The Known Unknowns: Missing Pieces in \textit{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 \textit{in vivo} models of FXS for drug discovery.

This review endeavours to consolidate the information on all available \textit{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 \textit{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 \textit{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.
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. 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. FMRP is an RNA binding protein, a well-known regulator of translation, and is known to interact with well over 800mRNAs in the adult neuron. 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. 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. 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). 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, 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

<table>
<thead>
<tr>
<th>Clinical Presentations</th>
<th>Suggested Mechanisms of Pathobiology</th>
<th>Reference/s</th>
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</thead>
</table>
| Fragile X-associated neuropsychiatric disorders (FXAND) Attention-Deficit Hyperactivity Disorder (ADHD) Anxiety Autism Spectrum Disorder (ASD) Developmental Intellectual Disability Language Deficits Seizures (rarely) | Dysregulation of excitatory and inhibitory neurotransmission by:  
- mGluR1.mGluR5 (glutamatergic) enhanced signal transduction  
- Deficits in GABA signalling  
Indirect glutamatergic mechanisms that modulate mGluR:  
- Dysregulation of N-methyl-D-aspartate receptor (NMDAR)  
- Altered expression, trafficking, and functions of Alpha-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) | [5]–[8] |
| Fragile-X associated tremor/ataxia syndrome (FXTAS) | Excess mRNA from FMR1 premutation may lead to FXTAS in the progeny through the following mechanisms:  
- Toxic FMR polyG protein production  
- Formation of ubiquitin-positive inclusion bodies through protein & RNA sequestration  
- 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, hnRNPs, Sam68, and drosha, thus affecting some of the normal cell function in both FXAS and FXPOI. | [9], [10] |
| Fragile X-Associated Primary Ovarian Insufficiency (FXPOI) | Repeat-associated non-AUG (RAN)-translated poly-glycine species (FMR polyG) leading to intracellular inclusions bodies affecting ovarian function. There is an overlap of mechanisms suggested for FXTAS and FXPOI. | [11], [12] |
| Spine abnormalities | FMRP loss-related dendritic abnormalities due to the following:  
- FMRP2-Cofilin pathway disruption  
- Matrix metalloproteinase 9 (MMP9) upregulation  
- Excess soluble amyloid precursor protein (APP) levels | [5], [13], [14] |
| Macroorchidism | Increase in hydrophilic glycoprotein granules causing testicular edema followed by scattered collagen fibres and bundles of microfibril. Exact pathway has not been elucidated but may be attributed to connective tissue dysregulation. | [15], [16] |
| Other Physical Characteristics | Loss of FMRP can lead to dysregulation of the following extracellular matrix and connective tissue components:  
- Elastin downregulation  
- Bone morphogenic protein receptor 2 (BMPR2) downregulation  
- Matrix metalloproteinases (MMPs)upregulation | [16], [5] |

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

<table>
<thead>
<tr>
<th>Species, Strain, Model</th>
<th>Technology used to create model</th>
<th>Phenotypes</th>
<th>Mechanisms</th>
<th>Reference/s</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primate</strong></td>
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<tr>
<td>Non-human primate, Macaca mulatta, Spontaneous</td>
<td>Zinc-finger-nuclease mediated knockout of Fmr1 (exon 8) in SD or LEH background</td>
<td>Macroorchidism, alterations in density of dendritic spines; Impaired associative learning and memory.</td>
<td>Loss of FMRP, altered translation profile and development of prefrontal cortex circuitry, altered mGluR5 signalling</td>
<td>[18]–[21]</td>
<td>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</td>
<td>Instance of FXS was not observed; No further studies have been reported using macaques</td>
</tr>
<tr>
<td>Sprague Dawley, Fmr1 in1KO</td>
<td>Zinc-finger-nuclease mediated knockout of Fmr1 in SD or LEH background</td>
<td>Macroorchidism, altered spatial learning and memory; impaired social interaction (novelty recognition)</td>
<td>Loss of FMRP, altered translation and synaptic plasticity</td>
<td>[22]</td>
<td>Clear evidence of deficits in hippocampus-dependent spatial learning and memory (unlike murine models)</td>
<td></td>
</tr>
<tr>
<td>LEH, Fmr1&lt;sup&gt;1&lt;sup&gt;exon8&lt;/sup&gt;&lt;/sup&gt;PWc</td>
<td>Zinc-finger-nuclease mediated knockout of Fmr1 in SD or LEH background</td>
<td>Macroorchidism, altered spatial learning and memory; impaired social interaction (novelty recognition)</td>
<td>Loss of FMRP, altered translation and synaptic plasticity</td>
<td>[22]</td>
<td>Clear evidence of deficits in hippocampus-dependent spatial learning and memory (unlike murine models)</td>
<td></td>
</tr>
<tr>
<td>Sprague Dawley, Fmr1 exon4 KO</td>
<td>CRISPR-Cas9, exon 4 mutation leading to truncation</td>
<td>Macroorchidism, altered protein levels; impaired spatial learning and memory</td>
<td>Loss of FMRP, altered translation and synaptic plasticity</td>
<td>[22]</td>
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### Mouse

<table>
<thead>
<tr>
<th>Knock-outs: C57BL6/J or FVB/N, Sighted FVB, *Fmr1&lt;sup&gt;transcriber&lt;/sup&gt;</th>
<th>Homologous recombination with disruption in exon 5</th>
<th>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)</th>
<th>Loss of FMRP, altered translation profile and altered mGluR5 signalling</th>
<th>[23]–[25] and others, summarized in [26]</th>
<th>Most commonly used model; Very well studied and shows most of the clinical phenotypes.</th>
<th>Residual <em>Fmr1</em> 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.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditional KOs: Sighted FVB or C57BL6/J, <em>Fmr1</em>&lt;sup&gt;KO&lt;/sup&gt;, <em>Fm-&lt;sub&gt;C57BL6/J&lt;/sub&gt;</em> or <em>C57BL6/J</em>, <em>Fmr1</em>&lt;sup&gt;Rescue&lt;/sup&gt; models: <em>Fmr1</em>&lt;sup&gt;C57BL6/J&lt;/sup&gt; and <em>FVB/N</em>, Point mutation: <em>cON</em>, <em>CKO</em>, <em>KO2</em>&lt;sub&gt;C57BL6/J&lt;/sub&gt; or <em>FVB or C57BL/6J</em>, Conditional KOs: Sighted C57BL6/J or FVB/N, <em>Knock In/Repeat Expansion</em></td>
<td>Cre-lox recombination with disruptions in exon/intron 1</td>
<td>Phenocopies KO models</td>
<td>Missense mutation in the KH domain of FMRP prevents RNA as well as ribosome binding, leads to reduced FMRP levels.</td>
<td>[27]</td>
<td>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).</td>
<td>Not very widely used, offers no specific advantages over the KO models for general behavioural tests.</td>
</tr>
<tr>
<td>Rescue models: C57BL6/J <em>Fmr1</em> or <em>Fmr1&lt;sup&gt;KO&lt;/sup&gt;</em>&lt;sub&gt;Tg&lt;sup&gt;206&lt;/sup&gt;&lt;/sub&gt;, <em>Fmr1&lt;sup&gt;Tg&lt;sup&gt;206&lt;/sup&gt;&lt;/sup&gt;</em>&lt;sub&gt;Tg&lt;sup&gt;206&lt;/sup&gt;&lt;/sub&gt;</td>
<td>Cre-lox recombination</td>
<td>YAC transgenesis of human <em>FMR1</em> alleles with 20/92 repeats, into WT</td>
<td>Altered anxiety related phenotypes contrary to those observed in FXS KO</td>
<td>Overexpression of FMRP (~10 fold over endogenous); Presence of multiple copies of a transgene.</td>
<td>[29]</td>
<td>Reveals phenotypes associated with FMRP overexpression; Useful to study gene therapy and titrate expression for optimal phenotypic rescue; Provides evidence for rescue of mouse phenotype by human FMRP.</td>
</tr>
<tr>
<td>Knock In/Repeat Expansion C57BL6/J or FVB/N, <em>Fmr1&lt;sup&gt;premutation&lt;/sup&gt;</em>&lt;sub&gt;CGG&lt;sub&gt;4&lt;/sub&gt;_K1 CGG&lt;sub&gt;8&lt;/sub&gt; KI&lt;/sub&gt;</td>
<td>Homologous recombination</td>
<td>Impaired locomotion, altered anxiety, spatial memory and learning deficits</td>
<td>Moderate repeat expansion and instability, leading to reduced FMRP and increased <em>Fmr1</em> mRNA, intranuclear inclusions in the neurons and astrocytes</td>
<td>Summarized in [31]</td>
<td>Only model to study mechanisms of pathogenic repeat expansion similar to that observed in humans with FXS premutation</td>
<td>Repeat expansion is not accompanied by promoter methylation and silencing, even though FMRP level is reduced; Not useful for screening methylation-modulation therapies.</td>
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### Zebrafish

<table>
<thead>
<tr>
<th>Knockdown (transient)</th>
<th>Morpholino</th>
<th>Morphological changes in the brain, altered neurite morphology, neural crest specification defects</th>
<th>Reduction in Fmrp, increased mGluR5, perturbed calcium distribution and signalling</th>
<th>[32]</th>
<th>Simple, robust knockdown produces strong morphological changes; Rescued by pharmacological inhibition of mGluR5, suited for high throughput screening</th>
<th>Morpholino based induction of artefacts may confound some findings; No behavioural studies.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>fmr1&lt;sup&gt;transcriber&lt;/sup&gt;</em>&lt;sub&gt;stop&lt;/sub&gt;; <em>fmr1&lt;sup&gt;12398&lt;/sup&gt;</em>&lt;sub&gt;splice&lt;/sub&gt;</td>
<td>ENU, (N-ethyl-N-nitroso)urea induced mutations and screening for <em>fmr1</em>knock out</td>
<td>No anatomical defects. Anxiolytic behaviour, hyperactivity, impaired inhibitory avoidance, altered synaptic plasticity (LTP and LTD)</td>
<td>Loss of Fmrp, increased mGluR5</td>
<td>[33]–[35],</td>
<td>Loss of function model illustrates possible mechanism. Demonstrates social deficits.</td>
<td>Mild phenotypes, not suitable for screening. Potential compensatory mechanisms in play.</td>
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Knock down (transient) DNAzyme

<table>
<thead>
<tr>
<th>dfmr1 mutant</th>
<th>P-element insertion</th>
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<tbody>
<tr>
<td>Drosophila</td>
<td>dfmr1 mutant</td>
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<tr>
<td>- fmr1+ mutant</td>
<td>CRISPR/Cas9</td>
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<tr>
<td>- Knock down (transient) DNAzyme</td>
<td></td>
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<tr>
<td>- dfmr1 mutant</td>
<td>DNAzyme</td>
</tr>
<tr>
<td>- Zebra finch, Taeniopyia guttata</td>
<td>No model yet</td>
</tr>
<tr>
<td>- Craniofacial abnormalities, Hyperactivity, Memory deficit</td>
<td></td>
</tr>
<tr>
<td>- Craniofacial abnormalities, Anxiety, Attention deficit, Irritability</td>
<td></td>
</tr>
<tr>
<td>- Expression of varying CGG repeat length containing reporters.</td>
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<tr>
<td>- Vocalization &amp; Language development is dependent on fmr1 expression.</td>
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<tr>
<td>- Loss of Fmrp, changes in the expression of genes involved in learning and memory and cartilage development.</td>
<td></td>
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<tr>
<td>- Loss of Fmrp, increased mGlur5, altered p-ERK and p-Akt</td>
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<tr>
<td>- Repeat containing RNA acts through miRNA-binding Argonaute ALG-2, to impact adaptive olfactory response (a readout for reduced neuronal plasticity)</td>
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<tr>
<td>- Potential model to study olfactory plasticity and subversion in neuronal function, and investigate the molecular link between expanded repeats and plasticity in a simplified system.</td>
<td></td>
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<tr>
<td>- Potential model to study the mechanisms behind speech and language pathology.</td>
<td></td>
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<tr>
<td>- First targeted knockout model showing phenotypes similar to observed human FXS symptoms. Shows rescue with fmr1 mRNA;Suited for drug screening and longitudinal studies</td>
<td></td>
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<tr>
<td>- Simple, inexpensive transient model for drug screening; Reveals the effect of limited loss of Fmrp during early development on later phenotypes</td>
<td></td>
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<tr>
<td>- Transient knock down. Social deficits cannot be appreciated in short duration knock down.</td>
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</table>

| Drosophila |
|  - fmr1+ mutant | CRISPR/Cas9 |
|  - Knock down (transient) DNAzyme |
|  - dfmr1 mutant | DNAzyme |
|  - Zebra finch, Taeniopyia guttata | No model yet |
|  - Craniofacial abnormalities, Hyperactivity, Memory deficit |
|  - Craniofacial abnormalities, Anxiety, Attention deficit, Irritability |
|  - Expression of varying CGG repeat length containing reporters. |
|  - Vocalization & Language development is dependent on fmr1 expression. |
|  - Loss of Fmrp, changes in the expression of genes involved in learning and memory and cartilage development. |
|  - Loss of Fmrp, increased mGlur5, altered p-ERK and p-Akt |
|  - Repeat containing RNA acts through miRNA-binding Argonaute ALG-2, to impact adaptive olfactory response (a readout for reduced neuronal plasticity) |
|  - Potential model to study olfactory plasticity and subversion in neuronal function, and investigate the molecular link between expanded repeats and plasticity in a simplified system. |
|  - Potential model to study the mechanisms behind speech and language pathology. |
|  - First targeted knockout model showing phenotypes similar to observed human FXS symptoms. Shows rescue with fmr1 mRNA;Suited for drug screening and longitudinal studies |
|  - Simple, inexpensive transient model for drug screening; Reveals the effect of limited loss of Fmrp during early development on later phenotypes |
|  - Transient knock down. Social deficits cannot be appreciated in short duration knock down. |

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. The phenotypes observed are a result of these cumulative changes. Even though FMRP 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 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 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 stages in which the premutation stage (55-200 repeats) is associated with an increase in 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 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.

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