UNIVERSITY OF CRETE

SCHOOL OF MEDICINE

GRADUATE PROGRAMME IN NEUROSCIENCE

LABORATORY OF MOLECULAR AND CELLULAR NEUROSCIENCE

Study of Mena-RNP complex in the developing brain

Μελέτη του Mena-RNP συμπλόκου στον αναπτυσσόμενο εγκέφαλο

Daklada Elisavet

Supervisor: Marina Vidaki

Ηράκλειο 2022

Examination Committee

Karagogeos Domna
Professor of Molecular Biology-Developmental Neurobiology

Vidaki Marina
Assistant Professor of Cellular Molecular Biology

Charalampopoulos Ioannis
Associate Professor of Pharmacology

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Finally, I must express my very profound gratitude to my parents and to friends for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

Curiculum Vitae

Personal Information ──────────────────────────■

Elisavet Daklada

◴ 00306984599740
Nikomideias 19, Haidari, Athens, Greece
eliswdakl@gmail.com

Date of birth:
08/09/1995
Nationality:
Greek

Education and Qualifications ──────────────────■

10/2019-2/2022	**MSc Neurosciences**
	Medical School, University of Crete
	Grade (until now): 8,37 of 10
1/2014 – 11/2019	**BSc Biology**
	(4 years program-240 ECTS)
	Department of Biology, Faculty of Sciences and Engineering-
	University of Crete, Greece. Grade : 6,63 of 10
09/2010 – 06/2013	Graduation from 1st Lyceum (Senior High School) in Haidari, Athens, Grade: 17,9/10 of 20 (excellent)
9/2006- 6/2013	Secondary education
	Music Junior High School of Ilion, Athens

Laboratory experience

2/2021-2/2022   **Master Intership**
Project Title: "The Mena-RNP complex in the developing mouse brain"
Supervisor: Marina Vidaki, Assistant Professor of Cellular Molecular Biology, School of Medicine, University of Crete

11/2020-1/2021   **Master's 3 months Rotation Practice**
Project Title: "Study of Hey regulation through 3'-UTR from miRNAs"
Supervisor: Maria Monastirioti, Senior Staff Scientist, IMBB-FoRTH
       Grade: 9 out of 10

10/2018-1/2019   **Erasmus Mobility Fellowship (placement)**
        Research Training
Project Title: "The role of Mtg8 in cortical interneuron development"
Supervisor: Nikoletta Kessaris, Professor of Neuroscience, UCL, Wolfson Institute for Biomedical Research
Grade: 9 out of 10

8/2017- 8/2018   **Bachelor Internship**
Neurology Laboratory, Medical School, University of Crete
Project Title: "Viability control in HEK293 Cell Lines,   Overexpressing hGDH1 and hGDH2" Supervisor: Ioannis Zaganas, Assistant Professor of Neurology at University of Crete, Medical School
Grade: 10 out of 10

Research skills ━━━━━━━━━━━━━━━━━━━━━━━━━━━━━━■

Handling, collection and maintenance of fruit flies (Drosophila melanogaster)	Enzyme Kinetics Assay measuring enzymatic activity	GFP+ cell preparation for Fluorescence- activated cell sorting (FACs) with MoFlo Cytometer
Management of cell cultures (passing, freezing, thawing), using aseptic techniques and avoiding microbial contamination	Trypan Blue assay	Watched and explained how FACs machine works
Cell cultures of HEK293 and primary cortical neurons	MTT assay	Use of Cryotome
Isolation of eukaryotic DNA, RNA from cells and tissues	Animal anatomy (mouse) ✓ Mouse brain dissection (including specific brain regions from the whole brain) ✓ Mouse cortex dissection	Immunohistochemistry in cells and tissues
Isolation of human DNA from blood	Cortical Interneurons isolation	Immunoprecipitation
Quantitation, electrophoresis and digestion of human/eukaryotic DNA	X-Gal staining assay	CLIP (Crosslinking and Immunoprecipitation)

Bradford assay	Standard PCR/ Real Time PCR	Oligod(T) pull-down
Western-Blot analysis	cell lysis / protein isolation	cDNA synthesis

Publications

2018   Mathioudakis L, Bourbouli M, **Daklada E**, Kargatzi S, Michaelidou K, Zaganas I. Localization of Human Glutamate Dehydrogenases Provides Insights into Their Metabolic Role and Their Involvement in Disease Processes. Neurochem Res 44: 170-187, 2019.

Conferences and Seminars

2016   25-27 November 2016, attended the 67th Panhellenic Congress of the Hellenic Society of Biochemistry and Molecular Biology in Ioannina

2019   4-6 October 2019, attended the 28th Meeting of the Hellenic Society for Neuroscience, Heraklion, Crete

Additional interests and Skills

Education
  Teaching of Biology in secondary education- Certificate provided by University of
  Crete

Languages
  Greek: Native
  English: 2011 First Certificate in English (B2-
  Cambridge degree) Grade: C French: self-studying
  for 3 years

Computer Skills
  Very good knowledge of computer use- Certification provided by the University of
  Crete, equivalent to the ECDL Diploma
  Using Programming
  language R MS
  Windows
  7/10/XP/Vista
  Microsoft Office software (Word –
  Excel - Power Point) Using e-mail and
  the internet
  Using GraphPad Prism
  Familiar with PyMOL Molecular Graphics System, Version 1.4
Personal Interests
  Music: Piano lessons for 12 years
  Flute lessons for 4 years
  Banjo (tampouras) lessons for 3 years
  Taekwondo for 13 years

Abstract

Development and growth of the nervous system are complex processes that require neurons to extend their axons, reach their targets and form functional networks. Any mistake at these processes can lead to neurodevelopmental or cognitive disorders. Local mRNA translation is one of the main molecular mechanisms for axonal guidance, synaptogenesis and maintenance of neuronal networks and understanding the regulation of this mechanism will uncover many other aspects of the development and regeneration of the nervous system as well as neurodevelopmental diseases. Studying the actin-regulatory protein Mena and its RNP complex in the regulation of local translation of mRNAs that are involved in the ribonucleoprotein complex in the developing axons, may reveal important information about the process of axonal translation in the developing neurons.

Keywords: mRNAs, RBP, Mena, local translation, axonal development

Περίληψη

Η ανάπτυξη του νευρικού συστήματος είναι μία περίπλοκη διαδικασία που προϋποθέτει την επιμήκυνση των αξόνων των νευρώνων, την σύνδεσή τους με τα κύτταρα στόχους και τελικά την σύνθεση ενός λειτουργικού δικτύου. Οποιοδήποτε λάθος σε αυτήν την διαδικασία μπορεί να οδηγήσει σε νευροαναπτυξιακές και γνωσιακές διαταραχές. Η τοπική μετάφραση mRNA είναι ένας βασικός μηχανισμός για τον προσανατολισμό και την καθοδήγηση των νευρώνων, την συναπτογένεση και την διατήρηση των νευρωνικών δικτύων. Η κατανόηση της ρύθμισης του μηχανισμού αυτού θα αποκαλύψει πολλές πτυχές της ανάπτυξης και της αναγέννησης του νευρικού συστήματος καθώς και των νευροαναπτυξιακών ασθενειών. Η μελέτη της Mena και του ριβονουκλεοπρωτεϊνικού συμπλόκου της (RNP), στη διαδικασία της ρύθμισης της τοπικής μετάφρασης των mRNAs που εμπλέκονται στο RNP σύμπλοκο στους αναπτυσσόμενους άξονες,

ενδεχομένως να παρέχουν σημαντικές πληροφορίες για τη διαδικασία της τοπικής μετάφρασης στους άξονες στους αναπτυσσόμενους νευρώνες.

Λέξεις κλειδιά: mRNAs, RNP, Mena, τοπική μετάφραση , ανάπτυξη αξόνων

Contents

Introduction

During development, processes of polarization, outgrowth of the axon and the growth cone and axonal guidance in order to find its target and form a synapse with a target cell, are crucial for the establishment of a functional nervous system (NS)(Corradi, 2020).Abnormal axonal navigation and connectivity result in multiple neurodevelopmental disorders, including epilepsy, intellectual disabilities, autism and schizophrenia (Wegiel *et al.*, 2010; M. Sahin and M. Sur,2016,;Disorders,2016).

Axon guidance includes axon elongation and attractive turning of the growth cone, as well as axon retraction and growth cone collapse whenever that is necessary. All these steps require a continuous and autonomous respond of the developing axons to micro-environmental cues, (Verma *et al.*, 2005; Filipa *et al.*, 2017). For this rapid responses, the process of local translation is vital(Filipa *et al.*, 2017). An axon can receive a stimulus at any site all along it. Free mRNAs and ribosomes are distributed in the axon and after the stimulus they are activated to start the translation process. The newly synthesized protein can find its target either close to the site of the stimulus or far from it(Rangaraju, Dieck and Schuman, 2017). Impaired local translation can lead to multiple disorders, such as the Fragile X syndrome that is a genetic condition with a range of developmental deficits, including learning disabilities and cognitive impairments, as a result of aberrant local translation (C.Opin *et al.*, 2013; Ciaccio *et al.*, 2017).

Our understanding of local protein synthesis regulation is still very limited with respect to the molecular mechanisms underlying it. One such mechanism that was recently uncovered to regulate axonal local translation is that of Mena(Vidaki *et al.*, 2017). Mena is a member of the Ena/Vasp protein family, characterized by its high expression in the developing and adult nervous system and its involvement in integrin-mediated signaling as well as its regulatory activity in actin dynamics(Bear and Gertler, 2009). Apart from those well-characterized functions, Mena was recently shown to form a ribonucleoprotein complex (RNP) with RNA-binding proteins (RBPs) PCBP1 and HnrnpK, and cytosolic mRNAs. Mena is necessary for the disassembly of the RNP complex and the translation of the associated mRNAs. In the absence of Mena the translation of those mRNAs is attenuated, potentially through the action of HnrnpK

and PCBP1 that can function as translational inhibitors when they remain bound to the mRNAs (Vidaki *et al.*, 2017).
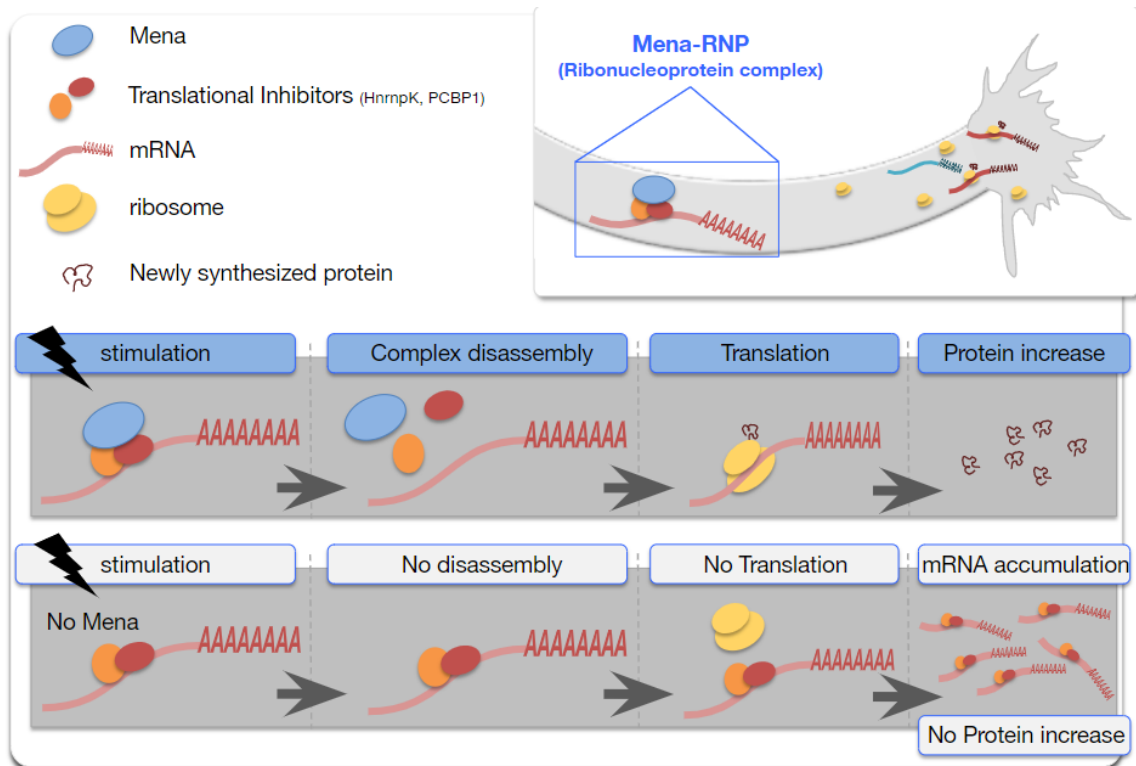
Schematic representation of Mena-RNP complex as a translational regulatory machinery

The importance of Mena has been previously reported through the studies of Mena-KO mice. Those mice exhibit abnormal navigation of calossal fibers in the developing cortex during the formation of corpus callosum; The axons are unable to cross the midline in order to form a normal corpus callosum structure(Menzies *et al.*, 2004). Similar deficiencies seem to be present in the developing spinal cord of those animals, where commissural axons fail to cross the ventral midline(Mcconnell *et al.*, 2016). Those phenotypes can be highly associated with Mena's role in actin cytoskeleton re-arrangements. However, the other 2 members of the Ena/VASP protein family, VASP and Evl, can compensate for the absence of Mena in actin regulation to a large extent. Therefore, the role of Mena in local translation regulation may be primarily responsible for the Mena-KO phenotypes in axon development.

Hundreds of mRNAs have been found associated with the Mena-RNP complex (Vidaki *et al.*, 2017). Gene set enrichment analysis showed that most of those mRNAs associated with Mena are involved in axon guidance and developmental processes(Vidaki *et al.*, 2017). Most of them are bound with Mena-RNP complex through exon regions while a small amount of them are bound through their 5' or 3'-UTR region. 3'-UTR contains regions for the localization of the mRNA or the post-transcriptional regulation of it(Vidaki *et al.*, 2017). This clue, lead us to focus on mRNAs that are associated with Mena-RNP complex through their 3'-UTR region and investigate whether or not the Mena-RNP complex can influence their translational regulation. One mRNA that is associated with the Mena-RNP complex is *dyrk1a*, a dual specificity kinase that is related to Down syndrome phenotypes and other autism spectrum disorders(Roak *et al.*, 2013; Krumm *et al.*, 2014; Witherspoon *et al.*, 2016). In these disorders, *dyrk1a*, occurs in high levels and published work revealed that its translation in developing axons occurs in a Mena-dependent manner(Vidaki *et al.*, 2017).

mRNAs of interest	
1. *ablim-1*	12. *nudt21*
2. *acd*	13. *ppplt3e*
3. *bcr*	**14. *rgag4***
4. *bmt2*	**15. *rit1***
5. *carmil2*	**16. *rnf41***
6. *ctbp1*	17. *sec22c*
7. *igf2bp3*	**18. *tanc2***
8. *jun*	**19. *trmt1***
9. *junas*	**20. *ttf1***
10. *malsu1*	21. *usp49*
11. *nhsl2*	

Table 1. mRNAs that were found to be associated with Mena-RNP complex through their 3'-UTR and having an interesting functional role.

AIM of the STUDY: Based on these evidence we wanted to study the potential regulation, of other mRNAs that are in association with the Mena-RNP through their 3'-UTR region(Vidaki *et al.*, 2017). Some of those mRNAs (Table 1) seem to be linked with neurodevelopmental diseases and syndromes that cause intellectual disabilities, mental retardation and other neuronal deficiencies, indicating the importance of their normal regulation process. Studying those molecules could provide a mechanistic insight into the axonal defects resulting from Mena-deficiency.

Materials and Methods

Immunofluorescence

Subbed microscope slides were left for 10 min at RT after -20°C and were permeabilized with PBS-Triton X 0.2% for 15min at RT. Blocking with blocking serum (5% NGS and 3% BSA in PBS - Triton X 0.2%) was performed for 1h at RT followed by incubation with primary antibodies diluted in antibody solution (1% BSA in PBS-Triton X 0.1%)O/N at 4°C. The next day, after 5 washes with PBS-Triton X 0.1% for 10min each, slides were incubated with secondary antibodies for 2h at RT. Primary antibodies used: Ms-anti-Mena (1:500, homemade at Frank Gentler's laboratory), Rb-anti-Mena (1:500, homemade at Frank Gentler's laboratory), ms-anti-4D7 /TAG1 (1:100, DSHB), Ms-anti-2H3 (1:1000, DSHB) . Secondary antibodies were used were conjugated to 594, 488, 405 and and 654nm fluorophores and were used diluted 1:500.

Oligo dT pull-down

E15.5 mouse brain lysates were prepared exactly as previously described in Mena CLIP protocol. Half of them were X-linked. After lysis, all samples were placed on ice for 15' to rest and then were centrifuged for 20min at 14000rpm at 4°C. Then, lysates were treated with DNAse and RNAseA for 15' at 37°C and then at 65°C for 5'and placed on ice. Oligo-d(T) beads were added to lysates for 15min at RT and then placed in magnet followed by three washes with 1M NaCl lysis buffer and 4x laemli buffer for beads' extraction and Western Blot analysis, respectively.

Mena CLIP (Immunoprecipitation after Crosslinking)

Mena IP was performed to E15.5 brains after UV X-linking in 254nm in order to maintain the RBP-mRNA complex. Embryonic brains were triturated in cold RNase free-PBS and were irradiated 3 times, at 400 $/cm^2$, in UV (254nm), using Stratalinker. The tissue was collected by brief centrifugation and lysed in mild lysis buffer (5M NaCl, 1% Triton, 0,5% DOC, 1M Tris pH8) supplemented with EDTA-free protease and phosphatase inhibitor tablets (Rosche).

Primary Neuron Stimulation with BDNF

At first, primary cortical neurons were starved for 4 hours in L15-Leibowitz medium (Invitrogen). After starvation, neurons were stimulated with BDNF (50ng/ml; R&D systems) for 15min.

Primary Neurons on Transwell Filters/Axotomy

Cortical neurons from E15.5 mouse brains were isolated and plated on the top compartment of a 6-well hanging inserts with 1um membrane pores (Cortical neurons form E15.5 mouse brains were plated on the top compartment of 6-well hanging cell cultures inserts with 1μm membrane pores (Millipore,PET), which was primary coated with PDL (0,4mg/ml) on both sides. Primary neurons were cultured for 2 days in Neurobasal medium supplemented with B27 and Pen/Strep.

At first, neurons were starved as previously described, with the cell bodies at the top compartment of the filter and the axons at the bottom. BDNF was added at the top compartment of the transwell filter for 15min. After stimulation cell bodies were washed with cold PBS, scraped in order to separate them from the axons and both cell bodies and axons were lysed for protein and RNA extraction.

Western Blot

Lysates were resolved by SDS-PAGE and after proteins were separated, were transferred to nitrocellulose membranes and immunoblotted. Membranes were incubated with primary antibodies in PBS + Tween-20 0.1% overnight at 4°C after blocking, which was performed with 5% milk in PBS in RT for 1h. The next day, after three 5min washes with PBS + Tween-20 0.1% ,

membranes were incubated with secondary antibodies, conjugated with HRP, at 1:5000 dilutions and were visualized by enhanced chemiluminescence (SuperSignal West Pico Chemluminescent HRP substrate –ThermoFisher).

cDNA synthesis and Quantitative PCR

cDNA synthesis was performed using the Invitrogen Superscript III First Strand Synthesis for RT-PCR kit, with Random Hexamer primers. Quantitive PCR was performed using Biorad iQ SYBR Green Supermix, with the following gene-specific primers:

mouse Mena 5'-gggcagaaagattcaagacc   ; mouse Mena 3'-gcgaagacattggcatcc

mouse Dyrk1a 5'-caaacggagtgcaatcaaga   ; mouse Dyrk1a 3'-agcacctctggagaccgata

mouse Ctbp1 5' -tagccagggaccccttaaa   ; mouse Ctbp1 3' -atggcactgggaacaatc

mouse Nudt21 5'-gggtatggacccatcatttc   ; mouse Nudt21 3'-caccactatctcggtcatattc

mouse Jun 5'- cagctgagcccaatatac   ; mouse Jun 3'-agcacatgccacttcatac

mouse Acd 5'-gagttccagtgctccttcaa   ; mouse Acd 3'-tgtcccagtgactcctcata

mouse Rgag4 5'-gagaaggaacttgaggaagag ; mouse Rgag4 3'-ctggaatctgaggtacaagac

mouse Rit1 5'-gggaacaagtctgacctaaag   ; mouse Rit1 3'-gtactagctccttctctttcttac

mouse Rnf41 5'-ctggaggagacaatcgaatac   ; mouse Rnf41 3'-agcatcaggagtggagat

mouse Sec22c 5'-ctctcccttgttctcaacatc   ; mouse Sec22c 3'-caggccacagaaggaataaa

mouse Tanc2 5'-tgtctcctacctacttgatctt; mouse Tanc2 3'-cgaacagtgctgaatagagg

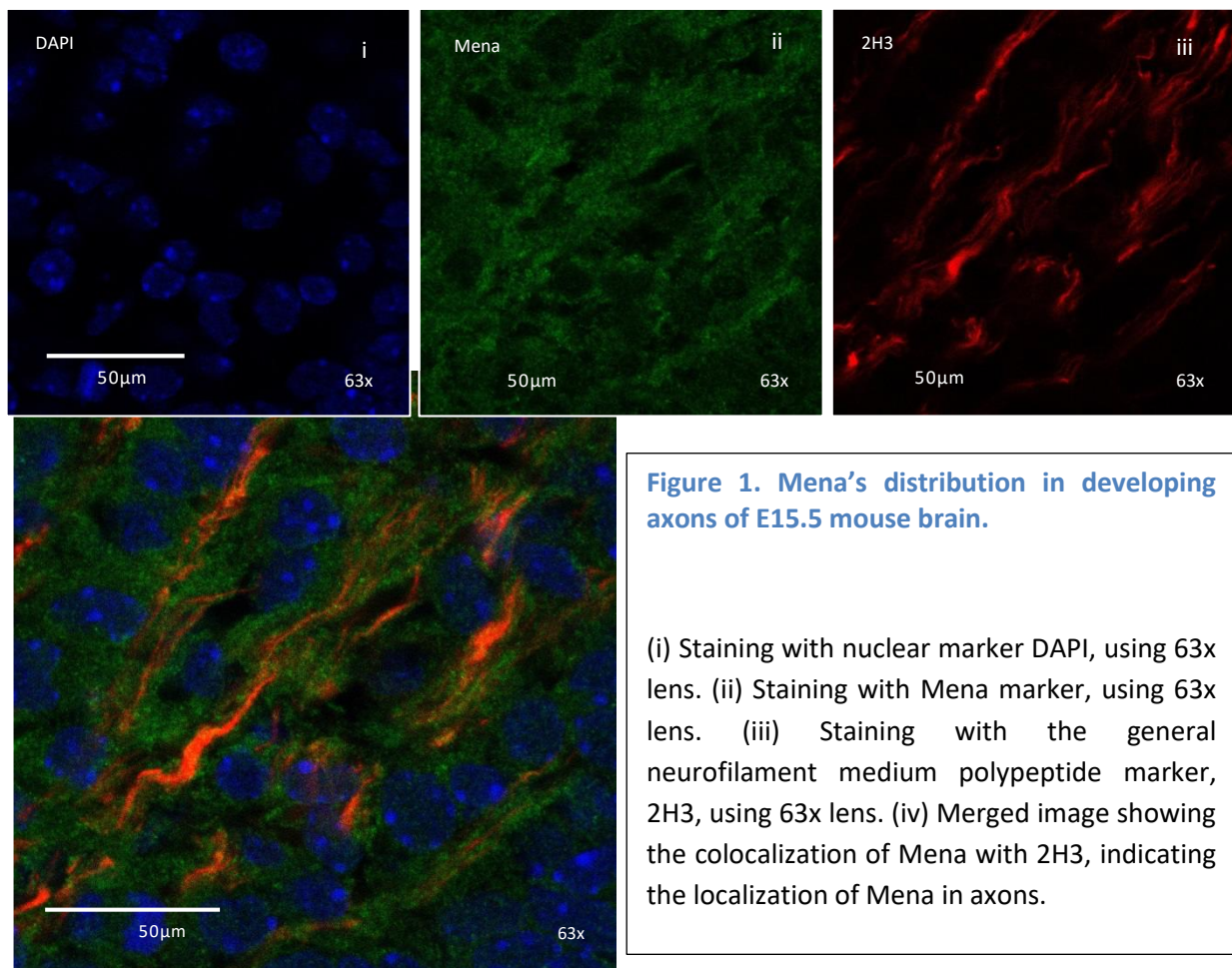mouse Trmt1 5'-ccgaattcttgctgtggag   ; mouse Trmt1 3'-ccagttggcttctggattg

mouse Ttf1 5'- agctcccaaacaagactttc   ; mouse Ttf1 3'-cagcacaggagaggtatca

mouse Gapdh 5'-gagtgtttatttggccgtattg   ; mouse Gapdh 3'-catgtagaccatgtagttgagg

Results

Distribution of Mena protein in developing brains (Immunofluorescence)

Immunofluorescence experiments were performed in order to study the distribution of Mena in developing brains *in vivo.* For the experiments, E15.5 mouse coronal brain's sections were used from the forebrain and the images were obtained from the cerebral cortex, where numerous axon types are developing at this stage. The colocalization of Mena with axonal markers, indicate the presence of Mena in different subsets of axons. The colocalization of Mena with 2H3, a marker of neurofilaments that is present in most axonal populations throughout the nervous system, indicates the presence of Mena in axons *in vivo* (Fig.1). Additional specific markers were

Figure 1. Mena's distribution in developing axons of E15.5 mouse brain.

(i) Staining with nuclear marker DAPI, using 63x lens. (ii) Staining with Mena marker, using 63x lens. (iii) Staining with the general neurofilament medium polypeptide marker, 2H3, using 63x lens. (iv) Merged image showing the colocalization of Mena with 2H3, indicating the localization of Mena in axons.

used, to determine the presence of Mena in specific axonal subpopulations, like the corticothalamic axons (stained with TAG1 in Fig.2) and thalamocortical axons (stained with L1 in Fig.3).
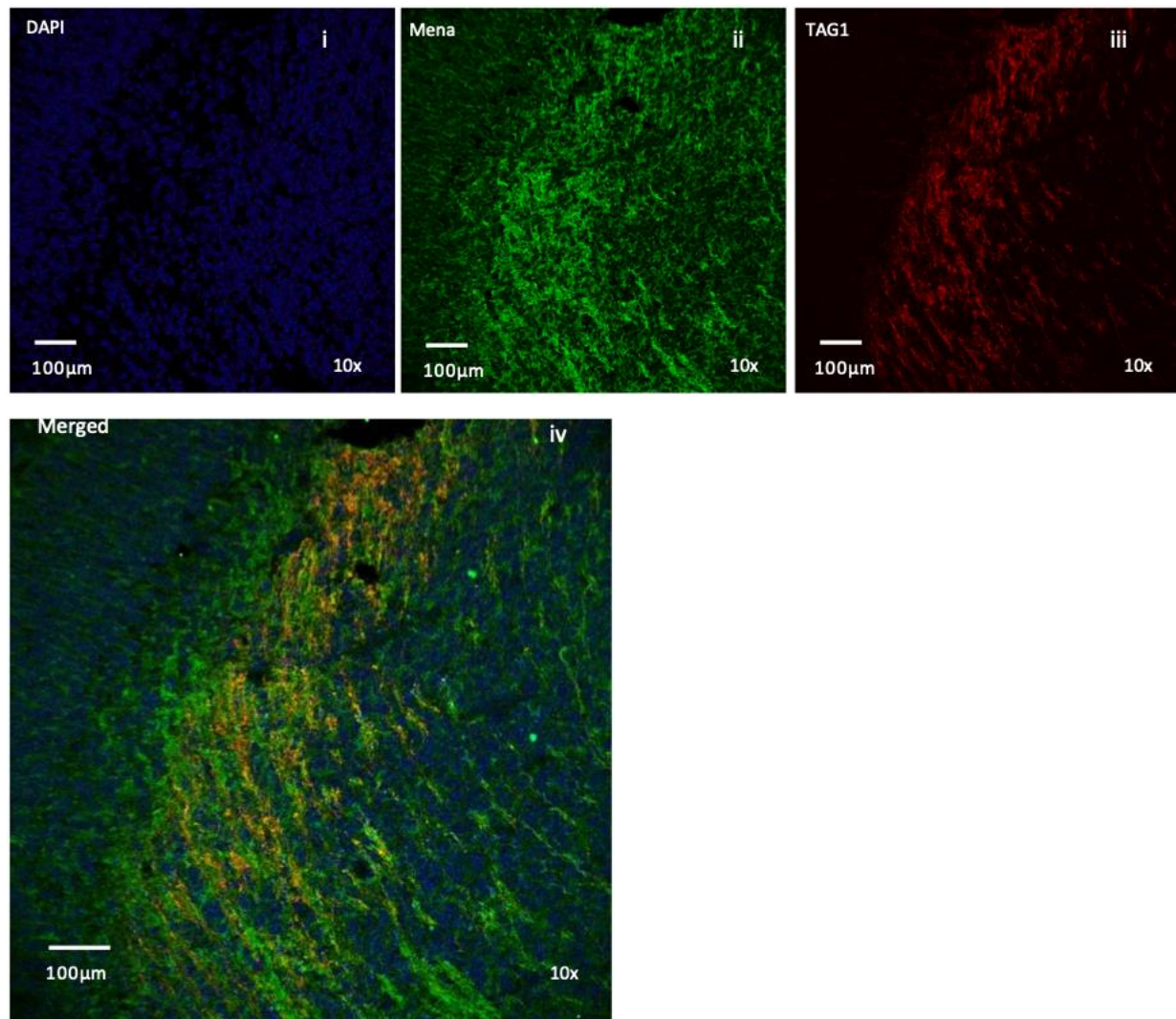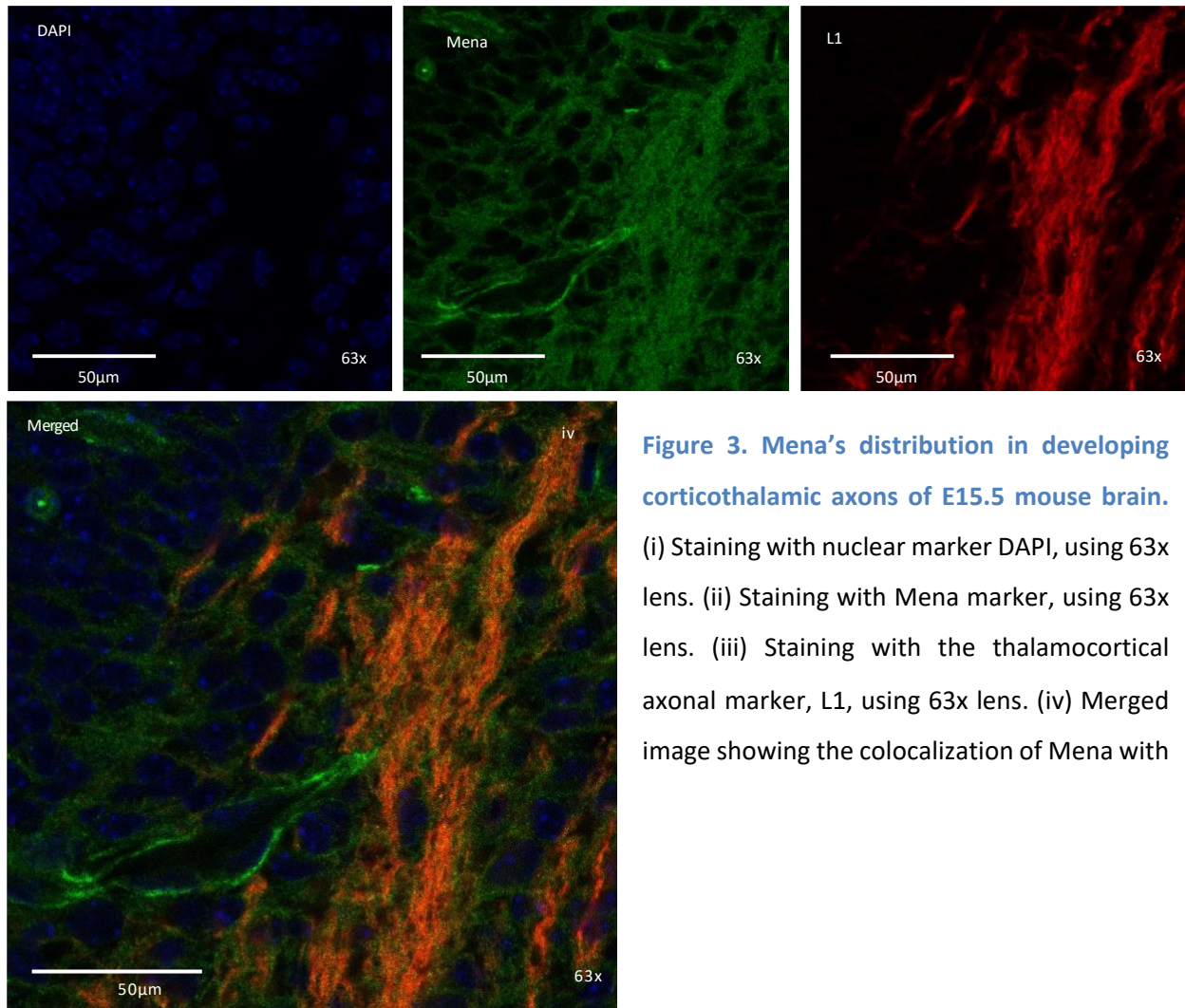

Figure 2. Mena's distribution in developingcorticothalamic axons of E15.5 mouse brain.

(i) Staining with nuclear marker DAPI, using 10x lens. (ii) Staining with Mena marker, using 10x lens. (iii) Staining with the corticothalamic axonal marker, TAG1, using 10x lens. (iv) Merged image showing the colocalization of Mena with TAG1, indicating the localization of Mena in corticothalamic axons.

Figure 3. Mena's distribution in developing corticothalamic axons of E15.5 mouse brain. (i) Staining with nuclear marker DAPI, using 63x lens. (ii) Staining with Mena marker, using 63x lens. (iii) Staining with the thalamocortical axonal marker, L1, using 63x lens. (iv) Merged image showing the colocalization of Mena with

Distribution of Mena protein in primary cortical neuron cultures (Immunofluorescence)

Further immunofluorescence experiments were performed in order to study the distribution of Mena in axons of primary cortical cell cultures *ex vivo,* from E15.5 mouse brains plus 2 days *in vitro.* As expected, Mena is colocalized with beta-actin in cortical neurons (Fig.4), as well as intermediate filament markers like Vimentin (Fig.5). Interestingly, Vimentin is one of the first proteins that are locally translated in adult axons in response to injury (Willis *et al.*, 2005). In order to examine the colocalization of Mena with the translation machinery in axons, we detected the presence of the protein in combination with ribosomal component S6 (a protein of the small ribosomal subunit which catalyzes the translation of the mRNA), in developing axons of cortical neurons in culture (E15,5+2DIV). Our results show clearly that Mena and S6

20

are colocalized in axons, further supporting a role for Mena in translation regulation during development (Fig.6).
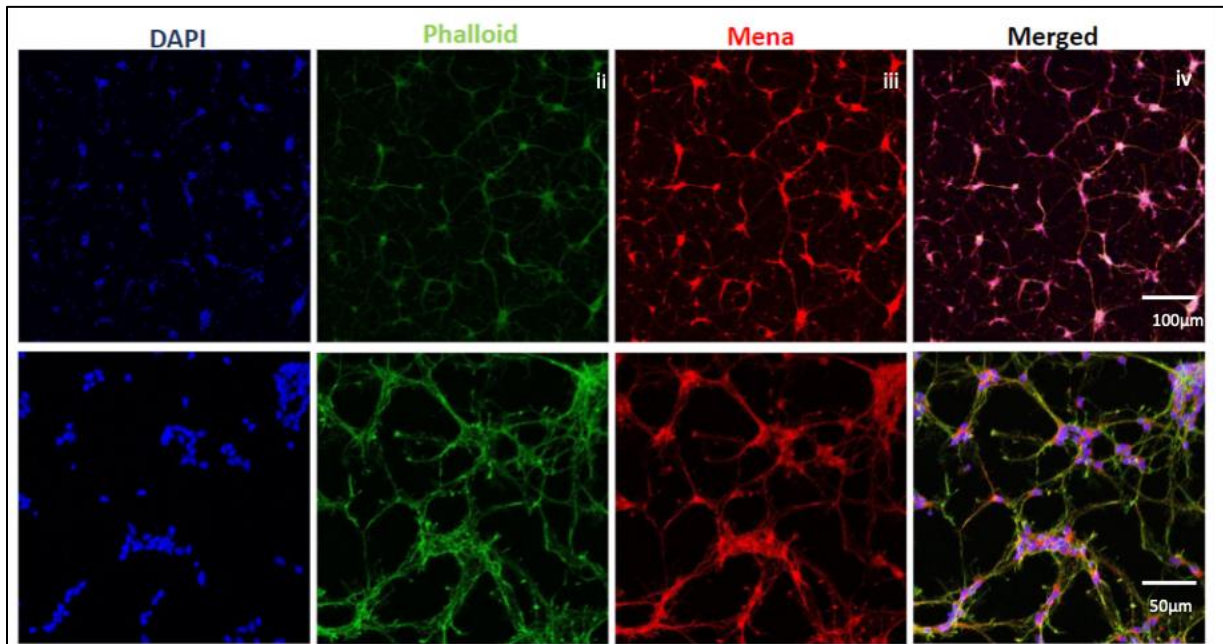
Figure 4. Mena localization in developing axons of E15.5+2DIV of cortical cell cultures
D. (i) Staining with nuclear marker DAPI. (ii)Staining with beta-actin marker. (iii) Staining with Mena marker. (iv) Merged image showing the colocalization of Mena and beta-actin in axons
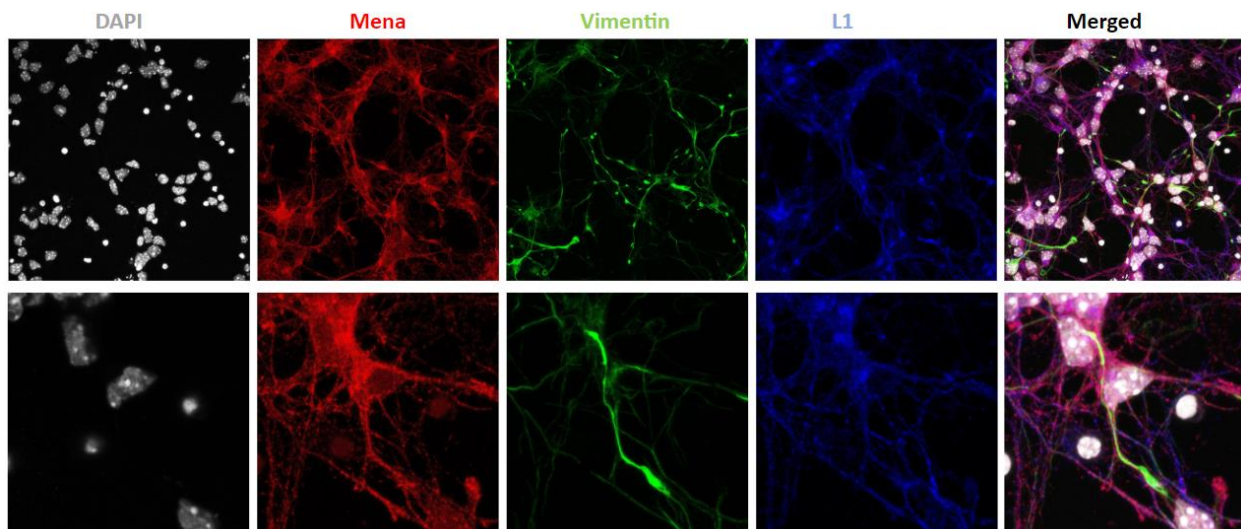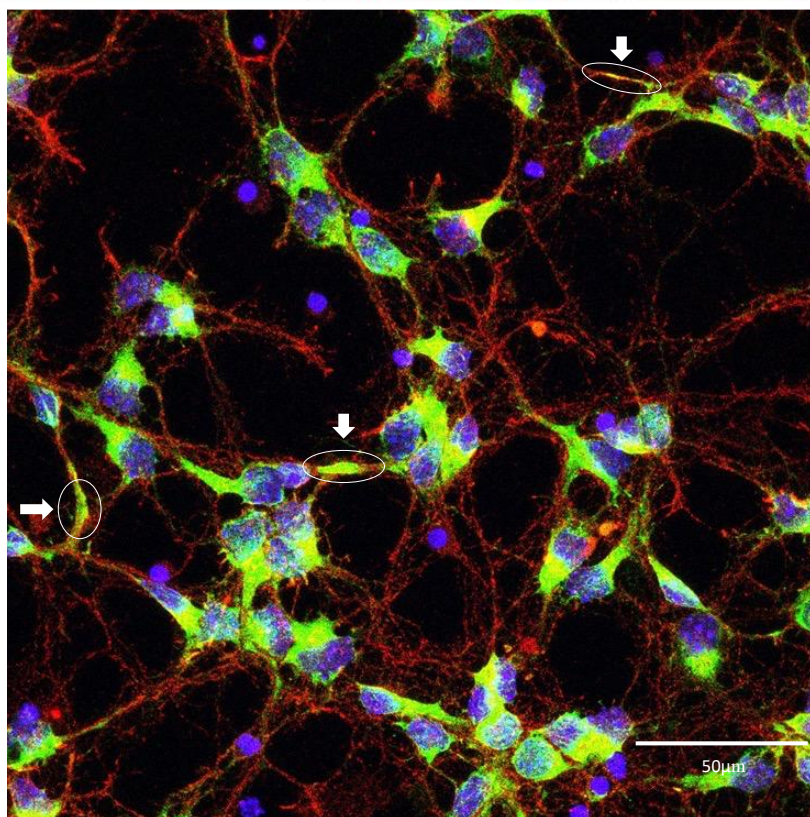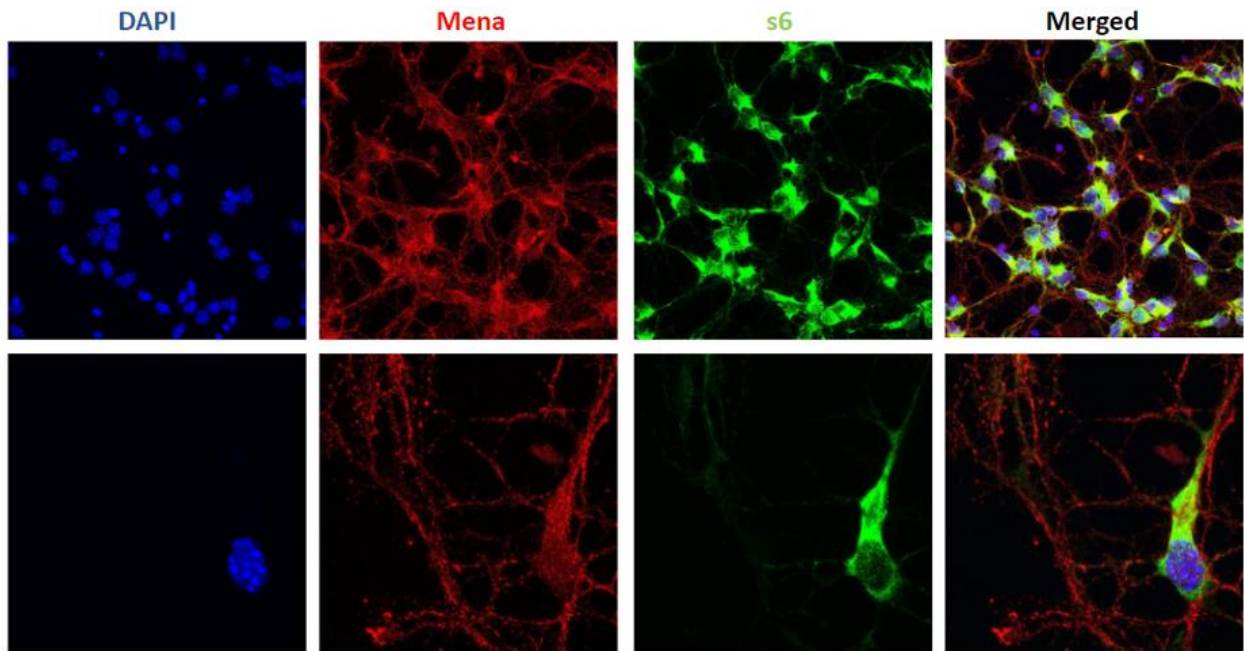

Figure 5. Mena colocalization with Vimentin in developing axons of E15.5+2DIV of cortical cell cultures. (i) Staining with nuclear marker DAPI. (ii) Staining with Mena marker. (iii) Staining with Vimentin marker. (iv) Staining with thalamocortical axonal marker L1. (v) Merged images showing the colocalization of Mena with Vimentin in axons.

Figure 6. Mena colocalization with S6 in developing axons of E15.5+2DIV of cortical cell cultures. (i) Staining with nuclear marker DAPI. (ii) Staining with Mena marker. (iii) Staining with S6 marker. (iv) Merged images showing the colocalization of Mena with S6 in developing axons and cell bodies. (v) Image (iv) zoomed showing more clear with the white arrows the colocalization of Mena with S6 in developing axons.

Mena Association with mRNAs

Oligo-d(T) pull-down

The first step to examine the mRNAs of interest involved in Mena-RNP complex, was to verify that Mena-RNP complex is associated with mRNAs. For that reason, oligo-d(T) pull-down and Western Blot analysis was performed with lysates from E15,5 whole mouse brains. Western Blot analyses, verify that all Mena isoforms (140kDa & 80kDa) with HnrnpK (60kDa), were pulled down with mRNAs, both in crosslinked and in non-crosslinked samples (Fig.7).

A
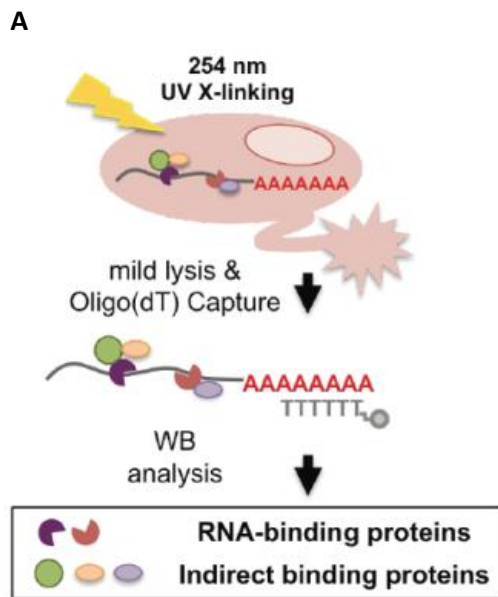
Figure 7. Oligo-d(T) pull-down verify that Mena-RNP complex is associated with mRNAs (A) Schematic representation of Oligo-d(T) capture protocol. E15.5 mouse brains were triturated and UV crosslinked in order to preserve RNP complexes. Poly(A) tails of mRNAs were captured to oligo-d(T) beads, containing all associated proteins. (B) Western Blot analysis of the beads containing all associated proteins with mRNAs, showing that Mena and HnrnpK are present after mRNA pull-down, indicating their association with mRNAs.
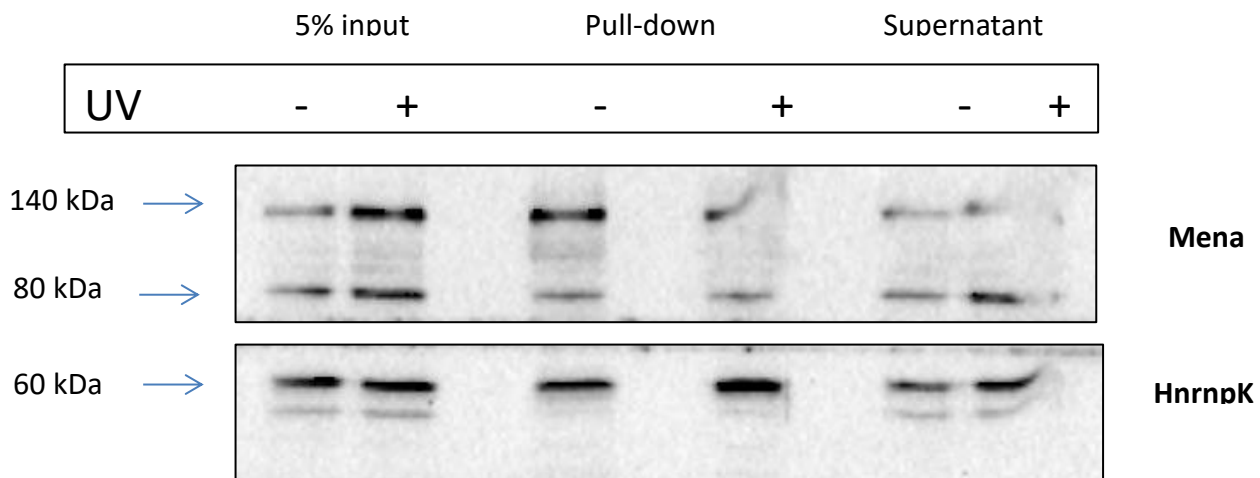
B

Mena Co-Immunoprecipitation (CoIP)

Mena Immunoprecipitation experiments were performed, using whole brains from E15,5 mice, in order to standardize the protocol and verify it can work properly. IgG2b bound to magnetic beads was used as a negative antibody-control, and supernatants of each sample were used, to validate the efficiency of the method. In case of the Mena supernatant, we verified that all of Mena protein found in the lysate is bound to the beads, while in the IgG2b supernatant Mena is still present, thus our negative control has worked successfully. The results show that Mena Immunoprecipitation protocol is successful, since Mena seems to be enriched in Mena IP sample and Vasp, which was used as a positive control since it is a known Mena interactor, is also Co-immunoprecipitated. GAPDH was used as a non-interacting negative control, and to ensure that all samples were equally loaded (Fig.8).
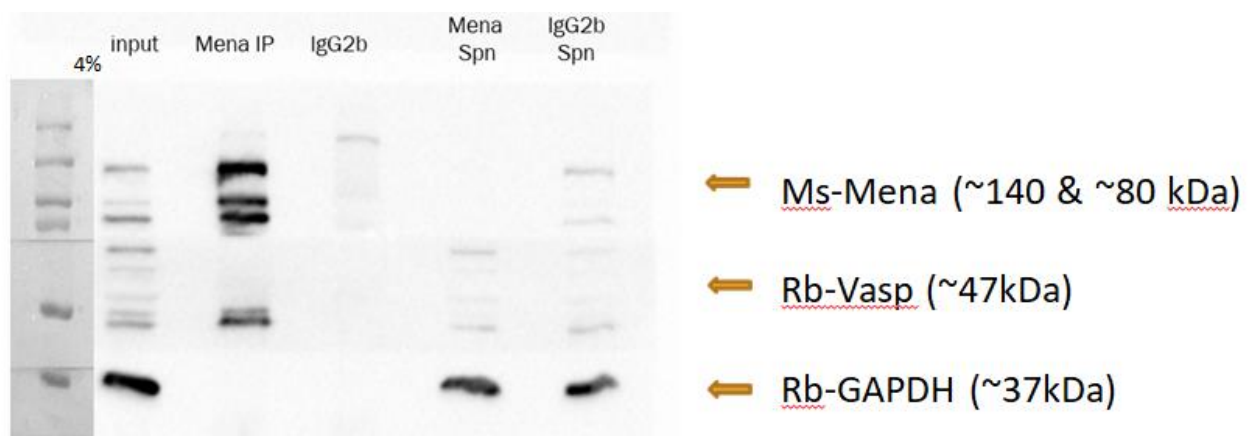
Mena Cross-Linked Immunoprecipitation (CLIP)

The next step was to proceed to Mena CLIP, in order to isolate the Mena-RNP complex associated mRNAs of interest and quantify them with qPCR. For that reason, E15.5 mouse brain tissues were UV crosslinked in order to stabilize irreversibly the bonds of the RNP complexes and were subjected to RNAse treatment in order to get rid of the rest RNA. Then we proceed to Mena Immunoprecipitation to precipitate the Mena-RNP complex with its associated mRNAs, RNA isolation, cDNA synthesis and quantitative PCR (Fig.9A). The Western Blot analysis shows the success of the Mena CLIP, since we see no Mena in Mena CLIP sample, indicating that most of Mena protein and its complex were bound on the beads (Fig.9B).
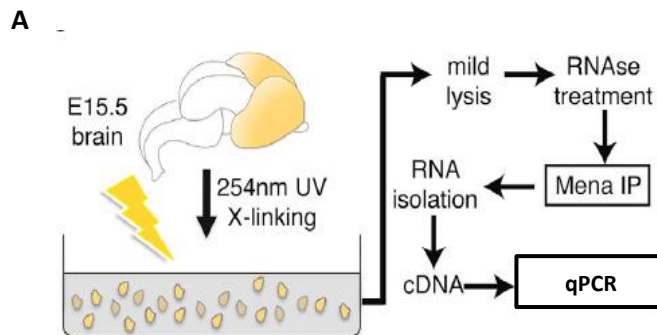
A

B

Figure 9. Mena Crosslinked Immunoprecipitation. (A) Schematic representation of CLIP protocol. Tissues of E15.5 mouse brain were UV crosslinked and lysed in lysis buffer in order to proceed to Mena IP. CoIPed RNA was isolated, cDNA was synthesized and quantified.

qPCR results

Quantification of mRNAs in Mena-RNP complex reveals significant enrichment for some of them. More specifically, *Ctbp1, Rgag4, Rit1, Rnf41* and *Ttf1*, exhibit the highest association with the Mena-RNP, indicating a possible importance in their translational regulation during development (Fig.10). *Mena* mRNA in Mena-RNP complex presents a 305 fold enrichment, indicating a possible positive loop of Mena regulating its own mRNA (Fig.11).
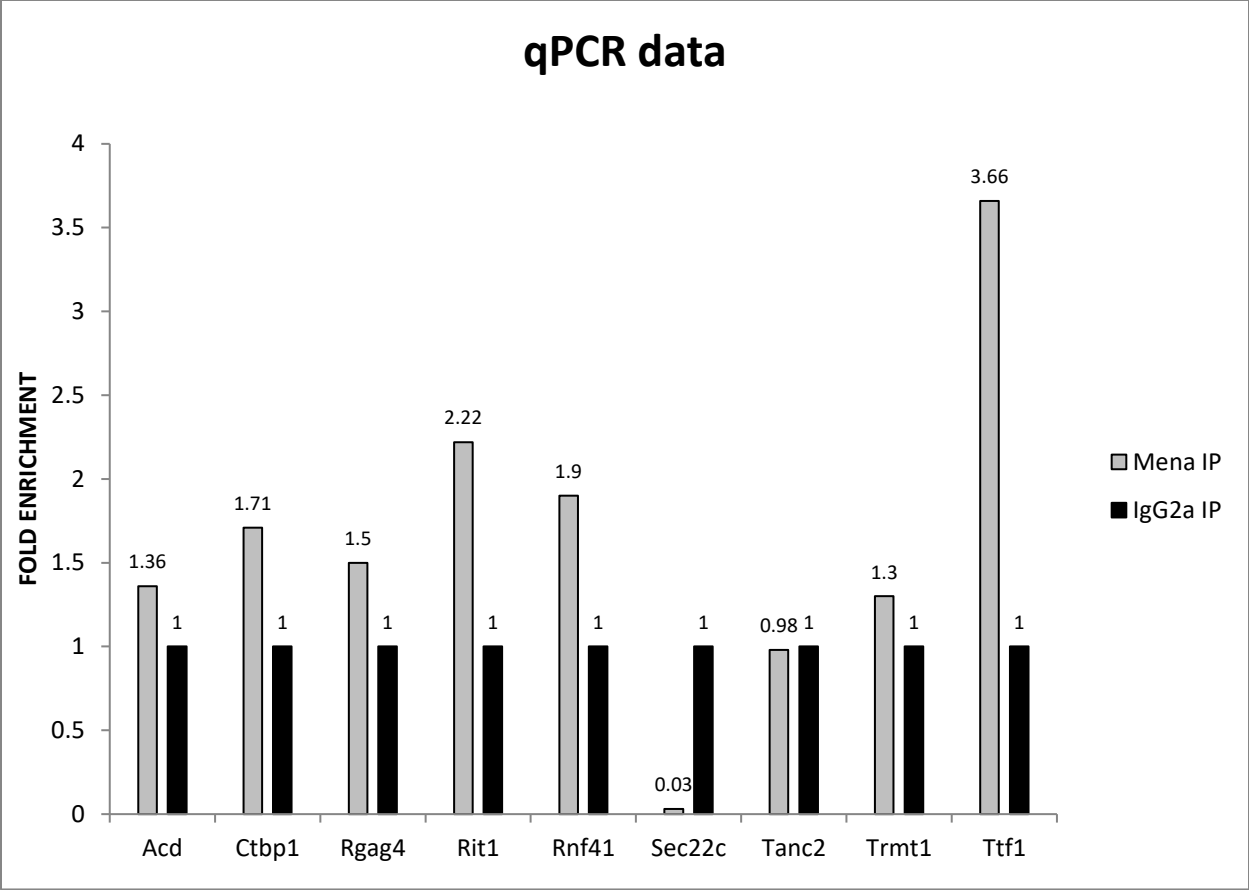
Figure 10. qPCR results reveal fold enrichment to some of mRNAs of Mena-RNP complex Mena IP mRNAs normalized to IgG2b mRNAs reveal fold enrichment to all of them except *Sec22c* and *Tanc2*.
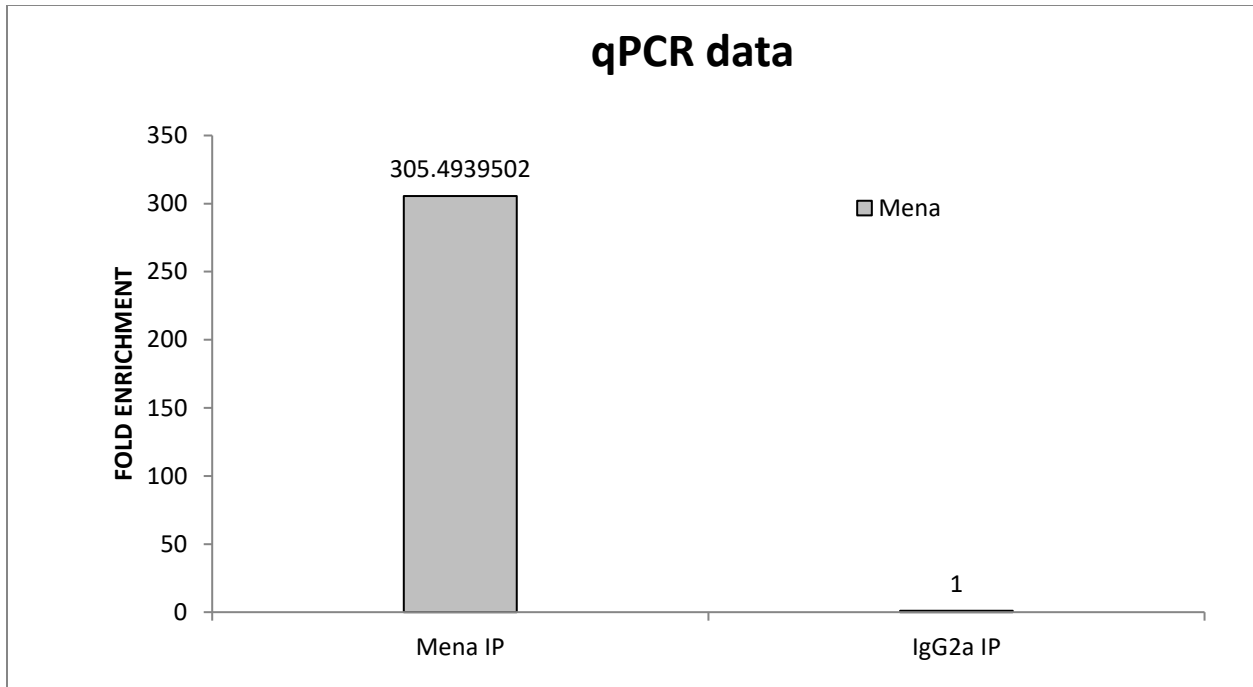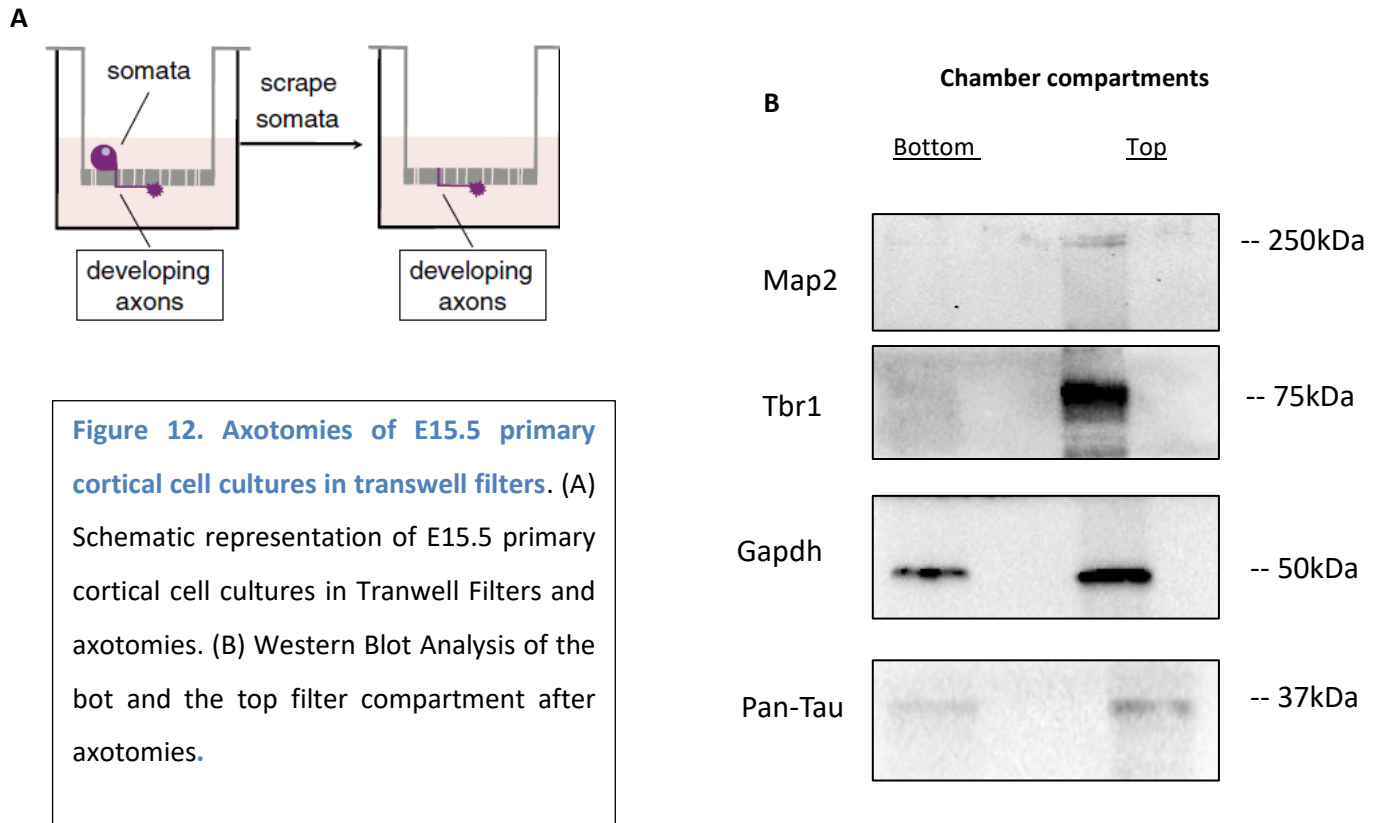
qPCR data

Figure 11. qPCR results show that Mena mRNA is highly enriched in the Mena-RNP complex
Mena mRNA seems to be 305 times fold enriched in Mena-RNP complex normalized to IgG2b IP.

Transswell Filters/Axotomies and qPCR

Transwell Filters/Axotomies

The next step, was to investigate further the presence of these mRNAs in the different neuronal compartments. For that reason we proceeded to cortical cell cultures form E15.5 mouse brain cortex in transwell filters and axotomies. Developing neurons were cultured in transwell filters with 1μm pores, allowing only axons to pass through and reach the bottom compartment of the filter, while cell bodies or whole cells remain mostly at the top compartment. For the axotomies, somata were scraped and RNA and protein extraction were accomplished for both compartments (Fig.12A). Western Blot analysis of lysates of bottom and top compartment, ensure the successful separation of cell bodies and axons after axotomies since we can see

what was expected in each compartment using markers Map2, a dendritic marker, Tbr1, a nuclear one and Pan-Tau, an axonal marker. On the top compartment Map2, Tbr1 and Pan-Tau seem to be present, while Pan-Tau is present on the bottom compartment (Fig. 12B).

A

B Chamber compartments

Bottom   Top

Map2 — -- 250kDa

Tbr1 — -- 75kDa

Gapdh — -- 50kDa

Pan-Tau — -- 37kDa

Figure 12. Axotomies of E15.5 primary cortical cell cultures in transwell filters. (A) Schematic representation of E15.5 primary cortical cell cultures in Tranwell Filters and axotomies. (B) Western Blot Analysis of the bot and the top filter compartment after axotomies.

qPCR results

After RNA extraction and cDNA synthesis, qPCR was performed to study the presence of mRNAs of interest in each compartment. The results show a possible absence of *Acd, Rit1* and *Rgag4* in axons while as expected, the quantity of all the other mRNAs it higher in cell bodies than in axons (Fig.13).

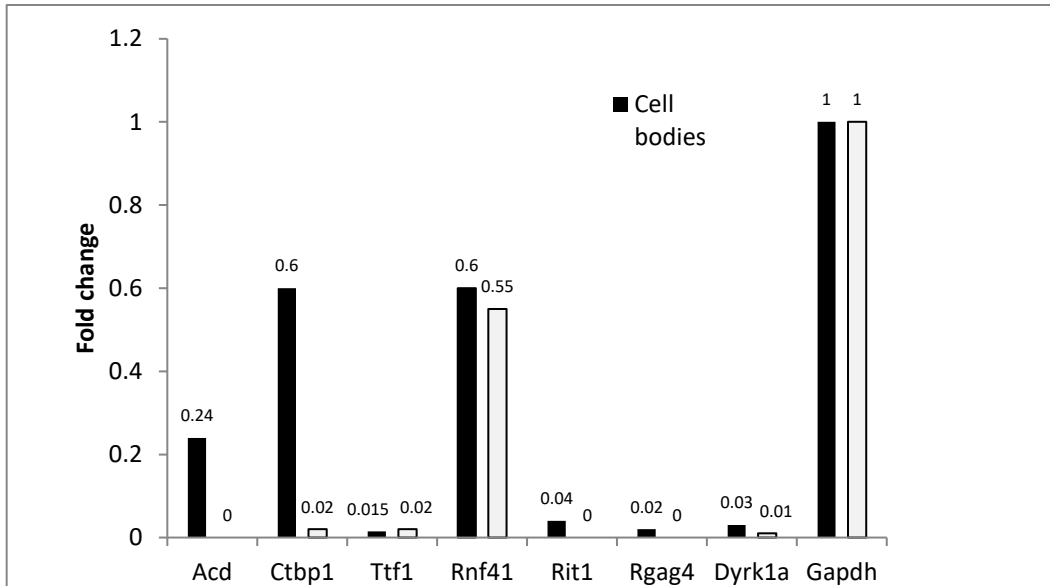**Figure 13. qPCR results of mRNAs in each filter compartment**. mRNAs in axonal compartment compared to them in cell bodies, all normalized to GAPDH.
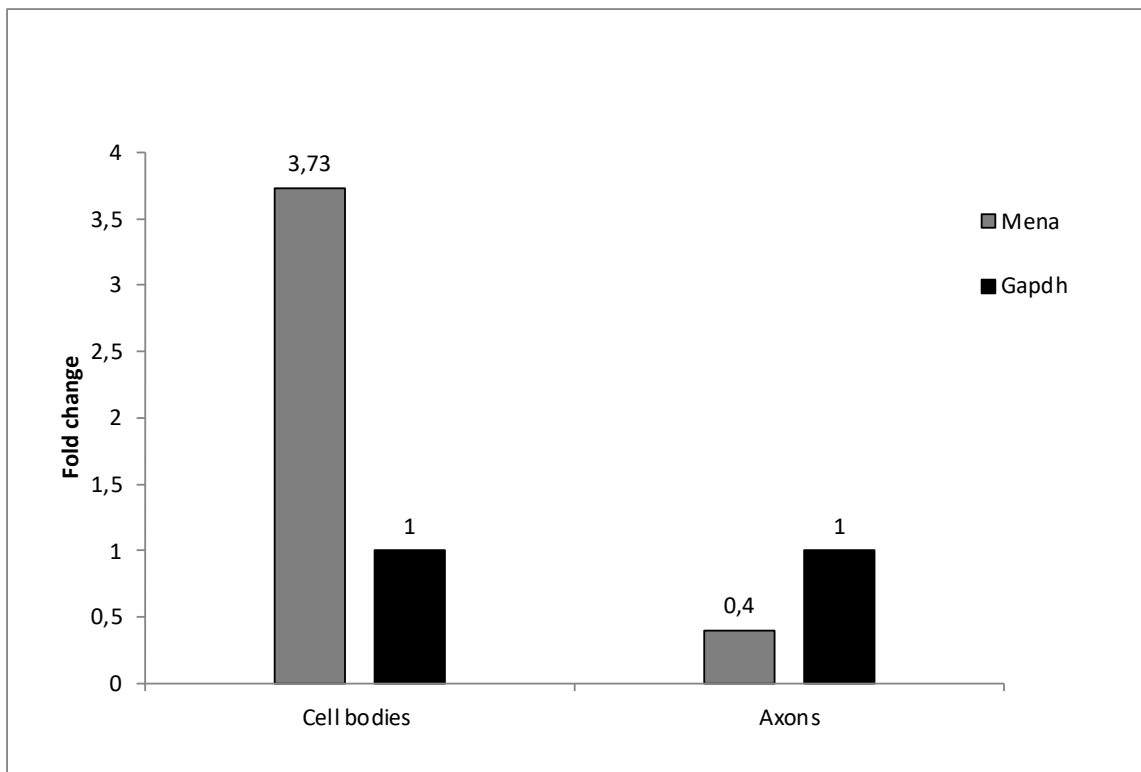

Figure 14. qPCR results of Mena mRNA in each compartment. Mena mRNA in cell bodies compared to Mena mRNA in axons, normalized to GAPDH.

BDNF stimulation

Furthermore, it would be interesting to investigate the way these mRNA respond to BDNF stimulation in the different neuronal compartment. BDNF is a neurotrophic factor that supports the survival of existing neurons and has a crucial role in the development, growth and differentiation of new ones, by enhancing vital processes like this of transcription and translation whenever and wherever that is necessary (Miranda *et al.*, 2019). For that reason, primary cortical neurons were cultured in transwell filters and BDNF was added
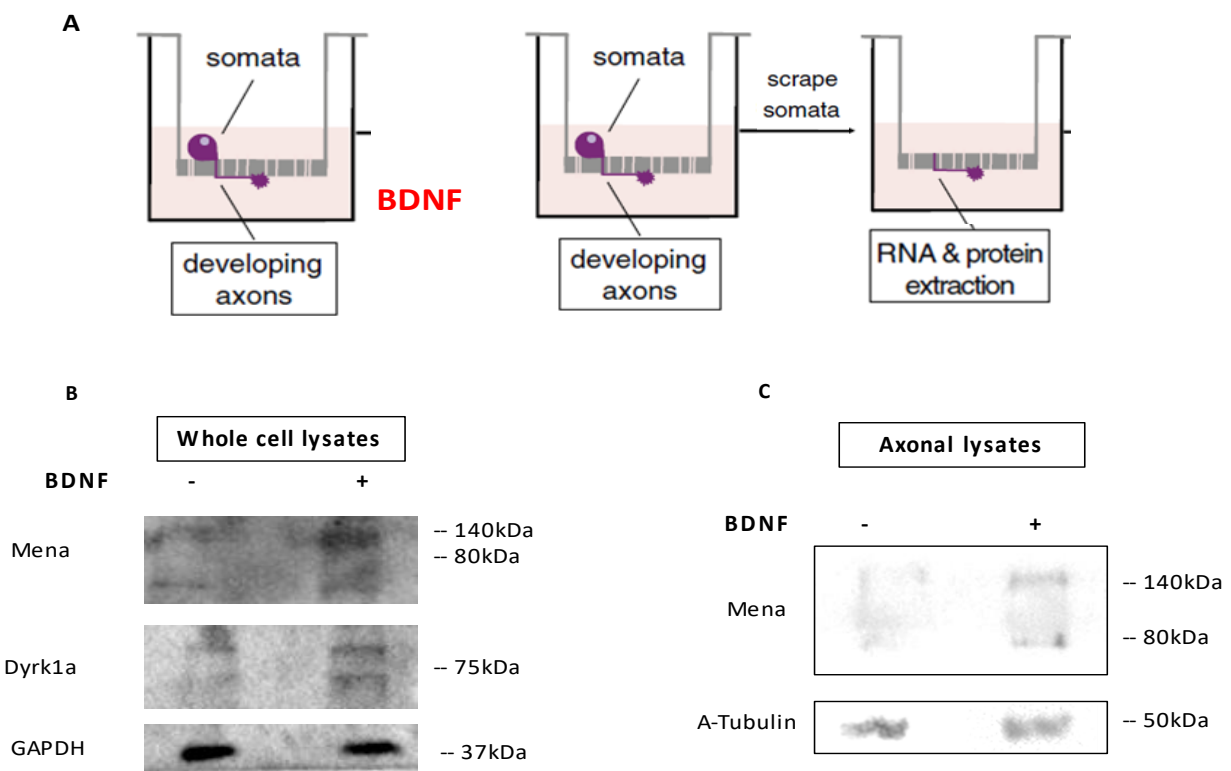

Figure 15. BDNF stimulation in E15.5 primary cortical cell cultures in transwell filters and axotomies (A) Schematic representation of BDNF stimulation assay. (B) Protein levels of Mena and Dyrk1a increased after BDNF stimulation in whole cell lysates. (C) Protein levels of Mena increased after BDNF stimulation in axons

for 15' on both compartments (Fig.15A). After BDNF stimulation, somata were scraped and RNA and protein isolation was perfomed from both top and bottom compartment. Western Blot analysis verifies that BDNF assay worked successfully since protein levels of Mena and Dyrk1a are increased in whole cell lysates and Mena protein levels are increased in axons after BDNF stimulation (Fig.15B, C).

qPCR results

qPCR results show Increased levels of specific mRNAs of interest upon BDNF stimulation in each neuronal compartment. Specifically, in cell bodies it is clear that *Mena, Rnf41, Rit1, Ctbp1* and *Ttf1* are highly increased after stimulation. In axons *Mena, Dyrk1a, Rnf41, Ctbp1* and *Ttf1* also seem to be increased after BDNF stimulation.
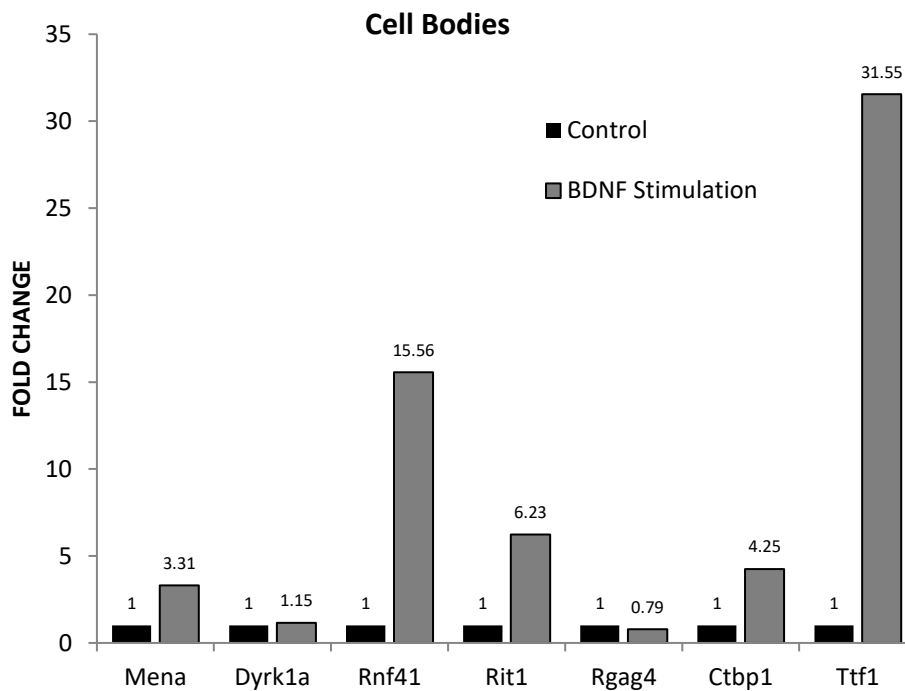

Figure 16. qPCR results of mRNAs of interest upon BDNF stimulation compared to control in cell bodies. Fold change of mRNAs of interest reveals an induced-BDNF increase to mRNA levels in cell bodies, with more significant increase to *Rnf41* and *Ttf1*.
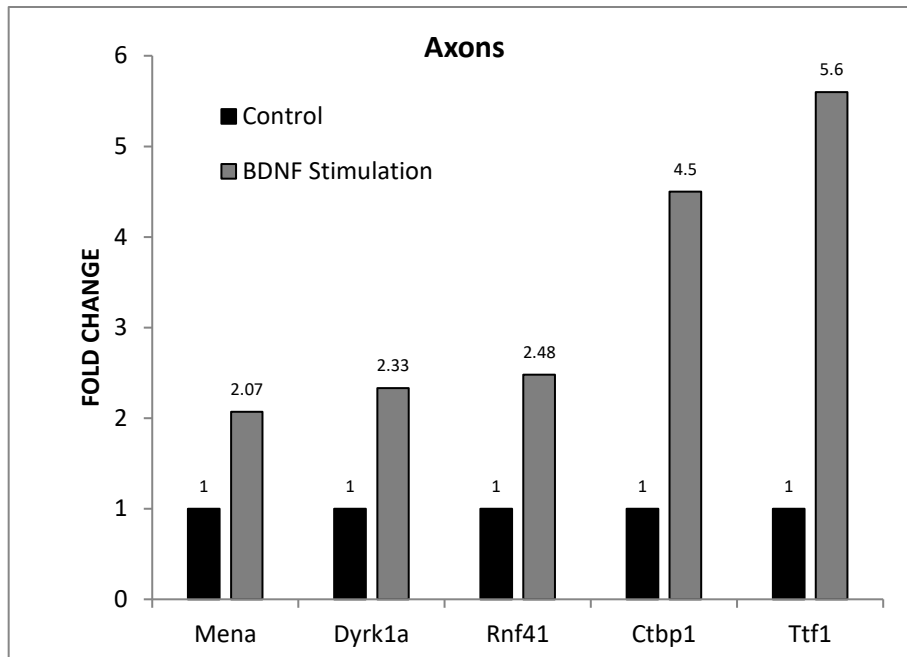
Figure 17. qPCR results of mRNAs of interest upon BDNF stimulation compared to control in axons. Fold change of mRNAs of interest reveals an induced-BDNF increase to mRNA levels in axons, with more significant increase to *Ctbp1* and *Ttf1*.

Discussion

Through immunofluorescence experiments in developing cortex *in vivo* and *in vitro,* the expression of Mena seems to be present in the axons of different subsets of neurons and also colocalized with Vimentin as well as with S6, supporting the proposal that Mena could interact with the translation machinery in developing axons for local translation regulation. Mena CLIP and qPCR experiments showed the enrichment of some mRNAs in Mena-RNP complex indicating their possible regulation during development and after axotomies. Based on our BDNF stimulation and qPCR data, we have some first evidence that some of these mRNAs that are localized in axons, have increased quantity after BDNF stimulation, indicating a possible important role in a developmental process. The genes of the mRNAs that were found on cell bodies and axons seem to have very crucial and interesting functional roles. Specifically, Ctbp1 encodes a transcriptional regulator to modulate gene expression, Rnf41 is connected to the

regulation of degradation of target proteins and Ttf1, which is the one that seems to have interesting quantities, encodes a transcriptional termination factor. Their functional role seems important in developmental processes and their existence in axons combined to their increased levels after BDNF stimulation may indicate their possible translational regulation. Also, due to their interesting functional role and importance in developmental processes it is likely that the abnormal phenotypes of Mena deficient mice could be partially linked to the lack of translational regulation of these genes.

However, these experiments are still in progress and thorough studies of these mRNAs one by one need to be completed in order to reach to a safe conclusion about their possible local translation and the possible Mena-dependent translational regulation.

Some future experiments can be performed for further studies of these mRNAs and their products. More specifically, FISH (fluorescence *in situ* hybridization) experiments could be performed to investigate the distribution of Mena-RNP complex-related mRNAs in neurons during development, and biochemical experiments to check protein expression levels of these mRNAs, before and after BDNF stimulation in cell bodies and axons. Additionally, extensive studies will be performed with Mena deficient mice that will provide the opportunity to compare with FISH and biochemical experiments the distribution of these mRNAs and their protein levels with and without Mena. Such experiments could uncover step by step both their possible Mena-dependent translational regulation and their functional role during development.

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