# Analyzing the Effects of FMR1 Mutations on Reproductive Function in A Mouse Model of Fragile X Syndrome

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ABSTRACT

Fragile X syndrome (*FXS*) is caused by mutations in the fragile X mental retardation gene (*FMR1*), leading to a variety of developmental disorders. It affects 1/7000 males and 1/11,000 females and is the most common monogenic cause of autism and intellectual disabilities. Individuals with FXS also experience reproductive disorders, such as premature ovarian failure in females and macroorchidism in males. Reproduction is controlled by GnRH neurons in the hypothalamus of the brain. *FMR1* is highly expressed in the brain, however, the role of *FMR1* in the hypothalamus has not been addressed. We used a mouse model that lacks the Fmr1 gene (*Fmr1*-KO) and determined that female mice stop reproduction early similarly to women with mutations. *Fmr1-KO* also have increased corpora lutea number, larger litter sizes, increased follicle-stimulating hormone and luteinizing hormone levels, and increased synaptic molecules in GnRH neurons. Since we observed altered GnRH neuron connectivity and gonadotropin levels, we wanted to investigate how FMR1 mutations affect GnRH pulsatile activity. Additionally, we wanted to determine if the brain or the ovaries are the specific site of reproductive dysregulation in individuals with FMR1 mutations. Overall, this study will give us insight into the mechanisms underlying reproductive disorders linked to *FMR1* mutations.

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## Introduction

Fragile X syndrome (FXS) is a genetic disorder caused by mutations of the fragile x mental retardation gene (FMR1) found on the X-chromosome. FMR1 mutations lead to the decrease or lack of fragile x mental retardation protein (FMRP). Under normal conditions, the FMR1 gene encodes FMRP, which is an mRNA binding protein that plays an important role in protein translation, mRNA transport, and RNA degradation (Edbaur, 2010). Normally, the 5 prime untranslated region of the FMR1 gene contains around 6 to 40 CGG trinucleotide repeats in the DNA sequence. With FMR1 mutations, the 5 prime untranslated region expands to more than 200 CGG trinucleotide repeats leading to hypermethylation, silencing of the gene, and the loss of FMRP (2). FXS is a developmental disorder because FMRP plays an essential role in brain development. Recently it was discovered that premutations of FMR1 (about 55 to 200 CGG repeats) are linked to fragile x associated premature ovarian failure (POF) in women and fragile x- associated tremor/ataxia syndrome (FXTAS) (Cornelia, 2016). POF is an infertility disorder that affects 1% of reproductive aged women. This disorder is characterized by infertility and irregular menstrual periods before the age of 40. It is also associated with increased serum follicle stimulating hormone (FSH) levels (Fink, 2018). Women with POF show premature depletion of ovarian follicles and it causes them to lose ovarian function before the age of 40 (Fink, 2018).

FMR1 is a ubiquitously expressed gene with its highest expression being in the brain. Specifically, in the brain FMRP binds mRNA that encodes synaptic proteins, and contributes to the development of intellectual disabilities (Darnell, 2011, Ascano, 2012). In a study comparing GABA A receptor inhibitory activity within the hippocampus and cortex of control and FMR1-knockout (Fmr1-KO) male mice, it was found that GABA A receptor subunits were decreased in

KO mice (El Idrissi, 2005). Another study analyzing how the lack of FMRP affects synaptic plasticity within the brain determined that FMR1 mutation caused an increase in excitatory NMDA receptor subunits NR1 and NR2A in the hippocampus and cortex (Schutt, 2009). This reveals how the lack of FMRP leads to decreased inhibitory signaling and changes in neuronal connectivity within the brain, which could explain the neuronal hyperexcitability and dysregulation that is present in individuals with FXS. The role of FMR1 in regions of the brain, such as the cortex and hippocampus, is increasingly being investigated, however, the role in the hypothalamic are not. Since FMR1 mutations result in the reproductive disorders and the hypothalamus is an important region for regulating reproduction, it is essential that we investigate the effects of FMR1 mutations on the hypothalamus and analyze the underlying mechanisms causing these issues.

As stated before, FXS is not only associated with intellectual disabilities and autism, but it has been linked to reproductive disorders, such as premature ovarian syndrome (POF) in women carriers and macroorchidism (enlarged testes) in men (Ascano, 2012). However, the mechanisms behind these disorders are not known. Normally, reproduction is controlled by hypothalamic GnRH neurons that release gonadotropin-releasing hormone (GnRH), a neuropeptide essential for regulating reproduction, in a pulsatile manner. The pulsatile frequencies of GnRH are controlled by upstream regulatory networks, such as afferent GABAergic neurons that reside in the medio basal hypothalamus (Wang, 2020). GABA neurotransmitters are predominantly inhibitory within the adult brain; however, they have been seen to have an excitatory effect on the GABA A receptors that activate GnRH neurons (Moore, 2015). GnRH acts on the anterior pituitary via the hypophyseal portal system, which leads to the secretion of pituitary gonadotropin hormones: follicle stimulating hormone (FSH) and luteinizing

hormone (LH) (Coss, 2018). FSH regulates follicular growth and recruitment of follicles in the ovary to the growing pool, while LH is an essential hormone for stimulating ovulation and steroid hormone production. The ratio of FSH and LH secreted is controlled by GnRH pulse frequencies. High pulsatile frequencies predominately stimulate the release of LH and low pulsatile frequency stimulates the release of FSH (Coss, 2018). Both FSH and LH then act on the gonads to stimulate the release of sex hormones, such as estrogen and progesterone for females and androgens for males (Coss, 2018).

Specifically in females, gonadotropins act on the ovaries, which are organs that produce mature oocytes and the hormones that are essential for reproductive function. The ovary houses a reserve of follicles that contain an oocyte surrounded by granulosa cells and theca cells. Granulosa cells are stimulated by FSH to produce estrogen and theca cells are stimulated by LH to produce androgens (Young, 2010). These cells work together to promote the development of ovarian follicles from the initially immature primordial follicles to mature follicles that release the oocyte during ovulation. An LH surge triggers ovulation, which is the release of a mature oocyte from its follicle. After ovulation has occurred both granulosa and theca cells go through a process called luteinization, which is where they are formed into the corpus luteum (Young, 2010). This structure secretes high levels of progesterone and estrogen to help prepare the body for pregnancy and prime the uterus for implantation (Young, 2010).

Since the effects of FMR1 mutations are most commonly linked to autism and intellectual disabilities, studies have predominantly focused on how regions like the cortex and hippocampus are affected by these mutations. However, since the effects of FMR1 mutations on the hypothalamus are not investigated and it plays an essential role in reproduction, prior studies within Dr. Coss' lab done by Pedro Villa, Biomedical Sciences PhD student, sought to identify

the underlying mechanisms behind the development of reproductive disorders in individuals with FXS. To accomplish this, an Fmr1-KO mouse model was used to examine how the lack of FMRP affects reproductive function of female mice. Additionally, they wanted to determine if the reproductive changes seen in Fmr1-KO female mice correspond to reproductive pathology seen in women with FMR1 mutations. In a fertility study, where 8-week-old wildtype (WT) and FMR1-KO mice were paired with a control male and allowed to reproduce until reproductive function ceased, , Fmr1-KO female mice exhibit early cessation of reproductive function. This is similar to women with FMR1 mutations, who experience premature ovarian failure. Additionally, compared to WT, the Fmr1-KO mice experienced earlier vaginal opening (an external sign of puberty) and gave birth to larger litter sizes.

Since KO mice exhibited early cessation of reproduction and larger litter sizes, the Coss lab wanted to analyze the number of pre-pubertal primordial follicles in the WT and KO mice to see if these effects are due to initially diminished follicle reserve within the ovary or due to an accelerated loss of follicles. It was found that there was no difference in primordial follicle numbers between WT and Fmr1-KO mice, which indicates that the development of follicles is not affected by mutations. Then, corpora lutea numbers is adults were counted to investigate ovulation. Fmr1-KO mice had more corpora lutea per ovary than the WT mice, indicating that KO mice have ovulated more than WT, which corresponds to larger litters. This may indicate that KO mice recruit more follicles to grow in each cycle, since they have more pups. Coss lab postulates that this may lead to early loss of follicles.

These findings prompted the Coss lab to investigate how FMR1 mutations impact the LH and FSH levels, since they are gonadotropin levels responsible for regulating follicular growth and ovulation. Fmr1-KO mice exhibited elevated levels of FSH, which correlates with high FSH

levels seen in women with FMR1 mutations. Additionally, KO mice showed elevated levels of LH. High FSH could be causing increased follicular recruitment and high LH could be increasing ovulation resulting in the early depletion of follicles seen in Fmr1-KO mice.

Since Fmr1-KO mice exhibit increased LH and FSH the Coss lab sought to determine if FMR1 mutation affects ovarian function, which could result in the lack of ovarian feedback and increased gonadotropin levels. To do this they specifically analyzed ovarian steroid hormones that feedback to the hypothalamus and inhibit it. Compared to WT, Fmr1-KO mice showed no difference in inhibin subunits and circulating estradiol. However, they did see that Fmr1-KO mice had higher levels of circulating testosterone, which shows that opposite to expectations, Fmr1-KO mice do not lack ovarian feedback. However, increased testosterone can alter gonadotropin levels and GnRH pulse frequency in women with polycystic ovarian syndrome.

Fmr1 mutation has been seen to impact neurocircuitry within regions like the hippocampus. This prompted the Coss lab to analyze the effects of Fmr1 mutations on synaptic molecules within the hypothalamus since it is not investigated. Fmr1-KO exhibited higher levels of GABARgamma2, an obligatory subunit of GABA A receptors. GABA transmission is essential for GnRH function and acts excitatory within the brain so increased GABAR2 could possibly result in increased GnRH neuron activity, which could explain the elevated gonadotropin levels seen in Fmr1-KO mice.

These studies determined that Fmr1 mutations result in higher recruitment of follicles, early cessation of reproduction, elevated gonadotropin levels, and altered GnRH neuron circuitry. Since we observed altered LH and FSH levels and GnRH pulsatility is extremely important in the regulation of gonadotropin hormones my project sought to determine how FMR1 mutations are impacting GnRH neuron activity.

Additionally, since we observed adverse effects of Fmr1 mutations in both hypothalamic and ovarian function, we sought to determine the specific site of reproductive dysregulation. To determine this an ovariectomy procedure was done on prepubertal WT and Fmr1-KO female mice. Ovarian hormones secreted during puberty and adulthood play an essential role in regulating the secretion of gonadotropins from the anterior pituitary via negative and positive feedback systems. By performing an ovariectomy pre-pubertally we will be able to analyze differences in gonadotropin levels before ovarian hormone exposure can alter HPG axis activity. If gonadotropin levels between Fmr1-KO and WT female mice are significantly different in the absence of ovaries we can determine that the brain is a possible site of dysregulation. However, if the absence of ovaries eliminates differences in gonadotropin level between Fmr1-KO and WT, we can conclude the ovaries are a potential site of reproductive dysregulation.

Studies from epidemiology, fertility clinics, and reproductive biology determined that about 10% of women could be at risk for reproductive decline before the age of 35 years old (Fink, 2018). Since women with FMR1 mutations make up 80% of individuals with a known genetic cause of POF, this research will give us more insight on the underlying mechanisms of reproductive disorders in individuals with FMR1 mutations, which could potentially open the doors for the development of future treatments.

## Methods

### Animals

All procedures performed on these mice were performed with the approval of the University of California, Riverside Animal Care and Use Committee. These procedures were done in accordance with the National Institutes of Health Animal Care and Use Guidelines. Breeding pairs of FVB.129P2-Fmr1tm1Cgr/*J* (*Fmr1* KO) and their respective control (FVB WT)

mice were obtained from the Jackson Laboratory. These mice are bred in house and are maintained under a 12-hour light/12-hour dark cycle to create an optimal environment and minimize stress. Additionally, the mice received water and food *ad libitum*. Since we are analyzing the effects of Fmr1 mutations on reproductive function, specifically relating to POF, we used female Fmr1-KO and WT mice for these experiments.

Studies have shown that mice under psychosocial stress showed disruption in their reproductive neuroendocrine function and rapid suppression of LH pulsatile suppression (13). So, in order to conduct an accurate LH pulsatile analysis and minimize stress, we habituated both the WT and FMR1-KO mice used. Habituation is a process where mice are exposed to repeated stimuli so eventually their response to that stimuli is minimized. In this case, we wanted to habituate the mice to certain parts of our procedure that could cause stress, such as removing them from their cages or removing blood from their tails. Everyday for 3 weeks before the procedure, we habituated each mouse in the room we would use for the procedure, by massaging their tails for ten minutes and getting them used to handling. The estrous cycles of mice were staged via vaginal lavage and LH pulsatile analysis was taken when they were in Diestrus. Diestrus is a good stage to use because there is less fluctuation of hormones compared to other stages, which allows for better repeatability.

We also aim to investigate if the brain or ovaries are the specific site causing reproductive dysregulation in individuals with FMR1 mutations. To do this we performed ovariectomies on pre-pubertal WT and Fmr1-KO female mice and then allowed them to grow till they were 8 weeks. To begin the procedure the mice were put in an isoflurane chamber to be anesthetized. Once immobile the mice were laid in a prone position and an isoflurane mask supplied by an isoflurane vaporizer was placed on their nose to maintain anesthesia at 2 mg/mL.

We then shaved and disinfected the flank area of the mice, and also administered an analgesic for pain management. Incisions were made above the iliac crest on the left and right dorsal areas of the mice. A small internal incision was made in the fascia to allow visualization of the ovary. Once the ovary was identified we clamped the uterine horn, applied a ligation, and removed the ovary. The wound was then cauterized to prevent internal bleeding. Next the uterine horn was placed back into the abdominal cavity, the internal incisions were sutured using dissolvable sutures, external incisions were closed using wound clips, and the mice were placed on a heating pad for recovery. For 10 days after the procedure post-op protocol was followed. This included checking the incision site for infection or inflammation, administering an analgesic for pain management, and observing the animal for common signs of pain.

**Hormone Assay**

To analyze gonadotropin levels in ovariectomized mice, we needed to collect serum. For FSH analysis, WT and FMR1-KO mice were sacrificed via isoflurane inhalation and blood was obtained from the inferior vena cava. The blood was allowed to coagulate for 15 minutes and then centrifuged at 2000 RCF for minutes so serum could separate. For LH pulsatile analysis blood serum was obtained from the tail from live freely moving mice . For 3 weeks before serum collection the mice were habituated to our procedure and their tails were massaged daily in order to minimize stress and stimulate blood circulation in the tail. On the day of collection, the tail tips of WT and FMR1-KO mice were cut and 10 microliters of blood was collected every eight minutes for a total of three hours.

LH levels were measured using an in-house method that used an ultrasensitive mouse LH ELISA. The capture monoclonal antibody (anti-bovine LH beta subunit) used was provided by Janet Rosen, University of California, Davis. The detection polyclonal antibody (rabbit LH

antiserum) was provided by the National Hormone and Peptide Program. The secondary HRP-conjugated polyclonal antibody (goat anti-rabbit) was purchased from DakoCytomation. LH reference prep was used as the assay standard. The Hormone assay performed to measure FSH levels was performed by the University of Virginia, Ligand Core. The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is a fee-for-service core facility and is supported in part by the Eunice Kennedy Shriver Grant. FSH was assayed by RIA using reagents provided by Dr. A.F. Parlow and the National Hormone and Peptide Program. Mouse FSH reference prep was used for assay standards. Each animal used to analyze these gonadotropin levels are represented by a dot on the graphs.

**Histological Analysis and Immunohistochemistry**

WT and FMR1-KO female mice were anesthetized, perfused with 20ml PBS and 20ml 4% paraformaldehyde, and tissues were collected. Ovaries were fixed in 4% paraformaldehyde on ice for an hour. They were stored in sucrose, flash frozen in isopentane on dry ice, and coronally cryosectioned at 30 microns. Free-floating immunohistochemistry was then performed on the ovaries in order to visualize FMRP expression and ovarian vascularization.

To analyze FMRP expression WT ovary sections were washed twice for 5 minutes using .1% PBS-T. Antigen retrieval was done for 30 minutes using .5% PBS-T and the tissues were blocked using 10% goat serum with 2 drops of Avidin. The sections were then biotinylated for 15 minutes and incubated for 48 hours at 4℃ with primary antibody anti-mouse FMRP made in rabbit at a concentration of 1:1000. After two 15-minute washes with .1% PBS-T the sections were incubated in anti-rabbit secondary antibody at 4℃ overnight. The sections were mounted and cross-stained using vectamount with dapi.

To visualize vascularization WT ovaries immunohistochemistry protocol remained the same, however, the sections were incubated with the primary antibody anti-mouse CD31 made in rabbit at a concentration of 1:1000. CD31 is a well known vascular marker. Additionally, they were treated with a secondary anti-rabbit antibody at a concentration of 1:1000. Staining for FMRP and vascularization was done by UCR undergraduate student Ashvathi Sivanesan.

**Statistical analysis**

Statistical differences between WT and Fmr1-KO mice were determined using the t-test. Significance ($p < .05$) is indicated on graphs with an asterisk (*).

## Results

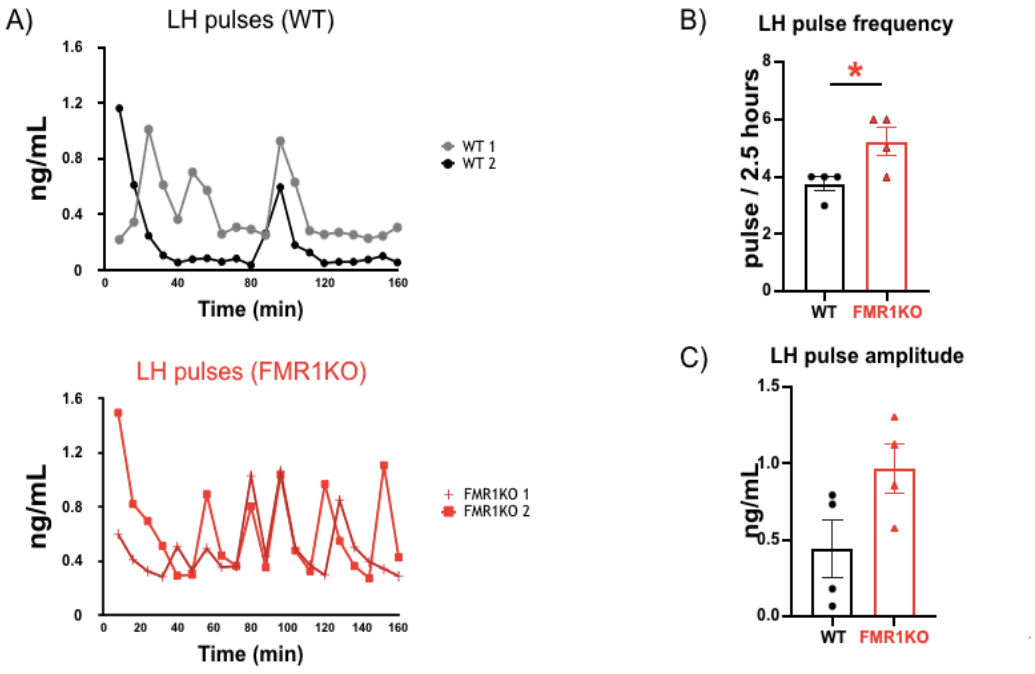


**Figure 1:** A) LH pulsatile pattern of WT (A, black) and Fmr1-KO (A, bottom, Red lines) mice for the span of 3 hours. B) Difference in LH pulse frequency between WT and Fmr1-KO mice.

LH pulse frequency is significantly higher in Fmr1-KO mice compared to WT. Significance is indicated by an *. C) LH pulse amplitude in WT and Fmr1-KO mice. There was no significant difference in LH pulse amplitude between WT and Fmr1-KO mice. Four mice from each group were used for this analysis.

**Fmr1-KO female mice exhibit increased LH pulse frequency**

Since GnRH pulsatile secretion is essential in regulation of LH and FSH secretion and Fmr1-KO mice show early cessation of reproductive function, larger litter sizes, increased synaptic molecules on GnRH neurons, and altered gonadotropin hormone levels we were prompted to investigate how FMR1 mutations are affecting GnRH neuron activity. Since GnRH neuron pulsatile activity is impossible to measure, an LH pulsatile analysis was conducted. For every pulse of GnRH from neurons there is a direct and matching pulse of LH, which is why measuring LH pulsatility is a great representation of GnRH neuron function.

To analyze this, we staged mice and when they were in Diestrus we collected 10 microliters of tail tip blood every 8 minutes for a span of 3 hours. The amplitude of LH pulses was calculated by subtracting the lowest point of the LH pulse from the highest point of the LH pulse. LH pulse amplitude was analyzed and we determined that there was no difference between WT and Fmr1-KO mice (Figure 1C). Since amplitude indicates the amount of hormone secreted per pulse and there was no significant difference between KO and WT mice, we can assume that Fmr1 mutation is not affecting the amount of LH secreted per pulse. We also analyzed LH pulse frequency by observing how many LH pulses occurred in the span of 3 hours. We determined that Fmr1-KO mice exhibited higher LH pulse frequency compared to WT mice (Figure 1B). Increased frequency without a change in amplitude tells us that increased LH levels in Fmr1-KO

mice is due to increased instances of LH secretion rather than an increased amount of LH secreted per pulse. Overall, these results indicate that Fmr1 mutations cause an increase in GnRH pulsatility, resulting in increased LH pulsatility, which in turn results in higher circulating LH and could explain the increased corpora lutea and higher rate of ovulation seen in prior studies.

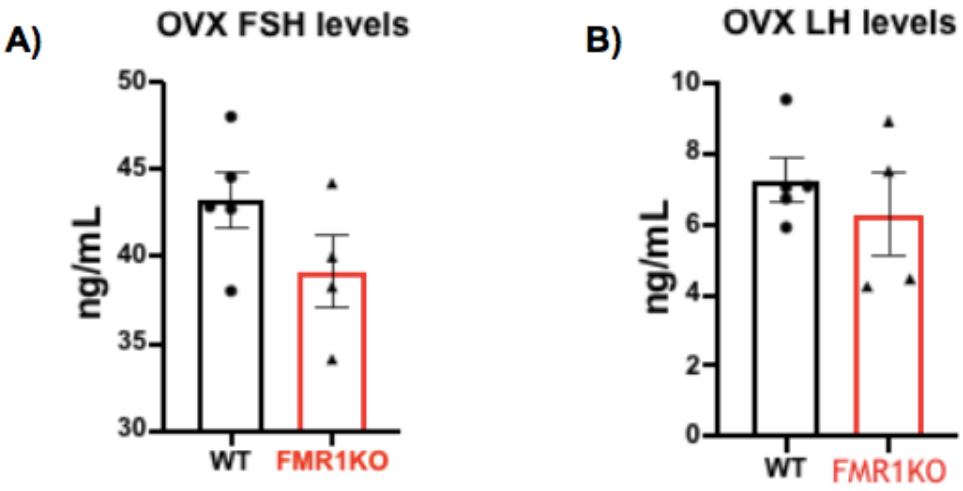


**Figure 2:** Gonadotropin levels in ovariectomized WT and Fmr1-KO mice. FSH levels detected in WT and Fmr1-KO mice were 43.24 ng/mL and 39.13 ng/mL, respectively. LH levels detected in WT and Fmr1-KO mice were 7.25 ng/mL and 6.28 ng/mL, respectively. There was no significant difference in gonadotropin levels between genotypes after ovariectomy.

**Ovariectomized Fmr1-KO mice exhibit normal gonadotropin levels compared to WT**

Although we know FMR1 mutation is affecting GnRH neuron activity, we wanted to determine if the brain or ovaries are the specific site causing this reproductive dysregulation. To do this, we surgically ovariectomized prepubertal WT and Fmr1-KO mice then allowed them to grow till they were 8 weeks old. These mice were sacrificed and their blood serum was collected. Prepubertal mice were used so we could specifically observe the effect of FMR1 mutations on gonadotropin levels without any confounding effects or changes that could result from sex

hormone exposure. Ovariectomy was used to see if gonadotropin levels would differ between Fmr1-KO and WT mice when the ovaries were absent. If FSH and LH levels differed in ovariectomized Fmr1-KO mice compared to WT we can assume the brain is the possible site of reproductive dysregulation in individuals with FMR1 mutations. However, if FSH and LH levels were similar in WT and Fmr1-KO mice in the absence of ovaries we could identify the ovaries as the potential specific site causing reproductive dysregulation. We analyzed both FSH and LH levels and determined that there were no significant differences in adult gonadotropin levels after ovariectomy between WT and KO mice. Since prior studies showed elevated gonadotropin levels in Fmr1-KO mice compared to WT in the presence of ovaries and the absence of ovaries led to no difference in gonadotropin hormones between Fmr1-KO and WT, we suggest that the ovaries could potentially be driving this increase in hormone levels in Fmr1-KO female mice. This allowed us to identify the ovaries as the potential site for reproductive dysregulation in individuals with FMR1 mutations.

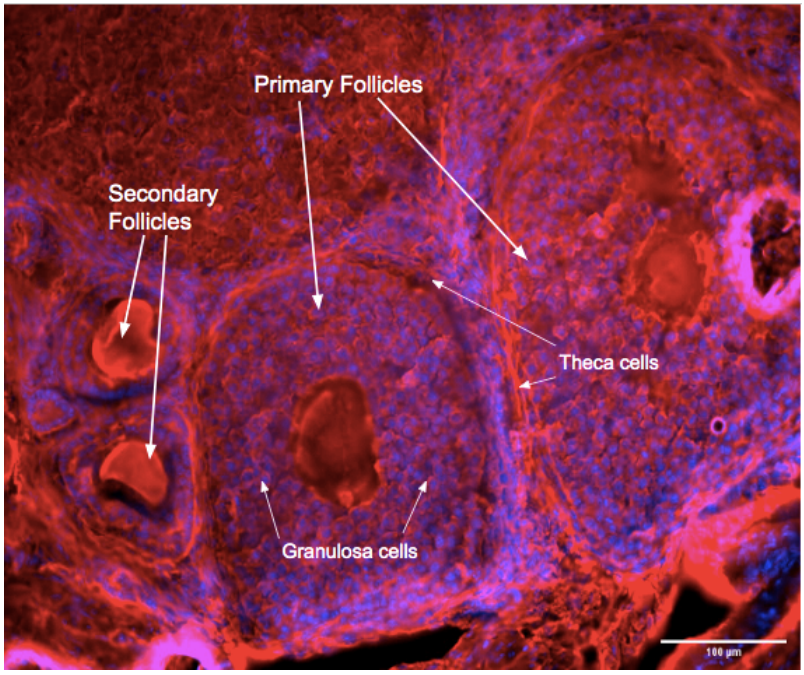

**Figure 3:** Cryosectioned ovary of a WT mouse that illustrates FMRP expression through the ovary. The image is 20x magnification and in the image we can see 2 primary follicles, 2 secondary follicles, granulosa cells within the follicles, and theca cells on the surface of the follicles. FMRP expression is reflected by the red color and the nuclei is stained blue using dapi.

**FMRP is expressed in both the granulosa and theca cells of the ovary**

Since we have identified the ovaries as a potential site of reproductive dysregulation we wanted to investigate FMRP expression within the ovary using immunohistochemistry. We determined that FMRP is expressed throughout the ovary, and specifically in both the granulosa and theca cells, that represent endocrine portion of the ovary (Figure 3). FSH stimulates granulosa cells to synthesize estrogen within granulosa cells (Coss, 2018). Theca cells respond to LH activation and synthesize androgens. FMRP expression in both these cells suggests that lack of FMR1 could be affecting ovarian function which could be driving the increased testoterone leading to the reproductive dysregulation observed in KO mice.

## Discussion

Premature ovarian failure is the phenomenon where ovarian function ceases in women before the age of 40. Many women who experience POF show signs of irregular period, reduced sex drive, and infertility . They are also predisposed to other illnesses, such as osteoporosis, cardiovascular disease, depression, and anxiety (CDC, 2022). There are currently no official screening methods to indicate if women are at risk for POF, which has resulted in women not knowing they are at risk of POF until their symptoms have already started (Sukur, 2014). Although the cause of POF is often idiopathic, women with FMR1 mutations make up about 80% of individuals with a known genetic cause for POF. However, since FMR1 mutations are

more severe in males and are commonly linked to cognitive and intellectual developmental disorders, the effects of FMR1 mutations in women are NOT studied. Nor, are the effects on reproduction or the hypothalamus examined.

Prior studies in the Coss lab examined the underlying mechanisms causing reproductive dysregulation within individuals with FMR1 mutations, specifically regarding POF. They were able to determine the Fmr1-KO female mice exhibited early cessation of reproductive function, early depletion of follicles, and elevated FSH levels, which all correspond to women who experience POF. Additionally, they investigated the hypothalamus and determined that Fmr1-KO mice contained increased synaptic molecules in GnRH neurons, indicating that FMR1 mutations cause altered GnRH circuitry and connectivity. These results correlated with the observed elevated gonadotropin levels prompted me to determine how FMR1 mutations are affecting GnRH pulsatility. Our results showed that Fmr1-KO mice showed increased LH pulsatility compared to WT mice. Since LH pulsatility is an indicator of GnRH neuron activity, we can determine that FMR1 mutations could be causing increased GnRH pulsatility leading to elevated LH and FSH levels. Increased GnRH activity is also supported by a prior study in the lab that observed higher levels of excitatory $GABA_A$ and NMDA receptors in GnRH neurons of Fmr1-KO mice.

We next sought to determine the specific site of reproductive dysregulation causing POF in individuals with FMR1 mutations. We ovariectomized prepubertal female KO and WT mice, observed gonadotropin levels, and saw no difference in gonadotropin levels between Fmr1-KO and WT mice. This allowed us to determine that the ovary is potentially the site of reproductive dysregulation in women with Fmr1 mutations since prior results determined elevated gonadotropin levels in Fmr1-KO with ovaries intact. Additionally, prior research within the lab

determined that Fmr1-KO mice contained increased levels of circulating testosterone, which has also been hypothesized to disrupt GnRH neuron function (Dulka, 2020). High testosterone levels were shown to contribute to other reproductive disorders, such as PCOS. Additionally, studies have shown that increased prenatal androgen exposure resulted in changes in the excitatory GABAergic innervation of GnRH neurons (Dulka, 2020). Possibly FMR1 mutations could be affecting ovarian function leading to increased ovarian secretion of testosterone, which could be leading to upstream reproductive dysregulation. If high testosterone affects GnRH neuronal activity this could also possibly explain the increased LH pulsatility and elevated gonadotropin levels seen in Fmr1-KO mice.

Since we determined the ovary as the potential site of reproductive dysregulation in individuals with Fmr1 mutations we decided to analyze FMRP expression within the ovary. We saw that FMRP was expressed throughout the ovary. These results allow us to assume that the absence of FMR1 could alter ovarian function, such as testosterone secretion, which could result in the reproductive dysregulation seen in Fmr1-KO mice. Future studies will analyze how FMR1 mutations affect ovarian vasculature, since it is essential in providing the ovary with hormones and nutrients needed for proper function. In an optimization trial we used Immunohistochemistry to stain a control ovary for CD31, a common marker for vasculature. We observed vasculature surrounding developing follicles and throughout the corpora lutea (Figure 4). Since we determined FMRP is expressed throughout the ovary and if it's knocked out we can assume that it would affect ovarian function, we hope to examine if ovarian vasculature is altered by the lack of FMRP and if it is contributing to the reproductive dysregulation witnessed.

Additionally, since we observed altered GnRH activity in FMR1-KO mice and FXS is a developmental disorder, in the future we will investigate GnRH neuron development and how it

is affected by the lack of FMRP. GnRH neuron development begins at the nasal placode around embryonic age 10 to 11 then they migrate towards the nasal forebrain junction (Mulrenin et. al., 1999). By embryonic age 15.5 GnRH neuron migration rates decrease and neurons reach their final destination in the hypothalamus (Cho et. al., 2019). I will analyze the development of GnRH neurons in wildtype and Fmr1-KO mice at the embryonic day 15.5, which is a critical stage of GnRH neuron migration to their correct location in the hypothalamus. I will then quantify the differences in GnRH neuron number and migration patterns to determine if Fmr1 mutation causes differences in neuronal development.

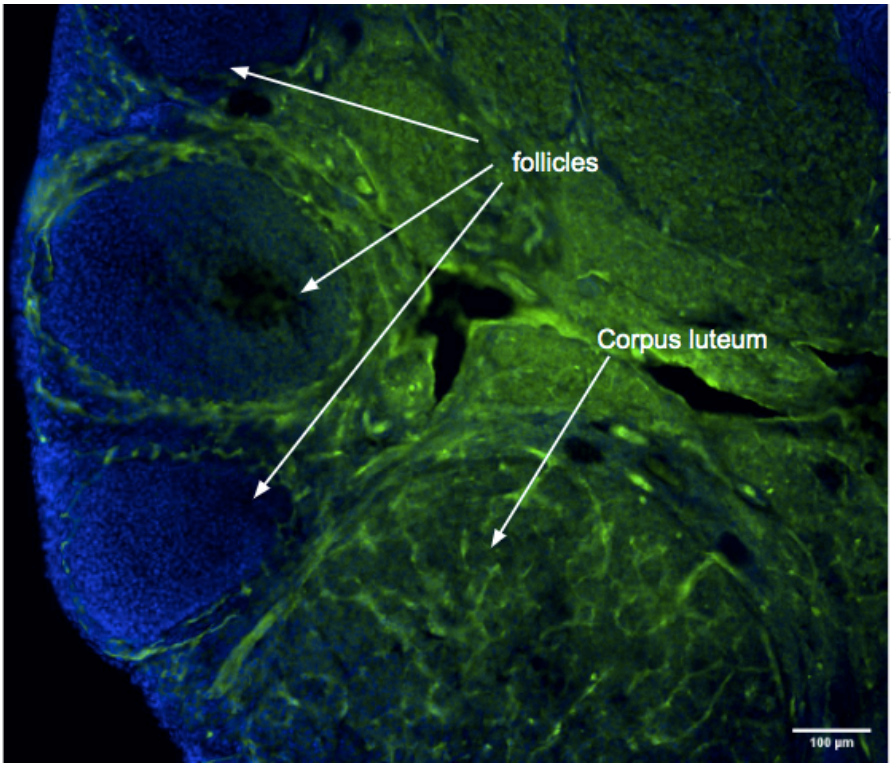


**Figure 4:** Immunohistochemistry staining of a WT ovary at a magnification of 20x. Within the image one corpus luteum and 2.5 follicles can be seen. Vasculature is represented by the color green. Vasculature is seen surrounding developing follicles and throughout the corpus luteum. The blue represents nuclei stained with dapi.

Overall, these studies aim to get a better understanding of how FMR1 mutations affect reproductive function. Hopefully our findings can contribute to the overall knowledge of FXS related reproductive disorders leading to the potential development of treatments and screening methods.

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