Theme 3 In Vitro Experimental Models

P55 A CELLULAR MODEL OF ALS WITH SQSTM1 MUTATIONS EXHIBITS AUTOPHAGY DEFECTS; A NEW PLATFORM FOR EVALUATING GENETIC INTERACTIONS AND DRUG SCREENING

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Keywords: SQSTM1/p62, TBK1, autophagy

Background: Growing histological and genetic evidence supports the notion that defects in autophagy may underlie some cases of ALS-FTLD. Consistent with this proposal we recently reported an autophagy phenotype in motor neuron-like cells (NSC-34) expressing an ALS mutant SQSTM1/p62 sequence. Specifically, L341V-mutant EGFP-mCherry-SQSTM1/p62 was less readily incorporated into acidic autophagic vesicles than the wild-type, due to defective recognition of the autophagy adapter protein LC3B (1).

Objectives: To determine if other ALS/FTLD mutations of SQSTM1/p62 impact on autophagy in the NSC-34 cell model and if other ALS/FTLD gene products or autophagy-targeting drugs can modulate the cellular autophagy phenotype.

Methods: EGFP-mCherry-SQSTM1/p62 sequences and TBK1 (an ALS gene product and SQSTM1/p62 kinase) were expressed in NSC-34 cells and the former detected by live cell imaging, in the presence or absence of autophagy-targeting drugs.

Results: Autophagy defects in the NSC-34 cell model extend to ALS mutations affecting different regions of the SQSTM1/p62 protein, including within the cargo-recognition UBA domain (T430P). The cellular autophagy phenotype associated with L341V-SQSTM1/p62 can be rescued by the expression of wild-type TBK1 and by the archetypal autophagy activator rapamycin.

Discussion and conclusions: Our findings provide further evidence of functional interactions in autophagy and ALS between TBK1 and SQSTM1/p62, and indicate the NSC-34 cell model represents a new platform to screen for autophagy-enhancing compounds that might be considered as leads for use in ALS patients with autophagy defects.

Acknowledgements: RL, MSS and AG were supported by the UK MND Association [Ref:6095].

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DOI: 10.1080/21678421.2016.1232054/001

P56 ALTERATION OF OLIGOMERIC STATES AND SUBCELLULAR LOCALIZATION OF ALS2 MUTANTS UNDERLIE THE PATHOGENESIS OF ALS2-LINKED MOTOR NEURON DISEASES

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Keywords: ALS2, pathogenic mutation, oligomeric protein complex

Background: Loss-of-function mutations in ALS2 account for a number of recessive motor neuron diseases (MNDs). Thus, the ALS2 gene product, ALS2/alsin, plays an important role in the maintenance and/or survival of motor neurons. Previously, we have demonstrated that ALS2 acts as a guanine nucleotide exchange factor (GEF) for Rab5 and modulates endosome dynamics. ALS2 interacts with ALS2 itself through the C-terminal region and forms a homophilic oligomer. This homo-oligomerization is crucial for the Rab5-GEF activity and for the ALS2-mediated endosome enlargement. Moreover, the intracellular distribution of ALS2 is drastically shifted from cytosol to the membrane compartments by the activation of Rac1, thereby inducing endosome enlargement in its Rab5-GEF activity-dependent manner. Recently, several missense and in-frame deletion mutations, which preserve the C-terminal Rab5-activating domain, in ALS2 have been identified. However, it is still unknown as to how these mutations cause loss of the ALS2-mediated normal functions in cells.

Objectives: To investigate whether the conformational changes in the ALS2 complex and differences in its intracellular distribution were associated with the pathogenesis, we examined the oligomerization and subcellular localization of missense/in-frame deletion ALS2 mutants in cultured cells.

Methods: We generated the ALS2 expression-plasmid constructs for FLAG-tagged wild-type (ALS2_WT), wild-type variants (ALS2_P132L, ALS2_E1173K), Rab5-GEF defective mutant (ALS2_P1603A, ALS2_L1617A), and pathogenic mutants (ALS2_S100I, ALS2_C157Y, ALS2_G540E, ALS2_A861_T904del, and ALS2_R1611W). We transfected these constructs into COS-7 cells. Oligomeric states of the ALS2 complexes...
were determined by immunoprecipitation and gel-filtration analysis with the use of Superose 6 10/300 GL column (GE Healthcare), followed by SDS-PAGE and western blot analysis using anti-ALS2 polyclonal antibody. FLAG-tagged ALS2 or its mutants was ectopically co-expressed with HA-tagged Rac1_Q61L in HeLa cells. Subcellular localization of ALS2 and its mutants in HeLa cells was immunocytochemically analyzed by a laser scanning confocal microscope (LSM700 Carl Zeiss) and ImageJ.

**Results:** Gel-filtration analysis revealed that majority of ALS2 WT, ALS2_P132L, ALS2_E1173K, and ALS2_P1603A were eluted at an apparent molecular masses of ~669kDa (tetramer) with a minor peak at ~975kDa, suggesting the presence of two different oligomeric ALS2 complexes. Importantly, the ratios of ~975/~669kDa observed in all pathogenic mutants were significantly higher than that of ALS2 WT, indicating that the pathogenic mutants exist as higher-molecular weight complexes in cells. Further, ALS2_R1611W and ALS2_L1617A exhibited different gel-filtration profiles, in which, in addition to two commonly observed peaks, lower-molecular-mass complex (400–600 kDa) was evident. Subcellular localization study revealed that tetramer-dominated ALS2 variants were predominantly localized to EEA1-positive early endosomes, while pathogenic mutants were widely distributed throughout cytoplasm.

**Discussion and conclusions:** These results indicate that the pathogenic mutations in ALS2 alter the oligomeric states and subcellular localization of ALS2, which might lead to loss of the ALS2-mediated cellular functions and ALS2-linked MNDS.

**Acknowledgements:** This work was supported by Grant-in-Aid for Scientific Research from JSPS.

DOI: 10.1080/21678421.2016.1232054/002

**P57 AN OPTICAL METHOD FOR DETECTING ENDOPLASMIC RETICULUM AND MITOCHONDRIAL-ASSOCIATED MEMBRANES**

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**Keywords:** VAPB, mitochondria, ER

**Background:** Interactions between the ER and mitochondrial membrane play a critical role in a variety of processes including calcium exchange, lipid metabolism, and autophagy. Close apposition is achieved by the formation of protein complexes that tether the organelles at specialized junctions termed mitochondrial-associated membranes (MAMs). The distance between the membranes at these junctions is heterogeneous and abnormal changes to ER-mitochondria contacts have been associated with neurodegeneration. Studies have shown that VAPB is enriched at these junctions and forms a complex with the outer mitochondrial membrane protein PTPIP51 to regulate ER-mitochondrial interactions (1). A missense point mutation vapBP56S is associated with ALS8 (2). It is suggested that the disease may result, in part, from mitochondrial dysfunction resulting from a failure to maintain functional MAMs.

**Objectives:** To develop a biomolecular fluorescence complementation assay (BiFC) for visualizing and detecting changes to ER-mitochondrial contact sites in living cells and relate any changes to physiological responses such as calcium signaling and mitochondrial function. This will provide a platform for investigating the effect of VAPB mutations on the formation and regulation of MAMs and identify the resulting cellular consequences.

**Methods:** Split fluorescent venus proteins fused to the ER-targeted C-terminal transmembrane domain of VAPB and the outer mitochondrial membrane anchor of TOMM20 were transfected in different cell lines that were then exposed to various stress conditions. Cells were imaged by confocal microscopy and subsequently analyzed to detect any changes in the number of ER-mitochondria contacts.

**Results:** Split venus expression constructs using C-terminus of VAPB (V1-VAPB-CT) and the outer mitochondrial membrane anchor of TOMM20 (V2-TOMM) were correctly targeted to cellular membranes. Co-expression of these fusion proteins generated punctate fluorescence signals at regions of tight juxtaposition (<3nm) between ER and mitochondrial membranes, consistent with a MAM localization. The induction of apoptosis via serum starvation caused a significant increase in the mean number of ER-mitochondria contacts per cell in NSC34 (p value 0.0006, n=36) and COS-7 (p value 0.0091, n=31 to 36) cell lines as previously reported in the literature (3).

**Discussion and conclusions:** These results suggest that this BiFC method will allow the detection and characterization of MAMs in living cells. Further work will allow us to directly correlate the features of MAM with mitochondrial activity and the regulation of Ca2+ homeostasis. Furthermore, we may establish how mutations in proteins such as VAPB influence the activity of MAMs and how this might lead to neurodegeneration.

**Acknowledgements:** This research is supported by a joint studentship between the University of Edinburgh and Zhejiang University.

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DOI: 10.1080/21678421.2016.1232054/003
P58 MUTANT CYCLIN F INHIBITS ENDOPLASMIC RETICULUM (ER)-ASSOCIATED DEGRADATION (ERAD), ER-GOLGI TRAFFICKING AND AUTOPHAGY, PERTURBING ER PROTEOSTASIS, AND INDUCING TOXICITY IN ALS

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Keywords: endoplasmic reticulum, protein degradation, cyclin F

Background: We recently identified missense mutations in the CCNF gene, encoding cyclin F, in familial and sporadic ALS/FTD (1). However, the pathogenic mechanisms associated with CCNF remain unknown. Cyclin F forms part of an E3 ubiquitin-protein ligase complex that mediates degradation of proteins via the ubiquitin proteasome system (UPS). The UPS is closely linked to autophagy and endoplasmic reticulum-associated degradation (ERAD), whereby proteins are retro-translocated from the ER to the cytosol and degraded, and this is the primary mechanism for clearing misfolded proteins from the ER. ER ubiquitin ligases therefore are important in maintaining homeostasis of the ER, but dysfunction in ERAD induces ER stress and cell death. ER stress is now implicated as a key event leading to apoptosis in ALS, and we have recently demonstrated that this is also linked to impairment of ER-Golgi trafficking in cells expressing mutant FUS, TDP-43 or SOD1 (2). Stress granule formation is also linked to pathology in ALS.

Methods/Results: Cyclin F is normally a nuclear protein, and the cellular localization of wild-type (WT) and ALS mutant S621G cyclin F were analyzed in human SH-SY5Y cells. Quantification revealed that few cells expressed WT cyclin F in the cytoplasm (<10%), whereas significantly more cells expressed mutant cyclin F (p<0.001) in the cytoplasm. Similarly, a significantly higher percentage of cells expressing mutant cyclin F associated with stress granules compared to WT (p<0.05), suggesting that in ALS, mutant cyclin F becomes redistributed to the cytoplasm, where stress granules are formed. Next, using a specific ERAD substrate, mutant A1AT-NHK-Venus, S621G cyclin F significantly impaired ERAD degradation of NHK (p<0.001) compared to wild-type cyclin F. Autophagy was also examined by quantifying the levels of LC3-II and p62 by immunoblotting and immunocytochemistry: significantly more LC3-II and p62 accumulated in cells expressing mutant cyclin F, revealing impairment of autophagy (p<0.05). Next, using specific marker VSVG, impairment of ER-Golgi transport was detected in cells expressing mutant cyclin F compared to wild-type (p<0.05). Consistent with dysfunction in the ER, ER stress sensors IRE1 and spliced Xbp1, and ER stress-pro-apoptotic marker CHOP, were significantly upregulated in cells expressing mutant cyclin F compared to WT (p<0.05, p<0.001). Finally, nuclear morphology and Sytox Blue staining revealed that significantly more mutant cyclin F cells were undergoing apoptosis compared to WT cells (p<0.001), demonstrating that ALS mutant cyclin F induces toxicity in neuronal cells.

Discussion: This study describes novel pathogenic mechanisms triggered by mutant cyclin F, and suggests that ER homeostasis and protein degradation are key processes that cause dysfunction in neuronal cells expressing ALS-mutant cyclin F.

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DOI: 10.1080/21678421.2016.1232054/004

P59 PRIMARY MOTOR NEURONS FROM A NOVEL TDP-43-ASSOCIATED ALS MOUSE AS A PLATFORM FOR HIGH-THROUGHPUT SCREENING.

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Keywords: TDP-43, mouse model, primary motor neurons

Background: TDP-43 is the pathological hallmark protein of ALS, becoming depleted from the nucleus and mislocalized to the cytoplasm, where it forms the major protein component of insoluble, ubiquitinated pathological inclusions (1). We have previously developed a novel bacterial artificial chromosome (BAC) transgenic mouse expressing human TDP-43 at physiologically relevant levels. TDP-43M337V mutant mice develop distinct motor deficits (rotarod and grip strength), survival deficits in the second year and pathological phenotypes (TDP-43 cytoplasmic mislocalization, NMJ deficits) recapitulating the key features of human ALS.

Objective: Characterization of the in vitro phenotype of MNs from TDP-43M337V transgenic mice to optimize TDP-43 cytoplasmic mislocalization as an assay for high content automated image-based screening.

Methods: Primary motor neurons (MNs) were generated from E13.5 lumbar spinal cord from nontransgenic (NTg), TDP-43WT, and TDP-43M337V heterozygous embryos. TDP-43 was detected using a panTDP-43 antibody with equal affinity for mouse and human forms of the protein. Human-specific TDP-43 and GFP (Ypet)
antibodies were used to detect the human-specific form. Oxidative stress was induced by treating MNs with 0.05M sodium arsenite for 0, 30, and 60 min prior to fixation.

**Results:** TDP-43M337V-derived primary MNs recapitulate the characteristic cytoplasmic mislocalization of TDP-43 with concomitant nuclear depletion, under basal culture conditions. A significantly increased proportion of TDP-43M337V MNs display cytoplasmic mislocalization of TDP-43 compared to NTg and TDP-43 controls, and overall there is a shift in cytoplasmic localization of the protein. Data suggests the TDP-43WT vs TDP-43M337V cytoplasmic distribution of TDP-43 staining is robust (Z Factor1 for the cytoplasmic localization = 0.65 (n=3)). In response to increasing exposure to oxidative stress, TDP-43M337V MNs show a reduction in the number of cells containing stress granules, reduced recruitment of mutant TDP-43 to stress granules, deficits in stress granule dissolution following the removal of stress and significantly reduced survival.

**Discussion and conclusions:** With the Oxford Target Discovery Institute we will develop an automated computerized imaging system to establish high-throughput screening (HTS) of FDA approved compounds and a “druggable” siRNA library. We aim to identify drugs and drug targets that promote nuclear retention of TDP-43, which can then be validated through detailed analysis of phenotypic and transcriptional changes in primary MNs from our TDP-43 BAC transgenic mice, and iPSC-derived motor and cortical neurons from ALS patients carrying TDP-43 mutations.

**Acknowledgements:** This work was funded by the Motor Neurone Disease Association

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**DOI:** 10.1080/21678421.2016.1232054/005

**P60 ANALYSIS OF SUMOYLATION AS A POST-TRANSLATIONAL MODIFICATION OF TDP-43 PROTEIN**

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**Keywords:** TDP-43, SUMOylation, post-translational modification

**Background:** TDP-43 protein represents the major component of the pathological cytoplasmic inclusions observed in ALS affected brains. The molecular events that lead to TDP-43 aggregate formation are not clear yet. Post-translational modifications (PTM), including ubiquitinylation, phosphorylation, and acetylation, are associated with TDP-43 in ALS brains and seem to influence TDP-43 protein aggregation. SUMOylation is a PTM mediated by small ubiquitin modifier proteins (SUMO) whose family consists of five members of about 100 amino acids. SUMO conjugation to lysine residues of target proteins controls a variety of biological activities, including response to stress, nuclear body formation, transcription, nucleocytoplasmic transport, protein stability, and aggregation. Previous studies suggest that a short splicing isoform of TDP-43 is conjugated to SUMO-2/3 proteins in the insoluble fraction and that in response to heat shock TDP-43 represents a target of SUMOylation.

**Objectives:** To study and characterize the TDP-43 SUMOylation as a PTM potentially able to regulate its function and aggregation in the cytoplasm.

**Methods:** Immunoprecipitation experiments were carried out on HEK and human neuroblastoma SK-N-BE cell lysates. Modulation of SUMOylation was obtained by overexpressing SUMO-1 and the E2-conjugation enzyme UBC9. Distinct TDP-43 deletion constructs were used to map the SUMOylation region.

**Results:** We tested the hypothesis that TDP-43 is a substrate of SUMOylation by immunoprecipitation (IP) assays in human HEK and neuroblastoma cells. Our data show that a fraction of endogenous TDP-43 protein is conjugated to SUMO-1 covalently, but that TDP-43 interacts also noncovalently with SUMO-1 protein. The use of antibodies against different amino acidic regions of TDP-43 clearly suggests that the SUMOylation sites reside in the N-terminal part of TDP-43 protein. We also found that TDP-43 SUMOylation can be increased upon overexpression of SUMO-1 and of the E2-conjugation enzyme UBC9. To define TDP-43 SUMOylation sites, we performed a computational analysis using different prediction algorithms. The analyses indicate that Lysine136 has the highest prediction score, and that the 106–110 amino acidic region likely represents a SUMO-interacting motif (SIM) through which the noncovalent interaction with SUMO may occur. By overexpressing SUMO-1 and TDP-43 wild-type and deletion constructs in the RRM1 domain and in the C-terminal region (DC), we confirmed that the SUMOylation site is comprised within the RRM1 domain. We also observed that the absence of the C-term region increases the SUMOylation pattern of the mutant protein, suggesting a potential role of this region in the regulation of TDP-43 modification by SUMO-1.

**Discussion and conclusions:** Our results indicate that SUMOylation occurs as a physiological modification of TDP-43 protein in its RRM1 domain. This PTM deserves further investigation as a potential mechanism regulating TDP-43 function and/or aggregation in ALS.

**DOI:** 10.1080/21678421.2016.1232054/006
P61 IMPAIRED STRESS GRANULE DYNAMICS IN PRIMARY MOTOR NEURONS FROM A SYMPTOMATIC TDP-43M337V TRANSGENIC MOUSE

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Keywords: TDP-43, stress granules, mouse model

Background: ALS is characterized by selective death of motor neurons, cells which are intrinsically more vulnerable to stress. TDP-43 is the pathological hallmark protein of ALS, where it is aggregated and mislocalized in affected neurons. TDP-43 is an RNA binding protein (RBP) which is normally nuclear, but can shuttles into the cytoplasm to participate in stress granule (SG) formation. SGs are aggregates of mRNA and RBPs which form in response to stress, facilitating cell survival through repression of translation and inhibition of stress-induced apoptosis. SG markers have been identified in TDP-43-containing aggregates post-mortem (1), and TDP-43 knockdown leads to impaired SG assembly in cell lines (2). Together these suggest that disrupted SG dynamics might be a central pathogenic mechanism in ALS. Using bacterial artificial chromosome (BAC) technology we developed a novel mutant TDP-43M337V-expressing mouse, which has delayed-onset progressive motor deficits characteristic of ALS. We used this to examine the relationship between TDP-43 and SGs.

Objectives: Our objectives were to use primary motor and cortical neurons and neuropathological tissue to determine the impact of a disease-associated mutation in TDP-43 on SG dynamics and cell survival.

Methods: Primary neurons were cultured from non-transgenic and BAC-derived lines expressing normal human TDP-43 (hTDP-43WT), or mutant TDP-43M337V. Cells were stressed with 0.5mM sodium arsenite, then immunostained for total TDP-43, hTDP-43 and SG markers TIA-1, PABP1 and G3BP1. Images were acquired using a confocal microscope and analyzed for colocalization. Survival assays were performed. Neuropathological tissue from 12-month-old mouse spinal cord was also analyzed for evidence of TDP-43 and SG markers TIA-1, PABP1 and G3BP1. Images were acquired using a confocal microscope and analyzed for colocalization. Survival assays were performed. Neuropathological tissue from 12-month-old mouse spinal cord was also analyzed for evidence of TDP-43 and SG markers TIA-1, PABP1 and G3BP1. Images were acquired using a confocal microscope and analyzed for colocalization.

Results: TDP-43 was predominantly cytoplasmic in TDP-43M337V motor neurons, compared to hTDP-43WT and nontransgenic controls (p<0.05). Fewer cells formed TIA-1 and PABP1-positive SGs (both p<0.05) with reduced colocalization between TDP-43M337V and TIA-1 (p<0.01). Furthermore, in vitro survival was significantly reduced in mutant motor neurons following stress (p<0.05). Preliminary data suggests that PABP1-positive SG formation is delayed in TDP-43M337V motor neurons, with slower SG disassembly after stress. In mutant primary cortical neurons, TDP-43 localization, survival, and stress responses did not differ significantly from controls.

Conclusions: In this model, we demonstrate characteristic TDP-43 mislocalization and reduced survival of motor neurons. Our data suggest that impaired stress responses may underlie the link between TDP-43 mislocalization and selective motor neuron vulnerability.

Acknowledgements: This work was funded by the Motor Neurone Disease Association (DG) and a National Institute for Health Research Clinical Fellowship (LF).

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DOI: 10.1080/21678421.2016.1232054/007

P62 OXIDATIVE STRESS IN PATIENT-DERIVED ALS ASTROCYTES AND THERAPEUTIC APPROACHES

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Keywords: astrocytes, RNA, therapeutic approaches

Background: Oxidative stress is a major cellular insult and is implicated in multiple neurodegenerative diseases, including Motor Neurone Disease (MND) (1). As a consequence of persistent nucleic acid oxidation, downstream senescence/apoptosis pathways can be activated. We have developed a coculture system with astrocytes derived from MND patients and mouse wild-type GFP+ motor neurons (MNs). In this in vitro system ALS astrocytes induce MN death (2). Our hypothesis is that oxidative stress is a major component of MND astrocyte pathology and therapeutic approaches aiming at decreasing this pathogenic component will lead to MN rescue in coculture.

Objectives: 1. To identify the level of oxidative stress in (A) MND patient brain and spinal cord as well as cerebrospinal fluid (CSF); (B) MND astrocytes derived from patients carrying different mutations and gender and age-matched controls. 2. To perform a drug screening to identify antioxidant drugs that can decrease the level of oxidative stress and lead to MN rescue.

Methodology: Nucleic acid damage was detected by the 8-OHdG marker which is considered to be the predominant marker of oxidative stress. Immunostaining and ELISAs were used to detect the level of RNA/DNA oxidation in postmortem tissues from patients and controls. Cocultures of human astrocytes derived from MND patients and controls with GFP+ MNs were used to assess the effect of three activators of Nrf2, a master
regulator of oxidative stress response, on MND astrocyte toxicity.

**Results and discussion:** ELISA results showed a significant increase in oxidized RNA in CSF from patients compared to controls. Consistently, immunohistochemistry of 8-OHdG also showed a high level of reactivity in neurons and glia in the cortex and spinal cord of patients, reflecting the involvement of this pathway in MND pathology. Levels of cellular oxidation in astrocytes derived from patients were also significantly higher than in controls using the 8-OHdG as a marker of direct oxidative damage. Consistently, treatment of ALS astrocytes with drugs known to activate the antioxidant response pathway led to a decrease in 8-OHdG reactivity accompanied by significant MN rescue in coculture. This also corresponded to a decrease in the levels of oxidized RNA released by patient-derived astrocytes. Interestingly, ALS patients carrying different mutations respond to different antioxidant drugs, thus creating the bases for personalized medicine approaches.

**Acknowledgments:** This study was funded by the National Neuroscience Institute, King Fahad Medical City, Riyadh, Kingdom of Saudi Arabia and the MND Association.

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DOI: 10.1080/21678421.2016.1232054/008

**P63 THE ROLE OF LOW COMPLEXITY DOMAIN MUTATIONS IN RNA-BINDING PROTEINS ASSOCIATED WITH MOTOR NEURON DISEASE PATHOGENESIS**

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**Keywords:** TDP-43, RNA processing and dysregulation

**Background:** Most patients with motor neuron disease (MND) display TDP-43 protein aggregates, which gives TDP-43 a central role in the development of this disease. Aggregation of TDP-43 is promoted by its low-complexity (LC) domain, which is also the region containing most of the disease-causing mutations. While most studies suggest that the LC domain mainly mediates protein–protein interactions, its function is poorly understood. We therefore examined the role of the TDP-43 LC domain in protein–protein and protein–RNA interactions, and the functions of these interactions in regulating pre-mRNA processing.

**Methods:** For this purpose, we created cell lines where the endogenous RBP is replaced by mutants lacking specific portions of the LC domain and assessed them with the use of individual-nucleotide resolution UV cross linking and immunoprecipitation (iCLIP), RNA-Seq, PolyA-Seq, and mass spectrometry.

**Results and conclusion:** We found that a partial deletion of the LC domain in TDP-43 leads to a loss of binding to several RBPs, many of which contain long LC domains. This indicates that LC domain serves as docking-platforms for protein–protein interactions, thereby potentially forming RNP granules. Finally, we show that deletions in the LC domain affect the function of TDP-43 in splicing and 3’ end processing. In conclusion, our research uncovers a crucial role of the LC domain in protein–protein interactions, which affects its regulatory functions.

DOI: 10.1080/21678421.2016.1232054/009

**P64 DECODING THE NONCODING SIDE OF FUS-ALS**

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**Keywords:** mESC-MN, FUS, IncRNAs

**Background:** Long noncoding RNAs (IncRNAs) are emerging as essential players in cellular physiopathology. The identification of mutations in FUS/TLS, TDP-43, and hexanucleotide repeat expansion in C9ORF72 points to an essential role of abnormal RNA metabolism in the puzzle of ALS neurodegeneration. In this study, we intend to highlight the biological relevance of noncoding transcriptome alterations induced by mFUSP517L mutation, the murine equivalent of the juvenile ALS-associated hFUSP525L mutation.

**Methods:** To this aim, we took advantage of HB9::GFP mouse embryonic stem cells (mESCs) derived either from wild type or FUS knock-out (mFUS/C0) or knock-in mice harboring different FUS genotypes (mFUSP517L/P517L, mFUSP517L+/C0), and hexanucleotide repeat expansion in C9ORF72 points to an essential role of abnormal RNA metabolism in the puzzle of ALS neurodegeneration. In this study, we intend to highlight the biological relevance of noncoding transcriptome alterations induced by mFUSP517L mutation, the murine equivalent of the juvenile ALS-associated hFUSP525L mutation.

**Methods:** To this aim, we took advantage of HB9::GFP mouse embryonic stem cells (mESCs) derived either from wild type or FUS knock-out (mFUS/C0) or knock-in mice harboring different FUS genotypes (mFUSP517L/P517L, mFUSP517L+/C0). GFP-expressing motoneurons (MNs), in vitro differentiated from mESCs, were purified by FAC-sorting and a triplicate of RiboMinus RNA for each genotype was sequenced (Illumina, ca.40 million reads) for transcriptome analysis of long and short RNAs. Real-Time PCR was used for RNA-Seq data validation and further gene expression analyses.

**Results:** With respect to wild-type background, bioinformatics analyses revealed alterations of 87 and 189 genes in mFUS/C0 and in mFUSP517L/P517L, respectively (q value < 0.05); more than 90% of genes show a fold change between +2 and −2 (log2), which is not surprising considering the late onset of the disease. Upon FUS...
knockout, expression changes were symmetrically distributed between up and downregulation, whereas 87% of genes were downregulated in mFUSP517L/P517LMNs. Finally, 17 genes were deregulated in both conditions, which suggest that gene expression was mainly affected by a mechanism of FUS gain-of-function rather than by nuclear FUS loss-of-function. LncRNA expression was also affected by FUS mutation: in spite of a poor annotation of this class of transcripts in mouse, we could identify 19 deregulated lncRNAs (q value < 0.05) uncharacterized in the ALS context. These candidate RNAs were (i) bioinformatically confirmed to be noncoding, (ii) showed an increased expression along mESC-MN differentiation, and (iii) were mainly localized in the cytoplasm.

Discussion: This approach will allow us to get a global view of the FUS-dependent transcriptome changes in the cell type specifically affected by ALS. Our data will provide us the opportunity to get insights into the noncoding side of the pathology, which is still poorly characterized. Based on genomic organization, MN-specific expression and validation data, we will point our attention on interesting lncRNA candidates that will be phenotypically and functionally screened starting with reverse genetics and inspections of downstream gene expression alterations.

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DOI: 10.1080/21678421.2016.1232054/0010

P65 HUD REGULATION OF SOD1 AND FUS MRNAS IN SPORADIC ALS.

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Keywords: RNA, FUS, SOD1

Background: Altered RNA metabolism has been defined as a central pathogenic mechanism in Amyotrophic Lateral Sclerosis (ALS). Neuronal ELAV RNA-binding proteins (RBPs), in particular HuD, have been previously associated with neurodegenerative diseases (NDs), and in vitro and in vivo studies demonstrated the involvement of HuR in the regulation of SOD1 (1) and FUS (2) mRNAs.

Objectives: Bioinformatics analysis of SOD1 and FUS 3’UTRs demonstrated the presence of HuD consensus binding sequences in these mRNAs. We aimed to test whether HuD levels are altered in ALS and how this affects levels and localization target mRNAs and proteins.

Methods: Using human neuroblastoma SH-SY5Y cells as an in vitro model of ALS pathophysiology and post-mortem tissues from sporadic ALS patients we evaluate HuD and its targets level by qRT-PCR, WB and immunofluorescence (IF) analyses. Through RNA immunoprecipitation (RIP) assays, we tested HuD binding on SOD1 and FUS 3’UTR.

Results: RBP accumulation in stress granules (SGs) and processing bodies (P-bodies) is a hallmark of ALS pathology. In our cellular model we found that HuD colocalization with SG and P-body markers is increased after H2O2 treatment. By immunoprecipitation, we confirmed that HuD interacts with Argonaute and GW182 proteins. Moreover, the induction of a neuronal-like phenotype by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) differentiation triggers a significant increase in HuD mRNA levels and an oxidative stress-dependent overexpression of SOD1 and FUS mRNAs. Correspondingly, the overexpression of HuD in the in vitro cellular model leads to increased SOD1 and FUS mRNA levels. The increase in target mRNA levels is likely due to the binding of HuD to their 3’UTRs and consequent stabilization, as demonstrated by the significant reduction of SOD1 and FUS mRNAs after the overexpression of a dominant negative HuD mutant protein lacking the RNA Recognition Motif 3 (RRM3) required for target stabilization. By in vitro RNA immunoprecipitation (RIP) assays, we demonstrated that HuD binds and stabilizes SOD1 and FUS mRNAs leading to increased mRNA levels. By Immunohistochemistry (IHC) experiments in post-mortem tissues from sporadic ALS patients, we found that HuD protein levels were increased in the motor cortex compared to the healthy controls. We also found increased HuD mRNA levels in the posterior frontal cortex from ALS patients, along with increases in mRNAs due to HuD binding on its 3’UTR.

Discussion and conclusions: Uncovering HuD post-transcriptional regulation of SOD1 and FUS mRNAs and defining the potential involvement of additional intracellular pathways and trans-acting molecules will open novel perspectives for ALS research and the identification of new therapeutic targets.

Acknowledgements: This work is supported by 1R01NS089633-01A1 and 5R01DA034097-05 to NPB.

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DOI: 10.1080/21678421.2016.1232054/0011
P66 IMPACT OF INTERFERON-GAMMA ON NEUROTOXICITY AND ER-MITOCHONDRIA COUPLING CYCLE IN ALS

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Keywords: proinflammatory cytokine, neurotoxicity, ERMCC

Background: Perturbation of the Endoplasmic Reticulum-Mitochondria-Coupling Cycle (ERMCC) appears to be a hallmark of ALS. On the other hand, interferon-gamma, a proinflammatory cytokine, directly triggers calcium transients. In neurons, it induces neurotoxicity in vitro by forming a calcium-permeable neuron-specific receptor complex of IFNGR and GluR1. IFN-gamma control of ERMCC could provide an important direct molecular link between neuroinflammation and neurodegeneration and pave way to therapeutic targets.

Objectives: We aim to investigate (i) the distribution and (ii) expression level of IFNGR and AMPAR, (iii) the effects of IFN-gamma with and without kainite-induced excitotoxicity on (iv) neuronal survival and on ERMCC.

Methods: Motor neuron co-cultures from E13 mouse ventral spinal cords with (TG) or without (NT) overexpression of mutant hSOD1G93A were used for immunofluorescent staining. Neuronal cell survival was assessed after IFN-gamma treatment with or without kainate for 24 h.

Results: Basal immunofluorescence studies show similar levels of IFNGR and AMPAR distributed across the membranes in both NT and TG motor neurons. Toxicity tests for neuronal survival show that TG neurons appear to be more tolerant to the combined insult from IFN- and kainate unlike the WT neurons. Also, they show a better rescue response when IFNGR is blocked. Further tests will be run to confirm the preliminary findings.

Conclusion: Interferon-gamma, an important regulator of neuroinflammation, enhances calcium influx in the neurons. But, surprisingly, hSOD1G93A neurons appear to show lesser susceptibility to neurotoxicity induced by the cytokine. Further investigation is required to understand this observation better.

Acknowledgment: This research is supported by a BMBF (the Bundesministerium für Bildung und Forschung) grant PYRAMID in the framework of the ERANET E-RARE program (http://www.e-rare.eu) and was undertaken in cooperation with the BMBF funded MND-NET.

DOI: 10.1080/21678421.2016.1232054/0012

P66A TRYPToPHAN-32 OF SOD1 IS AN AGGREGATION MODULATING RESIDUE

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Keywords: SOD1, aggregation, misfolding

Background: The strikingly high number of mutations in SOD1 that are associated with familial ALS (183 http://alsod.iop.kcl.ac.uk/), indicates that the common toxic property of these mutations may be protein misfolding and aggregation. Furthermore, the curious observation that endogenous murine SOD1 does not co-aggregate with overexpressed human SOD1 in ALS mouse models (1) suggests that the sequence difference between these isoforms (83% sequence identity) could be key to understanding SOD1 associated misfolding in ALS. One critical residue identified to be nonconserved between mice and humans, but conserved across ALS patients, is position 32, which is tryptophan in humans, and serine in mice (2).

Objectives: We sought to elucidate the role that Trp-32 plays in the aggregation of SOD1 in both an in vitro and cellular context.

Methods: We purified recombinant SOD1 WT, G93A, and V148G protein, as well as W32S variants of each protein (i.e. SOD1-G93A-W32S), from E. coli. The purified proteins were aggregated at 37°C in the presence of DTT and EDTA and thioflavin-T with and without preformed aggregate seed. The structural consequences of DTT and EDTA treatment was assessed using analytical size exclusion chromatography and native mass spectrometry. In addition, aggregation was examined in a cellular context by transfecting NSC-34 cells with EGFP tagged SOD1 fusion proteins and counting the number of cells containing inclusions 48 h post-transfection. We tested the seeding of our W32S mutants in a cellular context by co-transfecting NSC-34 cells with EGFP and tdTomato-tagged fusion proteins. We compared the fluorescence intensity of the EGFP channel to that of the tdTomato to determine the level of EGFP fusion that had coaggregated with the tdTomato fusion.

Results: All W32S variants had significantly decreased aggregation propensities when compared to their disease mutant counterparts. The addition of seed to assays significantly increased the aggregation of our disease mutants, but not the W32S mutants (n=3 assays). Our native mass spectrometry analysis revealed that all W32S variants were more destabilized by DTT and EDTA treatment, when compared to disease only mutants. In all cases, transfection of NSC-34 cells with W32S variants yielded a significant decrease in the number of cells containing inclusions, as well as W32S EGFP and tdTomato cotransfections showing a significantly lower EGFP signal in cellular inclusions (n=3 transfections).

Discussion and conclusions: It is clear from these results that aggregation of SOD1 is highly dependent on Trp-32, and that even seeded aggregation is impeded by the W32S substitution. This suggests that the cell to cell propagation of misfolded/aggregated SOD1, may be...
mediated by Trp-32, and that targeting this residue could yield effective therapeutics.

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DOI: 10.1080/21678421.2016.1232054/0013

P67 INVESTIGATING NON-CELL AUTONOMOUS EFFECTS ON METABOLISM IN ALS THROUGH METABOLOMIC ANALYSIS OF ASTROCYTE-NEURON CO-CULTURES

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Keywords: non-cell autonomous effects, astrocytes, metabolism

Background: Non-cell autonomous processes involving astrocytes have been shown to contribute to motor neuron degeneration in amyotrophic lateral sclerosis. Expression of mutant SOD1G93A in astrocytes is selectively toxic to motor neurons in coculture, even when the mutant protein is expressed only in the astrocytes and not in the neurons.

Objectives: The exact causes and mechanisms leading to the development of noncell autonomous neurotoxic effects in ALS continue to be unclear. We aimed to determine whether there were distinctive metabolic signatures characteristic of this process, and to identify which metabolic processes were perturbed by the presence of mutant SOD1 in cocultures and potentially affect neuronal survival.

Methods: To examine metabolic changes that occur in astrocyte-spinal neuron cocultures, we carried out a metabolomic analysis by 1H NMR Spectroscopy of media from astrocyte-spinal neuron cocultures after 3 and 6 days in co-culture, as well as astrocytes in single culture.

Results: We observed metabolic profiles in differentiating co-cultures with SOD1G93A astrocytes after 3 days in culture, and differentiating co-cultures with SOD1G93A neurons after 6 days in culture. In addition, we observed that glucose uptake was increased with SOD1G93A, but, while cocultures with SOD1G93A neurons had lower lactate release, those with SOD1G93A astrocytes exhibited the reverse. Reduced uptake of branched-chain amino acids and increased release of the branched-chain keto acid 3-methyl-2-oxovalerate in cocultures with SOD1G93A neurons was also observed alongside reduced release of glutamine and glutamate with both SOD1G93A astrocytes and neurons. As these processes are coupled, a block in glutamate processing impacting neuronal survival may be present. We also observed shifts in methionine and cysteine metabolism, which may be due to altered responses to oxidative stress.

Discussion and conclusions: Overall, the metabolic response to SOD1G93A was different between the astrocytes and spinal neurons. Our results highlight a number of changes in functional metabolic cooperation between these cell types. This requires further examination of the specific metabolic changes that occur, and their impact on motor neuron survival.

Acknowledgments: This work was supported by the European Community’s Health Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 259867 (Euro-MOTOR).

DOI: 10.1080/21678421.2016.1232054/0014

P68 METABOLIC INFLUENCE OF GLUTAMATE EXPOSURE AND SOD1 MUTATION IN ASTROCYTES USING A METABOLIC APPROACH

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Keywords: astrocytes, SOD1, metabolomics

Background: Selective motor neuron (MN) death during ALS may be a noncell autonomous process in which astrocytes induce and/or contribute to MN injury (1–3). Even if astrocytes play a crucial role in ALS (4), the mechanisms of toxicity are partially unknown. We suggest that metabolism alteration largely described in ALS, may play a key role in pathogenesis of MN death, in particular through metabolism disturbance in astrocytes.

Objectives: We aimed to elucidate, for the first time, the mechanisms of changes in astrocyte metabolism in the presence of MN in ALS conditions using a metabolomics approach. First, we assessed the impact of MN on astrocyte metabolism, in particular superoxide dismutase 1 (SOD1) G93A mutant astrocytes. Then, we evaluated the metabolic alterations linked to the human SOD1G93A mutant expression and glutamate exposition in astrocytes cocultured with MN.

Method: In vitro model systems of SOD1-related ALS were performed using mono and coculture of SOD1G93A newborn mice cortical astrocytes with...
embryo spinal cord MN. Metabolomics studies were carried out using mass spectrometry coupled with liquid chromatography (LC-MS) and nuclear magnetic resonance (NMR) analytical techniques. We performed supervised and unsupervised multivariate analysis to highlight the metabolic pathways involved in the discrimination between groups. We assessed these findings in ALS mice and human published data.

Results: First we provided evidence of metabolic dysregulation in SOD1G93A astrocytes after interaction with MN (p < 0.05). Dysfunction of the purine metabolism, phenylalanine, tryptophan, and tyrosine metabolism, cysteine and methionine metabolism and glutathione metabolism pathways were particularly observed. Second, we showed that SOD1G93A mutant expression and glutamate exposure significantly modified metabolic fingerprinting of astrocytes (scores on PC1: 40.2% and PC2: 22.2%) such as arginine and proline metabolism, lysine degradation, Acetyl CoA transfer, and pyrimidine metabolism pathways. We also noted a prominent impact of SOD1 mutation, glutamate, and MN on the cellular shuttling of lactate between astrocytes and MN. The involvement of most of these pathways has been confirmed in mouse models of ALS and in previous human ALS data.

Discussion: This work is the first metabolomics study performed on primary astrocytes and MN cells to better understand the metabolic role of astrocytes in ALS conditions. We highlighted some metabolic pathways linked with pathophysiology of ALS including excitotoxicity, mitochondrial dysfunction, and oxidative stress and importantly we characterized the role of astrocytes in these dysfunctions. This work opens up the prospect of therapeutic targets though focuses on these metabolic pathways.

Acknowledgment: INSERM, ARSLA and “Région centre”

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DOI: 10.1080/21678421.2016.1232054/0015

P69 IN VIVO AND IN VITRO CHARACTERIZATION OF SOD1 IN EARLY STAGES OF ALS AS A PRECURSOR TO INSOLUBLE AGGREGATES

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Poster Communications In Vitro Experimental Models 127

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Keywords: Cu/Zn-superoxide dismutase, protein misfolding, metal ions

Background: The accumulation of insoluble Cu/Zn superoxide dismutase (SOD1) aggregates within motor neurons is a pathological hallmark of SOD1-linked ALS. While it still remains unknown how natively folded SOD1 undergoes conformational changes to form insoluble aggregates in vivo, our previous in vitro evidence suggests that metal-free SOD1 with the disulfide bond (apo-SOD1S–S) is a precursor to insoluble aggregates. Thus, the development of an antibody specifically recognizing apo-SOD1S–S will provide a useful tool for understanding the pathogenic misfolding process of mutant SOD1 in vivo.

Objectives: The aim of the present study was to develop an antibody that specifically recognizes apo-SOD1S–S and to examine the involvement of apo-SOD1S–S in the pathological process of SOD1-linked ALS.

Methods: A rabbit was immunized with a peptide corresponding 24–83 residues of human SOD1, which includes metal-binding residues and the disulfide-bonding cysteine residue. The antisera were first absorbed with the peptide and then affinity-purified using immobilized Protein G resins. The reactivity of the purified antibody (anti-apoSOD1) against various forms of SOD1 species in vitro was evaluated using indirect ELISA. In vivo test for the reactivity of anti-apoSOD1 antibody against pathological SOD1 proteins was done by sandwich ELISA using soluble fractions of the tissue lysates (spinal cord, brainstem, and cerebellum) prepared from SOD1G93A mice at different disease stages. Temporal changes of insoluble SOD1 aggregates in SOD1G93A mice were also quantified by Western blotting. Furthermore, the soluble fractions of the lysates were prepared from spinal cord ventral and dorsal horn areas of ALS patients with SOD1 mutation and analyzed by sandwich ELISA using anti-apoSOD1 antibody.

Results: The ELISA using various forms of purified SOD1 in vitro assured the recognition specificity of our anti-apoSOD1 antibody toward apo-SOD1S–S with ALS mutations. The epitope of the antibody appears to cover the region in the zinc-binding loop containing metal-binding histidine residues and disulfide-bonding cysteine residues, which corroborates the specificity of this antibody toward the apo form of SOD1 with the disulfide bond. By using the anti-apoSOD1 antibody, we have found that apo-SOD1S–S exists in the spinal cords of pre-symptomatic SOD1G93A mice but disappeared with the disease progression. In sharp contrast, insoluble SOD1 aggregates were not observed in pre-symptomatic SOD1G93A mice but were detectable after the disease symptoms appeared. Also, we observed the species reactive with anti-apoSOD1 antibody in the ventral but not dorsal horn of the spinal cords of the ALS patients with SOD1 mutation.

Conclusion: Based on these results, apo-SOD1S–S probably accumulates specifically in the region affected by the disease. We thus propose that apo-SOD1S–S exists in the early pathological process and acts as a precursor for the formation of insoluble SOD1 aggregates.

DOI: 10.1080/21678421.2016.1232054/0016
P70 A GFP-FUSION LIBRARY SCREEN REVEALS RESPONSES IN THE PROTEOSTASIS NETWORK TO MUTANT SOD1

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Keywords: ubiquitin-proteasome system, gene expression, protein homeostasis

Background: A striking feature of ALS is its molecular heterogeneity, both in terms of the genes harboring mutations in different kindred with familial ALS and also the distinct types of inclusion bodies containing different aggregated proteins. Within this heterogeneity, however, evidence points to dysfunctional protein homeostasis (proteostasis). Hundreds of proteins are involved in proteostasis; a crucial step in understanding ALS pathogenesis is focusing on the responses of key proteostasis genes to ALS gene mutants.

Objectives: To screen for genes that are differentially expressed in response to heterologous expression of human A4V mutant SOD1 in Saccharomyces cerevisiae.

Methods: Query strains expressing either DsRed-tagged wild-type (WT) or A4V SOD1, or DsRed alone as control were crossed with 120 selected strains from the GFP-fusion yeast library (1). Criteria for selection were either involvement in conserved proteostasis pathways or yeast orthologs of human proteins found to be aggregated in ALS patients’ motor neurons. Arrays of the strains were grown in a fluorescent plate reader to monitor the expression of the GFP-fusion genes (GFP fluorescence), expression of SOD1 (DsRed fluorescence) and growth (absorbance at 600nm) throughout logarithmic growth and into stationary phase. The GFP fluorescence intensity normalized to absorbance at each time point was used to calculate fold change in gene expression in strains expressing WT or A4V SOD1-DsRed relative to control strains. Strains exhibiting a difference in gene expression (p<0.05) were selected for repeat screening. “Hits” were defined as those exhibiting differential expression only in strains expressing A4V SOD1.

Results: Five yeast genes were differentially expressed in strains expressing A4V SOD1-DsRed relative to both WT SOD1 and control strains. Four of these were upregulated: PRE2, UBA1, DCP2, UF1D (p<0.05). Interestingly, although the β5 proteasome subunit (PRE2) was upregulated, β1 (PRE3) was downregulated. Upregulation was observed for another five genes, although statistical significance was not reached; RAD6 (p = 0.052), SCJ1 (p = 0.058), DSK2 (p = 0.062), ERV25 (p = 0.064), UFD4 (p = 0.086). The differentially expressed list of genes is enriched for the involvement in the ubiquitin–proteasome system (UPS) (p = 0.0024).

Discussion and conclusions: Basic proteostasis mechanisms are highly conserved among eukaryotic species. The upregulated genes identified in this study highlight key proteostasis mechanisms integral in the response to mutant SOD1, in particular, the UPS. The human orthologs of the identified yeast genes and their pathways are likely to be compromised in motor neurons expressing mutant SOD1, and will be interesting to examine in mammalian model systems.

Acknowledgements: Australian Rotary Health, Rotary Club of Dural, NHMRC, Wellcome Trust. Special thanks to Lu Cao and Daniel Bean for their help in different stages of this study.

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DOI: 10.1080/21678421.2016.1232054/0017

P71 ROLE OF MCU IN A SOD1 MODEL OF ALS

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Keywords: ERMCC, MCU, KN-62

Background: Disruption of the ER-mitochondria-calcium-cycle (ERMCC) in the G93AhSOD1 model of ALS is characterized by Ca2+ depletion of the ER and Ca2+ overload of the mitochondria (1–3). Ca2+ is channeled through the inner mitochondrial membrane by the mitochondrial calcium uniporter complex (MCU) and through the outer mitochondrial membrane by the voltage dependent anion channel (VDAC). In extreme stress situations, as mitochondrial Ca2+ overload, the mitochondrial permeability transition pore (mPTP) is formed and the apoptotic cell death is activated due to the release of cytochrome c.

Objectives: Our objectives were (i) to investigate the expression of MCU, VDAC, and Cyclophilin D (CyPD, modulator of mPTP) in primary nontransgenic and G93AhSOD1 mouse motor neurons, (ii) to influence ERMCC by pharmacological manipulation of mitochondrial Ca2+ uptake as a possible rescue strategy.

Methods: Embryonic mouse spinal motor neurons and nonmotor neurons were seeded on a glial feeder layer. Immunocytochemistry and qRT-PCR were done to investigate mRNA levels and expression of target proteins.
For survival assay we challenged our cultures with kainate (100 μM, 12h) to induce excitotoxicity and applied KN-62 (10 μM) and kaempherol (KMF, 25 μM) as possible rescue drugs. The corrected total cell fluorescence (CTCF) of the target proteins was calculated from immunocytochemistry z-stacks, for native and treated motor neurons.

Results: MCU was over expressed in G93AhSOD1 motor neurons (p<0.05). MCU modulation by CaM kinase inhibitor KN-62 protected G93AhSOD1 motor neurons from kainate-induced excitotoxicity (p<0.01), however, MCU activation by kaemferol failed to show protective effect. KN-62 decreased MCU overexpression in G93AhSOD1 motor neurons (p<0.01).

Conclusion: MCU is overexpressed in G93AhSOD1 motor neurons confirming the involvement of MCU in mitochondrial Ca2+ overload (4). Rescue effect of KN-62 against KA-induced excitotoxicity suggests MCU as target for therapeutic strategies within ERMCC in ALS. The mechanism underlying a potential protective effect of KN-62 has to do with the reduction of MCU in G93AhSOD1 motor neurons. Functional consequences of KN-62 and kaempherol on ERMCC will be investigated in future using calcium imaging.

Acknowledgment: This research is supported by BMBF (Bundesministerium für Bildung und Forschung) in the framework of the E-RARE programme (PYRAMID) and JPND (SOPHIA) of the European Union.

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DOI: 10.1080/21678421.2016.1232054/0018

P72 ROLE OF THE MITOCHONDRIAL NA/CAL/LI-EXCHANGER (NCLX) IN THE PATHOPHYSIOLOGY OF ALS

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Keywords: ERMCC, calcium homeostasis, NCLX

Background: Disturbance of the ER-mitochondria-coupling-cycle (ERMCC) appears to be an important feature in the pathophysiology of ALS. This includes ER calcium depletion and overload in mitochondria which take up large amounts of calcium. Calcium release is mediated by the sodium-calcium-lithium-exchanger (NCLX) localized at the inner mitochondrial membrane. It is the main calcium eliminating pathway of excitable cells. As a part of the ERMCC modulating the NCLX can provide a new therapeutic principle.

Objectives: To investigate (i) the distribution and (ii) expression level of NCLX, to determine effects of NCLX modulators on (iii) neuronal survival and (iv) ERMCC.

Methods: Western blot analyses were carried out using SOD1-G93A and SOD1-WT over expressing NSC34 cells. Murine motor neuron cocultures without (NT) or with (TG) overexpression of hSOD1G93A were used for immunofluorescent staining, survival assay, and calcium-imaging experiments. Survival assays were performed in the presence or the absence of CGP35157 (NCLX inhibitor) or forskolin (activator) alone or in combination with kainate (KA) for 12h. Calcium imaging with fura-2AM was used to assess changes in intracellular calcium concentrations.

Results: Immunofluorescent quantification of basal protein levels of the NCLX in hSOD1G93A motor neurons were not significantly changed compared to NT. Application of forskolin to the NSC G93A cell line generated a significant upregulation of NCLX protein level. Preliminary results from survival assays with forskolin did not show an increased survival after KA induced excitotoxicity. CGP35157 treatment did not show effects on NCLX expression, but modulated ERMCC in motor neurons and improved survival of motor neurons in KA toxicity. Further results about the effects of NCLX modulators on the ERMCC will be provided.

Conclusions: ERMCC dysregulation can induce disruptions in different cell processes finally leading to cell death. A dysfunction of NCLX can contribute to an impaired ERMCC. Hence, NCLX modulation may be beneficial to the viability of motor neurons.

Acknowledgment: This research is supported by BMBF (Bundesministerium für Bildung und Forschung) in the framework of the E-RARE programme (PYRAMID), JPND (SOPHIA) of the European Union and IZKF Jena (Interdisziplinäres Zentrum für klinische Forschung Jena).

DOI: 10.1080/21678421.2016.1232054/0019

P73 MUTANT PROTEIN AGGREGATION, MITOCHONDRIAL IMPAIRMENT, AND CALCIUM DYSREGULATION IN IPSC-DERIVED MOTOR NEURONS FROM ALS PATIENTS CARRYING DIFFERENT SOD1 MUTATIONS

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Keywords: induced pluripotent stem cell, SOD1 gene mutation, mitochondrial dysfunction

Introduction: Abnormal protein accumulation and mitochondrial dysfunction have been observed in amyotrophic lateral sclerosis (ALS) patients in central nervous
tissue, as well as in animal models. Investigations of the pathogenic mechanisms in motor neurons derived from ALS disease-specific induced pluripotent stem (iPS) cell lines could generate understanding of the issues affecting motor neurons (MN). Therefore, we explored mutant superoxide dismutase (SOD1) protein expression and mitochondrial function in motor neurons derived from the iPS cell lines of ALS patients carrying different SOD1 mutations.

Methods: We generated iPSC lines from three familial ALS patients with SOD1-V14M, SOD1-G16A, and SOD1-C111Y mutations, and then differentiated them into motor neurons. We investigated levels of the SOD1 protein in iPSCs and MNs and measured mitochondrial membrane potentials, intracellular Ca2+ levels in MNs, and lactate dehydrogenase (LDH) activity in the process of differentiation into the MNs derived from the controls and ALS patients’ iPSCs.

Results: The iPSCs from the three familial ALS patients were able to differentiate into motor neurons carrying different SOD1 mutations and different expressed MN markers. We detected high SOD1 protein expression, decreased mitochondrial membrane potential, and high intracellular calcium levels in both the MN and iPSCs that were derived from the three SOD1 mutant patients. However, at no time did we see greater LDH activity in the patient lines compared with the control lines.

Conclusions: Motor neurons derived from patient-specific iPSC lines can recapitulate key aspects of ALS pathogenesis, providing a cell-based disease model to further elucidate disease pathogenesis and to explore gene repair coupled with cell-replacement therapy. Incremental alterations in intracellular calcium signaling that selectively associate with mutations in C9orf72 in iPSC-derived MNs, using an engineered iPS line where the hexanucleotide expansions have been corrected.

Methods: To investigate the physiological phenotypes associated with the C9orf72 mutation in motor neurons, we used induced pluripotent stem cells derived from the skin fibroblasts of three C9orf72 patients and two healthy controls. As additional controls, we have generated isogenic iPSC lines where the (GGGGCC) n repeat were excised using the CRISPR/Cas9 system. The differentiated neurons were loaded with Fura 2-AM or Rhod 2-AM for live calcium experiments and exposed to various stimuli. Cocultures of iPSC-derived MNs and C2C12 muscle cells were plated in microfluidic chambers, the axons were allowed to extend and connect to the myotubes and analyzed after 2 weeks.

Results: In this study, we assessed calcium signaling in C9orf72 MNs. Live calcium imaging showed that exposure to 50 mM KCl led to high release and intake of Ca2+ in all C9 MNs. The clearance of Ca2+ ions from the cytosol was significantly delayed compared to controls and both phenotypes were rescued in the CRISPR/Cas9 edited iPS-derived MNs (p<0.01). Glutamate excitotoxicity was investigated by measuring (1) the levels of cytosolic Ca2+; (2) the recovery period, and (3) mitochondrial uptake of Ca2+ following stimulation. While cytosolic Ca2+ amplitude was not significantly higher in C9 MNs, the recovery time to baseline was up to three times longer in C9 MNs (p<0.01) and it was not rescued in the edited MNs. When stimulated with 100 μM kainate, all MN lines showed similar Ca2+ amplitudes, but recovery was significantly delayed in C9 MNs. In C9 MNs, we also found that mitochondrial uptake of cytosolic Ca2+ was significantly lower compared to healthy controls (p<0.001). In addition, we found reduced levels of the calcium-buffering protein calbindin in the C9 MNs. When cocultured with C2C12 muscle cells, we detected a reduced number of neuromuscular junctions formed by C9 MNs when compared to healthy MNs.

Conclusions: Our cellular model of C9orf72 iPSC-derived motor neurons reveals low Ca2+ buffering in the diseased MNs, which may be partially attributed to mitochondrial dysfunction in Ca2+ uptake and low levels of calcium-buffering proteins.

Acknowledgments: This work is funded by the MND Association.

DOI: 10.1080/21678421.2016.1232054/0020

P74 C9ORF72 IPSC DERIVED MOTOR NEURONS SHOW FUNCTIONAL DEFICITS IN CALCIUM BUFFERING

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Keywords: induced pluripotent stem cells, C9orf72, calcium signalling

Background: An expanded hexanucleotide (GGGGCC) n repeat in chromosome 9 open reading frame 72 (C9orf72) is a major cause of familial amyotrophic lateral sclerosis (fALS) and frontotemporal dementia (FTD). The function of the C9orf72 gene and its pathogenic mechanisms are currently unknown.

Objectives: The aim of this study is to identify functional alterations in intracellular calcium signaling that selectively associate with mutations in C9orf72 in iPSC-derived MNs, using an engineered iPS line where the hexanucleotide expansions have been corrected.

Methods: To investigate the physiological phenotypes associated with the C9orf72 mutation in motor neurons, we used induced pluripotent stem cells derived from the skin fibroblasts of three C9orf72 patients and two healthy controls. As additional controls, we have generated isogenic iPSC lines where the (GGGGCC) n repeat were excised using the CRISPR/Cas9 system. The differentiated neurons were loaded with Fura 2-AM or Rhod 2-AM for live calcium experiments and exposed to various stimuli. Cocultures of iPSC-derived MNs and C2C12 muscle cells were plated in microfluidic chambers, the axons were allowed to extend and connect to the myotubes and analyzed after 2 weeks.

Results: In this study, we assessed calcium signaling in C9orf72 MNs. Live calcium imaging showed that exposure to 50 mM KCl led to high release and intake of Ca2+ in all C9 MNs. The clearance of Ca2+ ions from the cytosol was significantly delayed compared to controls and both phenotypes were rescued in the CRISPR/Cas9 edited iPS-derived MNs (p<0.01). Glutamate excitotoxicity was investigated by measuring (1) the levels of cytosolic Ca2+; (2) the recovery period, and (3) mitochondrial uptake of Ca2+ following stimulation. While cytosolic Ca2+ amplitude was not significantly higher in C9 MNs, the recovery time to baseline was up to three times longer in C9 MNs (p<0.01) and it was not rescued in the edited MNs. When stimulated with 100 μM kainate, all MN lines showed similar Ca2+ amplitudes, but recovery was significantly delayed in C9 MNs. In C9 MNs, we also found that mitochondrial uptake of cytosolic Ca2+ was significantly lower compared to healthy controls (p<0.001). In addition, we found reduced levels of the calcium-buffering protein calbindin in the C9 MNs. When cocultured with C2C12 muscle cells, we detected a reduced number of neuromuscular junctions formed by C9 MNs when compared to healthy MNs.

Conclusions: Our cellular model of C9orf72 iPSC-derived motor neurons reveals low Ca2+ buffering in the diseased MNs, which may be partially attributed to mitochondrial dysfunction in Ca2+ uptake and low levels of calcium-buffering proteins.

Acknowledgments: This work is funded by the MND Association.

DOI: 10.1080/21678421.2016.1232054/0021
P75 TRANSLATING RIBOSOME AFFINITY PURIFICATION FROM C9ORF72-ALS/FTD PATIENT-DERIVED IPS MOTOR NEURONS

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Keywords: iPS motor neurons, C9orf72, TRAP

Background: A hexanucleotide repeat expansion in intron 1 of the C9orf72 gene is the most common cause of amyotrophic lateral sclerosis (ALS) in both sporadic and familial patients. Transcriptomic analysis of patient-derived iPS motor neurons is challenging due to the presence of up to 30% of cells in culture being of unknown or nonneuronal identity. Using the translating ribosome affinity purification (TRAP) method (1) of RNA purification to achieve more accurate analysis of the translatome/translatome of this disease model, without skewed results, is likely to enhance understanding of the pathogenesis of C9/ALS.

Objectives: Generation of a TRAP vector to facilitate RNA extraction from iPS motor neurons in a heterogeneous culture. RNA sequencing of purified RNA from at least three control and C9/ALS patient lines, with C9orf72 LNA gapmer-treated and CRISPR/Cas9-edited patient motor neurons as further controls, to analyze translatome profiles and find key pathways associated with early changes of neurodegeneration in C9orf72 motor neurons.

Methods: Molecular cloning was used to generate a lentiviral vector containing a major large ribosomal subunit, RPL22, fused with a FLAG affinity tag, and bicistronic-enhanced green fluorescent protein (eGFP) gene, under the control of a choline acetyl transferase (ChAT) promoter. Generation of motor neurons from control/patient-derived iPSCs has previously been optimized within the Talbot group (2). Magnetic beads coated with anti-FLAG antibodies will be used to capture FLAG-tagged RPL22 subunit-containing polysomes from iPS motor neuron lysates and standard RNA extraction will be used to purify motor neuron-specific mRNA. Following quality control steps, these RNA will be sent for RNA Sequencing at the Oxford Wellcome Trust Centre for Genomics.

Results: Live GFP fluorescence and immunostaining with a FLAG antibody, in HEK293-T cells transfected with constructs containing the components for TRAP vector cloning showed efficient expression of reporters GFP and RPL22. Confocal imaging of fixed iPS motor neurons transduced with lentiviral particles containing constructs under ChAT promoters have shown successful expression of eGFP reporter and costained ChAT and SMI-32 antibodies (common markers for mature motor neurons). This was found in both control and patient lines.

Conclusions: Completion of the TRAP vector will allow us to extract mRNA exclusively from iPS motor neurons in culture. This will allow us to analyze the C9/ALS patient translatome and simultaneously assess whether correction of the C9 repeat expansion is a viable method of therapy.

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DOI: 10.1080/21678421.2016.1232054/0022

P76 INVESTIGATING THE ROLE OF C9ORF72 IN AUTOPHAGY USING HUMAN IPS-DERIVED MOTOR NEURONS FROM ALS/FTD PATIENTS

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Keywords: C9ORF72, iPSC, autophagy

Background: An intronic repeat expansion (GGGGCC) in the C9ORF72 gene is the most common cause of familial frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) identified to date. Although three nonmutually exclusive mechanisms have been proposed to explain the neuronal degeneration induced by the hexanucleotide repeats, little is known about the normal molecular and cellular functions of C9ORF72.

Objectives: As autophagy is known to be implicated in many neurodegenerative diseases, we investigated this pathway in motor neurons (MNs) carrying the C9ORF72 expansion. In addition, we investigated possible interactions between C9ORF72 protein and key elements of the autophagic and degradation pathways.

Methods: To investigate the role of C9ORF72 in autophagy, we used MNs derived from induced pluripotent stem cells of patients carrying the C9ORF72 expansion (C9ORF72 iPSC-derived MNs). We examined defects in autophagy and measured the levels of pro- and antiapoptotic markers in C9ORF72 MNs and controls. The cells were examined by immunoblotting at baseline and following treatment with the autophagy inhibitors 3-methyladenine, bafilomycin A1 and chloroquine, the proteasome inhibitor MG-132, the SERCA-pump inhibitor thapsigargin and the mTOR inhibitor rapamycin. We also investigated the colocalization of C9ORF72-GFP with Rab5 and Rab7 in the iPSC-derived MNs, to understand whether there is a direct association between C9ORF72 and Rab proteins.
**Results:** At baseline, control and C9ORF72 patient MNs show differences in the level of apoptotic and autophagic markers. Lower levels of antiapoptotic proteins, such as Bcl-2, were detected in the C9ORF72 MNs compared to healthy controls. Immunoblotting revealed elevated p62, a marker of autophagic flux and proteosome degradation, in C9ORF72 MNs compared to healthy control MNs. Compared to the other treatments, the inhibition of mTOR, which leads to the induction of autophagic flux, did not increase the level of p62 in C9ORF72 MNs. Moreover, the mutant MNs showed higher levels of the autophagosomal marker LC3II. These differences were maintained following all autophagic treatments. Colocalization assays indicated significant interaction between C9ORF72 and the endosomal markers Rab5, but without differences between control and C9ORF72 patient MNs. Rab7 did not colocalize with C9ORF72 in our iPS-derived MNs.

**Conclusions:** Taken together these results suggest that autophagic flux is reduced in C9ORF72 iPS-derived MNs. Differences between control and patient lines after all autophagic treatments could indicate which pathways are directly affected as a consequence of the C9ORF72 expansion. As C9ORF72 MNs showed lower levels of the anti-apoptotic marker Bcl-2, we will examine the role of mitochondria in the autophagic pathway, in accordance with our previous work (1). The interaction between C9ORF72 and Rab5 in iPS-derived MNs confirms bioinformatic analysis and previous studies suggesting a direct interaction.

**Acknowledgment:** This project is funded by Motor Neurone Disease Association (Dr Ruxandra Dafinca).

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DOI: 10.1080/21678421.2016.1232054/0023

**P77 THE C9ORF72 PROTEIN INTERACTS WITH RAB1A AND THE ULK1 COMPLEX TO REGULATE INITIATION OF AUTOPHAGY**

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**Keywords:** C9ORF72, autophagy, Rab GTPase

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**Background:** A GGGGCC hexanucleotide repeat expansion in the first intron of the C9orf72 gene is the most common genetic cause of amyotrophic lateral sclerosis and frontotemporal dementia (C9ALS/FTD). Reduced levels of C9orf72 expression have been reported in C9ALS/FTD patients and although C9orf72 haploinsufficiency has been proposed as contributing to C9ALS/FTD, its significance is not yet clear. Data from C9orf72 knockout mice have shown that C9orf72 is required for macrophage and microglial function in vivo but the neuronal function of C9orf72 remains unclear. C9orf72 encodes two C9orf72 protein isoforms with structural homology to the Differentially Expressed in Normal and Neoplasia (DENN) proteins that are implicated in membrane trafficking events as GDP/GTP exchange factors (GEF) of Rab GTPases. Hence, it has been suggested that C9orf72 regulates endosomal/lysosomal trafficking. C9ALS/FTD patients show specific ubiquitin and p62 positive but TDP-43 negative, neuronal cytoplasmic and intranuclear inclusions in the cerebellum and hippocampus, which is indicative of impaired autophagy. As C9orf72 haploinsufficiency may contribute to C9ALS/FTD by a loss-of-function mechanism we hypothesized that C9orf72 may be involved in autophagy.

**Objectives:** To investigate the role of C9orf72 in autophagy.

**Results:** We report that C9orf72 interacts with Rab1a and the Unc-51-like kinase 1 (ULK1) autophagy initiation complex. As a Rab1a effector, C9orf72 controls initiation of autophagy by regulating the Rab1a-dependent trafficking of the ULK1 autophagy initiation complex to the phagophore. Accordingly, reduction of C9orf72 expression in cell lines and primary neurons attenuated autophagy and caused accumulation of p62 positive puncta reminiscent of the p62 pathology observed in C9ALS/FTD patients. Finally, basal levels of autophagy were markedly reduced in C9ALS/FTD patient-derived neurons.

**Discussion and conclusion:** Our data identifies C9orf72 as a novel regulator of autophagy. We propose a model in which C9orf72 acts as an effector of Rab1a that recruits active Rab1a to the ULK1 complex, promoting translocation of the ULK1 complex to the phagophore during autophagy initiation. Furthermore, our data indicate that C9orf72 haploinsufficiency and associated reductions in autophagy might be the underlying cause of C9ALS/FTD-associated p62 pathology.

**Acknowledgements:** This work was funded by grants from the Thierry Latran Foundation (Project RoCIP), MRC (MR/K005146/1 and MR/M013251/1), Alzheimer's Society (260 (AS-PG-15-023)), the University of Sheffield Moody Family Endowment, and an EU Framework 7 Award (Euromotor No 259867). PJS is supported as an NIHR Senior Investigator. EFS is supported by a Motor Neurone Disease Association Prize Studentship (DeVos/Oct13/870-892)

DOI: 10.1080/21678421.2016.1232054/0024
P78 INTRANUCLEAR (G4C2)N RNA FOCI INDUCE FORMATION OF PARASPECKLE-LIKE STRUCTURES

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Keywords: C9orf72, RNA foci, paraspeckles

Background: Expansion of GGGGCC hexanucleotide repeat in the gene C9ORF72 is the most common pathogenic mutation in families with autosomal dominant FTD, FTD/ALS and ALS (1). The expanded repeat is transcribed and can form intranuclear RNA foci, which sequester and deplete RNA-binding proteins and may consequently cause neurodegeneration (2).

Objectives: Identify new RNA-binding proteins that bind (G4C2)n RNA in vitro and colocalize with intranuclear RNA foci in cells transfected with expanded repeat and postmortem brain tissue.

Methods: We performed an RNA-pull down assay using (G4C2)48 RNA and rat brain lysates. Proteins specifically bound to (G4C2)48 RNA were identified by mass spectrometry and results were further confirmed by immunoblotting. To evaluate the relevance of interacting proteins in disease pathomechanism we performed in situ hybridization followed by immunocytochemistry in cells transfected with expanded repeat and postmortem brain tissue.

Results: Six proteins (Prx-III, SFPQ, NONO, NPM1, EF1z2, and hnRNP H) were identified to specifically bind (G4C2)48 RNA in vitro. Among them SFPQ and NONO also colocalize with intranuclear RNA foci in cells transfected with expanded repeats and to a lesser extent in postmortem brain tissue. Indeed 54% of foci colocalized with SFPQ in transfected cells, while up to 5.6% of foci per case colocalized with SFPQ in post-mortem brain tissue.

Discussion: Newly formed paraspeckle-like structures could function in similar fashion as NEAT1 positive paraspeckles. It is possible that paraspeckle proteins assembled on repeated RNA are capable of retaining adenosine to inosine hyperedited mRNA in the nucleus. This would lead to increased nuclear retention of mRNA in C9ORF72 mutant cells and consequently reduced the expression of proteins encoded by these mRNAs. Our study identifies a new mechanism through which RNA foci could cause an increase in the nuclear retention of mRNA and lead to toxicity.

Acknowledgements: This project was funded by the Slovenian Research Agency (ARRS), Slovene human resources development and scholarship fund, The Wellcome Trust, Medical Research Council, the Motor Neuron Disease Association and the American ALS Association.

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DOI: 10.1080/21678421.2016.1232054/0025

P79 C9ORF72 DPRS UNDERGO LIQUID-LIQUID PHASE SEPARATIONS AND PERTURB RNA GRANULE METABOLISM

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Keywords: C9orf72, stress granules, phase transitions

Background: Neurodegenerative diseases are characterized by the presence of protein inclusion bodies with different protein content depending on the type of disease. Amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) are no exceptions to this common theme. In most ALS and FTLD cases, the predominant species of aggregated proteins are RNA-binding proteins (RBPs). Yet the exact processes underlying this pathological aggregation remain unknown.

Objectives: In this project, we wanted to investigate the underlying mechanisms of RBP mislocalization and aggregation. To this end we studied the hexanucleotide repeat expansions in C9orf72 since they are the most common genetic cause of the disease, and patients present with the hallmark RBP pathology. We specifically focused on a potential role of dipeptide repeat (DPR) pathology in these processes.

Methods: We have previously generated and characterized different DPR models based on codon-optimized PR expression constructs. Combined with in vitro work using synthetic peptides, these allow us to probe the physical determinants of DPR toxicity.

Results: In previous work we have shown that nuclear transport factors are modifiers of arginine-rich DPR toxicity. These data suggest that DPRs could perturb the nucleocytoplasmic transport system, eventually resulting in cytoplasmic RBP mislocalization. We wondered whether DPRs also could play a direct role in the aggregation of these DPRs. Recent studies have shown that RBP aggregation is likely initiated from a liquid-like
phase separated state. We now show that arginine-rich DPRs can undergo such phase transition themselves in vitro. This process is length- and dose-dependent, and is mediated by counterions. Moreover, PR and GR are capable of affecting the phase separation and aggregation behavior of disease-relevant RBPs in vitro. Lastly, we found that PR and GR perturb stress granule metabolism in cells.

**Discussion and conclusions:** As suggested besides perturbing RBP subcellular localization, arginine-rich DPRs could also directly affect the phase separation and aggregation of RBPs in C9 ALS/FTLD pathology. In recent work, we are further exploring the physical underpinnings of arginine-mediated phase separations and its involvement in both health and disease.

DOI: 10.1080/21678421.2016.1232054/0026

**P80 SCREENING ALS PATIENT CSF TOXICITY TO SPINAL MOTOR NEURONS DERIVED FROM INDUCED PLURIPOTENT STEM CELLS**

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**Keywords:** stem cells, induced-motor neurons, cerebrospinal fluid

**Background:** Human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), offer a powerful model system to generate and differentiate motor neurons (MNs) for screening toxicity and potential therapeutic agents in ALS. The overall aim of this study was to adapt a reproducible protocol to generate highly pure human spinal MNs for testing the sensitivity to toxicity of cerebrospinal fluid (CSF) from MND patients and controls.

**Objectives:** To characterize the toxicity of patient CSF (MND, neurological, and non-neurological control) to human iPSCs-derived MNs and identify the toxic factor(s) responsible.

**Methods:** Spinal MNs were generated and differentiated from H9 hESC cells using a published protocol (1) which produces MNs in the shortest time frame (28 days), includes four differentiation stages, with slight modifications, and is characterized by morphological and immunocytochemical analyses for neuroepithelial, MN progenitor, and mature MN markers. Cells at different steps of differentiation were also characterized for the expression of HOX and MN genes by quantitative PCR (qPCR). To prescreen CSF toxicity, NSC-34 cells were treated with 30% CSF (n=8 MND, n=8 MS, n=8 neurological control, n=8 non-neurological controls) in growth medium for 24 or 72 h. Cell death was examined using Annexin V/propidium iodide labeling and FACS analysis.

**Results:** Initial differentiations were carried out to determine the optimal density for differentiation. Immunocytochemistry for SOX1 and HOXA3 revealed higher densities were optimal for differentiation. Preliminary data shows neuroepithelial cells (NEPs) were successfully induced from hESCs, which stain positive for SOX1 and these NEPs were caudal NEPs, staining positive for HOXA3. These cells were then successfully differentiated into MN progenitors (MNP)s, which stained positive for OLIG2. These MNP)s can be passaged and expanded at least twice and stained positive for OLIG2 and showing similar morphology with initial differentiation. Differentiations were then continued for another 17 days and staining showed ChAT and TUJ1 positive cells. qPCR data showed these cells expressed NEP gene (SOX1), caudal spinal MN genes (HOXA3, hindbrain; HOXC6, cervical; HOXC9, and thoracic), MNP)s, and MN gene (OLIG2, HB9, and ISL1).

**Discussion and conclusions:** Spinal MNs identified by ChAT and TUJ1 expression were generated from H9 hESCs in 28 days. These mature spinal MNs also expressed HOXC6 and HOXC9, consistent with a mixed population of cervical and thoracic MNs. This iPSC-derived spinal MN model will serve as an important platform to screen the toxicity or CSF from MND patients, identify factor(s) responsible for this toxic and agents that mitigate toxicity.

**Acknowledgements:** Stafford Fox Medical Research Foundation; MND Research Institute of Australia; Bethlehem Griffiths Research Foundation

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DOI: 10.1080/21678421.2016.1232054/0027