Specific mutations in Methyl-CpG-Binding Protein 2 confer different severity in Rett syndrome

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ABSTRACT

Objective: To determine if a relationship exists between the clinical features of Rett syndrome, an X-linked dominant neurodevelopmental disorder, and specific mutations in MECP2.

Method: Cross-sectional study of 245 girls and women with typical Rett syndrome seen between 1990 and 2004 in tertiary academic outpatient specialty clinics and who had complete MECP2 mutation analysis. A structured clinical evaluation was completed for each participant. The results were grouped by MECP2 mutation and compared.

Results: Participants with the R133C mutation are less severely affected than those with R168X or large DNA deletions (p < 0.05). Likewise, individuals with the R168X mutation are more severely affected than those with R294X and late carboxy-terminal truncating mutations (p < 0.05). Clinical differences are notable in ambulation, hand use, and language (p < 0.004), three cardinal features of Rett syndrome. Individuals with R168X are less likely to walk (p = 0.008), retain hand use (p = 0.002), or use words (p = 0.001). In contrast, those with carboxy-terminal truncations are more likely to walk (p = 0.007) and use words (p < 0.001). The R306C mutation, previously found to confer milder features, adversely affects only one clinical feature, language (p < 0.05).

Conclusions: Specific mutations in MECP2 confer different severity. These results allow the design of therapies targeted toward the amelioration of expected problems. Furthermore, the distinct effects of MECP2 mutations on clinical severity must be considered in clinical intervention trials. Neurology® 2008;70:1313–1321

GLOSSARY

BCM – Baylor College of Medicine; CSS – Clinical Severity Score; MLPA – multiple ligation-dependent probe amplification; RTT – Rett syndrome; TCH – Texas Children’s Hospital; UAB – University of Alabama–Birmingham; XCI – X-chromosome inactivation.

Rett syndrome (RTT) is an X-linked dominant neurodevelopmental disorder which occurs in 1.09/10,000 females¹ and is characterized by regression of language and hand use.² Hand stereotypies are characteristic, occurring during wakefulness and interfering with purposeful hand use. Ambulation is often disturbed. Furthermore, a number of other neurologic problems including tremor, chorea, dystonia, and epilepsy are common. Affected individuals have decreased somatic and brain growth and autonomic abnormalities such as breathing irregularities and cold, blue extremities.³ Typical RTT is diagnosed based on a set of clinical criteria (table e-1 on the Neurology® Web site at www.neurology.org).⁴ Atypical RTT, which can be milder or more severe than typical RTT, is diagnosed when some, but not all, of the typical RTT clinical criteria are present.⁴

Mutations in the gene encoding methyl-CpG-binding protein 2 (MECP2) cause the majority of cases of typical RTT.⁵ Although over 200 unique mutations in MECP2 cause RTT (Rettbase; http://mecep2.chw.edu.au/), eight common mutations (R106W, R133C, T158M,
R168X, R255X, R270X, R294X, R306C) account for more than 60% of typical RTT cases. In addition, a number of small insertions/deletions in the 3’ end of the gene lead to carboxy-terminal truncations (C-terminal truncations). The molecular similarity of these mutations justifies considering them as a group, accounting for 5 to 10% of typical RTT (Rettbase). Mutations in MECP2 have also been discovered in atypical RTT* and in other neurodevelopmental disorders, such as autism,7-10 Angelman-like syndrome,11,12 and nonspecific mental retardation.13,14

Although the features of typical RTT are distinctive, affected individuals display clinical variability. For example, some individuals with typical RTT are able to walk unassisted whereas others are completely nonambulatory. One proposed explanation for this variability is nonrandom X-chromosome inactivation (XCI). XCI is the inactivation of one of the two X-chromosomes in every female cell which usually results in a random distribution of active X-chromosomes in the adult. Although variation in XCI can explain some of the variance in severity of RTT (approximately 20%),15 it does not fully account for the range of clinical severity seen in typical RTT.

This begs the question: Do specific MECP2 mutations result in particular clinical features in typical RTT? The fact that some mutations are more common in mild atypical RTT8-9,16-21 supports the notion that specific MECP2 mutations contribute significantly to the clinical variation in typical RTT. If these MECP2 mutations are more frequently present in mild atypical RTT, they may also represent some of the less severe typical RTT cases. Indeed, individuals with R133C are less severely affected.22 Previous genotype/phenotype correlations lacked the discerning power to detect differences between specific mutations.23-30 In this study, we present a large, cross-sectional cohort of strictly defined individuals with typical RTT* (table e-1). Common point mutations, large deletions, and C-terminal truncations are compared and distinct differences in clinical severity identified. The comparison of clinical features in RTT resulting from specific MECP2 mutations provides information that will be useful in guiding therapeutic interventions, designing clinical intervention trials, and understanding the molecular nature of the MeCP2 protein.

METHODS Patients and clinical evaluation. The protocol and consent form were approved by the Institutional Review Boards of Baylor College of Medicine (BCM) and the University of Alabama–Birmingham (UAB). Parents or legal guardians of the participants gave informed consent. Participants were seen at the Blue Bird Circle Rett Center at Texas Children’s Hospital (TCH), BCM, or UAB Rett Center between 1990 and 2004. Forty-five participants from UAB25 and 69 from TCH26 were included in previous studies. A history and structured examination was performed on each girl by experienced examiners (D.G., A.P.) to confirm the diagnosis using consensus criteria.4 Disease severity was determined using a clinical rating (Clinical Severity Score [CSS]; table e-2) which was developed specifically for RTT. The CSS is a composite score based on 13 individual, ordinal categories measuring clinical features common in Rett syndrome. All scores range from 0 to 4 or 0 to 5 with 0 representing the least severe and 4 or 5 representing the most severe finding. A simplified scoring system was used to compress the ordinal category measures into a binary measurement with 0 representing “mild” or “retained function” and 1 representing “severe” or “lost/absent function.” The clinical interpretation for the score 0 is given in table e-3. From this compressed clinical severity score, the percentage of individuals with mild/retained function can be generated for each clinical category. Individuals were included if they meet the consensus criteria for typical RTT (requires all the main criteria listed in table e-1 except deceleration in head growth), had complete testing for MECP2 mutations (as outlined below, including testing for large DNA rearrangements), and had a complete CSS assessed. We excluded 40 individuals who had complete MECP2 mutation analysis and a complete CSS but did not meet the criteria for typical Rett syndrome but rather atypical RTT. The rationale for excluding individuals with atypical RTT is that they often represent the extreme ends of the phenotypic spectrum, both milder and more severe than typical RTT. The overall results were unchanged when these individuals were included in the analysis (data not shown).

MECP2 mutation analysis. Participants in this study had complete MECP2 mutation analysis performed including sequencing exon 1 and evaluation for large DNA rearrangements by Southern blotting or by multiple ligation-dependent probe amplification (MLPA) analysis. MECP2 mutation analysis was performed either by the Baylor DNA Diagnostic Laboratory17,24 or by the Greenwood Genetics Laboratory (http://www.ggc.org/diagnostics/molecular/rett_syndrome.htm). X-inactivation analysis was performed based on the protocol described previously.31

Statistical analysis. Analyses were performed using SPSS v.12 (SPSS, Chicago IL) or SAS (SAS Institute, Cary, NC).
MECP2 mutation groups were compared on continuous variables (age, total CSS) using analysis of variance with post hoc tests conducted using Tukey honestly significant difference test. For comparisons of the individual CSS categories, which are ordinal data, nonparametric statistics (Kruskal-Wallis test for K groups, Mann–Whitney U test for pairwise comparisons) were used. Proportional data were analyzed using Pearson χ² analysis (or where the expected number of cell counts was less than 5, a 2 × K Fisher exact test), with a Bonferroni adjusted significance level for the overall difference (p < 0.004) followed by a Tukey-style procedure (SAS Macro implementing procedure[^35]) to detect pairwise differences. Relative risk was calculated for those pairwise differences that were significant (p < 0.05).

Two approaches were used to minimize Type I errors. First, defined procedures to account for multiple comparisons were used. For example, parametric analyses were followed by defined post hoc procedures. Similarly, post hoc testing of proportional data was analyzed using Tukey-style multiple comparisons of proportions.[^36] When no formal correction method exists, we adjusted the significance threshold on those clinical categories that were different overall and only between those mutation pairs that were different in total CSS. Thus, the pairwise comparisons were limited to 12 tests ([3 overall clinical category differences] × [4 pairwise mutation differences in total CSS]) using Mann–Whitney U test with a Bonferroni adjusted significance level (p = 0.05/12 = 0.004).

**RESULTS** Description of participants. A total of 245 girls and women seen at TCH or UAB met the criteria for typical RTT[^4] (table e-1), had a CSS (table e-2) assessed, and complete MECP2 mutation analysis performed. Ninety-seven percent (236/245) have a mutation in MECP2. To determine if differences in clinical severity exist between different MECP2 mutations, participants were placed into 12 mutation groups. Eight mutation groups consist of the most common point mutations (R106W, R133C, T158M, R168X, R255X, R270X, R294X, R306C). One individual with R133P and one with S134C were included in the R133C group and one participant with R306H was included in the R306C group. These common point mutations account for 67.4% of the total group. Two additional groups consist of clusters of molecularly similar mutations: large deletions (deletions of exon 3 and 4 or exon 4 alone, or complex insertions/rearrangements that disrupt the entire coding sequence), and mutations that cause late carboxy-terminal truncations (C-terminal truncations). The final two groups consist of all the remaining mutations (other mutations) and those participants with no MECP2 mutations. The number, percentages, mean age at examination, and mean total CSS for each mutation group is shown in table 1. We observe no difference in the mean age of examination between mutation groups [F(11,233) = 1.664, p = 0.083]. Furthermore no correlation was found [F(1,243) = 0.126, p = 0.723] between CSS and age.

Overall clinical severity depends on MECP2 mutation. The total CSS is different [F(11,233) = 3.33, p = 0.0003] between the mutation groups (figure 1, table 1). Post hoc analyses show pairwise differences (p < 0.05) between both R133C and R168X and between R133C and large rearrangements. Similarly, pairwise differences exist between

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No.</th>
<th>%</th>
<th>Mean age, mo (95% CI)</th>
<th>Mean total CSS (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R106W</td>
<td>9</td>
<td>3.7</td>
<td>173 (111–236)</td>
<td>24.8 (20.7–28.8)</td>
</tr>
<tr>
<td>R133C</td>
<td>12</td>
<td>4.9</td>
<td>125 (71–177)</td>
<td>18.1 (14.6–21.6)</td>
</tr>
<tr>
<td>T158M</td>
<td>30</td>
<td>12.2</td>
<td>105 (71–169)</td>
<td>22.3 (20.1–24.6)</td>
</tr>
<tr>
<td>R168X</td>
<td>29</td>
<td>11.9</td>
<td>133 (98–167)</td>
<td>27.0 (24.8–29.3)</td>
</tr>
<tr>
<td>R255X</td>
<td>32</td>
<td>13.1</td>
<td>100 (67–132)</td>
<td>24.2 (22.1–26.4)</td>
</tr>
<tr>
<td>R270X</td>
<td>18</td>
<td>7.3</td>
<td>146 (102–190)</td>
<td>23.6 (20.7–26.4)</td>
</tr>
<tr>
<td>R294X</td>
<td>14</td>
<td>5.7</td>
<td>184 (134–234)</td>
<td>19.7 (16.5–23.0)</td>
</tr>
<tr>
<td>R306C</td>
<td>21</td>
<td>8.6</td>
<td>142 (101–182)</td>
<td>21.6 (19.0–24.3)</td>
</tr>
<tr>
<td>C-terminal truncations</td>
<td>17</td>
<td>6.9</td>
<td>128 (82–173)</td>
<td>19.9 (17.0–22.9)</td>
</tr>
<tr>
<td>Large deletions</td>
<td>17</td>
<td>6.9</td>
<td>101 (56–146)</td>
<td>26.1 (23.1–29.0)</td>
</tr>
<tr>
<td>Other mutations</td>
<td>37</td>
<td>15.1</td>
<td>105 (75–136)</td>
<td>22.4 (20.4–24.4)</td>
</tr>
<tr>
<td>No mutation</td>
<td>9</td>
<td>3.7</td>
<td>173 (111–235)</td>
<td>24.7 (20.6–28.7)</td>
</tr>
<tr>
<td>All</td>
<td>245</td>
<td>100</td>
<td>135 (121–148)</td>
<td>22.9 (20.0–23.7)</td>
</tr>
</tbody>
</table>

CSS = Clinical Severity Score.
There is an overall difference \( p = 0.0007 \) in the Clinical Severity Score between different mutations (listed along the x-axis). The asterisk (*) and the number sign (#) show significant \( p < 0.05 \) post hoc pairwise differences. Values represent mean ± SEM.

R168X and R133C, R294X, and C-terminal truncations for total CSS.

To determine if the effect of specific MECP2 mutations is independent of XCI status, we performed XCI analysis on mutation groups that represent extreme ends of the clinical severity spectrum (R133C, R168X, R306C, and large deletions). We assessed the total CSS score for those participants that had random (less than 80:20%) XCI and found that R168X (\( n = 12 \) with random XCI) has a higher total CSS (26.6) compared with R133C (\( n = 7 \) with random XCI, CSS = 18.1, \( p = 0.011 \)) or with R306C (\( n = 8 \) with random XCI, CSS = 20.1, \( p = 0.014 \)). Furthermore, R133C shows a trend toward being less severe than large deletions (\( n = 8 \), CSS = 26.4, \( p = 0.053 \)). These results indicate that, in general, the overall clinical severity conferred by specific MECP2 mutations is independent of XCI status.

MECP2 mutations confer differences in ambulation, hand use, and language. To determine if specific MECP2 mutations result in differences in any of the clinical categories that make up the overall CSS, we analyzed each of the 13 categories. Analyses reveal differences \( p < 0.004 \) between the MECP2 mutations in the following categories: ambulation, hand use, and language. Pairwise analyses, performed only between those mutation pairs that were different on the overall CSS score, reveal that ambulation is different between R168X and C-terminal truncations, and between R168X and R294X (table 2), and trends toward difference between R168X and R133C (\( p = 0.005 \)). Hand use is different between R168X and R133C, and between R294X and C-terminal truncations (table 2). Hand use also trends toward difference between R133C and large rearrangements (\( p = 0.0044 \)). Language is different between R168X and R133C, and between R294X and C-terminal truncations (table 2).

Retention of function depends on specific MECP2 mutations. We assessed the percentage of individuals with a given mutation who retain meaningful skills or are only mildly affected. For example, in the ambulation category, a score of 2 or less indicates the ability to walk alone, whereas a score of 3 or higher indicates that the individual cannot walk unaided or is completely unable to walk (table 2, table e-2). Similar divisions can be made for all the clinical categories in the CSS to reflect whether the individual is mildly affected (i.e., retains function or symptomatically mild) or severely affected (i.e., lacks function or symptomatically severe). Table e-3 outlines the clinical definition for the mildly affected criteria for each category used to generate a compressed clinical severity scoring system. Using this simplified system, we determine the percentage of individuals with a specific MECP2 mutation who retain function or are minimally impaired for each clinical category. Implementing this approach, we find that individuals with specific MECP2 mutations differ in their ability to walk independently, use their hands, and use words (\( p < 0.004 \)). The percentages of individuals with retained function for these categories are shown in table 3.

Pairwise tests confirm that language is more retained in R133C compared with R168X, in R294X compared with R168X, in other mutations compared with R168X, in C-terminal truncations compared with R168X, in C-terminal truncations compared with R306C, and in C-terminal truncations compared with large deletions (all \( p < 0.05 \)). The absolute risk and relative risk for each of these comparisons is shown in table 4. In summary, the milder mutations (R133C, R294X, other mutations, c-terminal truncations) confer between 6.0- and 20.5-fold increased relative risk of having some word use compared to those with the severe mutations (R168X, large deletions, R306C).

The ability to walk alone is more likely in individuals with C-terminal truncations compared to those with R168X and in R294X compared to those with R168X (table 3). There is approximately a threefold increase in the relative risk that the individuals with mild (C-terminal truncations or R294X) mutations will be able to walk alone compared to those with R168X (table 4). Individuals with R168X also have decreased use of hands compared with R133C, C-terminal truncations,
and other mutations (table 3). Mild mutations (R133C, C-terminal truncations, other mutations) confer between 2.1- and 2.4-fold increased relative risk of retained hand use compared to R168X (table 4).

Using this simplified scoring system, an interesting observation concerning the correlation of retained function within specific mutations is apparent. Whereas some of the mutation groups have a low percentage of individuals with retained skills, such as R168X and large deletions, and other mutations have a high percentage of individuals with retained skills, such as R133C and C-terminal truncations, an interesting dissociation of the retained function exists in those with R306C (figure 2). Although the R306C group tends to be less affected in total CSS (figure 1) and a high percentage of these individuals can walk alone (67%) and have some hand use (52%), only a small percentage (10%) use words. This percentage is different ($p < 0.05$, table 3) from the percentage of those with retained language in the C-terminal truncations group (RR, 7.4; table 4). This unexpected dissociation of the degree of clinical severity among these three categories suggests that the molecular nature of R306C is unique and disrupts distinct functions of the MeCP2 protein.

Although the differences in clinical severity are important in understanding the molecular nature of different MECP2 mutations, assessing the likelihood of retaining function given the presence of a specific mutation could provide meaningful clinical information to assist in prediction of clinical features. This information could help guide interventions, such as physical therapy, to ameliorate a specific symptom. With this goal, we compared among the five specific mutations (R133C, R168X, R294X, R306C, and large deletions) the percentage of individuals with a specific mutation

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Specific MECP2 mutations cause differential severity in three clinical features: Ambulation, hand use, and language</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation</td>
<td>Hand use</td>
</tr>
<tr>
<td></td>
<td>0 (conserved)</td>
</tr>
<tr>
<td>R133C*</td>
<td>17</td>
</tr>
<tr>
<td>R294X†</td>
<td>0</td>
</tr>
<tr>
<td>C-term‡</td>
<td>0</td>
</tr>
<tr>
<td>Large deletions</td>
<td>0</td>
</tr>
<tr>
<td>R168X*†</td>
<td>0</td>
</tr>
</tbody>
</table>

Values represent percentages of individuals with a specific mutation and a Clinical Severity Score on three of the clinical categories: hand use, language, and ambulation. Severity subscale ranges from 0 (mild) to 5 (severe). The clinical meaning for the numeric score is shown below the score. Symbols in column 1 (*, †, ‡) indicate pairwise differences between mutation groups on post hoc testing ($p < 0.004$). The total number of participants with a given mutation is shown in table 1.

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The absolute and relative risk were calculated for the pairwise differences shown in table 3.

All 13 compressed subscales (table e-3) were compared and differences between all mutation groups were observed for ambulation, hand use, and language. Pairwise testing between specific mutation groups revealed differences (p<0.05) shown by the asterisk (*) or the number sign (#).

who retained function to the percentage of individuals without that mutation who retain function. Using a significance cutoff of p < 0.01, individuals with R168X are approximately one half as likely to be able to walk, one half as likely to be able to use hands, and one tenth as likely to be able to use words as those without R168X (table 5). On the other hand, those with C-terminal truncations are almost twice as likely to walk alone and nearly three times as likely to use words as those without C-terminal truncations (table 5). Individuals with R294X were also almost twice as likely to walk as those without R294X.

**DISCUSSION** This study consists of a large series of individuals with typical RTT, formal clinical assessment, and complete MECP2 mutation analysis. This allows comparisons of the severity between participants with common specific mutations. In general, specific MECP2 mutations confer different clinical severity in typical RTT. Furthermore, specific mutations (R133C, R294X, C-terminal truncations) are less severe than other mutations (R168X, large deletions). These differences seem to be largely independent of XCI status. The difference in severity appears to result primarily from variation in three clinical features: ambulation, hand use, and language.

One particular mutation, R306C, has an unusual dissociation of the amount of preserved function. A large percentage of those with R306C are able to walk but very few are able to use words. In contrast, a previous study found that individuals with R306C have a milder phenotype and specifically had better language skills. A significant difference between this study and the previous work is the inclusion of atypical RTT in that study and the exclusion of such atypical individuals in this study. The improved lan-
guage skills in the R306C group in the prior study may be due primarily to inclusion of atypical RTT. This argues that the R306C mutation can either severely affect language thus causing typical RTT, or does not dramatically affect language and allows the expression of milder atypical RTT or non-RTT neurodevelopmental disorders. The clinical variability of this mutation suggests that it plays a major role in the function of the MeCP2 protein.

This study presents two features of clinical relevance. First, it provides the basis for clinical counseling. Although the study lacked the power to discern the severity of any specific common mutation, it does distinguish five mutations that represent the extremes of the severity spectrum. Furthermore, within these five mutations, predictions for the possible clinical outcomes can be made. For example, this series shows that R168X confers an increased risk that the affected individual will not be able to walk, will not use words, and will not have any retained hand use, whereas individuals with C-terminal truncations are much more likely to use words and walk alone. Such knowledge provides the framework for clinical counseling and assists in tailoring therapies toward the problems that result more frequently from particular MECP2 mutations.

The second clinically relevant finding concerns the design of clinical trials for Rett syndrome. Because clear differences in clinical severity exist between mutations in Rett syndrome, any intervention trial must take this into account in study design. Without such planning, false negative and false positive results might occur due to a skewed distribution of mutations among the treatment groups. The simplest way to account for this is to design trials that compare individuals pre- and post-treatment.

A major point of this work is the need for a larger cohort to analyze for these genotype effects. Although this study has the largest clinical population of typical RTT published to date, the smallest mutation group (R106W) only represents 3.7% of the total population, necessitating a very large sample size to acquire an adequate number of individuals for this group. Because a number of genotype–phenotype comparisons have been performed in the past, it is possible to perform a meta-analysis on the published data to look for specific mutation effects. A challenge with such an analysis is the variation in the clinical rating systems used across studies. We propose that use of the simplified, compressed system presented here would allow an easy method to perform a meta-analysis of previously published genotype–phenotype comparisons in RTT and the collation of existing international data sets for de novo analysis.

Beyond clinical relevance, this work highlights a MECP2 allelic series which indicates that particular regions of the MeCP2 protein have unique genetic and protein interactions that determine

<table>
<thead>
<tr>
<th>Walks alone</th>
<th>With mutation (%)</th>
<th>Without mutation (%)</th>
<th>p Value</th>
<th>Absolute risk reduction (95% CI)</th>
<th>Relative risk (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R168X vs all other mutation groups</td>
<td>28</td>
<td>54</td>
<td>0.008</td>
<td>-0.26 (-0.41 to -0.07)</td>
<td>0.5 (0.1-0.9)</td>
</tr>
<tr>
<td>R294X vs all other mutation groups</td>
<td>86</td>
<td>49</td>
<td>0.006</td>
<td>0.37 (0.10-0.49)</td>
<td>1.8 (1.4-2.3)</td>
</tr>
<tr>
<td>C-terminal truncations vs all other mutation groups</td>
<td>82</td>
<td>48</td>
<td>0.007</td>
<td>0.34 (0.10-0.48)</td>
<td>1.7 (1.4-2.2)</td>
</tr>
</tbody>
</table>

| Uses hands |
|------------------|------------------|-----------------|---------|---------------------------------|----------------------|
| R168X vs all other mutation groups | 38 | 67 | 0.002 | -0.29 (-0.46 to -0.10) | 0.6 (0.1-0.9) |

| Uses words |
|------------------|------------------|-----------------|---------|---------------------------------|----------------------|
| R168X vs all other mutation groups | 3 | 33 | 0.001 | -0.30 (-0.37 to -0.15) | 0.1 (0.01-0.7) |
| C-terminal truncations vs all other mutation groups | 71 | 26 | <0.001 | 0.45 (0.20-0.62) | 2.7 (2.3-3.9) |
dissociable functions of MeCP2. For example, the common missense mutations (R133C, R306C, T158M, and R106W) are dissimilar with respect to the overall clinical severity conferred by these mutations. Although R106W appears to disrupt interaction with methylated cytosines, the other mutations do not.37,38 This suggests that these mutations alter distinct functional and possible physical interactions. An alternative explanation is that these mutations may have different effects on the mRNA or protein stability, which could be tested experimentally. An interesting comparison is between R133C and R306C, both of which are relatively mild but have differential effects on language. Understanding the interactions disrupted by these specific mutations will help elucidate the molecular mechanisms involved in the acquisition and maintenance of important neurodevelopmental skills and will identify key proteins important for control of ambulation, language, and hand use.

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