hnRNPM Deficiency Deteriorates Cognitive Function by Decreasing Synaptic Proteins

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Abstract

Heterogeneous nuclear ribonucleoproteins (hnRNPs), a wide sub-family within the diverse RNA-binding proteins family, contribute to multiple aspects of nucleic acid metabolism including alternative splicing, mRNA stabilization, transcriptional, posttranscriptional and translational regulation. In this study, we report that alteration to the endogenous level of Heterogeneous nuclear ribonucleoprotein M (hnRNPM) in pyramidal cells of the CA1 hippocampal region of young wildtype mice brain leads to cognitive dysfunction. We further show that deficit to hnRNPM level impedes synaptic transmission while affecting the expression levels of pre- and post-synaptic markers following its binding to the 3'UTR of synaptophysin (SYP) and Postsynaptic Density Protein 95 (PSD95). In addition, we gathered that knockdown of hnRNPM affects the expression level of NeuN (Rbfox3), a protein essential for hippocampal neurogenesis and synaptogenesis. These further suggest that diminished level of hnRNPM could lead to synaptic dysfunction or deficits in neurogenesis and impaired cognitive functions as reported in NeuN homozygous knockout mice. Furthermore, we then show that hnRNPM binds directly and significantly to the 3'UTR of NeuN, synaptophysin and PSD95 to stabilize their mRNA. Our findings present a novel insight into the regulatory role of hnRNPM in synaptic transmission and cognitive functions.

Introduction

Cognitive abilities occur primarily in the cerebral cortex. Embedded within the deep structures of the temporal lobe of the cerebral cortex are, the hippocampus and amygdaloid nuclei whose functions have been linked to learning, memory, and emotion. The hippocampus proper is divided into two major regions, CA3 and CA1 based on the size and connections of the resident pyramidal cells. Hippocampus is important for the conversion of short- to long-term memory and its role have been implicated in memory and cognitive impairments [1-5], learning [6-8], and more recently in multiple sclerosis [9].

RNA-binding proteins (RBPs) regulate RNA processing at multiple levels including mRNA stability, mRNA localization and translation efficiency. RBPs can function in the regulation of mRNA localization, mRNA stability or regulate the efficiency with which specific mRNAs are translated. RNA-binding protein function at synapses has been shown in Patients with neurological and neurodegenerative disorders where genetic mutations or deletions of genes encoding RNA-binding proteins result in loss of synaptic plasticity and neuron function. Amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) are neurological diseases that have been linked with RBPs [10, 11].

Heterogeneous nuclear ribonucleoproteins (hnRNPs) assist in controlling the maturation of newly formed heterogeneous nuclear RNAs (hnRNAs/pre-mRNAs) into messenger RNAs (mRNAs), stabilize mRNA during their cellular transport and control their translation [12]. The expression level of hnRNPs is altered in many types of cancer, suggesting their role in tumorigenesis. In addition to cancer, many hnRNPs were also linked to various neurodegenerative diseases, such as spinal muscular atrophy (SMA), amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD) and fronto-temporal lobe dementia (FTLD). The hnRNPs frequently undergo post-translational modifications, leading to changes in biological activity and subcellular localization. Till date, the functional classification of the role of hnRNPs in cognition, learning and memory is still elusive and vague.

This study took advantage of the knowledge gap on the report of hnRNPM role in the nervous system, especially within the brain despite its abundance in this region, and examined its role. Through hnRNPM shRNA Adenoviral delivery targeting the CA1 region of the hippocampus, we repressed hnRNPM in young wildtype mice and behavioral tests present significant cognitive impairment, compared to the control mice. hnRNPM being an RNA binding protein harbors a number of predicted binding targets, we then set out to evaluate targets enriched in synaptic/neuronal specific functions, with significant and considerable enrichments observed following RNA immunoprecipitation. From these

targets, hnRNPM binds to the 3'UTR and stabilizes the mRNAs of PSD95, Synaptophysin and NeuN (Rbfox3). Together, these consequently affect neuronal/synaptic transmission evident as cognitive dysfunction in hnRNPM deficient mice.

Material And Methods

Reagents

Anti-hnRNPM (WB, 1:1000, Elabscience); anti-GAPDH (WB, 1:5000, Proteintech); anti-PSD95 (WB, 1:2000, Proteintech); anti-Synaptophysin [SY38] (WB, 1:1000, abcam); anti-NeuN (WB, 1:1000; IF, 1:500, Proteintech); anti-FLAG Tag (WB, 1:5000; IP, 1:1000, Enogene), Goat anti-Mouse and anti-Rabbit IgG-HRP (WB, 1:5000, Elabscience), Goat anti-rabbit IgG Alexa Fluor 594 (1:400, Molecular Probes); AceQ qPCR SYBR Green Master Mix (Vazyme). WB: Western blotting; IP: Immunoprecipitation; IF: Immunofluorescence

Animals

C57BL/6J mice were purchased from Model Animal Research Center of Nanjing University. Experimental protocols were approved by the Animal Studies Committee, University of Science and Technology of China, Hefei, China.

Cell lines

Mouse N2a cells (ATCC, CCL-131) and HEK293T cells (ATCC, CRL-3216) were cultured under standard conditions in DMEM (GIBCO) supplemented with 10% FBS (GIBCO) and 1% penicillin (Invitrogen) and incubated in 5% humidified CO₂ incubator at 37°C. All cell lines were tested negative for mycoplasma contamination.

Primary cultured neurons

Primary cultured neurons were prepared from wildtype C57BL/6J mice at day 17 of embryonic period as previously described by Liu et al., 2011 [13]. Neurons were seeded at a confluence of 500,000 cells per 6cm dish or 350,000 per 6 well plate. Cultured neurons were grown in Neurobasal medium that was supplemented with B27 (Invitrogen).

Plasmid construction and Transfection

hnRNPM coding sequence was amplified from mouse hippocampus derived cDNA by PCR, and subcloned into a pLenti-Puro lentivector (SBI) along with Xba1/Mlu1 restriction sites. A Kozak sequence was inserted to ensure proper translation. Lentivirus was then prepared for overexpression in primary cells. For knockdown of hnRNPM, sequences that target both mouse and human hnRNPM mRNA were obtained from Damianov et al., 2016 [14]. The target shRNA sequences were subcloned into a pGreenPuro hairpin (H1) backbone lentivector (SBI), and lentivirus was produced for primary cells knockdown. The H1 promoter and hnRNPM shRNA sequences were amplified from the above cloned SBI vector and subcloned into pAAV-EF1a-DIO-Gcamp6s vectors (Addgene, 67526). The EF1a promoter was replaced with a CamkIIa promoter, followed by a zsGreen sequence for neuron-specific visualization of the injected area. Correct insertion was confirmed by gel electrophoresis and DNA sequencing. Plasmids transfection was conducted with PEI (Sigma) according to manufacturer's protocol.

RNA Immunoprecipitation

N2a cells were transfected with hnRNPM+FLAG tagged plasmid. Cells were lysed in PBS containing 1% Triton X-100. Lysates were sonicated for 2 min to break down tangled DNAs, followed by 15 min incubation on ice and centrifugation at 14,000 rpm for 10 min at 4°C. Supernatant was collected and the protein concentrations were determined by BCA protein assay kit (Thermo Scientific). Equal amounts of lysates were incubated with protein A/G beads (BioTools)

conjugated with FLAG Tag antibody or control IgG. Beads were washed three times each, with buffers A, B and C. RNAs were collected from protein A/G beads using Trizol (Invitrogen), and followed by qPCR analyses. Primers for the detection of PSD95, Synaptophysin, NeuN and GAPDH are described in Table 1.

Table 1:

Primers for plasmid construction (Introduced restriction sites underlined, mutated sites in RED)		
Target	Primer	Sequence (5' to 3')
pLenti-Puro-hnRNPM	F	<u>GGTCTAGACCGCC</u> ACCATGGCGGCAGGGGTCGA
pLenti-Puro-hnRNPM-FLAG	F	<u>GGTCTAGACCGCC</u> ACCATGGATTACAAGGATGACGACGATAAGATGGCGGCAGGGGTCGA
	R	<u>CGACGCGTCGT</u> TAAAGCATTTCTATCAATTCGAA
pmirGlo-PSD95 (S1)	F	<u>CTAGCTAGCTAG</u> CGCGCTCTCCCGAGGCAGGG
	R	<u>GCTCTAGAGC</u> AGTTAGACCTTCCACTCATGC
pmirGlo-PSD95 (S2)	F	<u>CTAGCTAGCTAG</u> TTCTGGGATTTTATTTCAATTTTA
	R	<u>GCTCTAGAGC</u> TGTCTGTCTCTTCCTTTCACTC
pmirGlo-PSD95 (S1 mutagenesis)	F	CTTTTCTTCTCAAAAACGATTCTTGGGGGGGAGAAGC
	R	GCTTCTCCCCCAAGAATCGTTTTTGAGAAGAAAAG
pmirGlo-PSD95 (S2 mutagenesis)	F	GGCAAAGGGTCAGGAATTGAACGTCCGGGAGCCAGG
	R	CCTGGCTCCCGGACGTTCAATTCCTGACCCTTTGCC
pmirGlo-Synaptophysin (S1)	F	<u>CTAGCTAGCTAG</u> GCGCCCCAGAAAAGCAACCAG
	R	<u>GCTCTAGAGC</u> GTGGGCGCACCCCTGTTGGCC
pmirGlo-Synaptophysin (S2+S3)	F	<u>CTAGCTAGCTAG</u> ATGTGGAGCCTAAAAGGGAAG
	R	<u>GCTCTAGAGC</u> GCCTTGCTCAAGCTCTTTGG
pmirGlo-Synaptophysin (S2 mutagenesis)	F	GGCTTAAACACATGGTAGATTCCGGAGTGGGCAG
	R	CTGCCCCACTCCGGAATCTACCATGTGTTTAAGCC
pmirGlo-Synaptophysin (S3 mutagenesis)	F	CCGGAGTGGGCAGGTTTCGAGTTGGATCCAGCATC
	R	GATGCTGGATCCAACTCGAACCTGCCCCACTCCGG
pmirGlo-NeuN (S1)	F	<u>CTAGCTAGCTAG</u> CTATGTGAGAAAGCTTCCGGC
	R	<u>GCTCTAGAGC</u> GAGCCTACAGAAGGGAGTGGA
pmirGlo-NeuN (S1 mutagenesis)	F	GCCCTCGGGCACAAAGATCTTCGTCGTGGCAGCC
	R	GGCTGCCACGACGAAGATCTTTGTGCCCGAGGGC

hnRNPM shRNA target sequences highlighted RED		
Target	Primer	Sequence (5' to 3')
hnRNPM-shRNA-1	top strand	GATCCGCATCTTGTTGACATCGAATCTTCCTGTCAGAATTCGATGTCAACAAGATGCTTTTTG
	bottom strand	AATTCAAAAAGCATCTTGTTGACATCGAATTCTGACAGGAAGATTCGATGTCAACAAGATGCG
hnRNPM-shRNA-2	top strand	GATCCAGATTGCAATGTGCGCAATTCTTCCTGTCAGAAATTGCGCACATTGCAATCTTTTTTG
	bottom strand	AATTCAAAAAAGATTGCAATGTGCGCAATTTCTGACAGGAAGAATTGCGCACATTGCAATCTG

Primers for qPCR detection		
Gene	Primer	Sequence (5' to 3')
hnRNPM	F	GCGACAGAACCCAAAATGGAG
	R	TGGAATATGGCTCAAAGCGATTG
NeuN	F	ATCGTAGAGGGACGGAAAATTGA
	R	GTTCCCAGGCTTCTTATTGGTC
PSD95	F	TGAGATCAGTCATAGCAGCTACT
	R	CTTCCTCCCCTAGCAGGTCC
Synaptophysin	F	CAGTTCCGGGTGGTCAAGG
	R	ACTCTCCGTCTTGTTGGCAC
GAPDH	F	AGGTCGGTGTGAACGGATTTG
	R	TGTAGACCATGTAGTTGAGGTCA

Production of lentivirus and transduction of primary cells

293T cells were transfected with lentivirus plasmids, along with packaging plasmid (pHR⁺ 8.2deltaR) and envelope plasmid (pCMV-VSVG). Virus-containing medium was harvested 24 h after the transfection, followed by ultracentrifugation to precipitate virus. The virus was re-suspended in PBS and viral titer was determined by qPCR.

Production of Adeno-associated virus and hippocampus CA1 injection

Adeno-associated virus (AAV) was produced following protocol described by Guo et al., 2012 [15]. Briefly, target plasmid, pHelper, and helper 2/9 were co-transfected into 293T cells in a ratio of 2:1:1. PEI (Sigma) was used as transfection reagent with ratio of PEI: total DNA maintained at 4:1 (weight/weight). 24 h after the transfections, the culture medium was changed to DMEM plus 2% FBS. 72 h after the transfection, cells were collected and lysed in buffer containing 50mMTris-HCl, 150mMNaCl, and 2mMMgCl₂ at pH 8.0. AAV particles were released from cells with three freeze/thaw cycles, after which incubated with 50 U/ml benzonase nuclease (Mkbio) and 10 U/ml RNase I (Vazyme) at 37°C for 30 min, and 0.5% sodium deoxycholate (Sigma) treatment for another 30 min. Cell debris were removed by centrifugation at 2,500 g for 10 min, 40% PEG8000 and 2.5MNaCl were added to precipitate the virus. Thereafter, pellet was re-suspended in 1% PBS, followed by chloroform and (NH₄)₂SO₄ extraction to remove

contaminated proteins. Viral titer was determined by qPCR. Male WT C57BL/6J mice at 10 weeks of age (n=15 each group) were stereotactically injected with adenovirus expressing either hnRNPM-shRNA (0.5 μ l, 1.53×10^{12} TU ml⁻¹) or control AAV (0.5 μ l, 1.43×10^{12} TU ml⁻¹) into the bilateral CA1 area of hippocampus with an air pressure injector system (KDS). The coordinates used for stereotaxic injections were AP -2.3, ML 2.0, DV -1.5 and AP -2.3, ML -2.0, DV -1.5. Behavioral tests were performed 4 weeks after the injections.

Luciferase reporter assay

The luciferase reporter plasmids were constructed by subcloning the corresponding hnRNPM binding motifs on PSD95, Synaptophysin and NeuN 3'UTR into NheI/XbaI restriction sites of pmirGlo dual luciferase vector (Promega). Mutagenesis of PSD95 (S1 and S2), Synaptophysin (S2 and S3) and NeuN (S1) 3'UTR were carried out using Site-Directed Mutagenesis Kits (Thermo Scientific). The reporter plasmids were introduced into hnRNPM administered or deficient N2a cells, as well as their respective controls. Cell lysates were harvested 24 hours later and incubated with luciferase substrate (Promega). Luciferase activities were measured and plotted as fold change of overexpressed or knockdown over the controls.

RNA isolation, Reverse transcription, and Quantitative PCR (qPCR).

Total RNAs were extracted from N2a, primary neurons, or hippocampus tissue using Trizol (Invitrogen) and subjected to DNaseI digestion for genomic DNA removal. Reverse transcription was done using Hiscript II Reverse Transcriptase (Vazyme), following the manufacturer's instructions. Quantitative PCR was performed with AceQ qPCR SYBR Green Master Mix (Vazyme) on a Light Cycler 96 (Roche) instrument according to standard procedures. The real-time value for each sample was averaged and compared using the CT method, where the amount of target RNA ($2^{-\Delta\Delta CT}$) was normalized to an endogenous reference (ΔCT) and related to the amount of target gene in tissues or cells, which was set as the calibrator at 1.0. Student's t tests were applied as statistical analysis. qPCR detection primers are listed in Table 1.

Western blot and Densitometry Analysis

Proteins were extracted from cells or tissues in 1% PBS, containing 1% Triton X-100 and 1% cocktail protease inhibitor (Roche). Protein concentrations were determined using BCA Protein Assay kit (Thermo Scientific). Equal amounts of proteins were separated on SDS-PAGE electrophoresis. Proteins from SDS-PAGE gel were transferred to nitrocellulose membrane (Millipore), followed by primary and secondary antibody incubations. The immunoreactive bands were visualized by enhanced chemiluminescence (Thermo Scientific) and detected by ChemiScope (CLiNX). Immunoreactive bands were quantified using ImageJ software (NIH).

Immunofluorescence staining

Mice were perfused transcardially with 1% PBS solution, followed by 4% paraformaldehyde (PFA) in PBS. Brains were dissected and fixed in 4% PFA for 24 hours, then cryo-protected in gradient of 20% and 30% sucrose for 48 hours, followed by OCT compound embedding and tissue sectioning with microtome at 40 μ m thickness (Leica CM1860). Brain tissue sections were then subjected to permeabilization with PBS containing 0.2% Triton X-100, followed by incubation with primary antibody at 4°C overnight. Primary antibody was then visualized using Alexa-conjugated secondary antibody, followed with a nuclear specific stain for DAPI (4',6-diamidino-2-phenylindole) (Invitrogen).

Behavioral Tests

For the Morris water maze, construction of the test apparatus, test procedures, data collection, and analysis were performed following a standard protocol, as described previously [16]. Behavioral parameters were recorded by a video camera set on top of the circular pool and data were analyzed using Ethovision XT 11 software (Noldus).

Novel object recognition test was conducted as described previously [17]. A cognition index was calculated as the ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects.

Open field test was performed according to the specifications provided by Tsuda and Ogawa, 2012 [18]. During the test, a mouse was placed in a corner square with its head facing the corner of the open field apparatus, allowing 5 min to record its exploratory activities. Crossing square line with all four paws was counted as one unit of exploratory movement (square traveled). Chambers were cleaned with 70% ethanol before and after each use.

Elevated plus maze test was performed according to description by Leo and Pamplona, 2014 [19], with minor modifications. In brief, a customized apparatus is consisted of two open arms (30 cm x 5 cm), two closed arms (30 cm x 5 cm x 15.25 cm), that is connected to an open center platform (5 cm x 5 cm). The apparatus was set at the middle of the room with equal level of illumination on both arms. Mice were housed in groups (5 per cage) and brought to the testing room one week before test day, to acclimatize to the test environment. On test day, each mouse was placed at the center of the maze, facing an open arm opposite to the experimenter. Allowing mice to explore the maze for 5 min. The number of entry to open arm and time spent in open arms were recorded.

Image Acquisition and Analysis

Fluorescent signals were viewed and captured with CCD camera mounted on U-CB5S microscope system (Olympus). Fluorescence intensities were analyzed using ImageJ software.

Quantification and Statistical Analysis

All quantified data represent an average of at least triplicate samples. Error bars represent Mean $\pm$ standard error of the mean. Statistical significance was determined by Student's t test in GraphPad Prism 7. $p < 0.05$ was considered significant (indicated by * in the figures), $p < 0.01$ (indicated by ** in the figures), $p < 0.001$ (indicated by *** in the figures), $p < 0.0001$ (indicated by **** in the figures).

Results

hnRNPM Loss Induces Cognitive Deficit in Mouse Brain

In order to establish the role of hnRNPM in the brain, since none is available till date; we conducted hnRNPM knockdown (KD) in the CA1 region of the hippocampus of 10 weeks-old C57BL/6J wild-type mice using adeno-associated virus (AAV) delivered short hairpin RNA (shRNA) technology. After 4 weeks, successful injection was confirmed through the Green Fluorescence Protein (GFP) signal observed across the entire length of the hippocampus proper (Supplementary Figure S1a). In addition, mRNA levels of hnRNPM were determined to indicate a successful injection of AAV (Supplementary Figure S1b). We found that direct injection of AAV containing hnRNPM shRNA and subsequent loss of hnRNPM in the hippocampus of young wildtype mice resulted in significant display of learning and memory deficits, as indicated by a series of behavioral tests. In the Morris water maze test, mice with hnRNPM deficiency in the hippocampus took significantly longer time to locate the hidden platform, when compared to controls, during the training phase (Fig. 1a). In the probe trial, hnRNPM-deficient mice spent less time in the target quadrant (Fig. 1b), where the platform had been located during training, crossed the platform location less frequently (Fig. 1c), and spent significantly more time to locate the initial positioning of the platform (Fig. 1d). In the novel object recognition

test, mice with deficiency of hnRNPM in the hippocampus spent less time exploring for novel objects compared with the control (Fig. 1e). Interestingly, we further show that mice with hnRNPM deficiency displayed similar spontaneous locomotor activity and anxiety-like or emotional behavior as their respective controls, as indicated by the open field test (Figs. 1f and 1g) and elevated plus maze (Figs. 1h and 1i). The essence of these tests was to be certain of the ineffectiveness of locomotor activity and anxiety on the readout of the Morris water maze test. Together, these reports suggest that hnRNPM endogenous abundance in the brain, especially within the hippocampal formation is essential for cognitive purposes.

hnRNPM regulates PSD95 along with Synaptophysin and NeuN levels *In Vivo* and *In Vitro*

Shortly after the behavioral studies, we then investigated how deficiency of hnRNPM in the hippocampus would result in cognitive deficit as seen in Fig. 1. Several reports have linked disease conditions such as; schizophrenia or autism [20, 21, 22], Alzheimer's disease [23, 24], Obesity [25] and Down syndrome [26] to cognitive dysfunction. In addition, traumatic brain injury [27] and aging [28, 29] have all been reported to be linked to cognitive disorder. Essentially, these reports have revealed alterations to cluster of genes frequently associated with intellectual and cognitive disabilities, especially those encoding proteins involved in synaptic function. We then hypothesize that changes occurring in this cluster of genes would interfere with interconnected downstream signaling pathways to cause the resulting cognitive deficits observed in hnRNPM deficient mice. Hence, we took interest in the presynaptic protein (Synaptophysin) and the postsynaptic density protein 95 (PSD95), as well as the highly conserved NeuN (Rbfox3) protein.

PSD95 is exclusively located in the post synaptic density of neurons and plays an important role in synaptic plasticity and long-term potentiation [30]. It is almost exclusively located in the post synaptic density of neurons [31] and is involved in anchoring synaptic proteins. PSD95 plays an important role in the stabilization of synaptic changes during long-term potentiation. In this study, we found that PSD95 levels were clearly decreased in the hippocampus of hnRNPM deficient mice (Fig. 2a). This decrease was further substantial in the mRNA levels and densitometry analysis (Figs. 2b and 2c). In order to validate our findings *in vivo*, we then check PSD95 levels following knockdown of hnRNPM in primary cultured neuron using lentiviral delivered antisense hnRNPM shRNA to knockdown hnRNPM *in vitro*. Similar to the results obtained *in vivo*, *in vitro* cultured neuron deficient of hnRNPM show substantial diminished level of PSD95; both in protein and mRNA levels (Figs. 3a, 3b and 3c). Conversely, to investigate the gain of function of hnRNPM, we introduced hnRNPM into primary cultured neuron using lentiviral delivery technology (Fig. 3d). hnRNPM enriched primary cultured neuron show significant increase in the protein level, indicated by Western blot and densitometric analyses, and subsequent increase in the mRNA levels of PSD95 (Figs. 3d, 3e and 3g).

Synaptophysin, also known as the major synaptic vesicle protein p38, is a protein that is encoded by the SYP gene [32]. It is a synaptic vesicle glycoprotein, with ubiquity at the synapse. Elimination of synaptophysin in mice has shown to result in impaired object novelty recognition and reduced spatial learning [33]. We report that knockdown of hnRNPM in the hippocampus of young wildtype mice results in diminished level of presynaptic protein (Synaptophysin) (Fig. 2a), with resultant consequences on the mRNA levels and densitometry analyses (Figs. 2b and 2c). *In vitro* primary cultured neuron deficient of hnRNPM show substantial deficit in the levels of synaptophysin mRNA and protein levels (Figs. 3a, 3b and 3c), similar to the outcomes observed *in vivo*. Furthermore, overexpression of hnRNPM by lentiviral system in cultured neuron enhances resultant increase in the protein and mRNA levels of synaptophysin (Figs. 3d, 3e and 3g).

Together, dysregulation to the levels of presynaptic protein (synaptophysin) and postsynaptic protein (PSD95) following alteration to the endogenous level of hnRNPM both *in vivo*, in the hippocampus of young wildtype mice and *in vitro* in primary cultured neuron, suggest that the RNA binding protein, hnRNPM is vital in neurogenesis, synaptogenesis and neuronal transmission.

In an attempt to validating these findings; and based on the phenotype of cognitive decline displayed by hnRNPM hippocampus deficient mice (Fig. 1), with subsequent diminished levels of proteins essential for synaptic and neuronal functions; we then related these results with the report by Wang et al., 2015 [34], where NeuN (Rbfox3) was shown to be essential for hippocampal circuit balance and function, by reporting anatomical and functional abnormality in Rbfox3 knockout mice. More so, Lin et al., 2016 [35] show that NeuN homozygous knockout mice displayed deficits in neurogenesis and impaired cognitive function. Hence, comparing our findings with these previous reports, we then check the possibility of hnRNPM regulating the level of NeuN. Interestingly, we found that *in vivo* knockdown of hnRNPM in the hippocampus diminished the endogenous enrichment of NeuN drastically (Fig. 2a), with consequential decrease in the mRNA levels and densitometry (Fig. 2b and 2c). Furthermore, our *in vitro* model of primary cultured neuron reveals substantial diminished protein and mRNA levels of NeuN following lentiviral hnRNPM shRNA delivery (Fig. 3a, 3b and 3c). In addition, similar to the response of PSD95 and synaptophysin following overexpression of hnRNPM in primary cultured neuron, Figs. 3d, 3e and 3g show consequential increase in the levels of NeuN.

hnRNPM Modulates PSD95 together with Synaptophysin and NeuN levels by Affecting Their mRNA Stability

With the changes observed in the mRNA levels of PSD95, synaptophysin and NeuN in cultured neuron transduced with hnRNPM for overexpression or its shRNA for knockdown using a lentiviral delivery system (Figs. 3a and 3d), we next determined the mRNA levels of PSD95, synaptophysin and NeuN in N2a cell line with deficient and enriched hnRNPM (Figs. 4a, 4b and 4c). Furthermore, we found results consistent with the observed changes *in vivo* in hippocampus, and *in vitro*, in primary cultured neuron. RNA binding proteins (RBPs) comprise a large and diverse group [36] that lie at the center of posttranscriptional regulation by governing the fate of mRNA transcripts from biogenesis, stabilization, and translation to RNA decay [37]. The regulation of mRNA decay is a major control point in gene expression by RBPs [28]. We then proceeded to examine the mRNA stability of PSD95, synaptophysin and NeuN in hnRNPM deficient and administered N2a cells, by treating N2a cells with 0.2µg/ml of actinomycin D (ACTD) [38] and analyzing mRNA levels at different time intervals after treatment. We found that hnRNPM-deficient N2a cells exhibited shorter mRNA half-lives for both PSD95, synaptophysin and NeuN (Figs. 4d, 4e and 4f). On the other hand, prolonged mRNA half-lives were observed for PSD95, synaptophysin and NeuN in hnRNPM-overexpressed N2a cells (Figs. 4g, 4h and 4i). For clarity on these findings, binding of hnRNPM to PSD95, synaptophysin and NeuN was then revealed by RNA immunoprecipitation (RIP). Prior to this, the binding specificity was validated by Flag Tag antibody pull down, followed by detection/blotting with Flag Tag antibody (Fig. 4j). For the RIP, N2a cell lysates enriched with hnRNPM+Flag Tag were incubated with anti-Flag tag antibody and hnRNPM-bounded RNA was thereafter subjected to qPCR analyses. Following these procedures, the RIP results unveil significant enrichment of PSD95, synaptophysin and NeuN mRNA compared to the control IgG. On the contrary, the GAPDH mRNA levels bear no significant enrichment (Fig. 4k). From these results, we notice that hnRNPM binds specifically to PSD95, synaptophysin and NeuN, and furthers their mRNA stability.

hnRNPM Binds directly to the 3' UTR of PSD95, Synaptophysin and NeuN mRNAs

The binding motif of hnRNPM has been previously reported to be poly U and poly G [12, 14, 39]. In addition, Paz et al., 2014 [40] through the RNA binding protein map predicted that the 3' untranslated region (3'UTR) of both PSD95 (Fig. 5a) and NeuN (Fig. 5c) bear two (2) and one (1) poly U and poly G binding motifs respectively for hnRNPM. Whereas, the prediction for synaptophysin bears a binding motif for hnRNPM on its last exon (Exon 6), with two other poly U and G binding motifs for hnRNPM on the 3' UTR (Fig. 5b).

In other to explore the possibility of hnRNPM binding to PSD95 mRNA through these predicted binding sites, we then subcloned each putative hnRNPM binding site along with a total of 200 bp upstream and downstream sequences into PGK promoter driven pmirGlo luciferase vector. The plasmid construction was done per binding site. Thereafter, constructed plasmids containing each binding site were then introduced into hnRNPM deficient or supplemented N2a

cells, along with their respective controls. Luciferase activities were measured in these cells, and the results show substantial decrease in the luciferase activity of hnRNPM KD cells with introduced S1 and S2 binding sites (Fig. 5d). Conversely, cells with enhanced level of hnRNPM show significant increase in the measured luciferase activities (Fig. 5e). Furthermore, luciferase plasmid constructs containing mutated variants of the S1 and S2 motifs were introduced into hnRNPM KD or overexpressed N2a cells. Our results show no significant difference of luciferase activities in hnRNPM KD or overexpressed cells and the controls (Figs. 5d and 5e). Essentially, these findings suggest that hnRNPM binds directly to the 3' UTR of PSD95 mRNA through the S1 and S2 binding motifs.

Synaptophysin mRNA was predicted to contain one hnRNPM binding motif in its last exon, exon6, and two on its 3' UTR. Similar to PSD95, we subcloned each predicted binding site into pmirGlo luciferase vector. We constructed the plasmids per binding site, S1 for the site in exon6 (Figure 5B). However, S2 and S3 are overlapping sites. Plasmids containing hnRNPM binding motifs were then introduced into hnRNPM KD or overexpressed N2a cells, and control. Measured luciferase activities show that S2 and S3, but not S1 were significantly reduced or increased in cells deficient or enriched with hnRNPM respectively (Figs. 5f and 5g). Subsequently, when constructs with mutant variants of S2 and S3 were introduced into hnRNPM KD or overexpressed N2a cells, S3 but not S2 showed no significant luciferase activities compared with the controls (Figs. 5f and 5g). These results therefore suggest that hnRNPM binds specifically to the S3 binding motif in the 3' UTR of synaptophysin.

Paz et al., 2014 [40] predicted two but overlapping poly U and G binding motifs of hnRNPM on the 3' UTR of NeuN, with single nucleotide difference in their positions (positions 3777 and 3778) (Fig. 5c). We therefore took these putative sites as one motif, S1, and subcloned with a total of 200 bp upstream and downstream sequences into PGK promoter driven pmirGlo luciferase vector. S1 was introduced into hnRNPM KD or overexpressed N2a cells, with luciferase activities measured afterwards. Figs. 5h and 5i show significant decrease or increase, respectively in the hnRNPM knockdown or overexpressed cells. In addition, mutation to S1 shows no significant difference in luciferase reporter activities in cells deficient or administered hnRNPM (Figs. 5h and 5i).

Together, we report that hnRNPM binds precisely to the S1 and S2 binding sites in the 3'UTR of PSD95 mRNA; S3 motif in the 3'UTR of synaptophysin mRNA and S1 binding site in the 3'UTR of NeuN mRNA (Figs. 5a, 5b and 5c); and through these binding targets; hnRNPM regulates the stability of these genes.

Discussion

hnRNPs is essential in several aspects of nucleic acid metabolism such as; alternative splicing, mRNA stabilization, and transcriptional and translational regulation [12]. hnRNPs have many common features with other RBPs, such as the SR proteins and ELAV (embryonic lethal abnormal vision)-like proteins. Structurally, they comprise multiple RBDs, in particular the RRM, and often also contain auxiliary domains [41]. Physiologically, they have diverse and overlapping roles in the regulation of alternative splicing, mRNA stability, mRNA export and other aspects of post-transcriptional processing [42, 43]. More so, hnRNPs are expressed ubiquitously, and most of them shuttle between the nucleus and cytoplasm [44-47]. More recent studies have shown that hnRNPs have the potential to play diverse, but important roles in maintaining mRNA homeostasis in the nucleus and cytoplasm. Given their central roles in the regulation of gene expression, it is not surprising that the hnRNPs have been linked to numerous diseases. Many of the hnRNPs have been directly or indirectly implicated in cancer development, and aberrant expression of hnRNPs is characteristic of various types of cancer [48, 49]. Dysregulation of hnRNP function contributes to carcinogenesis in multiple ways. Contrary to previous views, more recent findings have shown that the functions of hnRNPs extend far beyond the packaging of nascent RNA, and they are now viewed as a highly divergent group of proteins with roles in many important aspects of nucleic acid metabolism [41].

The hnRNPM is an abundant protein composed of three RRM domains and is mostly present in the nucleus where it acts as a component of the spliceosome complex [50]. Since the first report where hnRNPM was identified as a splicing regulator for fibroblast growth factor receptor 2 (FGFR2) [51], many reports followed and identified new targets: carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1) [52], dopamine D2 receptor (D2M) [53] and survival motor neuron 1 and 2 [54]. Meanwhile, despite the distribution abundance of hnRNPM in the brain (Human Protein Atlas; Allen Brain Atlas), no study is yet available on the role of hnRNPM in this region, especially within the hippocampus. Hence, this study appreciates the gap in knowledge and examines the role of hnRNPM in the brain, precisely in the hippocampus of young wildtype mice.

To elucidate the precise role of hnRNPM in the brain, we show that the depletion of hnRNPM in the hippocampus of young wildtype mice results in learning and memory dysfunction. Prior to this study, a couple of studies have linked RBP to cognitive function or dysfunctions, including diseased conditions and aging [55, 56]. We hereby report that hnRNPM plays a vital role in cognitive functions. Precisely, our data shows that hnRNPM deficits in the hippocampus affect cognitive functions without an impedance on the locomotor activity and anxiety-like or emotional behavior. Our next goal was to then determine the reality of these findings. As an RNA binding protein, hnRNPM has numerous predicted binding targets. Our primary goal was to identify the binding and regulatory targets of hnRNPM and further appreciate how these targets could influence cognitive functions. Interestingly, our *in vivo* and *in vitro* data show that levels of pre and post synaptic proteins such as; post-synapse density protein 95 (PSD95) and synaptophysin were substantially altered in response to level changes of hnRNPM. In addition, we observed significant changes in the level of NeuN (Rbfox3); an RNA binding protein that has been largely used as neuronal cell body marker, but recently identified essential in synaptogenesis and neurogenesis [35]. Furthermore, through immunoprecipitation and subsequent analysis, we showed that these proteins have direct binding targets for hnRNPM. Our results revealed that hnRNPM binds precisely to the 3' UTR of PSD95, synaptophysin and NeuN, to regulate their mRNA stability to then influence cognitive functions; such as learning and memory.

hnRNPM has been implicated and relatively studied in oncology. However, to our knowledge, the role of hnRNPM in the nervous system, especially in brain function is yet to be unveiled. In this study, we demonstrate that hnRNPM plays a key role in learning and memory, by regulating the mRNA stability of PSD95, synaptophysin and NeuN, all being essential targets for synaptogenesis and neurogenesis. This study elucidates a novel function of hnRNPM in cognition, and subsequently identifies downstream interacting proteins, specific to synaptic functions. Hence, our study establishes a significant role that hnRNPM plays in brain disease and functions, especially as it concerns cognition.

Abbreviations

hnRNPs: Heterogeneous nuclear ribonucleoproteins, hnRNPM: Heterogeneous nuclear ribonucleoprotein M, OV: Overexpression, KD: Knockdown, RBP: RNA Binding Protein, CA1: Cornu Ammonis 1, CA3: Cornu Ammonis 3, SYP: Synaptophysin, PSD95: Postsynaptic Density Protein 95, 3'UTR: 3' Untranslated Region, SMA: Spinal Muscular Atrophy, ALS: Amyotrophic Lateral Sclerosis, AD: Alzheimer's disease, FTLD: Frontotemporal Lobe Dementia, WT: Wildtype, DAPI: 4',6-diamidino-2-phenylindole (Invitrogen), AAV: Adeno-Associated Virus, HEK: Human Embryonic Kidney Cell, N2a: Mouse Neuroblastoma Cell, SR: Serine/arginine proteins, GFP: Green Fluorescence Protein, ACTD: Actinomycin D, RIP: RNA Immunoprecipitation.

Declarations

Acknowledgements

Not applicable

Authors' contributions

Q.L. supervised the project; A.R.A, J.Z and Q.L. conceived and designed the study; A.R.A performed the experiments; A.R.A and D.L analyzed data; A.R.A, J.Z. and Q.L. wrote the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

All animal experimental protocols were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and Animal Studies Committee, University of Science and Technology of China, Hefei, China.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

hnRNPM Loss Induces Cognitive Deficit in Mouse Brain (a) During the training days for Morris water maze test, mice deficient of hnRNPM in their hippocampus spent significantly longer time to locate the platform compared to the control mice. (b–d) In the probe trial, hnRNPM hippocampal knockdown mice spent significantly less time in target quadrant (b), crossed the platform less frequently (c), and took longer time to locate the hidden platform (d). (n = 15 per group). (e) Represents the novel object recognition test, where cognitive index was calculated as the ratio of the amount of time spent exploring the novel object over the total time spent on exploring both objects. Data were presented as fold difference of hnRNPM KD over controls, expressed in percentage (n = 15 per group). (f–g) Show the analysis of hnRNPM hippocampus deficient and control mice when subjected to open field test. (f) Represents the distance traveled while (g) reports the time spent in the center of the compartment (n = 15 per group). (h–i) In the elevated plus maze; (h) is the number of open arm entries, while (i) represents time spent in the open arms for both knockdown and control groups (n = 15 per group). For this and subsequent figures, KD represents knockdown, and OV represents overexpression. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.001 by two-tailed Student's t test. Error bars represent SEM.

Figure 2

hnRNPM regulates PSD95 along with Synaptophysin and NeuN levels in vivo (a) Levels of hnRNPM, PSD95, Synaptophysin and NeuN in Control and hnRNPM knockdown hippocampus of young wildtype mice were determined by Western blot. GAPDH was included as input controls. (b) Levels of hnRNPM, PSD95, Synaptophysin and NeuN mRNA in hnRNPM knockdown hippocampus, and their respective controls, as determined by qPCR. Data were normalized to GAPDH and plotted as fold difference over controls. (c) Densitometric quantification and analyses representing protein levels of hnRNPM, PSD95, Synaptophysin and NeuN in hnRNPM knockdown hippocampus and their controls.

Figure 3

hnRNPM regulates PSD95 together with Synaptophysin and NeuN levels in vitro (a and d) Levels of hnRNPM, PSD95, Synaptophysin and NeuN in Control and hnRNPM knockdown (a) and hnRNPM administered (d) primary cultured neuron were determined by Western blot. GAPDH was included as input controls. (b, e and f) Levels of hnRNPM, PSD95, Synaptophysin and NeuN mRNA in hnRNPM deficient (b) and hnRNPM administered (e-f) primary cultured neuron. (f) Represents level of hnRNPM mRNA relative to control only in hnRNPM administered neuron, as determined by qPCR. Data were normalized to GAPDH and plotted as fold difference over controls. (c and g) Densitometric quantification and analyses representing protein levels of hnRNPM, PSD95, Synaptophysin and NeuN in hnRNPM knockdown (c) and administered (g) cultured neuron and their controls.

Figure 4

. hnRNPM Modulates PSD95 together with Synaptophysin and NeuN levels by Affecting Their mRNA Stability (a-c) mRNA Levels of PSD95 (a), Synaptophysin (b) and NeuN (c) in hnRNPM-deficient, hnRNPM-administered, and their respective control cells, as determined by qPCR. Data were normalized to GAPDH and plotted as fold difference over controls. (d-f) Levels of PSD95 (d), Synaptophysin (e) and NeuN (f) mRNA in hnRNPM-deficient and control N2a cells that were subjected to 0.2μg actinomycin D (ACTD) treatment. Data were plotted as fold difference over time 0 hours. (g-i) mRNA levels of PSD95 (g), Synaptophysin (h) and NeuN (i) in hnRNPM overexpressed and control N2a cells that were subjected to 0.2μg actinomycin D (ACTD) treatment. Data were plotted as fold difference over time 0 hours. (j) N2a cell lysates administered with hnRNPM+FLAG Tag were subjected to immunoprecipitation with FLAG Tag antibody or control IgG, followed by immunoblotting with FLAG Tag antibody. (k) hnRNPM+FLAG Tag administered N2a cell lysates were subjected to immunoprecipitation with anti-FLAG antibody or control IgG, as control. Total hnRNPM-

bounded RNA was isolated and subjected to qPCR analyses. Data were shown as the enrichment by FLAG Tag antibody over control IgG. GAPDH was included as a negative control.

Figure 5

hnRNP Binds directly to the 3'UTR of PSD95, Synaptophysin and NeuN mRNAs to Stabilize them (a-c) Schematic of hnRNP binding sites on the 3'UTR of PSD95 (a), exon6 and 3'UTR of Synaptophysin (b), and 3'UTR of NeuN (c). (d-e) PSD95 S1, PSD95 S1 mut, PSD95 S2, and PSD95 S2 mut variants in pmirGlo luciferase vectors were introduced into hnRNP-deficient (d) and hnRNP administered (e) or control N2a cells. Binding affinities were determined by luciferase assay. Luciferase activities were presented as relative difference over controls. (f-g) synaptophysin S1, synaptophysin S2+S3, synaptophysin S2 mut and synaptophysin S3 mut variants were introduced into hnRNP-deficient (f) and administered (g) or control N2a cells. Binding affinities were determined by luciferase assay. (h-i) NeuN S1 and NeuN S1 mut variants in pmirGlo luciferase vectors were introduced into hnRNP-deficient (h) and hnRNP

administered (i) or control N2a cells. Binding affinities were determined by luciferase assay. Luciferase activities were presented as relative difference over controls.