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Investigating the Functions of *FMR1* mRNA and FMRP Protein in Human Neurodevelopment

By

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The Wisconsin Initiative for Science Literacy

I believe science should be made readily accessible to the world so everyone can understand the amazing discoveries that are being made. This begins with scientists taking the time to communicate research to people outside of their field. In this chapter, I have summarized my findings from my doctoral studies so they can be understood by a broad, non-scientific audience. I thank the Wisconsin Initiative for Science Literacy (WISL) at UW-Madison for giving PhD students this wonderful opportunity to create an accessible chapter for their theses. I especially thank Professor Bassam Shakhashiri for creating this program, Cayce Osborne for facilitating it and answering my questions, and my editor, Elizabeth Reynolds, for her invaluable feedback and support throughout the writing of this chapter.

Autism Spectrum Disorders

Autism spectrum disorders (ASD) are on the rise with 1 in 31 children in the US identified with ASD. As you are reading this, you can probably think of a friend, family member, or acquaintance that has been diagnosed with ASD. Previously confused for schizophrenia in 1911, ASD was later formalized as a diagnosis in 1980. Even today, the cause and symptoms of ASD are very complex, leaving physicians and researchers confused as to what should be and should not be classified as ASD. This creates a large gap in how we approach research and treatment options for these patients.

ASD is a neurodevelopmental disorder characterized by challenges in social behavior, repetitive behaviors, and delays in speech, language, and development. However, ASD has a broad and complex spectrum ranging from individuals that are able to overcome learning obstacles and go on to perform well in school and hold jobs in

adulthood, to individuals that are non-verbal and have a difficult time navigating society on their own. As the saying by Dr. Stephen Shore goes “If you’ve met one person with autism, you’ve met one person with autism.” I personally am the youngest sister of two brothers with ASD and can attest to how diverse their speech and behavior symptoms are despite them being in the same family. My brothers have what is called idiopathic autism, when the cause of their autism is not known. This accounts for a surprising 80-90% of autism cases. ASD exemplifies the complexity of brain function and development, and illustrates how a slight interruption in developmental timing can cause cascading consequences. Curiosity to learn more about the molecular mechanisms behind brain development and the causes of ASD, and the desire to develop therapeutics is what drove me to pursue my doctoral studies in neuroscience.

Fragile X Syndrome

Because I was interested in studying neurodevelopment, I joined the laboratory of Dr. Xinyu Zhao. Our lab has a broad range of projects focused on understanding basic brain development as well as neurodevelopmental disorders such as fragile X syndrome (FXS), Rett syndrome, and autism. For my PhD project, I focused on understanding the developmental deficits found in FXS, the most commonly inherited form of autism.

In 1943, James Purdon Martin and Julia Bell identified the first case study of intellectual disability that appeared to be inherited and linked to the X-chromosome. They first called it Martin-Bell syndrome, but in 1969 Dr. Herbert Lubs observed a characteristic fragile site on the long arm of the X chromosome (**Figure 1**), which later gave rise to the name fragile X syndrome. Children with FXS typically display language impairment, anxiety, increased seizure risk in childhood, and also have physical features such as a

broad forehead, long face, and large ears. Since FXS is an X-linked disorder, it mainly affects boys over girls, currently occurring in 1 in 7,000 males and 1 in 11,000 females according to the Centers for Disease Control and Prevention.

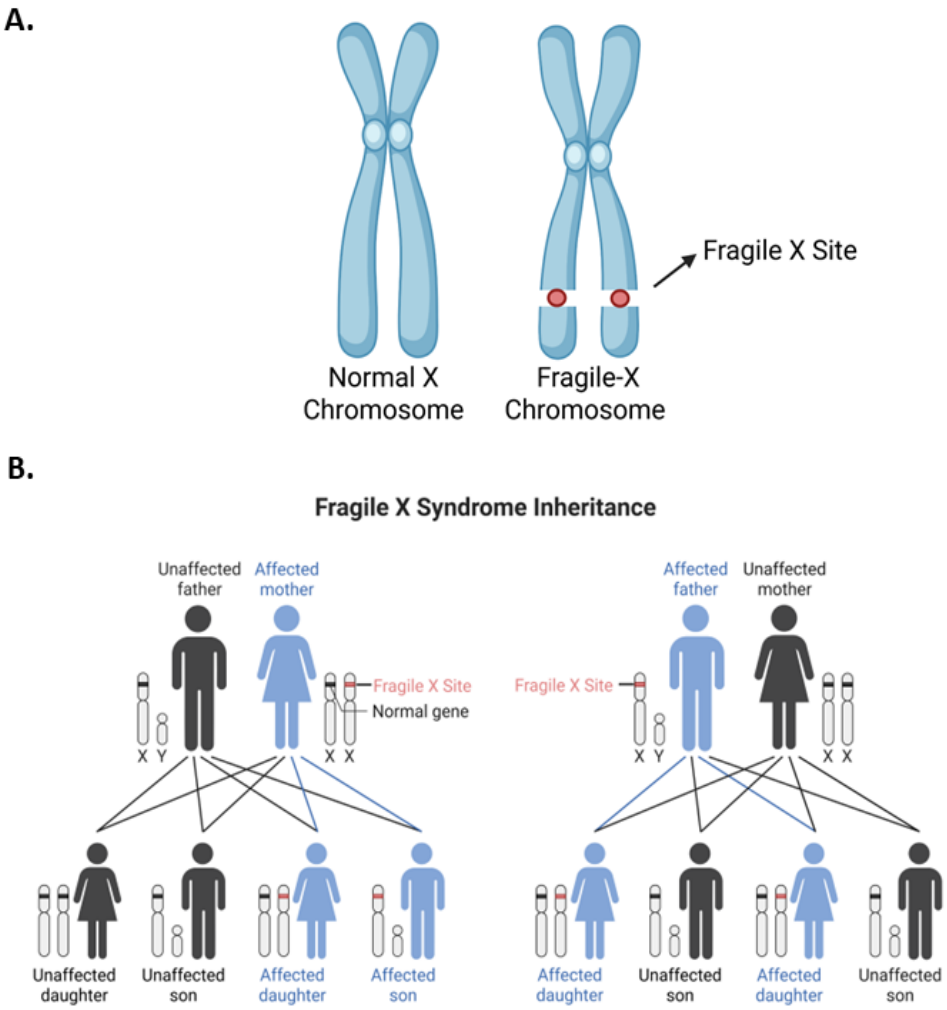

Figure 1.Fragile X Site and inheritance A) Depiction of the fragile X site discovered by Dr. Hubert Lubs in 1969 on the X chromosome. B) Diagram depicting the chances an affected mother will pass on the fragile X site to their daughter or son (50/50 chance) or affected father pass the fragile x site to their daughters. Figure made in Biorender.

Central Dogma of Biology

Our body goes through an orchestration of steps during development. It has to know when and where to develop our hands, our feet, organs, etc. For this, the body uses instructions from the blueprint of life - the DNA, which contains a set of genes that are turned on and off when necessary. When a gene is turned on, that DNA produces RNA, which in turn provides the instructions to produce the building blocks of proteins called amino acids. Proteins are the functional molecules that control activation, repression, and modifications of other genes to maintain a balanced environment and facilitate proper development. This relation of DNA to RNA to Proteins is referred to as the central dogma of biology (**Figure 2**).

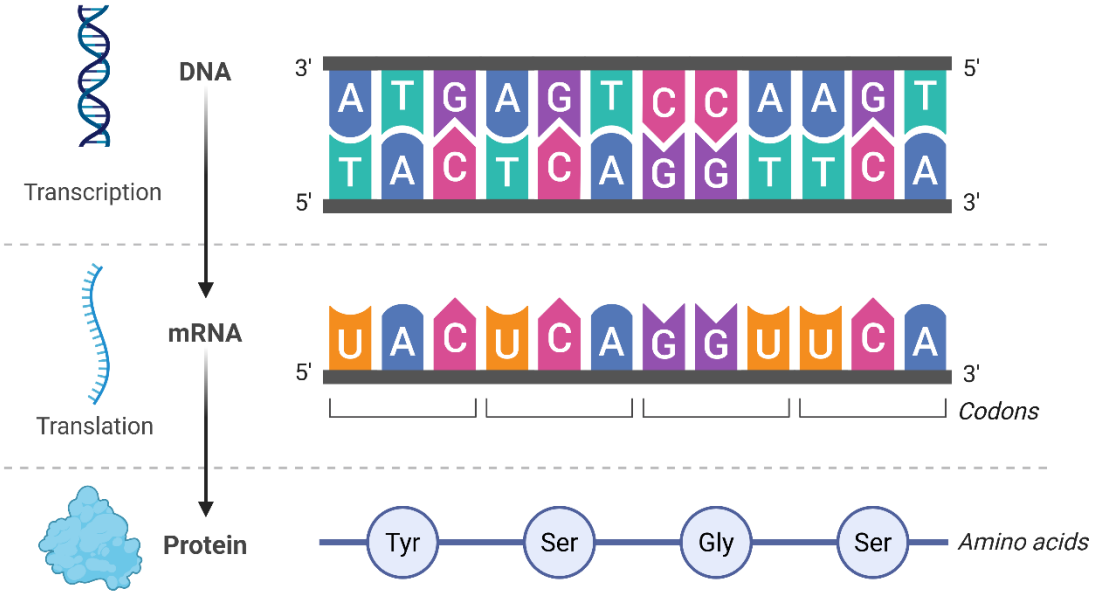

Figure 2. The central dogma of biology is the process of DNA being converted to RNA and subsequently Protein. Our DNA consists of the units Adenosine (A), Thymine (T), Guanine (G), and Cytosine (C) and it is double stranded so these units make pairs (A-T and G-C). RNA is single stranded and the T is replaced to Uridine (U). RNA then provides the code to make amino acids which make protein molecules.

Cause of FXS

Our DNA consists of the units Adenosine (A), Thymine (T), Guanine (G), and Cytosine (C). At the beginning of the fragile X messenger ribonucleoprotein (*FMR1)* gene in the X chromosome, there is a sequence of DNA “CGG” that repeats less than 44 times in healthy individuals. This number of repeats is important, as when this sequence repeats over 200 times, the gene is silenced (**Figure 3**). And when this gene is silenced, the body does not have instructions to produce the essential protein, fragile X messenger ribonucleoprotein (FMRP), thus leading to the development of FXS.

Since FXS is an X-linked disorder, women carriers have a 50/50 chance of passing it to their offspring, depending on which chromosome they pass on. A male carrier would pass it to all of his daughters, but not his sons, since a son only gets the Y chromosome from his father and the X chromosome from his mom (**Figure 1**). In order to test patients suspected of having FXS, physicians and families first identify symptoms similar to autism, then families take their children to get their blood drawn, get cheek swabs, or give saliva samples to test for CGG repeat size and *FMR1* gene levels.

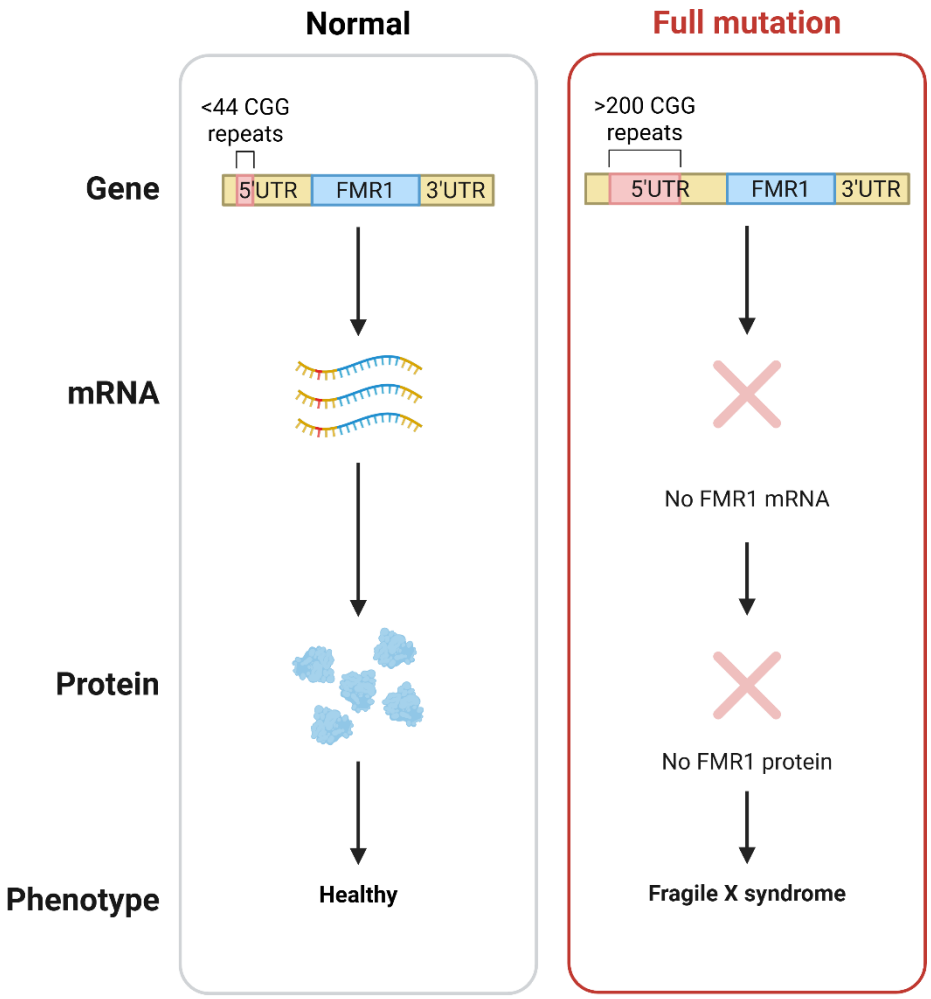

Figure 3. FMR1 Polymorphic CGG Repeat Length in Fragile X Syndrome. A normal individual contains less than 44 CGG repeats in the *FMR1* gene, typically around 30 and lives a healthy life. An individual with more than 200 CGG repeats in the *FMR1* gene has complete silencing of the *FMR1* gene and no FMRP protein leading to FXS. Figure adapted from Rosario and Anderson, 2020.

Brain Development

Brain development requires precise timing - particular cell types must be born in and migrate to the right place at the right time. Think about travelling through an airport to catch a flight. In order for you to get on your flight, you need to complete a series of steps: packing your bags, getting a ride to the airport, then going through security and to the gate. Hopefully, your flight made it to the airport on time so you are not delayed catching your connection to your final destination. If anything stalls you during any of these steps, such as an accident on the way to the airport, a security threat that closes the airport, or a plane malfunction, this not only delays you, requiring alternate plans, but also has cascading consequences: the airline and airport have to relocate passengers to new flights or to hotels. Similarly, the brain has different cell types, which keep everything running smoothly and efficiently and make connections at the correct time.

Some of these cell types include excitatory neurons, inhibitory neurons, and non-neuronal cells such as oligodendrocytes, microglia, and astrocytes (**Figure 4**). We can think of excitatory neurons as green-light cells that tell other neurons to keep sending messages, while inhibitory neurons are red lights that tell other neurons to slow down or stop firing. Oligodendrocytes are important for the formation of myelin, which acts as an insulator in neurons to allow for more efficient transmission of electrical signals. While microglia and astrocytes are the surveillant cells of the brain that usually respond to injury and clear out toxicity to prevent neuroinflammation. They also play important roles during development, including refinement to allow for proper neuronal maturation. FMRP is an essential protein in regulating many of these cell types, which is why its loss is the main cause of FXS.

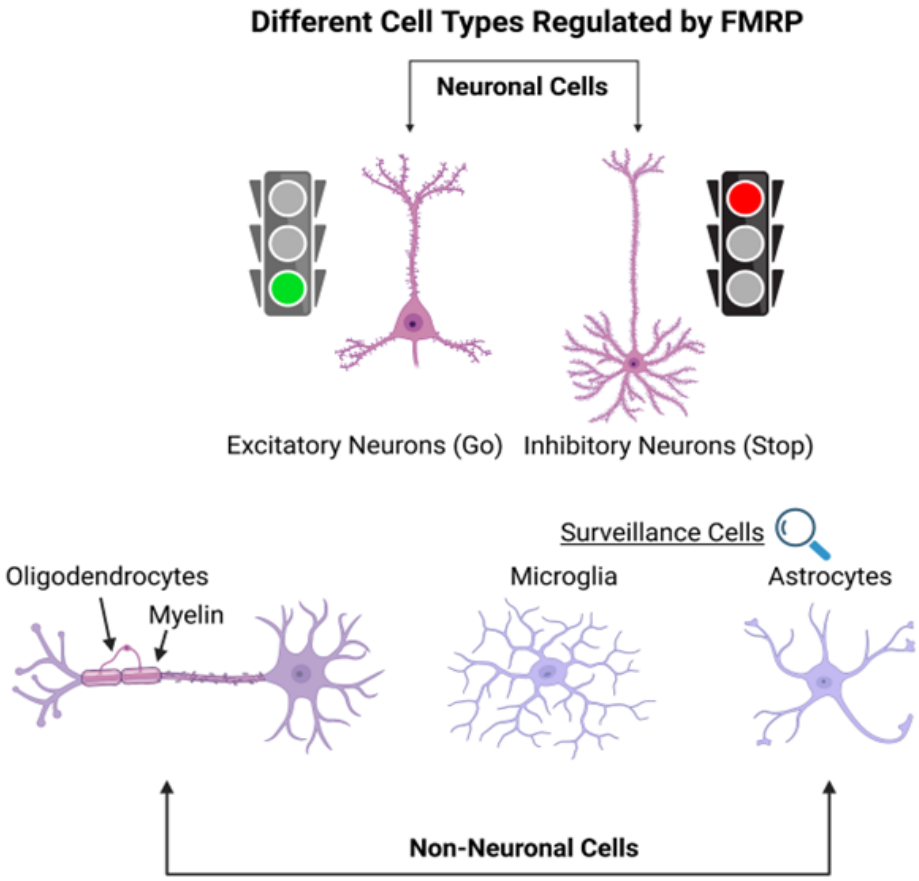

Figure 4. Different cell types in the brain. FMRP has roles in regulating the function of all these cell types and more**.** Excitatory neurons send signals to go and inhibitory send signals to stop when communicating with other neurons in the brain. Other cells types include non-neuronal cells usually referred as glia and these include oligodendrocytes important for myelination of the neuron, and the surveillance cells microglia and astrocytes that scan the environment to make sure injury and toxicity is removed. Figure made in BioRender.

FXS models

Although FXS is a single gene disorder, its set of symptoms is complex and difficult to study in a developing brain. In order to try to understand what is occurring early in development, in our lab we have to use different models of FXS. These different models include drosophila (fruit flies), zebrafish, non-human primate slices, mouse, and human stem cells. These models are made by scientists deleting the *FMR1* gene from animal or cell models, or extracting blood or skin samples from patients to derive FXS patient stem cells.

While no model is perfect, each has its own advantages and disadvantages. For example, with the mouse model we are able to do genetic studies on different brain regions to understand how FMRP loss leads to changes in brain size and volume in different areas. Another advantage of mouse models is that we are able to study the interactions between neurons and glial cells like astrocytes and microglia. Additionally, we are able to perform behavioral studies to correlate with patient symptoms, which is useful to understand the impact of FMRP loss across developmental time-points. Not only that, but with mice we are able to test therapeutic targets before testing it in the FXS patients. However, mouse and human biology are different, and the mouse model includes a deletion of the *FMR1* gene, while the human mutation is due to CGG repeats. Because of this biological difference, some molecular discoveries in the mouse model may not apply to humans. This is why human stem cell models are necessary.

For my PhD project, I used human stem cells reprogrammed from FXS patient cells and stem cells that had the FMRP protein silenced (FMRP-KO). Human stem cells are useful for understanding human diseases and for developing drug screens before

testing compounds on a patient. They are also a good way to study the differences across groups of patients, helping us develop personalized medicine. We obtain these cells from blood or skin cells of FXS patients and then add molecules that reprogram these cells to stem cells to be able to be turned into various cell types such as neurons, lungs, cardiac cells, etc. In our case, I was interested in studying the function of the *FMR1* gene in human excitatory neurons. In the following sections, I will describe how I investigated the role of *FMR1* in excitatory neurons.

Can *FMR1* serve a dual-function role?

As mentioned above, the important protein missing in FXS is FMRP, which binds to RNA and controls expression. For many years, the field has focused on understanding the many functions of FMRP, which RNAs it targets and the function of those RNAs. The ultimate goal was to understand why, when FMRP is lost, we observe certain cell changes. This has led to the identification of novel molecular pathways that can be targeted with molecules to be able to use as medicine. However, recently, more research has started to investigate genes that can function as RNA *and* as a protein, thereby serving a dual purpose. So, this prompted the question: Does the *FMR1* gene have a non-coding RNA role in neurodevelopment that can be contributing to FXS deficits?

Investigating the role of *FMR1* in neurodevelopment besides its coding function to FMRP

In order to answer this question, I used three sets of cells 1. a set of FXS patient-derived stem cells, 2. a set of stem cells that were gene edited to have the FMRP protein silenced, but retained the expression of *FMR1* RNA (FMRP-KO), and 3. a set of healthy

control cells (**Figure 5**). I added molecules to these cells to mimic gradients that occur during brain development to produce neural cells that would turn into excitatory neurons. I then grew the excitatory neurons from each condition for either a week or three weeks to investigate different aspects of development.

When the neurons were 1 week-old, I measured their neuronal morphology (the shape of the neuron) by assessing their number of processes and cell body area. Later in development, at 3 weeks, I measured neuronal activity by introducing a calcium sensor into the neurons. When calcium enters an active neuron, the sensor causes the cell to glow bright green, allowing us to detect its activity. At 3 weeks, I also assessed gene, protein, and metabolic expression to determine what might be driving cellular changes in morphology and activity between FXS, FMRP-KO, and control neurons.

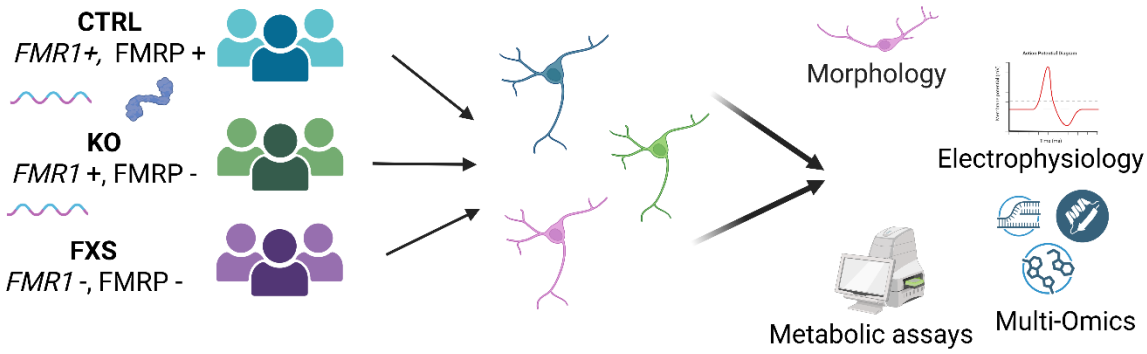

Figure 5. Experimental design. Three sets of stem cells were differentiated to excitatory neurons and assessed for morphology, electrical activity (electrophysiology), gene, protein, and metabolite expression, and metabolic assays.

Considering our lab had already studied the shape and activity of FXS neurons, my first step in testing my hypothesis if *FMR1* has a non-coding function was to test the different cell characteristics present in the FMRP-KO neurons. I first performed the morphology analysis, and interestingly, I observed that although the FXS neurons were less complex, the FMRP-KO neurons appeared normal. I then assessed their activity, and found that the FXS neurons had increased activity, whereas the FMRP-KO neurons appeared normal. This was very exciting for me because it meant that although FMRP was silenced, the presence of *FMR1* RNA could be allowing these functions to be normal in the neurons.

To verify if the presence of *FMR1* is attenuating the drastic cellular changes observed in FXS, I performed a set of experiments that broadly looks at gene expression, protein expression, and metabolites (small molecules important for energy production). To look at gene expression (RNA levels in the cell), I performed transcriptomics by first isolating the RNA from the neurons, then sent those samples to an outside company for RNA sequencing. To look at protein expression I performed proteomics and sent frozen cells to our collaborator lab (Lingjun Li's lab) to extract the protein from the neurons and quantify which proteins were present in the cells. To examine metabolite expression, I followed a similar approach to proteomics, where I first froze the cells, then sent them to a company to extract the metabolites and quantify which were present in the neurons (a process called metabolomics).

To my surprise, there were very few differences at the RNA level when I compared FMRP-KO neurons to control neurons; however, I observed more differences when comparing FXS neurons to control neurons, indicating that *FMR1* may influence

gene expression. I saw more differences in protein levels, which is consistent with FMRP's role in regulating protein expression.

I then integrated all three data sets to identify common differences in our transcriptomics, proteomics, and metabolomics when comparing the FXS neurons to the FMRP-KO neurons. I noticed there was a hub of genes and proteins related to metabolic pathways, which are the series of steps our body needs to convert nutrients to energy. In particular, the gene and protein lactate dehydrogenase A (LDHA) was more highly expressed in the FXS neurons compared to FMRP-KO and control neurons.

LDHA is a protein that is important in breaking down sugar to produce energy in the cells, a process called glycolysis. Most of the research on LDHA has been done in cancer cells since they exhibit increased levels of LDHA. This makes sense for cancer cells, since they need rapid energy to continue dividing. However, neurons usually rely on the energy produced by the mitochondria, a tiny organ inside the cell. Previous research in our lab has seen that the FXS neurons have deficient mitochondrial function. It is possible that the FXS neurons have to compensate by using another energy source, which explains why we see an increase in glycolysis in the FXS neurons.

To confirm this is the case, I investigated mitochondrial function and glycolytic levels in the FXS, FMRP-KO, and control neurons. Interestingly, both FMRP-KO and FXS neurons have reduced mitochondrial function, but only the FXS neurons have increased glycolysis. So, this made me wonder whether FMRP is essential for mitochondrial function (as we showed in our lab before) and whether *FMR1* plays a role in maintaining glycolytic levels.

To figure out how losing *FMR1* leads to higher *LDHA* levels and increased glycolysis, I looked into what other proteins bind to the *FMR1* gene. I identified a protein called IGF2BP2 that binds to both *FMR1* and *LDHA*, and in cancer cells, it's known to enhance *LDHA* activity. This led me to wonder whether *FMR1* normally keeps IGF2BP2 in check - preventing it from over-activating *LDHA*. If my hypothesis is correct, when *FMR1* is present, IGF2BP2 should bind less to *LDHA*; when *FMR1* is gone, it should bind more. My experiments confirmed this (Figure 6), supporting my theory that *FMR1* regulates *LDHA* through its control of IGF2BP2.

Everything at this point was leading to an exciting new discovery never seen in the FXS field before: I found a role for the *FMR1* gene that was irrelevant to its coding function to FMRP. I next wanted to check if restoring *FMR1* RNA levels in the FXS neurons rescued the glycolytic levels, morphological deficits, and activity. For this experiment, I infected cells with a virus that expressed *FMR1* RNA but did not express FMRP protein (similar to the FMRP-KO neurons), and another virus as a control that expressed *FMR1* and FMRP (similar to our control neurons). Shockingly, the FXS cells with only *FMR1* expression had reduced glycolytic levels, similar to those that also had FMRP expression. I also observed that *FMR1* expression restored neuronal activity to normal levels and partially restored neuronal shape. This was surprising because it means that *FMR1* RNA expression alone appears sufficient to reverse cellular changes in FXS, without the need of the protein FMRP. This may open a new avenue for treatment - delivering only parts of the RNA, may be easier than delivering the full protein.

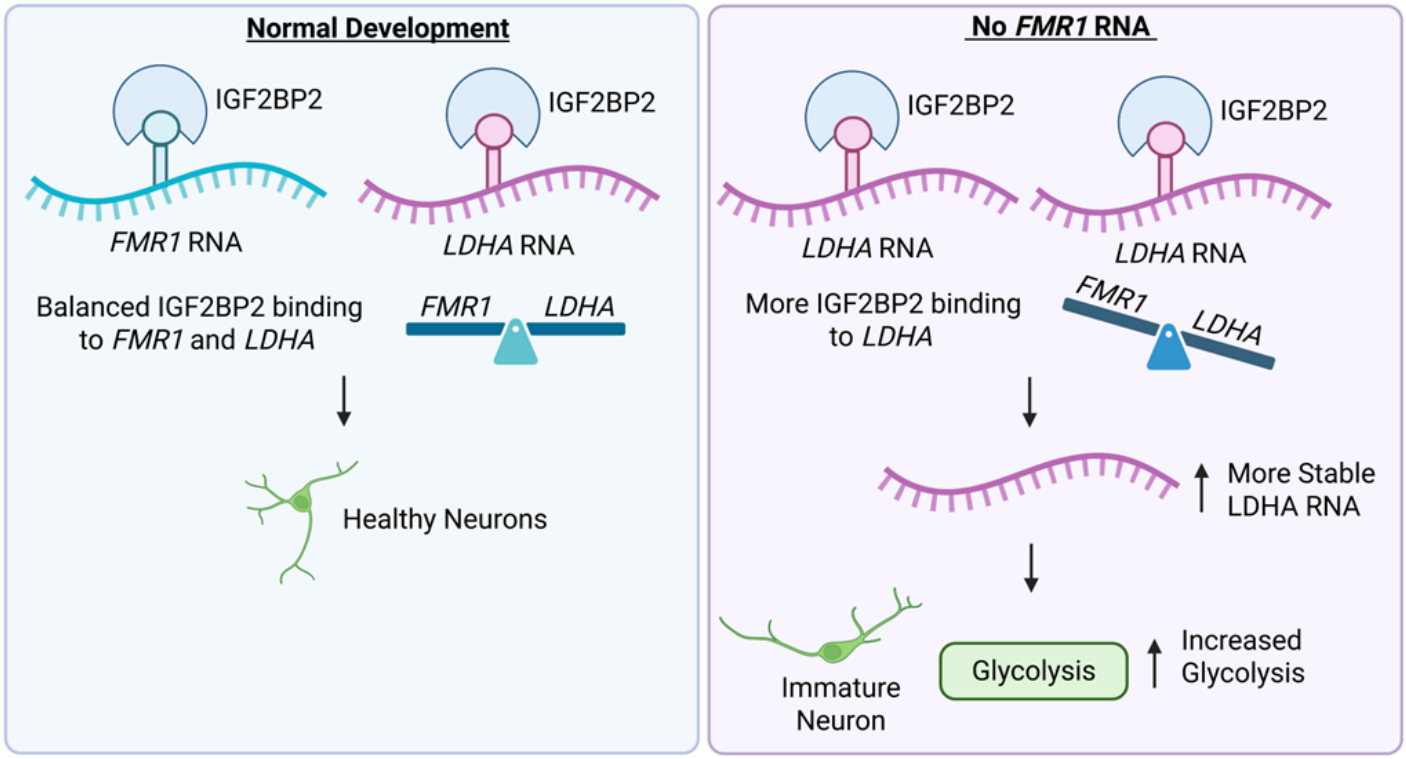

Figure 6. Summary of IGF2BP2 binding to *FMR1* and *LDHA*. In normal conditions IGF2BP2 binds to both *FMR1* and *LDHA* creating a balanced environment and healthy neuronal development. When we lose *FMR1*, now there is more IGF2BP2 available to bind to more *LDHA* and increase its stability, which in turn increases glycolysis leading to immature neuronal development. Figure made in BioRender.

Clinical significance

Overall, for my PhD project, I have studied *FMR1* from a new angle and have made an exciting new discovery in the field of FXS. To date, there have not been many studies investigating the dual-function of coding RNAs, and the finding we have made here can apply to many other genes affected in other neurodevelopmental disorders, neuropsychiatric disorders, and other diseases as well. The goal of our research in the lab is to build new knowledge that can hopefully be paired with other studies around the world, so as a research community we can one day translate our findings to a successful therapeutic approach for FXS patients. While my research is only the beginning, we have identified a novel mechanism and other dysregulated genes and proteins in FXS that can be researched further, and hopefully spell real results for patients. More importantly, full length FMRP restoration to a patient is extremely difficult, so knowing that only needing *FMR1* RNA for partial restoration of some neuronal abnormalities can be a game changer to how we approach some of these clinical solutions.

Limitations and Future Directions

While I have tried my best over these years to do great research and produce rigorous and reproducible results, this project comes with its limitations. First, I have focused on one cell-type, excitatory neurons. As mentioned above, the brain is composed of many different cell-types and it is possible that even though I rescued some phenotypes in this cell type, it may not apply to other cell-types. Second, I used an *in-vitro* model system (outside of a living organism grown in tubes or petri dishes), as opposed to an *in-vivo* system (inside a living organism, such as animal models), so I am not able to assess how *FMR1* expression may affect behavioral outcomes. Lastly, I did not examine which

areas of *FMR1* are functional and necessary to rescue FXS deficiencies, which would be useful to be able to translate my research to a clinical setting.

In the future, it would be useful to expand this research to other models, including mouse models to investigate the effects of *FMR1* expression on behavior, and 3D human cell models to examine other cell types over a longer developmental period. Translating these findings to patients will require identifying the functional pieces of *FMR1* and determining the optimal concentration of *FMR1* to achieve therapeutic goals without detrimental side effects. Much work is still needed, but with each new finding we get closer to understanding how our brain develops in hopes of helping people with neurodevelopmental disorders.

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