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**14. ABSTRACT**

Fragile X Syndrome (FXS) is a single gene disorder caused by loss of *FMR1* gene function. This disease leads to cognitive impairment and is the most common genetic cause of autism, accounting for 2-6% of all diagnosed cases (Hagerman et al 2008). In previous studies of a *Drosophila* model for FXS, we identified pharmacological treatments that rescued phenotypes relevant to this syndrome such as social, neuroanatomical and cognitive deficits (McBride et al., 2005; Choi et al., 2010). These results have been translated to the mouse model of FXS leading to the impetus to initiate clinical trials with Fragile X patients (Yan et al., 2005; Dolen et al., 2007; de Vrij et al., 2008; Choi et al., 2011). The fact that clinical trials of two distinct compounds identified in flies and tested in mice have reported some level of efficacy highlights the relevance of *Drosophila* and mouse-based disease modeling to identify potential treatments for developmental brain disorders and other diseases (Berry-Kravis et al., 2008; Berry-Kravis et al., 2009;

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## Introduction

Fragile X syndrome is the leading cause of intellectual disability resulting from a single gene mutation. Previously, we characterized social and cognitive impairments in a *Drosophila* model of Fragile X syndrome and demonstrated that these impairments were rescued by treatment with metabotropic glutamate receptor (mGluR) antagonists or lithium. In the mouse model of Fragile X a well-characterized phenotype is enhanced mGluR-dependent long-term depression (LTD) at Schaffer collateral to CA1 pyramidal synapses of the hippocampus. Last year we have reported the use of PDE-4 inhibitors in rescuing social, and memory phenotypes in the mouse as well as the enhanced-LTD phenotype observed in the *Fmr1* mouse KO. Last year we also reported the finding that metformin treatment also rescues the memory phenotype in the fly model of Fragile X. In this year we have focused on metformin treatment in the fly model and prepared to perform metformin treatment in the mouse to determine if it can also rescue memory and other phenotypes in the mouse model.

Metformin is an important drug to test in the fly and mouse models of Fragile X. Most importantly, metformin is an FDA approved drug that has a very safe and long clinical history. It is commonly used to treat type II diabetes in humans and has recently been used to treat weight gain in patients treated with anti-psychotics. It is safe enough to prescribe to children and is now routinely prescribed to children as young as 5 years of age both to control weight gain and to treat type II diabetes. If metformin is effective in the fly and mouse model, clinical trials with Fragile X patients would clearly be warranted.

There are two known targets of metformin action that should help ameliorate the increased insulin signaling that we observed in our Fragile X fly model. First metformin is known to increase the activity of AMPK. AMPK in a known activator of the TSCI/II complex that represses Rheb activity. Since Rheb is a known activator of mTOR, the increased activation of AMPK should result in a decrease in mTOR activity (Figure 1). Another activity that metformin has is the transcriptional activation of PTEN, increasing PTEN activity levels. PTEN antagonizes PI3K activity which reduces the activation of mTOR (Figure 1). Thus both activities of metformin should correct the increased signaling observed in the fly fragile X model.

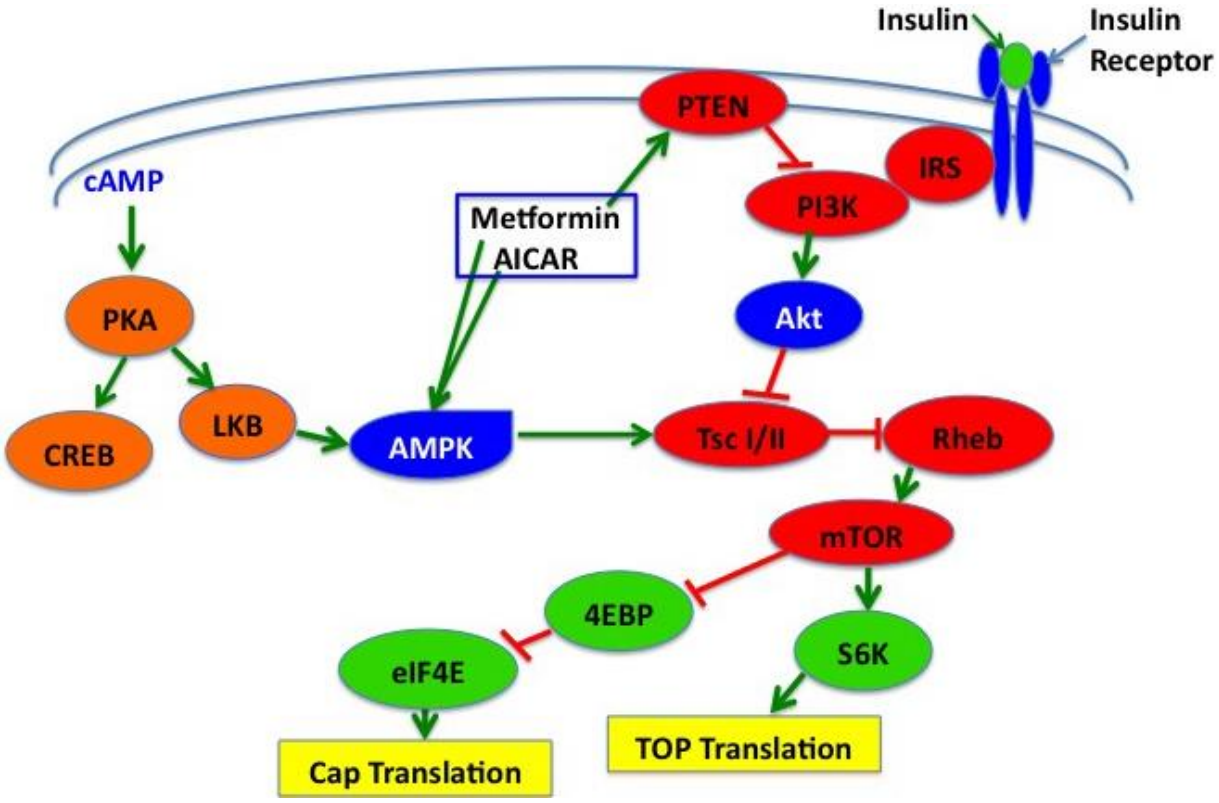


**Figure 1. Insulin, mTOR signaling pathway the activity of metformin.** Metformin has two activities that act to reduce mTOR signaling activity. First it acts to activate AMPK which increases the activity of the TSCI/II complex and represses Rheb activity more, thus activating mTOR less. Metformin also activates the transcription of PTEN, which results in increased repression of PI3K and less activation of Akt and thus less repression of TscI/II and again adding to the repression of Rheb and thus less activation of mTOR.

## Reportable outcomes:

### Task 8

To study the efficacy of metformin in more detail, we have tested the effect of treating *dfmr1* mutants during development, during adulthood or both and tested for short-term memory as well as for rescue of circadian behavior. We have found that even with adult treatment alone we can rescue the memory phenotype in the *dfmr1* mutant. We however could not rescue the circadian defect, however we feel that this is due to an accessibility problem. In temporal experiments we have found that *dfmr1* activity is required during pupal development for proper circadian regulation. Unfortunately we currently cannot provide the drug treatment during the pupal period (the flies/larvae do not eat and are covered by a hard shell) and have enough adults hatch to test for circadian behavior. Nonetheless the success that we have had in rescuing memory warrants testing in the mouse Fragile X model.

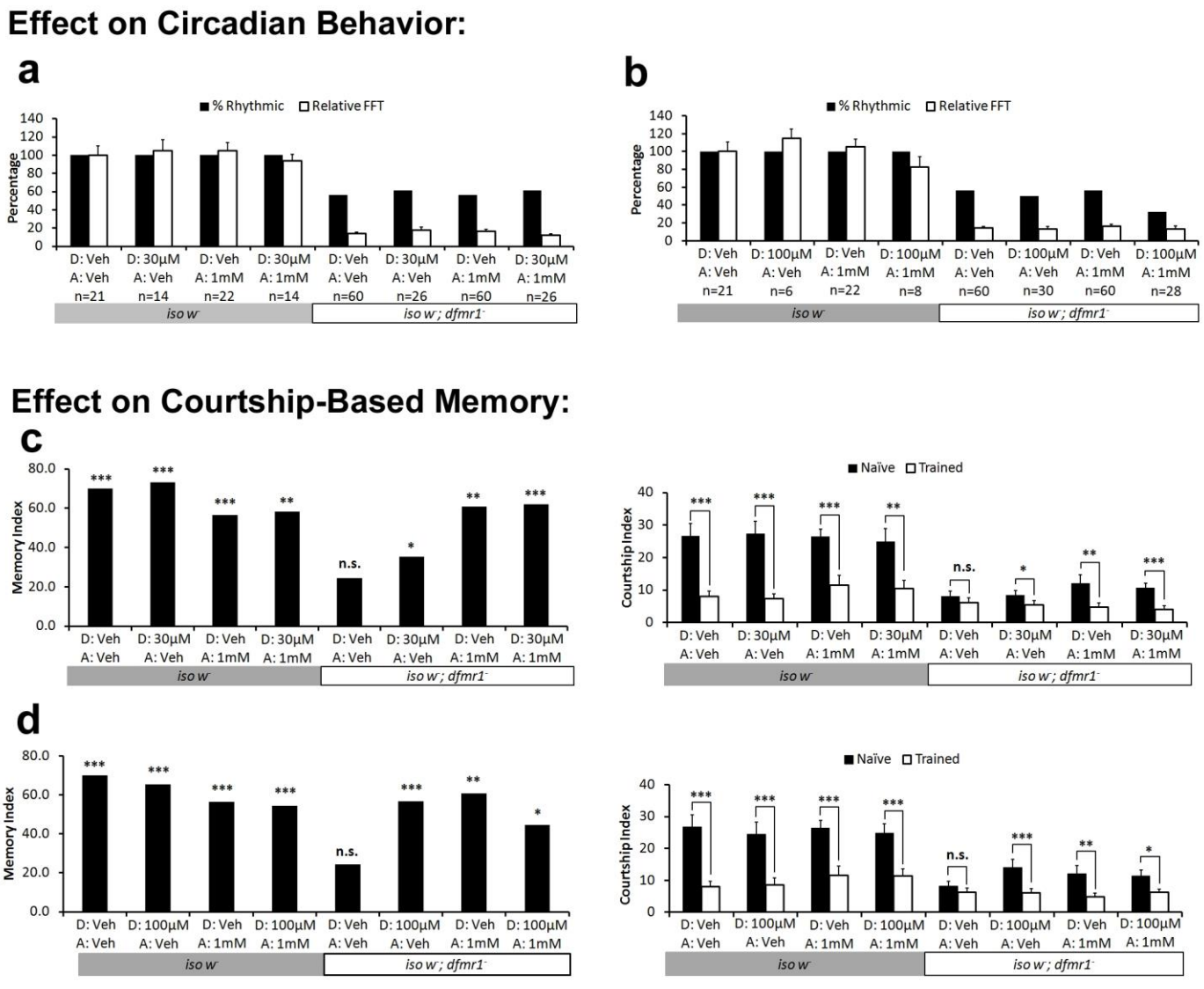


**Figure 2. Effect of developmental and adulthood metformin treatment on circadian behavior and courtship-based memory. a-b,** The circadian behavior of flies raised on **a,** 30µM or **b,** 100µM metformin and moved to 1mM metformin or vehicle control food within 24 hour of eclosion was examined. Metformin treatment did not improve the rhythmicity of *dfmr1* mutants. **c-d,** Flies raised on **c,** 30µM metformin or **d,** 100 µM metformin and moved to 1mM metformin or vehicle control food within 24 hours of eclosion were tested in the conditioned courtship paradigm. Treatment with either 30µM or 100µM metformin in development alone, or paired with 1mM metformin treatment in adulthood rescued STM in *dfmr1* mutant flies. Both MIs and CIs are displayed for each experiment. N ranged between 17-27.

## Task 13

To prepare for studies to determine the efficacy of metformin treatment in the mouse we have tested the effect of a high dose of metformin on mice to determine how well it is tolerated and if there are any adverse side effects. As shown in Figure 3 we observed that the *dfmr1* maintain a relatively normal weight profile during from weaning and well into adulthood.

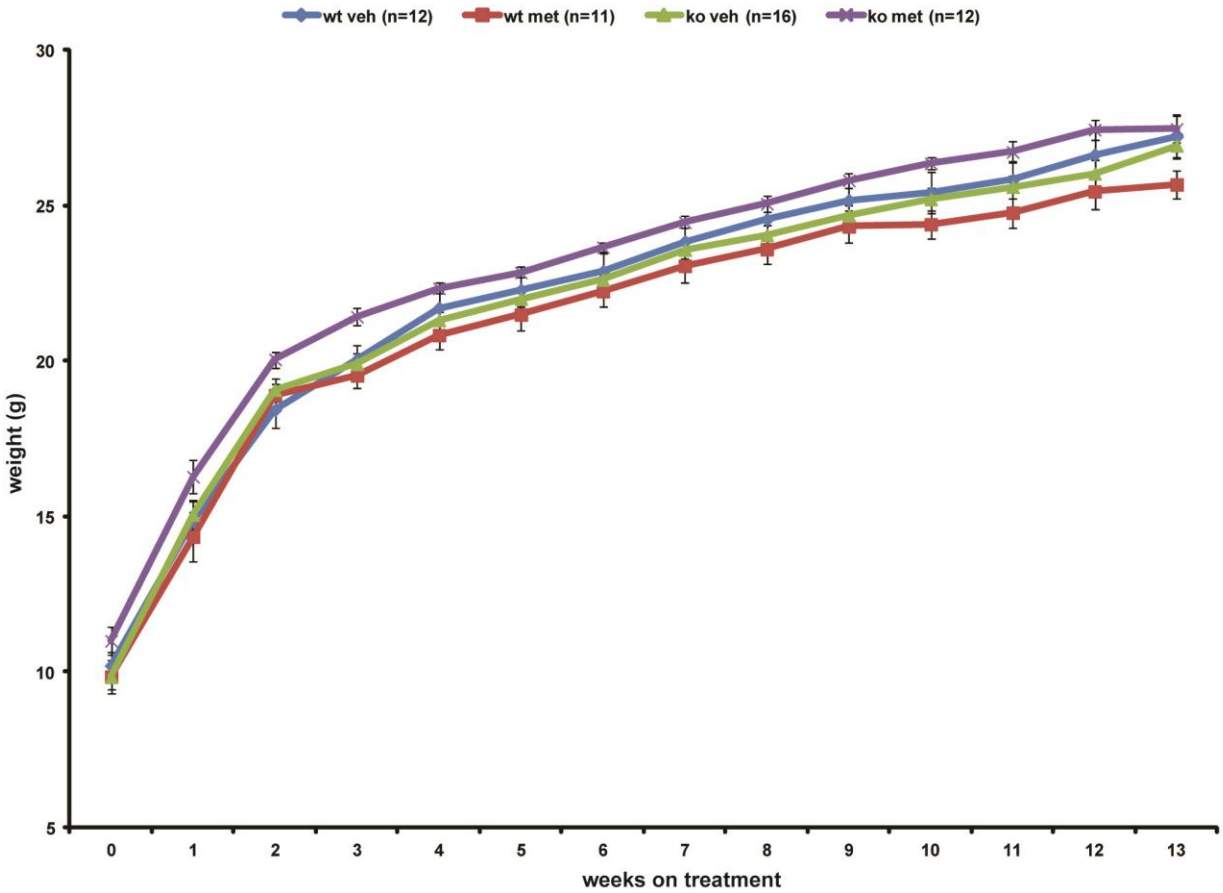



**Figure 3. Effect of high dose metformin on *Fmr1* KO and control mice to determine how well the drug is tolerated.** We find that mice that were given a high dose of metformin (2.0mg/ml) (a comparable dose for type II diabetes patients would be 200mg/ml) maintain normal weight and do not show any obvious negative effects of metformin treatment. The mice were placed on metformin at 4 weeks of age.

## continued studies for Task 12

To prepare for testing the mice for rescue of phenotypes with metformin, we have obtained reproducible phenotypes with the novel object recognition test, the rotorod with several protocols and we have also noted a significant hypoactivity phenotype in the *Fmr1* KO mice during their active phase.

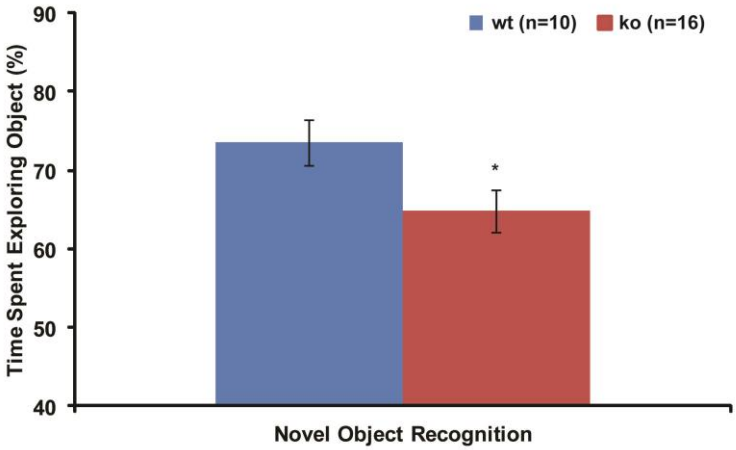
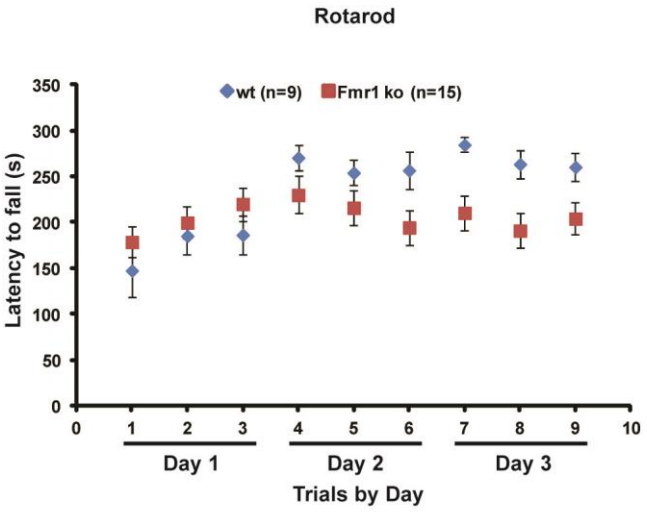



**Figure 4. The *Fmr1* KO mice display a deficit in the novel object recognition task.** In this assay mice are habituated to a chamber that contains two objects for several trials. The following day one of the objects is replaced with a novel object. Control mice display a significant exploratory preference for the novel object. The *Fmr1*KO mice display a significantly reduced preference for the novel object displaying a reduced memory of the two initial objects in the chamber. This task is dependent on hippocampal and perirhinal cortex function, and the *Dfmr1* mutants display a reproducible memory deficit in this task.

We have also established a reproduced a locomotor memory deficit in the rotorod test (Figure 5). We have found that in a test where mice are give three trials a day for three consecutive days that the *Fmr1* KO mice fail to continue to learn to stay on the rod during trials during days 2 and 3. This task requires a combination of cerebellar and hippocampal function (Figure 5).



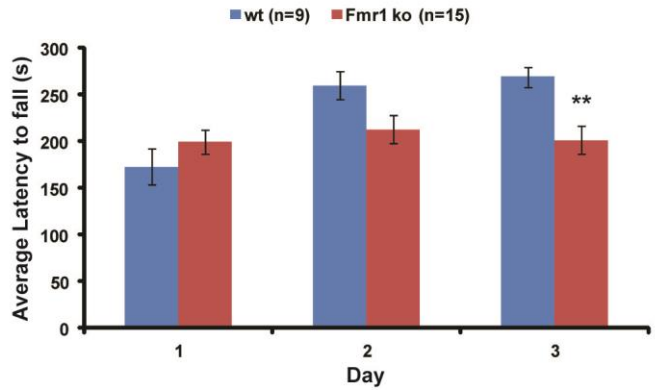
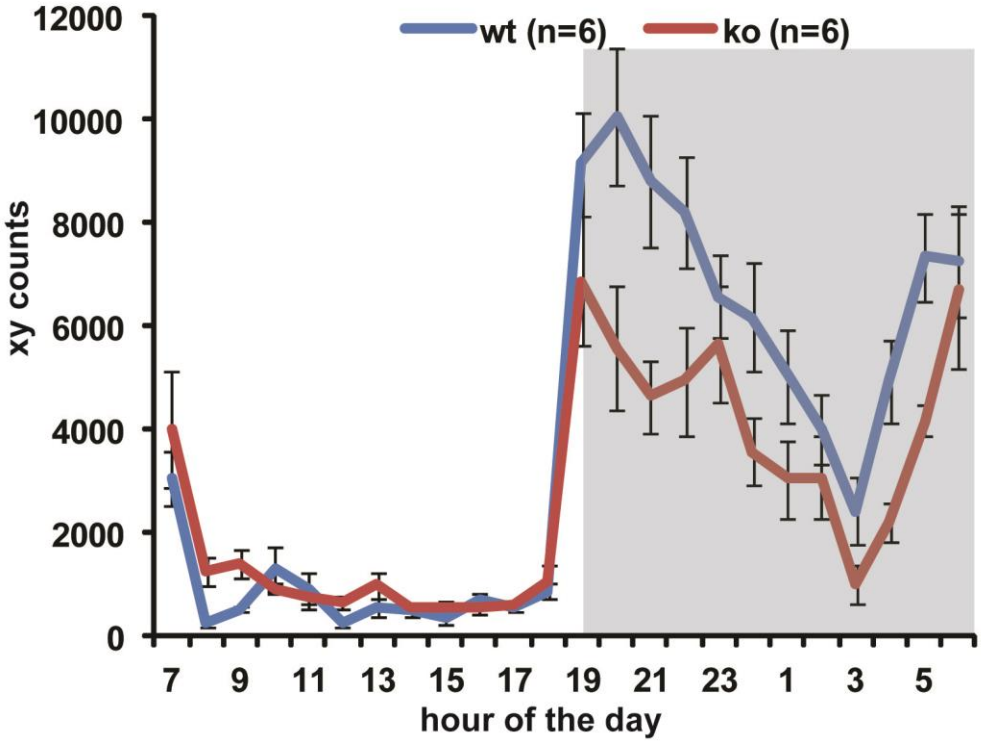



**Figure 5. *Fmr1* KO mice display reduced locomotor learning in the rotorod assay.**

In activity monitoring of the *Fmr1* KO and control mice, we have observed that the *Fmr1* KO mice display an activity deficit during their active phase(night time) relative to control mice. Although this is not a cognitive task, we will explore what effect metformin treatment has on this phenotype.

**Novel Finding not listed in original tasks**




**Key Research Accomplishments:**

**Task 8**-We have completed timeline testing for metformin treatment of the *dfmr1* mutants and have found that adult only treatment is sufficient to rescue the memory phenotype with both courtship conditioning and olfactory based memory testing.

**Task 12.** Continued development of cognitive and behavioral phenotypes to test the efficacy of drug treatments. We also currently have *Fmr1* KO and control mice on metformin and will be testing there abilities on the rotorod and in the novel object recognition assay shortly, see 13c.

**Ongoing tasks:**

**Task 1c.** Perform biochemical analysis to determine effects of PDE-4 inhibition on PI3K and Akt activity and smRP6 levels.

Using an elisa assay to quantitate cAMP levels, we have now established that the *dfmr1* mutants have reduced resting levels of cAMP. We have also determined that treatment with rolipram can rescue the deficit of cAMP. Therefore we are in a position to now examine the effect of PDE-4 inhibition on PI3K, Akt and smRP6 levels.

We are also initiating biochemical tests to determine the effect that meformin treatment has on the insulin-signaling pathway of the *dfmr1* mutants. We expect that we will observed increased levels of p-AMPK (activated AMPK), decreased activity of S6K which is downstream of mTOR and is the next proximal antibody that we can use (Figure 1) in Drosophila. We will also

determine the levels small ribosomal protein 6 whose expression is regulated by the mTOR pathway. Increased mTOR activity leads to increase smRP6 levels. Also to examine the effect on PTEN activity, we will determine whether metformin treatment also reduces Akt levels (e.g. p-Akt).

**8a.** Examine naïve courtship, learning during training and memory in *dfmr1* mutants and controls treated with AICAR and vehicle during development alone, adulthood alone and during both times. This ongoing test will provide validation for the efficacy of metformin, as AICAR also activates AMPK activity.

**12c.** Perform behavioral testing battery on *FMR1* KO and control mice.

**13c.** Perform behavioral testing on *FMR1* KO and control mice that are treated with metformin or vehicle.

**Manuscripts Accepted:**

1) Deciphering discord: How Drosophila research has enhanced our understanding of the importance of FMRP in different spatial and temporal contexts. Weisz ED, Monyak RE, Jongens TA. Exp Neurol. 2015 May 28. pii: S0014-4886(15)30001-7. doi: 10.1016/j.expneurol.2015.05.015. [Epub ahead of print] Review. PMID: 26026973

2) Increased expression of the PI3K enhancer PIKE mediates deficits in synaptic plasticity and behavior in fragile X syndrome. Gross C, Chang CW, Kelly SM, Bhattacharya A, McBride SM, Danielson SW, Jiang MQ, Chan CB, Ye K, Gibson JR, Klann E, Jongens TA, Moberg KH, Huber KM, Bassell GJ. Cell Rep. 2015 May 5;11(5):727-36. doi: 10.1016/j.celrep.2015.03.060. Epub 2015 Apr 23. PMID: 25921541

3) PDE-4 inhibition rescues aberrant synaptic plasticity in Drosophila and mouse models of fragile X syndrome.
Choi CH, Schoenfeld BP, Weisz ED, Bell AJ, Chambers DB, Hinchey J, Choi RJ, Hinchey P, Kollaros M, Gertner MJ, Ferrick NJ, Terlizzi AM, Yohn N, Koenigsberg E, Liebelt DA, Zukin RS, Woo NH, Tranfaglia MR, Louneva N, Arnold SE, Siegel SJ, Bolduc FV, McDonald TV, Jongens TA, McBride SM.
J Neurosci. 2015 Jan 7;35(1):396-408. doi: 10.1523/JNEUROSCI.1356-12.2015. PMID: 25568131

4) Modulation of cAMP and ras signaling pathways improves distinct behavioral deficits in a zebrafish model of neurofibromatosis type 1. Wolman MA, de Groh ED, McBride SM, Jongens TA, Granato M, Epstein JA.
Cell Rep. 2014 Sep 11;8(5):1265-70. doi: 10.1016/j.celrep.2014.07.054. Epub 2014 Aug 28. PMID: 25176649

**Papers under revision:**

Insulin Misregulation underlies Behavioral and Cognitive Deficits in a *Drosophila* Fragile X Model
Monyak, R., Emerson, D, Zheng, X., Schoenfeld, B., McBride, S.M.J, Sehgal, A., and Jongens, T.A.

**Conclusions:**

The overall objective of the work we have accomplished so far was to examine the efficacy of pharmacologically inhibiting PDE-4 activity to correct synaptic plasticity impairments in the fly and

mouse models of Fragile X syndrome. Now we have added metformin to the treatment testing. Since metformin has a much better clinical history than any recently FDA approved PDE-4 inhibitors, we have reprioritize our studies to focus on the efficacy of metformin treatment on the fly and mouse fragile X models. The *Drosophila* Fragile X model recapitulates the most debilitating aspect of the disease in humans, namely impaired cognitive function. In our further dissection of the proteins involved in the mGluR signaling cascade, we have identified metformin as a potential therapy for treatment of Fragile X. The data from the fly model indicate that this drug can rescue several memory phenotypes displayed by the *dfmr1* mutant and this is with adult only treatment. This is important as this indicates that by changing the physiology of the *dfmr1* mutants we can rescue memory. We are currently moving to test whether metformin treatment can rescue the locomotor and novel object recognition task deficits that we have established with the *Fmr1* KO mice. If we can successfully rescue these or other cognitive tasks with the *Fmr1* KO mouse, this will provide necessary data to warrant clinical testing with Fragile X patients.

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