Duquesne University

Annual Progress Report: 2014 Formula Grant

Reporting Period

January 1, 2015 – June 30, 2015

Formula Grant Overview

The Duquesne University received $65,557 in formula funds for the grant award period January 1, 2015 through December 31, 2016. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Project for Cognitive Advancement in Infants with Neuromotor Disorders:The CAN-DO Project
The purpose of this longitudinal study is to examine the ongoing interaction between the domains of cognitive and motor development in infants with neuromotor disability, and to compare outcomes of two groups of infants receiving two different types of home-based, parent-delivered intervention, in order to determine which intervention is more effective in advancing cognitive as well as motor development. Knowledge of the effectiveness of two types of intervention will lead to improved early intervention for children with developmental disabilities, as well as future studies to examine ongoing outcomes.

Anticipated Duration of Project

1/1/2015 – 12/31/2016

Project Overview

The long term goal of our research is to determine the most effective early intervention for infants with neuromotor disability to grow productively, and provide that intervention in a cost-effective manner. Despite extensive evidence that motor skill has a driving influence on cognitive advancement in infants, the translation of these findings to intervention with at-risk infants is limited. Thus, early intervention for infants with neuromotor disorders is not based on research findings linking motor skills to cognitive skills, and is actually based on outdated practices. New, proven methods for early intervention are desperately needed.

We plan, in this proposed longitudinal trial, to compare two parent-delivered interventions for infants with neuromotor disorders. By comparing two approaches that are currently in wide use, we can obtain pilot data for a future randomized controlled trial. One approach focuses on helping the child to problem solve new motor strategies without physical assistance, while the

Note: This project is funded, in part, under a grant with the Pennsylvania Department of Health. The Department specifically disclaims responsibility for any analyses, interpretations or conclusions.

other approach focuses on physically assisting the infant to move. Of great significance in this study, we will link any motor changes with cognitive change using new techniques for assessing cognition and information processing in infants, such as eye-tracking, along with other clinically available tests. We will combine measures of cognitive functioning, visual attention, focused attention and early problem solving skills with motor skill measures. *The specific aims for this study are*:

1. To measure alignment changes of the head, trunk and pelvis during the achievement of sitting and the transition to crawling in infants with neuromotor disability.
2. Quantify the changes in problem-solving and cognitive abilities of infants with neuromotor disability over time and compare between intervention groups.
3. Using eye-tracking technology, quantify the evolution of focused attention in infants with neuromotor disability as the motor skills of sitting and the transition to crawling emerge.
4. Compare motor skill, attention and cognitive change between infants receiving two different interventions, and determine which is more effective overall.

Principal Investigator

Regina T. Harbourne, PhD, PT
Assistant Professor
Duquesne University
104 Rangos School of Health Sciences
600 Forbes Avenue
Pittsburgh, PA 15282

Other Participating Researchers

Jana Iverson, PhD; Klaus Libertus, PhD – employed by University of Pittsburgh

Expected Research Outcomes and Benefits

The findings of this study have the potential to create a paradigm shift in early intervention for infants with neuromotor disorders that accentuates the co-dependence and co-emergence of motor and cognitive skill. Understanding this relationship in this special population of infants may improve the overall developmental outcomes of children with early movement problems. It is expected that both interventions will help advance gross motor skills in infants, and that both groups will show significant differences from baseline to follow-up [Specific Aim 1]. We expect differences between the groups in that the motor skill + problem solving group is expected to have greater change scores in the test of problem solving skills (EPSI) and in focused attention than the group participating in a motor skill intervention only [Specific Aim 2]. Using eye-tracking technology, we expect to discover strategies for problem solving and how these strategies are linked to improving motor skill [Specific Aim 3]. And finally, we will use this pilot data indicating efficacy of the best approach to advance development [Specific Aim 4] to further define and create a larger clinical trial to examine differing interventions. The outcome from this study will launch a proposal for an R21 or R01 NIH grant for a randomized clinical trial to examine the efficacy of early intervention for infants with neuromotor disability. This project

will improve the health status of infants with developmental neuromotor disabilities by determining which type of intervention, out of 2 commonly delivered approaches, is more effective in advancing overall skill. Advances in developmental skills in infancy will contribute to overall health status as these infants attain school age, and build the capacity for future healthy outcomes.

Summary of Research Completed

Validity

The first step in executing this project was to determine if our planned procedures to collect and extract data for our measured outcomes are accurate and viable in the settings we are using. Because our aims reflect infant behaviors in natural settings, we collected trial data in the possible settings for young infants including homes, day cares, and a neutral play area (such as in a relative's home). Data collection in all settings resulted in the acquisition of adequate data for our research questions, including eye-tracking data, two concurrent video recordings of sitting and movement behavior, and attention of the infant to the problem-solving tasks presented. All the data recordings (video of movement and eye scene/tracking) were able to be synchronized to within several milliseconds, which is adequate for the sampling of the measured outcomes in our aims. The entire data collection session can be accomplished within 45 minutes from set-up to take-down, allowing ease for the families and children participating in the study. The data collection involving the infant takes only 15 minutes at the most, which is well within the tolerated time for infants in the age range of this study.

Reliability

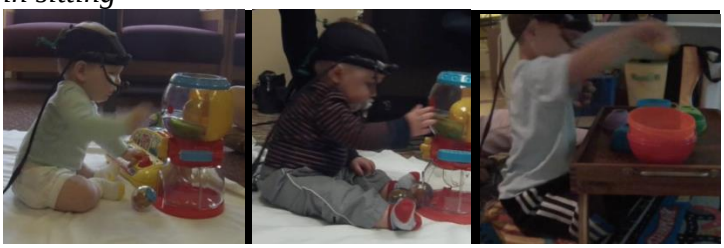
The second step in executing this project was to establish reliability of our coding of behaviors for the outcome measures. After 15 hours of training, the graduate assistant was tested for reliability. Using data from this project, as well as data from a previous project, the graduate assistant coded 20 sets of data, and achieved a high level of agreement, 97%. This is well above our target of 90% agreement for code-recode reliability. This dedicated student for this project will code all videos using a well-established coding software, *DATAVYU*.

Added Measures

The consultants (Iverson and Libertus) met with the primary investigator several times to review the initial data collection procedures and data set. Two measures were added enhancing the description of infant behaviors elicited during the tasks. We added a measure for novelty preference (related to how quickly and how long the infant switches gaze to a new object placed in the visual field), which is directly correlated with cognitive measures, as well as a measure coding for vocalization that occurs when the infant looks at the targeted toy. These added measures will improve our understanding of the targeted developmental skills in this project, and may provide a springboard for additional collaborative projects in the future. These added measures will not add to the initial procedures described in the grant, they are merely additions to coding.

Relationship to additional research collaboration that will aid this project
The initiation of this project has led to one additional funded project, and an additional submission of a proposal. A faculty development fund was awarded to Dr. Harbourne to work with a motion analysis company, ***SIMI***, to create a mobile application for measuring sitting posture and trunk control in infants in a natural home environment. The mobile application will both enhance this project (allowing more accurate measurement of posture, aim 1), and will help to translate the findings of this project more easily to a clinical tool useful for physical therapists working with infants and children with developmental disabilities. Another proposal was submitted for a PInCH grant by Dr. Libertus, on behalf of our team, to create "Motor Coach", a video instruction series that will show parents how to foster sitting and reaching development in their young children. Thus, the early stage of this CURE grant has already provided a springboard for future clinical research projects and tool development.

Preliminary Results
These results are very preliminary, and based on data used to establish reliability and validity. These data will not be used in the final analysis, but are helpful to understand developmental trajectories and how we might expect children to change over time within our intervention paradigm. It is important to note that these data are from typically developing children, who were used to establish some norms and to refine our methods.
Specific Aim 1: Over time, infants gain a more vertical orientation of the head, trunk and pelvis in sitting



In the above photos taken from our videos with children at different stages of sitting skill, clear progression to vertical alignment of the trunk can be noted. We do not yet have enough data to do any meaningful group analysis, but we have established that we can clearly and reliably code the changes in sitting postural behavior during a dynamic sitting task in early infancy.
Specific Aim 2: Changes in problem solving will be related to changes in gross motor scores.
Because we have not yet started the intervention stage of our project, we do not have data of this relationship for this project, although previous research has suggested that we will find this linkage.
Specific Aim 3: Changes in problem solving will be measured by eye-tracking technology pre and post intervention.
Although we have not started the intervention portion, our testing of infants at different developmental stages indicates a linear trend in focused attention. Infants who are less skilled in sitting have many switches of gaze to a novel object presented in the visual field, and this progresses to few switches of gaze as

the child becomes more skillful in sitting. This result is consistent with previous developmental research showing that as cognitive skills improve over time, children need fewer opportunities and less time to extract important information from the visual field.

Specific Aim 4: Comparisons between intervention groups will show greater problem solving skill gain in the group training for motor skill + problem solving. Because the intervention portion is just starting, we do not have data for this aim yet.

Recruitment

Brochures for this study have been distributed and several early interventionists are currently recruiting for the study. A media story is also in production, which we anticipate will bring further subjects to the study, as has been our experience in the past. Because we only received the grant award money in May, we were unable to begin recruiting for patients until this month, but we anticipate adequate recruitment due to the interest from our community clinical contacts.

Research Project 2: Project Title and Purpose

RNA-protein Interactions: G quadruplex RNA Structure Involvement in Neurodegeneration – The goal of this project, which will employ a novel combined biochemical/biophysical experimental and computational biophysics approach, is to investigate the detailed mechanisms of RNA G quadruplex structure interactions with specific proteins and their involvement in neurodegeneration, in the context of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). The results of the project will contribute to our understanding of the molecular mechanisms that cause ALS/FTD, potentially providing the basis for drug design.

Anticipated Duration of Project

1/1/2015 – 12/31/2016

Project Overview

Eukaryotic gene expression requires the coordination of several processes: transcription to produce pre-messenger RNA (mRNA), its processing to the mature mRNA, whose stability, transport, and finally translation lead to the successful expression of a functional protein. These tightly coupled processes are mediated by interactions between various proteins and DNA/RNA, which in turn rely on the proper folding and localization of the interacting partners. Consequently, errors in the folding, localization and in the correct identity of the interacting proteins and/or DNA/RNA could lead to gene expression miss-regulation, and in many cases to disease. Altered RNA-protein interactions have been linked to neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS, also known in the U.S as Lou Gehrig's disease), frontotemporal dementia (FTD), Alzheimer disease and Parkinson disease. Among the structures available to mRNA, the G quadruplex structure has received special attention in the past decade due to its involvement in the regulation of transcription, RNA processing and translation of many neuronal genes.

ALS is a fatal neurodegenerative disorder resulting in motor neuron loss in the brain and spinal cord. FTD is a degenerative condition of the brain frontal and anterior temporal lobes that results in progressive abnormalities in personality, behavior and language. Mutations of the DNA/RNA binding proteins Tar-DNA binding protein 43 and fused in sarcoma/translocated in liposarcoma protein (FUS/TLS) are causative of ALS and FTD. In 2011 it was also discovered that a hexanucleotide (GGGGCC) expansion in the C9ORF72 gene is a common genetic cause for ALS and FTD. The current project, which advances the hypothesis that the G-quadruplex RNA structure plays an essential role in the pathogenic mechanisms of both FUS and C9ORF72 hexanucleotide expansion in ALS and FTD, has the following specific aims:

Specific Aim 1. Analysis of wild type and ALS/FTD-linked mutant FUS protein interactions with neuronal mRNA G quadruplex structures.
Specific Aim 2. Characterization of the hexanucleotide expansion (GGGGCC) structure(s) in C9ORF72 mRNA and of their interactions with RNA binding proteins.

Principal Investigator

Mihaela Rita Mihailescu, PhD
Associate Professor
Duquesne University
Department of Chemistry & Biochemistry
308 Mellon Hall
600 Forbes Ave.
Pittsburgh, PA 15282

Other Participating Researchers

Jeffry D. Madura, PhD; Damian McAninch, BS – employed by Duquesne University

Expected Research Outcomes and Benefits

The project will explore if the RNA G quadruplex structure is implicated in the pathology of ALS/FTD. Its results will contribute to our understanding of the molecular mechanisms that lead to ALS/FTS in the context of the FUS protein mutations, as well as in the case of GGGGCC expansions in the *C9ORF72* gene, the most common genetic cause of ALS/FTD. This is novel from a mechanistic point of view, as previously this pathway has not been proposed and studied. Moreover, the link between RNA G quadruplex and ALS/FTD will open new possible targets for drug design. Although not the object of the current proposal, the PIs have already established additional collaborations in which peptide nucleic acids that target such RNA G quadruplex structures will be designed and analyzed. In addition, the computational studies will provide for the first time the tools to analyze the RNA G quadruplex structure by computational methods, opening the possibilities to apply such methods to other biological processes in which these structures are involved.

Summary of Research Completed

Specific aim 1. Analysis of wild type and ALS/FTD-linked mutant FUS protein interactions with neuronal mRNA G quadruplex structures.

Although the grant's starting date is 01/01/2015, the funds were not received until 06/2015, hampering to some extent our ability to perform more experiments. Nonetheless, we have made significant progress under this specific aim. We have analyzed the binding of the FUS wild type RGG peptide to three mRNA G quadruplex targets previously characterized in the lab: $(G_4C_2)_4$ derived from the C9ORF72 mRNA, Shank1 mRNA and the Q2 quadruplex from the PSD-95 mRNA. Specifically, after determining that the FUS RGG peptide binds to each of these G quadruplexes by electromobility shift assay, we have employed fluorescence spectroscopy to obtain quantitative information about these interactions. We have labeled the RNA molecules by either 2-aminopurine (2-AP), a fluorescent purine analog, or pyrollo C, a florescent cytosine analog and performed binding experiments by titrating increasing amounts of the FUS RGG peptide into a fixed concentration of mRNA, in the presence of a 5 fold excess of a non-related

peptide to reduce non-specific binding. As seen in Figure 1 A-C, the FUS RGG peptide binds to each of these RNA G quadruplexes, with dissociation constants K_d ranging from 92 to 451 nM. Experiments are in progress now to measure the dissociation constants of the complexes formed by the full-length FUS with the three mRNA G quadruplex targets.
Additionally, we have expressed and purified one of the FUS mutants associated with ALS/FTD, FUS R495X. This mutation is located within the RGG box domain of FUS and we postulated that it will affect the ability of the protein to bind to G quadruplex RNA.

Specific Aim 2. Characterization of the hexanucleotide expansion (GGGGCC) structure(s) in C9ORF72 mRNA and of their interactions with RNA binding proteins.

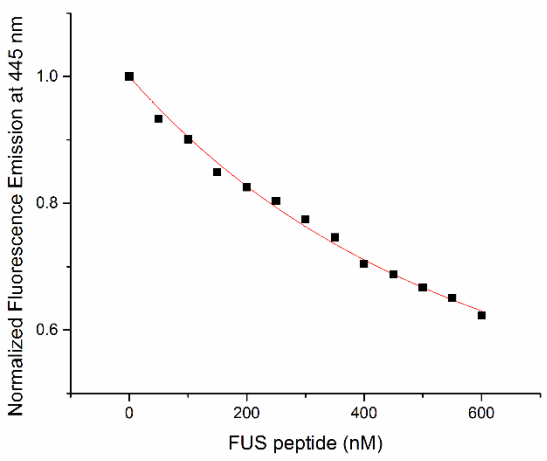
We have first produced the C9ORF72 4 repeat $(G_4C_2)_4$ and analyzed its conformations in the presence of increasing KCl conformations by native gel electrophoresis. As seen in figure 3, a single conformation is present at 0 mM KCl (lane 1), whereas as the KCl concentration increases, additional conformations become apparent as evidenced by the new upper bands with the concomitant decrease in the intensity of the lower band (lanes 2-7). Since KCl stabilizes the G quadruplex conformation we attribute the new bands to the G quadruplex structure.

To obtain higher resolution information about these conformations we employed ^{1}H NMR spectroscopy. As seen in figure 4, at 0 mM KCl, there are intense resonances in the 12-14 ppm region, corresponding to imino protons of guanines and uracils involved in Watson-Crick base pairs, but also a broad resonance centered around 11 ppm, corresponding to guanine imino protons involved in G quadruplex formation. As the KCl concentrations increases the intensities of the resonances in the 12-14 ppm region decreases with the concomitant increase in intensity for the broad resonance centered around 11 ppm. This result is consistent with the native gel electrophoresis results, together indicating that the $(G_4C_2)_4$ adopts a G quadruplex conformation, in equilibrium with the hairpin conformation that involves Watson-Crick base pairs. When the sample was boiled in the presence of 150 mM KCl, the G quadruplex conformation becomes dominant (figure 4, top spectrum).

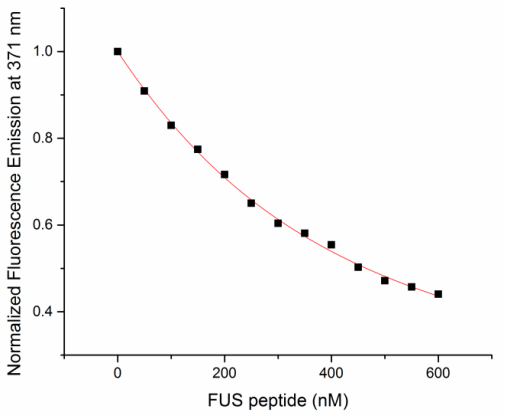
The proposed computational chemistry studies were also initiated, and we have built an initial G quadruplex structure using as a starting point an experimental G quadruplex structure (figure 5).

Figure 1

A.



B.



C.

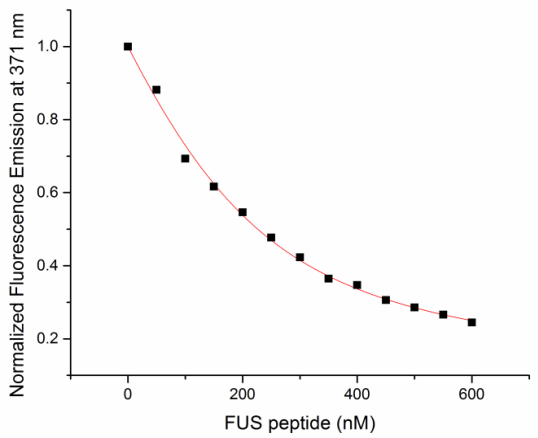


Figure 1. FUS RGG peptide binding to the following G quadruplexes: **A.** $(G_4C_2)_4$ derived from the C9ORF72 mRNA, K_d = 451± 57 nM; **B.** Shank1 mRNA, K_d = 271± 21 nM; **C.** PSD-95 Q2 mRNA, K_d = 92± 9 nM;

Figure 2.

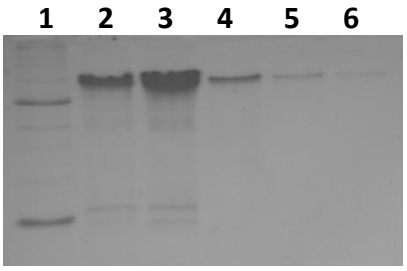


Figure 2. SDS PAGE of eluted fractions from the purification of the FUS R495X mutant. Lane 1: molecular weight markers, lanes 2-6 eluted fractions. Fractions 4-6 were pooled and concentrated to yield a protein concentration of 22 μM.

Figure 3

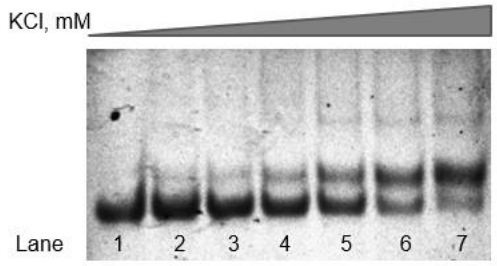


Figure 3: Conformational changes within $(G_4C_2)_4$ under increasing KCl concentration (0, 5, 10, 25, 50, 100, 150 mM). RNA concentration was kept constant at 10 μM.

Figure 4

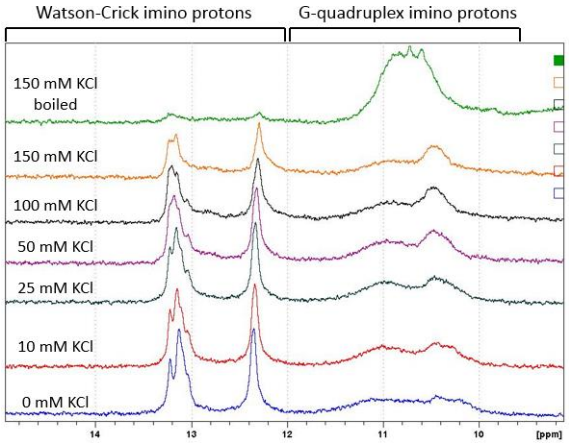


Figure 4: 1D ^{1}H NMR spectra of $(G_4C_2)_4$ at increasing KCl concentrations. All spectra with exception of the top spectrum were obtained for non-denatured RNA samples.

Figure 5

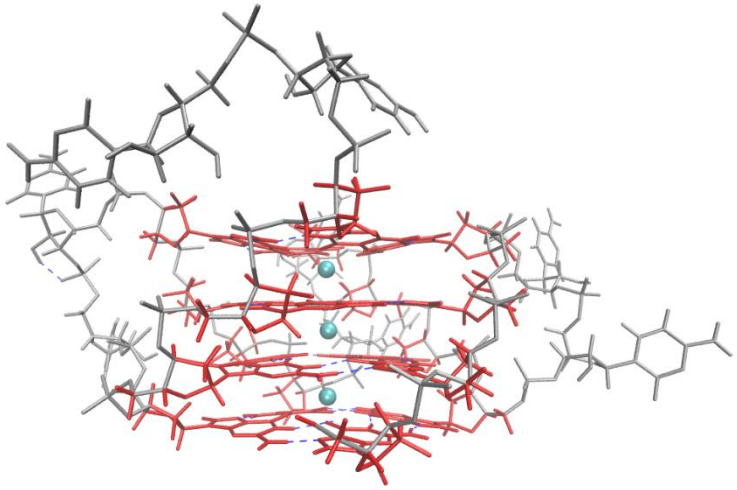


Figure 5. Initial intramolecular G quadruplex built from an experimental intermolecular G quadruplex. The phosphate-sugar backbone is colored grey while the guanine bases are colored red. The potassium ions between the guanine bases are represented as blue spheres. This initial system has remained stable for 32+ nanoseconds of molecular dynamics simulations.