Testing Efficacy of Sensorimotor Enrichment in Ameliorating Symptoms of Rett Syndrome in a Mouse Model

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Rett Syndrome (RTT) is a genetic, neurodevelopmental disorder that primarily affects girls (Hagberg, Aicardi, Dias, & Ramos, 1983). A mutation of the *Mecp2* gene has been implicated as a cause of RTT (Amir et al., 1999). Between 6-18 months of age, individuals with RTT experience deterioration in various cognitive, motor and social skills such as walking, speaking, and pointing (Naidu, 1997). Recent research of motor recovery hints that sensorimotor enrichment helps to improve the motor skills of *Mecp2* null mice (Kondo et al., 2008). Our study was performed using wild type mice and mutated RTT mice. Some of the mutated mice were exposed to daily enrichment. Our study not only used novel enriched-environments targeted at particular motor skills, but also crafted two motor tests/criteria for scoring that directly assess specific component motor skills necessary for functional activity. Our findings show that sensorimotor enrichment may ameliorate the motor deficiencies seen in this RTT mouse model. In addition, the results suggest that discontinuing enrichment may lead to deterioration of motor skills and reinstitution of enrichment may support motor skill recovery as assessed by these two tests. These results suggest that enrichment might be a viable treatment method for enhancing motor skill performance in girls with RTT.

Rett syndrome (RTT) is a neurodevelopmental disorder that affects 1 in 10,000 females (Amir et al., 1999). The clinical manifestations of RTT include the deterioration of acquired cognitive, motor and language skills between the ages of 6 and 18 months and the loss of purposeful hand use (Naidu, 1997). Initially, it was suggested that RTT might be an X-linked genetic disorder that mainly affects girls (Hagberg et al., 1983). A familial analysis of monozygotic and dizygotic twins provided compelling evidence that the disorder was in fact associated with the X-chromosome (Percy, 1992). However, it was not until 1999 that the specific locus for a mutation in the X-chromosome that might potentially dictate the pathogenesis of RTT was reported (Amir et al., 1999). Amir et al. (1999), identified mutations in the gene for methyl CpG-binding protein 2 (MeCP2) at the locus Xq28 as the cause of most RTT cases. *Mecp2* is a transcription regulator that targets many genes such as the *bdnf* gene (Chen et al., 2003).

The *bdnf* gene produces the protein brain-derived neurotrophic factor (BDNF), which helps to develop and maintain dendritic connections in immature and mature nervous systems (Chen et al., 2003). The regulation of BDNF is complex, involving the phosphorylation of MeCP2. Mutations of MeCP2 inhibit it from being phosphorylated, thereby preventing the eventual production of the BDNF protein (Sun & Wu, 2006). Due to the mutation in the *Mecp2* gene in people with RTT, low levels of brain BDNF levels have been reported (Moretti & Zoghbi, 2006).

There are multiple invasive ways to increase BDNF levels in the CNS. One noninvasive method seems to arise when environmental enrichment is present. Effects of enrichment have shown to be beneficial in the recovery of lost motor skills in multiple rat and mouse models of disease/injury (Diener, 2002;

Klinstova et al., 1998: Berrocal et al., 2007; Kondo, et al. 2008). Currently, there is no standard intervention for the treatment of RTT. However, environmental enrichment has been suggested as an option to ameliorate the symptoms experienced by *Mecp2* knockout mice via increasing BDNF levels (Kondo, et al. 2008). Sensorimotor or environmental enrichment, in this specific context, means housing the mice in an environment that is stimulating and includes play items with different textures. The present study tested whether the symptoms experienced by *Mecp2* knockout mice could be minimized or eradicated after multiple days of exposure to varied play environments, each providing sensorimotor enrichment. An additional purpose of the study is to create and evaluate two novel testing batteries to determine how effectively they can assess the performance of mice with RTT-like symptoms.

In previous studies using mouse models of neurologic diseases (Rett Syndrome, Spinal Cord Injury, Huntington's disease), assessment of motor control was conducted using various tests such as the accelerating rotarod (Kondo et. al, 2008; Tabuse, Yaguchi, Ohta, Kawase & Toda, 2010; Berrocal et al., 2007). The rotarod is an automated cylinder that accelerates at a fixed rate. For testing, the mouse is placed on the surface and time spent on the rod is recorded in order to examine how long the animals remain on the surface. We opted against using the rotarod because it is designed to test generalized motor abilities such as coordination and balance (Shiotsuki et al., 2010) rather than isolating and challenging the recovery of specific components of skilled function as has been previously reported in another model of immature nervous system injury (Diener and Bregman, 1998). In addition, testing using the rotarod shows that sometimes mice remain on the accelerating rod while gripping it tightly, rotating around with the rod, without actually moving their forelimbs and hindlimbs to remain balanced upright on the rod. Since the assessment scale for this battery is mainly time spent on the rod rather than measuring motor skills used to remain on the rod, we believe that the rotarod may not be effective in measuring improvements in/recovery of motor skills in mouse models of Rett Syndrome. Hence, we assessed performance using modified alternate batteries i.e., the Grid Walk and Grid Hang (Carlson, 2012) in which we developed a scoring criteria to meticulously analyze the movement and patterns of movement.

METHOD

Animals Male strain B6.128S-MeCP2$^{tm1Hzo/J}$ mice (Jackson Labs, Maine) were obtained after weaning. There were three groups of mice: *Mecp2* mutant mice exposed to the sensorimotor enrichment environment (SMEE) (E-group; n=5), *Mecp2* mutant mice that were not exposed to SMEE (N-group; n=4), and wild type mice, which were also not exposed to SMEE (W-group; n=6). The mice were housed in the Georgetown University Division of Comparative Medicine (DCM) and had access to unlimited food and water. Two mice in the E-group died during the study of causes seemingly unrelated to Rett syndrome. The Georgetown University Animal Care and Use Committee approved all animal protocols.

Study Design Baseline measurements were obtained four days after the mice arrived at the DCM facility. SMEE started three days after baseline testing. Mice were placed into the enriched environments five days a week, for four hours a day during the intervention period of the study. The study design includes an enrichment period for the first 7 months (March 19-October 1) followed by three month period when enrichment is discontinued (October 2 – Dec 28) and a final third period when enrichment is reinstituted (December 29-February 15). Behavioral testing took place every 4 weeks throughout the entire study, except in February when the final testing was scheduled 6 weeks after reinstitution of enrichment.

Environmental Enrichment Different tactile cues and motivating toys that elicited play and digging behaviors along with varied textured terrain and climbing equipment to encourage exploration were used within the 10 different sensorimotor enriched environments. Each environment was designed to spare the deterioration of or promote the recovery of different aspects of motor behavior typically lost in RTT mutant mice. Each mouse was exposed to each of the environments for a 4 hour time period for 5 days /week. This typically increased novelty and encouraged the mouse to explore the environment throughout the time that each was within, rather than exploring and then falling asleep in the corner of a cage. The animals were constantly monitored to ensure that they were engaged actively in the novel environment and daily notes of activity in each SMEE were recorded.

Behavioral Testing and Measures All testing protocols were videotaped with Canon Vixia HG20 camcorder with a shutter speed of 1/1000 sec and later analyzed using MacBook Pro laptops QuickTime player through frame-by-frame analysis. This study looks at two specific kinds of behavioral testing. To focus more specifically on testing particular aspects of motor skills and posture/balance, we chose to modify tests that would give a clear interpretation of recovered motor skills (or recovered components of motor skills).

Based on previous work, the tail hang test was used to validate *Mecp2* mutation through stereotypical forelimb movements (Young & Zoghbi, 2004). During this test, the researcher suspended each mouse by his tail to observe for a forelimb clasping. If forelimb clasping was identified, the animal received a score of 2. If clasping was absent, a score of 1 was given.

We modified two tests of motor skills that are widely used to study the rodent's ability to maintain grip against gravity, *Grid Hang* and achieve precise foot placement during ambulation, *Grid Walk*.

A) Grid Hang The behavioral test of grid hang was modified from Tillerson and Miller's study (2003) assessing mice affected with Parkinson's Disease. The grid hang test measures the core strength, coordination and skilled limb movements of mice. Our testing apparatus was constructed from a grid rack (37 cm x 50 cm; each opening of grid is 1 cm x 1 cm). The test begins by placing the mouse on the grid. Once the mouse grips the grid, the grid is inverted over a large bin filled with multiple layers of bedding materials. The mouse is required to hold its body on the grid while hanging upside down. The grid size was chosen because it was big enough to allow the animals to grasp the bars without difficulty but small enough for the mice to transition between the bars with ease and to move around on the grid, if desired. To compare the performance of the three groups of mice, we measured the time (in seconds) that the animals gripped the grid bars to maintain their body position against gravity. Testing was terminated after 60 seconds or when they fell onto the cushioned surface below them, whichever occurred first. This test appears to be a good measurement of the core strength of the animal. Testing was repeated three times to maximize the number of results collected and to minimize a change being due to an error.

B) Grid Walk This protocol was also adapted from a study that used the testing for other mouse models (Cummings, Engesser-Cesar, & Anderson, 2003; Merkler, 2001; Pajoohesh-Ganji, Byrnes, Fatemi, & Faden, 2010). The behavioral testing apparatus was constructed like the rungs of a ladder using six-inch long wooden dowels spaced 1 cm apart on a runway that was approximately 1 meter long. The object was for each mouse to walk from one end of the grid to the other. The spacing between the dowels was determined in random order (A coin was flipped to determine whether the dowel remained or was removed) so that the mouse could not predict what would happen next on each testing days. Testing took place in horizontal, ascending and descending planes. To test equal loading of forelimbs and hindlimbs, the grid was placed along a horizontal surface. The two other testing scenarios involved a grid declined or inclined 55 degrees. Hence, when the animal walked up the ladder-like structure, the hindlimbs were loaded. In contrast, when the animal descended the grid, the forelimbs were loaded. This test was constructed primarily to test for coordination and proprioception deficits.

This study used a unique measurement scale to evaluate the method of grip and non-grip on the grid walk within each of three planes. Non-grips were operationalized as a stutter (step into the air/space between rods), repeated attempts for forward progression to the same rod, flexing of fingers without a dowel (gripping the air), and a full or partial limb under the dowel misjudging the location of the dowel resulting in limb placement directly below the dowel or finally, a limb dropping through an empty space between the dowels. It is important to note that we evaluated the animals only in a forward in an attempt of forward movement/ progression. For example, neither placement of an animal's limb/paw onto the cardboard sidewall of the grid walk nor reaching/moving backwards for a prior rung were considered a non-grip because both failed to meet the precise non-grip criteria.

RESULTS

Tail Hang Test The E-group and N-groups' both received a score of 2 for forelimb clasping during each month of testing. On the other hand, the W-group received a score of 1 during each month of testing indicating that they did not grasp their forepaws.

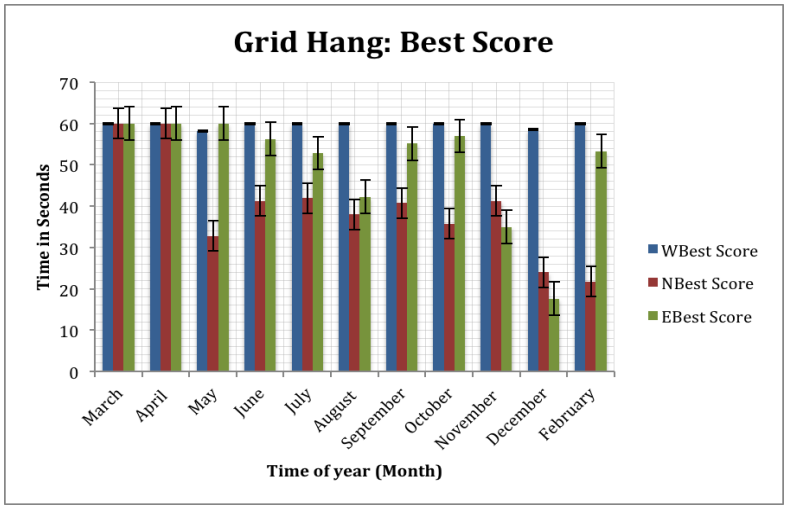

FIGURE 1. Core strength is measured through grid hang test. The results for grid hang indicate that, during the time period that the enrichment was provided (September to October), the enriched (E-group) mice hung from the grids for longer time periods than the non-enriched mice and more similar to that of wildtype (W-Group) mice. During November and December when no enrichment was provided, the E-group's grid hang score declined significantly, becoming more comparable to the nonenriched (N-Group) mice. After enrichment was reinstituted, the E-group animals' performance improved (see February) back to levels approaching the W-Group.

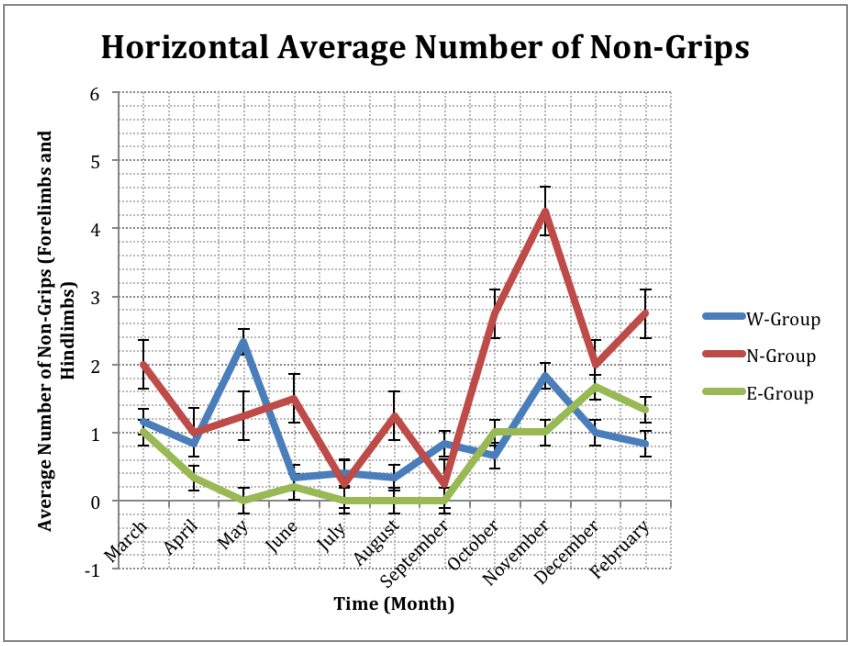

FIGURE 2. Performance on Horizontal Grid Walk, Forelimb and Hindlimb Non-Grips. While engaged in the enriching intervention, with the exception of July and September, the mice in the E-group made significantly fewer errors (less non-grips) than the N-group. Errors in the E-Group increased during the months when intervention ceased and showed improvement in February when intervention was reinstituted. While the N-Group continued to increase the number of errors as they aged, the E-group was more consistent with the W-Group.

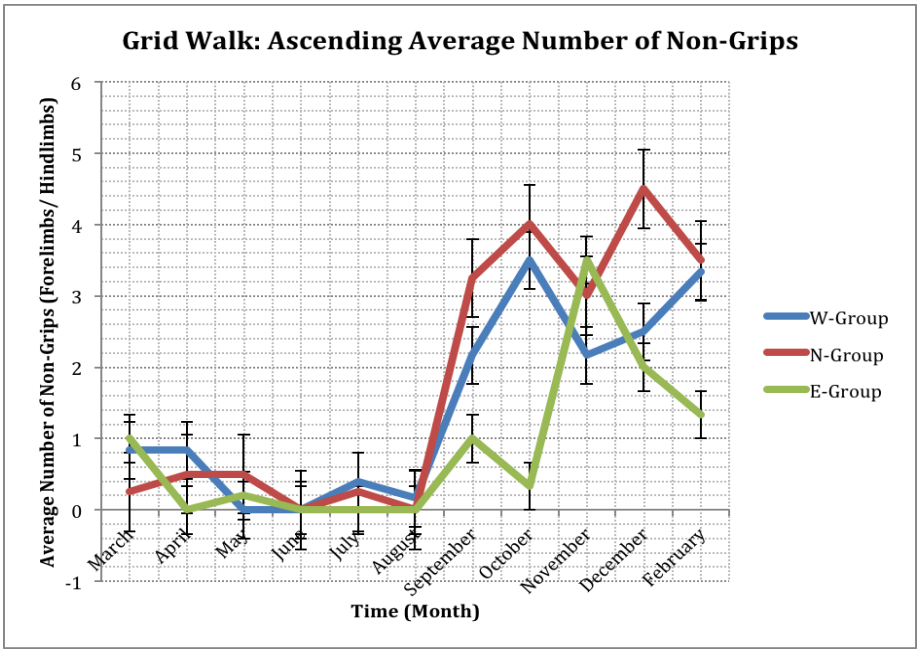

FIGURE 3. Performance on Ascending Grid Walk, Hindlimbs and Forelimbs Non-Grip Combined Score. The average number of non-grips from the months of March through February are shown. During the month of November when no enrichment was provided, the E-group mice had an unusually high average number of non-grips. By February's testing, the E-group returned to significantly fewer non-grips as compared to the N-group.

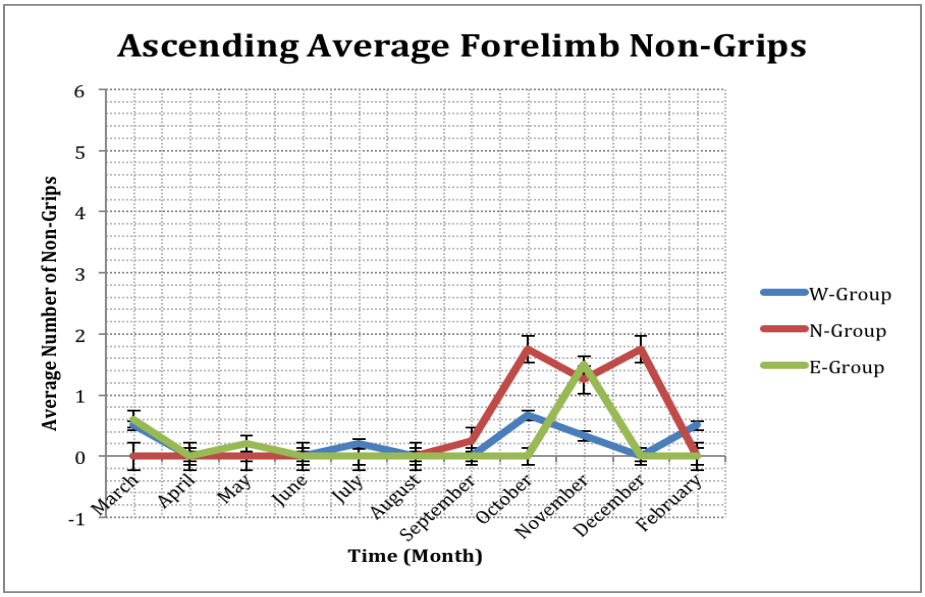

FIGURE 4. Performance on Ascending Grid Walk, Forelimb Non-Grips. Except for October through December for the N-Group and the month of November for the E-Group, most mice forelimb nongrips contributed minimally to the errors made during precise locomotion as tested by the ascending grid walk (typically receiving a score of zero nongrips).

Grid Hang Considering the size of the sample, the data was analyzed mainly through trend observation (Figure 1). To calculate all means, the best score from the three trials was used since we were interested, at this time, in what each animal's/ group's capabilities were, not necessarily how many times they performed at that level or how the fatigue factor may or may not have influenced performance. Through the first testing period (March 19-October 1) the mean times of the W-group, E-group and N-group were 59.77, 55.44 and 43.81 seconds, respectively (Figure 1). There was a significant group effect during the first testing period (F=11.29, P=0.000). The E-groups maintained their grip on the grid hang significantly longer than the N-group. However for the first month of testing, a time period before the onset of RTT symptoms, the average time spent on the grid hang for the three groups was not significantly different (Figure, 1). Another set of average scores for the best performance of the animals in each group was computed for the second testing period (October 2-December 28) when the animals did not receive any treatment ($\mu_{W\text{-}group}$=59.25, $\mu_{N\text{-}group}$=32.62, and $\mu_{E\text{-}group}$=26.33). For this period the N-group and E-group's performance were not significantly different (F=0.05, P=0.828). Finally, the last set of testing was performed in February after enrichment had been reinstituted ($\mu_{W\text{-}group}$=60, $\mu_{N\text{-}group}$=21.75, and $\mu_{E\text{-}group}$=53.33). During this testing, the performance of the E-group animals was significantly better than the N-group animals (Figure 1).

Grid Walk Trend analysis was also used to investigate the difference between the performances of the three groups in the behavioral testing of grid walk. Each animal's number of non-grips was tallied for the three subsets of tests: horizontal, ascending and descending. The results were compared between the three groups. The average rather than total number of non-grips were compared per group since the number of animals in each group differed.

A) Horizontal Grid Walk The horizontal grid walk loaded both the forelimbs and hindlimbs equally. After computing forelimb and hindlimb non-grips, the data from the 11 months was compiled (Figure 2). The observed trend shows that for the period between March and September, the N-group had in general more non-grips than the E-group animals (Figure 2). Although this trend continues in October and November, the E-group animals had significantly more non-grips than the previous months. This is consistent with the time period where the E-group did not receive enrichment.

B) Ascending Grid Walk The ascending grid walk was used as a testing battery that loaded the hindlimbs. The average numbers of non-grips (both forelimb and hindlimb non-grips) were compared. For the months March through August, all three groups of animals performed similarly and there does not appear to be a significant difference in their motor performance (Figure 3). Performance begins to worsen for all three groups in the months of September and October. Further, in the month of November, while enrichment was discontinued, the E group's performance was inferior to that of the N-group, noted by the increased number of failed grips than the N and W groups. But in the month of February, testing revealed that the E group had the fewest number of non-grips as compared to the N and W groups (Figure 3).

To analyze the specific performance of individual limbs, separate graphs showing the errors of the forelimbs and hindlimbs have been constructed (Figure 4 and Figure 5). The hindlimbs comparison graph shows that during the months of March through September motor abilities fluctuated. From September to December the N-group performed worse than the period from March to September. However, except for the month of November, the E-group had fewer non-grips than the N-group suggesting that they had better limb placement (Figure 4).

C) Descending Grid Walk The descending grid walk was used as a testing battery that loaded the forelimbs. This test reveals numerous differences between the performances of the three groups. The average number of non-grips (combined forelimb and hindlimb non-grips) shows that unlike the grid-hang test, all three groups of the animals generally had more non-grips at the beginning-testing month of April (Figure 5), which may be due to the more difficult test parameter of the downhill walk. However, on average, over the subsequent months the E-group animals made fewer mistakes than the N-group, demonstrated by the fact that they had a lower number of non-grips compared to the N-group animals ($\mu_{E\text{-}group}$= 1.89, $\mu_{N\text{-}group}$=3.25) during the 11 testing months. To check for a change in performance after the enrichment ceased, separate means were computed for the first testing period and the second testing period ($\mu_{Period1}$=1.75, $\mu_{Period\ 2}$=2.75). Although performing a t-test would be meaningless due

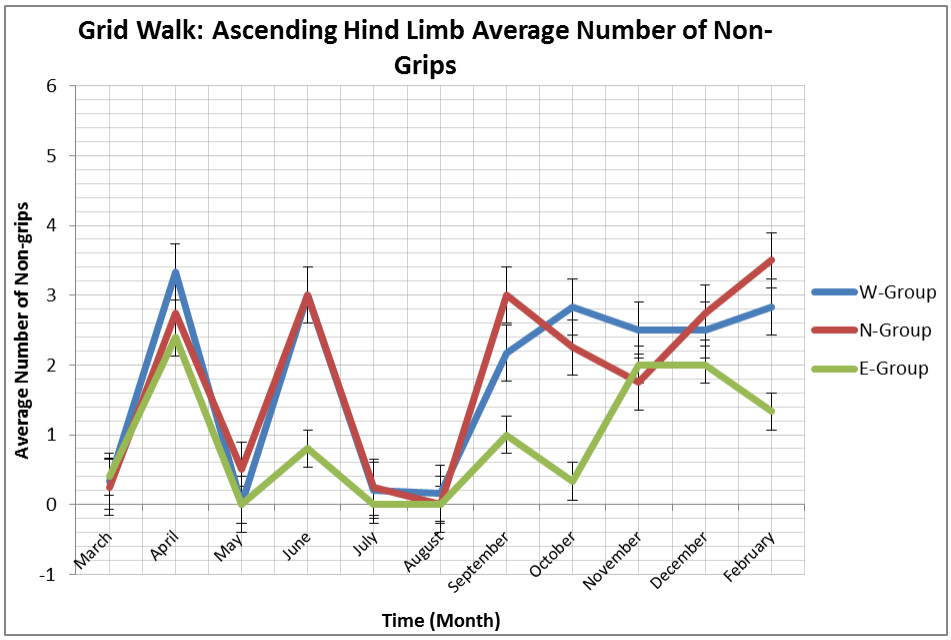

FIGURE 5. Performance on Ascending Grid Walk, Hindlimb Non-Grips. As the mice matured (September – February), the E-group experienced fewer non-grips compared to the N- or W-group. During November, when no intervention was provided, the E-Group had more difficulty with hindlimb placement (increased number of non-grips).

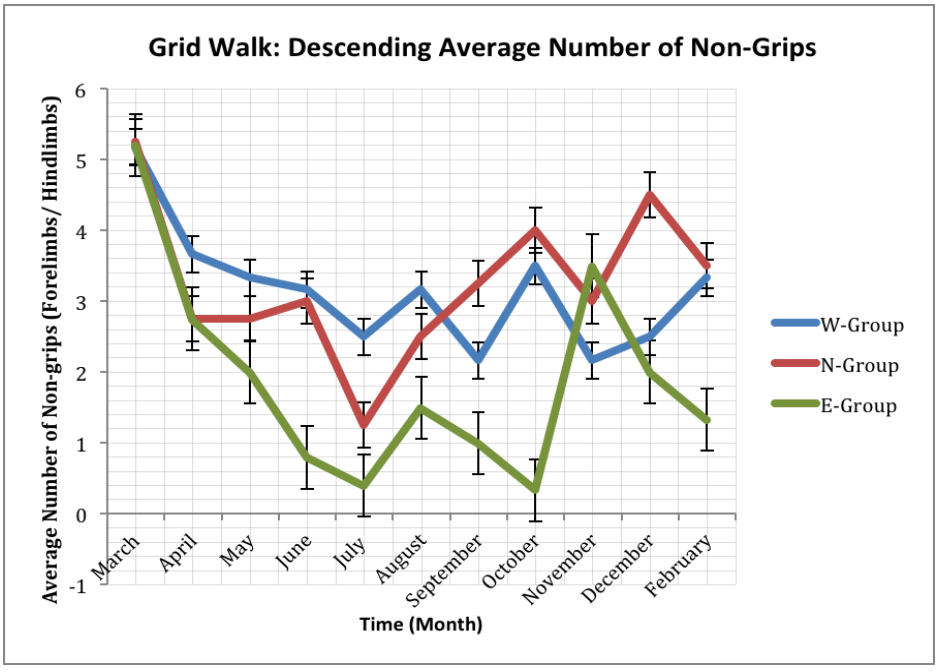

FIGURE 6. Performance on Descending Grid Walk, Forelimb and Hindlimb Non-Grips. The E-group outperformed mice in the N- and W-Group for least number of errors in grip. The one exception was during the month of November, when errors were equivalent to the N-Group. Scores returned to significantly better than all groups when enrichment was reinstituted prior to February testing.

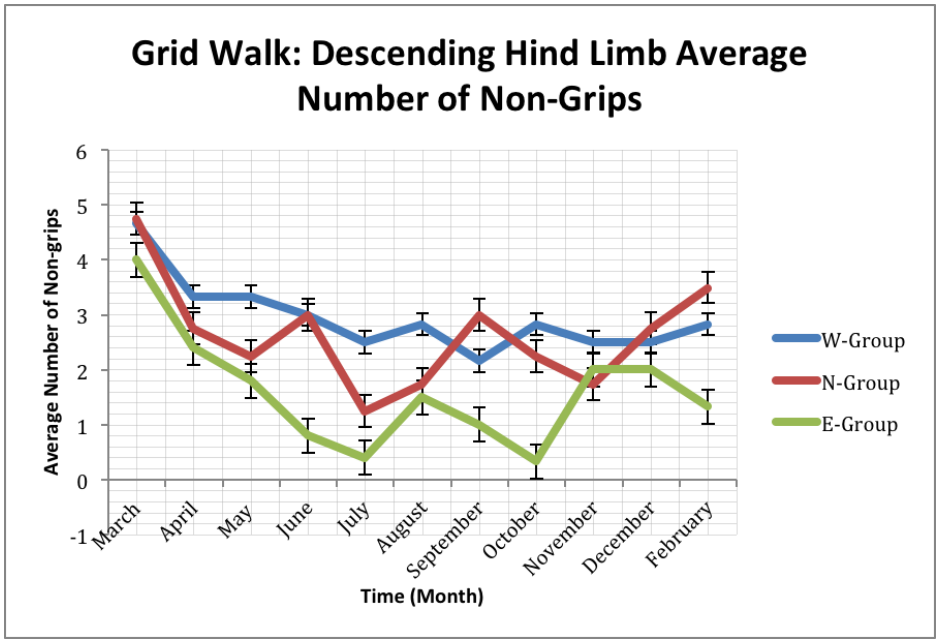

FIGURE 7. Performance on Descending Grid Walk, Hindlimb Non-Grips. The E-group mice generally exhibited significantly less hindlimb gripping errors throughout the study period. Non-grips spiked during the month of November when no enrichment was provided.

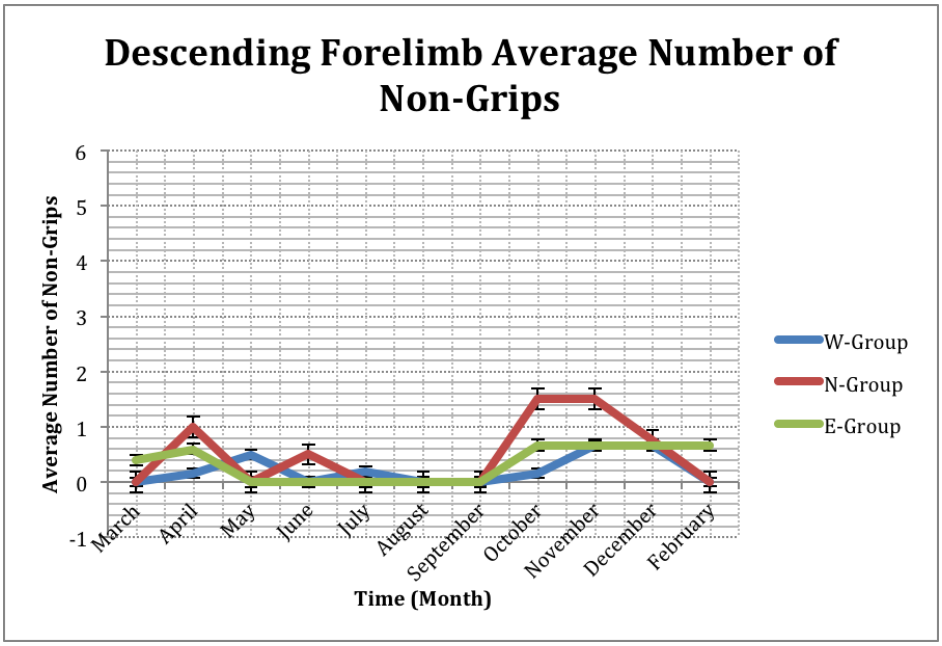

FIGURE 8. Performance on Descending Grid Walk, Forelimbs Non-Grips. All groups of mice performed similarly, generally exhibiting less than 1 forelimb gripping error throughout the testing period, with the exception of testing in October and November when the N-group made significantly more errors than the E- or W-Groups. While the E-Group made significantly more errors, on average, than the W- or N-Group in February, they continued to exhibit, on average, less than 1 non-grip.

to the small sample size, the trend suggests that E-group mouse performance worsened during the testing period when enrichment was withdrawn from the mice (Figure 5).

Further trend analysis was used to detect performance differences between the hindlimb and forelimbs when walking in a descending plane. The hindlimb analysis (Figure 7) shows that the enriched group, in general, had the least number of non-grips. The E-groups had a lower number of hindlimb non-grips compared to N-group ($\mu_{E\text{-}group}$=1.60, $\mu_{N\text{-}group}$=2.64) computed for all testing months (Figure 7). The forelimb analysis shows that, in general, for every month the E-group had lower number of non-grips compared to the N-group (Figure 8). However, the number of non-grips increased significantly for the E-group testing during the months of November and December reaching the level of the N-group during November.

DICUSSION

The results of the tail hang assessment confirmed that the Mecp2 mice possessed a defining phenotypic characteristic of forelimb grasp when held by the tail, inverted in space. This characteristic was maintained throughout the study in the two Mecp2 groups. The wild-type animals did not display these movements, again suggesting that such movements are only typical in RTT mice.

The results of the Grid Hang test suggest that enrichment helped increase the core strength of the mice. In fact, during select months, performance of the E-group and the W-group were not significantly different. This supports the hypothesis that sensorimotor enrichment can ameliorate the phenotypic behavioral symptoms of *Mecp2* null mice. It is also notable that when enrichment stops during the second testing period, the performance of the E-group animals worsened such that there was no significant difference in the months November and December in the performances between the E-group and N-group. Behavior deteriorated as early as one month after treatment ceased and recovered when the intervention was reinstituted (December 15- February 29). Since there was only one month of behavioral testing after treatment restarted (February), it is difficult to determine if a trend exists. Nonetheless, the February results suggest that a certain extent of stimulation might be needed to allow for continued progress in the maintenance of motor skills in RTT.

Kondo et al. (2008) reported an improvement in performance of the hemizygous male animal model using the standard accelerating rotarod that tests coordination and balance. Kondo et al. (2008) saw limited improvements with the rotarod while we saw generalized motor skill improvements with our more sensitive testing measures. Our results suggest that the grid hang testing apparatus may be a more sensitive testing measure for detecting subtle differences in core strength, skilled motor activity, and coordination and postural/balance reactions. Kondo et al. (2008) report housing the mice in an enriched environment but do not report monitoring the animals to ensure that they were engaging with their environment. In this study, enrichment was extensive (4hrs/day, 5times/wk.). When the animals were placed in the enriched environment, they were also monitored carefully to ensure that they were actively engaged. When lack of interaction within the environment occurred, toys were moved around to increase the activity of individual mice. Thus, the difference in the level of improvement reported by Kondo et al. (2008) and this study may be as a consequence of multiple targeted sensorimotor enriched environment and more sensitive testing measures.

Similar to the grid hang test, the results from the horizontal grid walk also indicate that enrichment may help improve RTT like symptoms. The average number of total non-grips in the horizontal grid walk test show that the enriched animals generally had fewer non-grips compared to the non-enriched *Mecp2* null mice. Moreover, the results of the performance of E-group animals on the horizontal grid walk test were significantly lower during the months of November and December when intervention ceased, reiterating the importance of continuing sensorimotor enrichment and testing for extended time periods to achieve and maintain gains in proprioceptive and limb placement abilities.

A separate analysis of the forelimb and hindlimb non-grips for the horizontal grid showed no significant difference. A possible explanation could be that the horizontal grid walk loads both forelimbs and hindlimbs equally. Hence, we only report the average number of total non-grips (forelimb+ hindlimb non-grips).

For the ascending grid walk test, it was expected that the hindlimbs would be loaded, which enhances the mouse's awareness of those limbs in space to allow for precise paw

placement. If it is true that the hindlimbs were not able to balance and handle the weight of the animal well, potentially due to poor reception of proprioception/body sense in space as appears to be the case in RTT (Kerr, 2006), we would expect to see more hindlimb non-grips compared to forelimb non-grips. The results confirmed this notion, indicating, in general, that there were more hindlimb errors. Again, our hypothesis that enrichment improves phenotypic manifestations of RTT was supported because the E-group performed better than the N-group. Although there was a fluctuation in performance in the months of March through July, the increase in the number of hindlimb errors resembles the result from the horizontal grid walk. The decline in performance in the months of November and December once more implies that when the mice are not exposed to an enriching intervention, they make more mistakes while walking up the grid walk.

For the descending grid walk test, the average number of forelimb non-grips was expected to be more than the average number of hindlimb non-grips for each animal since the design of the test loads the forelimbs more. However, the results show that on average there were more hindlimb non-grips for each group of animals. When mice were descending the grid walk, their whiskers were pointed forward, so they may have been better at detecting the position of the next grip even though they are loading their forelimbs more.

It is interesting to note that during some of the months the W-group had more non-grips than both the E-group and N-group. Although it may appear that the wild type mice are performing worse than the other groups, during the video analysis it was observed that the W-group were very quick in crossing the grid walk. In our analysis, we did not take into consideration the duration of time the animal took to cross the grid. Increased speed, however, may have compromised accuracy for the W-group of animals. Nonetheless, the comparison between the N-group and E-group is still meaningful because we did not observe considerable speed difference between these two groups.

In agreement with others (Kondo et al., 2008), the mice in this study that were allowed to actively explore enriched environments improved motorically, which suggests that enrichment is effective in multiple models of RTT. Further exploration will need to be carried out to determine if, in our model, we also see an up-regulation of BDNF as others have shown (Kondo et al., 2008).

CONCLUSIONS

The results of this study contribute to empirical findings (Kondo et al, 2008; Tabuse et al., 2010; Diener, 2002) that enrichment improves performance of multiple rodent models of neuromuscular disorders including Mecp2$^{-/y}$ mice. Moreover, the testing batteries assessed were able to identify subtle differences in performance. We argue that our assessment batteries test the mice in environments that resemble the natural places they inhabit and therefore have more functional relevance for analyzing motor recovery. It was especially enlightening to observe the animals recuperating when enrichment was reinstituted after withholding it from them in the months of October through December. Our pilot study results suggest that exposure to or active exploration within multisensory environments may be beneficial when used to treat RTT.

Nonetheless, this study has several limitations. We have primarily reported on trends since the sample size of the animals is very small. Assessing the effect of enrichment on mice should be repeated to increase the sample size and better report on the sensitivity of our testing battery to assess for motoric changes as a result of the enrichment intervention. Second, the results reported for the grid walk are preliminary data since one person has coded them. Once secondary coding is completed for all months we will reconcile scores. This would allow us to report on the reliability and validity of the novel testing protocol. Finally, the results of the testing in February suggest that the time frame for analysis of motor recovery following cessation of treatment should be extended.

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