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FELLOWSHIP FINAL REPORT

Targeting synaptic dysfunction using SINEUP ncRNA enhancer in neurodegenerative diseases

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ABSTRACT

Synaptic transmission is of critical importance for the neurons to communicate, and abnormalities are observed in neurodegenerative diseases, psychiatric disorders, and intellectual disability. Loss of the synaptic vesicle proteins is shared among these disorders and is being noted as one of the earliest hallmarks of neurogenerative diseases. Therefore, novel therapeutics targeting synapses are fundamental to improve brain plasticity and maintain a healthy brain function. Here, we propose to normalize synaptic protein levels by targeting unstable synaptic mRNAs using antisense RNA enhancer molecules with the 'long-term goal' of developing a therapy for patients with synaptic dysfunction, specifically in Alzheimer's Disease (AD) and Amyotrophic Lateral Sclerosis (ALS). Our 'hypothesis' is that stabilization of unstable synaptic mRNA's by antisense RNA molecules will be effective in enhancing and restore the levels of downregulated synaptic proteins in AD and ALS. As a 'proof of concept' antisense RNA molecules targeting 5'UTR regions of unstable synaptic genes (synapsin and synaptophysin) fused to enhancer elements such as SINE. To explore the efficacy and specificity, three different binding domains that span the 5'UTR region and transcription start sites (-40/+32, -40/+4, -14/+4) per gene were prepared and screened in a cell line that endogenously expresses the target genes. Our preliminary results show that SINEUP elements enhanced protein translation of the synapsin dimer by 80% and the monomers by 40%. This significant enhancement can stimulate synaptogenesis, synaptic vesicle recruitment, and maintain the mature synapses. An increase in synaptophysin was also observed. Ex vivo studies using a diseased cell model are in progress to assess phenotype and function. This is a promising step toward targeting synapses in neurodegenerative diseases.

1- INTRODUCTION

Neurodegenerative diseases such as Alzheimer's disease (AD) and Amyotrophic lateral sclerosis (ALS) are characterized by degeneration of neurons in the brain affecting millions of people worldwide. All AD patients and up to 50% of ALS patients present an early cognitive decline due to a decrease of the synaptic vesicle's proteins reducing synaptic transmission (Bae & Kim, 2017; Henstridge et al., 2017; Honer, 2003). This synaptic reduction has also been observed in psychiatric disorders such as schizophrenia and Bipolar Disorder (Porton & Wetsel, 2007; Vawter et al., 2002) as well as in patients with intellectual disability (Qiao et al., 2014). Down regulation mechanisms of these synaptic genes are not fully understood due to the complexity of the brain, but it has been anticipated that lack of mRNA stability might be occurring in these diseases, therefore stabilization of mRNAs of synaptic genes is essential to restore and maintain the synapses. Therefore, tackling this early sign in the disease (Jackson et al., 2019) by stimulating synaptogenesis and synaptic vesicle recruitment in the presynaptic terminals it may allow the brain to remodel itself and rewire by generating new neuron-neuron interactions thus increasing brain plasticity.

Synapsins. Synapsins play a crucial role in the central nervous system (CNS) regulating these important mechanisms for neuron-neuron communication, which also include, vesicle recruitment, synaptic vesicle docking, neurotransmitter release, vesicle recycling and axon development (Ferreira et al., 1994) (See Figure 1) (Longhena et al., 2021; Mirza & Zahid, 2018). Reductions of synapsins has been significantly observed in Alzheimer's Disease (AD), specifically in the dentate gyrus, a brain region important for learning and memory formation (Qin et al., 2004; Terry et al., 1991). These reductions affect crucial interactions of the synapsins with other proteins altering the brain protein network leading to a dysfunction of the central nervous system. Thus, reduction of synapsins reduce the vesicular glutamate transporters (VGLUTs) in a mouse model, these reductions abolishes glutamatergic neurotransmission which lead to severe cognitive malfunctions and lethality (Bogen et al., 2006). Other vesicular membrane proteins are also affected such as synaptophysin and synaptobrevin which are important for synaptic vesicle docking and neurotransmitter release. Also, a loss of synapsin-SORL1 interaction impair neuronal endosomal trafficking (Hartl et al., 2016). Synapsins are also associated with other disorders such as ALS, epilepsy, autism, parkinson's disease, multiple sclerosis, and psychiatric disorders such as attention deficit/hyperactivity disorder (ADHD), bipolar disorder and schizophrenia. Thus, targeting synapsins not only will target one disease, but other diseases.

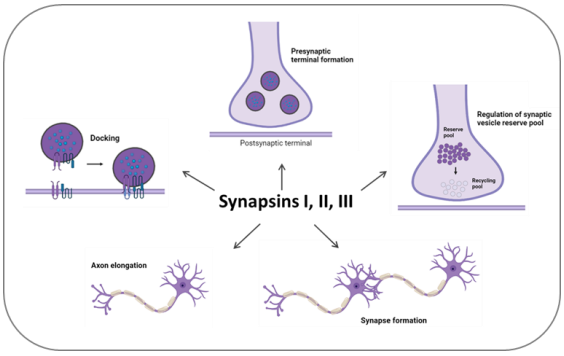

Figure 1| Functions of synapsins: Preysnaptic terminal formation, regulation of the synaptic vesicle reserve pool, synapse formation, axon elongation and synaptic vesicle docking.

Antisense RNA Therapeutics. COVID19 mRNA vaccine technology is changing the way on how to treat diseases. Although challenges remain using non-viral delivery of RNA molecules specially in the heart and the central nervous system (CNS), efforts by biotechnology companies such as Ionis pharmaceuticals have been successful on getting FDA approval and put on the market the RNA-based drug Spiranza, an antisense RNA drug that treats spinal muscular atrophy (SMA), which has showed that 60% of the infants treated with Spiranza improve motor function. RNA antisense therapies have been developed to treat other neurodegenerative diseases such as ALS, Huntington's disease (HD) and Alzheimer's disease to reduce protein aggregation, which is a hallmark of these diseases and they are currently in clinical trials (for ALS and HD). Although these are very encouraging news, studies in a mouse model have shown that clearance of protein aggregates can also cause excessive loss of synapses (Paolicelli et al., 2017) which it may also occur

during these therapies and it may be one of reasons why so many therapies for neurodegenerative diseases fail during clinical trials. Therefore, a combination therapy will be need it to restore and maintain the synapses loss during these treatments. Herein, we present our approach to enhance protein translation of synaptic genes at the early stage and at the advance stage of disease with synaptic dysfunction using a SINEUP approach (Carrieri et al., 2012; Espinoza et al., 2020; Long et al., 2017; Yao et al., 2015; Zucchelli et al., 2015) Our results show that SINEUPs enhanced protein translation of the synapsin dimer by 80% and the monomers by 40%. This significant enhancement can stimulate synaptogenesis, synaptic vesicle recruitment, and maintain the mature synapses. An increase in synaptophysin was also observed. This is a promising step toward targeting synapses in neurodegenerative diseases.

2- MATERIALS AND METHODS

Binding domains (BDs) design and cloning in pcDNA 3.1(-). Binding domains were designed as antisense molecules to target the 5' UTR and the transcription start site region of the mRNA of synapsin1 (SYN), synaptophysin (SYP) and the E3 ubiquitin ligase HECW1. Each binding domain was designed using different genome browsers such as Zenbu (Fantom5), UCSC genome browser, Ensembl and NCBI as previously described x with the goal of having consistent information of the target sequence. Three different binding domains were designed per gene with the following sizes: -40/+32, -40/+4, -14/+4. Binding domains fused with the SINE element were synthetically prepared (GenScript) and cloned into the XhoI and HindIII restriction sites of the pcDNA 3.1(-) plasmid vector (Invitrogen).

Cloning of Binding domains (BDs) in pAAV-CAG-miniSINEUP-mPGK-eGFP. Binding domains were synthetically prepared (Eurogentec) as double stranded with XhoI and PstI. pAAV-CAG-miniSINEUP-mPGK-eGFP plasmid was linearized with XhoI and PstI (NEB) and gel purified. Quick ligation (NEB M2200S) was performed by using 50 ng (0.020 pmol) of the linearized plasmid and 500 ng of the insert. Sanger sequencing was done for diagnostic.

SINE-Binding domain Screening. NSC-34 cells were plated in 6-well plates at $4x10^6$ cells per plate, next day cells with 70% confluency are ready for transfection. Transfection was prepared by mixing 10 ug of plasmid in 300 ul of OPTIMEM and 20ul of lipofectamine 2000 in 300 ul of OPTIMEM in separate tubes. The two tubes were mix and incubated for 15 min. Media was removed from cells and the 630 ul mix was added directly to the cells and incubated for 5 hours at the 37ºC CO2 incubator. After incubation, OPTIMEM media was removed and 2 ml media without selection was added to the cells for transient transfection. Cells were incubated for 48 hrs. at the 37ºC, 5% CO2 incubator and collected for protein and RNA analysis

Protein extraction. In a cold setting, media was aspirated from 6-well plates and cells were scrapped using a cell lifter. Cells were collected by adding 500 ul of cold 1x PBS to scrapped cells and transferred to a 1.5 ml Eppedorf tube. Tubes were centrifuged at 3000xg for 10 min at 4ºC, PBS was pipette out, and cell pellet was resuspended in 75 ul RIPA lysis buffer (RIPA lysis buffer recipe: 1 ml of Ripa buffer (ThermoFisher cat 89901), 10 ul of 100x Protease Inhibitor -Halt (ThermoFisher, cat 1862209), 10 ul of 100x PMSF (Sigma, cat 93482-50 ml), 10 ul of 100x Phosphatase Inhibitor (Sigma, cat P5726-1ml), 1 ul of 1000x DTT (Sigma, cat 43816-10 ml). Cell pellets resuspended in Ripa lysis buffer were incubated for 30 min or 1 hour on ice. Tubes were centrifuged at 16000xg for 20 minutes at 4ºC and supernatant was transferred to new labeled tubes. Bradford assay was used for protein quantification.

Western Blot protein analysis. Protein samples were prepared at 10 ug final concentration with, 1x Laemmli Sample Buffer (Biorad cat 161-0747) and 10 mM DTT reducing reagent final concentrations and incubated at room temperature for 15 min (samples were not boiled). Protein samples were loaded in a pre-cast gel (BIORAD Mini Protean TGX -stain free gels 4-20%, cat 4568094) and run at 150 V, 45 min, AMP 0.07A

(70mA) using 1x of Tris/Glycine/SDS Running buffer (BioRad, cat 161-0772). Once the run was completed, the gel was assembled in the transfer sandwich (Biorad, Mini format 0.2 um PVDF, cat 1704156) according to the Trans-Blot Turbo Transfer System Manual from BIORAD. Transfer was done in 7 minutes using Biorad transfer system. After completion, the sandwich was dissembled, and the blot was rinse briefly with TBST (1x TBS + 0.1% Tween 20) for 5 min. Blot was block with non-fat milk (5% non-fat milk in 1x TBST = 2g non-fat milk in 40 ml TBST) for 1 hr. at room temperature with gently shaking. Primary antibody was probe in 10 ml of 5% non-fat milk/TBST at room temperature for 1 hr. with gently shaking. Blot was washed three times with 1x TBST for 5 minutes and probe with the secondary antibody in 10 ml of 5% non-fat milk/TBST at 4ºC overnight with gently shaking. Blot was washed three times with 1x TBST and develop with Clarity™ Western ECL substrate (BIORAD cat# 170-5061). Image was done with Biorad ChemiDoc™ Touch Imaging System.

Antibodies. Synaptophysin, 1:5000 (2ul/10 ml 5% non-fat milk/TBST) (Thermo Scientific, cat PAI-1043), Synapsin I, 1:5000 (2ul/10 ml) (Abcam, cat Ab18814), Synapsin II, 1:2000 (5ul/10 ml) (Sigma, cat S2822), HRP-Conjugated b-actin mouse 1:50000 (1ul/50 ml) (ProteinTech, cat HRP-60008), Goat anti-rabbit IgG (H+L) Superclonal™ secondary antibody HRP, 1:2500 (4ul/10 ml) (Thermo Scientific, cat A27036). All antibodies were diluted in 5% non-fat milk/TBST

In vitro transcription. Plasmid linearization was carried out using 10 ug of plasmid DNA containing binding domains fused to SINE element using Bgl II. Digestion was done in 1.5 Eppedorf tubes at 37ºC for 2 h in the thermoblock and clean up using PCR clean up kit. For IVT, 1 ug of linearized plasmid was subjected to RNA synthesis using T7RiboMax Express Large-Scale RNA (Promega cat# P1320) following manufacturer instructions. RNA was purified using 7.5M LiCl precipitation solution. For diagnostic, 1 ug of RNA was analyzed in 1% agarose gel.

Isolation and culture of primary neurons. Cortex was isolated from four E17 embryos and collected in a 60 x 15mm petri dish containing cold PBS. Cortex was triturated and transferred to papain solution and incubated at 37ºC for 20 minutes. Enzyme digested cortexes were transferred to a new 15 ml falcon tube and resuspended in 2 ml neurobasal media to homogenize cells. Homogenization was done by pipetting up and down gently followed by adding 8 ml of media to wash cells and remove excess of papain. Cells were centrifuge for 5 minutes and resuspended in 12 ml neurobasal media (49 ml of 1x Neurobasal media, 1 ml of 50x B27+ supplement, 125 ul of 200Mm Glutamax and 500 ul of 1x Pen/Strept) and cultured in a 6-well plate. Media was changed every four days. Cells were observed under the microscope for assess morphology and quality.

AAV9-CAG-miniSINEUP-mPGK-eGFP virus preparation. AAV virus expressing SINEUP fused to synaptic genes were outsourced and prepared by Vigene Biosciences, a Charles River company (Rockville MD).

3- RESULTS AND DISCUSSION

Natural antisense (AS) ncRNAs have emerged as important regulators of the stability and translation of coding (sense) mRNAs by either protect them from degradation or cause their decay.

In this work we present our approach to enhance protein translation of synaptic genes at the post-transcriptional using the SINEUP system, a technology with an unique mechanism of action that utilize a non-coding RNA (ncRNA) with an overlapping antisense sequence targeting the mRNA 5' untranslated region which enhances translation of the corresponding protein at the post-transcriptional level without increasing mRNA transcripts (Carrieri et al., 2012). This is due to pairing of the ncRNA with the corresponding mRNA strand which increase ribosome binding and mRNA stability. The SINEUP system consist of two domains: the binding domain that range from 40-73 nucleotides length that targets a specific mRNA molecule and the SINE element (Figure 2A), which act as an enhancer element. The system works when mRNA and SINEUPS are

transcribed in the nucleus and their hybridization occurs in the 5' regions. In the cytoplasm, the secondary structure of SINE elements recruits' ribosomes resulting in greater translation enhancement (Figure 2 B-C).

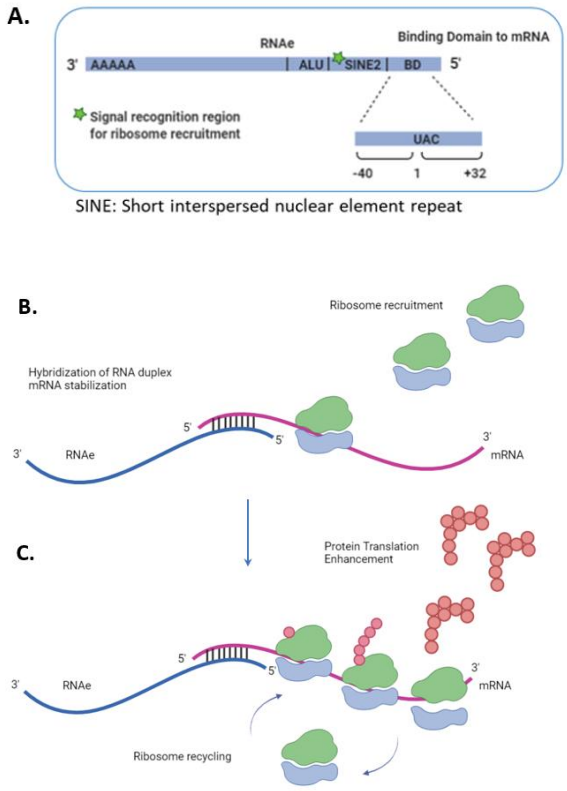

Figure 2| A. Hybridization of RNAe with mRNA occurs in the 5' regions increasing mRNA stability. B. The hybridized RNA duplex is then transported to the cytoplasm where the SINE elements recruits' ribosomes for translation enhancement.

RNA Screening and Selection. Protein translation enhancement studies were performed in the mouse cell line NSC-34 that endogenously express synapsin and synaptophysin genes. Antisense RNA molecules were designed to specifically target the 5'UTR regions of synapsin and synaptophysin fused to SINE enhancer elements. To explore the efficacy and specificity, three different binding domains that span the 5'UTR region and transcription start sites (-40/+32, -40/+4, -14/+4) per gene were prepared and screened in NSC-34 cells.

Our results show that SINEUPs enhanced protein translation of the synapsin heterodimer SYNI-SYNII by 80% and the monomer SYNIIa by 30%. (See Figure 3 and 4). This significant enhancement of the heterodimer can stimulate synaptogenesis, synaptic vesicle recruitment, and maintain the mature synapses. An increase of synaptophysin was also observed (Figure 5). This is a promising step toward targeting synapses in neurodegenerative diseases.

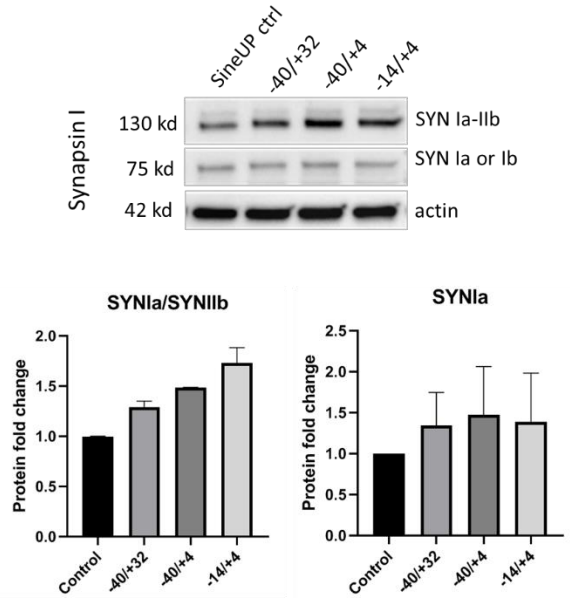

Figure 3 | Protein analysis of synapsin I. SINEUP fused to mouse SYN -14/+4 showed 80% protein enhancement of the dimer SYN1a-SYNIIb, while the monomers SYN1a or SYN1b didn't showed a significant enhancement. N=3

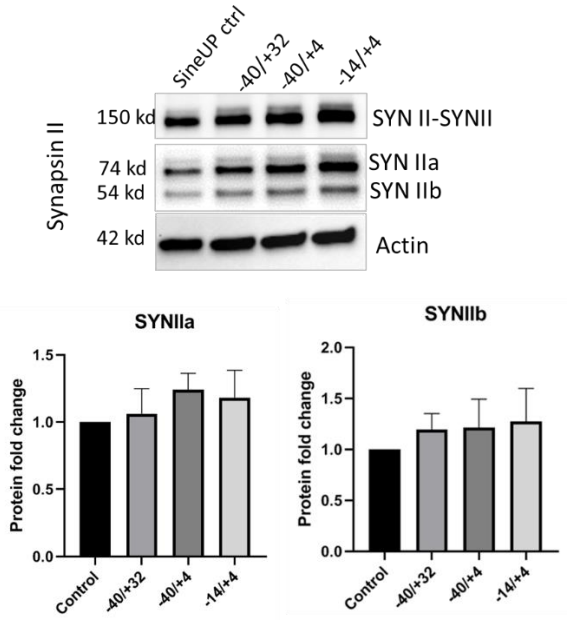

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Figure 4 | Protein analysis of synapsin II. SINEUP fused to mouse SYN -40/+4 showed 30% protein enhancement of the monomer SYNIIa, but not for SYNIIb. N=3

Figure 5 | Protein analysis of synaptophysin I. SINEUP fused to mouse SYP -14/+4 showed no significant protein enhancement of synaptophysin monomers or dymer. N=4

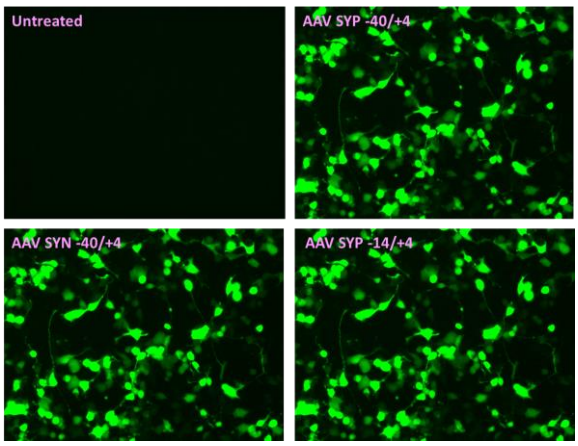

Figure 6 | Cloning of the Syn and SYP binding domains to AAV9-CAG-miniSINEUP-mPGK-eGFP and tested in NSC-34 cell line.

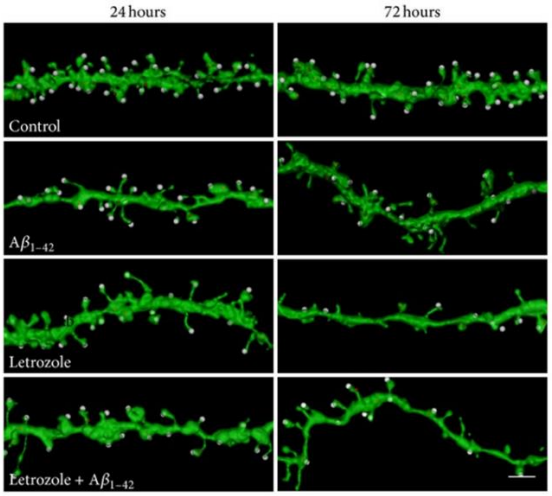

Figure 7 | Future Experiments: Asses the formation of dendritic spines after treatment with SINEUP fused to SYN and SYP binding domains. (Chang et al., 2013).

4- SUMMARY

In this study, we present data that supports protein translation enhancement of the heterodimer SYNIa-SYNIIb (130 kd) by 80% (Figure 3), homodimer SYNII-SYNII (150 kd) by 40%, (Figure 4) and the monomer SYNIIa by 30% (74 kd)) using a SINEUP approach. The same was observed for synaptophysin. Thus, having higher enhancement of the dimers is crucial for vesicle clustering, vesicle docking and for interactions with the actin filaments, which are important to stimulate synaptic transmission mechanisms. Currently, SINEUPs fused to SYN and SYP binding domains have been cloned and prepared in AAV serotype 9 (Figure 6). This study will be continuing in embryonic neurons where we will assess the formation of dendritic spines after SINEUP treatment (Figure 7).

5- AUTHOR CONTRIBUTIONS

Dr Kathia Zaleta-Rivera conceived the research performed the experiments and analyzed data; Frederic Laumonnier isolated neurons from mouse embryos. Drs Stefano Espinoza, Roberto F. Delgadillo, Emmanuel Astoul, Sylviane Marouillat, Frederic Laumonnier, Stefano Gustincich, and Patrick Vourc'h contributed tools and reagents and critical discussion. Dr. Kathia Zaleta-Rivera, wrote the report.

6- GRANTS

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