Improvement in motor and exploratory behavior in Rett syndrome mice with restricted ketogenic and standard diets

John G. Mantis a, Christie L. Fritz a, Jeremy Marsh a, Stephen C. Heinrichs b, Thomas N. Seyfried a,*

Abstract

Rett syndrome (RTT) is a rare X-linked autistic-spectrum neurological disorder associated with impaired energy metabolism, seizure susceptibility, progressive social behavioral regression, and motor impairment primarily in young girls. The objective of this study was to examine the influence of restricted diets, including a ketogenic diet (KD) and a standard rodent chow diet (SD), on behavior in male Mecp2 308/y mice, a model of RTT. The KD is a high-fat, low-carbohydrate diet that has anticonvulsant efficacy in children with intractable epilepsy and may be therapeutic in children with RTT. Following an 11-day pretrial period, adult wild-type and mutant Rett mice were separated into groups that were fed either an SD in unrestricted or restricted amounts or a ketogenic diet (KetoCal) in restricted amounts for a total of 30 days. The restricted diets were administered to reduce mouse body weight by 20–23% compared to the body weight of each mouse before the initiation of the diet. All mice were subjected to a battery of behavioral tests to determine the influence of the diet on the RTT phenotype. We found that performance in tests of motor behavior and anxiety was significantly worse in male RTT mice compared to wild-type mice and that restriction of either the KD or the SD improved motor behavior and reduced anxiety. We conclude that although both restricted diets increased the tendency of Rett mice to explore a novel environment, the beneficial effects of the KD were due more to calorie restriction than to the composition of the diet. Our findings suggest that calorically restricted diets could be effective in reducing the anxiety and in improving motor behavior in girls with RTT.

1. Introduction

Rett syndrome (RTT) is an X-linked dominant neurodevelopmental disorder that affects about 1 in 9000 girls [1]. Girls with RTT develop normally for about 6–18 months after birth before exhibiting signs of speech and behavioral regression in addition to progressive motor impairment [2–4]. Many symptoms of RTT are age dependent and include hand wringing, reduced muscle tone, sensory, anxiety, microencephaly, indications of mental retardation, and seizures, among other autistic-like behaviors [5–8]. Although RTT patients show abnormal neuronal morphology, no neuronal loss is evident. The relatively low incidence of RTT in humans often results from misdiagnosis of the disorder as autism or, to a lesser extent, as Angelman syndrome [9].

About 80% of girls with RTT have a mutation in the Mecp2 (methyl-CpG-binding protein 2) gene [10–12], which encodes a protein involved in transcriptional regulation and, more specifically, histone deacetylation and methylation-dependent gene silencing [13–15]. Males with mutations in the Mecp2 gene often die before birth or in infancy owing to severe neonatal encephalopathy [16]. A small number of males with a Mecp2 mutation, however, have developed signs and symptoms similar to those of classic Rett syndrome [17–19]. Some of these boys have an extra X chromosome in many or all of the body’s cells. Several mouse Mecp2 gene mutants have been generated, including a partially truncated form of the MeCP2 protein (Mecp2 308/y) that is commonly found in girls with RTT [3,20,21]. In contrast to humans, male Rett mice exhibit the classical RTT phenotype much earlier in life than female mice and thus are predominately used for animal studies. Importantly, Mecp2 308/y mice exhibit several symptoms associated with RTT in humans, including behavioral abnormalities and impaired social interactions [3,20–23]. More specifically, around 6 weeks of age Mecp2 308/y mice begin to display learning and memory deficits that are indicative of synaptic dysfunction, as well as other symptoms of RTT progression. The RTT phenotype in the female Mecp2 mice is milder and shows greater variability, presumably due to differences in the pattern of X-chromosome inactivation [24,25]. Skewed X inactivation is...
also believed to be responsible for the mild and hardly recognizable RTT phenotype in girls with RTT [26,27].

RTT children are generally smaller than normal children and these differences become increasingly exaggerated over time [28]. Girls with RTT tend to be disinterested in social interactions and are often emotionally withdrawn [29]. They also have elevated circulating levels of pyruvate, lactate, and glucose, which could be indicative of an abnormal metabolic phenotype [30–32]. Interestingly, Mecp2-null mice also have reduced levels of brain glutamine, glutamate, choline, N-acetyl aspartate, and ATP, further indicating that RTT could be associated with normal neuronal and glial cell metabolism [33,34]. These findings, viewed together, indicate that abnormal energy metabolism may contribute to the growth failure associated with RTT and also suggest that diet therapies, and in particular restricted diet, could help delay the onset or at least mitigate the severity of the RTT phenotype [28,35–37]. This hypothesis has been confirmed in (some) girls with RTT who demonstrated modest improvements in behavior and motor performance when maintained on a ketogenic diet [30,38].

The ketogenic diet (KD) is a high-fat, low-carbohydrate diet that has been shown to have antiepileptic, anticonvulsant, and other neuroprotective effects in both rodents and humans [39–45]. We previously showed that although an unrestricted KD could delay the onset of seizures in EL mice with a genetic predisposition to epileptic seizures [46], greater seizure control could be achieved in these mice when fed a calorically restricted KD (KD-R) [40]. These findings suggest that a KD-R has a greater neuroprotective effect than an unrestricted KD, at least in rodent models of epilepsy [40].

Calorie restriction (CR) is a natural dietary therapy that has long been recognized to improve health, promote longevity, and reduce the incidence as well as delay the onset and/or severity of symptoms associated with a variety of neurochemical and neurobehavioral disorders, including epilepsy [47–51]. CR differs from acute fasting or starvation in that CR reduces total caloric intake without causing anorexia (appetite loss) or malnutrition [40]. Although the mechanisms underlying the neuroprotective effects of CR are unknown, it is believed that neuroprotection is associated with reduced circulating glucose levels and elevated ketone body levels. With regard to epilepsy, the metabolic transition from glucose to ketone bodies as the primary cerebral energy source under CR conditions has been shown to reduce seizure frequency in epileptic rodents and humans by inducing synaptic changes that ultimately attenuate neuronal hyperexcitability, thus increasing the extent to which these hyperexcitable foci are inhibited [40,48].

KetoCal (KC) is a nutritionally balanced soy oil-based KD that has been evaluated and classified by the FDA as a medical food for the management of seizures in children with intractable epilepsy [52]. In addition, our recent findings in experimental mouse brain tumor models indicate that KC could inhibit tumor growth only when administered in restricted amounts, suggesting that the full neuroprotective effects of KC could be achieved only under CR conditions [52]. Thus, in light of the evidence described above, we propose that CR could have a positive influence on the anxiety behavior and motor characteristics of a mouse model of RTT. Our preliminary results indicate that calorically restricted diets can be of clinical importance since CR improved symptoms of behavioral abnormalities in Rett mice, particularly with respect to reduced anxiety involving exploratory activity within an unfamiliar environment.

2. Methods and materials

2.1. Mice

The inbred B6.129S-Mecp2<sup>am1Hzo</sup> J (Mecp2<sup>208/y</sup>) Rett mice were originally obtained from The Jackson Laboratory (JAX; Bar Harbor, ME, USA). Mice were generated as previously described by Shahbazi et al. [21]. The mice were maintained through brother–sister inbreeding and kept in the Animal Care Facility of Boston College with all procedures in strict adherence with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care Committee. The mice were group housed (prior to initiation of study) in plastic cages with Sani-Chip bedding (P.J. Murphy Forest Products Corp., Montville, NJ, USA) and kept on a 12–12 light/dark cycle at approximately 22 °C. Cotton nesting pads were provided for warmth when animals were individually housed. All cages and water bottles were changed once per week. Only males were used for these studies since female Rett mice have a less severe disease phenotype [21].

2.2. Genotyping Rett mice

DNA from 30-day-old Rett mice was isolated from ~3 mm of tail using the Qiagen DNeasy tail tissue protocol. The PCR was set up similar to that of the JAX genotype protocol for the Mecp2<sup>208/y</sup> mouse with the following modifications as previously described [53]. Briefly, 1 µl of DNA (~50–100 ng) was amplified with 5 µl of 5X buffer, 0.5 µl dNTPs, 5 µl forward primer (10 nM), 2.5 µl AR primer (10 nM), 2.5 µl BR primer (10 nM), 0.25 µl GoTaq DNA polymerase (Promega), and 8.25 µl water for a 25-µl total reaction volume. The DNA PCR amplification protocol used was 94 °C for 2 min, followed by 31 cycles of 94 °C for 45 s, 62 °C for 45 s, and 72 °C for 45 s, with a final extension at 72 °C for 5 min following the last cycle. The forward and AR primer set amplified a 396-bp fragment from the wild-type allele, whereas the forward and BR primer set amplified a 318-bp fragment from the knockout allele. PCR products (5–15 µl) were separated on 1% agarose gels containing ethidium bromide, visualized with UV light.

2.3. Diets

All mice were fed a standard Prolab RMH3000 chow diet (SD) prior to experimentation (LabDiet). The SD is a nutritionally balanced low-fat, high-carbohydrate diet that delivers 4.1 kcal/g of gross energy [40]. The KetoCal diet (Nutricia North America) is a nutritionally balanced soy oil-based high-fat, low-carbohydrate KD that delivers 7.2 kcal/g of gross energy and has a ketogenic ratio (fats/proteins + carbohydrates) of 4:1 [52]. KC was used in this study because it is a more palatable form of the KD. The feeding regimens for the SD-R and the KC-R mice were previously described [40,52]. Briefly, both the SD and the KC diets were calorie restricted to reduce mouse body weights by 20–23%. Water was provided ad libitum to all mice throughout the study. The energetic composition of the SD and KC diets is shown in Table 1.

2.4. Pretrial testing period for Rett mice

Twelve wild-type Mecp2<sup>+/+</sup> (control) and 18 Mecp2<sup>208/y</sup> (Rett) mice (188 days of age) were selected for the study and were individually housed for an 11-day pretrial period. Young adult symptomatic male Mecp2 mice were selected instead of female Mecp2 mice because of the greater degree of RTT phenotypic similarities

<table>
<thead>
<tr>
<th>Component</th>
<th>Standard diet</th>
<th>KetoCal diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>65</td>
<td>3.3</td>
</tr>
<tr>
<td>Fat</td>
<td>6.9</td>
<td>80</td>
</tr>
<tr>
<td>Protein</td>
<td>28.1</td>
<td>16.7</td>
</tr>
<tr>
<td>Energy (kcal/g)</td>
<td>4.1</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Values were obtained from normalizing energy content (see Section 2.3).

Table 1 Composition (%) of the standard diet and the KetoCal ketogenic diet.
of the male Mecp2 mice to RTT children. All mice were fed the SD ad libitum during the pretrial period and the daily food intake of each mouse was determined [40]. This pretrial period was used to establish baseline physiological (metabolism) and behavioral (motor coordination, proprioception, and exploration) parameters for each mouse. The experimental protocols for each behavioral test used are summarized below.

2.5. Testing battery

All behavioral testing was conducted before body weights or food/water intakes were determined for each mouse. Only one behavioral test was performed on a given mouse in a given day. The following behavioral tests sensitive to motor and sensory function were employed: (1) grip strength, (2) incline latency, (3) righting reflex, (4) visual placing, (5) light–dark compartment, (6) rotarod, and (7) open field.

2.5.1. The grip-strength test

The grip-strength test examined defects in motor neurodevelopment related to muscle strength [54]. The test was performed in triplicate with 60 s being the maximum allowable time for mice to grab/hold with their forelimbs and/or hind limbs onto a wire suspended 60 cm above a soft, padded surface. Only the maximum grab/hold time for a mouse to accomplish the task was considered for statistical analysis.

2.5.2. The incline latency test (negative geotaxis)

The incline latency test was performed to examine proprioceptive neurodevelopment and the ability to sense gravitational forces [55]. The test was performed in triplicate with 60 s being the maximum allowable time for mice to reorient themselves 180° (head facing upward) after being placed head facing downward on a soft, high friction surface with a negative 40° from horizontal slope. Only the maximum time for a mouse to accomplish the task was considered for statistical analysis.

2.5.3. The righting latency test

The righting latency test was also performed to examine proprioceptive neurodevelopment necessary to restore the body to an upright spatial position [56]. The test was performed one time unless a mouse demonstrated a reduced ability to turn over onto its belly (position itself in an upright position—all four limbs) after being placed gently on its back atop a flat padded surface. Only the maximum time for a mouse to accomplish the task (60-s trial) was considered for statistical analysis.

2.5.4. The placing latency test

The placing latency test examined neurodevelopmental defects in visual proprioception necessary to see and grasp an approaching solid surface. Mice were lifted gently by the tail, suspended briefly in midair, and then lowered slowly toward the edge of a table/mouse cage rack that the mice were able to reach by extending their forelimbs. The test was performed one time unless a mouse demonstrated a reduced ability to grasp/extend forelimbs toward an edge 2–3 cm away. Only the maximum time for a mouse to accomplish the task (60-s trial) was considered for statistical analysis.

2.5.5. The light–dark latency test

The light–dark latency test examined anxiety and the propensity of a mouse to explore a novel environment [57–59]. The test was performed one time for each mouse. The testing apparatus consisted of two compartments: a dark compartment and a light compartment. The dark compartment, a standard mouse cage covered with a solid box, served as the control environment and the light compartment, an uncovered mouse cage, served as the novel environment. The mouse was initially placed in the dark compartment and was allowed to move freely between the light and the dark compartments. The length of time that it took for a mouse to completely enter the lighted compartment, the amount of time that the mouse spent in the lighted compartment, and the total number of times that the mouse entered and exited this compartment were considered for statistical analysis. Each test lasted for 5 min.

2.5.6. The rotarod test

The rotarod test examined defects in mouse motor neurodevelopment related to coordination and balance [60]. The test was performed in duplicate at four different speeds (20, 30, 40, and 60 rpm) with 60 s being the maximum allowable time for the mice to stay on a rotating bar/rough edge cylinder positioned over mouse bedding. Mice were allowed to rest for 30 s between trials at the same speed and for 2 min between trials at different speeds. The average length of time that a mouse remained on the bar for a given speed was considered for statistical analysis.

2.5.7. The open-field test

The open-field test examined defects in mouse locomotor/exploratory activity, anxiety, and rearing events using the Smart-Frame Cage Rack System (Kinder Scientific, San Diego, CA, USA) [61,62]. Photobeams along the frame of the system track mouse movement within the cage and register mouse location, distance, and rearing capabilities. A mouse was placed in the center of the open-field apparatus and behavior was measured for 15 min. The data were analyzed using the MotorMonitor software (Kinder Scientific). Locomotor activity was measured as the total distance traveled in either the center or in the periphery (in cm), as well as the basic and fine movements the mouse performed during the 15-min period. Rearing events were measured as the number of times the mouse stood on its hind legs. Anxiety was measured as the degree of avoidance the mouse showed in exploring the center of the apparatus (number of entries in the center).

2.6. Dietary regimen

On the 11th day of the pretrial period, the mice were separated into the following diet groups: (1) a wild-type (Mecp2+/+) mouse group fed the standard diet ad libitum, or unrestricted (SD-UR), (2) a wild-type mouse group fed the KetoCal diet restricted (KC-R), (3) a wild-type mouse group fed the standard diet restricted (SD-R), (4) a Rett (Mecp2308+/-) mouse group fed the SD-UR diet, (5) a Rett mouse group fed the KC-R diet, and (6) a Rett mouse group fed the SD-R diet. Mice in each of the wild-type mouse groups were matched for body weight (29.5 ± 1.7 g), as were the mice in each of the Rett mouse groups (30.5 ± 1.5 g). All mice were then fasted for 17 h before the diets were initiated in order to establish a similar metabolic starting point. The feeding regimen for all KC-R and SD-R group mice was designed to reduce mouse body weights by 20–23% compared to each mouse’s individual pretrial body weight as we previously described [40,52]. The recommended body weight reduction was achieved and maintained during the dietary treatment period by adjusting the food intake of the R-fed mice every 3 days. Mice in the SD-UR groups were provided with ~200 g of fresh SD food pellets on a weekly basis. The body weights and food intakes of all mice were measured every 3 days. No KC-UR groups were included in these studies because this particular feeding regimen was not found to be neuroprotective in mouse models of brain cancer [52], nor was the KD-UR found as effective as the KD-R in reducing seizure frequency in a mouse model of epilepsy [40]. At the end of the dietary treatment period the same battery of behavioral tests was performed for each
mouse to evaluate the effect of the diet on the behavior of these mice.

2.7. Statistical analysis

Both ANOVA and a two-tailed t-test were used to evaluate the significance of differences in body weight and each behavioral parameter between unrestricted and restricted groups (SPSS software). Differences were considered significant at $p < 0.05$. The three-way mixed factor ANOVA statistical analysis was also performed to verify any significant effect between the diet, the mice, and the performance of the mice on the various rotorod speeds and the open field. All values are expressed as means ± SEM. All statistical data are presented according to the recommendations of Lang and Secic [63].

3. Results

3.1. Influence of diet on behavior

All mice were tested prior to the initiation of the dietary treatment period (see Section 2) in order to establish baseline information pertaining to their behavioral features. At the end of the 1-month dietary treatment period, all mice from each of the six groups were subjected to the same battery of behavioral tests to evaluate the effects of the calorically restricted KC diet or the restricted SD on their behavior. Consistent with the well-recognized health benefits of mild to moderate calorie restriction in rodents, no adverse effects were observed in either mouse group fed a calorically restricted diet. Despite a 20–23% body weight reduction, all R-fed mice appeared healthy and more active than mice in the groups fed ad libitum, as assessed by ambulatory and grooming behavior. Furthermore, nesting behavior was similar for all dietary groups (empirical observation). It is important to mention that no epileptic seizures were observed throughout this study in the Rett mice.

3.2. Influence of diet on body weight and food intake

All Rett (Mecp2<sup>308/y</sup>) and wild-type (Mecp2<sup>+/y</sup>) mouse groups were matched for age (~199 days) and body weight at the beginning of the dietary treatment period. The average daily food intakes for the wild-type and Rett groups over the pretreatment period were 4.2 and 4.6 g, respectively. All mice lost approximately 8–13% body weight over the course of the 17-h fast at the beginning of the treatment period. Mice in both the KC-R and the SD-R groups achieved the desired 20–23% reduction in body weight within 2–3 weeks of the initiation of dietary treatment (Fig. 1). The degree of CR needed to maintain the 20–23% body weight reduction was approximately 50%. No significant differences in body weight were observed between the wild-type (29.3 g) and the Rett (30.0 g) SD-UR mouse groups (Fig. 1).

3.3. Influence of diet on grip strength

The grip-strength test was used to distinguish motor neurodevelopment deficits between the Rett mice and the wild-type control mice. The suspension time was significantly less in the Rett SD-UR mice (16.7 s) than in the control SD-UR mice (47.5 s) (Fig. 2). Restriction of the KC diet or the SD did not improve the performance of the Rett mice on the wire (21.9 and 23.7 s, respectively) compared to the Rett mice fed SD-UR (Fig. 2). Moreover, CR of either diet had no effect in improving the performance of wild-type mice in the grip-strength test. These findings indicate that the Rett mice have a motor deficit and that restriction of either the KC or the SD diet does not improve this Rett phenotype.

3.4. Influence of diet on incline latency

The incline latency test measured the ability of a mouse to orient (face upward) itself against gravitational forces when placed facing downward on a negative 40° slope. No significant differences in the incline latency to face upward were observed between the wild-type (40.2 s) and the Rett (41.4 s) SD-UR mouse groups (Fig. 3). The incline latency of both KC-R mouse groups was significantly reduced relative to their respective SD-UR groups (Fig. 3). In addition, the latency of the wild-type SD-R mouse group was significantly reduced compared to the wild-type SD-UR mouse group. Although CR of the SD had no significant effect ($p = 0.058$) on improving the incline latency in the Rett mice (Fig. 3), a definite trend of improved behavior was evident in these mice as well. These findings suggest that restriction of either the KC or the SD diet improves overall reorientation (face upward) to negative geotaxis.

3.5. Influence of diet on righting reflex

The righting reflex test was used to measure each mouse’s proprioception and reflex response to revert back on its four limbs after being placed on its back. No differences were found between the Rett and the wild-type mice (all mice performed the task within 0–2 s). In addition, restriction of KC or the SD had no effect on this behavior (data not shown).

![Fig. 1. Influence of diet on body weight in Mecp2<sup>+/y</sup> and Mecp2<sup>308/y</sup> mice. Body weights of the R-group mice were significantly lower ($p < 0.001$) than those of their respective SD-UR groups; $n = 4$ for all Mecp2<sup>+/y</sup> mouse groups and for the Mecp2<sup>308/y</sup> SD-R group, whereas $n = 7$ for both Mecp2<sup>308/y</sup> (Rett) SD-UR and KC-R mouse groups.](image1.png)

![Fig. 2. Influence of diet on the wire suspension latency in Mecp2<sup>+/y</sup> and Mecp2<sup>308/y</sup> mice. Grip strength of the Rett mouse groups was significantly lower ($p < 0.05$) than that of the wild-type SD-UR group. Diet had no effect on improving the ability of the Rett mice to suspend from the wire. Other conditions were similar to those shown in Fig. 1.](image2.png)
of the SD increased the total time the wild-type mice spent in the light compared to their respective wild-type SD-UR mice (Fig. 5). The transition frequency between the two compartments was significantly greater in the wild-type SD-UR mice than in the Rett mice fed the SD-UR (Table 2). Restriction of the SD significantly increased the transition frequency between the light and the dark compartments for both the Rett and the wild-type mice (Table 2). It is important to mention that although the restricted KC diet moderately increased the number of transitions between the two compartments in both the wild-type and the Rett mouse groups, these differences were statistically significant only for the Rett mice (Table 2). Overall, these findings suggest that the Rett mice have an exploratory deficit and that restriction of either the KC or the SD diet reduces that deficit by increasing the activity of the Rett mice compared to Rett mice fed the SD unrestricted.

3.8. Influence of diet on rotarod performance

The rotarod was used to measure motor development, coordination, and balance in the Rett mice. At the three lower speeds (20, 30, and 40 rpm), the performance of the Rett SD-UR mice was significantly worse than that of the wild-type SD-UR mice (Table 3). Restriction of KC did not increase the time the mice spent on the bar (Table 3). On the other hand, restriction of the SD signifi-
cantly increased the time that the Rett mice spent on the bar compared to the Rett SD-UR mice (Table 3). No significant differences were found between the four groups in the rotorad performance at 60 rpm. The three-way mixed ANOVA further verified that the Rett SD-UR mice performed significantly worse on the rotorod compared to the wild-type SD-UR mice; however, the restricted KC diet had no significant effect on the rotorod performance. These findings indicate that the Rett mice have a motor coordination/balance deficit. Although the restricted KC diet had no effect on improving this deficit, the restricted SD was able to improve the time the Rett mice spent on the bar.

### 3.9. Influence of diet on open-field performance

The open-field test was performed to measure motor defects in locomotor activity and rearing events in the Rett mice during a 15-min testing period. No significant differences were observed between the Rett SD-UR mice and the wild-type SD-UR mice in all the behavioral parameters we measured (Table 4). Total time and rest time in each zone (center and periphery) were also similar between the Rett and the wild-type mice (data not shown). The restricted KC diet significantly increased the number of entries into the center and the periphery of the open-field apparatus as well as the number of rearing events in the Rett mice compared to the Rett mice fed the SD-UR (Table 4). The restricted KC diet had no effect in the total distance traveled in the periphery or in the center of the Rett mice (Table 4). Furthermore, the restricted KC diet significantly increased all behavioral parameters measured in the wild-type mice compared to the wild-type mice fed the SD-UR (Table 4). Similarly, the restricted SD significantly increased the total distance in the center as well as the number of entries in the center and the periphery of the Rett mice compared to the Rett mice fed the SD-UR (Table 4). Restriction of the SD also improved both the number of entries into the center and into the periphery of the open-field apparatus as well as the number of rearing events of the wild-type mice compared to the wild-type mice fed the SD-UR (Table 4). Although the average total distance traveled in the center of the open-field apparatus by the wild-type SD-R mice was greater than that traveled by the wild-type SD-UR mice, this difference did not reach statistical significance (p = 0.058) (Table 4). The restricted SD had no effect in improving the total distance traveled in the periphery of either the Rett or wild-type mice. Furthermore, although restriction of either diet significantly increased the basic movement in all R-fed groups compared to respective UR-fed mice, fine movement was not significantly different (data not shown). These findings indicate that the locomotor activity is similar in normal and Rett mice and that restriction of either the KC or the SD diet increases the locomotor activity in mice.

## 4. Discussion

Therapeutic diets, such as the KD, have been shown to have a wide range of neuroprotective effects (i.e., antiepileptic, anticonvulsant, antitumorigenic) both in humans and in rodent disease models [40-42,64-67], as well as improving the behavior of some girls with RTT [30,38]. The KD can also positively influence the behavior of autistic children [68] and can produce metabolic alterations in the brain and in the body that enhance energy expenditure and ultimately reduce body weight [69]. A reduction in circulating glucose levels coupled with an elevation of circulating ketone body levels is thought to underlie the therapeutic effects of the KD [40,48,52]. These neuroprotective effects of the KD suggest that a restricted KD could improve behavioral abnormalities and motor dysfunction in mouse models of RTT [30,38-40,68]. Our current findings support our prior evidence that the neuroprotective effects of either the KD or CR stem primarily from a reduction in total calorie intake rather than caloric origin (i.e., from carbohydrates, protein, or fats) [40,70]. Administration of the KD in restricted amounts also reduces the adverse effects of the diet’s high fat content (i.e., weight gain, hypercholesterolemia, diabetes, kidney stones, and cardiovascular disease) if the diet were to be administered ad libitum for extended periods of time [71-73]. Hence, we considered a restricted KD to be more therapeutic for the management of behavioral abnormalities in Rett mice than the unrestricted KD. This observation further supports our rationale for omitting an unrestricted KetoCal mouse group.

In agreement with the findings previously reported by Shahbazian et al., our results show that adult Rett (Mecp2<sup>308/y</sup>) mice are deficient in their ability to hang on a suspended wire or perform adequately on the rotarod motor test compared to control wild-type (Mecp2<sup>+/y</sup>) mice [21]. In contrast, the performance of the Rett mice on the open-field test was not significantly different from that

### Table 3

Mean walking time on the bar.

<table>
<thead>
<tr>
<th>Group</th>
<th>Time on bar (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mecp2&lt;sup&gt;+/y&lt;/sup&gt; SD-UR</td>
<td>41.38 ± 10.52</td>
</tr>
<tr>
<td>Mecp2&lt;sup&gt;+/y&lt;/sup&gt; KC-R</td>
<td>52.25 ± 5.42</td>
</tr>
<tr>
<td>Mecp2&lt;sup&gt;+/y&lt;/sup&gt; SD-R</td>
<td>59.62 ± 0.37</td>
</tr>
<tr>
<td>Mecp2&lt;sup&gt;308/y&lt;/sup&gt; SD-UR</td>
<td>18.14 ± 3.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mecp2&lt;sup&gt;308/y&lt;/sup&gt; KC-R</td>
<td>27.50 ± 9.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mecp2&lt;sup&gt;308/y&lt;/sup&gt; SD-R</td>
<td>43.00 ± 11.68&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM for all six groups of mice. 

- Mecp2<sup>308/y</sup> significantly different from control Mecp2<sup>+/y</sup> SD-UR group at p < 0.05 (as determined from the ANOVA). 
- Mecp2<sup>308/y</sup> significantly different from control Mecp2<sup>+/y</sup> SD-UR group at p < 0.01 (as determined from the ANOVA). 
- Mecp2<sup>308/y</sup> SD-R significantly different from Mecp2<sup>308/y</sup> SD-UR group at p < 0.05 (as determined from the ANOVA). 

### Table 4

Performance of Mecp2<sup>+/y</sup> mice in the open-field task.

<table>
<thead>
<tr>
<th>Group</th>
<th>Total distance in periphery (cm)</th>
<th>Total distance in center (cm)</th>
<th>No. of entries into periphery</th>
<th>No. of entries into center</th>
<th>Rearing events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mecp2&lt;sup&gt;308/y&lt;/sup&gt; SD-UR</td>
<td>859.15 ± 86.80</td>
<td>410.21 ± 61.43</td>
<td>36.00 ± 3.70</td>
<td>35.25 ± 3.70</td>
<td>68.00 ± 10.45</td>
</tr>
<tr>
<td>Mecp2&lt;sup&gt;308/y&lt;/sup&gt; KC-R</td>
<td>1225.55 ± 96.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>737.23 ± 149.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>74.25 ± 8.57</td>
<td>73.75 ± 8.82</td>
<td>128.50 ± 16.76</td>
</tr>
<tr>
<td>Mecp2&lt;sup&gt;308/y&lt;/sup&gt; SD-R</td>
<td>954.40 ± 120.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td>646.43 ± 65.57</td>
<td>36.29 ± 5.76</td>
<td>35.71 ± 5.84</td>
<td>58.86 ± 10.72</td>
</tr>
<tr>
<td>Mecp2&lt;sup&gt;308/y&lt;/sup&gt; SD-R</td>
<td>812.80 ± 64.02</td>
<td>502.67 ± 81.46</td>
<td>36.00 ± 3.70</td>
<td>35.71 ± 3.70</td>
<td>68.00 ± 10.45</td>
</tr>
<tr>
<td>Mecp2&lt;sup&gt;308/y&lt;/sup&gt; SD-R</td>
<td>953.95 ± 45.16</td>
<td>528.32 ± 28.93</td>
<td>54.29 ± 5.76</td>
<td>53.71 ± 5.84</td>
<td>93.71 ± 11.66</td>
</tr>
<tr>
<td>Mecp2&lt;sup&gt;308/y&lt;/sup&gt; SD-R</td>
<td>835.66 ± 52.22</td>
<td>725.17 ± 27.76</td>
<td>63.25 ± 3.33&lt;sup&gt;++&lt;/sup&gt;</td>
<td>62.75 ± 2.78&lt;sup&gt;++&lt;/sup&gt;</td>
<td>55.25 ± 12.00</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM for all six groups of mice (n = 4–7 mice per group). 

- Mecp2<sup>308/y</sup> KC-R or Mecp2<sup>308/y</sup> SD-R group significantly different from control Mecp2<sup>+/y</sup> SD-UR group at p < 0.01 (as determined from the ANOVA). 
- Mecp2<sup>308/y</sup> KC-R group significantly different from control Mecp2<sup>+/y</sup> SD-UR group at p < 0.05 (as determined from the ANOVA). 
- Mecp2<sup>308/y</sup> SD-R group mice are approaching significance level compared to control Mecp2<sup>+/y</sup> SD-UR group mice at p = 0.058 (as determined from the ANOVA). 
- Mecp2<sup>308/y</sup> KC-R group mice are significantly different from Mecp2<sup>308/y</sup> SD-UR group at p < 0.05 (as determined from the ANOVA). 
- Mecp2<sup>308/y</sup> SD-R group mice are significantly different from Mecp2<sup>308/y</sup> SD-UR group mice at p < 0.01 (as determined from the ANOVA).
of the wild-type mice. Although this particular finding is not consistent with that previously observed [21], it is important to mention that the length of the testing period between the two open-field tests in the two studies was different and could hence explain this discrepancy in our findings. More specifically, our test was performed for a total of 15 min, whereas Shahbazian et al. performed the test at 10-min intervals for a total of 30 min. In the aforementioned study, the performance of the Rett mice was not significantly different from that of the wild-type mice after 10 min, but differences were observed at the 20- and 30-min time points [21]. Consistent with prior evidence that the Mecp22lox/y mice display interaction deficiencies [21,22], our findings from the light–dark latency test also suggest that Mecp22lox/y mice express deficits in the exploration of a novel environment. The failure of the Rett mice to explore novel environments may reflect a heightened level of anxiety [62]. It is important to mention that the performance of the Rett SD-UR mice on the light–dark compartment test is consistent with previous findings in rodents [74–76].

Previous studies showed that both CR and the KD increase the activity and exploratory behavior of rodents [77,78]. Our current findings in the Rett mice support these observations and suggest that CR underlies the mechanism of the increased activity observed in mice fed either the restricted KC diet or the restricted SD. Interestingly, we observed that calorie restricted Rett mice exhibited not only an increased ability or tendency to explore a novel environment (i.e., the light–dark paradigm test) but also an increased number of entries into the center of the open-field apparatus (i.e., the open-field test) compared with Rett mice fed an SD-UR. These findings are consistent with prior evidence in rodents that CR increases the number of entries into, and the total amount of time spent in the center of the open-field apparatus [79,80]. It is important to mention that both the light–dark test (the emergence time into the light and the total time spent in the lit environment) and the open-field test (the entry into the center of the open-field apparatus) are measures of anxiety [62]. Hence, these data suggest that restriction of either the KC or the SD could reduce anxiety associated with the RTT phenotype. Furthermore, CR of either diet enhanced the performance of Rett mice on both the incline latency test (proprionception) and the number of rearing events in the open-field test (motor function) relative to Rett mice fed the SD-UR.

Although seizure susceptibility was assessed using our established handling-induced seizure susceptibility protocol for the epileptic EL mice [40,49], no myoclonic episodes were observed in the Mecp22lox/y mice that we studied. Consequently, we are unable to determine if the restricted KD could reduce seizure susceptibility in Rett mice as was shown previously in girls with RTT [30,38]. As an aside, althoughnesting is a measure of home–cage activity related to both social behavior and motor function [22], it is important to point out that neither the restricted KC diet nor the restricted SD was able to improve nesting behavior in the Rett mice (empirical observation). In general, R-fed mice spent significantly less time interacting with their nesting material compared to mice fed an unrestricted diet due to a persistent search for food. Thus, our data, viewed together, suggest the possibility that the increased activity observed in the Rett mice may be associated with increased hunger resulting from CR. Nevertheless, the restriction of either the KD or the SD can be of clinical importance since the diet improved symptoms of certain behavioral abnormalities in Rett mice, particularly those with respect to reduced anxiety involving exploratory activity within a novel environment and the number of entries into the center of the open-field apparatus.

It seems likely that the beneficial effects of the KD in human patients with RTT are similar to those observed in Rett mice in this study. Because girls with RTT are withdrawn emotionally and hes-


