RESEARCH ARTICLE

Reversal learning paradigm reveals deficits in cognitive flexibility in the *Fmr1* knockout male mouse [version 1; referees: 1 approved, 2 approved with reservations]

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Abstract

Background: Loss of *FMR1* is associated with Fragile X syndrome, amongst the most prevalent inherited intellectual disability. Despite extensive research in this area, previous studies have failed to detect consistent evidence of cognitive impairments in the Morris water maze (MWM) task in the *Fmr1* knockout (KO) mouse. However, few studies have examined cognitive flexibility in a reversal form of the MWM task, which may illuminate subtle learning deficits.

Methods: Adult male *Fmr1* wildtype (WT) and KO mice were bred and tested in the MWM reversal paradigm. The testing paradigm consisted of two blocks per day, with 4 trials per block to locate a hidden platform. After the last trials on the fourth day of testing, the animals were given a probe trial with the platform removed. The following week, the location of the platform was switched to the opposite quadrant and the animals received 2 more days of testing, with 4 blocks in total.

Results: As expected, *Fmr1* KO mice did not display a learning deficit during the acquisition phase, $F_{genotype}$ (1, 24) = 0.034, p = 0.854, and performed similarly on the probe trial, $F_{genotype}$ (1, 23) = 0.024, p = 0.877. However, during the reversal phase of learning, *Fmr1* KO mice showed deficits in their ability to learn the new location of the platform, $F_{genotype}$ (1, 23) = 3.93, p = 0.059. Further independent samples t-testing revealed that KO animals displayed significantly higher latency to reach the hidden platform during the third trial, $t(23)$ = -2.96, $p < 0.01$.

Conclusions: While previous studies have not demonstrated deficits in spatial memory in the *Fmr1* KO model, it is possible that the acquisition phase of the task is less sensitive to deficits in learning. Future studies using this model to evaluate therapeutic interventions should consider utilizing the MWM reversal paradigm.

Keywords

Cognitive flexibility, Autism, Morris water maze, Prefrontal cortex, FMRP

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Referee Status: ? ? ✓

	Invited Referees 1	2	3
version 1 published 07 Jun 2018	? report	? report	✓ report

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Comments (0)

Corresponding author: Joaquin N. Lugo (joaquin_lugo@baylor.edu)

Author roles: Nolan SO: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Lugo JN**: Conceptualization, Formal Analysis, Funding Acquisition, Methodology, Project Administration, Validation, Writing – Review & Editing

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Introduction

Fragile X syndrome (FXS) is a neurodevelopmental disorder, caused by a trinucleotide expansion mutation in the *FMR1* gene, and is also one of the most prevalent inherited forms of intellectual disability[1]. FXS is often modeled using the *Fmr1* knockout (KO) mouse, which can be characterized by several behavioral phenotypes, including alterations in sociability and deficits in fear memory[2–4]. Aside from deficits in spatial and non-spatial learning, one understudied facet of intellectual disability is the ability to incorporate new information into existing learning, termed cognitive flexibility. Cognitive flexibility can be studied in rodents using a variant of the Morris water maze (MWM) paradigm[5,6]. In the MWM reversal paradigm, the location of the hidden platform is moved, and the latency to adjust to the new location is measured. As expected, several reports find evidence of impairments in reversal learning in the *Fmr1* KO mouse across multiple strains, the C57BL/6J backcrossed strain[7,8] and the albino C57BL/6J background[2]. However, other studies have been unable to replicate these findings, and further investigation points to the possibility of background strain differences[9]. Previous reports have not detected any impairments in reversal learning in the FVB.129 strain[10]. However, it may be that this paradigm is perhaps even more sensitive to methodological differences. The current study adds to this literature by using the FVB.129 strain in a previously utilized paradigm.

Methods

Animals

Male *Fmr1*$^{+/+}$ and female *Fmr1*$^{+/-}$ FVB.129P2-Pde6b+Tyrc-ch Fmr1tm1Cgr/J (Stock No: 004624, The Jackson Laboratory, Bar Harbor, ME, USA) mice were used as breeders (9 total breeding pairs) to produce the following groups: male WT and male KO pups. Breeding pairs were of the following groupings: WT Female/WT Male (n = 2), KO Female/KO Male (n = 5), WT Female/KO Male (n = 2). Genotype was determined from toe clippings taken prior to age postnatal day (PD) 12 (Mouse Genotype, Escondido, CA, USA). The final sample sizes were as follows: $n_{male\ WT} = 10$, $n_{male\ KO} = 16$. Target samples sizes (n = 10) were calculated *a priori* using a power calculation in G*Power 3.1 with the following parameters: f = 0.50 (large effect), $\alpha = 0.05$, power $(1 - \beta) = 0.80$, for the F family of tests with two groups and 8 repeated measures (trials). All pups were housed in individual cages (Allentown Caging PC7115HT, Allentown, PA, USA), filled with sani-chip bedding (7090 Teklad, Envigo, Somerset, NJ, USA). Prior to weaning on PD21, pups were housed with parents (1 male and 2 females) and littermates (up to 12 pups). Following weaning, subjects were housed with mixed genotype littermates, no more than 5 to a cage. The light cycle was kept at 12 hr. light, and the colony room was kept at an ambient temperature of 22° C. Animals had *ad libitum* access to food and water. All procedures performed were in accordance with *Baylor University Institutional Animal Care and Use Committee* (Animal Assurance Number A3948-01), as well as the *National Institutes of Health Guidelines for the Care and Use of Laboratory Animals*. All efforts were made to ameliorate any stress and harm to the animals, specifically by habituating animals to the testing apparatus and room prior to trial recordings.

Morris water maze

All behavioral testing was conducted during the light cycle, specifically between 8 am and 5 pm. The methods for the current study were adapted as closely as possible from earlier studies of this behavior in the *Fmr1* KO mouse (represented in Figure 1)[9]. Briefly, a 1.3 m diameter white pool was filled with water and made opaque through the addition of non-toxic white paint (Item LT3010, S&S Worldwide, Connecticut). The hidden platform measured 14.5 cm × 14.5 cm and was submerged approximately 2 cm below the water level. The testing paradigm consisted of two blocks per day for 4 days, with 4 trials in each block, for each mouse to test the ability to locate a hidden platform. The mice were habituated to the testing room in their holding cages for 30 minutes prior to the onset of testing. The amount of time spent in each quadrant for each trial was recorded with a ceiling-mounted video camera (Ganz YCH-02, Cary, NC, USA), and analyzed using automated tracking software (Ethovision XT 6, Noldus, Wageningen, Netherlands). After the last trial on the fourth day of testing was completed, the animals were given a probe trial. The probe trial involved removing the platform and

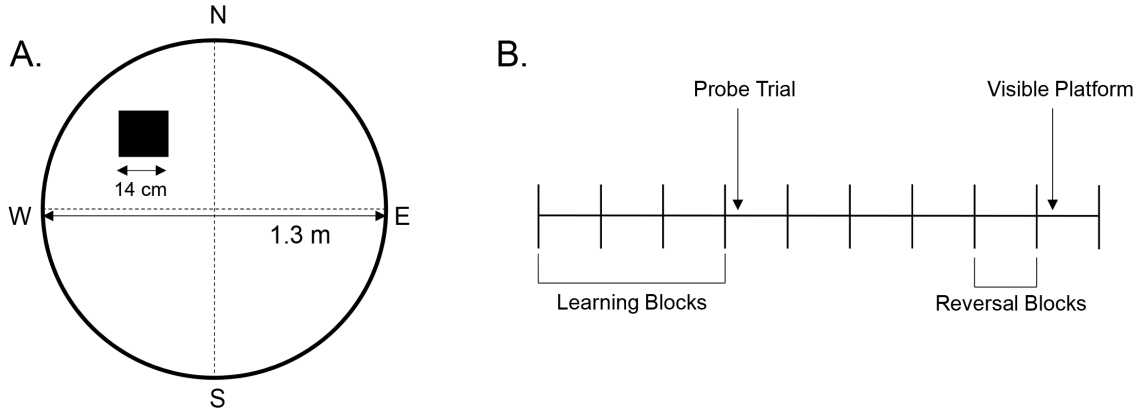

Figure 1. Overview of the testing paradigm. A. An overview of the set-up of the testing arena. **B**. An overview of the progression of testing days.

allowing the subjects to explore the maze for 60 seconds. During the probe trial, the number of times the animal crossed the location of the hidden platform and the duration of time in each quadrant was calculated. Testing resumed on day 8 after a 3-day rest period. On day 8, the platform was placed in the opposite quadrant from the previous location that housed the hidden platform. Testing progressed as with the initial acquisition phase, with 2 blocks per day for 2 days. On the final day of testing, a visible platform was used to evaluate visual performance as well as swim speed. The visible platform was a two-tiered platform similar to the initial platform, with a second higher tier platform that extended 9.5 cm above the lower platform, allowing the animal to see the platform. The differences in methodology from the cited source were as follows: only four days of acquisition were conducted and the testing paradigm was lengthened to account for a consolidation period between the learning and reversal trials (See Figure 1 for a description of the testing paradigm). One KO animal was excluded from analysis due to a seizure during this task.

Statistical analysis

Statistical analysis was performed in the form of a one-way analysis of variance (ANOVA) with one between-subjects factor (Genotype [wildtype, knockout]) and one within-subjects factor (Trial). All data were analyzed using GraphPad Prism Software 7.0 (San Diego, CA, USA) or IBM SPSS Statistics 23 (Aramonk, NY, USA).

Results

Fmr1 KO mice show no impairment in acquisition of spatial memory

To investigate the effect of genotype on hippocampal spatial memory, animals were tested in the MWM paradigm (Dataset 1). During the 8 blocks of learning trials (Figure 2A), there was a significant within-subjects effect of trial for latency to reach the platform, $F(3.56, 85.47) = 30.15$, $p < .0005$. Trial results did not interact significantly with genotype, $F(3.56, 85.47) = 1.33$, $p = 0.24$, suggesting both groups learned the location of the platform similarly. Between-subjects analyses indicated no effect of genotype, $F(1, 24) = 0.03$, $p = 0.85$. Further independent samples t-testing revealed no differences between WT and KO at any of the 8 different trials, $p > 0.05$.

For the probe trial (Dataset 2), as expected, male KO mice demonstrated similar time spent in the target quadrant, $F(1,23) = 0.02$, $p = 0.88$ (Figure 2B), compared to male wildtype (WT). Further independent samples t-testing revealed no differences in duration in any of the quadrants, $p > 0.05$.

Loss of *Fmr1* impairs ability to update existing learning with new platform location

The week following the initial learning trials, animals were tested in the reversal learning paradigm (Dataset 3). During the 4 blocks of learning trials (Figure 2C), a one-way ANOVA with repeated measures revealed a significant within-subjects effect of trial, $F(3, 69) = 3.8$, $p < 0.05$. Trial results did not interact significantly with genotype, $F(3, 69) = 1.28$, $p = 0.29$. However, between-subjects analyses indicated a marginal effect of genotype, $F(1, 23) = 3.93$, $p = 0.059$ (Figure 2C). Further independent samples t-testing revealed that KO animals displayed significantly higher latency to reach the hidden platform during the third trial, $t(23) = -2.96$, $p < 0.01$. Together, these results demonstrate that *Fmr1* KO males demonstrate decreased learning and altogether a lack of cognitive flexibility across all trials of the MWM reversal task.

Impairments were not due to deficits in vision or motor capabilities

Visible platform information was also assessed to ensure differences were not due to deficits in vision (Dataset 4). Results were analyzed using a repeated-measures ANOVA across the four visible platform trials on latency to the platform across the two blocks of trials. Results indicated no effect of block, $F(1, 24) = 1.341$, $p = 0.26$, nor an interaction of block and genotype, $F(1, 24) = 0.0005$, $p = 0.98$. There was not a significant effect of genotype on latency to the platform during these trials either, $F(1, 24) = 0.98$, $p = 0.33$ (Figure 3A). Altogether, these data suggest that differences in latency to the platform could not be attributed to deficits in visual perception in the *Fmr1* KO male mouse. Moreover, differences in latency to the platform on the previous trials could not be attributed to impairments in swimming abilities, as no differences in swim speed were detected during the visible trials, $t(11.17) = 1.526$, $p = 0.16$ (Figure 3B).

Dataset 1. Learning Trial Data

http://dx.doi.org/10.5256/f1000research.14969.d206068

This datasheet contains the raw data exported from the Ethovision program (Columns A – G) as well as the transformed dataset that was used for analysis for the learning trials (Columns J – T)

Dataset 2. Probe Trial Data

http://dx.doi.org/10.5256/f1000research.14969.d206069

This datasheet contains the raw data exported from the Ethovision program (Columns A - AB) as well as the transformed dataset that was used for analysis for the probe trial (Columns AC – AI)

Dataset 3. Reversal Trial Data

http://dx.doi.org/10.5256/f1000research.14969.d206076

This datasheet contains the raw data exported from the Ethovision program (Columns A – G) as well as the transformed dataset that was used for analysis for the reversal trials (Columns J – O)

Dataset 4. Visible Platform Data

http://dx.doi.org/10.5256/f1000research.14969.d206077

This datasheet contains the raw data exported from the Ethovision program (Columns A – H) as well as the transformed dataset that was used for analysis of the visible platform trials (Columns K – S)

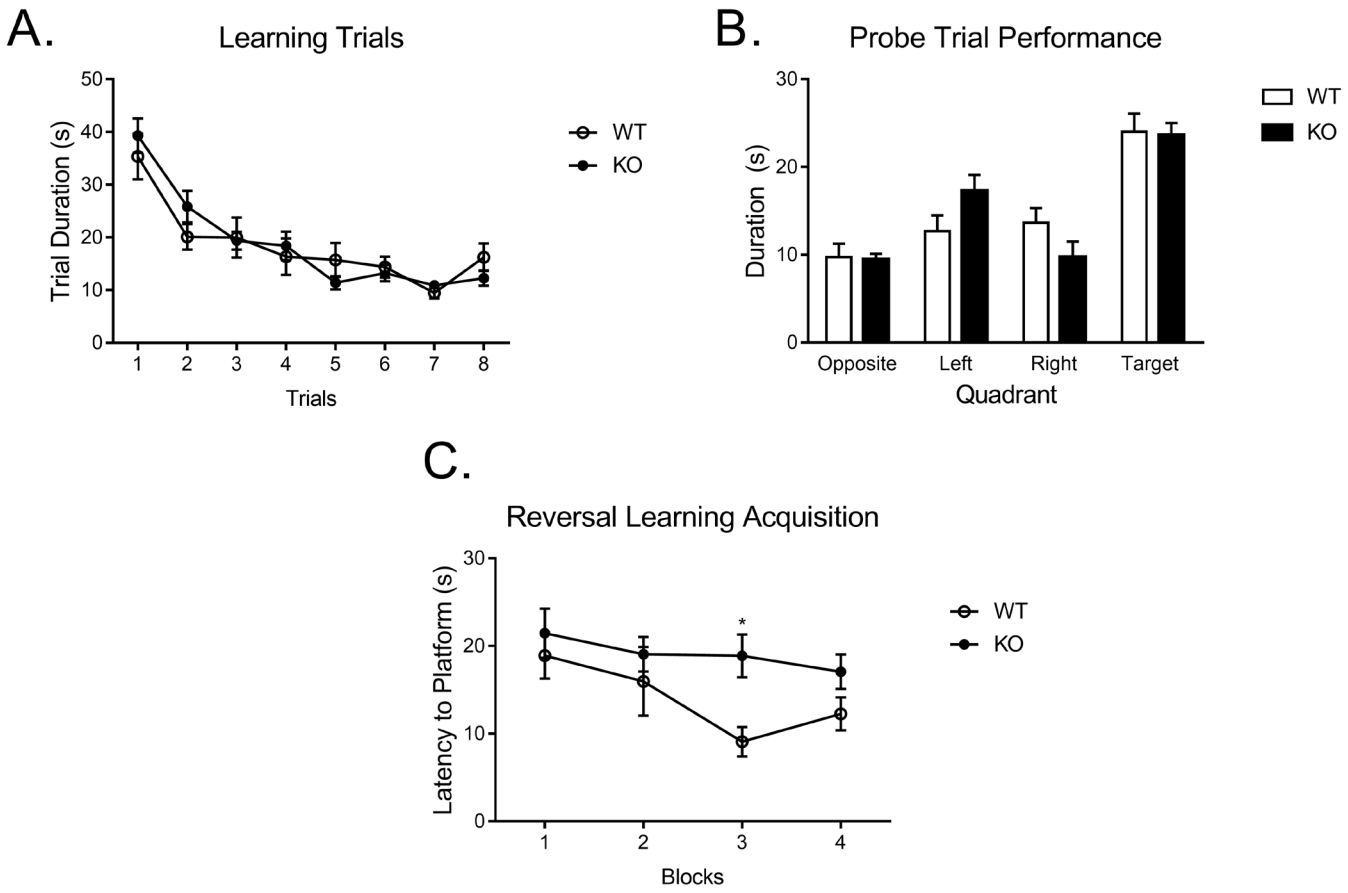

Figure 2. Performance in the Morris water maze in *Fmr1* knockout males. A. *Fmr1* knockout males show no deficits in acquisition in performance. **B**. Performance during the probe trial is not impaired in the *Fmr1* knockout males. **C**. When subjected to a reversal learning paradigm, *Fmr1* knockout mice display increased latency to the new platform location, demonstrating deficits in cognitive flexibility. Knockout – KO, Wild type - WT.

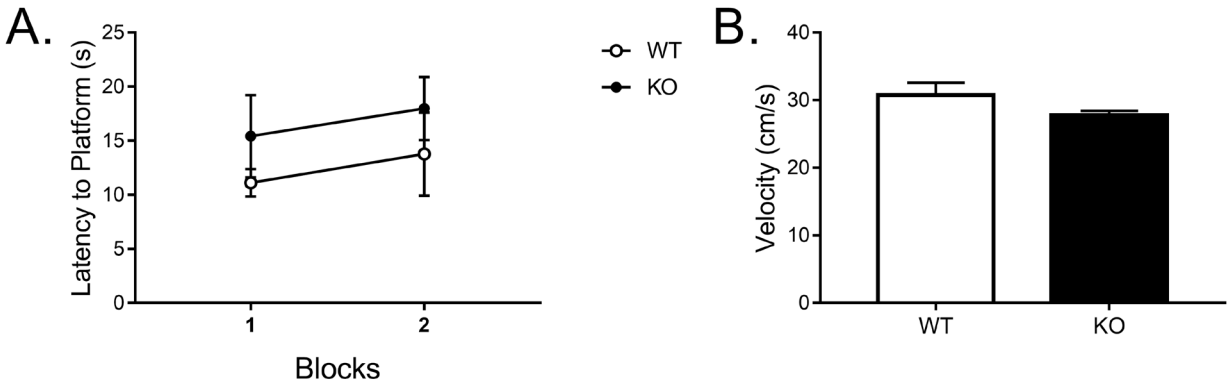

Figure 3. Performance in the Morris water maze visible trials. A. *Fmr1* knockout males showed no deficits in latency to the platform across the two blocks of the visible platform test. **B**. *Fmr1* knockout males showed no deficits in swim speed across the visible platform test. Knockout – KO, Wild type - WT.

Discussion

As expected, deletion of *Fmr1* did not impact initial spatial learning in the MWM. The current study did, however, demonstrate impairments in cognitive flexibility in the *Fmr1* KO. As previously mentioned, other studies have not before detected such changes, and this discrepancy could be attributed to methodological differences[10]. In the aforementioned study, training occurred over 8 days, with only 3 training trials per day, and the reversal paradigm consisted of 4 days, with 3 trials per day. Furthermore, in support of our findings, deficits in long-term potentiation in the prefrontal cortex have been demonstrated in the *Fmr1* KO mouse, the area on which the ability to adapt to a new location in this task is dependent on 11–13. Moreover, this ability to adapt to a new location is mediated through multi-synaptic connections between the hippocampus and the prefrontal cortex[13]. This proposed mechanism further supports our findings of no change to the initial spatial learning phase, as lesions to this area did not impact initial learning performance in spatial navigation[11].

The current study provides preliminary evidence that could be applied to tease apart subtle differences between the male and female *Fmr1* KO phenotype, which has been difficult to conclusively evaluate. Future studies should expand upon these findings in females, as many studies have demonstrated a sex-specific effect of loss of *Fmr1* on behavior (discussed in a recent review[14])[2–4,15]. Overall, this study corroborates and extends previous evidence of impaired cognitive flexibility in the male *Fmr1* KO mouse.

Data availability

Dataset 1 – "Learning Trial Data – CSV.csv"

This datasheet contains the raw data exported from the Ethovision program (Columns A – G) as well as the transformed dataset that was used for analysis for the learning trials (Columns J – T). 10.5256/f1000research.14969.d206068[16]

Dataset 2 – "Probe Trial Data – CSV.csv"

This datasheet contains the raw data exported from the Ethovision program (Columns A - AB) as well as the transformed dataset that was used for analysis for the probe trial (Columns AC – AI). 10.5256/f1000research.14969.d206069[17]

Dataset 3 – "Reversal Trial Data – CSV.csv"

This datasheet contains the raw data exported from the Ethovision program (Columns A – G) as well as the transformed dataset that was used for analysis for the reversal trials (Columns J – O). 10.5256/f1000research.14969.d206076[18]

Dataset 4 – "Visible Platform Data – CSV.csv"

This datasheet contains the raw data exported from the Ethovision program (Columns A – H) as well as the transformed dataset that was used for analysis of the visible platform trials (Columns K – S). 10.5256/f1000research.14969.d206077[19]

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

We would like to thank Gregory Smith for his initial training guidance. We would also like to thank Conner Reynolds, Samantha Hodges, Matthew Binder, and Andy Holley for their critical review of the paper.

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16. Nolan SO, Lugo JN: **Dataset 1 in: Reversal learning paradigm reveals deficits in cognitive flexibility in the *Fmr1* knockout male mouse.** *F1000Research.* 2018.
Data Source

17. Nolan SO, Lugo JN: **Dataset 2 in: Reversal learning paradigm reveals deficits in cognitive flexibility in the *Fmr1* knockout male mouse.** *F1000Research.* 2018.
Data Source

18. Nolan SO, Lugo JN: **Dataset 3 in: Reversal learning paradigm reveals deficits in cognitive flexibility in the *Fmr1* knockout male mouse.** *F1000Research.* 2018.
Data Source

19. Nolan SO, Lugo JN: **Dataset 4 in: Reversal learning paradigm reveals deficits in cognitive flexibility in the *Fmr1* knockout male mouse.** *F1000Research.* 2018.
Data Source

Open Peer Review

Current Referee Status: ? ? ✓

Version 1

Referee Report 16 July 2018

doi:10.5256/f1000research.16297.r34793

✓ **Lisa Monteggia**
Department of Neuroscience, University of Texas Southwestern Medical Center, Dallas, TX, USA

The current study examines male Fmr1 knockout mice in the Morris Water maze. This group focuses on the FVB strain as previous work has shown background strain differences can impact the phenotype of Fmr1 knockouts. The authors show that the male Fmr1 knockout mice have no impairment in acquisition of spatial memory but do show differences in the reversal learning paradigm that are not attributed to vision or motor differences. In the reversal learning paradigm, the authors show that during the 4 blocks of reversal learning the Fmr1 KOs show a rather stable latency to find the platform in contrast to the WT mice that show the expected decrease to reach the platform with increasing trials. The authors highlight the finding that male Fmr1 knockout mice have deficits in reversal learning as assessed in the Morris water maze but not in acquisition, demonstrating a link to a specific form of learning in this paradigm. The study is straightforward and the data clearly presented.

A couple of comments the authors should further clarify:
The authors should elaborate on the breeding scheme as they are not directly comparing littermate controls.

Based on the authors discussion point regarding potential sex differences between male and female Fmr1 KO mice, is there data to suggest differences in cognitive flexibility between the males and female KOs?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?

No source data required

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 02 July 2018

doi:10.5256/f1000research.16297.r34795

Andre Fenton
Center for Neural Science, New York University, New York, NY, USA

I am afraid that I did not find this manuscript and the results to be compelling.
The experimental design is straightforward but the analysis is questionable and the report is not as scholarly as it should be.

Statistics are used questionably. 1) t tests are used for post-hoc comparisons, when they are not justified, For example, post-hoc t tests are performed after ANOVA has failed to find relevant differences. 2) Furthermore, the alpha level for post-hoc t tests seem not to be corrected for multiple comparisons. The reported significant difference on trial 3 may not maintain after correcting alpha for multiple comparisons 0.05/4, although it might - a reader can't know. Regardless, what is the justification for the t tests after the ANOVA results were not significant?

Dataset 1: Given no effects of genotype or interaction in the ANOVA, what justifies data snooping as follows: "Further independent samples t-testing revealed no differences between WT and KO at any of the 8 different trials, $p > 0.05$." ?

Dataset 2: Given the ANOVA was not significant for a 1-way ANOVA, there is no justification for a t test and in fact it is numerically equivalent to the F-test, so even more so not warranted.
"Further independent samples t-testing revealed no differences in duration in any of the quadrants, $p > 0.05$."

The manuscript states that "Fmr1 KO mice showed deficits in their ability to learn the new location of the platform, $F_{genotype}(1, 23) = 3.93$, $p = 0.059$." Why is $p = 0.059$ indicative of a deficit?

The Introduction is written as if the water maze is the only way to test spatial memory and cognitive flexibility, when of course it is not.

The Discussion states "The current study did, however, demonstrate impairments in cognitive flexibility in the Fmr1 KO. As previously mentioned, other studies have not before detected such changes"
Remarkably, the authors fail to consider the work from the Fenton lab which explicitly has investigated cognitive flexibility in Fmr1 KO mice (Radwan et al., 2015 Neurobiol. Dis.; Dvorak et al., 2018, PLoS Biol[1].) along with electrophysiological correlates of the ability.

Something is wrong with the initial statement: "Male Fmr1+/+ and female Fmr1+/- FVB.129P2-Pde6b+Tyrc-ch Fmr1tm1Cgr/J (Stock No: 004624, The Jackson Laboratory, Bar Harbor, ME, USA) mice were used as breeders (9 total breeding pairs) to produce the following groups:" because WT mice cannot contribute to breeding involving a KO male.

Fig 1B should have the days indicated, as well as the rest period.

Fig. 2C shows poor learning even in WT, despite the significant effect of trial. Could the paradigm simply not be robust in mice? There is a literature on this possibility, see Wolfer et al., 1998 News Physiol Sci[2].).

What justification is there for the assertion that "Moreover, this ability to adapt to a new location is mediated through multi-synaptic connections between the hippocampus and the prefrontal cortex13."? What is the mechanism that is referenced by the statement "This proposed mechanism further supports our findings of no change to the initial spatial learning phase, as lesions to this area did not impact initial learning performance in spatial navigation11."?

The final statement seems untrue given that the authors claim to find a reversal deficit but say there had not been one detected previously "Overall, this study corroborates and extends previous evidence of impaired cognitive flexibility in the male Fmr1 KO mouse."

What is "t-testing" should this not simply be stated as "t tests" ?

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Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 20 June 2018

doi:10.5256/f1000research.16297.r34794

? **Laura Smith**
Department of Neuroscience & Experimental Therapeutics, Texas A&M Health Science Center, Bryan, TX, USA

The study authored by Nolan and Lugo investigates the ability of *Fmr1* KO mice on the FVB.129 background to demonstrate proper reversal learning in the Morris water maze (MWM). Background strain appears to make a large difference in observed cognitive deficits in mice lacking expression of the *Fmr1* gene, and this study makes a valuable contribution to the literature by assessing MWM reversal learning in *Fmr1* KO mice on the pure FVB background.

Suggested revisions:

1. The authors refer to papers showing impaired reversal learning in *Fmr1* KO mice on the *C57BL/6J* strain and cite D'Hooge et al., 1997[1] and Kooy et al., 1996[2] However, in each of these studies the authors specify that only pigmented offspring were selected for testing, suggesting that these mouse lines were not fully backcrossed to C57BL/6J. Of note, stem cells used in the creation of transgenic mice have often been derived from the 129 strain. In any case, the authors should likely revisit this statement, and instead may wish to report Paradee et al. (1999)[3] which used fully backcrossed C57BL/6 KO mice.
2. The statement that "few studies have examined cognitive flexibility in a reversal form of the MWM task" (presumably in *Fmr1* KO mice) seems inaccurate, as most studies using MWM to assess *Fmr1* KO mice actually include this task (Paradee et al., 1999; Kooy et al., 1996; D'Hooge et al., 1997; The Dutch-Belgian Fragile X Consortium paper, 1994[4]; Baker et al., 2010)[5]. However, as the authors state, it had not been properly assessed on the FVB background.
3. The abstract conclusion says that "previous studies have not demonstrated deficits in spatial memory in the *Fmr1* KO model." However, multiple previous assessments of *Fmr1* KO mice in the MWM have shown minor acquisition differences compared to WT mice (e.g., Paradee et al., 1999; Kooy et al., 1996, *Am J Med Genet*; The Dutch-Belgian Fragile X Consortium paper, 1994). Please consider these and also deficits observed in the plus-shaped water maze (e.g., Dobkin et al., 2000[6]; Van Dam et al., 2000[7]) and radial arm maze (Mineur et al., 2002[8]) to perhaps soften this statement appropriately.
4. Consider changing "disability" to "disabilities" in first sentence of the abstract.
5. Please add more detail about the Morris water maze task. For instance, did mice receive consecutive trials within each block? Was entry quadrant varied or static over each mouse's trials within (and across) blocks? Was the temperature of the water controlled? What were the visual

cues, where were they positioned with respect to each quadrant, and were they proximal or distal? If animals were artificially colored for detection in the maze, please specify. Were animals dried and/or warmed between or after trials?

6. It may be helpful to label days on Fig. 1B.
7. In the first Results paragraph-- in the absence of a significant main effect or interaction involving genotype, further independent samples t-testing between WT and KO should probably not be reported.
8. In the Discussion, the authors point to deficits in *Fmr1* KO prefrontal cortex LTP with regard to the observed reversal learning deficit, which is fine. However, using this same logic to claim support for *lack* of observed differences in acquisition, based on findings that PFC is not relevant to acquisition, is somewhat flawed. It may be more appropriate (e.g., in the sentence regarding PFC and learning a new location) to simply acknowledge that "PFC is not required for acquisition" and allow the reader to intuit the point (that seems to be intended) more subtly. If this topic will be discussed, other brain regions likely involved in MWM acquisition (e.g., hippocampus), and that also show significant plasticity-related variations in *Fmr1* KO compared to WT mice, should be included.
9. The authors' point that this investigation should be made in females is well-received. However, it is not immediately clear how these data in male *Fmr1* KO mice provide "preliminary evidence that could be applied to tease apart subtle differences between the male and female *Fmr1* KO phenotype." Perhaps this point can be further clarified or removed?
10. In the attached data sets, it is not clear why a few animal IDs are repeated. Specifically, please see the "Learning trial" set, and the "transformed" data on the right-hand side of the screen. Subjects 124 and 134 are listed twice, but all other IDs appear once. Also, a little more explanation, either directly on the data sheets or elsewhere in the manuscript, for the trial names, etc. seen here may be beneficial to users of these data.

References

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2. Kooy R, D'Hooge R, Reyniers E, Bakker C, Nagels G, De Boulle K, Storm K, Clincke G, De Deyn P, Oostra B, Willems P: Transgenic mouse model for the fragile X syndrome. *American Journal of Medical Genetics*. 1996; **64** (2): 241-245 <241::AID-AJMG1>3.0.CO;2-X">Publisher Full Text
3. Paradee W, Melikian HE, Rasmussen DL, Kenneson A, Conn PJ, Warren ST: Fragile X mouse: strain effects of knockout phenotype and evidence suggesting deficient amygdala function.*Neuroscience*. 1999; **94** (1): 185-92 PubMed Abstract
4. Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium.*Cell*. 1994; **78** (1): 23-33 PubMed Abstract
5. Baker KB, Wray SP, Ritter R, Mason S, Lanthorn TH, Savelieva KV: Male and female Fmr1 knockout mice on C57 albino background exhibit spatial learning and memory impairments.*Genes Brain Behav*. 2010; **9** (6): 562-74 PubMed Abstract | Publisher Full Text
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7. Van Dam D, D'Hooge R, Hauben E, Reyniers E, Gantois I, Bakker CE, Oostra BA, Kooy RF, De Deyn PP: Spatial learning, contextual fear conditioning and conditioned emotional response in Fmr1 knockout mice.*Behav Brain Res*. 2000; **117** (1-2): 127-36 PubMed Abstract
8. Mineur YS, Sluyter F, de Wit S, Oostra BA, Crusio WE: Behavioral and neuroanatomical

characterization of the Fmr1 knockout mouse.*Hippocampus*. 2002; **12** (1): 39-46 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Referee Expertise: fragile X; autism; addiction; dendritic structure and plasticity

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.