ABSTRACT

Title of Document: DEVELOPMENTAL ALTERATIONS IN INHIBITORY NEUROTRANSMISSION IN THE FRAGILE X SYNDROME MOUSE BASOLATERAL AMYGDALA

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Fragile X Syndrome, caused by Fmr1 gene inactivation, is characterized by symptoms including enhanced fear, hyperactivity, social anxiety, and autism, pointing to synaptic and neural circuit defects in the amygdala. Previous studies in Fmr1 knockout (KO) mice have demonstrated alterations in GABA_A receptor (GABA_AR) function in the basolateral amygdala during early postnatal development. In this study, we sought to determine whether these early defects in GABA_AR function are accompanied by changes in protein expression of GABA_AR α1, 2, and 3 subunits, the pre-synaptic GABA-synthesizing proteins GAD65 and 67 (GAD65/67), and the post-synaptic GABA_AR-clustering protein gephyrin. We found that the developmental trajectory of protein expression is altered in KO mice for all tested proteins except GABA_AR α3 and GAD 65/67. Our results suggest that alterations in the timing of inhibitory synapse protein expression in early postnatal development could contribute to observed inhibitory neurotransmission deficits in the KO mouse basolateral amygdala.
DEVELOPMENTAL ALTERATIONS IN INHIBITORY NEUROTRANSMISSION IN THE FRAGILE X SYNDROME MOUSE BASOLATERAL AMYGDALA

By

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Dedication

This research is dedicated to children affected by neurodevelopmental disorders and their families.
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Chapter 1: Introduction

1.1 Cause of Fragile X Syndrome

Fragile X Syndrome (FXS) is the most common inherited form of autism caused by an inactivation of the Fmr1 gene located on the X chromosome. When Fmr1 acquires more than 200 CGG trinucleotide repeats, it is consequently hypermethylated and the corresponding protein, Fragile X Mental Retardation Protein (FMRP) is not expressed [1]. FMRP is normally expressed during embryonic development and continues into adulthood. It is found throughout the body but is most highly expressed in the brain within neurons and glia [2-4]. In neurons, FMRP has been shown to interact with about 8% of synaptic mRNA [5-7]. It has been implicated in mRNA transport out of the nucleus and into dendrites and axons [8]. It has also been shown to regulate mRNA translation at synapses, controlling the amount, location, and timing of protein synthesis in response to neuronal activity [9-16].

1.2 FXS and the Basolateral Amygdala

Absence of FMRP in FXS leads to changes in the brain manifested in symptoms such as enhanced fear, hyperactivity, attention deficit, social anxiety, mental retardation, aggression, and sensory integration problems [17-26]. Some of the more prevalent but less-explored aspects of the syndrome are abnormalities in emotional processing seen in behaviors such as enhanced fear and increased social anxiety. These abnormalities point to deficits in the amygdala, a brain region thought
to tightly regulate emotionally relevant information. In this study, we specifically focused on the basolateral amygdala (BLA) since it is one of the major input and relay stations for emotionally salient information in the brain (Figure 1 A) [27-30]. In fact, the BLA is interconnected with the cortex, brainstem, thalamus, hippocampus, hypothalamus, the olfactory system, as well as other amygdala nuclei [27]. The BLA communicates the nature and strength of the emotional information via excitatory projection neurons which are tightly regulated by local inhibitory neurons that assert their control primarily through synapsing onto the excitatory projection neuron soma [31-37]. Therefore, the integrity of these inhibitory synapses is critical in manifestation of appropriate social and emotional behavior.

Figure 1. Alterations in inhibitory neurotransmission in the developing Fragile X Syndrome BLA.
Figure 1. Alterations in inhibitory neurotransmission in the developing Fragile X Syndrome BLA. A Movement of sensory input through the amygdala is controlled by inhibitory neurons which determine the intensity of sensory output such as fear. LA: lateral amygdala, BLA: basolateral amygdala, CE: lateral central amygdala, CEm: medial central amygdala B Axon of an inhibitory neuron synapses onto the cell body of an excitatory neuron forming the inhibitory synapse. The inhibitory synapse has a number of pre- and postsynaptic proteins which play an important role in inhibitory neurotransmission. C Patch clamp recording from the excitatory neuron soma in the BLA shows a decrease in sIPSC amplitude at P10 and P21, and an increase at P14 in KO mice. D Patch clamp recording from the excitatory neuron soma in the BLA shows a decrease in sIPSC frequency at P10 and P21, and an increase at P14 and P16. error bars: mean±SEM; *p<.05, **p <0.005 (unpaired, two-tailed t-test)

1.3 Neural Abnormalities in FXS Mouse Model

FXS studies in mice have been conducted using the Fmr1 KO model where the Fmr1 gene is fully knocked out to mimic the loss-of-function of the Fmr1 gene in the human FXS [89]. In the Fmr1 KO mouse model, molecular, morphological, and behavioral abnormalities including overactivation of the mGluR pathway [38-44], immature appearance and increased number of cortical dendritic spines [45-49], and an increase in hyperactivity and anxiety, have been observed [50-55]. Most recently, evidence for alterations in inhibitory neurotransmission was found in the BLA of 3-4 week-old KO mice, involving decreased frequency and amplitude of sIPSCs in
excitatory projection neurons and decreased GABA at inhibitory synapses [56]. Recording sIPSCs from the soma of excitatory neurons in the BLA, we have recently shown that these inhibitory neurotransmission deficits are preceded by dynamic changes in inhibition at P10 and 14. Furthermore, we found that the dynamic alterations in inhibition over early postnatal development are accompanied by functional changes in GABA\(_A\)Rs containing the \(\alpha1, 2,\) and 3 subunits [57, unpublished data].

1.4 Defects in Inhibitory Neurotransmission in FXS Mouse Model

Inhibitory neurotransmission precedes excitation in early postnatal development and plays a crucial role in shaping neuronal connections and in forming proper neural circuits [58]. Changes in expression of FMRP-regulated proteins in FXS could lead to alterations in synaptogenesis and, as a result, the precise timing of inhibitory neurotransmission during narrow critical periods in early postnatal development. In this study, we sought to illuminate any differences in the early postnatal developmental trajectory of the \(\alpha1, 2,\) and 3-containing GABA\(_A\)Rs, gephyrin, and GAD65/67—inhbitory synapse molecules responsible for GABAergic neurotransmission (Figure 1 B) [32-37, 59-61]. Changes in expression of different GABA\(_A\)Rs have in fact been observed in other areas of the brain in FXS flies and mice at different developmental stages [73, 82, 83, 85, 86]. In light of this evidence and our previous functional findings, we hypothesized that the absence of FMRP leads to changes in inhibitory neurotransmission early in postnatal development through altered expression of proteins responsible for GABAergic neurotransmission.
Our results indicate that, although expression of tested inhibitory synapse molecules was not significantly changed in KO versus WT mice, the precise timing of the expression was altered in KOs for a subset of proteins throughout early postnatal development. These results point to dynamic alterations in GABAergic protein expression which eventually lead to deficits in inhibitory neurotransmission in the BLA during the third week of life.
Chapter 2: Results

2.1 Functional Deficits in Inhibitory Neurotransmission in the Developing Fmr1 KO Mouse BLA

We have previously shown inhibitory neurotransmission deficits in the BLA in KO mice at P21 [56]. Recording from excitatory neuron soma in the BLA, we observed dynamic changes in the sIPSC amplitude and frequency in early postnatal development. At P10 and P21, we observed a statistically significant decrease in sIPSC amplitude and frequency while, intriguingly, we saw an increase in inhibition at P14 (Figure 1 C-D) [57, unpublished data]. Since α1, 2, and 3-containing GABA receptors are highly enriched on the post-synaptic surface and are major players in phasic inhibition [35, 62-64], we wanted to examine whether there are alterations in function of these GABA receptors. We took advantage of benzodiazepine drugs zolpidem, a GABA receptor α1 agonist, and clonazipam, a GABA receptor α2/3 agonist (Figure 2 A) [65-67]. We found that in the KO mice, there were deficits in GABA receptor α1 function at P16 and P21 while there were deficits in GABA receptor α2/3 function at P10. Interestingly, GABA receptor α2/3 function surpassed WT levels at P21 [57, unpublished data] (Figure 2 B-C). This data led us to the hypothesis that there might be alterations in GABA receptor subunit expression during early postnatal development in KO mice.
Figure 2. Functional deficits in α1, 2, and 3 subunit-containing GABAA Rs.  

A

The GABAA R is a pentamer containing a site where benzodiazepine drugs can bind and act as agonists.  

B Benzodiazepine drug zolpidem, specifically targeting α1 subunit-containing GABAA Rs, fails to enhance receptor function at P16 and P21.  

C Benzodiazepine drug clonazepam, specifically targeting α2/3 subunit-containing GABAA Rs, fails to enhance receptor function at P10 while increasing receptor function at P21.  error bars: mean±SEM; *p<.05, **p <0.005 (unpaired, two-tailed t-test)
2.2 Expression of GABA<sub>A</sub>R α1 Subunit Protein in the BLA of WT and Fmr1 KO Mice

To determine if changes in GABA<sub>A</sub>R function reflect alterations in protein expression, we examined the expression of GABA<sub>A</sub>R subunits α1, α2, and α3. Immunohistochemistry analysis was conducted at P10, 14, and 21 and revealed α1 subunit expression in the BLA at all three developmental time points. There was no qualitative difference in α1 expression in KO versus WT mice except at P21 where KO mice showed a decrease in the subunit (Figure 3 A-F). Quantification of α1 protein expression by immunoblotting did not reveal a statistically significant difference in KO versus WT α1 expression at each tested time point (Figure 3 G-H). However, within-genotype analysis over early postnatal development revealed differences in the timing of specific changes in protein expression in KO mice. In WT mice, α1 expression increased significantly from P14 to P21 (p=.048) while, in KO mice, the increase occurred from P10 to P14 (p=.016). Both genotypes show an increase in α1 from P10 to P21 (WT: p=.024, KO: p=.029). Our results suggest that defects in timing of α1 expression could be involved in changes in inhibitory neurotransmission over early postnatal development in KO mice.
Figure 3. Developmental trajectory of GABA$_{A}$R $\alpha$1 subunit protein expression.

A-F GABA$_{A}$R $\alpha$1 antibody staining of 50µm thick P10-P21 WT and KO mice brain sections reveals $\alpha$1 subunit protein expression in the BLA throughout development. G Immunoblot of protein from whole BLA tissue shows the amount of $\alpha$1 protein at P10, P14, and P21. H Quantification of the $\alpha$1 subunit, using actin as a loading control, reveals a statistically significant increase in the protein from P10 to P21 and P14 to P21 in WT mice. In KO mice, the developmental increase in expression is significant from P10 to P14 and P10 to P21. However, there is no statistically significant difference in KO versus WT expression at each developmental time point. Scale bar=200 µm, n=3 (each n is a pool of 3 BLAs), error bars: mean±SEM, *p<.05 (paired, two-tailed t-test), ns=not significant.

2.3 Expression of GABA$_{A}$R $\alpha$2 Subunit Protein in the BLA of WT and Fmr1 KO Mice
Using immunohistochemistry, we revealed that the α2-containing GABA<sub>A</sub>Rs are expressed throughout early postnatal development in the BLA. Qualitative assessment of protein expression revealed no statistically significant difference in α2 expression in KO versus WT mice except at P21 where the protein seemed to be increased in the KO mouse BLA (Figure 4 A-F). Immunoblot quantification of the α2 subunit demonstrated that there was no significant difference in protein expression between WT and KO mice at each time point. When the developmental trajectory of α2 was examined in WT mice, α2 expression did not increase significantly over development. In KO mice, however, the α2 significantly increased from P10 to P21 (p=.014), pointing to an early deficiency in the protein at P10 and/or to an increase in expression at P21 (Figure 4 G-H). This finding points to the α2 subunit involvement in observed inhibitory neurotransmission alterations in the KO mouse BLA.
Figure 4. Developmental trajectory of GABA$_A$R $\alpha$2 subunit protein expression.

A-F GABA$_A$R $\alpha$2 antibody staining of 50µm thick P10-P21 WT and KO mice brain sections reveals $\alpha$2 subunit protein expression in the BLA throughout development. 

G Immunoblot of protein from whole BLA tissue shows the amount of $\alpha$2 protein at P10, P14, and P21. 

H Quantification of $\alpha$2 subunit, using actin as a loading control, reveals a statistically significant increase in expression from P10 to P21 in KO mice. However, there is no statistically significant difference in KO versus WT expression at each developmental time point. scale bar=200 µm, n=3 (each n is a pool of 3 BLAs), error bars: mean±SEM, *p<.05 (paired, two-tailed t-test)
2.4 Expression of GABA₆R α3 Subunit Protein in the BLA of WT and Fmr1 KO Mice

We demonstrated that the α3 subunit is prominently expressed throughout early postnatal development in the BLA. Qualitatively, α3 protein seemed to be increased in expression at P10 and P14 in KO mice (Figure 5 A-F). However, quantification of α3 protein revealed that there was no statistically significant change in expression at all tested time points in KO versus WT mice. In addition, α3 expression timing did not significantly change over development in WT mice nor was it altered in KO mice (Figure 5 G-H). This result suggests that the α3 subunit does not seem to play a role in inhibitory neurotransmission defects seen in KO mice.

Figure 5. Developmental trajectory of GABA₆R α3 subunit protein expression.

A-F GABA₆R α3 antibody staining of 50µm thick P10-P21 WT and KO mice brain
sections reveals α3 subunit protein expression in the BLA throughout development. G Immunoblot of protein from whole BLA tissue shows the amount of α3 protein at P10, P14, and P21. H Quantification of α3 subunit, using actin as a loading control, reveals no statistically significant change in protein expression within each genotype over the tested time points. There is also no statistically significant difference in KO versus WT expression at each developmental time point. scale bar=200 µm, n=3 (each n is a pool of 3 BLAs), error bars: mean±SEM, *p<.05 (paired, two-tailed t-test)

2.5 Expression of Gephyrin Protein in the BLA of WT and Fmr1 KO Mice

Since gephyrin was demonstrated to directly interact with synaptic GABA_ARs [60, 61, 68-71], we wanted to examine any alterations in gephyrin protein expression in KO mice. We found that gephyrin is expressed in the BLA during the tested time points. We furthermore found that gephyrin staining was qualitatively less intense at P14 in KO mice (Figure 6 A-F). After immunoblot quantification, we did not find any significant differences in gephyrin expression in KO versus WT mice at each time point. In WT mice, gephyrin expression increased from P14 to P21 (p=.03) while KO mice failed to show this increase (Figure 6 G-H). Our observation reflects the developmental expression pattern of the α1 subunit in KO mice. Both gephyrin and the α1 subunit do not show a normal increase from P14 to P21 in KO mice, pointing to a possible link between the two proteins in the manifestation of inhibitory neurotransmission deficits.
Figure 6. Developmental trajectory of gephyrin protein expression.

A-F Gephyrin antibody staining of 50µm thick P10-P21 WT and KO mice brain sections reveals gephyrin protein expression in the BLA throughout development. G Immunoblot of protein from whole BLA tissue shows the amount of gephyrin at P10, P14, and P21. H Quantification of gephyrin, using actin as a loading control, reveals a statistically significant increase in the protein from P14 to P21 in WT mice but not KO mice. However, there is no statistically significant difference in KO versus WT expression at each developmental time point. scale bar=200 µm, n=3 (each n is a pool of 3 BLAs), error bars: mean±SEM, *p<.05 (paired, two-tailed t-test)
2.6 Expression of GAD65/67 Proteins in the BLA of WT and Fmr1 KO mice

To discern whether presynaptic changes contribute to defects in inhibitory neurotransmission, we examined GAD65/67 expression in the BLA. Immunohistochemistry revealed that GAD65/67 expression was prominent at all developmental stages in WT and KO mice. Qualitatively, there was no difference in GAD65/67 expression in KO versus WT mice (Figure 7 A-F). Protein quantification revealed that GAD65/67 expression increased in WT as well as the KO mice from P10 to P14 (WT: p=.01, KO: p=.005), P14 to P21 (WT: p=.025, KO: p=.013), and P10 to P21 (WT: p=.0003, KO: p=.002) (Figure 7 G-H). There was also no statistically significant change in expression at each time point in KO versus WT mice. Our results suggest that proteins GAD65/67 do not play a role in inhibitory neurotransmission changes we see in the BLA.
Figure 7. Developmental trajectory of GAD65/67 protein expression. A-F GAD65/67 antibody staining of 50µm thick P10-P21 WT and KO mice brain sections reveals GAD65/67 protein expression in the BLA throughout development. G Immunoblot of protein from whole BLA tissue shows the amount of GAD65/67 protein at P10, P14, and P21. H Quantification of GAD65/67, using actin as a loading control, reveals a statistically significant increase in the protein in WT and KO mice throughout early postnatal development. However, there is no statistically significant difference in KO versus WT expression at each developmental time point. Scale bar=200 µm; n=3 (each n is a pool of 3 BLAs); error bars: mean±SEM; *p<.05, **p <.005 (paired, two-tailed t-test)
Chapter 3: Discussion

3.1 GABA\(_A\)R \(\alpha1\) and Gephyrin Contribution to Defects in Inhibitory Neurotransmission in the Fmr1 KO BLA

Although we failed to find statistically significant changes in \(\alpha1\) expression in KO versus WT mice at each time point, we did find changes in how the protein progresses in expression over development in KO as compared to WT mice. The correct timing of GABAergic protein expression is essential in setting up proper neuronal connections and in wring up local neuron networks into correct brain circuits [70-73]. Failure in precise timing of protein expression in the BLA could lead to behavioral deficits we see in FXS patients, such as increased fear and anxiety. Although \(\alpha1\) expression in WT mice was similar to the expression in KO mice at P14, we only observed an increase in \(\alpha1\) expression from P10 to P14 in KO mice, an event which is reflected in the normal zolpidem drug enhancement and an increase in inhibitory neurotransmission at P14. Furthermore, the fact that \(\alpha1\) expression increases from P14 to P21 in WT mice but not in KO mice is reflected in the failure of zolpidam to enhance GABA\(_A\)R \(\alpha1\) function and in the decrease in inhibitory neurotransmission at P21 in KO mice.

Because there was no increase in \(\alpha1\) and gephyrin expression in KO mice from P14 to P21, there is a possibility that the homeostatic effort to increase these proteins in the post-synaptic membrane failed due to a smaller number of existing synapses at P21 [56]. The decrease in \(\alpha1\)-containing GABA\(_A\)R clusters on the postsynaptic surface could therefore be a reason for the decrease in inhibitory neurotransmission. Decrease in inhibition at P10 in KO mice could possibly be
explained by P10 and/or pre-P10 alterations in α2 or α3 expression. Furthermore, the increase in inhibition observed at P14 could be accounted for by homeostatic compensation in α2 protein after P10 and up to P14 [74-76]. It is most likely no coincidence that the culmination of the proposed homeostatic response occurs around P14, a peak time in synaptogenesis [77]. It is important to note that FMRP has not been found to directly target mRNA of the tested proteins and, as a result, translational regulation of these proteins likely occurs under different mechanisms which are accessible to homeostatic modifications.

3.2 GABA_4R α2 and α3 Contribution to Defects in Inhibitory Neurotransmission in the Fmr1 KO BLA

We did not observe significant changes in α2 and α3 subunit expression in KO versus WT mice at each time point; however, we detected differences in timing of α2 but not α3 subunit expression in KO mice. While α2 expression was unchanged over time in WT mice, there was a statistically significant increase in the α2 subunit from P10 to P21 in KO mice. This increase could be a consequence of an early deficiency in α2 at P10 and/or to an increase in expression at P21. The result matches our clonazepam drug data for KO mice, where the enhancement of α2/3 subunit-containing GABA_4R function undershoots at P10 and overshoots at P21 [57]. However, the α2/3 enhancement in function at P21 is not reflected in the strength of inhibitory neurotransmission which is significantly decreased. These seemingly contradictory results could be interpreted in the following ways: 1) although α2/3 function is intact and even greater in KOs at P21, the enhanced function is not enough
to compensate for the inhibitory neurotransmission deficits due to the functional defects and alterations in timing of expression of α1-containing GABA_ARS, 2) there is a decrease in GABA available at the synaptic cleft which, according to our findings, is not related to a decrease in GAD65/67 proteins, 3) since we have previously observed a decrease in inhibitory synapses in the P21 BLA, the enhancement of α2/3-containing GABA_ARS function could be due to the increased insertion of these receptors into the postsynaptic membrane in the existing synapses, and 4) the combination of the three scenarios could be at play. It has recently been demonstrated that α3-containing GABA_ARS are predominantly found in the extrasynaptic space on BLA excitatory neurons, participating mainly in tonic inhibition [79]. Another interpretation of our data showing increased function of α2/3-containing GABA_ARS at P21 in KO mice could be that due to the lack of the α1 subunit expression, α3 subunit replaces it at the postsynaptic membrane. Perhaps because of the lower affinity of GABA for the α2 and α3-containing receptors, inhibitory neurotransmission could not recover to the WT levels.

3.3 GAD65/67 Contribution to Defects in Inhibitory Neurotransmission in the Fmr1 KO BLA

We did not observe differences in GAD65/67 expression in KO versus WT animals at the tested time points nor did we observe changes in timing of expression in KO mice. Our findings therefore suggest that GAD65/67 did not contribute to the changes in inhibitory neurotransmission. However, deficits in GAD65/67 expression prior to P10 could have contributed to setting up the inhibitory deficits we see at P10.
At tested time points, inhibitory neurotransmission changes seem to be primarily driven by other factors. For instance, we observed an increase in inhibitory neurotransmission at P14 in KO mice but we did not see an increase in GAD65/67 protein expression. In addition, we did not observe a change in GAD65/67 expression at P21, yet there was a significant decrease in inhibition at that time point.

Since we have previously shown that there is a decrease in inhibitory synapses at P21, we can speculate that there was perhaps a homeostatic increase in GAD65/67 within the remaining inhibitory synapses. However, for a reason yet to be uncovered, the effort to maintain a certain level of inhibition failed at P21 despite the enhanced function of $\alpha_2/3$-containing GABA$_A$Rs, and was accompanied by a decrease in GABA at inhibitory synapses. Nonetheless, a decrease in GABA at synapses could have been caused by other downstream factors not involving GAD65/67 such as: 1) decrease in vesicle GABA content, 2) decrease in the number of docked and primed vesicles at the synaptic cleft, and 3) decrease in the amount of GABA released form vesicles in response to activity. One reason for a decrease in GABA release could have been a homeostatic response to strong enhancement in inhibition around P14. However, perhaps because of the loss of $\alpha_1$-containing GABA$_A$R function and because of the decrease in inhibitory synapses at P21, inhibitory neurotransmission decreased to levels below WT mice. Together, our results demonstrate that the events culminating into decreased inhibitory neurotransmission in KO mice are very dynamic in nature.
3.4 Conclusion and Implications for FXS Patient Therapy

Our results indicate that there are functional and protein changes in inhibitory neurotransmission in the BLA early in postnatal development. We have shown that α1 and α2/3-containing GABAARs have functional deficits at particular developmental time points [57]. On a protein level, we discovered that inhibitory synapse molecules GABAAR α1, GABAAR α2, and gephyrin have altered timing of expression in KO mice but do not significantly change in expression at each time point in KO versus WT mice. Existing studies concerning GABAergic protein expression and function have, for the most part, been conducted in adult FXS mice and have not included the amygdala and, therefore, our research has contributed and important piece to the FXS puzzle [73, 83, 85, 86]. Although we did not study developmental time points beyond P21, it would be interesting to see how these early deficits in inhibition influence GABAergic protein expression and function in adult FXS mice. Studying later developmental stages would be beneficial for examining long-term outcomes of drug therapies administered at early developmental stages.

Benzodiazepine drugs such as diazepam have been used in FXS patients to alleviate symptoms including increased fear and anxiety [67, 80]. Upon the discovery that different GABAAR subunits are responsible for unique subsets of diazepam effects, newer drugs such as zolpidam and clonazepam were developed, targeting specific subunits to minimize unnecessary side effects [67]. Our data suggests that these drugs could be used to treat some of the amygdala-associated FXS symptoms but the specific benzodiazepine drugs used and the timing of usage will have to be
carefully planned. For instance, since α1 expression and function appears to be normal at P10, zolpidem could be used to enhance inhibitory neurotransmission around 6 months after birth in humans which is comparable to mouse P10 [84, 87, 92]. Benefits of using zolpidem could be limited since α1 expression is relatively low in the BLA very early in development [78]. However, this initial boost in inhibition from zolpidam, no matter how small, could aid in alleviating increased fear and anxiety later in development. Clonazepam could be a more beneficial drug in humans around 5 to 10 years of age comparable to mouse P14 through P21, since α2/3-containing receptors are as or more functional and since both subunits are normally expressed at these stages in KO mice [84, 87, 92]. Dose-dependent studies will have to be performed in mice to assess functional benefits and side effects of this therapy. Even if zolpidem and clonazepam therapy proves to be beneficial in mice, another challenge will be adapting the therapy effectiveness to humans while minimizing adverse effects. Recent human and rodent studies have demonstrated that the use of GABA_A subunit-selective allosteric compounds modulating receptor function via the benzodiazepine site could substantially improve therapy outcomes in anxiety disorders while greatly decreasing side effects [67]. Collectively, our data support the idea that certain GABA_A subunit-selective benzodiazepine drugs could be effective in improving some of the amygdala-based FXS behavioral symptoms.

In addition, non-benzodiazepine drugs have been shown to be effective in treating symptoms such as hyperactivity and heightened fear in mice. For example, the neuroactive steroid ganaxolone, a δ subunit-containing GABA_A agonist, has been shown to be effective in decreasing anxiety, stress, and neuronal hyperexcitability
in FXS mice and is in the beginning stages of a clinical trial [80, 88]. Another δ subunit-targeting drug, the superagonist THIP, has been show to decrease hyperactivity, neuronal hyperexcitability, and sensitivity to auditory stimuli in FXS mice [56, 81]. Benzodiazepine drugs and other GABA A R-targeting drugs such as THIP and ganaxolone could be used in combination and at different developmental stages to enhance both phasic and tonic inhibition. GABA B R-targeting drugs have also been successful in ameliorating amygdala-based FXS symptoms such as social withdrawal in humans, and one such drug, arbaclofen, has reached stage II in human clinical trials [90, 91]. Taking into consideration the drugs’ known side effects, their GABA receptor specificity, and the best time for their administration, could help doctors design appropriate drug therapies that could have a major impact on FXS patient prognosis.
Chapter 4: Experimental Procedure

4.1 Animals

Fmr1 WT (stock no. 4828) and Fmr1 KO (Fmr1<sup>−/−</sup>) (stock no. 4624) mice on the FVB background were obtained from the Jackson Laboratory (Bar Harbor, MA, USA). In this study, mice at postnatal day 10, 14, and 21 were used for experiments. All protocols were approved by the animal committee at Children’s National Medical Center.

4.2 BLA Microdissection and Protein Isolation

WT and KO mice at P10, 14, and 21 were euthanized with an overdose of isoflurane. The brains were removed and placed in ice-cold 1X PBS. They were then cut into 300µm coronal slices using a vibratome. Slices containing the BLA (3-5 slices) were carefully dissected under the microscope using microscissors. The BLA tissue was immediately placed into RIPA buffer (Santa Cruz, sc-24948) and incubated on ice for 15 minutes. The tissue was homogenized by mixing the RIPA buffer and the tissue using a pipette until the tissue was completely dissolved. Homogenized tissue was sonicated for 5 minutes using a water sonicator filled with ice-cold water. The samples were then incubated on ice for 10 minutes before they were centrifuged for 3 minutes at 13,200 rpm at 4°C. The supernatant was transferred into new tubes and stored at −20°C for further use.
4.3 Immunoblotting, Quantification, and Statistics

Sample protein concentrations were determined using the BCA protein assay (Pierce, 23225). For each sample, 2 µg (for GABAAR α2, GABAAR α3, and GAD5/67) or 5 µg (for GABAAR α1) of protein was loaded on a 4-15% gradient SDS-polyacrylamide gel (Biorad, 456-1084) and transferred onto a PVDF membrane (Millipore, IPVH20200). Each sample was a pool of 3 BLA homogenates and three gels were run for each protein tested. The proteins were visualized using appropriate primary and secondary antibodies, ECL (Pierce, 32106), and Kodak film (VWR, 8689358). The film was scanned and TIFF images were analyzed using the ImageJ software (NIH website: rsbweb.nih.gov/ij/). Actin was used as a loading control to quantify the amount of protein on each blot. After samples were normalized to actin, positive control homogenate from three P21 hippocampi (2 or 5 µg) was used in order to normalize the amount of protein across different blots. Three values for each protein were averaged and standard error was calculated. Microsoft Excel was used to graph results and to perform statistical analysis. Two different statistical tests were used to analyze the results. First, a two-tailed, unpaired t-test was used to determine if there was a statistically significant difference between WT and KO mice at each time point. Second, a two-tailed, paired t-test was performed to analyze within-genotype progression of protein expression over development. The p-value was set to less than .05 (p<.05) or .005 (p<.005), and the error bars were defined as mean±SEM.
4.4 Immunohistochemistry

Mice at P10, 14 and 21 were deeply anesthetized with isoflurane and perfused through the heart with 4% paraformaldehyde. The brains were isolated and placed in 4% paraformaldehyde for up to 24 hours. The brains were washed with 1X PBS, mounted in 4% low-melting agarose, and cut in 50 µm thick coronal sections on a vibratome. Free-floating sections containing the BLA were blocked in 1X PBS, with .02% Triton X and 10% Normal Donkey serum. This solution was used for all subsequent antibody incubations. Blocked sections were then incubated with primary antibody overnight at room temperature. After three 10 minute washes with 1X PBS and .02% Triton X, the sections were incubated with secondary antibody for 2 hours at room temperature, and were protected from light. Sections were placed on positively charged slides, dried for 5 minutes, and mounted with CC/Mount (Sigma-Aldrich, C9369). 10X magnification pictures of the BLA were taken in the TIFF format on a Zeiss fluorescent microscope.

4.5 Antibodies

For immunoblotting, the following primary antibodies were used: GABA_{AR} \alpha1, 1:1000 (Millipore, 106-868); GABA_{AR} \alpha2, 1:250 (Abcam, ab72445); GABA_{AR} \alpha3, 1:1,000 (Sigma, G4291); gephyrin, 1:500 (Synaptic Systems, 147 003); GAD65/67, 1:5,000 (Abcam, ab11070); and actin, 1:5,000 (Millipore, MAB1501). The secondary antibody, goat rabbit polyclonal HRP-conjugated Ab, was obtained from Santa Cruz, 1:10,000 (SC-2004). For immunohistochemistry, the same primary
antibodies were used except GAD65/67 which was purchased from Millipore, 1:500 (AB1511). Dilutions used for primary antibodies were as follows: GABA$_{\alpha}$R $\alpha$1, 1:1000; GABA$_{\alpha}$R $\alpha$2, 1:250; GABA$_{\alpha}$R $\alpha$3, 1:200; and gephyrin 1:250. The secondary antibody, DyLight donkey anti-rabbit 549, was obtained from Jackson Immunoresearch, 1:100 (711-505-152).
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