Breeding and maintenance of an Mecp2-deficient mouse model of Rett syndrome

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Abstract

In this report, we present a retrospective assessment of our experiences in maintaining a colony of Mecp2-deficient mice, and present conditions that we have found to foster breeding success of this mutant mouse strain. Data from our colony of mutant Mecp2 mice show that the Mecp2-null allele is under-represented in the weaned litter population, that litters born to heterozygous Mecp2-null females are consistently smaller in number than wild-type, and that fewer litters from heterozygous Mecp2-null females survive to weaning age than wild-type. Our results also reveal that overall litter viability is significantly higher in heterozygous Mecp2-null females that frequently breed, and that the addition of sunflower seeds to the cages of expecting dams improves the overall breeding success of these mice. Taken together, these data highlight the breeding tendencies for this mutant mouse strain, and from these data, we suggest strategies to maximise their breeding efficiency.

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1. Introduction

Rett syndrome is an autism-spectrum disorder that was first described by Rett (1966), and is one of the most common genetic causes of profound cognitive impairment in girls and women (Percy, 2002). Mutations within the gene coding for the X chromosome residing methyl CpG-binding factor Mecp2 have been identified as the cause for the majority of clinical Rett syndrome cases (Amir et al., 1999). The clinical syndrome involves impairment of both cognitive and motor abilities, social withdrawal, communication dysfunction, breathing irregularities, and severe intractable seizures (Hagberg et al., 1983). Characteristically, Rett children are born without apparent abnormalities, but develop impairments typically between 12 and 18 months of age (Kerr and Engerstrom, 2001). At present, there are no effective treatments for this condition.

There are presently three predominant mutant mouse models of Rett syndrome. Two independent mouse lines were generated concurrently that contain loxP-flanked Mecp2 exons, and from these, several lines of complete and region-selective Mecp2 mutants have been generated through cre recombinase-mediated excision (Mecp2tm1.1Bird, Guy et al., 2001; Mecp2tm1.1Jae, Chen et al., 2001). In addition to these, a different mutant strain has been developed that expresses a truncating mutation of Mecp2 (Mecp2308, Shahbazian et al., 2002). Importantly, all of these mice develop a pathology that recapitulates many of the cardinal features of the clinical disease. However, in each of these lines, male hemizygous Mecp2-null mice better mirror the clinical progression of Rett syndrome than Mecp2 heterozygous females (Chen et al., 2001; Guy et al., 2001; Shahbazian et al., 2002; Tudor et al., 2002; Young and Zoghbi, 2004). Like Rett girls, these hemizygous male Mecp2-null mice appear normal at birth, exhibit normal motor function for about 4–5 weeks, but then develop many of the progressive neurological impairments that are common to Rett children.

Due to its early availability from Jackson Labs, the Mecp2-deficient mouse line developed by Adrian Bird’s group (Mecp2tm1.1Bird, Guy et al., 2001) is a widely used mouse model of Rett syndrome (Braunschweig et al., 2004; Kishi and Macklis, 2004; Makedonski et al., 2005; Samaco et al.,...
2005; Asaka et al., 2006). This specific mutant line was developed using a CMV promoter-driven cre recombinase, and the excision of exons three and four leads to the generation of an unstable mRNA transcript and the ablation of Mecp2 protein expression (Guy et al., 2001). When symptomatic, these mice display reduced movement, abnormal gait, hind limb clumping, low weight, uneven wearing of teeth, anxiousness, and a shortened life span. The expected lifespan of these males is approximately 60–80 days (Guy et al., 2001).

Mecp2<sup>−/−</sup> males display internal testes, and thus are infertile. Due to their infertility, heterozygous Mecp2<sup>−/−</sup>-deficient female mice must be mated with wild-type males to generate the Mecp2<sup>−/−</sup> affected males. Thus, the likelihood of obtaining a mutant male in any cross is one in four. In addition to the mating scheme required to generate mutant males, several groups have also noted difficulty in the rearing these mice. In particular, a high incidence of infanticide has been noted. While not unique to this particular mutant strain, this expected frequency highlights the need to maximize breeding efficiency and litter yield. As a result, a number of groups have attempted to share hints for improved breeding success. These suggestions include breeding-pair exclusiveness, minimizing cage disturbances of near term females, modifying vivarium light source, and adding irradiated sunflower seeds to the cages of expecting females (see information at http://www.rsrf.org/researchers/4.2.html). However, these suggestions have been provided on an ad hoc basis, and have yet to be examined for their effectiveness. Therefore, to begin to address the problems of breeding these Mecp2<sup>−/−</sup> null mice in an empirical manner, we have examined both the general breeding properties of Mecp2<sup>−/−</sup> mice, and also determined whether breeding success, as measured by increased number of weaned animals, can be improved by altering environmental conditions.

2. Materials and methods

2.1. Subjects

Mice were housed in our institutional facility after review and approval of animal experimentation protocols by the UHN Animal Care Committee in accordance with guidelines established by the Canadian Council on Animal Care. The experimental subjects used in all analyses were derived from female Mecp2<sup>−/−</sup> mice (Mecp2<sup>tm1.1Bird</sup>), obtained from The Jackson Laboratory, Bar Harbor, ME). Mecp2<sup>−/−</sup> mice were provided on a C57BL/6 background, and all crossing was done with wild-type C57BL/6 male mice. At approximately 8 weeks of age, male C57BL/6 and female C57BL/6 breeding pairs (wild-type breeders), or male C57BL/6 and female heterozygous Mecp2<sup>+/−</sup>- mice (Mecp2<sup>−/−</sup> breeders) were housed together as monogamous breeding pairs. The male remained with the female in the cage continually throughout the breeding life of the female. Mice were housed in individually ventilated and filtered home microisolator cages (29 cm x 18 cm x 12.5 cm), and individual cages changed weekly by the animal facility technicians. Food pellets and water were monitored daily, and provided ad libitum. The room housing the animals was kept on a 12 h light:12 h dark cycle, with lights on from 6:00 a.m. to 6:00 p.m. Ambient temperature was maintained at 21 ± 1 °C. Breeding pairs and litters were evaluated every 2–3 days, in the afternoon, throughout the duration of the investigations. Initial litter birth anticipation was based upon the days since initial breeder pairing, and from visual inspection, and placed under non-disturbance conditions, where the cages were not physically disturbed for a window that spanned approximately 3 days before until 3 days following the anticipated birth date. Nonetheless, all potentially pregnant mice were not-disturbed between 17 and 22 days post-initial pairing, in case they carried small litters not detected by the observation of a distended abdomen in the dam. Parturition was identified by visual inspection through the transparent cages during the non-disturbance period, without any physical disturbance, with the day of birth denoted postnatal Day 1. This method of visual litter detection does not allow for number of pups to be ascertained, and thus the exact number of pups per litter was not recorded until after the isolation period. Subsequent litter birth dates were predicted based on previous breeding patterns, and visual inspection, and placed under non-disturbance conditions as discussed above. The gender of the pups was determined on Day 14 ± 2, and a tail biopsy was also collected at this time for subsequent genotype determination (see below). The mice were weaned on Day 21 ± 2. After weaning, pups where housed in groups of three to five animals per cage until they reached breeding age, and were then separated by gender and genotype. Irradiated in-shell sunflower seeds (Advanced Protocol Picolab Natural Sunflower Seeds #5LP8) were purchased from Purina Mills through a local vendor (Ren’s Feed Supply, Oakville, Ont.), and aliquoted into sterile containers. Seeds were added to cages manually. Animals were bred and data collected from May 2003 to June 2005, representing 58 Mecp2<sup>−/−</sup> breeding pairs (215 litters), and 12 C57BL/6 wild-type breeding pairs (26 litters). Unanticipated litters born outside of non-disturbance periods did not meet our inclusion criteria, and thus were excluded from these analyses.

2.2. Genotyping

Mice were genotyped by PCR using DNA prepared from tail biopsies. Mouse tails were treated with topical anaesthetic and clipped on postnatal Day 14 ± 2, and digested in a solution of 50 mM Tris pH 8.0, 100 mM EDTA, and 0.5% SDS, with 100 µg/ml protease K, at 50 °C overnight. Tail genomic DNA was prepared via phenol/chloroform extraction. Genotyping was performed via the polymerase chain reaction using the primers and conditions provided by Jackson Laboratory. The common primer 5'-GTTAGAGACCCATGTGACCC-3' was used with 5'-GGCTTGGCACAGCACA-3 to detect the wild-type Mecp2 allele, or in a separate reaction with 5'-TCCACCTAGCCTGCCGTGAC-3' to detect the mutated Mecp2 allele. For each genotyping run, known Mecp2<sup>−/−</sup> and wild-type animal tissues were simultaneously assayed as positive and negative controls.

2.5. Data analysis

Statistical evaluations were based upon total breeding data collected. Statistical significance was evaluated by either
applying an one-way ANOVA, unpaired, two-tailed Student’s \( t \)-test followed by a post hoc Bonferroni correction for multiple comparisons, or a non-parametric Chi square test for bivariate tabular analysis. Significance levels were set at the \( p < 0.05 \) level. Correlation measurements between breeding criteria where made via Pearson product moment correlation coefficient, at the \( p < 0.05 \) level. Statistical analyses were performed in Microsoft Excel.

3. Results

3.1. Genotypes are not equally represented in Mecp2 mutant mouse litters at weaning

As discussed above, male Mecp2-deficient subjects from this strain are obtained by mating wild-type males with females heterozygous for the mutant Mecp2 allele (Guy et al., 2001). With such a cross, the predicted genotype representation for offspring is equivalent for wild-type males, hemizygous mutant males, heterozygous mutant females, and wild-type females. However, genotype analysis of the weaned pups from our crosses revealed that during the collection period, 29.8% were wild-type male, 21.8% were hemizygous mutant males, 19.3% were heterozygous mutant females, and 29.1% were wild-type females (Table 1). Chi square analysis revealed that although the total representation of males to females did not significantly differ \( (p > 0.20) \), Chi square = 0.024, critical value for \( p < 0.05 = 3.84 \), d.f. = 1), the presence of the mutant allele was significantly under-represented within this population \( (p < 0.01) \), Chi square = 8.14, critical value for \( p < 0.05 = 3.84 \), d.f. = 1). The under-representation of the mutant allele was not skewed towards gender, as mutant males and heterozygous mutant females were equally under-represented in the weaned populations.

3.2. Litters born to Mecp2-null dams are less likely to reach weaning age

We next examined whether litter viability would be affected by the Mecp2 mutation. For these analyses, a retrospective assessment of the litters born to heterozygous Mecp2-null females and wild-type C57Bl/6 breeders maintained under the same conditions (e.g., cages facing light source, without sunflower seed supplementation) was conducted. Only those litters detected following the non-disturbance period were evaluated in this study. On average, this cohort of heterozygous Mecp2-null breeders produced 4.82 ± 0.33 litters \( (n = 38 \) retired pairs; mean ± S.E.) during the typical 8 ± 1 months of monogamous pairing. Whereas 88% of the litters born to wild-type C57Bl/6 dams were viable at weaning age, only 53% of the litters born to heterozygous Mecp2-null dams are viable at weaning age (Fig. 1A). Of the litters born to heterozygous Mecp2-null dams, the average litter size was similar over the first 5 litters, but then progressively decreased (Fig. 1B). Analysis of individual litter
number revealed that the likelihood of litters reaching weaning age declines with subsequent litters, and is infrequent after the sixth litter (Fig. 1C).

3.3. Mecp2-null dams possess smaller weaned litter sizes

We then examined whether weaned litter sizes would differ between heterozygous Mecp2-null females and wild-type females. Litter sizes from heterozygous Mecp2-null dams crossed with wild-type males were found to be significantly smaller at weaning age (4.57 ± 0.18; n = 108 litters, mean ± S.E.) than C57BL/6 wild-type litters (7.38 ± 0.46 pups; n = 24 litters) (Fig. 2A). In fact, of all litters recorded over this study interval, no wild-type subject reared litters of smaller than four pups, while 25% of the total reared to Mecp2 mutants had fewer than four pups (Fig. 2B).

3.4. Litter viability is increased in mutant mice that have frequent litters

We then examined whether overall litter viability would be greater for mothers that were having frequent litters. These data revealed that, indeed, there was a significant correlation between the number of litters a dam produces and the overall viability of these litters (Fig. 3A). Further analysis revealed that litter viability also correlated with how rapidly she mated upon her first cage pairing with a male. As expected, mutant females that generated the largest numbers of litters over an 8 ± 1 month period were those which mated within the first 40 days of pairing with a male (Fig. 3B). In contrast, mutant females that required a larger window of time with the male for first mating showed consistently fewer overall litters (Fig. 3B). Interestingly, however, a correlation was also observed between the time a dam spent with a male until her first litter and the overall viability of the dam’s subsequent litters (Fig. 3C). Only 29% of the mothers who gave birth to their first litter after 40 days of initial pairing went on to wean more than half of their total litters, as compared to 66% of the mothers who delivered their first litter within 40 days following initial pairing.
3.5. Preceding birth interval does not vary, nor predict future viability

We then examined whether the average time period between subsequent litter births changes as subsequent litters are born. These data revealed that the average time between litter births does not significantly differ from the second to the seventh litter (Fig. 4A). We then examined whether the interval between two sequential births would be a predictor of the latter birth to weaning success outcome. No significant differences were observed, indicating that birth intervals do not appear to affect the success rate of litter weanings (Fig. 4B).

3.6. Sunflower seed supplementation improves breeding success

To investigate whether environmental differences might improve breeding success and/or the weaned litter viability rate, we examined whether light source position or the presence of sunflower seeds would affect breeding success. Although a trend towards improved litter viability was observed amongst the groups facing the light source compared to those facing away from the light source, the overall difference in outcome failed to reach statistical significance (Table 2A) (Chi square = 1.32, critical value for $p < 0.05 = 3.84$, d.f. = 1). We then tested whether the addition of a small handful of irradiated sunflower seeds to the cages of expecting dams would influence litter viability. For consistency, this examination was conducted only on mice whose cages faced the room light source. The irradiated seeds were added 3 days prior to predicted date of birth, and the cages were not disturbed for a 6-day window. The results from these examinations show that Mecp2-null breeding pairs supplemented with sunflower seeds displayed a significant increase in the proportion of litters that survive until weaning age, compared to those cages without supplementation ($p < 0.025$, Chi square = 6.05, critical value for $p < 0.05 = 3.84$, d.f. = 1). Percentages are expressed in parentheses.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Alive</th>
<th>Deceased</th>
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<tr>
<td>(A) Facing towards light source</td>
<td>51 (53.1)</td>
<td>45 (46.9)</td>
</tr>
<tr>
<td>Facing away from light source</td>
<td>29 (43.9)</td>
<td>37 (56.1)</td>
</tr>
<tr>
<td>(B) With seeds</td>
<td>25 (62.5)</td>
<td>15 (37.5)</td>
</tr>
<tr>
<td>Without seeds</td>
<td>14 (35)</td>
<td>26 (65)</td>
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(A) No significant difference in overall litter viability was observed between cages facing towards, or away from, the room light source ($p > 0.20$; Chi square = 1.32, critical value for $p < 0.05 = 3.84$, d.f. = 1). (B) Mutant Mecp2 breeders supplemented with sunflower seeds displayed a significant increase in the proportion of litters that survive until weaning age, compared to those cages without supplementation ($p < 0.025$, Chi square = 6.05, critical value for $p < 0.05 = 3.84$, d.f. = 1).

4. Discussion

The strategy used to generate male hemizygous Mecp2-null mice from the commonly employed Mecp2\textsuperscript{tm1.1Bird} strain requires crossing females heterozygous for the mutant Mecp2 allele with wild-type male mice. From such a cross, the genotype representation for offspring is expected to be equivalent for wild-type males, hemizygous mutant males, heterozygous mutant females, and wild-type females. Within our colony, however, we found that the mutant allele was significantly under-represented in our weaned litters. This diminution was not restricted to mutant males, as heterozygous mutant females were equally under-represented. The reasons for this observation are not clear. One possibility is that fewer mutant allele-containing offspring are born to these mothers. We have not attempted to examine early birth numbers, as due to our husbandry protocol, the home cages of expecting dams are not disturbed for the immediate 3 days preceding and following the anticipated parturition. However, selection against the mutant X allele has been noted in neurons cultured from heterozygous females in the related Mecp2\textsuperscript{308} mice (Young and Zoghbi, 2004), which tends to support the possibility of a skewing against the mutant allele. Additionally, it has also been noted that mice carrying the Mecp2 mutant allele are smaller in size at post-weaning ages (Guy et al., 2001). Thus, it is possible that these mutants may not compete for dam fostering as efficiently as their wild-type littermates, and as a result are culled from the litter. Additionally,
the presence of overgrown teeth in the pups, even at this perinatal stage, may have a negative influence on feeding from the dam, thus affecting pup viability. Regardless of reason, however, the apparent selection against the mutant allele, taken together with significantly smaller litter sizes, highlights the difficulty in generating sufficient numbers of mutant males for experimental investigation.

In terms of breeding efficiency, we found that females who became pregnant quickly generated more total litters over their lifetime than females that required a larger window of time with a male for their first litter, as would be anticipated. What was surprising was that females whose first pregnancy came quickly after being paired with a male showed a significantly higher likelihood of her subsequent litters reaching weaning age. We do not know why this is the case. However, it is possible that early onset of Rett-like symptoms in the heterozygous female dams could influence this pattern. Unfortunately, this characteristic was not recorded in this retrospective analysis. Nonetheless, these data suggest that females that are impregnated quickly after being paired with a male will not only have more litters over their lifetime, but also have a higher percentage of litters that survive past weaning age.

With respect to environmental conditions, although there appeared to be a trend towards improved litter viability when cages were placed facing the light-source, the difference in overall viability failed to reach the level of significance. Despite this, we still maintain our cages such that they face a light source. Our data do show clearly, however, that placing a small handful of irradiated sunflower seeds within the breeding cage a couple of days prior to the predicted date of birth correlates with a significant increase in litter survival. Whether this effect relates to the sunflower seeds providing a beneficial nutrient to the dam that attenuates aggressive behavior, or simply serves as a mastication distraction, remains unclear. In this regard it would be interesting to see if other cage environmental supplements would provide the same presumed calming effect. Interestingly, a recent study shows that perinatal choline dietary supplementation may attenuate some of the motor coordination deficits of symptomatic -deficient mice (Nag et al., 2005). It is also worth noting that sunflower seed supplementation was shown to calm the temperament of dystrophic pink-eyed RCS rat dams, and to improve their overall litter survival (Hess et al., 1981).

With the limited spatial and financial resources common to today's research laboratories, it is becoming increasingly important to maximize colony effectiveness. Based upon our data, we suggest the following to increase success with this mutant mouse strain. First, supplement the cages of expecting females with irradiated sunflower seeds from about 3 days prior to until 3 days following parturition. Second, face the cages of expecting females towards a light source. Although not reaching a level of statistical significance in our current study, the trend we observed supports this otherwise innocuous suggestion. Third, remove heterozygous mutant females from the breeder colony that do not become pregnant within the first 40 days of being paired with a fertile male. These heterozygous -null dams showed only a 14% likelihood of having more than three additional litters, and of these dams, only 31% of their litters survived to weaning. Fourth, retire heterozygous females from breeding stock following the weaning of their sixth litter. We found that the average size of subsequent litters at weaning from mutant females was $2.60 \pm 0.25$ pups, while the average size of the first 6 litters at weaning from these same dams was $4.12 \pm 0.29$ pups ($p < 0.01$, one-way ANOVA followed by post hoc test). In addition, although not addressed directly in this study, we also recommend refraining from disturbing the cages of expectant dams from 3 days preceding, to 3 days following, parturition. This will require alerting animal facility staff, and most animal facilities will allow a maximum of 6 days without bedding change for individually housed mouse pairs. These mutants appear to be highly sensitive to stress-induced infanticide, and minimizing physical contact during this time window would seem prudent.

Finally, it should be noted that our study only reports the outcome of litters that were visibly identified at the time following the period of non-disturbance. We did not include data from dams that appeared to be pregnant prior to the non-disturbance period but did not have pups in the cage at the end of the non-disturbance period. Similarly, we did not include data from dams that had been paired with a male and placed in isolation at the appropriate time window and had no pups at the end of the non-disturbance window. We do not know if the latter animals were pregnant with small litters that were not visibly evident and cannibalised their litter, or were simply not pregnant. If litters were lost prior to their being visibly recorded, our numbers would over-estimate the actual litter survival rate for this mutant strain. Even with this constraint, the data we present illustrate the outcomes for this mutant strain differ significantly from wild type, and highlight approximate yield expectations for this difficult to maintain mutant line.

In summary, in this report we present a retrospective assessment of our experiences in maintaining a colony of Mecp2-/- null mice maintained on a C57BL/6 genetic background, and my not, therefore, extend directly to mutants maintained on the 129 genetic background, or to other lines of Mecp2-/- deficient mice. Nonetheless, these data highlight the breeding tendencies for this frequently used mouse model of Rett syndrome, and present strategies for enhancing the breeding and maintenance of this mutant strain.

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