Targeting the PI3K signaling cascade: A therapeutic approach to fragile X syndrome

Intellectual disabilities and socioemotional diseases, such as autism spectrum disorders, can have a devastating impact on families, society, and the economy. Genetic, epigenetic, and environmental etiologies are thought to be highly diverse, and as a result, therapeutic strategies are lacking. The study of single-gene causes of these diseases has emerged as a promising research strategy that might further illuminate novel treatment approaches broadly applicable to larger groups of affected individuals.

Recent evidence suggests that many genetic defects linked to autism converge onto a specific intracellular signaling pathway that regulates protein synthesis – the phosphoinositide 3 kinase (PI3K)/mTOR signaling complex. For example, the most frequent inherited intellectual disability, fragile X syndrome (FXS), which is also the most common monogenic cause of autism, is characterized by excessive and dysregulated signaling through the PI3K/mTOR pathway. We hypothesize that genetic or pharmacological reduction of PI3K/mTOR signaling in mouse models of FXS will rescue FXS-associated phenotypes. To test this hypothesis, we propose a collaborative, two-pronged approach, which will characterize the effects of systemic reduction of PI3K signaling on synaptic signal transduction, synaptic protein synthesis, dendritic spine morphology, neuronal excitability, and cognitive function in mouse models of FXS.

FXS is caused by transcriptional silencing of the fragile X mental retardation gene 1 (fmr1), which leads to loss of the fragile X mental retardation protein (FMRP), an mRNA-binding protein involved in dendritic mRNA transport, stability, and translation. Fmr1 deficiency in mice was shown to recapitulate FXS symptomatology in patients. We will take advantage of these previous observations by using two different FXS mouse models: (1) germline knockout of Fmr1, and (2) brain region-selective short-hairpin RNA (shRNA)-mediated knockdown of Fmr1. We will utilize genetic and pharmacological strategies, and site-selective viral-mediated gene silencing to characterize the benefits of PI3K/mTOR-targeted therapies with the ultimate goal of developing novel treatment strategies that may be broadly applicable to larger populations, e.g., individuals with autism spectrum disorders unrelated to FXS. We propose the following specific aims:

**Aim 1. To identify the therapeutic effects of reducing PI3K signaling in Fmr1 knockout (KO) mice.** We will attempt to reverse FXS-associated phenotypes in Fmr1KO mice using multiple techniques:

**A. A gene-dosing approach.** In this sub-aim, we will test the hypothesis that reduced gene dosage of the PI3K-subunit p110β and the PI3K enhancer (PIKE) will rescue FXS phenotypes in Fmr1KO mice. Fmr1KO mice will be bred with p110βHET mice to generate Fmr1KO/p110βHET mice. Additionally, Fmr1KO mice will be bred with PIKEHET mice to generate Fmr1KO/PIKEHET mice. Both of these compound genetic crosses are expected to dampen PI3K/mTOR signaling and thereby rescue aberrant PI3K activity, synaptic protein synthesis, dendritic spine morphology, and seizure susceptibility in Fmr1KO mice.

**B. A pharmacological approach.** In parallel, we will administer p110β-selective antagonists to Fmr1KO mice with the expectation that these treatments will rescue aberrant PI3K activity, synaptic protein synthesis, dendritic spine morphology, and seizure susceptibility in Fmr1KO mice.

**Aim 2. To localize cognitive deficiencies in FXS and rescue these disabilities using site-selective in vivo gene silencing and behavioral pharmacological approaches.** Cognitive disabilities in FXS likely reflect the impact of FMRP deficiency in a brain region termed the “prefrontal cortex.” Empirical support is still limited, however, despite implications for targeted treatment approaches to intellectual disabilities in FXS and other developmental domains. We will simultaneously dissect the neuroanatomy and molecular biology of FXS-related intellectual disability using the following approaches:

**A. Site-selective shRNA delivery.** We will use in vivo stereotactic delivery of viral vectors expressing shRNAs directed against Fmr1 to generate mice with Fmr1 knockdown selectively in the prefrontal cortex. This manipulation is expected to confer cognitive deficiencies on an array of behavioral tasks that require goal-directed decision-making, complex planning, inhibitory control, and cognitive flexibility. We will then rescue deficiencies by simultaneously knocking down p110β or PIKE. Given the tight coupling between dendritic spine morphology and cognitive function, dendritic spines at the infusion site are expected to assume normative morphology. Normalization of synaptic signal transduction and protein synthesis will provide further evidence for overactive p110β and PIKE in FXS etiology.

**B. Site-selective pharmacological manipulation.** The antagonists described in aim 1B will be administered into the prefrontal cortex following Fmr1 knockdown with the expectation that targeted drug delivery will have therapeutic-like effects in behavioral, cellular, and physiological domains.

Together, these experiments will generate new insight into brain region-selective functions of FMRP; comprehensively test the suitability of PI3K reduction as a novel therapeutic strategy for FXS; and provide a platform from which to test the suitability of PI3K reduction in treating a range of developmental disabilities.
Background and Significance

A major goal of child health care is to provide medical treatment for children with developmental disabilities that will improve the quality of their lives through adulthood. So far, effective treatments that improve cognitive function in developmental brain diseases are scarce. A key obstacle impeding progress in treating or curing these disorders is the substantial variety of genetic or epigenetic causes; however, recent discoveries suggest that, despite of this diverse etiology, certain molecular networks and neuronal signaling pathways are “hot spots” of mutations and dysfunctions that are present in multiple developmental brain disorders such as autism and intellectual disability.\(^6,7\) Targeted treatments correcting these shared dysfunctions might therefore improve life quality for a large patient population. One of these molecular networks shown to be dysregulated in several developmental disabilities of diverse etiologies (including autism) is the PI3K/mTOR signaling pathway. Our proposed research will use genetic and pharmacological tools to explore the PI3K/mTOR pathway as a potential therapeutic target for developmental brain disorders.

We will analyze the therapeutic benefits of PI3K/mTOR-targeted treatments in an established model of developmental brain disorders, the fragile X syndrome (FXS), a single-gene intellectual disability with demonstrated PI3K/mTOR signaling defect.\(^5\) Fragile X syndrome is the most frequent inherited intellectual disability, and the leading monogenic cause of autism. Neurological dysfunctions arise from the absence of the fragile X mental retardation protein (FMRP), an important regulator of mRNA translation affecting synaptic function. Several studies over the past years have shown that loss of FMRP leads to dysregulated neurotransmitter-regulated signaling and activity-dependent protein synthesis. Recently, we and others discovered that FMRP directly and negatively regulates components of the PI3K/mTOR signaling complex, the PI3K catalytic subunit p110\(\beta\) and the PI3K enhancer PIKE.\(^2,3\) We will explore both p110\(\beta\) and PIKE as therapeutic targets in FXS to identify novel therapeutic strategies (fig. 1).

Animal models of FXS, e.g. Fmr1 knockout mice, are valuable for identifying and testing potential therapeutic strategies for human patients.\(^8\) Fmr1\(^{K0}\) mice phenocopy several neurological defects observed in human patients, including aberrant dendritic spine morphology and seizure susceptibility.\(^9,10\) Drugs rescuing cellular and behavioral phenotypes in mice also have beneficial therapeutic effects in human patients.\(^11-14\) For example, a prominent characteristic of all FXS animal models is exaggerated and dysregulated signaling through metabotropic glutamate receptors 1/5 (mGlu1/5)\(^15\), leading to increased and stimulus-insensitive protein synthesis.\(^2,16\) Based on these findings, several clinical trials using mGlu1/5 antagonists have been initiated with promising results.\(^17\) Recent work with Fmr1\(^{K0}\) mice and human patients suggests that targeting downstream signaling molecules, as proposed for this research, might also be therapeutic.\(^17\)

We will use this well-studied FXS mouse model to test our hypothesis that pharmacologic and genetic reduction of PI3K signaling can rescue FXS-associated phenotypes (aim 1). In addition, we will use a novel approach, in which we will knockdown the Fmr1 gene exclusively in the prefrontal cortex of wild type mice (aim 2), since core deficits of patients with FXS, such as attention disorders, and impaired cognitive flexibility, are associated with the prefrontal cortex. We will test how this knockdown affects biochemical, neuronal and behavioral phenotypes that model cognitive defects in FXS, and whether these phenotypes can be rescued by manipulating PI3K/mTOR signaling. A recent report showed that Fmr1\(^{K0}\) mice are impaired in prefrontal-associated molecular and cognitive function,\(^18\) but so far no study has addressed the question whether acute region-specific loss of FMRP underlies these deficits, and whether genetic or pharmacologic rescue strategies can correct them. By studying the efficiency of PI3K activity reduction in rescuing prefrontal cortex-associated deficits, this proposal will provide important information on the suitability of PI3K-targeted therapies to improve higher cognitive function in FXS.

The PI3K/mTOR signaling network is a promising therapeutic target with potentially rapid application in humans because it is also affected in many different forms of cancer,\(^19\) thus drugs targeting components of the PI3K/mTOR pathway have already been developed and tested in clinical trials with cancer patients. We will...
take advantage of this head start in cancer research by using PI3K subunit-selective antagonists, which were originally developed by the GlaxoSmithKline oncology department for the treatment of certain forms of tumors (see attached letter of collaboration). We will test these highly selective antagonists for their potential to rescue neuronal and cognitive dysfunctions, with the goal of ultimately developing novel therapeutic approaches to FXS. These subunit-selective antagonists do not appear to compromise cellular function in cells from unaffected individuals, yet they rescue in vitro phenotypes caused by excess PI3K activity in lymphoblastoid cell lines from FXS patients.

Taken together, this collaborative, multi-pronged research, using genetic and pharmacological approaches to rescue a wide array of molecular, cellular and behavioral deficits in chronic and acute mouse models for FXS will not only test PI3K/mTOR signaling as a novel therapeutic target, but also provide important new insight into the pathomechanisms underlying fragile X syndrome.

Experimental Design

A central hypothesis of this proposal is that FXS pathology reflects excessive and dysregulated signaling through the PI3K/mTOR cascade. Thus, we hypothesize that reduction of PI3K/mTOR signaling in mouse models of FXS will rescue FXS-associated phenotypes. To test this idea, we will use the following experimental approaches:

Specific Aim 1. To identify the therapeutic effects of reducing PI3K signaling in Fmr1KO mice.

FMRP is an mRNA binding protein shown to regulate translation and protein expression of numerous mRNA targets. Previous work of the Bassell lab has identified two novel FMRP targets, which have upregulated protein levels in Fmr1KO mice: the catalytic PI3K subunit p110β and the PI3K enhancer PIKE-L. Both proteins function within the PI3K signaling complex, and might thus cause excess PI3K signaling and synaptic protein synthesis observed in the absence of FMRP. It is expected that reducing protein levels and/or activity of p110β or PIKE will restore normal signal transduction and protein synthesis at Fmr1KO synapses. We further anticipate that this approach will rescue deficiencies in neuronal function in FXS, e.g. restore normal dendritic spine morphology and neuronal excitability.

Strategies to normalize p110β- and PIKE-associated activity in Fmr1 KO mice

Genetic rescue – To confirm that excess p110β and PIKE levels are the molecular mechanisms causing dysregulated signaling in FXS, the Bassell lab will use a genetic rescue strategy that will reduce p110β and/or PIKE protein in Fmr1KO mice to wild type levels. This will be accomplished by crossing Fmr1KO mice with mice that are heterozygous for p110β20 or PIKE21 to generate Fmr1KO mice with reduced expression of the respective proteins. We expect that molecular and neuronal FXS-associated phenotypes will be rescued in these compound mice. Importantly, similar strategies have been proven to be useful to validate mGlu5 and respective proteins. We expect that molecular and neuronal FXS-associated phenotypes will be rescued in these compound mice. Importantly, similar strategies have been proven to be useful to validate mGlu5 and respective proteins.

Pharmacological rescue – Previous work of the Bassell lab has shown that a broad spectrum PI3K inhibitor rescues stimulus-insensitive synaptic protein synthesis and aberrant dendritic spine morphology in Fmr1KO neurons, and a p110β-selective antagonist reduces excessive protein synthesis in synaptic fractions from Fmr1KO mice and in lymphoblastoid cells from human patients. Here, we will use p110β-selective inhibitors from the GlaxoSmithKline p110β program (see letter of collaboration) to correct neuronal phenotypes.

Biochemical, cellular and behavioral assays to test rescue of FXS phenotypes

Excessive signal transduction – We will use radioactive and ELISA-based PI3K-specific enzymatic assays, as well as western blotting with phospho-specific antibodies to assess basal and stimulus-induced PI3K/mTOR signaling activity in the FXS rescue models described above. These methods are well-established in the Bassell lab. We expect that genetic reduction of p110β and PIKE, as well as antagonizing p110β, will reduce p110β-specific PI3K enzymatic activity and downstream signaling and restore activation of signal transduction via mGlu1/5 receptors (as supported by preliminary data (fig. 2a, c)).

Dysregulated synaptic protein synthesis – We will use metabolic labeling with radioactive amino acids in synaptic fractions1, 2 and fluorescent tagging of bio-orthogonal amino acids in neuronal cultures (Click-it system, Invitrogen) to test whether rescue strategies restore stimulus-sensitive synaptic and neuronal protein synthesis. Published data1, 2 and preliminary results (fig. 2b, d) strongly suggest the success of this strategy.

Aberrant dendritic spine morphology – Defective dendritic spine morphology occurs in human patients with FXS, as well as in Fmr1KO mice. Rescue of this defect by reducing PI3K/mTOR signaling in the animal model would strongly corroborate the translational applicability of this rescue strategy in humans. Importantly, correction of dendritic spine morphology has served as a disease-relevant phenotype to evaluate therapeutic strategies targeting mGlu1/5 signaling, which are now being used successfully in clinical trials with patients with FXS. Neuronal morphology will be assessed in cultured primary cortical neurons from the genetic rescue mice...
or after drug treatment of Fmr1KO neurons using a fluorescent reporter (GFP-LifeAct24) and automated 3D analyses as described previously (2, 25 and fig. 2e,f).

Figure 2: Reducing p110β and PIKE gene dosage or PI3K activity rescues core deficits in Fmr1KO. (a-d) Decreased gene dosage of p110β (a, b) or PIKE (c, d) in p110β or PIKE heterozygous Fmr1KO mice normalizes PI3K activity (a, c) and protein synthesis rates (b, d) compared to Fmr1KO littermates. Preliminary results further suggest that PIKE reduction restores the stimulus-dependent increase of PI3K activity and protein synthesis (c,d). p110β-specific PI3K activity was quantified using ELISAs (Echelon Biosciences, described previously1), and protein synthesis rates were determined using radiolabeling of synaptic brain fractions2. In c and d, synaptic fractions were stimulated with the mGlu1/5 agonist DHPG (15 min, 50 μM). Error bars represent SEM. (b) two-way ANOVA, Tukey’s post-hoc analyses. c,d: 3-way ANOVA not statistically significant. We expect that repetition of these experiments will further support these promising preliminary results.

(e, f) A broad-spectrum PI3K inhibitor (LY294002, 3 days, 10 μM) rescues aberrant dendritic spine morphology in Fmr1KO cultured hippocampal neurons, but does not affect WT (figure modified from25). Dendritic spines were visualized by GFP-LifeAct transfection and analyzed using Imaris Filamenttracer (Bitplane), detailed methods described in25.

Higher susceptibility to audiogenic seizures – A major characteristic of Fmr1KO mice is neuronal hyperexcitability, which is testable by assessing the susceptibility to sound-induced epileptic seizures3. Loud sounds (2 min, 120dB) induce audiogenic seizures in about 40-60% of Fmr1KO mice, but not in wild type mice. Increased susceptibility to epilepsy is also observed in human patients, making this phenotype especially valuable to assess therapeutic strategies. In collaboration with Drs. Huber and Gibson (UT Southwestern), we recently showed that genetic reduction of PIKE in Fmr1KO mice rescued the prolonged duration and increased frequency of UP states normally observed in the Fmr1KO cortex26 (unpublished observation). UP states are spontaneously occurring neuronal network activities, which reflect neuronal excitability. We thus expect that reduction of PIKE and p110β in the context of this proposal will abolish seizure susceptibility in Fmr1KO mice.

Specific Aim 2. To localize cognitive deficiencies in FXS and rescue these disabilities using site-selective in vivo gene silencing and behavioral pharmacological approaches.

Cognitive disabilities in FXS likely reflect the impact of FMRP deficiency in the prefrontal cortex, a heterogeneous brain structure comprised of several subregions that together regulate complex decision-making, planning, and cognition. Empirical support for this model is still limited, however, despite implications for targeted treatment approaches to intellectual impairment in FXS and other developmental disabilities.

In this aim, we will use in vivo stereotactic delivery of viral vectors expressing shRNAs directed against Fmr1 to generate mice with Fmr1 knockdown selectively in the prefrontal cortex. We expect that site-selective knockdown will recapitulate cognitive deficiencies in FXS. In collaboration with Dr. Stephen Warren (Emory University), the Bassell lab has previously shown that acute Fmr1 knockdown in cultured neurons mimics neuronal dysfunctions observed in Fmr1KO 2, 27. In vivo, rather than in vitro, shRNA delivery will build on these findings by allowing for directed gene knockdown within discrete neurocircuits in awake, behaving mice. This technique is a powerful tool by which to simultaneously dissect the molecular biology and neuroanatomy of complex behavior. In this case, mice will be characterized on an array of tasks that require goal-directed decision-making, inhibitory control, and cognitive flexibility; deficiencies in these domains in FXS markedly diminish quality of life in affected individuals, as well as the likelihood that affected individuals will live independently, form meaningful interpersonal relationships, and maintain employment.

In mice, behavioral tasks are conducted in specialized behavioral testing chambers, in which animals must learn to make a response (nose poke, lever press, etc.) to obtain a favored food reinforcer. Once the response is acquired, mice must develop increasingly complex behavioral strategies that require planning, decision-
making and in some cases inhibitory control in order to earn food reinforcement. Successfully “winning” rewards relies on the prefrontal cortex across mammalian species, and deficiencies recapitulate higher-order cognitive deficits in FXS, mental retardation, and other forms of cognitive impairment.

These experiments require highly specialized chambers and carry a significant programming burden (to control the operating computers), but Dr. Gourley’s group operates the largest system for mice at the Emory University School of Medicine, and her group also routinely utilizes targeted intracranial manipulations to identify molecular mechanisms of psychiatric disease etiology. Thus, this group is exceptionally well-suited to conduct these experiments. For example, recent work in the Gourley lab has utilized viral-mediated brain-derived neurotrophic factor knockdown in order to show that site-selective knockdown of this single peptide implicated in adolescent-onset addiction fully recapitulates the behavioral consequences of surgically disconnecting a cortical-striatal network thought to be adversely impacted in addiction. Fmr1 knockdown is expected to confer cognitive deficiencies on tasks that require goal-directed decision-making, inhibitory control, and cognitive flexibility. Previous work implied a role for FMRP in attentional function, but did not identify an anatomical locus of action or strategies to prevent or reverse behavioral defects.

As a second component of this aim, we will rescue cognitive deficiencies by generating a viral vector cocktail that simultaneously knocks down Fmr1 and p110β or PIKE. Reduction of p110β or PIKE is expected to occlude the effects of Fmr1 knockdown to upregulate PI3K activity and thereby rescue behavioral deficiencies. Direct intracranial administration of p110β-selective antagonists to the virus infusion site will serve as secondary confirmation that dampening PI3K/mTOR signaling therapeutically mitigates the behavioral-cognitive effects of Fmr1 deficiency.

Given the likely tight coupling between dendritic spine morphology and cognitive function, dendritic spines at the Fmr1 knockdown site are expected to assume normative morphology after p110β/PIKE knockdown or pharmacological inhibition, providing further evidence that therapeutic-like effects reflect cellular morphological correction. All viral vectors in this proposal express Green Fluorescent Protein or mCherry, allowing for rapid, high-resolution post-mortem cell capture. We will enumerate dendritic spine density, volume, and head size in one hemisphere from each mouse, and in the other, the infusion site will be dissected and homogenized, allowing us to ascertain whether dampening PI3K/mTOR signaling normalizes synaptic signal transduction and protein synthesis using the techniques described in aim 1.

Together, these experiments will identify the neuroanatomical substrates of cognitive deficits in FXS; provide further evidence for overactive p110β and PIKE in FXS etiology; and further refine treatment strategies for these and potentially other developmental diseases.
References

26. Hays SA, Huber KM, Gibson JR. Altered neocortical rhythmic activity states in Fmr1 KO mice are due to enhanced mGluR5 signaling and involve changes in excitatory circuitry. J Neurosci 2011;31:14223-34.


Extramural Funding Plan and Leveraging of Resources description

i. Extramural Funding Plan

We expect that prior to completion of the proposed research we will have generated sufficient preliminary data to submit a competitive grant application to the NIH. The proposed research is justified and well-supported by recent literature and our preliminary work, however more extensive data corroborating the potential success of a PI3K/mTOR-targeted therapeutic strategy in fragile X syndrome and supporting the feasibility to mimic FXS-related phenotypes by brain region-specific knockdown will significantly increase our chances to acquire extramural funding for this multi-disciplinary project.

We intend to submit a multi-PI RO1 grant between Drs. Bassell, Gourley and Gross to the NIH, which will build on the proposed research, for the second application cycle of 2013 (deadline 6/5/2013, $250,000 direct costs p.a., for 5 years). In addition or alternatively, there are discussions underway with Stephen Warren about submission of an interdisciplinary NICHD Center Core grant (P30) between several labs at the Emory School of Medicine, including Dr. Stephen Warren (Human Genetics), Dr. Bassell (Cell Biology), Dr. Yue Feng (Pharmacology), Dr. Peng Jin (Human Genetics) and Dr. Gourley (Pediatrics), and the Emory School of Medicine Core Facilities (including Rodent Behavioral, Proteomics and Viral Vector Cores) to foster the use of these core facilities in collaborative projects between these investigators. Pilot funding by the Pediatrics Research Center for the proposed collaborative project between Drs. Gourley and Bassell would enable these labs to acquire important preliminary data to support an application for the proposed P30 center core grant.

Our proposed project to analyze the PI3K/mTOR pathway as therapeutic target in fragile X syndrome and other autism spectrum disorders fits well into the funding priorities of Autism Speaks and the Simons Foundation Autism Research Initiative. These agencies support research intended to improve life quality of individuals with fragile X syndrome or autism spectrum disorders. We thus also plan to apply for multi-PI grants from all three agencies when RFAs for 2013 will be announced. The allowed direct costs for the 2012 application cycle were up to $150,000 p.a. for three years (Autism Speaks) and up to $250,000 p.a. for 2 years (Simons Foundation). Funding of this pilot project would be instrumental to acquire comprehensive preliminary data necessary to submit competitive grant applications to these agencies.

ii. Leveraging of resource

This research proposal was initiated as a jump-start of a collaboration between the Bassell lab and the laboratory of Dr. Gourley, a new faculty member who recently joined the Department of Pediatrics at Emory School of Medicine. Together, the Bassell and Gourley labs combine a wide range of different, mutually complementing experimental techniques, including biochemical, molecular, cell biological, and imaging methods in disease mouse models (Bassell lab), as well as expertise in brain-region specific manipulations, and the study of goal-directed behavior in mice (Gourley lab). We expect that, in addition to the specific project proposed in this grant application, in general research in both labs will benefit from this interaction: The exchange of technical and scientific expertise and ideas will lead to a sustainable research collaboration between the Bassell lab and Dr. Gourley’s lab, which, in the future, could promote multi-PI collaborations including other labs at Emory School of Medicine interested in neurodevelopmental disorders, with which the Bassell lab has been collaborating since several years, e.g. Dr. Stephen Warren (Human Genetics) and Dr. Yue Feng (Pharmacology).

Specifically for this project, we will utilize the Viral Vector Core Facility (Center for Neurodegenerative Diseases). In the future, this research to identify PI3K/mTOR-targeted therapies for fragile X syndrome and other autism spectrum disorders will also greatly benefit from the planned Center for Drug Discovery at the Pediatric Research Center.

Also, Dr. Gourley has previously used the Grants Editing and Manuscript Support Core for feedback on grants and will continue to do so as the work described in this proposal matures and Drs. Bassell, Gross and Gourley prepare applications for independent funding. They will also take advantage of this service while preparing manuscripts for peer review.