Translational regulation of *Syngap1* by FMRP modulates NMDAR mediated signalling

Abhik Paul[1#], Bharti Nawalpuri[2#], Shruthi Sateesh[1], Ravi S Muddashetty[2], James P Clement[1*]

[1] Neuroscience Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore 560064. India Phone: +91-80-22082613

[2] The Institute for Stem Cell Biology and Regenerative Medicine, GKVK post, Bellary Road, Bangalore 560065. India

[#] Equal contribution

* Corresponding author: Email: chelliah@jncasr.ac.in Phone: +91-80-22082613

Number of Pages: 34 including figures

Number of Figure: 5 main and 4 extended Figures

Number of words for:

Abstract:157

Introduction: 452

Discussion: 854

Conflict of Interest: The authors declare no competing financial interests

Acknowledgement: This work was supported by grants to JPC by DST-SERB (SB/YS/LS-215/2013), and to RSM in part, by Dept. of Biotechnology, India (BT/PR8723/AGR/36/776/2013, and BT/IN/Denmark/07/RSM/2015-2016), and intramural funds from both the Institutes. We thank Bhavana Kayyar, Utsa Bhaduri, and Vijay Kumar M J for technical support in our bioinformatics analysis, and Sudhriti Ghosh Dastidar for technical advice on Figure 4. Further, we thank Dr Ravi Manjithaya and his group, and Prof. Kaustuv Sanyal's group for technical assistance. We also thank Prof MRS Rao, Prof Tapas K Kundu,

Dr Sheeba Vasu, Dr Sourav Banerjee, Dhriti Nagar, Natasha Basu, and JPC and RSM lab members for critical comments on this manuscript. We thank Dr Prakash, Animal house in-charge, JNCASR, for help with the animal facility. AP and BN performed all the experiments. JPC did mGluR-LTD in Figure 1A. SS performed part of the experiments in Figure 1B and 1D. AP, BN, RSM, and JPC designed the experiments and wrote the manuscript. SS edited the manuscript. Current address of SS is Department of Psychology, University of Otago, William James Building, Level1, 275 Leith Walk, Dunedin. 9016. New Zealand.

Key words: SYNGAP1, FMRP, NMDAR, Translation, eEF2, Polysome

Abstract

SYNGAP1, a Synaptic Ras-GTPase activating protein, regulates synapse maturation during a critical developmental window. *SYNGAP1*$^{+/-}$ is shown to cause Intellectual Disability (ID) in children. Recent studies have provided evidence for altered neuronal protein synthesis in a mouse model of *Syngap1*$^{+/-}$. However, the molecular mechanisms remain unknown. Here, we report the reduced expression of a known translation regulator, Fragile X Mental Retardation Protein (FMRP), during a specific developmental period in *Syngap1*$^{+/-}$ mice. Our results demonstrated that FMRP interacts with *Syngap1* mRNA and might regulate its translation. We further show that, during development, reduced translation of FMRP contributes to a compensatory increase of *Syngap1* translation in *Syngap1*$^{+/-}$. These developmental changes are reflected in the altered response of Eukaryotic Translation Elongation Factor 2 (eEF2) phosphorylation downstream of NMDA receptor signalling. Thus, we propose a cross-talk between FMRP and SYNGAP1 mediated signalling which can also explain the compensatory effect of impaired signalling observed in *Syngap1*$^{+/-}$ mice.

.

Introduction

SYNGAP1 is a synaptic RAS-GTPase Activating Protein (SYNGAP1), which acts downstream of N-Methyl D-Aspartate Receptors (NMDAR), and negatively regulates RAS GTPase (Kim et al., 1998; Komiyama et al., 2002). NMDAR activation leads to phosphorylation of SYNGAP1 by Ca^{2+}/Calmodulin-dependent Kinase II (CAMKII) (Krapivinsky et al., 2004). Phosphorylated SYNGAP1 is rapidly dispersed from dendritic spine to dendritic shaft leading to activation of its downstream molecules in dendritic spines (Araki et al., 2015). Removal of SYNGAP1 from spine leads to increased activity of ERK via RAS (Rumbaugh et al., 2006), which further allows insertion of α-amino 3-hydroxy 5-methyl 4-

isoxazolepropionic acid Receptors (AMPAR) on the post-synaptic membrane (Zhu et al., 2002).

Using mouse model, studies have shown that *Syngap1*$^{+/-}$ causes early maturation of dendritic spines in the Hippocampus (Clement et al., 2012) and altered critical period of development in Barrel cortex (Clement et al., 2013). Furthermore, these studies have shown altered excitatory synapaptic activity and increased AMPA/NMDA during Post-Natal Day (PND)14-16 in the hippocampus of *Syngap1*$^{+/-}$ mice. Consistent with its molecular function, studies from human patients have shown that loss of function mutations in *SYNGAP1* results in Intellectual Disability (ID) and epileptic phenotype (Hamdan et al., 2009; Hamdan et al., 2011; Rauch et al., 2012). All these studies suggest that SYNGAP1 is crucial for the development of excitatory circuit during a critical period of development (Jeyabalan and Clement, 2016).

Recent studies using *Syngap1*$^{+/-}$ mice and *Syngap1* knock-down in rat cultured cortical neurons demonstrated increased levels of basal protein synthesis in *Syngap1*$^{+/-}$ as compared to Wild-type (Wang et al., 2013; Barnes et al., 2015). The study also suggested that SYNGAP1 modulates insertion of AMPARs at the post-synaptic membrane, thereby regulating synaptic plasticity through protein synthesis (Wang et al., 2013). However, the molecular mechanisms of SYNGAP1-mediated regulation of protein synthesis, particularly during development, are unclear.

In order to regulate synaptic protein synthesis, SYNGAP1 may crosstalk with other translation regulators. One such potential candidate to consider is FMRP. Similar to *Syngap1*$^{+/-}$ mice, *Fmr1* knock-out results in exaggerated levels of basal protein synthesis and altered dendritic spine structure and function (Huber et al., 2002). Additionally, a recent report showed exaggerated

protein synthesis-independent mGluR-LTD in *Syngap1*$^{+/-}$ (Barnes et al., 2015), which is also a hallmark phenotype of FMRP associated synaptic deficit (Huber et al., 2002). Based on these findings, we hypothesise a possible cross-talk between SYNGAP1 and FMRP in regulating activity mediated protein synthesis at the synapse. Here we show that FMRP level was decreased during development, especially in PND21-23, in *Syngap1*$^{+/-}$. In addition, FMRP interacts with *Syngap1* mRNA and may regulate the translation of it and leads to a compensatory increase of *Syngap1* translation in *Syngap1*$^{+/-}$. Thus, modulation of the SYNGAP1 level by FMRP in *Syngap1*$^{+/-}$ could modulate NMDAR signalling.

Materials and Methods:

Animals: C57/BL6 mice (IMSR Cat# JAX:000664, RRID:IMSR_JAX:000664) and *Syngap1*$^{+/-}$ mice (IMSR Cat# JAX:008890, RRID:IMSR_JAX:008890) were obtained from The Jacksons Laboratory and bred and maintained in the Institute's animal house under 12-hour dark and light cycle. For more details on *Syngap1*$^{+/-}$ mice, please refer Kim et al., 2003 (Kim et al., 2003). All the experiments were performed in accordance with Institutional Animal Ethics Committee (IAEC), and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Preparation of hippocampal slices: Acute brain slices were prepared from PND>90 male and female Wild-type (WT) and *Syngap1*$^{-/+}$ mice. All mice were bred and maintained in the Animal facility, JNCASR. Mice were sacrificed by cervical dislocation, and brain was dissected out. The brain was kept in ice-cold sucrose based artificial cerebrospinal fluid (aCSF; cutting solution) comprising (mM): 189 Sucrose (S9378, Sigma), 10 D-Glucose (G8270, Sigma), 26 $NaHCO_3$ (5761, Sigma), 3 KCl (P5405, Sigma), 10 $MgSO_4.7H_2O$ (M2773, Sigma), 1.25 NaH_2PO_4 (8282, Sigma) and 0.1 $CaCl_2$ (21115, Sigma). The brain was glued to the brain holder of the vibratome (Leica #VT1200), and horizontal 350 µm thick slices were prepared. Cortex

was dissected out from each slice to obtain only the hippocampus. All the slices were kept in slice chamber containing aCSF comprising (mM): 124 NaCl (6191, Sigma), 3 KCl (P5405, Sigma), 1 $MgSO_4.7H_2O$ (M2773, Sigma), 1.25 NaH_2PO_4 (8282, Sigma), 10 D-Glucose (G8270, Sigma), 24 $NaHCO_3$ (5761, Sigma), and 2 $CaCl_2$ (21115, Sigma) in water bath (2842, ThermoScientific) at 37ºC for 45 minutes. Following recovery, slices were kept at room temperature (RT) till the completion of the experiment. Post-dissection, every step was carried out under constant carbogen (5% CO_2 and 95% O_2; Chemix, India) supply.

Extracellular Field Recordings: Field Excitatory Post-Synaptic Potential (fEPSP) were elicited from pyramidal cells of CA1 region of stratum radiatum by placing concentric bipolar stimulating electrode (CBARC75, FHC, USA) connected to a constant current isolator stimulator unit (Digitimer, UK) at Schaffer-collateral commissural pathway, and recorded from stratum radiatum of CA1 area of the hippocampus, with 3-5 MΩ resistance glass pipette (ID: 0.69mm, OD: 1.2mm, Harvard Apparatus) filled with aCSF. Signals were amplified using Axon Multiclamp 700B amplifier (Molecular Devices), digitized using an Axon Digidata 1440A (Molecular Devices) and stored in a personal computer using pClamp10.7 software (Molecular Devices). Stimulation frequency was set at 0.05 Hz. Metabotropic glutamate receptor-dependent LTD (mGluR-LTD) was induced by bath applying (*S*)-3,5-dihydroxyphenylglycine (DHPG; Cat# 0805, Tocris, UK) for 5 minutes. All measurements were performed by an experimenter blind to all experimental conditions.

Lysate Preparation: Brain lysates were prepared from Post-Natal Day (PND) 4-5, 7-9,14-16, 21-23 and adult mice (2-5 months). WT and *Syngap1*$^{+/-}$ mice were sacrificed by cervical dislocation, brain was dissected out, and hippocampus was separated in ice-cold Phosphate Buffered Saline (PBS) of pH 7.4 containing NaCl (137mM, S6191, Sigma), KCl (2.7mM, P5405, Sigma) Na_2HPO_4 (10mM, 10028-24-7, Fisher Scientific), KH_2PO_4 (1.8mM, GRM1188, HIMEDIA). The tissue was homogenised using RIPA buffer containing

NaCl (150mM, S6191, Sigma,), Tris-HCl (50mM, Tris: 15965, Fisher Scientific; HCl: HC301585, Merck) pH 7.4, EDTA (5mM, 6381-92-6, Fisher Scientific), Sodium-Deoxycholate (0.25%, Triton X (RM 845, HIMEDIA). Additionally, 1X Protease Inhibitor (P5726, Sigma,) and 1X Phosphatase Inhibitor Cocktail 2 and 3 (P5726 and P0044 respectively, Sigma) was added to the buffer to increase the stability of the lysate. Then, the homogenates were centrifuged at 16000g for 30 minutes at 4ºC. The supernatants were collected, aliquoted and stored at -80ºC and the protein was estimated using Bradford (5000006, Bio-Rad) assay.

SDS-PAGE & Western Blotting: The protein samples were electrophoresed onto SDS (161-0302, Bio-Rad) Polyacrylamide (161-0156, Bio-Rad) 5% stacking gel for 30 minutes and 8% resolving gel (for FMRP, SYNGAP1, MOV10, PSD95) for around 2 hours or 10% resolving gel (Phospho-eEF2, Total-eEF2 and RPLP0) for around 3 hours. The proteins were transferred for 3 hours at 80V at 4ºC onto Polyvinylidene Fluoride (PVDF) membrane (1620177, Bio-Rad) and blocked using 5% skimmed milk (GRM 1254, HIMEDIA) or 5% BSA (GRM105, HIMEDIA) in PBS for 1-hour in Room Temperature (RT) at 25ºC. BSA was used for blocking of all Phospho-Proteins. For MOV10, blocking was done for ~3-4 hours at RT. The blots were washed with 1% PBST (PBS+ Tween 20; GRM156 HIMEDIA) for 3 times for 10 minutes, and incubated with Primary Antibodies for FMRP (F4055, Sigma Aldriech, 1:1000 dilution, raised in rabbit), β-Actin (PA116889, Thermofischer, 1:15000 dilution, raised in rabbit), and MOV10 (ab80613, Abcam, 1:1000 dilution, raised in rabbit), PSD95 (MA1-046, Thermofisher, 1:1000 dilution, raised in mouse), Phospho-eEF2 (Thr 56) 2331S ,Cell Signalling Technology, 1:1000 dilution, raised in rabbit), Total-eEF2 (2332S, Cell Signalling Technology, 1:1000 dilution, raised in rabbit) and RPLP0 (ab101279, abcam, 1:1000 dilution, raised in rabbit) overnight. After primary incubation, blots were given PBST wash for 3 times for 10 minutes, then incubated with anti-Rabbit (1706515, Bio-Rad) / anti-Mouse (1706516,

Bio-Rad) HRP conjugated Secondary antibody (1:10000 dilution). After subsequent washes with PBST, the blots were developed by chemiluminescent method using ECL western clarity solution (1705060, Bio-Rad). Image was taken in Versa Doc (Bio-Rad) or in ImageQuant (LAS 4000 from GE) further merged using ImageLab version 5.2.1 and bands were quantified using ImageJ software.

Polyribosome Profiling assay: Hippocampus were dissected out from the Post-Natal Day (PND)21-23 and PND14-16 *Syngap1*$^{+/-}$ (HET) and Wild-type (WT; littermates) as described earlier. Tissue was homogenised using Lysis buffer containing Tris-HCl (200mM, Tris: 15965, Fisher Scientific; HCl: HC301585, Merck), KCl (100mM, P5405, Sigma), $MgCl_2$ (5mM, M8266, Sigma), Dithioerythritol (DTT, 1mM, 3483-12-3, Sigma), NP40 (1%) and 1X Protease Inhibitor cocktail (P5726, Sigma). All reagents were dissolved in Diethylpyrocarbonate (DEPC, D5758, Sigma) treated autoclaved water. Samples were aliquoted into two equal parts. Both the parts are treated with translation inhibitor like Cycloheximide (CHX, 10µg/ml, C7698, Sigma), and Puromycin (1mM, P9620, Sigma) respectively. After CHX and Puromycin addition, the lysates were kept at 37 ºC for 30 minutes and centrifuged at 4ºC for 30 minutes at 18213g. The supernatant was further loaded carefully on the sucrose gradient prepared in polysome tubes.

Sucrose (84097, SIGMA) gradient tubes were prepared 1-day before the day of the experiment. 15% to 60% gradients were made, and stored at -80 ºC. The samples (supernatants) were added to each polysome tubes (331372, BECKMAN COULTER) slowly, and ultra-centrifuged (Beckman, OptimaXL 100K) at 4ºC at 39000 RPM for 1 hour and 40 minutes. The tubes were then transferred to UV Visible spectrophotometer (Model: Type 11 Optical unit with reference Flowcell/No bracket, Serial No: 213K20162 at National Centre for Biological Sciences), and Fraction collector instrument (from TELEDYNE ISCO at NCBS). Fractions were collected at A254 spectra. The bottom of the tube was pierced using needle syringe attached to a pipe

holding 60% sucrose. Addition of 60% sucrose from the bottom created a positive pressure, which pushed the fractions to come out from the top of the polysome tube. Then, the fractions were collected in 1.5 ml tubes. Total of 11 fractions was collected from each tube, and these fractions were treated with SDS loading dye containing β-Mercaptoethanol (MB041, HIMEDIA). SDS-PAGE was done for these fractions and immunoblotted for RPLP0 protein.

Synaptoneurosome preparation and NMDA stimulation: The Hippocampus were dissected out as described earlier. Mouse hippocampus was homogenised in 1000 µl of Synaptoneurosome buffer containing NaCl (116.5mM, S6191, Sigma), KCl (5mM, P5405, Sigma), $MgSO_4$ (1.2mM, M7506, Sigma), $CaCl_2$ (2.5mM, C5670, Sigma), KH_2PO_4 (1.53mM, GRM1188, HIMEDIA), Glucose (3.83%, G8270, Sigma), 1X Protease Inhibitor Cocktail (P5726, Sigma). Homogenate was filtered through 3, 100 µm filter (NY1H02500, Merck Millipore), then through 11 µm filter once (NY1102500, Merck Millipore). The filtrate obtained was centrifuged at 1500g for 15 minutes at 4ºC. Pellet was resuspended in 1 ml Synaptoneurosome buffer. NMDA receptor stimulation was done by applying NMDA (40 µM, M3262, Sigma) for 1-minute and 2-minute respectively at 37ºC at 350 RPM. After stimulation, the synaptoneurosome was centrifuged at 11000 RPM for 21 Seconds, and the pellet was resuspended in Lysis buffer followed by centrifugation at 18213g at 4ºC for 30 minutes. The supernatant was taken and denatured with SDS and β-Mercaptoethanol (MB041, HIMEDIA) containing denaturing dye and western blot was done.

RNA extraction and qPCR: Total RNA was extracted from the polysome fractions by Trizol (15596026, Thermofisher Scientific) method and the mRNAs were reverse transcribed to cDNA using iScript cDNA synthesis kit (1708891, Bio-Rad). qPCR was performed for *Syngap1*, *Fmr1*, and *β-actin* (Primers were designed and obtained from Sigma) using CFX384 Real-Time System from Bio-Rad. SYBR green was obtained from Bio-Rad (1725122). Cycle

Threshold (C_t) values obtained from the reactions were converted to the copy number of the mRNA, and then the percentage of these copy number in each fraction was plotted. List of primers used is mentioned below.

Transcript	**Forward sequence (5'→3')**	**Reverse Sequence (5'→3')**
Psd-95	ATGGCAGGTTGCAGATTGGA	GGTTGTGATGTCTGGGGGAG
β-actin	GGCTCCTAGCACCATGAAGAT	AAACGCAGCTCAGTAACAGTC
Syngap1	CAACCGGAAGCTGGAAGAG	CATCAGCCTGCCAATGATGC
Fmr1	GCAGTTGGTGCCTTCTCTGT	GCTGCCTTGAACTCTCCAGT

Quantification, analysis, and statistical methods: All graphs were plotted in Graph Pad Prism 7 and Microsoft Excel 2016. Extracellular field recordings were performed and analysed using Clampfit10.7 and Excel 2016. Time course data were plotted by averaging every 2 minutes. Example traces are those recorded for 1-2 min around the time point indicated. Error bars correspond to ±SEM (Standard Error of Mean). Unpaired Student's *t-test* and 2-way ANOVA were performed to test for difference between groups and different age unless otherwise stated.

Results

Reduced FMRP level during development in *Syngap1*$^{+/-}$

Studies have shown that Group I mGluR and NMDA receptors interact via Homer-Shank that may regulate protein synthesis (Tu et al., 1999; Bertaso et al., 2010). To determine whether Group I mGluR activation in *Syngap1*$^{-/+}$ results in altered protein synthesis and hippocampal pathophysiology similar to the mouse model of Fragile X Syndrome, *Fmr1*$^{-/y}$, Group I mGluR-mediated LTD (mGluR-LTD) was induced in the Schaffer-collateral pathway in adult mice by bath applying DHPG, a Group I mGluR agonist, for 5 minutes. We observed significantly

increased mGluR-LTD in *Syngap1*$^{+/-}$ mice (*Syngap1*$^{+/-}$ referred as HET in Figures; 47±4% LTD) as compared to their Wild-type littermate controls (61±3% LTD; p=0.012; **Figure 1A**). This result suggests that mGluR-LTD in *Syngap1*$^{+/-}$ is similar to *Fmr1*$^{-/y}$ as shown by Barnes et al. (Barnes et al., 2015), and further reiterates the fact that protein synthesis downstream of synaptic signalling is likely to be altered in *Syngap1*$^{+/-}$ similar to *Fmr1*$^{-/y}$, which prompted us to investigate the role of FMRP in the pathophysiology of *Syngap1*$^{+/-}$ mutation.

We studied the expression of FMRP in the hippocampus of Wild-type and *Syngap1*$^{+/-}$ mice during different Post-Natal Days, starting from PND4 till 2-5 months old. Using quantitative immunoblotting, we observed that FMRP level (normalised to β-ACTIN) was reduced in *Syngap1*$^{+/-}$ mice (0.98±0.06) as compared to Wild-type in PND21-23 (1.31±0.09; p=0.0120; **Figure 1B-C**) but not in other age groups. Previous studies have shown that SYNGAP1 level was reduced significantly during development in *Syngap1*$^{+/-}$ mice that affected synaptic transmission (Vazquez et al., 2004; Clement et al., 2012). To study whether reduced level of FMRP is compensating for the altered SYNGAP1 level in *Syngap1*$^{+/-}$ mice, expression of SYNGAP1 in Wild-type and *Syngap1*$^{+/-}$ mice was quantified as shown in **Figure 1D (**Genotype: p<0.0001). Upon further analysis, we found that SYNGAP1 level was increased during PND21-23 (1.12±0.09) compared to PND14-16 in *Syngap1*$^{+/-}$ (0.83±0.05; p=0.0236; **Figure 1E**). In contrast, the level of SYNGAP1 was not altered significantly between PND21-23 (1.82±0.06) and PND14-16 (1.33±0.08) in Wild-type mice (p=0.0863; **Figure 1E**).

FMRP interacts with *Syngap1* mRNA in the hippocampus

FMRP is a known regulator of synaptic translation (Osterweil et al., 2010). A previous study using High Throughput sequencing of RNA isolated by Crosslinking Immunoprecipitation (HITS-CLIP) has reported that one of the FMRP targets is *Syngap1* mRNA (Darnell & Klann, 2013). G-quadruplexes are one of the structures present in RNA which can be recognised by FMRP (Darnell et al., 2001). To test whether *Syngap1* mRNA harbours signatures of being

targetted by FMRP, we did bioinformatics analysis using Quadraplex forming G-Rich Sequences (QGRS) Mapper that predicted the presence of multiple G-quadruplexes structures with high G-Score in *Syngap1* mRNA (**Figure 1-1A**). In addition, G-quadruplex forming residues were found to be conserved among mice, rat, and human *Syngap1* mRNA (**Figure 1-1B**). To further confirm the interaction of FMRP with *Syngap1* mRNA, we performed FMRP immunoprecipitation from mouse hippocampal lysates to investigate the enrichment of *Syngap1* mRNA by qPCR. We observed ~2-fold enrichment of *Syngap1* mRNA relative to *β-actin* mRNA in FMRP IP pellet over supernatant (2.16±0.18, p=0.0036; **Figure 1F**). *PSD-95* mRNA, a known FMRP target mRNA (Muddashetty et al., 2011) showed a significant 3.5-fold enrichment compared to *β-actin* mRNA (p=0.0001; 3.59±0.07; **Figure 1F**) which we used as a positive control. These results demonstrate that FMRP interacts with *Syngap1* mRNA.

***Syngap1* mRNA translation is differentially regulated in *Syngap1*$^{+/-}$**

To further understand the increase in SYNGAP1 levels during PND21-23 compared to PND14-16 in *Syngap1*$^{+/-}$, we analysed *Syngap1* mRNA translation status at PND14-16 and PND21-13. We studied translation by Polysome profile (**Figure 2A**), from hippocampal lysates of Wild-type and *Syngap1*$^{+/-}$ mice at PND14-16 and PND21-23 (Muddashetty et al., 2007). **Figure 2B** demonstrates that the A_{254} traces from cycloheximide-treated hippocampal lysate showed the distinct peaks which correspond to mRNP, monosome and polysomes respectively. Further, immunoblots for Ribosomal large subunit protein, RPLP0, has shown a shift in puromycin treated sample as puromycin disassemble the ribosome from translating mRNA (**Figure 2B**), along with a shift in *β-actin* mRNA (**Figure 2-1A**). In our experiments, fraction numbers 1 to 6 and 7 to 11 were considered as non-translating fractions and translating fractions or polysome (puromycin-sensitive) respectively (**Figure 2-1A, 2-1B**). Further, we estimated the RPLP0 distribution in translating/non-translating fractions of WT and *Syngap1*$^{+/-}$ during PND14-16 (WT=1.06±0.18, *Syngap1*$^{+/-}$=0.71±0.15, p=0.22) and PND21-23 (WT=1.27±0.21, *Syngap1*$^{+/-}$

=0.83±0.17, p=0.14), suggesting no significant change in the distribution of RPLP0 (**Figure 2C, 2-1C**). To understand the translation status of *Syngap1* mRNA during PND14-16 and PND21-23, we quantified *Syngap1* mRNA present in translating fraction by performing quantitative PCR from RNA isolated from both non-translating (Fraction 1-6) and translating fractions (Fraction 7-11). A significant reduction in translating *Syngap1* mRNA in *Syngap1*$^{+/-}$ mice during PND14-16 compared to WT (WT=84.32±4%; *Syngap1*$^{+/-}$=65.77±2%; p=0.0018; **Figure 2D**) was observed. On the contrary, this difference was absent during PND21-23 (WT=92.9±3.5%; *Syngap1*$^{+/-}$=87.9±2.5%; p=0.3033). As a control, distribution of *β-actin* mRNA in translating pool was quantified, and no significant difference was observed between Wild-type and *Syngap1*$^{+/-}$ at PND14-16 (WT=89.9±3%; *Syngap1*$^{+/-}$=83.6±2.9%; p=0.2039) and PND21-23 (WT=97.9±0.6%; *Syngap1*$^{+/-}$=89.8±3.8%; p=0.0697; **Figure 2-1B**). These results suggest that an increase in *Syngap1* mRNA translation leads to the corresponding increase in SYNGAP1 level observed in PND21-23 when compared to PND14-16 in *Syngap1*$^{+/-}$.

Reduced FMRP in polysome at PND21-23 in *Syngap1*$^{+/-}$

To understand if the changes in the levels of translating *Syngap1* mRNA is a result of the altered association of FMRP with polysomes, we estimated the distribution of FMRP in translating/non-translating fraction from polysome profiling. We observed that the level of FMRP was increased in PND14-16 in the polysomal fraction in *Syngap1*$^{+/-}$ (0.41±0.03) as compared to age-matched Wild-type (0.17±0.02) control (p=0.0011; **Figure 3A, 3B**). However, we observed reduced FMRP distribution in the polysomal fraction of *Syngap1*$^{+/-}$ (0.23±0.03) in PND21-23 compared to Wild-type (0.55±0.15; p=0.0473; **Figure 3A, 3B**). This might have a compounding effect on translation of FMRP target mRNAs during PND21-23 in *Syngap1*$^{+/-}$ as the overall FMRP level was also reduced. As a control, we analysed the PSD-95 levels during PND14-16 (WT=2.03±0.35; *Syngap1*$^{+/-}$=1.39±0.15; p=0.125) and PND21-23

(WT=0.98±0.05; *Syngap1*$^{+/-}$=0.98±0.12; p=0.96). However, we did not observe any change between Wild-type and *Syngap1*$^{+/-}$ (**Figure 3-1A, 3-1B**).

Our previous result showed reduced FMRP level during PND21-23 in *Syngap1*$^{+/-}$ as compared to its Wild-type counterpart (**Figure 1C**). We investigated whether the reduced level of FMRP is due to altered *Fmr1* mRNA levels or translation. We evaluated the levels of *Fmr1* mRNA from the hippocampal lysates of Wild-type and *Syngap1*$^{+/-}$ mice at PND14-16 and PND21-23. We did not observe any significant difference in *Fmr1* mRNA levels in PND14-16 (WT=0.019±0.008; *Syngap1*$^{+/-}$=0.029±0.008; p=0.4065) and PND21-23 (WT=0.009±0.001; *Syngap1*$^{+/-}$=0.020±0.009; p=0.3129) between Wild-type and *Syngap1*$^{+/-}$ (**Figure 3-1B**). This result suggests that reduction in FMRP levels in *Syngap1*$^{+/-}$ mice at PND21-23 could be due to a decrease in *Fmr1* translation.

To further understand the translation status of *Fmr1* mRNA during PND14-16 and PND21-23, *Fmr1* mRNA present in translating fraction was quantified by qPCR. We found that *Fmr1* mRNA present in translating pool was significantly reduced in *Syngap1*$^{+/-}$ mice, as compared to Wild-type (**Figure 3C**) in PND21-23 (WT=89.34±1.03%; *Syngap1*$^{+/-}$=73.38±4%; p=0.0257), but not in PND14-16 (WT=66.66±2.9%; *Syngap1*$^{+/-}$=66.03±4.1%; p=0.9058) indicating reduced FMRP level as a result of decreased *Fmr1* mRNA translation in PND21-23 in *Syngap1*$^{+/-}$.

Altered NMDAR-mediated translation response in *Syngap1*$^{+/-}$

Previous studies have shown increased levels of basal protein synthesis in *Syngap1*$^{+/-}$(Wang et al., 2013; Barnes et al., 2015). SYNGAP1 regulates synaptic maturation during a critical time window, and our results demonstrated the altered expression of FMRP during a specific developmental stage in *Syngap1*$^{+/-}$. Based on this, we hypothesised that the global translational status could be different at these developmental stages. To study this, the phosphorylation

status of eukaryotic Elongation Factor 2 (eEF2) was used as a read-out of translation response. Phosphorylation of eEF2 has been shown to repress global translation (Scheetz, Nairn, & Constantine-paton, 2000). We analysed phospho/total-eEF2 in hippocampal synaptoneurosomes in response to NMDAR stimulation from Wild-type and *Syngap1*$^{+/-}$ mice at PND14-16 and PND21-23 using immunoblotting analysis. Hippocampal synaptoneurosome preparation was evaluated by validating the enrichment of PSD-95 as shown by Muddashetty *et al.*, 2007 **(Figure 4-1A)**. As a proof of principle, we demonstrated that NMDAR stimulation of synaptoneurosomes from Wild-type mice showed ~1.5-fold increase in phospho/total-eEF2 after 1-minute of stimulation (Basal=0.84±0.11; Stimulated=1.3±0.12; p=0.0376; **Figure 4A**).

Further, we observed an increase in phospho/total-eEF2 in *Syngap1*$^{+/-}$ as compared to Wild-type under basal conditions in both PND14-16 (WT=0.84±0.11; *Syngap1*$^{+/-}$=1.6±0.22%; p=0.0245) and PND21-23 (WT=0.22±0.01%; *Syngap1*$^{+/-}$=0.9±0.11%; p=0.0233 with Welch's correction; **Figure 4B and 4C**). We found that, at PND14-16, NMDAR-mediated increase in phosphorylation of eEF2 was lost in synaptoneurosomes in *Syngap1*$^{+/-}$ (Stimulated/Basal values for WT=1.57±0.2; *Syngap1*$^{+/-}$=0.7±0.09; p=.0069). Further analysis of this data by normalising to Wild-type showed a significant reduction in phospho/total eEF2 on NMDA stimulation in *Syngap1*$^{+/-}$ synaptoneurosomes. (Stimulated/Basal values for WT=1.00±0.12; *Syngap1*$^{+/-}$=0.45±0.06; **Figure 4B).** That could be because of the increased level of phosphorylated-eEF2 at basal level in *Syngap1*$^{+/-}$ mice. Surprisingly, even though we observed an increase in the level of basal phospho/total-eEF2 in PND21-23 in *Syngap1*$^{+/-}$ synaptoneurosomes, NMDAR-mediated increase in phosphorylated-eEF2 in *Syngap1*$^{+/-}$ was recovered to Wild-type level (Stimulated/Basal values for WT=1.81±0.14; *Syngap1*$^{+/-}$ =1.92±0.45; p=0.8233; **Figure 4C**). The similar phenomenon was observed on 2-minute stimulation of NMDA (**Figure 4-1B, 4-1C**) This suggests that the NMDAR-mediated

translation upon NMDAR activation at PND21-23 in *Syngap1*$^{+/-}$ returned to the level comparable to that of Wild-type.

Discussion

Many synaptic plasticity mechanisms are dependent on activity mediated local protein synthesis in neurons (Klann et al., 2004; Pfeiffer and Huber, 2006). Protein synthesis is regulated stringently at the synapse. One such crucial regulator of synaptic protein synthesis is FMRP, which is encoded by *FMR1* gene, the absence of which leads to Fragile X Syndrome, a monogenic cause of ID similar to *SYNGAP1*$^{+/-}$ (Garber et al., 2008; Hamdan et al., 2009). Our observation of enhanced mGluR-LTD in the CA1 hippocampal region of *Syngap1*$^{+/-}$ is in line with the previous observation of enhanced basal protein synthesis in the heterozygous loss of *Syngap1* prompted us to investigate the role of FMRP in the pathophysiology of *Syngap1*$^{+/-}$ mutation (Wang et al., 2013; Barnes et al., 2015). Till date, only one study reports relationships between SYNGAP1 and FMRP (Barnes et al., 2015). The authors proposed that FMRP and SYNGAP1 leads to opposite effects on synapse development, with FMRP deficits resulting in delayed synaptic maturation, and *SYNGAP1* haploinsufficiency causing accelerated maturation of dendritic spines. Following this, Barnes et al. crossed *Fmr1*$^{-/Y}$ with *Syngap1*$^{+/-}$ but failed to rescue the electrophysiological deficit observed in *Syngap1*$^{+/-}$ (Barnes et al., 2015). This indicates that chronic depletion of these genes may not be an effective measure to rescue the pathophysiology observed in *Syngap1*$^{+/-}$, as both these genes are important for normal brain development. Since SYNGAP1 is known to regulate synaptic maturation during a specific developmental window (Clement et al., 2012; Clement et al., 2013), we hypothesised that the role of FMRP in *Syngap1*$^{+/-}$ could also be developmentally regulated. Hence, we looked at the developmental expression profile of FMRP in the hippocampus of *Syngap1*$^{+/}$ mice. Our results show reduced expression of FMRP specifically in PND21-23 in *Syngap1*$^{+/-}$. We demonstrate for the first time that FMRP interacts with *Syngap1* mRNA and probably regulate its

translation. We also show that the reduction in FMRP levels at PND21-23 in *Syngap1*$^{+/-}$ is required for the compensatory increase in SYNGAP1 levels via increased *Syngap1* mRNA translation. In polysome profiling assay, we did not observe any significant difference in the A_{254} traces or in the distribution of protein RPLP0 between Wild-type and *Syngap1*$^{+/-}$ animals indicating no difference in the basal translation in the hippocampus from *Syngap1*$^{+/-}$ animals at PND14-16 and PND21-23.

Studies have reported that NMDAR-mediated signalling is dysregulated in *Syngap1*$^{+/-}$ (Komiyama et al., 2002; Rumbaugh et al., 2006; Carlisle et al., 2008). These studies have further shown that SYNGAP1 associates with NR2B (Rockliffe and Gawler, 2006) and negatively regulates NMDA receptor-mediated ERK activation (Kim et al., 2005) and, hence, regulates insertion of AMPAR in the post-synaptic membrane (Rumbaugh et al., 2006). In line with this, studies have demonstrated increased basal levels of ERK phosphorylation in *Syngap1*$^{+/-}$ (Komiyama et al., 2002) which does not explain the deficits observed in NMDAR-LTP in *Syngap1*$^{+/-}$ mice as NMDAR stimulation resulted in a robust increase in ERK activation in slices from *Syngap1*$^{+/-}$ mice (Komiyama et al., 2002). Thus, to understand the deficits seen in NMDAR-mediated signalling in *Syngap1*$^{+/-}$ mice, we studied NMDAR-mediated translation repression. It has already been reported that NMDAR activation causes a reduction in global translation through phosphorylation of eEF2 (Scheetz et al., 2000). In our study, we measured the basal levels of phosphorylated eEF2 in hippocampal synaptoneurosomes from Wild-type and *Syngap1*$^{+/-}$ at PND14-16 and PND21-23 which showed increased phosphorylation of eEF2 at the basal condition in *Syngap1*$^{+/-}$ in these age groups. This increase in the basal level of phosphorylation of eEF2 could be due to enhanced excitatory neuronal activity in *Syngap1*$^{+/-}$ which might lead to an increase in Ca^{2+} levels and a subsequent increase in eEF2 phosphorylation via Ca^{2+}-Calmodulin kinase. We report that, at PND14-16, NMDAR activation fails to cause eEF2 phosphorylation in *Syngap1*$^{+/-}$ animals. Strikingly, at 3-week of

age, even though we observed an increased level of basal phospho/total-eEF2 in *Syngap1*$^{+/-}$ synaptoneurosomes, NMDAR-mediated increase in eEF2 phosphorylation was similar to Wild-type (**Figure 4C**). This observation suggests that NMDAR-mediated translation response at PND21-23 in *Syngap1*$^{+/-}$ is restored. This change observed in 3-week old mice could be due to a compensatory mechanism through increased NMDAR signalling. These findings further corroborate with the observations made by Clement et al. in which they have demonstrated increased synaptic transmission and increased AMPA/NMDA in PND14 but returns to normal level in the later age (Clement et al., 2012). Based on our findings, we propose a model in which increased NMDAR-mediated protein synthesis is compensating for the loss of SYNGAP1 during development in *Syngap1*$^{+/-}$ (**Figure 5**). We further propose that fine-tuned downregulation of *Fmr1* translation during a specific developmental window in *Syngap1*$^{+/-}$ mice might compensate for the dysregulation in NMDAR-mediated signalling.

In conclusion, our study suggests that altered response to activity mediated protein synthesis during development is one of the major causes of abnormal neuronal function in *Syngap1*$^{+/-}$. However, chronic depletion of two genes with common core pathophysiology may not be an effective measure to rescue the deficits observed in either of these mutations, *i.e*. *Fmr1*$^{-/y}$ and *Syngap1*$^{+/-}$, as both these genes are important for normal brain development. Therefore, modulating these proteins at a certain developmental window could be a potential therapeutic strategy for treating ID-related pathophysiology.

References:

Araki Y, Zeng M, Zhang M, Huganir RL (2015) Rapid dispersion of SynGAP from synaptic spines triggers AMPA receptor insertion and spine enlargement during LTP. Neuron 85:173-189.

Barnes SA, Wijetunge LS, Jackson AD, Katsanevaki D, Osterweil EK, Komiyama NH, Grant SG, Bear MF, Nagerl UV, Kind PC, Wyllie DJ (2015) Convergence of Hippocampal Pathophysiology in Syngap+/- and Fmr1-/y Mice. J Neurosci 35:15073-15081.

Bertaso F, Roussignol G, Worley P, Bockaert J, Fagni L, Ango F (2010) Homer1a-dependent crosstalk between NMDA and metabotropic glutamate receptors in mouse neurons. PLoS One 5:e9755.

Carlisle HJ, Manzerra P, Marcora E, Kennedy MB (2008) SynGAP regulates steady-state and activity-dependent phosphorylation of cofilin. J Neurosci 28:13673-13683.

Clement JP, Ozkan ED, Aceti M, Miller CA, Rumbaugh G (2013) SYNGAP1 links the maturation rate of excitatory synapses to the duration of critical-period synaptic plasticity. J Neurosci 33:10447-10452.

Clement JP, Aceti M, Creson TK, Ozkan ED, Shi Y, Reish NJ, Almonte AG, Miller BH, Wiltgen BJ, Miller CA, Xu X, Rumbaugh G (2012) Pathogenic SYNGAP1 mutations impair cognitive development by disrupting maturation of dendritic spine synapses. Cell 151:709-723.

Darnell JC, Jensen KB, Jin P, Brown V, Warren ST, Darnell RB (2001) Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. Cell 107:489-499.

Garber KB, Visootsak J, Warren ST (2008) Fragile X syndrome. Eur J Hum Genet 16:666-672.

Hamdan FF, Daoud H, Piton A, Gauthier J, Dobrzeniecka S, Krebs MO, Joober R, Lacaille JC, Nadeau A, Milunsky JM, Wang Z, Carmant L, Mottron L, Beauchamp MH, Rouleau GA, Michaud JL (2011) De novo SYNGAP1 mutations in nonsyndromic intellectual disability and autism. Biol Psychiatry 69:898-901.

Hamdan FF et al. (2009) Mutations in SYNGAP1 in autosomal nonsyndromic mental retardation. N Engl J Med 360:599-605.

Huber KM, Gallagher SM, Warren ST, Bear MF (2002) Altered synaptic plasticity in a mouse model of fragile X mental retardation. Proc Natl Acad Sci U S A 99:7746-7750.

Jeyabalan N, Clement JP (2016) SYNGAP1: Mind the Gap. Front Cell Neurosci 10:32.

Kim JH, Liao D, Lau LF, Huganir RL (1998) SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. Neuron 20:683-691.

Kim JH, Lee HK, Takamiya K, Huganir RL (2003) The role of synaptic GTPase-activating protein in neuronal development and synaptic plasticity. The Journal of neuroscience : the official journal of the Society for Neuroscience 23:1119-1124.

Kim MJ, Dunah AW, Wang YT, Sheng M (2005) Differential roles of NR2A- and NR2B-containing NMDA receptors in Ras-ERK signaling and AMPA receptor trafficking. Neuron 46:745-760.

Klann E, Antion MD, Banko JL, Hou L (2004) Synaptic plasticity and translation initiation. Learn Mem 11:365-372.

Komiyama NH, Watabe AM, Carlisle HJ, Porter K, Charlesworth P, Monti J, Strathdee DJ, O'Carroll CM, Martin SJ, Morris RG, O'Dell TJ, Grant SG (2002) SynGAP regulates ERK/MAPK signaling, synaptic plasticity, and learning in the complex with postsynaptic density 95 and NMDA receptor. J Neurosci 22:9721-9732.

Krapivinsky G, Medina I, Krapivinsky L, Gapon S, Clapham DE (2004) SynGAP-MUPP1-CaMKII synaptic complexes regulate p38 MAP kinase activity and NMDA receptor-dependent synaptic AMPA receptor potentiation. Neuron 43:563-574.

Muddashetty RS, Kelic S, Gross C, Xu M, Bassell GJ (2007) Dysregulated metabotropic glutamate receptor-dependent translation of AMPA receptor and postsynaptic density-95 mRNAs at synapses in a mouse model of fragile X syndrome. J Neurosci 27:5338-5348.

Muddashetty RS, Nalavadi VC, Gross C, Yao X, Xing L, Laur O, Warren ST, Bassell GJ (2011) Reversible inhibition of PSD-95 mRNA translation by miR-125a, FMRP phosphorylation, and mGluR signaling. Mol Cell 42:673-688.

Osterweil EK, Krueger DD, Reinhold K, Bear MF (2010) Hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hippocampus of a mouse model of fragile X syndrome. J Neurosci 30:15616-15627.

Pfeiffer BE, Huber KM (2006) Current advances in local protein synthesis and synaptic plasticity. J Neurosci 26:7147-7150.

Rauch A et al. (2012) Range of genetic mutations associated with severe non-syndromic sporadic intellectual disability: an exome sequencing study. Lancet 380:1674-1682.

Rockliffe N, Gawler D (2006) Differential mechanisms of glutamate receptor regulation of SynGAP in cortical neurones. FEBS Lett 580:831-838.

Rumbaugh G, Adams JP, Kim JH, Huganir RL (2006) SynGAP regulates synaptic strength and mitogen-activated protein kinases in cultured neurons. Proc Natl Acad Sci U S A 103:4344-4351.

Scheetz AJ, Nairn AC, Constantine-Paton M (2000) NMDA receptor-mediated control of protein synthesis at developing synapses. Nat Neurosci 3:211-216.

Tu JC, Xiao B, Naisbitt S, Yuan JP, Petralia RS, Brakeman P, Doan A, Aakalu VK, Lanahan AA, Sheng M, Worley PF (1999) Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. Neuron 23:583-592.

Vazquez LE, Chen HJ, Sokolova I, Knuesel I, Kennedy MB (2004) SynGAP regulates spine formation. J Neurosci 24:8862-8872.

Wang CC, Held RG, Hall BJ (2013) SynGAP regulates protein synthesis and homeostatic synaptic plasticity in developing cortical networks. PLoS One 8:e83941.

Zhu JJ, Qin Y, Zhao M, Van Aelst L, Malinow R (2002) Ras and Rap control AMPA receptor trafficking during synaptic plasticity. Cell 110:443-455.

Figure 1:

Altered expression of FMRP in the hippocampus of *Syngap1*$^{+/-}$ during development.

A. Application of 50 μm (*S*)-DHPG induced enhanced Group I mGluR mediated LTD in the Schaffer-collateral pathway of *Syngap1*$^{+/-}$ (HET) compared to Wild-type (WT) littermates. Sample traces obtained before and after induction of LTD as indicated by time points (*top*). WT=61±3% LTD, n=14; HET=47±4% LTD, n=15; * $p<0.05$

B. Representative immunoblot for FMRP level in the hippocampus during development (*top*). Pooled data of FMRP level normalised to β-ACTIN in the hippocampus during development (*below*). PND4-5 (WT: n=12; HET: n=7), PND7-9 (WT: n=8; HET: n=7), PND14-16 (WT: n=10; HET: n=8), PND21-23 (WT: n=14; HET: n=10), PND30-31 (WT: n=4; HET: n=7), PND>60 (WT: n=8; HET: n=10). *$p<0.05$; Two-way ANOVA, $F_{(5, 93)} = 0.7965$.

C. Bar graph derived from **1B** depicting FMRP level during PND21-23 in WT (n=14) and HET (n=10), *$p<0.05$, NS = not significant.

D. Representative Immunoblots for SYNGAP1 during development (*top*). SYNGAP1 level normalised to β-ACTIN during PND14-16 (WT: n=5; HET: n=5), PND21-23 (WT: n=4; HET: n=5), and adults *i.e*, PND>60 (WT: n=4; HET: n=3); ****$P<0.0001$, Two-way ANOVA, $F_{(1, 15)} = 0.1893$.

E. Bar graph derived from **1D** shows increased SYNGAP1 level in HET during PND21-23 (WT: n=4; HET: n=5) when compared to PND14-16 (WT: n=5; HET: n=5) while no significant change was observed in WT. *p<0.05, NS= not significant.

F: Immunoblot showing FMRP-IP, IgG-IP, and input probed for FMRP (*top).* Bar graph showing relative *Syngap1*, *Psd95* mRNA enrichment in FMRP IP pellet compared to supernatant after normalising to *β-Actin* (WT: n=3; *Below)* at PND14-16. One-way ANOVA followed by Dunnett's multiple comparisons test. **p<0.01, ****p<0.0001,

Figure 2. Altered *Syngap1* mRNA distribution in translating polysomal fractions of HET.

A. Schematic diagram depicting sucrose gradient method used for polyribosome profiling (translation assay)

B. Polyribosome profile obtained from Cycloheximide treated hippocampal lysate during PND14-16 in HET (*top*). Representative immunoblots for RPLP0 distribution in Cycloheximide and Puromycin treated polysome during PND14-16 (*below*).

C. Bar graph shows RPLP0 distribution in Translating/Non-translating fractions during PND14-16 (WT: n=5; HET: n=3; p=0.22) and PND21-23 (WT: n=4; HET: n=4; p=0.14). Unpaired Student's *t*-test was done for both age groups. NS = not significant.

D. *Syngap1* mRNA distribution in polysome in HET normalised to WT during PND14-16 (WT: n=4; HET: n=6; p=0.0018) and PND21-23 (WT: n=3; HET: n=3; p=0.3033).

Figure 3. **Decreased *Fmr1* mRNA and FMRP distribution in translating fractions of polysomes in HET during PND21-23**.

A. Representative line graphs showing percentage FMRP distribution in polysomes during PND14-16 (*top*) along with representative immunoblot for FMRP distribution (*below*).

B. Line graph showing representative percentage FMRP distribution in polysomes during PND21-23 (*top*) and the corresponding representative immunoblot for FMRP distribution (*below*).

C. Bar graph showing FMRP distribution in translating/non-translating fractions in HET normalised to WT during PND14-16 (WT: n=4; HET: n=4) and PND21-23 (WT: n=4; HET: n=5). *p<0.05, **p<0.01.

D. Bar graph depicting relative *Fmr1* mRNA in translating fractions of HET normalised to WT during PND14-16 (WT: n=3; HET: n=3) and PND21-23 (WT: n=3; HET: n=5). *p<0.05, NS= not significant.

Figure 4. **Dysregulated NMDAR mediated translation response is recovered during PND21-23 in HET**

A. Representative immunoblot for Phospho-eEF2 and Total-eEF2 showing increased phosphorylation on NMDAR stimulation for 1-minute (NMDA 1') in synaptoneurosomes from WT during PND21-23 (*left*). Pooled data of the same represented in the bar graph (*right,* Basal: n=4; Stimulated: n=4); *p<0.05.

B. Representative immunoblots of phospho- and total-eEF2 normalised to β-ACTIN during PND14-16 in WT and HET (*top*). The bar graph shows increased phosphorylation of eEF2 at basal conditions in synaptoneurosome obtained from the hippocampus of HET as compared to WT during PND14-16 in the (*middle,* WT: n=4; HET: n=3). Bar graph showing decreased phosphorylation of eEF2 in HET on NMDAR stimulation as compared to WT in PND14-16 (*below,* WT: n=4; HET: n=4). *p<0.05, **p<0.01, NS= not significant.

C. Representative immunoblots for phospho-eEF2 and total-eEF2 normalised to β-ACTIN during PND21-23 in WT and HET (*top*). Increased phosphorylation of eEF2 at the basal condition in HET synaptoneurosome as compared to WT during PND21-23 (*middle,* WT: n=3;

HET: n=3). Bar graph showing the extent of phosphorylation in HET is similar to WT during PND21-23 (*bottom*, WT: n=3; HET: n=4). *p<0.05, NS= not significant.

Figure 5: **Model is illustrating the regulation of *Syngap1* translation by FMRP during development:** This model shows that FMRP regulates *Syngap1* mRNA translation that in turn modulates NMDAR-mediated signalling. In WT, FMRP interacts with *Syngap1* mRNA and regulates its expression level. SYNGAP1 negatively regulates NMDAR-mediated signalling through phosphorylation of eEF2. Phosphorylation of eEF2 leads to inhibition on translation whereas dephosphorylated form allows translation. In HET, studies have shown that SYNGAP1 level is low, and thereby, cannot efficiently control the NMDAR mediated signalling leading to increased phosphorylation of eEF2 under basal condition (Shown as green dotted arrow). Based on our results, at PND21-23, FMRP level is reduced in HET which leads to increase in SYNGAP1 level that partially restores activity dependent NMDAR-mediated signalling.

Figure 1-1: *Syngap1* mRNA contains predicted G quadruplexes.

A. Multiple putative G-quadruplex was detected using QGRS Mapper in the validated sequence available for mouse *Syngap1* from NCBI (Gene ID: 240057). Three high G-score containing G- quadruplex sequences are highlighted in the red box. All these sequences have been mapped in the Coding Sequence (CDS).

B1-3. Multiple sequence alignment of the three high score G-quadruplexes of mouse *Syngap1* compared with Human and Rat. G scores: 82 (B1), 42 (B2), and 40 (B3) showing putative G-quadruplexes conserved among Human, Mouse, and Rat respectively.

Figure 2-1: RPLP0 distribution unaltered in polysome

A. *β-actin* mRNA distribution in polysome treated with cycloheximide and puromycin

B. Bar diagram showing *β-actin* mRNA distribution in Cycloheximide treated polysome HET normalised to WT in PND14-16 (WT: n=6; HET: n=6) and PND21-23 (WT: n=4; HET: n=5). NS = not significant.

C. Representative percentage RPLP0 distribution line graph in PND14-16 (*left*) and PND21-23 (*right*).

Figure 3-1: PSD-95 level in PND14-16 and PND21-23

A. Representative immunoblots for PSD-95 normalised to β-ACTIN in the hippocampus during PND14-16 and PND21-23 in WT and HET. *Below* Bar graph showing a no significant difference in the level of PSD-95 at PND14-16 (WT: n=6; HET: n=6) and PND21-23 (WT: n=10; HET: n=4) between WT and HET. *p<0.05, NS= not significant.

B. Bar graph depicting relative *Fmr1* mRNA normalised to *β-actin* from total hippocampal lysate at PND14-16 (*left*, WT: n=3; HET: n=3) and PND21-23 (*right*, WT: n=3; HET: n=3); NS= not significant.

Figure 4-1. Altered phosphorylation of eEF2 in HET

A. Representative immunoblot depicting the enrichment of PSD-95 in synaptoneurosome (S) compared to Total hippocampal lysate (T; *left*). Bar graph is showing quantified data normalised to α-TUBULIN (*right*).

B. Representative immunoblot images for Phospho-eEF2, Total-eEF2, and β-ACTIN synaptoneurosomes after 2-minute NMDAR stimulation during PND14-16 (*top*). Bar graph showing that 2-minute activation of NMDAR alters phosphorylation of eEF2 in HET (n=3) compared to WT (n=3) during PND 14-16 (*below*). *p<0.05, NS= not significant

C. Representative immunoblot for Phospho- and Total-eEF2 normalised to β-ACTIN synaptoneurosomes stimulated with NMDA for 2-minute in WT and HET during PND21-23

(*top*). Bar graph depicting unaltered phosphorylation of eEF2 in HET (n=3) compared to WT (n=3) post-2-minute activation of NMDAR. NS= not significant.

Figure 1

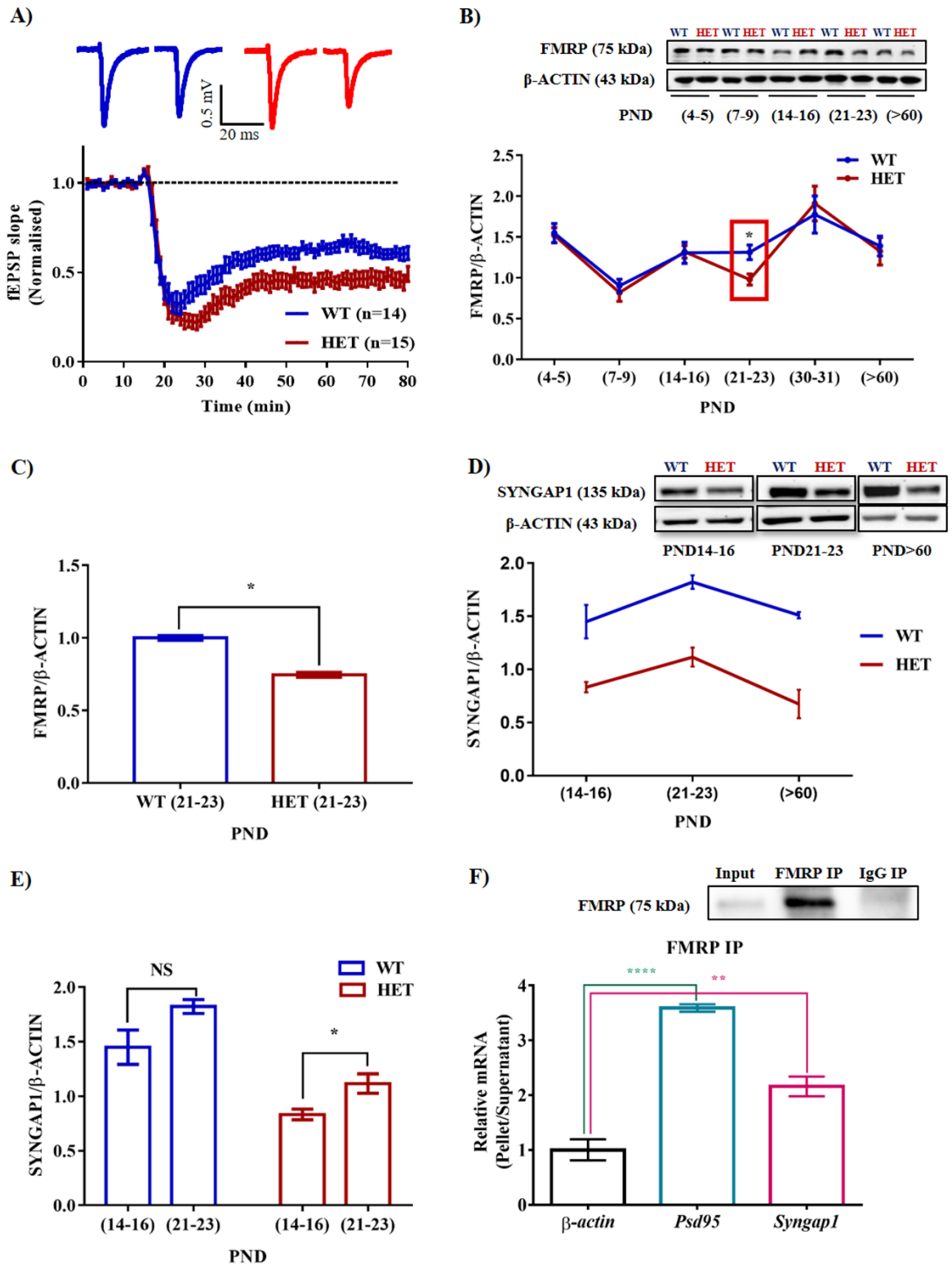

A)
0.5 mV
20 ms
fEPSP slope (Normalised)
1.0
0.5
0.0
0
10
20
30
40
50
60
70
80
Time (min)
WT (n=14)
HET (n=15)
B)
WT HET WT HET WT HET WT HET WT HET
FMRP (75 kDa)
β-ACTIN (43 kDa)
PND (4-5) (7-9) (14-16) (21-23) (>60)
FMRP/β-ACTIN
2.5
2.0
1.5
1.0
0.5
0.0
*
WT
HET
(4-5) (7-9) (14-16) (21-23) (30-31) (>60)
PND
C)
FMRP/β-ACTIN
1.5
1.0
0.5
0.0
*
WT (21-23)
HET (21-23)
PND
D)
WT HET WT HET WT HET
SYNGAP1 (135 kDa)
β-ACTIN (43 kDa)
PND14-16
PND21-23
PND>60
SYNGAP1/β-ACTIN
2.0
1.5
1.0
0.5
0.0
WT
HET
(14-16)
(21-23)
(>60)
PND
E)
SYNGAP1/β-ACTIN
2.0
1.5
1.0
0.5
0.0
NS
*
WT
HET
(14-16) (21-23) (14-16) (21-23)
PND
F)
Input FMRP IP IgG IP
FMRP (75 kDa)
FMRP IP
****
**
Relative mRNA (Pellet/Supernatant)
4
3
2
1
0
β-actin
Psd95
Syngap1

Figure 2

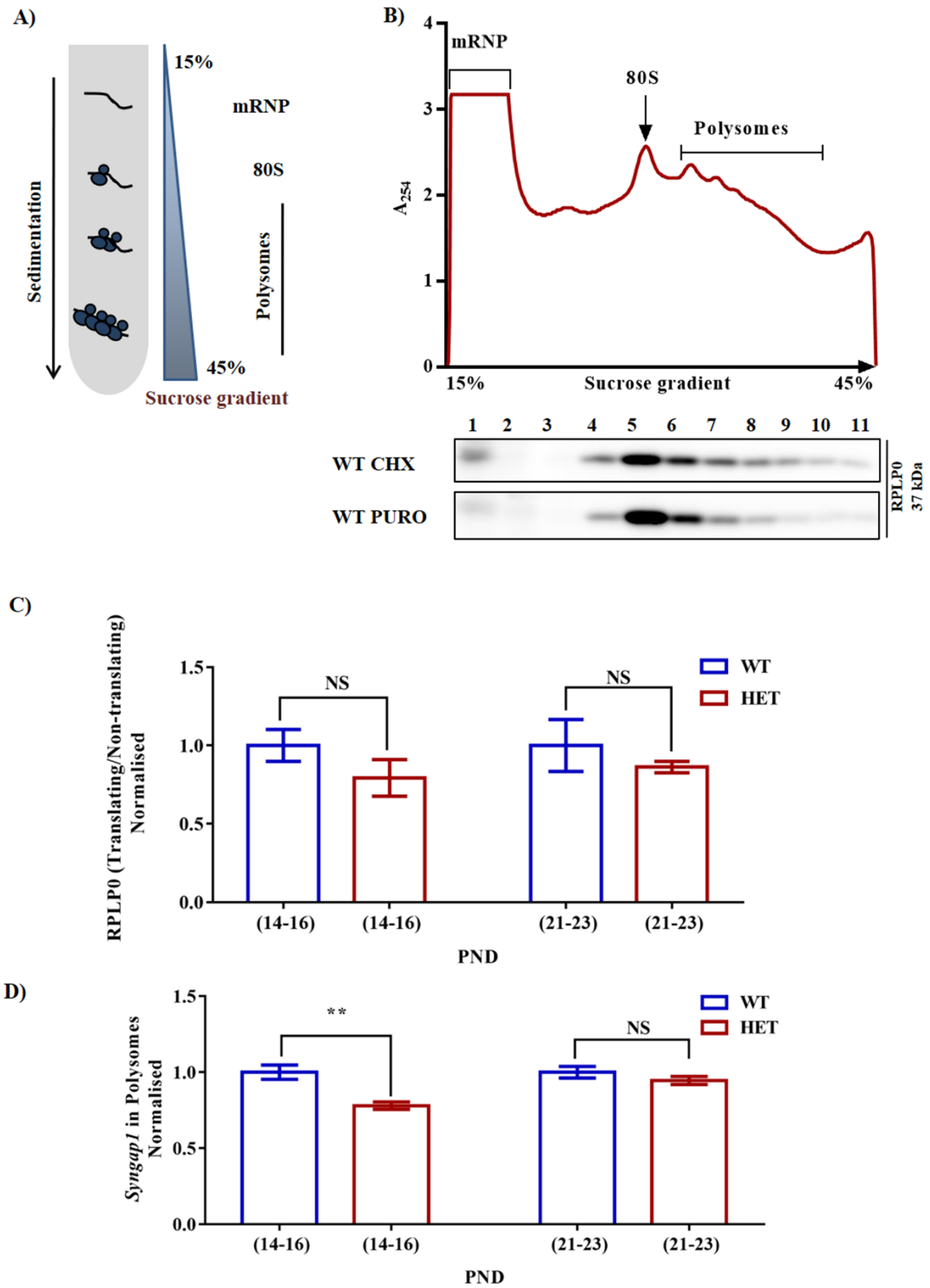

A)
15%
mRNP
80S
Polysomes
Sedimentation
45%
Sucrose gradient
B)
mRNP
80S
Polysomes
A_{254}
15%
Sucrose gradient
45%
1 2 3 4 5 6 7 8 9 10 11
WT CHX
WT PURO
RPLP0
37 kDa
C)
RPLP0 (Translating/Non-translating)
Normalised
NS
NS
WT
HET
(14-16)
(14-16)
(21-23)
(21-23)
PND
D)
Syngap1 in Polysomes
Normalised
**
NS
WT
HET
(14-16)
(14-16)
(21-23)
(21-23)
PND

Figure 3

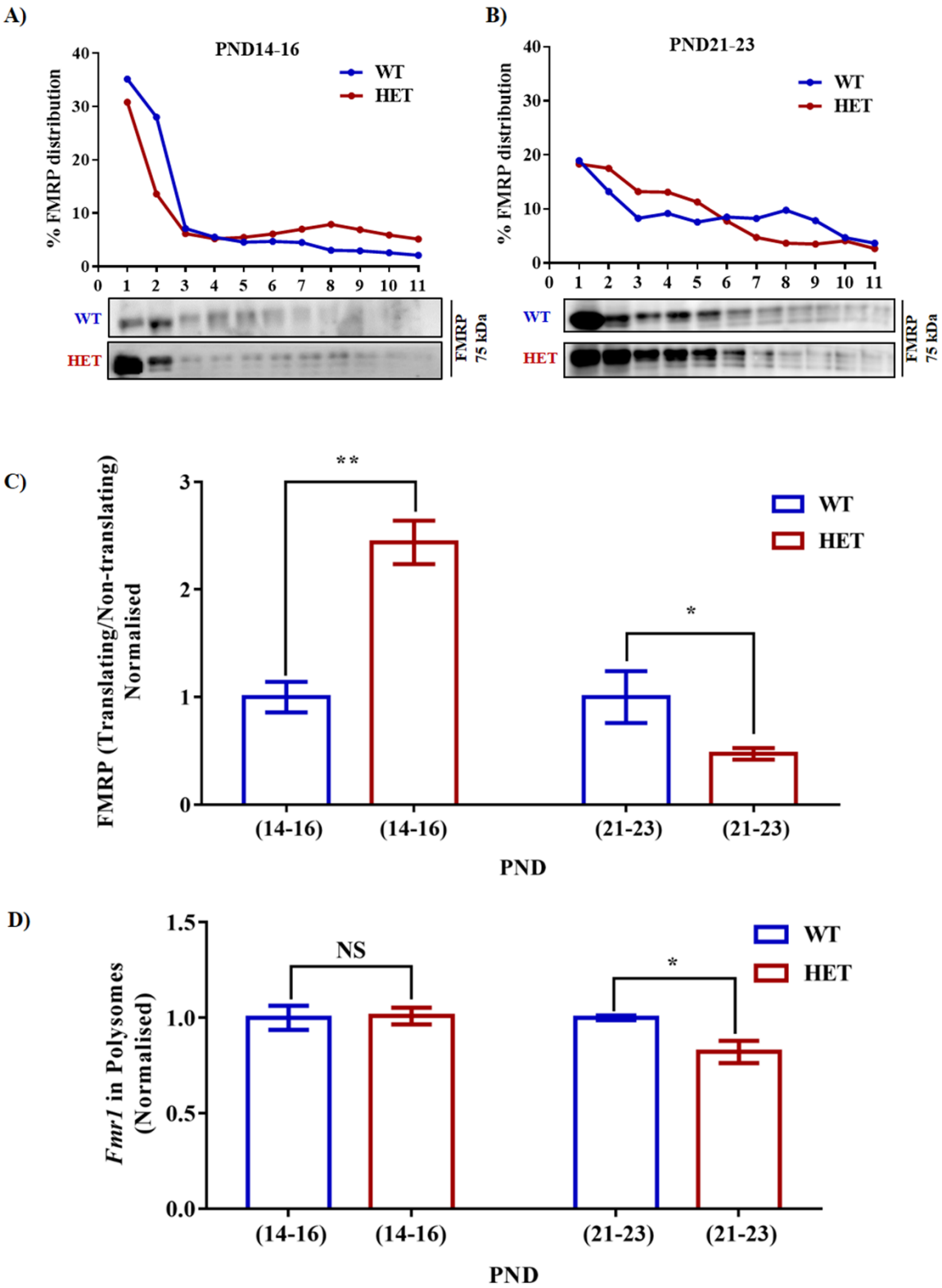

A)
PND14-16
WT
HET
% FMRP distribution
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
WT
HET
FMRP
75 kDa
B)
PND21-23
WT
HET
% FMRP distribution
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11
WT
HET
FMRP
75 kDa
C)
FMRP (Translating/Non-translating)
Normalised
3
2
1
0
**
*
WT
HET
(14-16)
(14-16)
(21-23)
(21-23)
PND
D)
Fmr1 in Polysomes
(Normalised)
1.5
1.0
0.5
0.0
NS
*
WT
HET
(14-16)
(14-16)
(21-23)
(21-23)
PND

Figure 4

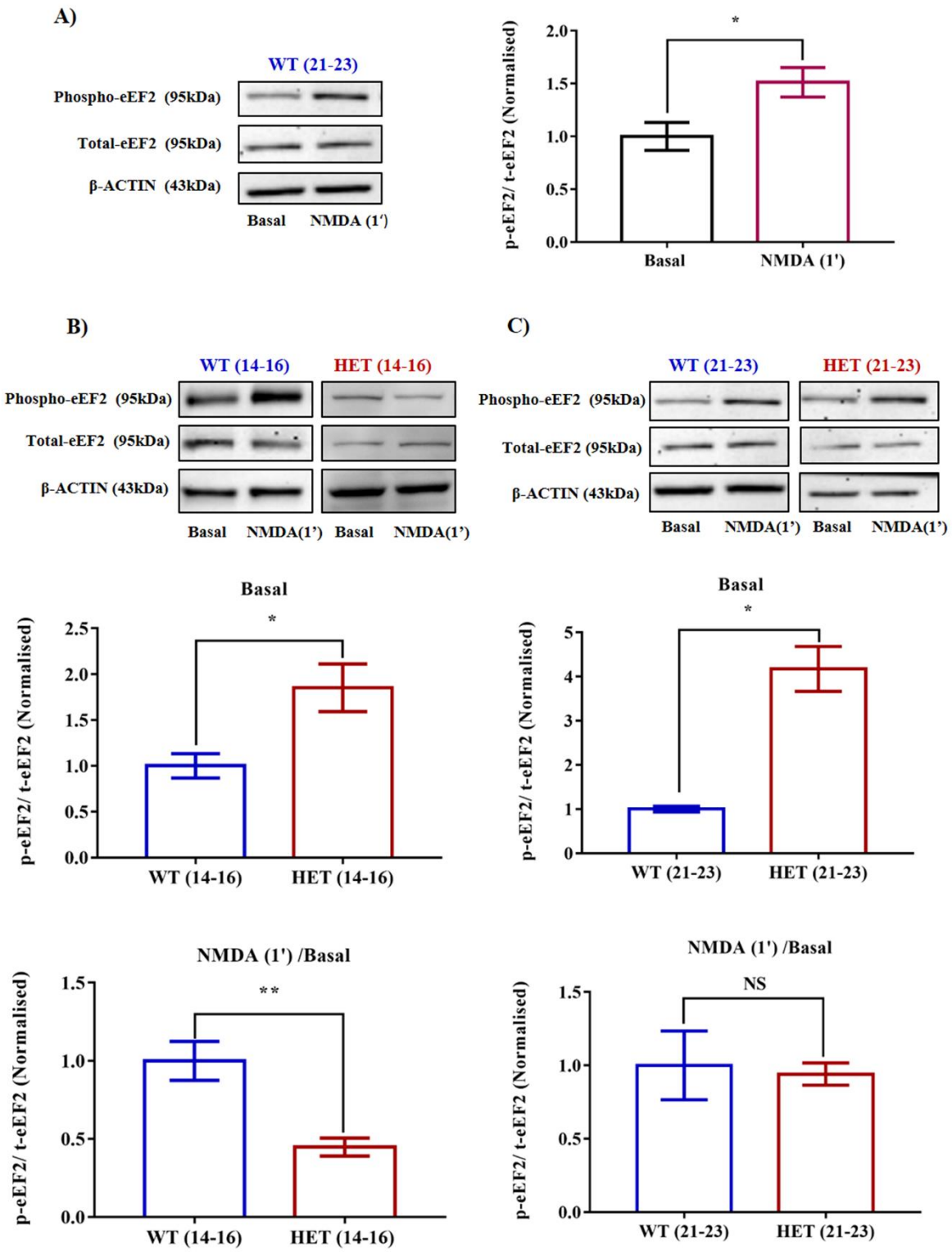

A)
WT (21-23)
Phospho-eEF2 (95kDa)
Total-eEF2 (95kDa)
β-ACTIN (43kDa)
Basal
NMDA (1')
p-eEF2/ t-eEF2 (Normalised)
2.0
1.5
1.0
0.5
0.0
*
Basal
NMDA (1')
B)
WT (14-16)
HET (14-16)
Phospho-eEF2 (95kDa)
Total-eEF2 (95kDa)
β-ACTIN (43kDa)
Basal
NMDA(1')
Basal
NMDA(1')
C)
WT (21-23)
HET (21-23)
Phospho-eEF2 (95kDa)
Total-eEF2 (95kDa)
β-ACTIN (43kDa)
Basal
NMDA(1')
Basal
NMDA(1')
Basal
*
p-eEF2/ t-eEF2 (Normalised)
2.5
2.0
1.5
1.0
0.5
0.0
WT (14-16)
HET (14-16)
Basal
*
p-eEF2/ t-eEF2 (Normalised)
5
4
3
2
1
0
WT (21-23)
HET (21-23)
NMDA (1') /Basal
**
p-eEF2/ t-eEF2 (Normalised)
1.5
1.0
0.5
0.0
WT (14-16)
HET (14-16)
NMDA (1') /Basal
NS
p-eEF2/ t-eEF2 (Normalised)
1.5
1.0
0.5
0.0
WT (21-23)
HET (21-23)

Figure 5

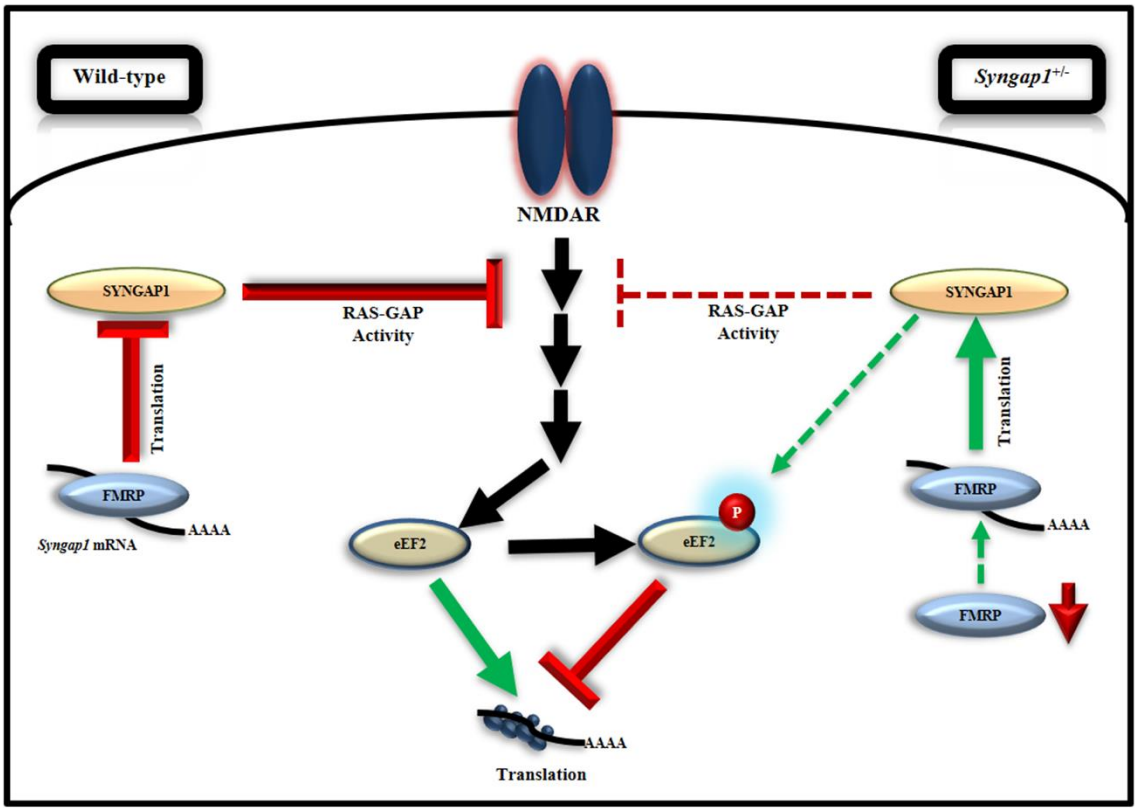

Wild-type
Syngap1+/-
NMDAR
SYNGAP1
RAS-GAP
Activity
RAS-GAP
Activity
SYNGAP1
Translation
Translation
FMRP
AAAA
Syngap1 mRNA
eEF2
P
eEF2
FMRP
AAAA
FMRP
AAAA
Translation

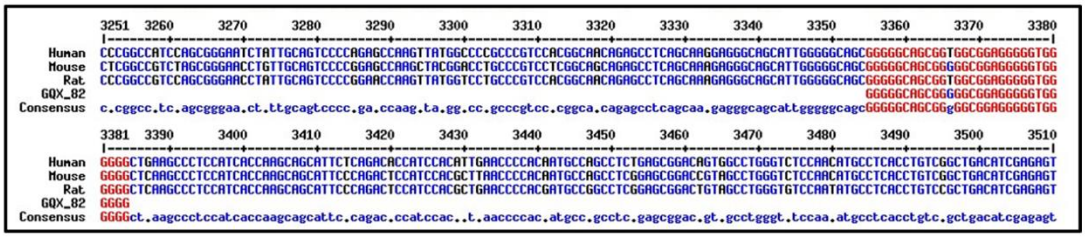
Figure 1-1

A)

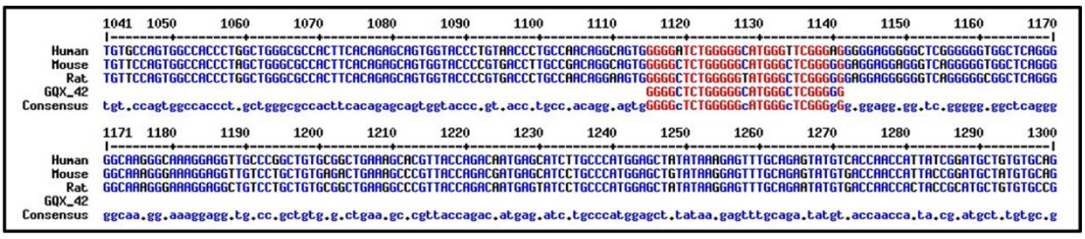
QGRS sequences found (overlaps not included)

Position	Length	QGRS	G-Score
131	29	GGAACCCTCGGTTCTGCATCATCTCGGGG	7
270	10	GGAGGGGCGG	20
307	29	GGTCGCTCGAGGAGGAAGAGTGTCCCAGG	10
336	23	GGGGAAACAGTACAGCATGGAGG	7
380	29	GGCCCTCGCAAGGCTTCCTGAGCCGGAGG	11
900	27	GGCTTCAGGAGACACCGTCTTTTGGGG	7
1004	28	GGAAGAAGGACAAGGCTGGCTACGTTGG	18
1115	27	GGGGCTCTGGGGGCATGGGCTCGGGGG	42
1150	30	GGGTCAGGGGGTGGCTCAGGGGGCAAAGGG	40
1327	16	GGCAAGGAAGAGGTGG	18
1698	22	GGAGGTGTTTGCATCTTGGCGG	10
1736	26	GGGGCCGAGAGGACATTGCTGACAGG	9
1959	15	GGAGCTGGAGTGGGG	17
2532	27	GGACCTTCAGGGCGACGGGCCTGGAGG	20
2666	22	GGCGCCTCTCCCAGGGGAGTGG	10
2830	26	GGCCATGGAGGGAGCAGTGGTCATGG	18
2902	19	GGGGGAGAACCCCCAGGGG	11
3142	24	GGGCCGGGAGGGGGCAGCGGCGGG	37
3166	14	GGCAGCGGTGGGGG	18
3346	30	GGGGGCAGCGGGGGCGGAGGGGGTGGGGGG	82
3563	18	GGCTGGACAGGGTGAAGG	19
3588	28	GGAGGAGATACATTCGCTGAAGGAGAGG	6
3632	26	GGAAGCTGGAAGAGTATGAGCGGAGG	10
3795	11	GGTGGAGGAGG	21
3924	23	GGCCCCACCTTGGAACGGCCTGG	15

B1)



B2)



B3)

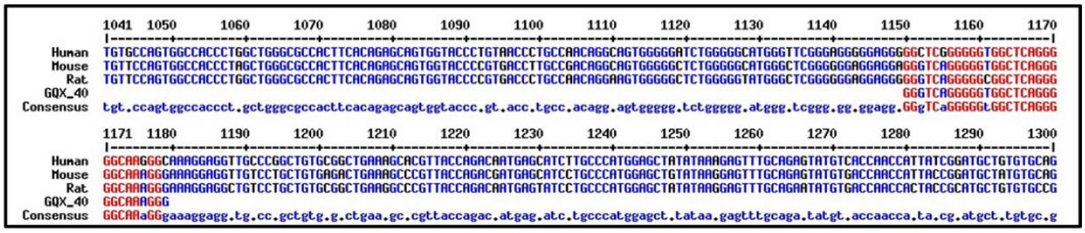


Figure 2-1

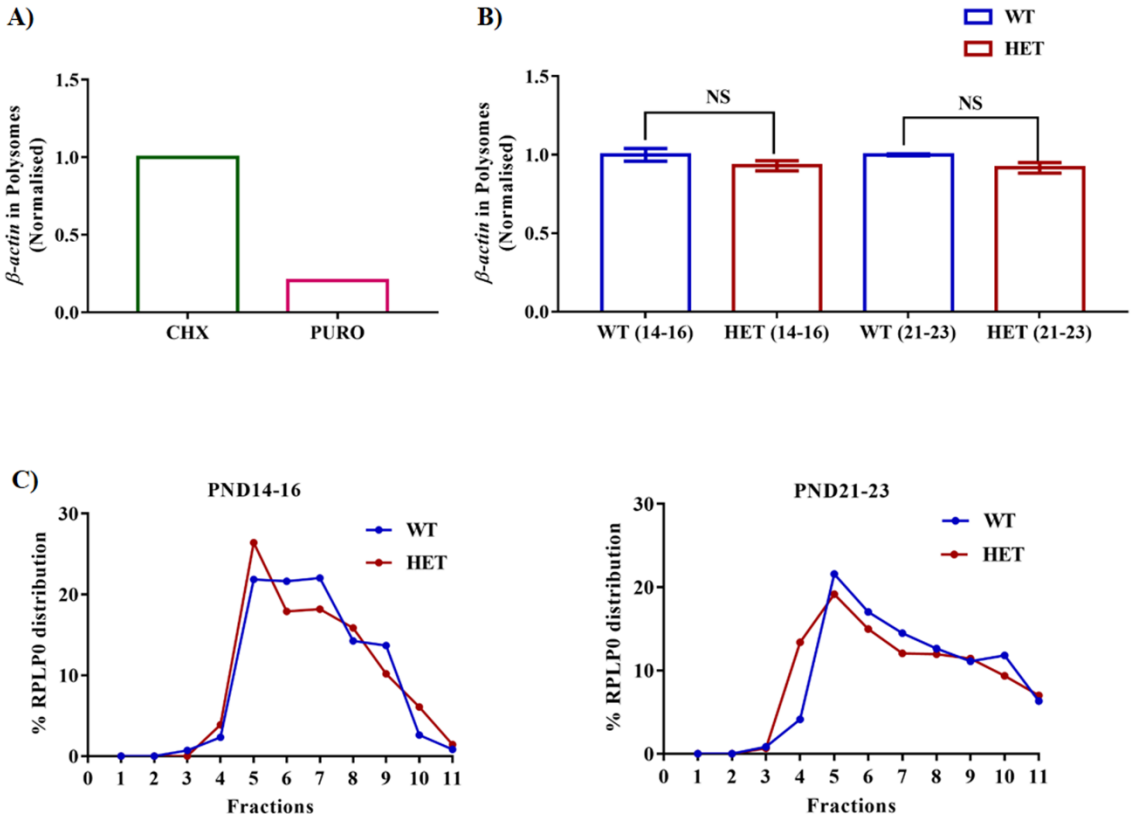
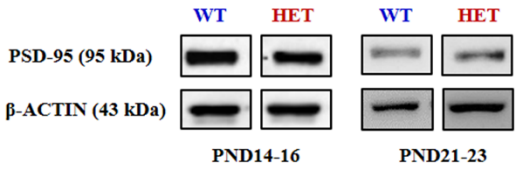
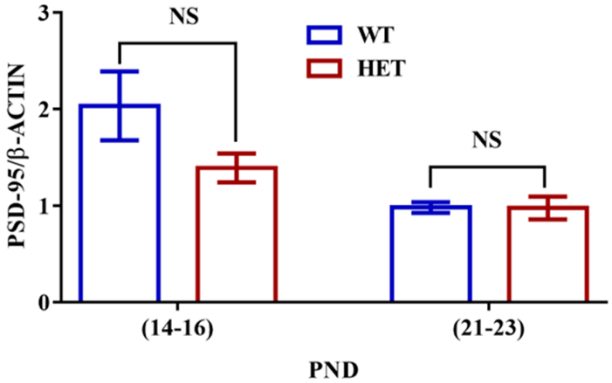

A)
β-actin in Polysomes (Normalised)
1.5
1.0
0.5
0.0
CHX
PURO
B)
WT
HET
NS
NS
β-actin in Polysomes (Normalised)
1.5
1.0
0.5
0.0
WT (14-16)
HET (14-16)
WT (21-23)
HET (21-23)
C)
PND14-16
% RPLP0 distribution
30
20
10
0
WT
HET
0 1 2 3 4 5 6 7 8 9 10 11
Fractions
PND21-23
% RPLP0 distribution
30
20
10
0
WT
HET
0 1 2 3 4 5 6 7 8 9 10 11
Fractions

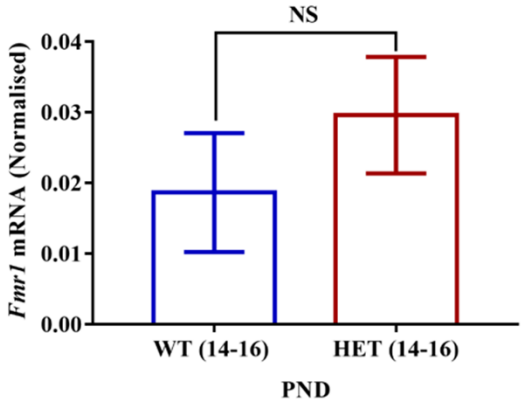
Figure 3-1

A)


WT
HET
WT
HET
PSD-95 (95 kDa)
β-ACTIN (43 kDa)
PND14-16
PND21-23


NS
WT
HET
NS
PSD-95/β-ACTIN
3
2
1
0
(14-16)
(21-23)
PND

B)


NS
Fmr1 mRNA (Normalised)
0.04
0.03
0.02
0.01
0.00
WT (14-16)
HET (14-16)
PND

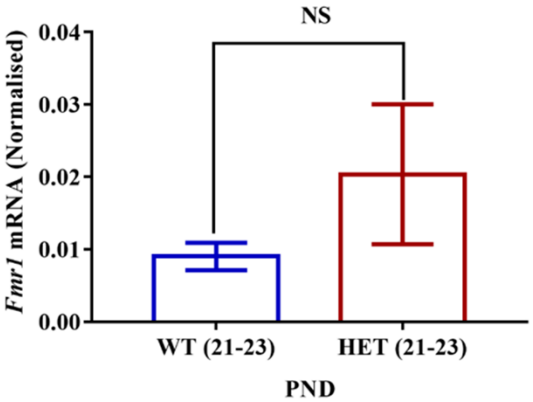
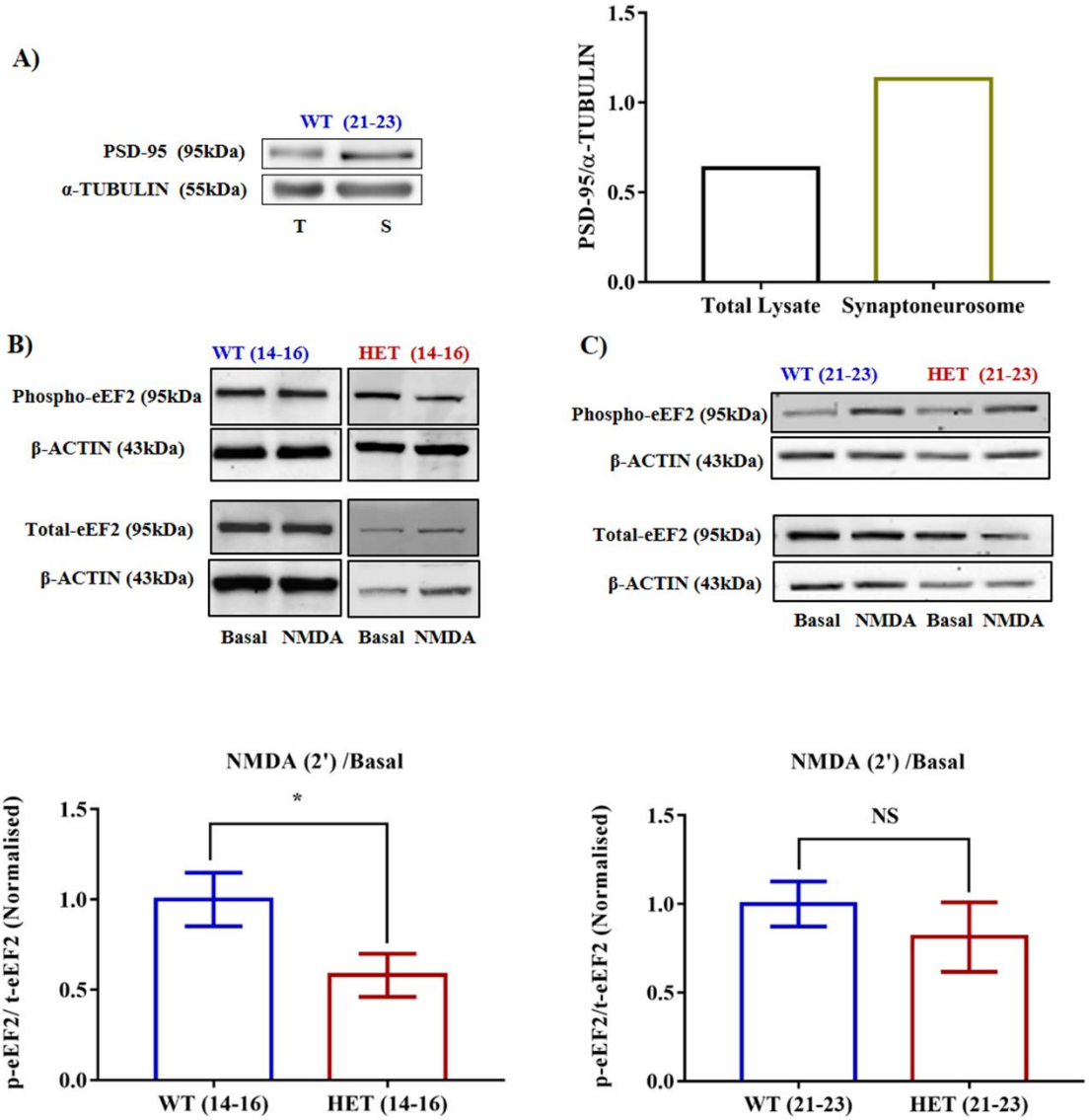

NS
Fmr1 mRNA (Normalised)
0.04
0.03
0.02
0.01
0.00
WT (21-23)
HET (21-23)
PND

Figure 4-1


A)
WT (21-23)
PSD-95 (95kDa)
α-TUBULIN (55kDa)
T
S
PSD-95/α-TUBULIN
1.5
1.0
0.5
0.0
Total Lysate
Synaptoneurosome
B)
WT (14-16)
HET (14-16)
Phospho-eEF2 (95kDa
β-ACTIN (43kDa)
Total-eEF2 (95kDa)
β-ACTIN (43kDa)
Basal
NMDA
Basal
NMDA
C)
WT (21-23)
HET (21-23)
Phospho-eEF2 (95kDa)
β-ACTIN (43kDa)
Total-eEF2 (95kDa)
β-ACTIN (43kDa)
Basal
NMDA
Basal
NMDA
NMDA (2') /Basal
*
p-eEF2/ t-eEF2 (Normalised)
1.5
1.0
0.5
0.0
WT (14-16)
HET (14-16)
NMDA (2') /Basal
NS
p-eEF2/t-eEF2 (Normalised)
1.5
1.0
0.5
0.0
WT (21-23)
HET (21-23)