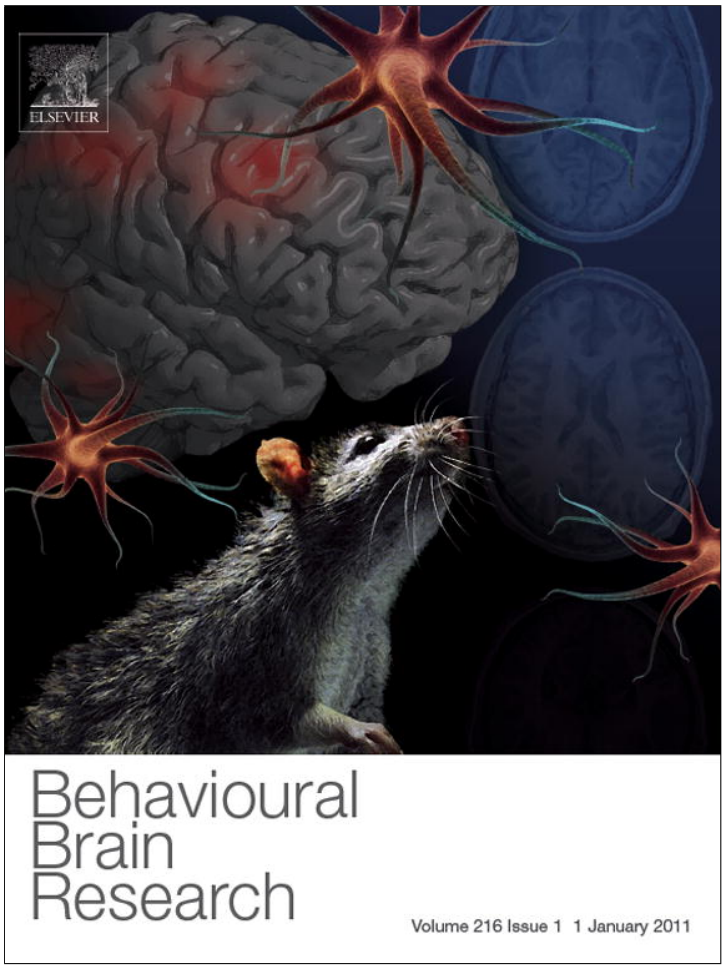

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Research report

Motor deficits on a ladder rung task in male and female adolescent and adult CGG knock-in mice

Michael R. Hunsaker[a,*], Ramona E. von Leden[a], Binh T. Ta[a], Naomi J. Goodrich-Hunsaker[b,c], Gloria Arque[b,c], Kyoungmi Kim[d], Rob Willemsen[b,e], Robert F. Berman[a,b]

[a] Department of Neurological Surgery, School of Medicine, University of California, Davis; Davis, CA, USA
[b] NeuroTherapeutic Research Institute, University of California, Davis; Davis, CA, USA
[c] Department of Psychiatry and Behavioral Sciences, University of California, Davis; Davis, CA, USA
[d] Division of Biostatistics, Department of Public Health Sciences, School of Medicine, University of California, Davis; Davis, CA, USA
[e] CBG-Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands

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ABSTRACT

The fragile X premutation is a tandem CGG trinucleotide repeat expansion on the *FMR1* gene between 55 and 200 repeats in length. A CGG knock-in (CGG KI) mouse with CGG trinucleotide repeat lengths between 70 and 350 has been developed and used to model the histopathology and cognitive deficits reported in carriers of the fragile X premutation. Previous studies have shown that CGG KI mice show progressive deficits in processing spatial and temporal information. To characterize the motor deficits associated with the fragile X premutation, male and female CGG KI mice ranging from 2 to 16 months of age with trinucleotide repeats ranging from 72 to 240 CGG in length were tested for their ability to perform a skilled ladder rung walking test. The results demonstrate that both male and female CGG KI mice showed a greater number of foot slips as a function of increased CGG repeat length, independent of the age of the animal or general activity level.

1. Introduction

The *FMR1* gene is polymorphic for the length of a CGG trinucleotide repeat in the 5′ untranslated region. In the general population there are <45 CGG repeats on the *FMR1* gene, while in the full mutation underlying fragile X syndrome (FXS) there are >200 CGG repeats and the *FMR1* gene is transcriptionally silenced. In the fragile X premutation there are between 55 and 200 CGG repeats and increased transcription of *FMR1* mRNA [29]. Additionally, the fragile X premutation has now been associated with a number of neurocognitive sequelae, such as working memory deficits and impaired spatial information processing [1,3,9,15,17,30,31,41–44]. The fragile X premutation can also result in the late onset neurodegenerative disorder called fragile X-associated tremor/ataxia (FXTAS), which occurs in upwards of 40% in males and 8–16% of female carriers of the fragile X premutation identified from known fragile X probands [14,38,46,52]. FXTAS sequelae include cerebellar gait ataxia and intention tremor that may be targetable symptoms for pharmacological intervention [33,52].

To further investigate the consequences of the fragile X premutation, a CGG knock-in (KI) mouse has been developed [6,11–13,65]. Behavioral characterizations of these mice have demonstrated subtle motor deficits on an accelerating rotarod as well as impaired spatial memory in the water maze, but only when mice were tested at greater than 12 months of age [53,62]. Subsequent studies have identified abnormal embryonic development as well as spatial memory deficits evident as early as 12 weeks of age in CGG KI mice [16,22,36,37]. Whether there are early motor deficits in CGG KI mice has yet to be determined.

To determine whether the CGG KI mouse model shows specific motor performance deficits at earlier ages than 12 months of age, more sensitive motor tasks are needed. Therefore, in the present study male and female mice ranging from 2 to 16 months of age were tested on a ladder rung task adapted from procedures reported by Soblosky et al. [58,59] and refined by Whishaw and co-workers [24,25,48,49,57–59], among others [4,21,56]. This task

Abbreviations: FXS, fragile X syndrome; FXTAS, fragile X-associated tremor/ataxia syndrome; *FMR1*, Fragile X mental retardation 1 gene; KI, knock-in; WT, wildtype.

* Corresponding author at: Department of Neurological Surgery, School of Medicine, University of California, Davis, 1515 Newton Court, Room 502C, Davis, CA 95616, USA. Tel.: +1 801 634 9463, fax: +1 530 754 5125.
E-mail addresses: ryanhunsaker@me.com, mrhunsaker@ucdavis.edu (M.R. Hunsaker).

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was chosen because it has been shown to be sensitive to subtle sensorimotor deficits in both mice and rats [24,58]. In the ladder rung task, mice were allowed to walk along a narrow walkway on a floor made of parallel thin rounded rods at a constant separation. Successful performance in this task requires the animal to precisely determine where to place a paw on a narrow rung, followed by a skillful limb advance and paw placement. In this task, foot slips, defined as the number of times the mouse's paw fell through the rung floor, were used as an index of skilled motor performance during walking. We found that both male and female CGG KI mice show a greater number of foot slips in this test than wildtype littermates. Furthermore, a CGG dosage effect was evident because within the CGG KI mice with expanded CGG trinucleotide repeats on the *Fmr1* gene, the number of foot slips showed a positive association with CGG repeat length, such that mice with long CGG repeat lengths had a greater number of foot slips than mice with more intermediate length CGG repeats.

2. Methods and materials

2.1. Animals

Forty-two male and 30 female CGG KI mice from 2 to 16 months of age as well as 41 male and 20 female wildtype mice of the same ages were used as subjects for this task. All wildtype mice were littermates with CGG KI mice included in the study. All CGG KI mice were bred onto a congenic C57BL/6J background as verified by microsattelite analysis from founder mice on a mixed FVB/N × C57BL/6J background [37,64,65]. Mice were housed in same sex, mixed genotype groups with between one and four mice per cage in a temperature and humidity controlled vivarium. A 12 h light–dark cycle was used with *ad libitum* access to water and food. All experiments were conducted during the light phase of the cycle and conformed to UC Davis IACUC approved protocols.

2.2. Genotyping

DNA was extracted from mouse tails by incubating with 10 mg/mL Proteinase K (Roche Diagnostics; Mannheim, Germany) in 300 μL lysis buffer containing 50 mM Tris–HCl, pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% SDS overnight at 55 °C. One hundred micro litre saturated NaCl was then added and the suspension was centrifuged. One volume of 100% ethanol was added, gently mixed, and the DNA was pelleted by centrifugation and the supernatant discarded. The DNA was washed and centrifuged in 500 μL 70% ethanol. The DNA was then dissolved in 100 μL milliQ-H2O. CGG repeat lengths were determined by PCR using the Expanded High Fidelity Plus PCR System (Roche Diagnostics). Briefly, approximately 500–700 ng of DNA was added to 50 μL of PCR mixture containing 2.0 μM/L of each primer, 250 μM/L of each dNTP (Invitrogen; Tigard, OR), 2% dimethyl sulfoxide (Sigma–Aldrich; St. Louis, MO), 2.5 M Betaine (Sigma–Aldrich), 5 U Expand HF buffer with mg (7.5 μM/L). The forward primer was 5′-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3′ and the reverse primer was 5′-AGCCCCGCACTTCCACCACCAGCTCCTCCA-3′. PCR steps were 10 min denaturation at 95 °C, followed by 34 cycles of 1 min denaturation at 95 °C, annealing for 1 min at 65 °C, and elongation for 5 min at 75 °C to end each cycle. PCR ends with a final elongation step of 10 min at 75 °C. DNA CGG band sizes were determined by running DNA samples on a 2.5% agarose gel and staining DNA with ethidium bromide [11,36,64,65]. Genotyping was performed twice on each animal, once using tail snips taken at weaning and again on tail snips collected at sacrifice. In all cases the genotypes matched.

2.3. Apparatus

The ladder rung apparatus was modeled on previously described ladder rung walk apparatus [24,48–50,57–59]. The apparatus consisted of two, 28 cm tall × 65 cm long black walls separated by 10 cm. The floor was elevated 10 cm from the bottom of the walls and was made from 43 parallel 1 mm diameter bars separated by 1.5 cm.

2.4. Ladder rung testing

All performance on the ladder rung test was recorded with a digital video camera (Sony Handycam; Sony, Inc., Tokyo, Japan) connected by a firewire connector to a PC laptop. The video camera was positioned at one end of the apparatus to record the full length of the beam floor. This allowed the experimenter to score whether the mouse's limbs extended below the beam floor as well as allowed the experimenter to observe the general posture of the mouse above the beam floor.

For testing, mice were gently placed in the apparatus and allowed to freely explore the apparatus and walk back and forth along the apparatus for 2 min. Mice typically explored the apparatus by walking the length of the apparatus, looking over the edge, and returning to the start position. During testing, the experimenter recorded how many times the mouse moved between the two ends of the apparatus as a general activity measure. All experiments were performed by the same experimenter. The digital recordings were later independently scored from the recordings by two experimenters blinded to the genotype of the animals (intraclass correlation coefficient = 0.94, $p < 0.001$).

2.5. Dependent measures and statistical analysis

Along with recording the number of times each mouse traversed the apparatus, the number of times the animals' fore or hind paws slipped below the ladder rungs was recorded as the dependent variable. A foot slip was recorded whenever the limb of the mouse passed below a rung sufficiently to clearly see the wrist of the animal. In this way, slipping was defined as the mouse completely missing or falling off a rung while walking across the apparatus.

To determine whether parametric analyses of variance (ANOVA) were appropriate for the data, tests of normality and homoscedasticity were performed. Once it was determined that parametric statistics were appropriate for the data, the data were plotted and placed into CGG repeat length groups as follows: the mice in the wildtype group all had between 8 and 12 CGG repeats (mean 10 ± 0.25 SEM; $n = 61$), mice included in the Low CGG repeat group ranged between 72 and 116 CGG repeats (mean 86 ± 3.1; $n = 20$), and the mice included in the High CGG repeat group ranged between 140 and 240 CGG repeats (mean 170 ± 9.2; $n = 52$). These groupings were used to categorize group by CGG repeat length because it was determined that the 24 CGG gap in the data between the Low (e.g., 116) and the High (e.g., 140) CGG repeat groups as well as the large gap between Wildtype (8–12 CGG repeats) and Low (e.g., 72) CGG repeat groups invalidated any using CGG repeat length as a continuous variable for statistical analysis. Similar groupings were also used in previous studies of the CGG KI mice [36]. To determine if age significantly contributed to ladder rung test performance, mice were further separated into two groups based upon a median split of age, with one group ≤6 months of age (mean 4.5 ± 0.99 months; range 2–6 months) and the other ≥7 months of age (mean 10.75 ± 2.1 months; range 7–16 months). The data were analyzed as follows. A 3 (Group) × 2 (Sex) × 2 (Age) analysis of covariance (ANCOVA) with locomotor activity as a potential covariate was used to determine which factors contribute to task performance. Subsequent analyses were performed to further characterize all main effects. All analyses were considered significant at $p < 0.05$. Statistical analyses were performed in R 2.7.1 language and environment (The R Foundation for Statistical Computing; Aukland University, Aukland, New Zealand; http://www.r-project.org/) and statistical power was calculated using both R and the statistical program G*Power 3 [26,27].

3. Results

For all mice, data were grouped by CGG repeat length (wildtype, Low CGG, High CGG), Sex (male, female), and Age (≤6 months, ≥7 months) and analyzed using three way ANCOVA with number of foot slips as the dependent variable and locomotor activity as a covariate. There was a main effect of CGG repeat length group ($F(2,120) = 5.4373$, $p = .005$), but no main effect of Age ($F(1,120) = 0.54$, $p = 0.47$) or Sex ($F(1,120) = 0.06$, $p = 0.94$), nor were there any significant interactions among variables (lowest p value $p = 0.45$). There was also no significant contribution of locomotor behavior for performance on the ladder rung task ($F(1,120) = 0.23$, $p = 0.63$). These results suggest that Age, Sex, and activity level did not contribute to task performance and only the CGG repeat length group factor significantly contributed to ladder rung task performance (Fig. 1A).

To further characterize the significant main effect of CGG repeat length group, a Tukey-Kramer post hoc pairwise comparisons test demonstrated that the wildtype mice showed significantly fewer foot slips (mean 12.8 ± 0.55) than the Low (mean 19.1 ± 0.88) or High (mean 25.3 ± 0.65) CGG repeat groups ($p < 0.005$, $p < 0.0001$ respectively), and that the Low CGG repeat groups had significantly fewer foot slips than the High CGG repeat group ($p < 0.005$) (Fig. 1A).

To characterize any possible relationship between CGG repeat length and performance on the ladder rung task in CGG animals with expanded CGG trinucleotide repeats, a partial correlation coefficient adjusted for Sex was calculated. A positive association was observed between the CGG trinucleotide repeat length and the number of foot slips during ladder rung task performance (Fig. 1B; Pearson's partial $r = 0.54$; $R^2_{(adj)} = 0.28$).

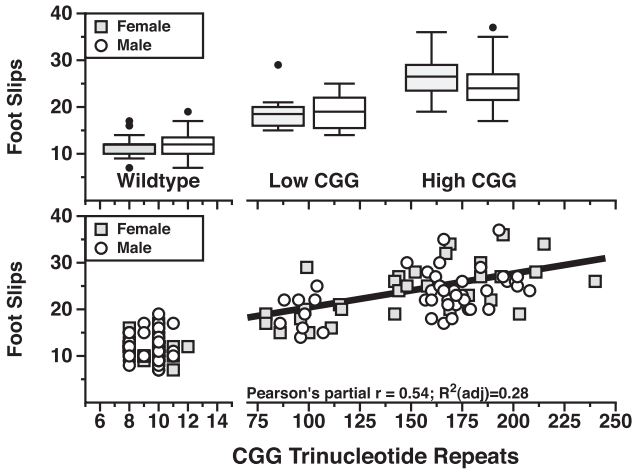

Fig. 1. CGG repeat length modulates ladder rung task performance. (A) Boxplots stratified by Sex and CGG repeat length group. Note that the High CGG repeat group showed a greater number of foot slips than mice in the Low CGG repeat group ($p < 0.005$). Both the High and Low CGG repeat groups had a greater number of foot slips than wildtype mice ($p < 0.0001$, $p < 0.005$ respectively). Wildtype male $n = 41$, female $n = 20$; Low CGG male $n = 10$, female $n = 10$; High CGG male $n = 32$, female $n = 20$. (B) Scatterplot of CGG repeat length and the number of foot slips for each animal included the present study. A partial correlation performed comparing the number of foot slips to CGG repeat length (adjusted for influence of Sex) within CGG KI mice with expanded CGG trinucleotide repeats demonstrated a positive association between CGG repeats and the number of foot slips (Pearson's partial $r = 0.54$; $R^2_{(adj)} = 0.28$).

4. Discussion

The current experimental results reveal that both male and female CGG KI mice are impaired in performance of a skilled ladder rung walking task compared to wildtype littermates. Specifically, CGG KI mice showed a greater number of foot slips with increasing CGG repeat length (Fig. 1B). These findings suggest that the length of an expanded CGG trinucleotide repeat on the *Fmr1* gene is related to impaired locomotor performance in CGG KI mice as observed in the ladder rung task. The present data also provide the first demonstration of motor deficits in CGG KI mice under 12 months of age. Interestingly, mice as young as 2 months of age appeared to show motor deficits similar to the mice over 12 months of age. Contrary to our initial hypotheses, age did not contribute to task performance, nor were there differences between sexes for skilled ladder rung performance. Male and female mice showed similar decrements in performance with increasing CGG repeat length (Fig. 1A and B). These data suggest that the ladder rung task is likely revealing an early motor deficit as opposed to directly modeling the late onset cerebellar gait ataxia reported in human cases of FXTAS.

The early appearance of motor deficits was not entirely unexpected, considering recent reports that embryonic cortical development is abnormal in CGG KI mice [22] and that dendritic complexity is reduced and synaptic structure is altered in cultured hippocampal neurons [16]. Furthermore, it has been found that CGG repeat length and age modulate performance on a spatial processing task in 23–43 year old human female fragile X premutation carriers [30].

The lack of differential performance between sexes was unexpected as it has been reported that fragile X syndrome and FXTAS are more prevalent in males than females, presumably due to a protective influence of a second non mutated *FMR1* gene on the second X chromosome [7,38]. Despite the reduced prevalence of FXTAS in female carriers of the fragile X premutation relative to males, females with FXTAS do not show reduced FXTAS symptoms once diagnosed [7,34]. Furthermore, it is possible that the ladder rung task is sensitive enough to probe the underlying motor networks that may be similarly disrupted in male and female CGG KI mice.

Although not quantified, CGG KI mice also showed a hunched posture all ages and a discernible shaking while walking along the ladder rung apparatus. Similar behaviors were not observed in wildtype mice. This tremoring during the performance of a motor task is of interest because no gross motor abnormalities or tremoring are apparent when CGG KI mice are observed in an open field. These results suggest that motor abnormalities in CGG KI mice may not be apparent until the mice are challenged by a difficult motor task, such as the ladder rung task, which may unmask a previously unidentified motor tremor. These postural tremor-like behaviors need to be further investigated and carefully described in CGG KI mice.

These results also indicate that the ladder rung task is a sensitive and robust assay that allows for a high throughput analysis of motor function in CGG KI mice. As each mouse was only exposed to the apparatus for approximately 2 min in the present experiment and there was no adaptation period preceding data collection, performance of the task served as a rapid assay of motor function without the potential confounds of motor learning that may mask between group effects, as suggested in earlier studies [24,58]. This point is important as the rotarod task used to test motor function in mice requires the mouse be placed on a rotating drum and the time to fall is typically used as the outcome measure. For the rotarod, there are often early training trials given to mice on the rotarod apparatus that may potentially mask any differences that are present in baseline motor function as mice are trained to set performance criteria before administration of accelerating rotarod testing [58,59].

The present experiment did not explicitly employ the subtle gait measurements described by Whishaw and co-workers [8,24,50]. This is because the sides of the apparatus used in this study were opaque and prevented the requisite recording the mouse's foot placement from the side for more precise analysis of limb movements. Such measurements could provide additional evidence for subtle motor deficits, as well as the precise nature of the observed missteps. For example, more sophisticated analyses of gait would reveal whether animals make predictable errors such as consistently under or overestimating the location of subsequent beams. Such errors could indicate a possible dysfunction in frontal-parietal network-dependent vector calculations underlying action in peripersonal space [23,54,63]. Alternately, if animals showed a trend toward a general clumsiness or lack of precision in motor performance that could indicate a more purely motor deficit [4,5]. The first possibility is intriguing in for the CGG KI mice as frontal-parietal network dysfunction is hypothesized to underlie spatiotemporal, arithmetic, and attentional deficits in FXS as well as the fragile X premutation [3,10,18–20,28,30,32,35,39,40,43,47,51,55,60,61], as well as being involved in skilled walking behaviors [2,4,5,23,45]. The latter possibility, based more directly on motor function is also important for the extension of the CGG KI mouse as a murine model of motor deficits present in FXTAS [62]. Such follow-up studies are currently underway to explore these possibilities in CGG KI mice as well as to correlate any performance deficits to neuropathological features, which are present throughout the neocortex and cerebellum of CGG KI mice [37,64,65].

The primary benefit of this modification of the ladder rung task is that extensive pre-training is not required and that testing times and trials are significantly shortened compared to the versions of the task reported previously [24,50]. Furthermore, spontaneous exploration is encouraged, and the effect of this behavior on potential error production can be considered. This results in a high throughput screen that is sufficiently sensitive to detect subtle motor impairments in transgenic mouse models.

In summary, the present experiment identified age-independent motor deficits in CGG KI mice. The detection of motor deficits in young CGG KI mice is important as performance on the ladder rung task may be used as outcome measures for behavioral or pharmacological therapeutic intervention in this mouse model as it pertains to FXTAS as well as other late onset neurodegenerative disorders. This modification might make the test more attractive to other groups. Because mice as young as 2 months of age show deficits, the need to limit testing to mice greater than 12 months of age to identify potential endpoints and outcome measures is mitigated.

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