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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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2012

Advisory Committee:
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Dedication

This research is dedicated to children affected by neurodevelopmental disorders and their families.

Acknowledgements

I would like to thank my advisor Dr. Joshua Corbin and my committee members Dr. Betsy Quinlan and Dr. Catherine Carr for their guidance and support with my research during my time in graduate school. I would also like to thank my colleagues at Children's National Medical Center for their company, technical expertise, and their experimental guidance. I am greatly indebted to my amazing friends for their unconditional support, kindness, and generosity which kept me reaching for my goal despite many setbacks. Finally, I would like to thank my family for their emotional support and their unshakable faith in me through the hurdles in my graduate school career.

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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 show 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.

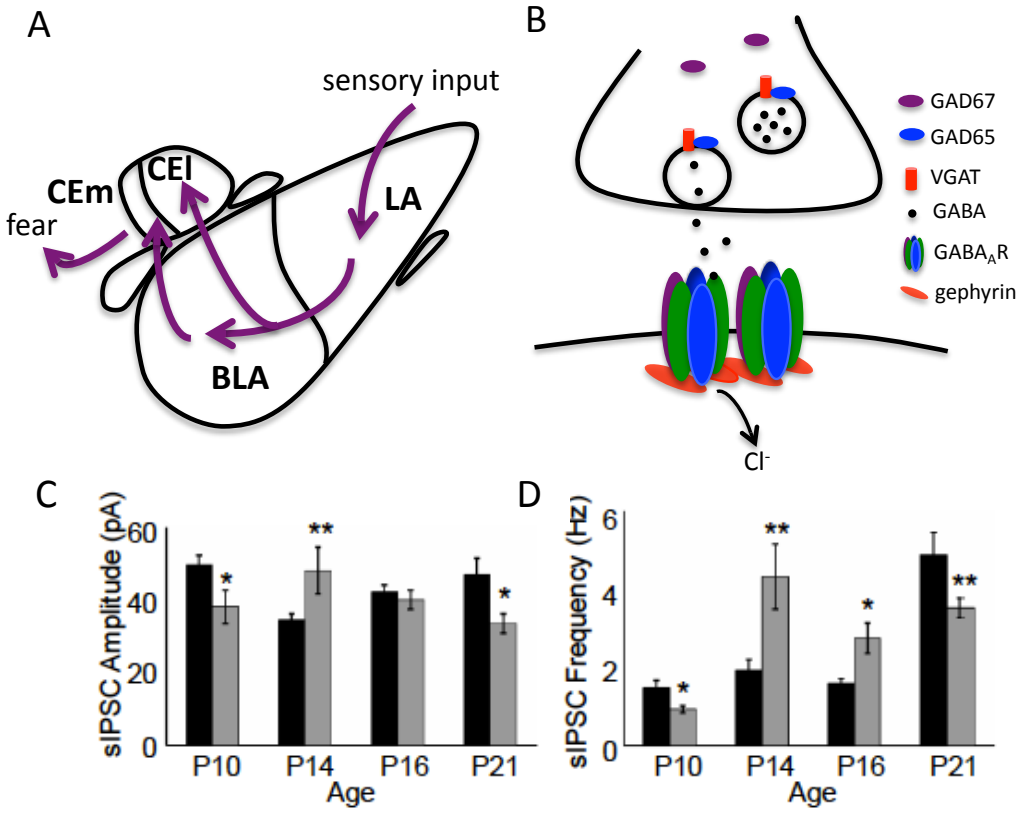

Figure 1. Alterations in inhibitory neurotransmission in the developing Fragile X Syndrome BLA. **A** Movement of sensory input through the amygdala is controlled by inhibitory neurons which determine the intensity of sensory output such as fear. LA: lateral amygdala, BLA: basolateral amygdala, CEl: 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 α1, 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 α1, 2, and 3-containing $GABA_A$Rs, gephyrin, and GAD65/67—inhibitory 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 $GABA_A$Rs 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 $GABA_A$Rs. We took advantage of benzodiazepine drugs zolpidem, a $GABA_A$R α1 agonist, and clonazipam, a $GABA_A$R α2/3 agonist (Figure 2 A) [65-67]. We found that in the KO mice, there were deficits in $GABA_A$R α1 function at P16 and P21 while there were deficits in $GABA_A$R α2/3 function at P10. Interestingly, $GABA_A$R 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 $GABA_A$R subunit expression during early postnatal development in KO mice.

Figure 2. Functional deficits in α1, 2, and 3 subunit-containing $GABA_A$Rs.

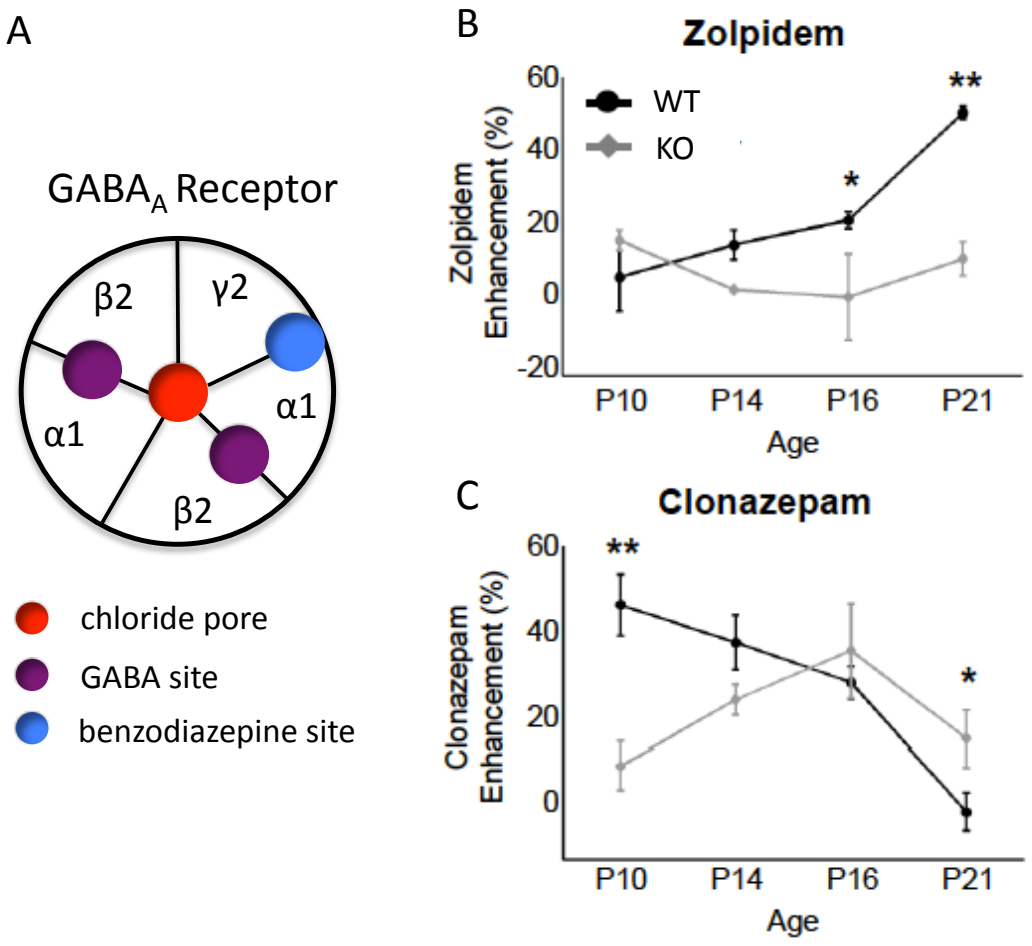

Figure 2. Functional deficits in α1, 2, and 3 subunit-containing $GABA_A$Rs. A The $GABA_A$R is a pentomer containing a site where benzodiazepine drugs can bind and act as agonists. **B** Benzodiazepine drug zolpidem, specifically targeting α1 subunit-containing $GABA_A$Rs, fails to enhance receptor function at P16 and P21. **C** Benzodiazepine drug clonazepam, specifically targeting α2/3 subunit-containing $GABA_A$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 $GABA_AR$ α1 Subunit Protein in the BLA of WT and Fmr1 KO Mice

To determine if changes in $GABA_AR$ function reflect alterations in protein expression, we examined the expression of $GABA_AR$ 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 $GABA_AR$ α1 subunit protein expression.

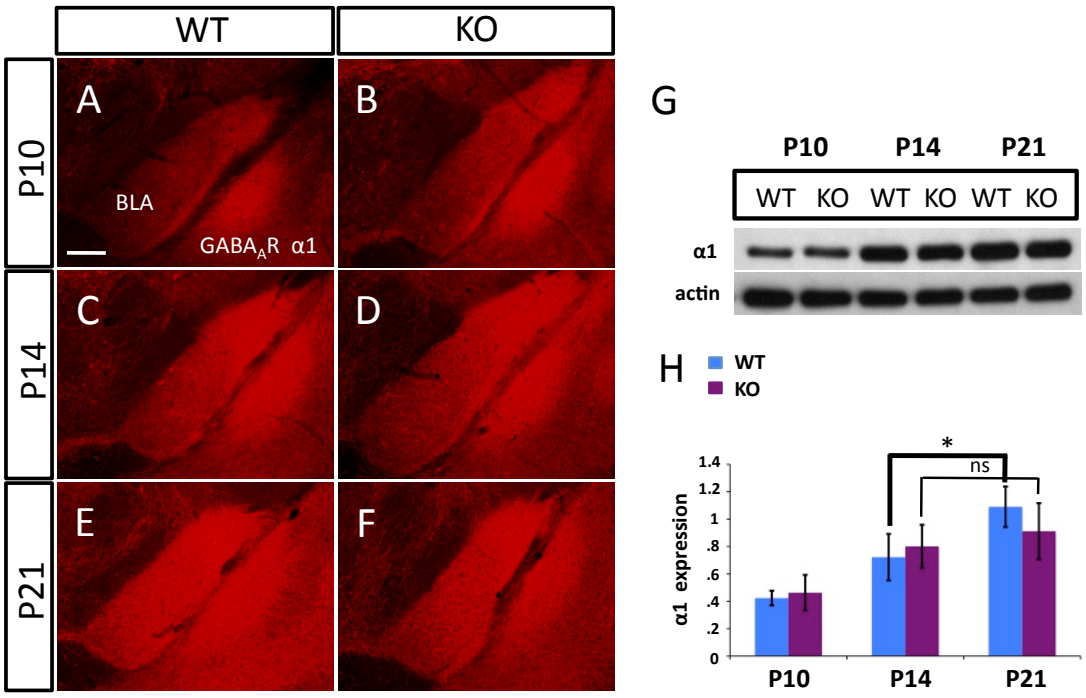

Figure 3. Developmental trajectory of $GABA_AR$ α1 subunit protein expression.

A-F $GABA_AR$ α1 antibody staining of 50μm thick P10-P21 WT and KO mice brain sections reveals α1 subunit protein expression in the BLA throughout development. **G** Immunoblot of protein from whole BLA tissue shows the amount of α1 protein at P10, P14, and P21. **H** Quantification of the α1 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 $GABA_AR$ α2 Subunit Protein in the BLA of WT and Fmr1 KO Mice

Using immunohistochemistry, we revealed that the α2-containing $GABA_A$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 $GABA_AR$ α2 subunit protein expression.

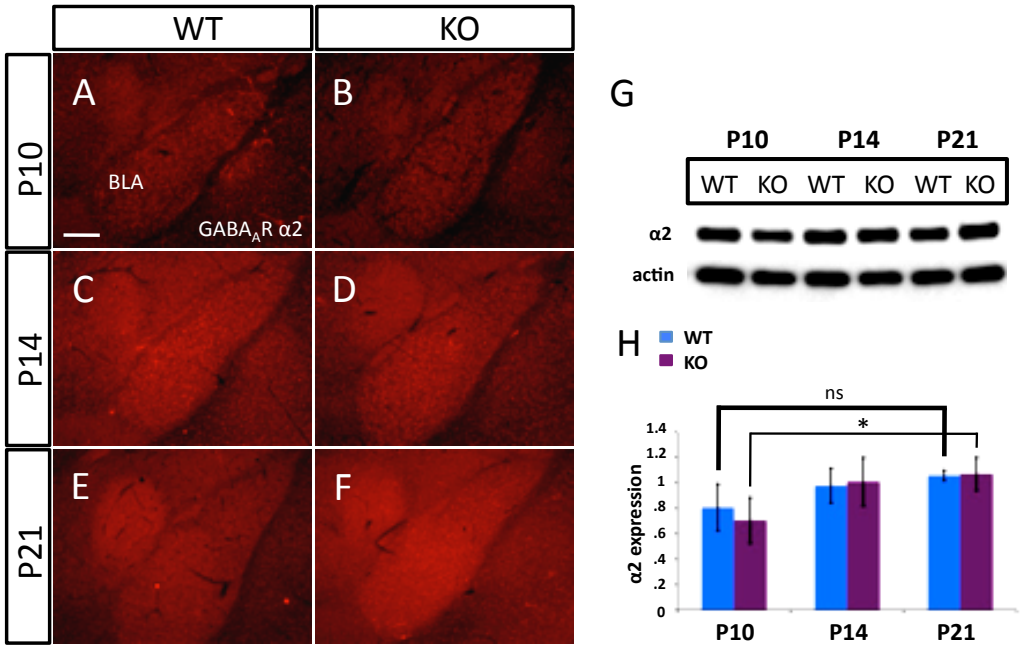

Figure 4. Developmental trajectory of $GABA_AR$ α2 subunit protein expression. **A-F** $GABA_AR$ α2 antibody staining of 50μm thick P10-P21 WT and KO mice brain sections reveals α2 subunit protein expression in the BLA throughout development. **G** Immunoblot of protein from whole BLA tissue shows the amount of α2 protein at P10, P14, and P21. **H** Quantification of α2 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 GABA$_A$R 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 GABA$_A$R α3 subunit protein expression.

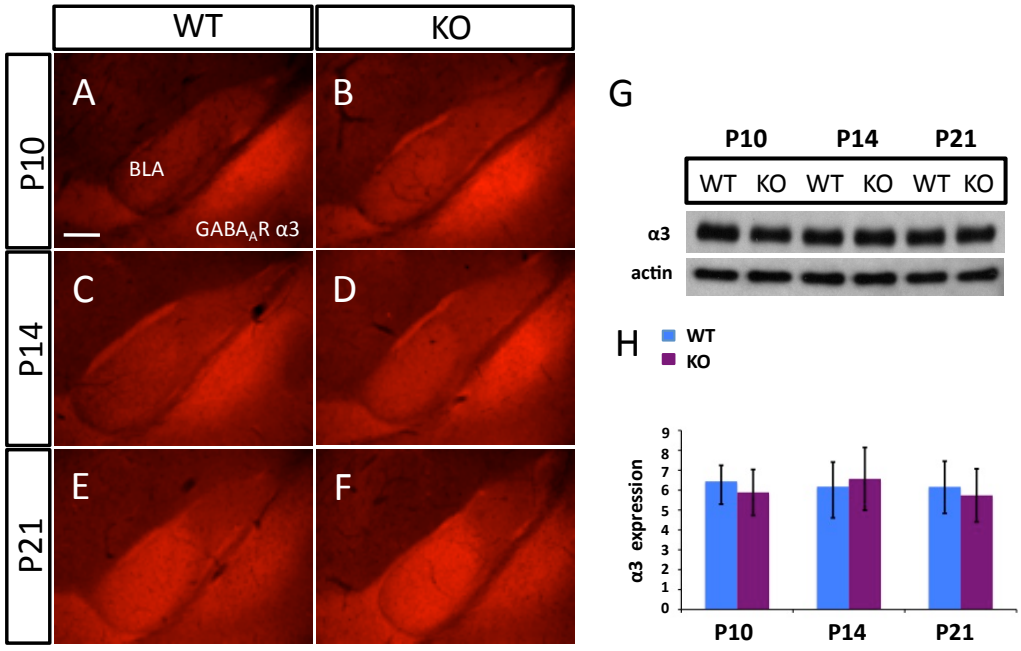

Figure 5. Developmental trajectory of GABA$_A$R α3 subunit protein expression.

A-F GABA$_A$R 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 $GABA_A$Rs [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.

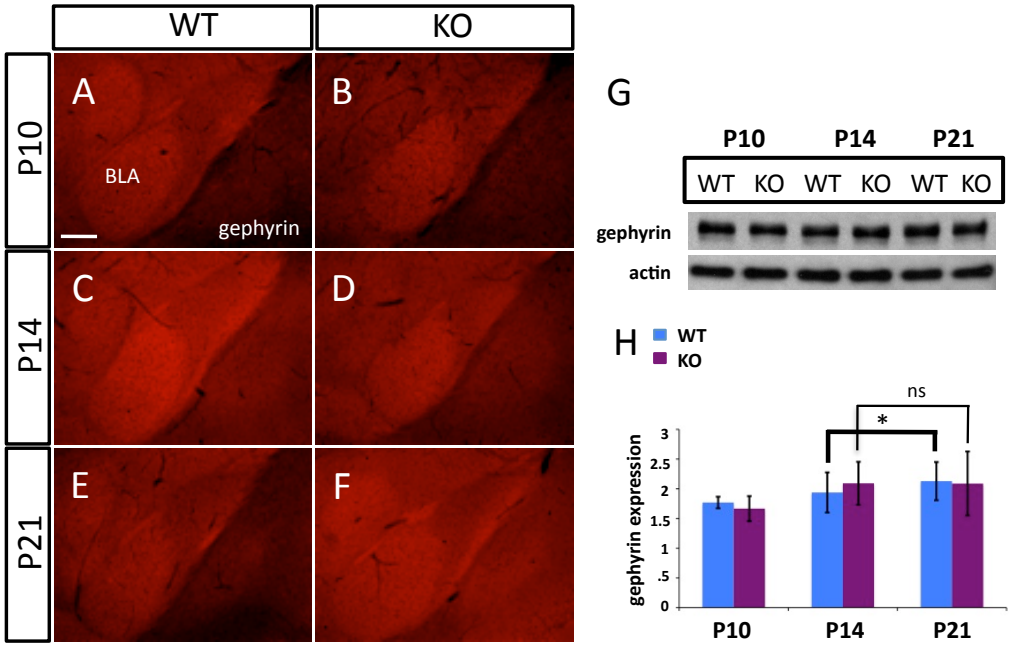

Figure 6. Developmental trajectory of gephyrin protein expression. A-F Gephyrin antibody staining of 50µm thick P10-P21 WT and KO mice brain sections reveals gephyrin protein expression in the BLA throughout development. **G** Immunoblot of protein from whole BLA tissue shows the amount of gephyrin at 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.

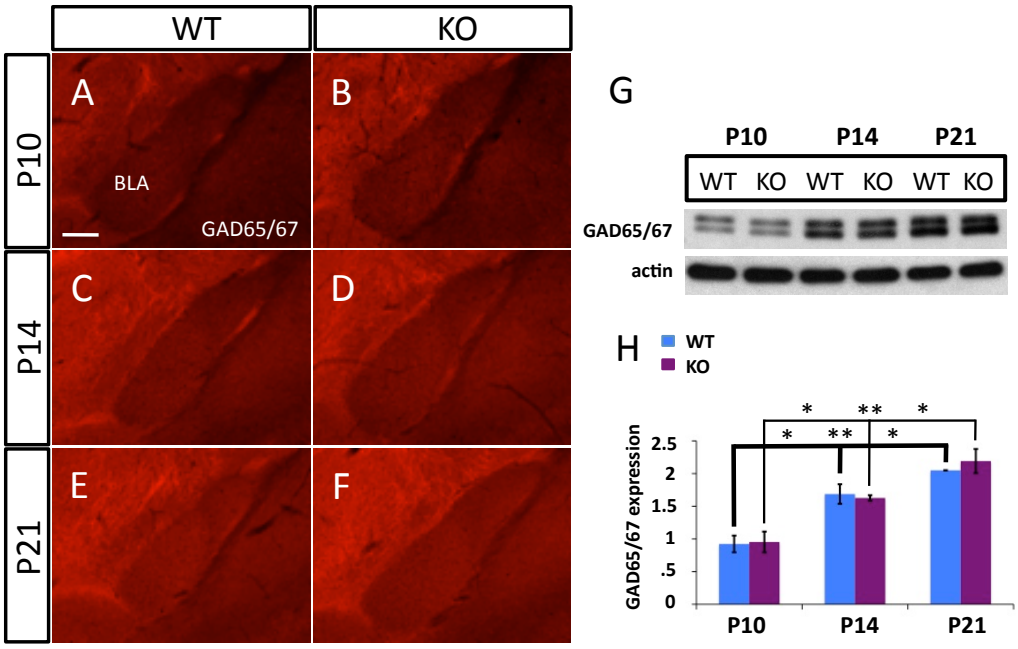

Figure 7. Developmental trajectory of GAD65/67 protein expression. A-F GAD65/67 antibody staining of 50µm thick P10-P21 WT and KO mice brain sections reveals GAD65/67 protein expression in the BLA throughout development. **G** Immunoblot of protein from whole BLA tissue shows the amount of GAD65/67 protein 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 <0.005 (paired, two-tailed t-test)

Chapter 3: Discussion

3.1 $GABA_AR$ α1 and Gephyrin Contribution to Defects in Inhibitory Neurotransmission in the Fmr1 KO BLA

Although we failed to find statistically significant changes in α1 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 α1 expression in WT mice was similar to the expression in KO mice at P14, we only observed an increase in α1 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 α1 expression increases from P14 to P21 in WT mice but not in KO mice is reflected in the failure of zolpidam to enhance $GABA_AR$ α1 function and in the decrease in inhibitory neurotransmission at P21 in KO mice.

Because there was no increase in α1 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 α1-containing $GABA_AR$ 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 $GABA_AR$ 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 $GABA_AR$ 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 $GABA_A$Rs, 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 $GABA_A$R 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 $GABA_A$Rs 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 $GABA_A$Rs 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 α2/3-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 α1-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 $GABA_A$Rs have functional deficits at particular developmental time points [57]. On a protein level, we discovered that inhibitory synapse molecules $GABA_A$R α1, $GABA_A$R 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 $GABA_A$R 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 $GABA_AR$ 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 date support the idea that certain $GABA_AR$ 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 $GABA_AR$ agonist, has been show 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 $GABA_AR$-targeting drugs such as THIP and ganaxolone could be used in combination and at different developmental stages to enhance both phasic and tonic inhibition. $GABA_BR$-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 ($Fmr1^{-/y}$) (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 Immunohistochemstry

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: $GABA_AR$ α1, 1:1000 (Millipore, 106-868); $GABA_AR$ α2, 1:250 (Abcam, ab72445); $GABA_AR$ α3, 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: $GABA_AR$ α1, 1:1000; $GABA_AR$ α2, 1:250; $GABA_AR$ α3, 1:200; and gephyrin 1:250. The secondary antibody, DyLight donkey anti-rabbit 549, was obtained from Jackson Immunoresearch, 1:100 (711-505-152).

References

1) Verkerk A., Pieretti M., Sutcliffe J.S., Fu Y.H., Kuhl D.P., Pizzuti A., Reiner O., Richards S., Victoria M.F., Zhang F.P., Eussen B.E., van Ommen G.B., Blonden L.A.J., Riggins G.J., Chastain J.L., Kunst C.B., Galjaard H., Caskey C.T., Nelson D.L., Oostra B.A., Warren S.T., 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65, 905–914.

2) Devys, D., Lutz, Y., Rouyer, N., Bellocq, J.P. and Mandel, J.L., 1993. The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat. Genet. 4, 335–340

3) Hinds, H.L., Ashley, C.T., Sutcliffe, J.S., Nelson, D.L., Warren, S.T., Housman, D.E. and Schalling, M., 1993. Tissue specific expression of FMR-1 provides evidence for a functional role in fragile X syndrome. Nat. Genet. 3, 36–43.

4) Wang H., Ku L., Osterhout D.J., Li W., Ahmadian A., Liang Z., Feng Y., 2004. Developmentally programmed FMRP expression in oligodendrocytes: a potential role of FMRP in regulating translation in oligodendroglia progenitors. Hum Mol Genet 13, 79–89.

5) Brown V., Jin P., Ceman S., Darnell J.C., O'Donnell W.T., Tenenbaum S.A., Jin X., Feng Y., Wilkin- son K.D., Keene J.D,. Darnell R..B., Warren ST., 2001.

Microarray identification of FMRP- associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell 107, 477–487.

6) Darnell J.C., Mostovetsky O., Darnell R.B., 2005. FMRP RNA targets: identification and validation. Genes Brain Behav 4, 341–349.

7) Darnell, J. C., Van Driesche, S. J., Zhang, C., Hung, K. Y. S., Mele, A., Fraser, C. E., Stone, E. F., et al., 2011. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. Cell, 146(2), 247-61.

8) Eberhart, D.E., Malter, H.E., Feng, Y., and Warren, S.T., 1996. The fragile X mental retardation protein is a ribonucleoprotein containing both nuclear localization and nuclear export signals. Hum. Mol. Genet. 5, 1083–1091.

9) Weiler, I.J., Irwin, S.A., Klintsova, A.Y., Spencer, C.M., Brazelton, A.D., Miyashiro, K., Comery, T.A., Patel, B., Eberwine, J. and Greenough, W.T., 1997. Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. Proc Natl Acad Sci 94, 5395–5400

10) Li Z. et al., 2001. The fragile X mental retardation protein inhibits translation via interacting with mRNA. Nucleic Acids Res 29, 2276–2283.

11) Laggerbauer B., Ostareck D., Keidel E.M., Ostareck-Lederer A., Fischer U.,

2001. Evidence that fragile X mental retardation protein is a negative regulator of translation. Hum Mol Genet 10:329–338.

12) Brown V., Jin P., Ceman S., Darnell J.C., O'Donnell W.T., Tenenbaum S.A., Jin X., Feng Y., Wilkinson K.D., Keene J.D., Darnell R.B., Warren S.T., 2001. Microarray identification of FMRP- associated brain mRNAs and altered mRNA translational profiles in fragile X syndrome. Cell 107, 477–487.

13) Miyashiro, K. Y., Beckel-Mitchener, A., Purk, T. P., Becker, K. G., Barret, T., Liu, L., Carbonetto, S., et al., 2003. RNA cargoes associating with FMRP reveal deficits in cellular functioning in Fmr1 null mice. Neuron, 37(3), 417-31.

14) Pfeiffer, B.E. and Huber, K.M., 2007. Fragile X mental retardation protein induces synapse loss through acute postsynaptic translational regulation. J Neurosci 27, 3120–3130.

15) Bassell G.J., Warren S.T., 2008. Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. Neuron 60, 201–214.

16) Liao, L., Park, S. K., Xu, T., Vanderklish, P., and Yates, J. R., 2008. Quantitative proteomic analysis of primary neurons reveals diverse changes in synaptic protein content in fmr1 knockout mice. Proc Natl Acad Sci, 105(40), 15281-6.

17) Kao, D.I., Aldridge, G. M., Weiler, I. J., & Greenough, W. T., 2010. Altered mRNA transport, docking, and protein translation in neurons lacking fragile X mental retardation protein. Proc Natl Acad Sci, 107(35).

18) Lachiewicz, A. M., Spiridigliozzi, G. A., Gullon, C. M., Ransford, S. N., and Rao, K., 1994. Aberrant behaviors of young boys with fragile X syndrome. Am J Ment Retard 98, 567–579.

19) Miller L.J., McIntosh D.N., McGrath J., Shyu V., Lampe M., Taylor A.K., Tassone F., Neitzel K., Stackhouse T., Hagerman R.J., 1999. Electrodermal responses to sensory stimuli in individuals with fragile X syndrome: a preliminary report. Am J Med Genet 83: 268–279.

20) Bailey, D. B., Jr., Hatton, D. D., Mesibov, G., Ament, N., and Skinner, M., 2000. Early development, temperament, and functional impairment in autism and fragile X syndrome. Am J Ment Retard, 30, 49–59.

21) Rogers, S. J., Wehner, E. A., and Hagerman, R., 2001. The behavioral phenotype in fragile X: Symptoms of autism in very young children with fragile X syndrome, idiopathic autism, and other developmental disorders. Res Autism Spectr Disord, 22, 409–417.

22) Kaufmann, W. E., Cortell, R., Kau, A. S. M., Bukelis, I., Tierney, E., Gray, R. M., et al., 2004. Autism spectrum disorder in fragile X syndrome: Communication, social interaction, and specific behaviors. Am J Med Genet A, 129A, 225–234.

23) Frankland, P. W., Wang, Y., Rosner, B., Shimizu, T., Balleine, B. W., Dykens, E. M., et al., 2004. Sensorimotor gating abnormalities in young males with fragile X syndrome and Fmr1-knockout mice. Mol Psychiatry, 9, 417–425.

24) Kau, A. S. M., Tierney, E., Bukelis, I., Stump, M. H., Kates, W. R., Trescher, W. H., and Kaufmann, W. E., 2004. Social behavior profile in young males with fragile X syndrome: Characteristics and specificity. Am J Med Genet A, 126A, 9–17.

25) Hatton, D. D., Sideris, J., Skinner, M., Mankowski, J., Bailey, D. B., Jr., Roberts, J., and Mirrett, P., 2006. Autistic behavior in children with fragile X syndrome. Prevalence, stability, and the impact of FMRP. Am J Med Genet A, 140A, 1804–1813.

26) Baranek GT et al., 2008. Developmental trajectories and correlates of sensory processing in young boys with fragile X syndrome. Phys Occup Ther Pediatr 28:79–98.

27) Sah, P., Faber, E. S. L., Lopez De Armentia, M., and Power, J., 2003. The amygdaloid complex: anatomy and physiology. Physiological Reviews, 83(3), 803-34.

28) Schultz, R. T., 2005. Developmental deficits in social perception in autism: the role of the amygdala and fusiform face area. International Society for Developmental Neuroscience, 23(2-3), 125-41.

29) Ehrlich, I., Humeau, Y., Grenier, F., Ciocchi, S., Herry, C., and Lüthi, A., 2009. Amygdala inhibitory circuits and the control of fear memory. Neuron, 62(6), 757-71.

30) Kleinhans, N. M., Richards, T., Weaver, K., Johnson, L. C., Greenson, J., Dawson, G., and Aylward, E., 2010. Association between amygdala response to emotional faces and social anxiety in autism spectrum disorders. Neuropsychologia, 48(12), 3665-70.

31) Haubensak, W., Kunwar, P. S., Cai, H., Ciocchi, S., Wall, N. R., Ponnusamy, R., Biag, J., et al., 2010. Genetic dissection of an amygdala microcircuit that gates conditioned fear. Nature, 468(7321), 270-6.

32) Fritschy, J. M., Johnson, D. K., Mohler, H. & Rudolph, U., 1998. Independent assembly and subcellular targeting of GABAA receptor subtypes demonstrated in mouse hippocampal and olfactory neurons in vivo. Neurosci. Lett. 249, 99–102

33) Klausberger, T., Roberts, J.D., and Somogyi,P, 2002. Cell type- and input-specific differences in the number and subtypes of synaptic $GABA_A$ receptors in the hippocampus. J Neurosci 22, 2513–2521.

34) Fritschy, J. M. and Brunig, I., 2003. Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. Pharmacol Ther 98, 299–323

35) Farrant, M., and Nusser, Z., 2005. Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nature reviews. Neuroscience, 6(3), 215-29.

36) Thomson, A. M., and Jovanovic, J. N., 2010. Mechanisms underlying synapse-specific clustering of GABA(A) receptors. Eur J of Neurosci, *31*(12), 2193-203.

37) Luscher, B., Fuchs, T., & Kilpatrick, C. L., 2011. GABAA receptor trafficking-mediated plasticity of inhibitory synapses. Neuron, 70(3), 385-409.

38) Dolen G., Bear M.F., 2008. Role for metabotropic glutamate receptor 5 (mGluR5) in the pathogenesis of fragile X syndrome. J Physiol (Lond) 586: 1502–1508.

39) Bear M.F., Huber K.M., Warren S.T., 2004. The mGluR theory of fragile X mental retardation. Trends Neurosci 27:370–377.

40) Yan Q.J., Rammal M., Tranfaglia M., Bauchwitz R.P., 2005. Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP. Neuropharmacology 49:1053–1066.

41) Nakamoto, M., Nalavadi, V., Epstein, M. P., Narayanan, U., Bassell, G. J., and Warren, S. T., 2007. Fragile X mental retardation protein deficiency leads to excessive mGluR5-dependent internalization of AMPA receptors. Proc Natl Acad Sci, 104(39), 15537-42.

42) Dolen, G., Osterweil, E., Rao, B. S. S., Smith, G. B., Auerbach, B. D., Chattarji, S., and Bear, M. F., 2007. Correction of fragile X syndrome in mice. Neuron, 56(6), 955-62.

43) Suvrathan, A., Hoeffer, C.A., Wong, H., Klann, E., & Chattarji, S., 2010. Characterization and reversal of synaptic defects in the amygdala in a mouse model of fragile X syndrome. Proc Natl Acad Sci, 107(25).

44) Hays S.A., Huber K.M., Gibson J.R., 2011. Altered Neocortical Rhythmic Activity States in Fmr1 KO Mice Are Due to Enhanced mGluR5 Signaling and Involve Changes in Excitatory Circuitry. J of Neurosci 31:14223–14234.

45) Comery T.A., Harris J.B., Willems P.J., Oostra B.A., Irwin S.A., Weiler I.J., Greenough W.T., 1997. Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. Proc Natl Acad Sci 94: 5401– 5404.

46) Nimchinsky, E.A., Oberlander, A.M. and Svoboda, K., 2001. Abnormal development of dendritic spines in FMR1 knock-out mice. J Neurosci 21, 5139–5146.

47) Michel, C. I., Kraft, R., and Restifo, L. L., 2004. Defective neuronal development in the mushroom bodies of Drosophila fragile X mental retardation 1 mutants. J of Neurosci, 24, 5798–5809.

48) McBride S.M.J. et al., 2005. Pharmacological rescue of synaptic plasticity, courtship behavior, and mushroom body defects in a Drosophila model of fragile X syndrome. Neuron 45:753–764.

49) Cruz-Martín A., Crespo M., Portera-Cailliau C., 2010. Delayed stabilization of dendritic spines in fragile X mice. J of Neurosci 30:7793–7803.

50) Paradee, W., Melikian, H. E., Rasmussen, D. L., Kenneson, A., Conn, P. J., & Warren, S. T., 1999. Fragile X mouse: Strain effects of knockout phenotype and evidence suggesting deficient amygdala function. Neuroscience, 94, 185–192.

51) Zhao, M.-G., Toyoda, H., Ko, S. W., Ding, H.-K., Wu, L.-J., and Zhuo, M., 2005. Deficits in trace fear memory and long-term potentiation in a mouse model for fragile X syndrome. J of Neurosci, 25, 7385–7392.

52) Spencer, C. M., Alekseyenko, O., Serysheva, E., Yuva-Paylor, L. A., and Paylor, R., 2005. Altered anxiety-related and social behaviors in the Fmr1 knockout mouse model of fragile X syndrome. Genes, Brain, and Behavior, 4, 420–430.

53) Moon, J., Beaudin, A., Weiskopf, M., Driscoll, L., Levitsky, D., Crnic, L., and Strupp, B., 2006. Attention dysfunction, impulsivity and resistance to change in a mouse model of fragile X syndrome. Behavioral Neuroscience, 120, 1367–1379.

54) McNaughton, C. H., Moon, J., Strawderman, M. S., Maclean, K. N., Evans, J., and Strupp, B. J., 2008. Evidence for social anxiety and impaired social cognition in a mouse model of fragile X syndrome. Behavioral Neuroscience, 122, 294–301.

55) Pietropaolo, S., Guilleminot, A., Martin, B., D'Amato, F. R., & Crusio, W. E., 2011. Genetic-Background Modulation of Core and Variable Autistic-Like Symptoms in Fmr1 Knock-Out Mice. PLoS ONE, 6(2), e17073.

56) Olmos-Serrano, J. L., Paluszkiewicz, S. M., Martin, B. S., Kaufmann, W. E., Corbin, J. G., and Huntsman, M. M., 2010. Defective GABAergic neurotransmission

and pharmacological rescue of neuronal hyperexcitability in the amygdala in a mouse model of fragile X syndrome. J of Neurosci, 30(29), 9929-38.

57) Vislay-Meltzer, Rebecca L., Olmos-Serrano, Jose Luis, Nelson, David L., Corbin, J. G., & Huntsman, M. M., 2012. Altered maturation of inhibitory neurotransmission in the Fragile X amygdala. (in revision, J of Neurosci)

58) Ben-Ari Y., 2002. Excitatory actions of GABA during development: the nature of the nurture. Nat Rev Neurosci 3: 726–739.

59) Sassoè-Pognetto, M., Frola, E., Pregno, G., Briatore, F., and Patrizi, A., 2011. Understanding the molecular diversity of GABAergic synapses. Front Cell Neurosci, 5(June), 4.

60) Marchionni, I., Kasap, Z., Mozrzymas, J. W., Sieghart, W., Cherubini, E., & Zacchi, P., 2009. New insights on the role of gephyrin in regulating both phasic and tonic GABAergic inhibition in rat hippocampal neurons in culture. Neuroscience, 164(2), 552-62.

61) Varley, Z. K., Pizzarelli, R., Antonelli, R., Stancheva, S. H., Kneussel, M., Cherubini, E., and Zacchi, P., 2011. Gephyrin regulates GABAergic and glutamatergic synaptic transmission in hippocampal cell cultures. J Biol Chem, 286(23), 20942-51.

62) Richards, J. G., Schoch, P., Haring, P., Takacs, B. and Mohler, H., 1987. Resolving $GABA_A$ /benzodiazepine receptors: cellular and subcellular localization in the CNS with monoclonal antibodies. J Neurosci 7, 1866–1886

63) Nusser, Z., Sieghart, W. and Somogyi, P., 1998. Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. J Neurosci 18, 1693–1703.

64) Brunig, I., Scotti, E., Sidler, C. and Fritschy, J. M., 2002. Intact sorting, targeting, and clustering of γ-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. J Comp Neurol 443, 43–55.

65) Hevers W., Lüddens H., 1998. The diversity of $GABA_A$ receptors. Pharmacological and electrophysiological properties of $GABA_A$ channel subtypes. Mol Neurobiol 18: 35–86.

66) Rudolph U., Crestani F., Benke D., Brunig I., Benson J.A., Fritschy J.M., Martin J.R., Bluethmann H., Mohler H., 1999. Benzodiazepine actions mediated by specific gamma-aminobutyric acid(A) receptor subtypes. Nature 401: 796-800.

67) Rudolph, U., and Knoflach, F., 2011. Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes. Nat Rev Drug Discov *10*(9), 685-97.

68) Essrich, C., Lorez, M., Benson, J. A., Fritschy, J. M. & Luscher, B., 1998. Postsynaptic clustering of major GABAA receptor subtypes requires the γ2 subunit and gephyrin. Nat Neurosci 1, 563–571.

69) Yu W., Jiang M., Miralles C.P., Li R.W., Chen G., De Blas A.L., 2007. Gephyrin clustering is required for the stability of GABAergic synapses. Mol Cell Neurosci 36: 484–500.

70) Saiepour, L., Fuchs, C., Patrizi, A., Sassoè-Pognetto, M., Harvey, R. J., and Harvey, K., 2010. Complex role of collybistin and gephyrin in GABAA receptor clustering. Journal Biol Chem, 285(38), 29623-31.

71) Selby L., Zhang C., Sun Q.Q., 2007. Major defects in neocortical GABAergic circuits in mice lacking the fragile X mental retardation protein. Neurosci Lett 412: 227–232.

72) Bureau I., Shepherd G.M.G., Svoboda K., 2008. Circuit and plasticity defects in the developing somatosensory cortex of FMR1 knock-out mice. J of Neurosci 28:5178–5188.

73) Paluszkiewicz, S. M., Martin, B. S., & Huntsman, M. M., 2011. Fragile X syndrome: the GABAergic system and circuit dysfunction. Dev Neurosci, 33(5), 349-64.

74) Turrigiano G.G., Nelson S.B., 2004. Homeostatic plasticity in the developing nervous system. Nat Rev Neurosci 5:97–107.

75) Davis G.W., 2006. Homeostatic control of neural activity: from phenomenology to molecular design. Annu Rev Neurosci 29:307–323.

76) Pozo, K., and Goda, Y., 2010. Unraveling mechanisms of homeostatic synaptic plasticity. Neuron, 66(3), 337-51.

77) Heinen K., Bosman L.W., Spijker S., van Pelt J., Smit A.B., Voorn P., Baker R.E., Brussaard A.B., 2004. GAB_AA receptor maturation in relation to eye opening in the rat visual cortex. Neuroscience 124: 161–171.

78) Fritschy J.M., Paysan J., Enna A., Mohler H., 1994. Switch in the expression of rat GABAA-receptor subtypes during postnatal development: an immunohistochemical study. J Neurosci 14: 5302–5324.

79) Marowsky, A., Rudolph, U., Fritschy, J.-M., and Arand, M., 2012. Tonic Inhibition in Principal Cells of the Amygdala: A Central Role for α3 Subunit-Containing GABAA Receptors. J Neurosci, 32(25), 8611-9.

80) D'Hulst, C., and Kooy, R. F., 2007. The GABAA receptor: a novel target for treatment of fragile X? Trends Neurosci, 30(8), 425-31.

81) Olmos-Serrano, J. L., Corbin, J. G., & Burns, M. P., 2011. The GABA(A) receptor agonist THIP ameliorates specific behavioral deficits in the mouse model of fragile X syndrome. Dev Neurosci, 33(5), 395-403.

82) D'Hulst, C., De Geest, N., Reeve, S. P., Van Dam, D., De Deyn, P. P., Hassan, B. a, and Kooy, R. F., 2006. Decreased expression of the $GABA_A$ receptor in fragile X syndrome. Brain Res, 1121(1), 238-45.

83) Adusei, D. C., Pacey, L. K. K., Chen, D., and Hampson, D. R., 2010. Early developmental alterations in GABAergic protein expression in fragile X knockout mice. Neuropharmacology, 59(3), 167-71.

84) Translating time across mammalian brains. University of Arkansas and Cornell University. <http://translatingtime.org/public/index>, 2012.

85) A. El Idrissi, X. H. Ding, J. Scalia, E. Trenkner, W. T. Brown, and C. Dobkin,

2005. Decreased GABA receptor expression in the seizure-prone fragile X mouse. Neurosci Lett, vol. 377, no. 3, pp. 141–146.

86) I. Gantois, J. Vandesompele, F. Speleman et al., 2006. Expression profiling suggests underexpression of the GABA receptor subunit δ in the fragile X knockout mouse model. Neurobiol Dis, vol. 21, no. 2, pp. 346–357.

87) Clancy, B., Finlay, B. L., Darlington, R. B., & Anand, K. J. S., 2007. Extrapolating brain development from experimental species to humans, Neurotoxicology 1-7.

88) Reddy D.S., 2010. Neurosteroids: endogenous role in the human brain and therapeutic potentials. Prog Brain Res 186: 113–137.

89) Mientjes, E. J., Nieuwenhuizen, I., Kirkpatrick, L., Zu, T., Hoogeveen-Westerveld, M., Severijnen, L., Rifé, M., et al., 2006. The generation of a conditional Fmr1 knock out mouse model to study Fmrp function in vivo. Neurobiol Dis *21*(3), 549-55.

90) Misgeld U., Bijak M., Jarolimek W., 1995. A physiological role for $GABA_B$ receptors and the effects of baclofen in the mammalian central nervous system. Prog Neurobiol 46: 423– 462.

91) Seaside Therapeutics, Inc. Study of Arbaclofen for the Treatment of Social Withdrawal in Subjects With Autism Spectrum Disorder. <http://clinicaltrials.gov/ct2/show/NCT01288716>, 2012.

92) Flurkey K., Currer J.M., Harrison D.E., 2007. The Mouse in Aging Research. In The Mouse in Biomedical Research 2nd Edition. Fox JG, et al (Eds.). American College Laboratory Animal Medicine (Elsevier), Burlington, MA. pp. 637–672.