each mouse and begin acceleration.
3. Record the time at which a mouse first spins with the rod (this will not happen for all mice).
4. End the trial and record the time at which a mouse falls off the rod, spins with the rod for two consecutive revolutions, or successfully remains on the rod for five minutes.
5. Repeat steps 2-4 for all mice in the cohorts. Clean the apparatus between cages.
6. Repeat steps 2-5 three times, so that all mice perform 4 trials.

Note: Wait half an hour between trials for any given mouse to ensure that a task ends due to lack of coordination rather than muscle exhaustion.

7. The next day, repeat steps 2-6 to complete trials five through eight.

Note: Not all accelerating rotarod protocols require as many as eight trials, nor two days of testing. In our experience, the greatest differences are observed in trials performed on day two. Improvements could be missed if fewer trials are performed.

SUPPORT PROTOCOL 2: Open Field Activity Assessment

As symptoms progress, *Mecp2*-mutant mice display decreased activity levels (Katz et al., 2012). This can be easily measured using an open field animal activity monitor, as described below. A detailed discussion of locomotor activity assessment, including additional protocols for open field activity plus wheel running assays, can be found in Thomas et al. (2011).

Note: The literature often misrepresents Mecp2-mutant mice as consistently hypoactive, but they can be hyperactive early in disease progression. Preliminary testing should be performed in your mutant line to determine when this switch occurs. Statins have been shown to increase activity levels at P70, when Mecp2-null mice are usually hypoactive (Buchovecky et al., 2013).

Materials

Treated and control, age and sex-matched *Mecp2*-mutant mice and wildtype littermates

Isolated room without noise or traffic

Adjustable light source

Light monitor

White noise source

Noise meter

Animal activity monitors – open field mouse configuration (AccuScan Fusion monitors, or equivalent)

Ethanol and paper towels

Protocol Steps

1. At the predetermined age (See Basic Protocol 1), set up the open field chamber room: clean chambers with ethanol, start software, adjust light and noise levels
Note: Open field chambers should be in an isolated room free from noise and traffic.
Note: Light and noise levels should be adjusted to decrease anxiety and the appropriate levels will depend on the genetic background of the mice being tested.
2. Move mice to be assessed into prepared room to acclimate for 30 minutes.
3. Follow manufacturer instructions, set up recording program for the first group of mice to record for 30 minutes.
4. Place one mouse in the center of each open field chamber and begin recording immediately.
Note: Leave the room while mice are in the open field to avoid distracting them.
5. Remove mice from open field after 30 minutes of recording.
6. Repeat steps 3-5 for all mice in the cohorts. Clean chambers with ethanol between mice.

SUPPORT PROTOCOL 3: Prepulse Inhibition of Acoustic Startle Reflex

Mecp2-mutant mice display deficits in prepulse inhibition of acoustic startle reflex. We have also observed decreased amplitude of startle (Buchovecky et al., 2013). A detailed protocol discussing prepulse inhibition of acoustic startle reflex can be found in Ouagazzal and Meziane (2011); a basic protocol is presented below.

Note: In our experience, the decreased amplitude of startle in Mecp2-mutant mice can sometimes be so severe that prepulse inhibition cannot accurately be measured. We did not observe improvement on either parameter with statin treatment (Buchovecky et al., 2013). However, as this assessment was performed only at P70 in Mecp2-null mice, we cannot rule out the possibility that statin treatment could delay startle symptom progression, though symptoms certainly fully progress eventually even with treatment. In future studies, we suggest performing longitudinal testing of startle response in your mutant line to determine the timing of symptom progression.

Materials

Treated and control, age and sex-matched *Mecp2*-mutant mice and wildtype littermates

Isolated room without noise or traffic

Startle Response Lab system (San Diego Instruments, or equivalent) and associated software

Ethanol and paper towels

Protocol Steps

1. At the predetermined age (See Basic Protocol 1), set up the room: clean chambers with ethanol, start software and perform a test run to ensure proper calibration.
Note: Chambers should be in an isolated room free from noise and traffic.
2. If necessary, move mice to be assessed into a separate nearby room to limit travel immediately before testing and acclimate for 30 minutes.
3. Follow manufacturer instructions, set up recording software for the first group of mice. Chose a program with multiple randomized trials including combinations of pulses of approximately 50 dB with prepulses of 4-12 dB, as well as each alone and trials with no audio stimulus.
4. Place one mouse in each chamber. Ensure proper alignment with the force plate.
5. Run startle response program and leave the room until the run is complete.
6. Remove the mice.
7. Repeat steps 3-6 for all mice in the cohorts. Clean chambers with ethanol between mice.

SUPPORT PROTOCOL 4: Unrestrained Whole-body Plethysmography

Mecp2-mutant mice display breathing abnormalities that are similar to those seen in human patients. Most prevalent are hyperventilation, interbreath irregularity, and increased apneas. The strength of these phenotypes has been shown to vary substantially with the age and genetic background of the mice (Ogier and Katz, 2008). A basic unrestrained whole-body plethysmography protocol is described below; additional details about performing the procedure are best obtained from the manufacturer of your particular plethysmography setup.

Note: In our experience, irregular breaths, particularly apneas, frequently do not pass quality control when using Buxco FinePointe software, thereby preventing accurate measurement unless done by hand by a blinded individual trained at reading plethysmography trace data. Unlike with previous software versions, the manufacturer does not allow changes to the algorithm parameters for what constitutes a breath (i.e. breaths with an expiratory time greater than a certain threshold are excluded and this threshold cannot be changed). Therefore, we cannot currently suggest use of FinePointe for this particular application, though previous BioSystem XA versions are appropriate.

Materials

Treated and control, age and sex-matched *Mecp2*-mutant mice and wildtype littermates

Isolated room without noise or traffic

Unrestrained whole-body plethysmography system for mouse (Buxco, EMMS PLY 310, or equivalent) with associated software

Chart for recording activity

Protocol Steps

1. At the predetermined age (See Basic Protocol 1), set up the room: clean chambers and start airflow and software. Calibrate chambers according to manufacturer instructions.

Note: Chambers should be in an isolated room free from noise and traffic.

2. Create a plethysmography program with at least 30 minutes of acclimation to the chambers, followed by at least one hour of data collection.

Note: Appropriate times will vary depending on how active the wildtype mice are and how long they take to settle to a resting state. Genetic background of the mice will have the greatest impact on this. Mice should not be kept in the chambers for greater than four hours, as they do not have access to food and water while inside.

3. Place one mouse in each chamber, enter their identifying information, and begin the plethysmography program.
4. Record the activity level of each mouse at 2-3 minute intervals. The data of interest are what is collected when each mouse is at rest (i.e., not exploring, grooming, or otherwise moving within the chamber).
5. Remove the mice at the end of the predetermined recording period.
6. Repeat steps 3-5 for all mice in the cohorts. Clean chambers with water between mice.
7. Analyze the data recorded during the resting phases for each mouse.

Note: Common measurements of interest for Mecp2-mutant mice include hyperventilation, interbreath irregularity, and number of apneas within a set duration (usually per hour). However, definitions for each of these parameters can vary between researchers. For a detailed discussion, see Ramirez et al. (2013).

COMMENTARY

Critical Parameters

Selection of Mouse Strain—This protocol could potentially be adapted to any *Mecp2*-mutant mouse models, nulls or those mimicking human mutations, with appropriate modifications to the timing of start of treatment and phenotyping assays, which can be determined with sufficient information about symptom progression in the mutant of interest. It should be noted that, symptom progression in males and females carrying the same *Mecp2* mutant allele will vary significantly because, as *Mecp2* is located on the X

chromosome, females are heterozygous mosaics, while males are hemizygous. Keep this in mind when searching the literature.

While a wealth of information exists in the literature about the timing of behavioral assays for a number of *Mecp2*-mutant lines, the cholesterol and lipid metabolism component of this disorder is a relatively new finding that has not been well studied. Therefore, we encourage researchers to measure cholesterol and triglyceride levels in blood and liver over the course of disease progression in their mutant prior to planning treatment with metabolic modulators. Researchers should also keep in mind that the phenotype of any *Mecp2*-mutant mouse depends not only on its *Mecp2* allele, but also on its genetic background strain. For example, weight gain in *Mecp2*-null mice varies in different strain backgrounds (Katz et al., 2012). Furthermore, both sex (Turley et al., 1998) and genetic background (Jolley et al., 1999) have been shown to influence cholesterol and lipid metabolism in wildtype mice. For example, we found that by 8 months of age *Mecp2*-mutant female mice do not develop levels of cholesterol as high as that of symptomatic males at ten weeks of age (Figure 3), but are storing nearly 2.5 times as much lipid in their liver (Buchovecky et al., 2013). Therefore, it is extremely important that this protocol be carried out on sex-matched mice on a pure or F1 genetic background. More generally, strain background can affect performance on behavioral assays and dictate the optimum setting for those assays. For instance, known anxiety or activity levels of a particular genetic background may influence your choice of behavioral assay, the length of the task, or the light and noise conditions under which the task is performed.

It should also be noted that although this protocol can be performed on *Mecp2*-conditional mutants, the researcher should be mindful that the necessary genetic control groups of the floxed allele without cre transgene, and cre transgene without the floxed allele will double cohort size. We do not suggest the use of this protocol in mice treated with tamoxifen to induce allelic recombination; as an estrogen receptor antagonist, tamoxifen injection could alter lipid metabolism and it is unclear how this would affect the activity of any compound being tested.

Considerations Regarding Compound of Interest—As discussed in the introduction, there are a number of compounds designed to modify cholesterol and lipid metabolism that an experimenter could choose to use in this protocol. For example, FDA-approved compounds exist that regulate both cholesterol and lipid metabolism upstream of HMGCR. Others in the development pipeline lower cholesterol levels by inducing the reuptake of blood cholesterol by the liver, where it can be converted into bile acids for excretion. When choosing a compound to test in this protocol, we suggest giving consideration to those that already have FDA-approval, for which a great deal of information about hydrophobicity, pharmacokinetics, transport, and metabolism of the compound can be found. This will both aid the researcher in determining the appropriate dosing and administration for the compound, and also maximize the speed at which successful compounds can be considered for repurposing for use in RTT patients. However, in instances in which this detailed information is not available, we suggest performing some preliminary studies to determine the lowest dose at which a given compound will overcome a challenge assay in wildtype mice. For example, if you expect your compound to decrease

serum cholesterol, you can follow existing protocols to ascertain its ability to lower chemically-induced high cholesterol (Sudha et al., 2011).

Once the compound of interest has been identified, questions of dosing, vehicle, and route of administration should be carefully considered. The answers to these questions are commonly interdependent. For example, we have found a good starting point for mg per kg body weight dosing of statins can be approximated based on the oral dose given to human patients when we are using subcutaneous injection as our route of administration. However, the efficiency of delivery, and therefore the necessary dose, as well as the desired pH and volume of a vehicle will differ between routes of administration. Considerations of the pharmacokinetics and pharmacodynamics of the compound will help determine the frequency of dosing. In our experience, drugs with half-lives as short as 8-10 hours in the mouse can be effective at shifting metabolism when given only twice weekly, though the lowest effective dose using this regimen is likely higher overall than if a daily dose were given. We would not suggest administration regimens less frequent than this except for drugs with exceptionally long half-lives. Furthermore, the safe volume in which a compound can be administered can vary depending on vehicle and route, and therefore compounds with low solubility or with high required doses can preclude certain options. Protocols and troubleshooting guides for your planned route of administration should be referenced in detail to ensure animal welfare (for example see Turner et al. (2011a, 2011b).

Creation of a concentrated stock solution at the beginning of a trial is recommended where possible, but be aware that some compounds may degrade if resuspended and stored for too long, or at the wrong temperature. Therefore, it is extremely important that you follow all manufacturer and MSDS guidelines when handling your compound of interest. The manufacturer will also commonly provide information about solubility of the compound in various solvents, which can be used in the vehicle solution. When choosing a vehicle, consider both solubility of the compound and bioactivity of the solvent. For example, you may be able to dissolve much more of your compound in ethanol than in sterile saline, but may not want the diuretic and behavioral effects of introducing relatively large amounts of ethanol to the animal. In some cases, if the desired dose will not dissolve in saline a mixed solution of saline and a more efficient solvent can be used. Furthermore, this example illustrates why using a true vehicle control is imperative in this protocol. Similarly, route of administration should also be consistent between treatment and vehicle control groups.

Selection of Behavioral and Metabolic Assessments and Timing—Subjective health assessments should be performed by a blinded observer at least weekly using the scoring system described in “development of the protocol”, or a similar established system with which the researcher is familiar. Assessments should be performed at the same time each week, both in relationship to time of day and time since last injection. Furthermore, these assessments should take place before any other manipulations of that mouse on the day of testing.

One-time assessments of behavioral functioning should include those for which metabolic modulators have already been shown to improve symptom profiles in a mouse model of your disease of interest. In the case of *Mecp2*-mutant mice, this would include open field

activity and accelerating rotarod assessments, as well as other established tests of motor function, such as grid-walking, where available (Samaco et al., 2012). Other tests can be added at the experimenter's discretion, often depending on the symptom profile of the disease and the proposed mechanism of cholesterol and lipid metabolism involvement in disease pathogenesis. Ideally, appropriate timing for each assay will be well established in the literature for your mutant of interest. Examples for *Mecp2*^{-/Y} (males) and *Mecp2*^{-/+} (females) can be found in Table 1. If this information is not available, it can be derived from cross-sectional studies comparing mutant mice with age and sex matched wildtype littermates on the assays of interest, following established protocols for the assays under consideration (for reference, see Wahlsten, 2010). Ideally, behavioral testing during the treatment with a metabolic modulator should be performed when a highly significant difference between mutant and wildtype mice is observed, but before the mutant mice reach their nadir at performance of the task, ensuring that there is sufficient room for improvement with treatment.

Metabolic assessments in the current protocol are limited to body weight or body composition analysis, serum cholesterol and lipid panels from regular blood draws and end of life studies. However, if evidence for lipid modulation by the compound is observed, additional assays for metabolic status, including intraperitoneal glucose tolerance tests (IPGTT), insulin tolerance (ITT) and calorimetry may be carried out.

Limitations—In some instances, it is possible that the morbidity and mortality of a particular *Mecp2*-mutant line is not strongly associated with the symptoms that have been successfully ameliorated with statin treatment. For example, some lines display a high incidence of malocclusion preventing them from consuming food well; treatment with metabolic modulators is not expected to increase longevity or significantly improve overall health in such lines.

The protocol described here as primarily been used to prevent or delay systemic dysregulation of cholesterol and lipid metabolism and motor symptoms. Treatment typically began after the earliest signs of sterol dysregulation, which occur in the brain, but before the *Mecp2*-mutant mouse model developed significantly higher levels of serum cholesterol or liver triglycerides than their wildtype littermates. This was, in part, a limitation of the primary model we studied; neurological and general health symptoms progress very rapidly in 129.*Mecp2*^{tm1.1Bird}/Y mice alongside systemic metabolic symptoms. However, we recognize such treatment might be most effective for patients if symptoms can successfully be ameliorated with treatment beginning when detectable systemic cholesterol and/or lipid symptoms arise. We therefore encourage researchers working with models in which there is a greater latency from symptom onset to mortality to alter this protocol to begin treatment shortly after high serum cholesterol or liver triglycerides are observed.

Troubleshooting

Please refer to Table 2 for Troubleshooting

Anticipated Results

Our protocol for treating *Mecp2*-mutant mice with metabolic modulators and evaluating the ability of those modulators to mitigate Rett-like symptoms through a variety of behavioral and biochemical assessments can be successfully applied to diverse compounds and adapted to many different mouse mutants. We have provided information that will allow the user to optimize to their compound and *Mecp2*-mutant model of choice, and that may even be extended to other neurological diseases for which there is sufficient information about the behavioral and metabolic symptoms of the rodent model.

Experience suggests that successful compounds will improve *Mecp2*-mutant symptoms that relate to cholesterol and lipid metabolism, motor function, and longevity. We have not seen improvements in acoustic startle or baseline breathing assays, but have provided suggestions for improved optimization if a researcher wishes to test these parameters in their own model. We hope this protocol will encourage researchers to extend the assessment of metabolic modulators in *Mecp2*-mutant mice to include additional phenotyping assays, such as those that test social activity, or learning and memory paradigms, which the authors have not yet studied.

When performing statistical analyses of the assessment results, we provide two cautions. First, in our experience, the *Mecp2*-mutant group typically showed increased variability compared to their wildtype littermates, so equal variances within groups should not be assumed. Second, because a protocol like this lends itself to testing multiple compounds, we remind experimenters to adjust for multiple comparisons when necessary.

Lastly, we wish to emphasize that we do not expect treatment of *Mecp2*-mutant mice with metabolic modulators will be sufficient to suppress all RTT-like symptoms. We anticipate that combination therapies that include existing treatments for RTT co-morbidities will be of further benefit and encourage researchers to attempt such combinations in their *Mecp2*-mutant mouse models.

Time Considerations

The entire protocol set takes place over a period of weeks or months, depending on time spent planning and the speed of symptom progression for the particular mutant model being studied. For *Mecp2*-null male mice, the protocol can be completed within seven weeks of weaning two cohorts (experimental and control) that contain at least six *Mecp2*-null males and four sex-matched littermates. The following timings for specific steps are approximate and assume the assessment of 20 mice:

Blood draws (Step 9): 6 hour fast plus 1-2 hours per repetition (varies based on method)

Subjective Health Assays (Step 10): 30 minutes per repetition

Weights (Step 11): 10 minutes per repetition

Body Composition Assay (Step 12): 30-60 minutes per repetition, depending on technology

Compound Administration (Step 13): 30-45 minutes per repetition, depending on route

End of Life Studies: 6 hour fast, plus necropsy time per mouse (varies with experience)

Accelerating Rotarod (Support Protocol 1): 5 hours divided between 2 days

Open Field Activity (Support Protocol 2): 3 hours (assuming 4 chambers)

Pre-Pulse Inhibition (Support Protocol 3): 3.5 hours (assuming 2 chambers)

Plethysmography (Support Protocol 4): Varies depending on background strain

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Figure 1.

A simplified schematic of cholesterol and lipid synthesis including major regulatory elements and feedback points. Pathway members are in black, enzymes in red, transcription factors in blue, and hormones in green. Both cholesterol and lipid synthesis are regulated by AMPK and downstream factors including the ACCs and SREBPs. Cholesterol itself is part of a negative feedback mechanism that adjusts levels of the rate limiting enzymes HMGCR and SQLE. Lipid synthesis is regulated in part by the accumulation of lipids in adipocytes, which produce adipokines, such as leptin and adiponectin. In turn, these adipokines are known to regulate AMPK levels.

Figure 2.
An example timeline used by the authors for cohorts of *Mecp2^{tm1.1Bird}/Y* going through treatment with metabolic modulators and phenotypic evaluation.

Figure 3. Comparison of (A) serum cholesterol levels and (B) liver lipid levels in 8-month-old female mice (striped bars) and ten-week-old male mice (solid bars). **A** Serum cholesterol levels do not differ between male and female wildtype mice, or between male and female statin treated *Mecp2*^{tm1.1Bird} mice. When not treated with statin drugs however, serum cholesterol levels in aged *Mecp2*^{tm1.1Bird}/₊ (female) mice are significantly lower than in the younger *Mecp2*^{tm1.1Bird}/_Y (male) mice. **B** Liver lipid levels are trend higher in 8-month-old female mice than ten-week-old male mice across all genotypes and treatment groups, likely due to age. However, this difference is only significant in *Mecp2*-mutant mice and most striking in those that have not been treated with statin drugs. (Derived from data in prior publication in Buchovecky et al. (2013))

Table 1

Summary of recommended phenotyping schedule for *Mecp2*-null mice.

TEST	MECP2-/Y MALES				MECP2-/± FEMALES		
Weekly body weight	+				+		
Weekly subjective health assessment	+				+		
	Week 4	Week 6	Week 8	Week 10	Week 8	Week 16	Week 32
Open field activity	-	-	-	+	-	-	+
Motor coordination	-	-	+	-	-	+	+
Clinical chemistry	+	+	+	+	+	+	+
Tissue lipid panels	-	-	-	+	-	-	+
Optional:	Week 4	Week 6	Week 8	Week 10	Week 8	Week 16	Week 32
Body composition	-	+	+	+	+	+	+
Plethysmography	-	+	-	-	+	-	-
Acoustic startle and Pre-Pulse Inhibition	-	-	+	-	-	+	-
Social activity	-	-	+	-	-	-	+

Table 2

Troubleshooting

Step	Problem	Possible Reason	Solution
Basic Protocol 1	5	Compound is insoluble	Vehicle is not an appropriate solvent
		Vehicle is too basic or acidic	(In)Organic compounds dissolve best in (in)organic solvents.
	6	Fighting in new cohorts	Carefully adjust to pH7-8 using HCL or NaOH.
		Combining males >6 weeks of age	Create cohorts when mice are younger.
	6	Symptom onset varies from initial observations	Handling improves symptoms
		Change in diet	Treat controls exactly like experimental group.
	9	Mutant mice do not survive overnight fast	Consistently use same chow with low (4-7%) fat content.
		<i>Mecp2</i> -null do not tolerate stress well	This is why only a six-hour fast is suggested. Six hours is sufficient to minimize dietary impact on serum cholesterol. A longer fast is not necessary and could harm the mice.
	9	Retroorbital bleeds difficult	Use a different method for bleeding mice
		<i>Mecp2</i> -null mice have eye abnormalities	(saphenous vein bleeds are suggested).
	9	Mice die under anesthesia	Choose a bleeding method that does not require anesthesia
		Breathing abnormalities put <i>Mecp2</i> -null mice at risk during anesthesia	OR use very low, controlled levels of isoflurane.
	10	Variable phenotyping results	Perform phenotyping assessments before bleeds in a day.
		Residual pain from bleeding method	
	12	Seize during body composition analysis	Perform stressful assays as quickly as possible, keeping the comfort of the mouse in mind. Note: This problem is rare
	13	Mice develop inflammation at the injection site	Vehicle is too basic or acidic
			Carefully adjust to pH7-8 using HCL or NaOH.
		Vehicle is a skin irritant	If possible, increase the percentage water or saline of vehicle.
		Compound is a skin irritant	Use intraperitoneal, rather than sub-cutaneous, injections.
Support Protocol 1 (Rotarod)	<i>Mecp2</i> -mutant mice do not grasp rotarod due to limb-clasping	Mouse holding bedding	Remove bedding and try again. This is the only time failure to perform should result in a repeated trial rather than a score of zero.

Step	Problem	Possible Reason	Solution
	Mice explore/sniff rotarod dividers	Scent residues of other mice present	Clean rotarod thoroughly with ethanol between cages.
	Weight correlates significantly with performance within genotypes	Known potential confound	Report this confound and provide statistical analysis of the correlation. AND/OR supplement with additional weight-neutral motor coordination assessments.
Support Protocol 2 (Open Field)	Variable open field results	Prior phenotyping skews results	Measure open field activity first in a given day.
	Wildtype control mice have low activity levels	Varies by background strain; anxiety related in certain strains	Use background strains known to display higher activity AND/OR adjust lighting and noise levels to reduce anxiety
	<i>Mecp2</i> -mutant mice do not move in open field	Mice are moribund	Test earlier (and euthanize moribund mice!)
		<i>Mecp2</i> -mutant mice freeze due to anxiety	Measure for full 30 minutes (mice may take 5 full minutes to move). AND/OR start all mice in the corner of the open field
	Mice focus on single point	Scent residues of other mice present	Clean chambers thoroughly with ethanol between mice.
Support Protocol 3 (PPI)	Startle response during the “no stimulus” trials	Setup is miscalibrated	Recalibrate according to manufacturer instructions.
		Startle response result of environmental stimulus	Place the chambers in a quiet room and leave while the program is running to avoid startles that result from noises independent of those presented by the program.
	Startle response not observed during any trial, even in wildtype mice	Setup is miscalibrated	Recalibrate according to manufacturer instructions.
		Chamber misaligned	Realign.
		Program is not producing acoustic stimulus	Contact the manufacturer.
Support Protocol 4 (Plethysmography)	Mice do not settle down to resting state during recording	Acclimation/recording session too short	Increase time in chamber
		Distractions present in the room	Minimize noise and movement; place opaque divider between chambers so mice cannot see each other.
	No apneas recorded in <i>Mecp2</i> -mutant mice	Mice too young	Test at a later age when respiration is known to be abnormal

Step	Problem	Possible Reason	Solution
		Apneas do not meet threshold for a breath in recording program	Adjust length of breath threshold until apneas visible in the trace are recorded as data.