The Known Unknowns: Missing Pieces in *in vivo* Models of Fragile X Syndrome

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ABSTRACT

Fragile X Syndrome (FXS) is a rare disease and the leading monogenic cause of Autism Spectrum Disorders (ASD). It is caused by the silencing of the Fragile X mental retardation (*FMR1*) gene and the subsequent reduction or loss of fragile X mental retardation protein (FMRP). The clinical effects seen in FXS patients are several and highly variable making it difficult to model them in a single model or even one organism. Furthermore, several human behaviours can be measured only through surrogate endpoints in animals. Therefore, it has been challenging to develop *in vivo* models of FXS for drug discovery.

This review endeavours to consolidate the information on all available *in vivo* models for FXS specifically with a focus on their suitability for drug development, with the objective of identifying gaps and potential solutions. To do so, we have summarised the major clinical characteristics and possible mechanisms underlying clinical phenotypes associated with FXS and other disorders that arise from abnormalities in the *FMR1* locus, such as fragile-X associated tremor/ataxia syndrome (FXTAS), fragile-X-associated neuropsychiatric disorders (FXAND) including ASD and fragile x-associated primary ovarian insufficiency (FXPOI). We then connect clinical features to phenotypes observed in available *in vivo* FXS models where possible, covering a wide range of organisms from primates, rats, mice, zebrafish, fruit fly and zebra finches. For each model organism, we list the technology of model creation, phenotypes/assays, mechanistic basis of disease manifestation and specific advantages or disadvantages of the model in the context of drug discovery.

Finally, we have highlighted the missing pieces in FXS modelling and propose strategies to address them, considering aspects of modelling spectrum disorders, repeat expansion and silencing, new functions of FMRP and identification of efficacious treatments.

Figure: Missing pieces in modelling of Fragile X Syndrome
Introduction

Fragile X Syndrome (FXS) is one of the most studied monogenic neurological syndromes over the past few decades. It is caused by silencing of the Fragile X mental retardation (FMR1) gene and the subsequent reduction or loss of FMR protein (FMRP). CGG repeat expansion in the 5'UTR of the FMR1 gene, followed by hypermethylation of the region is the basis of the observed silencing in FXS\(^1\). This event occurs sequentially in successive generations, beginning with small repeat expansion (55-200) causing pre-mutation in one generation with a toxic gain of function in the mRNA, followed by further expansion (>200) to full mutation in subsequent generations with complete silencing of the gene\(^2\). FMRP is an RNA binding protein\(^3\), a well-known regulator of translation, and is known to interact with well over 800mRNAs in the adult neuron\(^4\). Even though several of the mRNA targets have not been fully characterised, it can be said that, FMRP loss has a cascading effect on several pathways which result in the observed clinical features\(^5\,6\). Several model organisms have been used to model this disease, as discussed extensively in the following tables. The major challenges in modelling FXS are listed here (and substantiated in the rest of the review article):

a) The clinical presentation of the disease in humans is highly variable with different individuals showing different sets of clinical phenotypes.

b) Similar to other neurological diseases, the biochemical and pathological profiling of FXS in real time is practically impossible. This makes it extremely difficult to decipher the human pathobiology making it necessary to have in vivo models.

c) The human nervous system is the most evolved and mimicking it in lower mammals and other vertebrates is done only by using surrogate endpoints especially for cognitive and social behaviours.

d) Animal models show high variability in measurable phenotypes.

Therefore, modelling a complex neurological syndrome like FXS is a continuing process and will require a set of organisms to model all the clinical characteristics, to study various pathobiological aspects and to screen potential drug candidates.

Modelling most diseases and syndromes is necessary in disciplines of disease biology and drug discovery; however, given FXS's monogenic aetiology, these models can be potentially studied for understanding several other aspects including (but not limited to):

a) Pathways of development of various cognitive abilities

b) Specific connective tissue organogenesis

c) Pathways of behaviours like anxiety, depression, irritability, others.

Thus, modelling FXS and improving the models can have great value to the field of neurosciences. In the present review, we discuss clinical presentations and possible mechanisms, various in vivo models and their advantages and disadvantages, and propose the next set of models and methods that can be used to plug in the missing pieces.

Clinical characteristics of Fragile X Syndrome and the suggested mechanisms

Clinical characteristics that are most commonly noticed in patients with FXS and the suggested mechanisms have been presented below in Table 1.

In vivo models of Fragile X Syndrome

Various in vivo models of FXS have been described in Table 2. We have also provided the potential advantages and disadvantages of each of these models.

Missing pieces in modelling of FXS and potential solutions

Modelling spectrum disorders

Fragile X syndrome is the leading genetic cause of autism, and because of the implication of a single causal gene, animal models of FXS are numerous\(^26\). The characteristic clinical manifestation of FXS involves some or all of the following symptoms: long face, macrocephaly, prominent ears, prominent jaw, flat feet, joint hypermobility, macroorchidism (clinical); attention-deficit hyperactivity disorder (ADHD), anxiety autism spectrum disorder (ASD) (psychological), developmental intellectual disability, language deficits (developmental) and strabismus, recurrent otitis, gastrointestinal complaints, obesity and seizures (less prevalent)\(^44\). While the various models capture a subset of these phenotypes (see table 2 above), no single model has been able to mimic the spectrum of symptoms and deficits seen in human FXS patients, and this has severely impacted screening and drug discovery efforts. There could be two potential reasons for this:

i) The first is that the presence, severity and manifestation of FXS symptoms varies widely even in human patients\(^44\), and is likely influenced strongly by the genetic background and environmental factors. Therefore, one can argue that inconsistencies are expected in the models as well.

ii) The second stems from the nature and function of the FMR protein. FMRP is a regulator of translation and is expressed from very early on during development, which means that when FMRP is silenced, the levels of a number of proteins (several hundred in
### Table 1: Common clinical characteristics and their suggested mechanisms

| Clinical Presentations | Suggested Mechanisms of Pathobiology | Reference/s |
|------------------------|--------------------------------------|--------------|
| **Joint Hypermobility** | Dysregulation of excitatory and inhibitory neurotransmission by: a) mGluR1 mGluR5 (glutamatergic) enhanced signal transduction b) Deficits in GABA signalling Indirect glutamatergic mechanisms that modulate mGluR: a) Dysregulation of N-methyl-D-aspartate receptor (NMDAR) b) Altered expression, trafficking, and functions of Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPA-Rs) | [5]–[8] |
| **Flat Feet** | Excess mRNA from FMR1 premutation may lead to FXTAS in the progeny through the following mechanisms: a) Toxic FMR polyG protein production b) Formation of ubiquitin-positive inclusion bodies through protein & RNA sequestration c) DNA damage due to R-loop formation Its predicted that CCG repeats may lead to sequestration of specific RNA-binding proteins such as lamin A/C, PurA, hnRNP, Sam68, and drosha, thus affecting some of the normal cell function in both FXTAS and FXPOI. | [9], [10] |
| **Prominent Jaw** | Macroorchidism, alterations in dendritic spines; impaired associative learning and memory. | | |
| **Prominent Ears** | Loss of FMRP, altered translation profile and development of prefrontal cortex circuitry, altered mGluR5 signalling | [18]–[21] |
| **Long Face** | Loss of FMRP can lead to dysregulation of the following extracellular matrix and connective tissue components: a) Elastin downregulation b) Bone morphogenetic protein receptor 2 (BMPR2) downregulation c) Matrix metalloproteinases (MMPs) upregulation | [15], [16] |
| **Macrocephaly** | FMRP loss-related dendritic abnormalities due to the following: a) FMRP2-Cofilin pathway disruption b) Matrix metalloproteinase 9 (MMP9) upregulation c) Excess soluble amyloid precursor protein (APP) levels | [5], [13], [14] |

### Table 2: Description of various in vivo models of FXS and their advantages and disadvantages

| Species, Strain, Model | Technology used to create model | Phenotypes | Mechanisms | Reference/s | Advantages | Disadvantages |
|------------------------|--------------------------------|------------|------------|-------------|------------|---------------|
| **Non-human primate, Macaca mulatta, Spontaneous** | None, analysis from a population of macaque | Not measured | Not evaluated. Sequencing of FMR alleles carried out to assess CAG/CGG repeat distribution | [17] | CGG repeat distribution and propensity for expansion most similar to that in humans; Model most suited for behaviour studies | Instance of FXS was not observed; No further studies have been reported using macaques |
| **Rat** | Zinc-finger-nuclease mediated knockout of Fmr1 (exon 8) in SD or LEH background | Macroorchidism, alterations in density of dendritic spines; impaired associative learning and memory. | Loss of FMRP, altered translation profile and development of prefrontal cortex circuitry, altered mGluR5 signalling | [18]–[21] | Allows: Repeated, longitudinal testing of recognition memory paradigms during development; Identification of suitable treatment window for therapeutics; Differentiation between preventive and corrective MoA of a given drug | Rat models do not offer significant advantages over murine models with regard to behavioural phenotypes of FXS. Genetic background contributes significantly to variability in phenotypes |
| **LEH, Fmr1<sup>1st</sup>LEH<sup>F3</sup>** | Zinc-finger-nuclease mediated knockout of Fmr1 in SD or LEH background | Macroorchidism, altered LTD LTD, spatial learning and memory; impaired social interaction (novelty recognition) | Loss of FMRP, altered translation and synaptic plasticity | [22] | Clear evidence of deficits in hippocampus-dependent spatial learning and memory (unlike murine models) | |
| **Sprague Dawley, Fmr1 exon4 KO** | CRISPR-Cas9, exon 4 mutation leading to truncation | Macroorchidism, altered LTD LTD, spatial learning and memory; impaired social interaction (novelty recognition) | Loss of FMRP, altered translation and synaptic plasticity | | | |
| Mouse                                                                 | Knock-down (transient) | Homologous recombination with disruption in exon 5 | Macroroorchidism; Mild facial dysmorphism; Susceptibility to seizures; Neuroanatomical as well as functional brain abnormalities (reduced dendritic spine density, cognitive and behavioural deficits, altered LTD and LTP) | Loss of FMRP, altered translation profile and altered mGluR5 signalling [23]–[25] and others, summarized in [26] | Most commonly used model; Very well studied and shows most of the clinical phenotypes. | Residual Fmr1 mRNA present, repeat expansion and promoter methylation absent; Behavioural and cognitive deficits are mild, social behaviour modelling difficult; Genetic background contributes significantly to variability in phenotypes. |
|---------------------------------------------------------------------|------------------------|--------------------------------------------------|------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|
| Conditional KOs: Sighted FVB or C57BL/6J, Fmr1 KO CON, CKO, KO2   | Cre-lox recombination with disruptions in exon/intron 1 | Phenocopies KO models                            | Missense mutation in the KH domain of FMRP prevents RNA as well as ribosome binding, leads to reduced FMRP levels. |
|                                                                     |                        |                                                  | Additional model with a different genotype, same phenotype, for drug discovery; Absence of the neomycin cassette (present in the KOs) provides a minimally altered genome/transcriptome [28] | [27] Allows tissue or brain region specific function of FMRP (such as in the hippocampus, CA1 neurons etc) as well as temporal regulation (as yet unreported). | Not very widely used, offers no specific advantages over the KO models for general behavioural tests. | Models a very rarely observed patient mutation. Offers no significant advantages over the KO models. |
| Rescue models: C57BL6/J Fmr1 KO Tg26, Fmr1 Tg26                 | YAC transgenesis of human FMR1 alleles with 20/92 repeats, into WT | Overexpression of FMRP (~10 fold over endogenous); Presence of multiple copies of a transgene. |
|                                                                     |                        |                                                  | [29] Reveals phenotypes associated with FMRP overexpression; Useful to study gene therapy and titrate expression for optimal phenotypic rescue; Provided evidence for rescue of mouse phenotype by human FMRP. | [30] Rescue of FMRP deficit, with overexpression of FMRP; Small repeat expansions upon transmission. | No major repeat expansion observed; Only partial rescue of phenotypes observed, mechanism unclear. | No major repeat expansion observed; Only partial rescue of phenotypes observed, mechanism unclear. |
| Knok In/Repeat Expansion C57BL/6J or FVB/N, Fmr1 Tg26                     | Homologous recombination | Impaired locomotion, altered anxiety, spatial memory and learning deficits | Moderate repeat expansion and instability, leading to reduced FMRP and increased Fmr1 mRNA, intranuclear inclusions in the neurons and astrocytes | Summarized in [31] Only model to study mechanisms of pathogenic repeat expansion similar to that observed in humans with FXS premutation | Repeat expansion is not accompanied by promoter methylation and silencing, even though FMRP level is reduced; Not useful for screening methylation-modulation therapies. | Morpholino based induction of artefacts may confound some findings; No behavioural studies. |
| Zebrafish                                                           | Morpholino                  | Morphological changes in the brain, altered neurite morphology, neural crest specification defects | Reduction in Fmrp, increased mGluR5, perturbed calcium distribution and signalling [32] | [33]–[35], Loss of function model illustrates possible mechanism. Demonstrates social deficits. | [33]–[35], Loss of function model illustrates possible mechanism. Demonstrates social deficits. | [33]–[35], Loss of function model illustrates possible mechanism. Demonstrates social deficits. |

**Glossary**

- **fmr1** (stop): marginal defects, especially in anxiety and locomotion, observed in Fmr1 knock out. 
- **fmr1** (splice): premature truncation of Fmrp, resulting in increased mGluR5 expression, contributing to the phenotype. 
- **fmr1** knock out: the loss of Fmr1 results in severe phenotypic abnormalities, including brain abnormalities, altered neurite morphology, and cognitive deficits. 
- **fmr1** knock down: reduction in Fmr1 expression through morpholino injection, resulting in the loss of Fmrp and increased mGluR5 expression. 
- **fmr1** splice: altered splicing of Fmr1 mRNA, leading to the production of a truncated Fmrp protein. 
- **fmr1** repeat expansions: the expansion of CGG repeats in the Fmr1 gene, leading to the production of a truncated Fmrp protein. 
- **fmr1** knockout mimics the phenotype of FXS. 
- **fmr1** knock down provides a model for studying the effects of Fmrp depletion. 
- **fmr1** splice provides a model for studying the effects of altered splicing. 
- **fmr1** repeat expansions are thought to play a role in the pathogenesis of FXS. 

**References**

[1] Kulkarni P, Sevilimedu A. The Known Unknowns: Missing Pieces in in vivo Models of Fragile X Syndrome. J Rare Dis Res Treat. (2020) 5(1): 1-9.
an adult neuron, for example), and consequently a number of signalling pathways are likely to be altered, and differentially so at various stages during development\textsuperscript{45}. The phenotypes observed are a result of these cumulative changes. Even though FMRP \textit{per se} is fairly well conserved in all of the models, varying degrees of conservation across the target proteins, pathways and differentiation paradigms are likely to cause inconsistencies in phenotypes observed in the models. Restricting discovery programs to one model organism, or picking a single genetic background in a model, is unlikely to be beneficial, since heterogeneity is a feature of the disease, not the model. Therefore, we would like to propose that diversification, both in terms of model organisms and genetic backgrounds may be key to identifying robust and efficacious therapeutic interventions. Focussing on simpler genetic models like Zebrafish and Drosophila, may allow one to conduct high throughput screens in a genetically diverse population, and generate statistically significant results.

### Repeat expansion and silencing

In FXS, a repeat expansion in the 5’ UTR of the human \textit{FMR1} gene leads to hypermethylation and silencing, which results in a drastic reduction of FMRP. Therefore, control at the level of repeat expansion or methylation are the best therapeutic avenues. In humans, the \textit{FMR1} locus naturally contains 6-55 CGG repeats; however, all of the model organisms (except the primate) contain very few repeats, if any. The mechanism of repeat expansion involves different organisms (except the primate) contain very few repeats, if any. The mechanism of repeat expansion involves different stages in which the premutation stage (55-200 repeats) is associated with an increase in \textit{FMR1} RNA levels (toxic gain of function), which may be a prerequisite for the progression to full expansion (>200 repeats) leading to hypermethylation and subsequent silencing. Therefore, lack of a critical number of repeats at the outset, combined
with a lack of progressive expansion could explain why the knock-in models do not show any promoter methylation-dependent silencing. With a carefully chosen combination of artificial repeat insertion, control of FMR1 RNA levels and manipulation of the DNA replication or repair pathways to promote slippage, it may be possible to develop such a model. We believe that such studies can be conducted in simpler models like yeast, used to derive optimal conditions which may then be moved into higher models like Zebrafish and mouse, to create true FXS models. Interventions which aim to interfere with repeat expansion, or those that revert the hypermethylation-driven silencing (small molecules or genetic interventions) can then be screened in such a model, and will either significantly ameliorate the disease (even a two-fold increase in FMRP level is associated with a significantly higher IQ) or better still, prevent it.

**New functions of FMRP**

Since FXS is primarily seen as a disease of the brain, studies have focussed on FMRP’s neuronal function and neuronal phenotypes in the models. FMRP is ubiquitously expressed during early developmental stages, and it is increasingly clear that FMRP interacts with and regulates diverse cellular pathways in addition to its primary function as a translation regulator in neurons. These include regulation of RNA editing and splicing, chromatin structure, cellular differentiation kinetics, ion channel regulation and microRNA pathways. Therefore, exploring the molecular mechanisms underlying the contribution of these pathways to disease progression or phenotypes could be an important new avenue of research. Traditional translation targets of FMRP may also be influenced by disruptions in these other pathways (for example, AMPA receptor and RNA editing, microRNA and ion channels) and may be better rescued by novel treatments or combination therapies.

**Identifying efficacious treatments for FXS - moving the needle on the preclinical side**

While molecular mechanisms of FXS are fairly well understood, and small molecules targeting at least ten different pathways are able to rescue several molecular phenotypes such as protein synthesis, synaptic plasticity and calcium regulation, none of these have translated into clinical efficacy in humans. While one obvious explanation is that this is due to the complexity of, and our lack of understanding of the human system, there are alternative explanations which require due consideration. An important take-away from the clinical trials conducted so far, is that tests which measure core phenotypes like behaviour and cognition directly and not through surrogate indications, need to be developed in order to determine the true efficacy of drug treatment. However, there are many avenues for improvement on the preclinical side.

(i) First, newer models which capture the repeat expansion and methylation features should be developed, since targeting these upstream nodes will result in maximal impact (section above).

(ii) Second, varied genetic backgrounds and multiple model organisms should be employed in order to determine the robustness of the phenotypes or treatment being assessed. The molecular phenotypes are highly conserved from flies to human, and the small molecules being considered for clinical trials have been identified based on these conserved pathways. It may be prudent therefore to conduct such studies in models like *Danio rerio* (Zebrafish) where true “wild type” animals (wild caught) can be used (incorporating the genetic diversity present in the natural world), with as large a sample size as required to power the statistics. Such an approach may allow one to incorporate the varied baselines in the population (for example, the median increase in anxiety in wild-caught vs. lab-wild type strains) and multi-factorial influences on the neurological phenotypes during the screening process to make results more robust. Techniques for simple, rapid and inexpensive model creation, such as transient knockdown of gene expression (using DNAzymes or morpholinos in zebrafish, RNAi in *D. melanogaster*), and differentiation of patient derived cell lines will also aid in increasing the number of varied models available to assess the same phenotype and its treatment. A larger number of treatments (compounds and paradigms) can be tested in a more diverse set of assays in such models, and may drastically improve the chances of finding a drug that will translate well into humans compared to traditional approaches using the mouse model.

(iii) Third, multiple tests or assays which measure the same parameter should be employed in each study, and at least one of these should measure the same parameter as in a clinical trial (such as fMRI or EEG).

(iv) Fourth, drug screening should be conducted in models where the link between the molecular changes due to FMRP loss, to circuitry and behaviour are well-established (such as in olfactory system of *D. melanogaster*), and rescue at each stage should be assessed.

(v) Fifth, a number of treatment windows may need to be explored for each class of drug, coupled with longitudinal studies which measure impact over the long term, especially for treatments targeting behaviour. Given that the circuitry can be modulated
only in certain windows during development, earlier treatment windows need to be preferentially identified and studied.

(vi) Finally, FMRP appears to play a role in multiple unconnected pathways, therefore genetic studies in models like flies and zebrafish could be used to better understand these new molecular functions of FMRP. Subsequently, combination treatments to address more than a single target at a time may be prioritized for screening.

Disruptive platforms and niche models

Given that decades of therapeutic research using the available models of FXS have not led to the identification of a clinically efficacious drug, the possibility that the molecular landscape and regulatory network in the human brain is not sufficiently or completely replicated in any other model, has to be considered for the next phase of therapeutic research. In such a scenario, critical and validated endophenotypes may need to be used as a basis for screening directly in a “human” model. Brain organoids satisfy the need for “human origin” as well as provide the genetic, cellular and architectural features of the human brain and could therefore be a powerful platform for identification of critical endophenotypes as well as for screening. Brain organoids from patient derived cells (hiPSCs) have been used to study varied diseases of the central nervous system, and are likely to be relevant to FXS, where phenotypes are thought to stem from defective cross-talk between multiple cell types and altered neural circuitry. However, the current state of this technology in terms of the time, skill and expense involved, as well as the inability to sustain organoids in culture to model adult-phenotypes limits its applicability to drug discovery, as on date. Yet another strategy to conduct studies on human origin brain tissue, in an in vivo setting is the clever use of transplanted FXS brain tissue (iPSCs which differentiate after transplantation or neural precursor cells (NPCs)) into the mouse brain. While the chimeric setting has the same limitations as the mouse model in terms of screening for behavioural or cognitive end points, it is likely to reveal the most authentic, cell-type and microenvironment specific responses to drugs.

Conclusions and future outlook

Fragile X syndrome is a classic test case for a rare disease model, which despite the availability of numerous models and studies over the decades, has not yielded any therapeutic benefits for patients. We believe that part of the reason for this has been the use of approaches which were developed and standardized on the basis of what has worked for the more prevalent, non-monogenic diseases that have dominated the clinical research and drug development fields. In the case of drug development for rare monogenic disorders where disease manifestation is heavily influenced by the underlying genetic background and treatment needs are primarily symptomatic, radically different approaches like the ones described above may be more fruitful. Implementation of such strategies and the consequent identification of efficacious treatments may cause a paradigm shift in the way rare disease biology, modelling and drug development is practiced in the future.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Intellectual Property Statement: The method for creating zebrafish knock down models using DNAzymes is the intellectual property of Dr. Reddy’s Institute of Life Sciences and is covered by the following patent: Embryonic Zebrafish models using DNAzyme mediated knockdown. Sevilimedu A, Kulkarni P.Application No. PCT/IB2019/051255, Indian Application No. 01741031477. Complete specification filed for Dr. Reddy’s Institute of Life Sciences. The authors are affiliated to Dr. Reddy’s Institute of Life Sciences.

References

1. Oberlé I, Rousseau F, Heitz D, et al. "Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome," Science. 1991; 252(5009): 1097–1102, doi: 10.1126/science.252.5009.1097.

2. Fu YH, Kuhl DP, Pizzuti A, et al., "Variation of the GGG repeat at the fragile X site results in genetic instability: Resolution of the Sherman paradox," Cell. Dec 1991; 67(6): 047–1058, doi: 10.1016/0092-8674(91)90283-5.

3. Ashley CT, Wilkinson KD, Reines D, et al., "FMR1 protein: Conserved RNP family domains and selective RNA binding," Science. 1993; 262(5153): 583–586, doi: 10.1126/science.7692601.

4. Darneil JC, Driesche SJV, Zhang C, et al., "FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism," Cell. 2011; 146(2): 247–261, doi: 10.1016/j.cell.2011.06.013.

5. Sakoło-Arellano MJ, Dafour B, McLennan Y, et al. “Fragile X syndrome and associated disorders: Clinical aspects and pathology,” Neurobiology of Disease. Mar 01, 2020; 136. Academic Press Inc p. 104740, doi: 10.1016/j.nbd.2020.104740.

6. Hagerman RJ, Berry-Kravis E, Hazlett HC, et al. “Fragile X syndrome,” Nature reviews. Disease primers. Sep 29, 2017; 3(1). Nature Publishing Group. p. 17065, doi: 10.1038/nrdp.2017.65.

7. Hugger Toerb AK, Lundbye CJ, Banke TG. "Disregulated NMDA-receptor signaling inhibits long-term depression in a mouse model of Fragile X syndrome," J Neurosci. Sep 2016; 36(38): 9817–9827, doi: 10.1523/JNEUROSCI.3038-15.2016.
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8. Cheng GR, Xiang YD, Liu D, et al. "The Implication of AMPA Receptor in Synaptic Plasticity Impairment and Intellectual Disability in Fragile X Syndrome." Physiol. Res. 2017; 66(715–727), doi: 10.33549/physiolres.933473.

9. Giulivi C, Napoli E, Tassone F, et al. "Plasma metabolic profile delineates roles for neurodegeneration, pro-inflammatory damage and mitochondrial dysfunction in the FMR1 premutation." Biochim. Biophys. Acta. 2016; 1864(21): 3871–3880, doi: 10.1016/j.bbadis.2016.05.085.

10. Elzeur SE, Drahovzyl-Stombovska O, Derech-Haim S, et al. "FMR6 may play a role in the pathogenesis of fragile X-associated premature ovarian insufficiency." Gynecol. Endocrinol. Apr 2016; 32(4): 334–337, doi: 10.3109/09513550.2015.116508.

11. Fink DA, Nelson LM, Pyeritz R, et al. "Fragile X Associated Primary Ovarian Insufficiency (FXPOI): Case Report and Literature Review." Front. Genet. Nov 2018; 9: 529, doi: 10.3389/fgen.2018.00529.

12. Man L, Lekovich J, Rosenwalks Z, et al. "Fragile X-associated diminished ovarian reserve and primary ovarian insufficiency from molecular mechanisms to clinical manifestations." Frontiers in Molecular Neuroscience. Sep 12 2017; 10 Frontiers Media SA, doi: 10.3389/fnmol.2017.00290.

13. Pasciuto E, Ahmed T, Wahle T, et al. "Dysregulated ADAM10-Mediated Processing of APP during a Critical Time Window Leads to Synaptic Deficits in Fragile X Syndrome." Neuron. Jul 2015; 87(2): 82–398, doi: 10.1016/j.neuron.2015.06.032.

14. Bilousova TV, Dansie L, Davidian, et al. "PRENATAL PATHOGENESIS OF MACROORCHIDISM IN THE FRAGILE X SYNDROME." Pediatr. Res. Apr 1987; 21(4): 230A-230A, doi: 10.1203/00006450-198704010-00383.

15. I. R. Shapiro, P. L Wilmot, R. A. O’Meara, M. M. Davidian, and P. N. Chandler, “PRENATAL PATHOGENESIS OF MACROORCHIDISM IN THE FRAGILE X SYNDROME," Pediatr. Res. vol. 21, no. 4, pp. 230A-230A, Apr. 1987, doi: 10.1203/00006450-198704010-00383.

16. Ramírez-Cheny JA, Duque GA, Ayala-Zapata S, et al. "Fragile X syndrome and connective tissue dysregulation," Clin Genet. Feb. 2019; 95(2): 262–267, doi: 10.1111/cge.13469.

17. Ancena DG, Breece KE, Hagerman PJ. "Distribution of CGG repeat sizes within the fragile X mental retardation 1 (FMR1) homologue in a non-human primate population," Hum Genet. 2003; 113(5): 371–376, doi: 10.1007/s00439-003-4982-9.

18. Hamilton SM, Green JR, Veeraragavan S, et al. "Fmr1 and Nlgn3 knockout rats: Novel tools for investigating autism spectrum disorders," Behav. Neurosci. Apr 2014; 128(2): 103–109, doi: 10.1037/a0035988.

19. TILL SM, Asiminas A, Jackson AD, et al. "Conserved hippocampal cellular pathophysiology but distinct behavioural deficits in a new rat model of FXS," Hum Mol Genet. 2015; 24(21): 5977–5984, doi: 10.1039/h3mg22994.

20. Berzhanskaya J, Phillipa MS, Gorin A, et al. "Disrupted Cortical State Regulation in a Rat Model of Fragile X Syndrome," 2016, doi: 10.1039/c5cc03331.

21. Asiminas A, Jackson AD, Louros SR, et al. "Sustained correction of associative learning deficits after brief, early treatment in a rat model of Fragile X Syndrome," Sci Transl Med. 2019; 11(494): 1–11, doi: 10.1126/scitranslmed.aao4998.

22. Tian Y, Yang C, Shang S, et al. "Loss of FMRP impaired hippocampal long-term plasticity and spatial learning in rats," Front Mol Neurosci. Aug 2017; 10, doi: 10.3389/fnmol.2017.00269.

23. The Dutch-Belgian Fragile X Consortium, "Fmr1 Knockout Mice: A Model to Study Fragile X Mental Retardation," 1994.

24. Yan QL, Rammal M, Tranfaglia M, et al "Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP," Neuropharmacology. Dec 2005; 49(7): 1053–1066, doi: 10.1016/j.neuropharm.2005.06.004.

25. Dobkin C, Rabe A, Dumas R, et al. "Fmr1 knockout mouse has a distinctive strain-specific learning impairment," Neurosci. Sep 2000; 100(2): 423–429, doi: 10.1016/S0306-4522(00)00292-X.

26. Kooy RF, Jin P, Bao H, et al. "Animal models of fragile X syndrome," in Fragile X Syndrome: From Genetics to Targeted Treatment Rob Willemsen and R Frank Kooy Ed. Elsevier Inc. 2017; 123–147.

27. Mientes EJ, Nieuwenhuizen I, Kirkpatrick L, et al. "The generation of a conditional Fmr1 knock out mouse model to study Fmrp function in vivo," Neurobiol. Dis. Mar 2006; 21(3): 549–555, doi: 10.1016/j.nbd.2005.08.019.

28. Zang JB, Nosyreva ED, Spencer GM, et al. "A Mouse Model of the Human Fragile X Syndrome I304N Mutation," PLoS Genet. Dec 2009; 5(12): e1000758, doi: 10.1371/journal.pgen.1000758.

29. Peier AM, "(Over)correction of FMR1 deficiency with YAC transgenic mice," Genomics. 2002; 80(4): 423–432, doi: 10.1016/geno.2002.06.001.

30. Peier AM, Nelson DL, "Instability of a premutation-sized CGG repeat in FMR1 YAC transgenic mice," Genomics. 2002; 80(4): 423–432, doi: 10.1006/geno.2002.06.001.

31. Berman RF, Buijsen RA, Uddin K, et al. "Mouse models of the fragile X premutation and fragile X-associated tremor/ataxia syndrome," Journal of Neurodevelopmental Disorders. Jan 30 2014; 6(1): 1–16, Springer New York LLC, doi: 10.1186/1866-1955-6-25.

32. Tucker B, Richards RI, Lardelli M. "Contribution of mGluR and Fmr1 functional pathways to neurite morphogenesis, craniofacial development and fragile X syndrome," 2006; 15(23): 3446–3458, doi: 10.1093/hmg/dd422.

33. den Broeder MJ, van der Linde H, Brouwer JR, et al. "Generation and characterization of FMR1 knockout zebrafish," Hum Mol Genet. 2020; 0123456789, doi: 10.1007/s10519-020-09995-7.

34. Wu YJ, Hsu MT, Lu KT. "Behavioral and synaptic circuit features in a zebrafish model of fragile X syndrome," PLoS One. Jan 2013; 8(3): e51456, doi: 10.1371/journal.pone.0051456.

35. Medishetti R, Rani R, Kavati S, et al. "A DNaZyme based knockdown model for Fragile X-Related zebrafish reveals a critical window for therapeutic intervention," J Pharmacol. Toxicol. Methods. November 2019; 101: 106656, 2019, doi: 10.1016/j.jmbt.2019.127065.

36. Sevlimedu A, Kulkarni P. "Embryonic Zebrafish models using DNAzyme mediated knockdown." 2019, PCT/IB2019/051255.

37. Morales J, Hiesinger PR, Schroeder AJ, et al. "Drosophila fragile X protein, DFXR, regulates neuronal morphology and function in the brain." Neuron. Jun 2002; 34(6): 961–72, doi: 10.1016/s0896-6273(02)00731-6.

38. Dockendorff TC, Su HS, McBride SMJ, et al. "Drosophila lacking dfmr1 activity show defects in circadian output and fail to maintain circadian activity," Neuron. Jun 2002; 34(6): 973–984, doi: 10.1016/s0896-6273(02)00734-5.

39. Jiang BT, Ludwig AL, Benedetti KL, et al. "Expression of an expanded GGG-repeat RNA in a single pair of primary sensory neurons impairs olfactory adaptation in Caenorhabditis elegans." Hum Mol Genet. 2014; 23(18): 4945–4959.

40. Winograd C, Clayton D, Ceman S. "Expression of fragile X mental
