AriSLA
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Research, development and innovation in ALS

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ABSTRACT BOOK

AriSLA ETS

FONDAZIONE ITALIANA DI RICERCA PER LA SLA
SCLEROSI LATERALE AMIOTROFICA
ENTE DEL TERZO SETTORE

LECTIO MAGISTRALIS - DYSREGULATION OF ENERGY HOMEOSTASIS IN AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic lateral sclerosis (ALS) is associated with impairments of energy metabolism, including weight loss and decreased appetite that are negatively correlated with survival. Importantly, targeting weight loss through increased caloric intake was recently suggested to increase survival of fast progressing ALS patients, and decreased circulating neurofilament levels, suggesting neuroprotective potential of nutritional intervention. Neural mechanisms underlying metabolic impairment in ALS remain unknown, but ALS patients and presymptomatic ALS gene carriers have an early atrophy of the hypothalamus. Our most recent results suggest that the lateral hypothalamic area (LHA), an hypothalamic nucleus controlling metabolic homeostasis through the secretion of neuropeptides such as orexin/hypocretin and melanin-concentrating hormone (MCH), is particularly affected and could be involved in weight loss. Here, I will review metabolic abnormalities in ALS, their possible mechanisms and ways for intervention.

LECTIO MAGISTRALIS - DISREGOLAZIONE DELL'OMEOSTASI ENERGETICA NELLA SCLEROSI LATERALE AMIOTROFICA

La sclerosi laterale amiotrofica (SLA) è associata ad alterazioni del metabolismo energetico, tra cui la perdita di peso e la diminuzione dell'appetito, che sono correlati negativamente con la sopravvivenza. È importante sottolineare che è stato recentemente suggerito che si possa limitare la perdita di peso attraverso un maggiore apporto calorico per aumentare la sopravvivenza dei pazienti con SLA in rapida progressione e ridurre i livelli di neurofilamenti circolanti, indicando il potenziale neuroprotettivo dell'intervento nutrizionale. I meccanismi neurali alla base della compromissione metabolica nella SLA rimangono sconosciuti, ma i pazienti con SLA e i portatori presintomatici di geni correlati alla SLA mostrano un'atrofia precoce dell'ipotalamo. I nostri risultati più recenti suggeriscono che l'area ipotalamica laterale (LHA), un nucleo ipotalamico che controlla l'omeostasi metabolica attraverso la secrezione di neuropeptidi come l'orexina/ipocretina e l'ormone concentrante della melanina (MCH - melanin concentrating hormone), è particolarmente colpita e potrebbe essere coinvolta nella perdita di peso. Qui, esaminerò le anomalie metaboliche nella SLA, i loro possibili meccanismi e le modalità di intervento.

CALL 2016 - DDRNA&ALS, A ROLE FOR DNA DAMAGE RESPONSE RNA (DDRNA) IN NEURODEGENERATION IN ALS

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Endogenous DNA damage has been reported in motor-neurons of ALS patients with mutations in the genes encoding for TDP43 or FUS, only recently involved in the DNA damage response (DDR). We reported that DDR activation and DNA repair depends on the endoribonucleases DROSHA and DICER, cofactors of FUS and TDP43, for the biogenesis of the DNA damage response RNA (DDRNA). We have also reported that the small molecule enoxacin, by increasing DICER activity, promotes DNA repair by stimulating DDRNA production. We hypothesize that neurodegeneration of ALS patients with TDP43 or FUS proteinopathies is caused by altered DDRNA production and DDR activation, leading to progressive DNA damage accumulation.

In our AriSLA-funded project we confirmed that mutant FUS and TDP43 cytoplasmic inclusions (CI) reduce DROSHA activity. This is associated with DNA damage accumulation and aberrant activation of the DDR kinase ATM, leading to pan-nuclear accumulation of γH2AX and loss of cell viability. Treatment with specific ATM inhibitor reduces γH2AX signal in cells with TDP43 and FUS CI, indicating a causative role for such kinase in the observed DNA damage signalling hyperactivation. Importantly, increased DDRNA biogenesis by enoxacin improves DDR in cells with CI.

CI of FUSP525L and TDP-43 causes the accumulation of P62 of the autophagy pathway, which sequesters in the cytoplasm the nuclear DDR ubiquitin-ligase RNF168. Notably, P62 inactivation by RNAi in cells with FUSP525L CI partially restores DDR activation and DROSHA nuclear levels, thus reducing pan-nuclear γH2AX accumulation. Similar results were observed by overexpressing RNF168 and by inhibiting the de-ubiquitinase enzyme which counteracts RNF168 activity in DDR activation.

Finally, we extended our findings in a D. melanogaster TDP43 model of ALS, and observed that the inactivation of ATM counteracts neurodegeneration, further establishing a causal link between ALS and DDR by genetic means.

CALL 2016 - DDRNA&ALS, IL RUOLO DELLA RISPOSTA AL DANNO AL DNA NELLA NEURODEGENERAZIONE LEGATA ALLA SLA

Il danno endogeno al DNA è stato riportato nei motoneuroni di pazienti SLA con mutazioni nei geni che codificano per TDP43 o FUS, recentemente coinvolti nella risposta al danno del DNA (DDR). Abbiamo pubblicato che l'attivazione del DDR e la riparazione del DNA dipendono dalle endoribonucleasi DROSHA e DICER, cofattori di FUS e TDP43, per la biogenesi dell'RNA del DDR (DDRNA). Abbiamo anche visto che la molecola enoxacina, che aumenta l'attività di DICER, promuove la riparazione del DNA stimolando la produzione di DDRNA. La nostra ipotesi è che la neurodegenerazione nei pazienti SLA con proteinopatie di TDP43 o FUS sia causata da un'alterata produzione di DDRNA e dall'attivazione di DDR portando così ad un progressivo accumulo di danno al DNA.

Nel primo progetto finanziato da AriSLA abbiamo confermato che le inclusioni citoplasmatiche (IC) di FUS mutante e TDP43 riducono l'attività di DROSHA. Ciò è associato a danno del DNA e all'attivazione aberrante della chinasi apicale del DDR, ATM, che porta all'accumulo di γH2AX e perdita di vitalità cellulare. Il trattamento con un inibitore di ATM riduce il segnale di γH2AX nelle cellule con IC di TDP43 e FUS, indicando un ruolo cruciale di questa chinasi nell'iperattivazione del DDR. Anche l'enoxacina ripristina il corretto DDR nelle cellule con IC.

Inoltre, abbiamo osservato che le IC di FUS^{P525L} e TDP43 provocano l'accumulo di P62, che sequestra l'ubiquitin-ligasi nucleare del DDR, RNF168, nel citoplasma. In particolare, l'inattivazione di P62 tramite RNAi nelle cellule con IC di FUS^{P525L} ripristina parzialmente l'attivazione del DDR e i livelli di DROSHA, riducendo così l'accumulo pan-nucleare di γH2AX. Risultati simili si osservano over-esprimendo RNF168 e inibendo la de-ubiquitinasi che contrasta RNF168 nell'attivazione del DDR.

Abbiamo infine esteso i nostri risultati in un modello di SLA in D. *melanogaster*, in cui abbiamo osservato che l'inattivazione di ATM contrasta la neurodegenerazione, stabilendo un ulteriore legame genetico tra i geni SLA e DDR.

CALL 2018 - MLOPATHY, MEMBRANE-LESS ORGANELLE PATHOLOGY IN ALS: IDENTIFICATION OF CAUSES AND RESCUING FACTORS

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Biochemical and genetic studies show that protein aggregation, impaired function of protein-RNA complexes called stress granules (SGs), defective nucleocytoplasmic trafficking and nucleolar stress play a key role in ALS. These events are, at least in part, connected. We previously showed that defective ribosomal proteins (DRiPs) and ALS-mutated proteins can accumulate in SGs and convert SGs into a dysfunctional aggregated state. We also identified a protein quality control (PQC) system that avoids misfolded protein accumulation in SGs, maintaining their dynamics and functionality. The MLOpathy project studied if similar mechanisms occur on other membraneless organelles (MLOs), whose dynamic properties are essential to allow the cells to quickly respond and adapt to stressors, and how their malfunction could contribute to ALS.
We report that DRiPs rapidly diffuse into the nucleus and accumulate in nucleoli and PML bodies. We show that nucleoli and PML bodies act as dynamic overflow compartments that recruit PQC factors and store DRiPs for later clearance. Whereas nucleoli serve as constitutive overflow compartments, PML bodies are stress-inducible overflow compartments for DRiPs. If DRiPs are not properly cleared by chaperones and proteasomes due to proteostasis impairment, nucleoli undergo amyloidogenesis and PML bodies solidify. Solid PML bodies immobilize 20S proteasomes and limit the recycling of free ubiquitin. Ubiquitin depletion, in turn, compromises the formation of DNA repair compartments at fragile chromosomal sites, ultimately threatening cell survival. Finally, we report that PML bodies are impaired in ALS and we highlight the existence of an intricate cross-talk exists between PML bodies and cytoplasmic SGs. In conclusion, our results show how aberrant phase transitions of MLOs contribute to ALS.

CALL 2018 - MLOPATHY, STUDIO DEI MECCANISMI IMPLICATI NEL RIPRISTINO DELLA FUNZIONALITÀ DEI PROCESSI DI RISPOSTA ALLO STRESS E NELL'AGGREGAZIONE DI ORGANELLI CELLULARI NELLA SLA

Studi biochimici e genetici dimostrano che aggregazione proteica, alterato dinamismo di complessi ribonucleoproteici detti granuli da stress (SG), difetti del trasporto nucleo-citosol e stress nucleolare, processi fra loro in parte connessi, sono implicati nella SLA. Abbiamo dimostrato che i prodotti difettosi della sintesi proteica (DRiPs) e le proteine mutate nella SLA possono accumularsi nei SG, convertendoli in uno stato aggregato disfunzionale. Abbiamo identificato un sistema di controllo di qualità proteico (PQC) che evita l'accumulo di DRiPs nei SG, ripristinandone dinamismo e funzionalità. Il progetto MLOpathy ha studiato se meccanismi simili si verificano a carico di altri organelli senza membrana (MLO), le cui proprietà dinamiche sono essenziali affinché le cellule rispondano allo stress, nonché se alterazioni di questi MLO possano contribuire allo sviluppo della SLA.
Riportiamo che i DRiPs si accumulano nei nucleoli e nei corpi PML nucleari. Mentre i nucleoli agiscono come compartimenti di raccolta, i corpi PML reclutano componenti del sistema PQC per favorire la rimozione dei DRiPs. Se i DRiPs non sono correttamente rimossi da chaperoni e proteasomi, i nucleoli ed i corpi PML vanno incontro ad un processo di amiloidogenesi. La solidificazione dei corpi PML causa l'immobilizzazione di proteasomi e limita il riciclo di molecole di ubiquitina. La deplezione di ubiquitina, a sua volta, compromette la formazione di compartimenti per il riparo del DNA, riducendo la capacità delle cellule di sopravvivere a condizioni di stress. Infine, riportiamo una riduzione significativa del numero dei corpi PML nella SLA, che correla con alterazioni nel dinamismo dei SG. In conclusione, i nostri risultati dimostrano come alterazioni del dinamismo di MLO contribuiscano alla progressione della SLA.

CALL 2018 - PATHENSTDP, DEFINING THE ROLE OF HNRNP PROTEINS IN ENHANCING TDP-43 PATHOLOGY

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Pathological aggregation of TDP-43 is principally associated with Amyotrophic Lateral Sclerosis (ALS) but is also present in approximately 50% of all Frontotemporal dementia patients and is a co-pathology in ~40% of Alzheimer's disease cases and in Chronic traumatic encephalopathy (CTE). These observations suggest that TDP-43 pathological aggregation can occur in many different pathological conditions and may be affected by the presence of other RNA Binding Proteins binding to the same set of target RNAs. Many recent lines of evidence have highlighted the role played by heterogeneous nuclear ribonucleoproteins (hnRNPs) in ALS. In this study, we have aimed to identify transcripts co-regulated by TAR DNA-binding protein 43 kDa and highly conserved heterogeneous nuclear ribonucleoproteins which have been previously shown to regulate TAR DNA-binding protein 43 kDa toxicity (DAZ-associated protein 1, heterogeneous nuclear ribonucleoprotein -Q, -D, -K, and -U). Using transcriptomic analyses, we have uncovered that *Nitric Oxide Synthase 1 Adaptor Protein* mRNA (NOS1AP) is a direct TAR DNA-binding protein 43 kDa target, and in flies its modulation alone can rescue TDP-43 pathology. In primary mouse cortical neurons, we have shown that TDP-43 mediated downregulation of NOS1AP expression strongly affects the NMDA-receptor signaling pathway. In human patients, the downregulation of NOS1AP mRNA strongly correlates with TDP-43 proteinopathy as measured by *Stathmin-2* and Unc-13 homolog A cryptic exon inclusion. Overall, our results demonstrate that NOS1AP may represent a novel disease-relevant gene, potentially suitable for the development of new therapeutic strategies.

CALL 2018 - PATHENSTDP, STUDIO DEL RUOLO DELLE PROTEINE CHE REGOLANO LA COMPOSIZIONE DELL'RNA SULLA TOSSICITÀ CELLULARE LEGATA ALLA PROTEINA TDP-43

L'aggregazione patologica di TDP-43 è principalmente associata alla Sclerosi Laterale Amiotrofica (SLA), ma è presente anche in circa il 50% di tutti i pazienti con demenza frontotemporale ed è una co-patologia in circa il 40% dei casi di malattia di Alzheimer e nell'encefalopatia traumatica cronica (CTE). Queste osservazioni suggeriscono che la sua aggregazione patologica può verificarsi in condizioni molto diverse fra loro e quindi può essere influenzata dalla presenza di altre proteine che hanno gli stessi RNA bersaglio. Numerose evidenze recenti hanno evidenziato il ruolo svolto dalle ribonucleoproteine nucleari eterogenee (hnRNPs) nella SLA. In questo studio, abbiamo quindi mirato a identificare i trascritti co-regolati da TDP-43 e da hnRNPs che hanno precedentemente dimostrato di regolare la sua tossicità (DAZAP1 e le seguenti hnRNP: -Q, -D, -K e -U). Utilizzando le analisi del trascrittoma, abbiamo scoperto che l'mRNA della proteina chiamata Adattatore della Sintasi 1 dell'Ossido Nitrico (NOS1AP) è legata direttamente da TDP-43 e da sola è in grado di riparare il danno neurotossico indotto da TDP-43 in modelli di *Drosophila*. In neuroni corticali primari di topo, abbiamo inoltre dimostrato che la perdita di espressione di NOS1AP mediata da TDP-43 influisce fortemente sulla via di segnalazione del recettore NMDA. Infine, nei pazienti umani abbiamo osservato che la diminuzione dell'espressione dell'mRNA di NOS1AP correla fortemente con la proteinopatia da TDP-43 misurata sulla base dell'inclusione degli esoni criptici di Stathmin-2 e Unc-13A. Nel complesso, i nostri risultati dimostrano che la proteina NOS1AP può rappresentare un nuovo gene rilevante per la malattia, potenzialmente adatto per lo sviluppo di nuove strategie terapeutiche.

CALL 2017 - TDP-43-STRUCT, PURIFICATION AND DETERMINATION OF THE STRUCTURE, PHASE SEPARATION AND TOXICITY OF TDP-43

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Amyotrophic lateral sclerosis is associated with deposition of cytosolic inclusions of TAR DNA-binding protein 43 (TDP-43) in the motoneurons of the primary motor cortex, corticospinal tracts, brainstem, spinal cord, and also in the brain neurons in a subset of patients. We first purified full-length TDP-43 in a reproducible manner and to a high yield, finding that it is folded, dimeric and characterized by inter-domain interactions. We then induced its phase separation, under conditions in which it was initially unfolded and folded, respectively. In the first case TDP-43 formed filaments but retained a largely random-coil secondary structure and ability to bind amyloid diagnostic dyes, such as Congo red and thioflavin T (ThT). In the second case the protein formed rapidly round, 0.5-1.0 μm wide aggregates that had limited internal diffusion, as assessed with FRAP, and did not coalesce, but rather clustered into bunches with irregular non-filamentous morphology, enriched with α-helical/random-coil structure and without ThT binding. By monitoring with turbidimetry both initial aggregation and further clustering, we carried out a multiparametric analysis of the two phenomena.
Expression of TDP-43 in NSC-34 cells and application of last-generation probes to detect amyloid in the cytoplasm, Raman and Fourier transform infrared microspectroscopies to study in situ their secondary structure, and transmission electron microscopy to study their morphology, we confirmed the absence of amyloid dye binding, cross-β structure and fibrillar texture in the round cytoplasmic inclusions. We then monitored with STED microscopy the decrease of nuclear TDP-43 levels and the formation of various size classes of cytoplasmic aggregates with time. Using a quantitative biology approach and a simultaneous global fitting, we attributed neuronal dysfunction to the first (55-60%) and second (40-45%) factor, in particular with the formation of the largest inclusions in the latter case.

Call 2017 - TDP-43-STRUCT, PURIFICAZIONE E DETERMINAZIONE DELLA STRUTTURA, SEPARAZIONE DI FASE E TOSSICITÀ DI TDP-43

La sclerosi laterale amiotrofica (SLA) è associata alla deposizione di inclusioni citosoliche della proteina TDP-43) nei motoneuroni della corteccia motoria primaria, nei tratti corticospinali, nel tronco cerebrale, nel midollo spinale e anche nei neuroni cerebrali in un sottogruppo di pazienti. Abbiamo inizialmente purificato la TDP-43 completa in modo riproducibile e con alta resa, scoprendo che è ripiegata (*folded)*, dimerica e caratterizzata da interazioni inter-dominio. Abbiamo quindi indotto la sua separazione di fase, nelle condizioni in cui era inizialmente *unfolded* e *folded*, rispettivamente. Nel primo caso TDP-43 ha formato filamenti, conservando una struttura secondaria *random-coil* e la capacità di legare coloranti diagnostici amiloidi, come tioflavina T (ThT). Nel secondo caso ha formato rapidamente aggregati rotondi, larghi 0,5-1,0 μm che avevano una diffusione interna limitata (FRAP), e senza coalescere, ma formando grappoli con morfologia non filamentosa irregolare, arricchiti con struttura α-elica/*random* e senza legame con ThT. Monitorando con turbidimetria sia l'aggregazione iniziale che l'ulteriore *clustering*, abbiamo effettuato un'analisi multiparametrica dei due fenomeni.
Mediante espressione di TDP-43 in cellule NSC-34 ed applicazione di sonde di ultima generazione per rilevare amiloide nel citoplasma, di microspettroscopie a infrarossi a Raman per studiare *in situ* la loro struttura secondaria, e di microscopia elettronica per studiarne la morfologia, abbiamo confermato assenza di amiloide nelle inclusioni citoplasmatiche. Abbiamo quindi monitorato con microscopia STED la diminuzione dei livelli di TDP-43 nucleare e la formazione di varie classi dimensionali di aggregati citoplasmatici nel tempo. Utilizzando un approccio di biologia quantitativa e un *fitting* globale simultaneo, abbiamo attribuito la disfunzione neuronale al primo (55-60%) e al secondo (40-45%) fattore, in particolare alla formazione delle inclusioni più grandi nel secondo caso.

CALL 2018 - SPLICEALS, DISSECTING THE FUNCTIONAL INTERACTION BETWEEN FUS AND HNRNP A2/B1 IN THE PATHOGENESIS OF ALS

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Several genetic and experimental findings point to a crucial role of RNA dysfunction in the pathogenesis of ALS. In particular, evidence suggests that mutations in FUS, which are associated with genetic ALS, affect the regulation of alternative splicing of RNAs. We have previously shown that in transgenic mice expressing human wild-type FUS, which develop ALS-like phenotypes, the splicing of hnRNP A2/B1, a protein with key roles in RNA metabolism, is significantly affected. This suggests that a pathological connection between FUS and A2/B1 might contribute to motor neuron degeneration in ALS. To further characterize this process, we have analyzed the splicing of A2/B1 during the disease course and used isoform-specific antibodies to monitor the expression and distribution of A2/B1 splice variants in the affected tissues of FUS mice. Our results show that the disease in mice is marked by the progressive accumulation of A2/B1 splicing variants that lack exon 9 (A2/B1Δ9). Most interestingly, A2/B1Δ9 variants accumulate in the cytoplasm of degenerating spinal cord motor neurons, suggesting a role of cytoplasmic mislocalization in the pathological process. Experiments in cultured cells demonstrate that exon 9 is a key determinant of nuclear localization of A2/B1 and that cytoplasmic 9 isoforms accumulate into cytoplasmic stress granules, a condition that is further enhanced by the co-expression of P525L mutant FUS. Importantly, cytoplasmic localization is required for the toxicity induced by the expression of A2/B1 in cells. Finally, Drosophila orthologous of A2/B1 are modifiers of the degeneration of Drosophila eye induced by expression of wild-type FUS, further indicating that A2/B1 is a target of FUS toxicity in vivo. These findings suggest that cytoplasmic accumulation of A2/B1, as a consequence of alternative splicing defects, might be involved in the pathological process and that targeting these defects could contrast motor neuron degeneration in FUS-ALS.

CALL 2018 - SPLICEALS- INDAGINE SULL'INTERAZIONE TRA LA PROTEINA FUS E UNA PROTEINA COINVOLTA NELLA REGOLAZIONE DELLA MATURAZIONE DELL'RNA NELLA SLA

Mutazioni nel gene FUS, che sono responsabili di alcune forme genetiche di SLA, alterano i processi di regolazione dello splicing alternativo di specifici RNA. Utilizzando un modello transgenico murino di SLA associata al gene FUS, abbiamo osservato alterazioni nello splicing alternativo di hnRNP A2/B1, un gene con funzioni importanti nella regolazione del metabolismo dell'RNA ed associato a sua volta a forme genetiche di SLA. Per caratterizzare ulteriormente questo processo, abbiamo analizzato lo splicing di A2/B1 nel corso della progressione della malattia, ed utilizzato anticorpi isoforma-specifici per monitorare l'espressione e la distribuzione delle varianti di splicing di A2/B1 nei tessuti affetti. I risultati ottenuti mostrano che la malattia è caratterizzata da un progressivo accumulo delle isoforme di splicing di A2/B1 prive dell'esone 9 nel citoplasma dei motoneuroni in corso di degenerazione, ad indicazione di un possibile ruolo della de-localizzazione di A2/B1 nel processo patologico. Esperimenti in cellule in coltura dimostrano che l'esone 9 ha un ruolo importante nella localizzazione nucleare di A2/B1 e che le varianti citoplasmatiche di A2/B1 si accumulano all'interno di granuli di stress, una condizione che è esacerbata dalla espressione di mutanti di FUS associati a SLA. Inoltre, la localizzazione citoplasmatica è condizione necessaria alla tossicità cellulare indotta dall'espressione di A2/B1. Infine, la modulazione di geni ortologhi di A2/B1 modifica la degenerazione causata dall'espressione di FUS in Drosophila, ad ulteriore indicazione che A2/B1 è un possibile bersaglio della tossicità di FUS in vivo. Questi risultati suggeriscono che la delocalizzazione citoplasmatica di A2/B1, come effetto di alterazioni dello splicing alternativo, può essere coinvolta nel processo patologico, e che la correzione di questi difetti potrebbe contrastare la degenerazione dei motoneuroni nella SLA-FUS.

CALL 2018 - TARGET-RAN, TARGETING RAN TRANSLATION IN ALS

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Intronic GGGGCC (G4C2) hexanucleotide repeat expansion within the human C9orf72 gene represents the most common cause of familial forms of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) (C9ALS/ FTD). Repeat-associated non-AUG (RAN) translation of repeat-containing C9orf72 RNA results in the production of neurotoxic dipeptide-repeat proteins (DPRs). We aimed at the identification of small molecules able to modulate the DPR levels.
We performed a, fluorescence based, high-throughput (HTP) drug screen and identified positive and negative modulators of DPR levels and validated the hits by orthogonal assay in cell lines. We dissected the mechanism of action of the hits investigating degradative pathways, proteasome and autophagy, and the translation process, evaluating polysomal loading of the aberrant RNAs. Finally, we evaluated their efficacy in a Drosophila model of C9ALS/FTD and in C9ALS/FTD patient-derived iPSC motor neurons.
We identified i) forskolin (FSK, a cAMP-elevating compounds) as DPR levels enhancer, and ii) geldanamycin (GELD, an HSP90 inhibitor) and spironolactone (SPL, an aldosterone antagonist), as reducer of DPR levels. cAMP elevating compounds activate protein kinase A (PKA) activity and its inhibition, by both pharmacological and genetic approaches, reduced DPR levels in cells and rescued pathological phenotypes in a Drosophila model of C9ALS/FTD. Knockdown of PKA-catalytic subunits correlated with reduced translation efficiency of DPRs, while the PKA inhibitor H89 reduced endogenous DPR levels in C9ALS/FTD patient-derived iPSC motor neurons. Geldanamycin and Spironolactone reduced DPR levels by promoting protein degradation via the proteasome and autophagy pathways respectively as shown by the loss of efficacy observed when the degradative pathways were blocked.
Together, our results suggest new and druggable pathways modulating DPR levels in C9ALS/FTD.

CALL 2018 - TARGET-RAN, *IDENTIFICAZIONE DI PICCOLE MOLECOLE IN GRADO DI MODULARE IL PROCESSO ANOMALO DI TRADUZIONE DELLE SEQUENZE RIPETUTE DOVUTO A MUTAZIONI DEL GENE C9ORF72*

L'espansione ripetuta dell'esanucleotide GGGGCC (G4C2) all'interno del gene umano C9orf72 rappresenta la causa più comune di forme familiari di sclerosi laterale amiotrofica (SLA) e demenza frontotemporale (FTD) (C9ALS/FTD). La traduzione non-AUG (RAN) associata alla ripetizione dell'RNA C9orf72 contenente la ripetizione provoca la produzione di proteine dipeptidiche ripetute neurotossiche (DPR). In questo progetto abbiamo avuto l'obiettivo di identificare piccole molecole in grado di modulare i livelli di DPR.
Abbiamo eseguito uno screening farmacologico ad alta processività (HTP) ed identificato modulatori positivi e negativi dei livelli di DPR, convalidando i risultati mediante saggi ortogonali. Il meccanismo d'azione dei modulatori è stato valutato studiando i processi degradativi, il proteasoma e l'autofagia, e il processo della traduzione, valutando il carico polisomale degli RNA aberranti. Infine, abbiamo valutato la loro efficacia in un modello di Drosophila di C9ALS / FTD e in motoneuroni derivati da pazienti C9ALS / FTD.
Abbiamo identificato i) forskolina (FSK) come potenziatore dei livelli di DPR e ii) geldanamicina (GELD) e spironolattone (SPL), come riduttore dei livelli di DPR. FSK attiva l'attività della proteina chinasi A (PKA) e la sua inibizione, sia farmacologici che genetica, riduce i livelli di DPR nelle cellule e frena i fenotipi patologici in un modello di Drosophila di C9ALS / FTD. La sottoespressione della PKA diminuisce l'efficienza di traduzione dei DPR, mentre l'inibitore della PKA, H89, riduce i livelli di DPR endogeni nei motoneuroni derivati dal paziente C9ALS / FTD. GELD e SPL hanno ridotto i livelli di DPR promuovendo la degradazione delle proteine attraverso rispettivamente le vie del proteasoma e dell'autofagia.
Complessivamente, i nostri risultati suggeriscono percorsi nuovi e farmacologici che modulano i livelli di DPR in C9ALS / FTD.

CALL 2017 - HYPERALS, MODULATION OF HYPERMETABOLISM AND HYPEREXCITABILITY AS A STRATEGY TO COUNTERACT DEGENERATION IN ALS

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In ALS patients motor neurons loss is associated with increased neuronal excitability paralleled by defective energy metabolism. Whole-body energy dysregulation in ALS includes hypermetabolism with decreased fat stores, possibly associated with increased oxidation of lipids as energy source. The rationale of the study is that simultaneous decrease of neuron hyperexcitability and improvement of defective energy metabolism through modulation of hypermetabolism may be highly beneficial to ALS mice and possibly to ALS patients.
We aimed at investigating the potential therapeutic effect provided by chronic Ranolazine (RAN) administration in the SOD1-G93A mouse model. RAN is an FDA approved drug that inhibits β-oxidation and acts as a partial inhibitor of inward persistent Na+ currents (IpNa). To discriminate between the two RAN effects, we also separately targeted the inhibition of IpNa currents or the inhibition of β-oxidation by administering GS967 or Trimetazidine (TMZ) respectively.
We demonstrated that the alterations in energy metabolism and in cortical excitability of ALS mice predate the motor neuron degeneration. The administration of RAN and TMZ at symptoms onset decreased hypermetabolism improving significantly muscle strength. RAN improved disease phenotype as long as it decreased hypermetabolism. In fact, when the drug no longer had any effect on energy expenditure, the symptoms of the disease re-emerged without showing an impact on survival. TMZ, instead, showed a lasting effect on decreasing energy expenditure with the addition of a strong neuroprotective effect that was mirrored in the extension of life span of ALS mice. Finally, GS967 administration failed in improving neither muscle strength and life span.
The pharmacological targeting of Hypermetabolism may be useful for ameliorates disease phenotype as this metabolic dysfunction is related to a poor prognosis and to more aggressive form of ALS.

CALL 2017 - HYPERALS, MODULAZIONE DELL'IPERMETABOLISMO E DELL'IPERECCITABILITÀ COME STRATEGIA PER COMBATTERE LA NEURODEGENERAZIONE NELLA SLA

Nei pazienti la perdita di motoneuroni è associata a una maggiore eccitabilità e ad un metabolismo energetico difettoso. L'ipermetabolismo e la diminuzione delle riserve di grasso caratterizzano le disfunzioni metaboliche nella SLA. In tale contesto, la diminuzione dell'ipereccitabilità neuronale e dell'ipermetabolismo, potrebbe rappresentare una valida strategia terapeutica.
L'obiettivo primario del progetto è stato valutare il potenziale terapeutico fornito dalla somministrazione cronica di ranolazina (RAN) nel modello murino SOD1^{G93A}. La RAN è un farmaco approvato dalla FDA che inibisce la β-ossidazione e agisce come un inibitore delle correnti persistenti del Na+ (IpNa). Per discriminare tra i due effetti, abbiamo utilizzato altri due farmaci che inibiscono o le correnti IpNa o la β-ossidazione, rispettivamente il GS967 o la Trimetazidina (TMZ).
Abbiamo dimostrato che le alterazioni del metabolismo energetico e dell'eccitabilità corticale nei topi SLA precedono la degenerazione del motoneurone. La somministrazione di RAN e TMZ all'esordio dei sintomi ha ridotto l'ipermetabolismo migliorando significativamente la forza muscolare. La RAN ha migliorato la forza muscolare limitatamente al periodo in cui ha mitigato anche l'ipermetabolismo. Infatti, quando il farmaco non ha più avuto alcun effetto sul dispendio energetico, i sintomi della malattia sono riemersi senza mostrare un impatto sulla sopravvivenza. Il TMZ, invece, ha mostrato un effetto prolungato sulla diminuzione del dispendio energetico ed un forte effetto neuroprotettivo che si è riflesso nell'estensione della durata della vita dei topi SLA. Infine, la somministrazione di GS967 non è riuscita a migliorare né la forza muscolare né la durata della vita.
La modulazione farmacologica dell'ipermetabolismo può essere un'utile strategia terapeutica per migliorare il fenotipo della malattia anche in virtù del fatto che la disfunzione metabolica è correlata ad una prognosi sfavorevole e a forme più aggressive di SLA.

CALL 2017 - AXRIBALS, AXONAL TRANSLATOME AND FUNCTIONAL ALTERATIONS IN CELLULAR MODELS OF AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic lateral sclerosis (ALS) is a lethal neurodegenerative disorder affecting upper and lower motor neurons. Mounting evidence suggests that ALS is a dying-back neuropathy characterized by axonal degeneration that precedes the demise of the soma. Several ALS-related mutations affect RNA-binding proteins, including TDP-43, impairing transport of mRNAs along axons and their local translation; thus, altered RNA metabolism seems to be a key factor of this disease. While mutations in the TARDBP gene, encoding TDP-43, represent merely 5% of familial ALS patients, 97% of patients of sporadic and familial forms of ALS show TDP-43 proteinopathy, with nuclear depletion of TDP-43 and its accumulation in the cytoplasm.
In this work, we generated and characterized a cellular model of TDP-43 proteinopathy, consisting of mouse cortical neurons expressing lentivirally delivered wt or mutant human TDP-43.
Our primary cultures consist nearly exclusively of deep layer glutamatergic upper motor neurons, while containing hardly any GABAergic interneurons or glial cells. This model is characterized by the prominent formation of cytoplasmic RNA aggregates positive for RNA granule markers. Such aggregates are recruited to G3BP-positive granules under cellular stress conditions. Our cellular model was subjected to an extensive functional characterization that revealed profound abnormalities in ultrastructure, synaptic vesicle trafficking as well as neuronal activity. Neurons were cultured in microfluidic chambers, in order to physically separate axons from cell bodies. In this model, leveraging on the extensive systems biology analysis conducted by the Viero lab (see F. Lauria's poster), we identified a collection of differentially translated axonal mRNAs implicated in the response to oxidative stress and in synaptic function. Our results support the notion of ALS as a primarily axonal neuropathy and implicate axonal translation in the control of neuronal homeostasis and synaptic activity.

CALL 2017 - AXRIBALS, TRASLATOMA ASSONALE E ALTERAZIONI FUNZIONALI IN MODELLI CELLULARI DI SCLEROSI LATERALE AMIOTROFICA

La sclerosi laterale amiotrofica (SLA) è una malattia neurodegenerativa letale che colpisce i motoneuroni superiori e inferiori. Un numero crescente di risultati suggerisce che la SLA sia una neuropatia caratterizzata da degenerazione assonale che precede il danno a carico del soma neuronale. Diverse mutazioni che causano SLA compromettono la funzione delle proteine leganti l'RNA, inclusa TDP-43, riducendo il trasporto di mRNA lungo gli assoni e la loro traduzione locale; il metabolismo alterato dell'RNA sembra essere un fattore chiave di questa malattia. Mentre le mutazioni nel gene TARDBP, che codifica per TDP-43, causano solo il 5% dei casi di SLA a ricorrenza familiare, il 97% dei pazienti con forme sporadiche e familiari di SLA mostra una proteinopatia TDP-43, con deplezione nucleare di questa proteina, che si accumula invece nel citoplasma.
In questo progetto, abbiamo generato e caratterizzato un modello cellulare di proteinopatia TDP-43, costituito da neuroni corticali di topo che esprimono la TDP-43 umana normale o mutante.
Le nostre colture primarie sono costituite quasi esclusivamente da motoneuroni superiori glutammatergici dello strato profondo, mentre non contengono interneuroni GABAergici o cellule gliali. Questo modello è caratterizzato dalla formazione di numerosi aggregati citoplasmatici positivi per marcatori dei granuli di RNA. Tali aggregati vengono reclutati in granuli G3BP1-positivi in condizioni di stress cellulare. Il modello cellulare è stato sottoposto a un'accurata caratterizzazione funzionale che ha rivelato profonde anomalie nell'ultrastruttura, nel traffico delle vescicole sinaptiche e nell'attività neuronale. I neuroni sono stati coltivati in camerette microfluidiche, al fine di separare fisicamente gli assoni dai corpi cellulari. In questo modello, sfruttando la dettagliata analisi di *systems biology* condotta dal laboratorio Viero (vedi poster di F. Lauria), abbiamo identificato una collezione di mRNA assonali deregolati, implicati nella risposta allo stress ossidativo e nella funzione sinaptica.
I nostri risultati rafforzano la visione della SLA come neuropatia primariamente assonale e implicano la traduzione locale dell'mRNA assonale nel controllo dell'omeostasi neuronale e dell'attività sinaptica.

CALL 2019 - MUSALS-ACHR, STUDYING ACETYLCHOLINE RECEPTORS AND MUSCLE REGENERATION IN ALS TO DEVELOP PROGNOSTIC MARKERS AND POTENTIAL THERAPIES HAMPERING DISEASE PROGRESSION

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Amyotrophic lateral sclerosis (ALS) is a heterogeneous disease with high variability in the speed of progression, even in cases with a defined genetic cause, such as superoxide dismutase 1 (SOD1) mutations. Transgenic SOD1G93A mice with distinct genetic backgrounds (C57 and 129Sv) show consistent differences in the speed of disease onset and progression, resembling what is observed in ALS patients. We recently hypothesized that the difference in the peripheral neuromuscular system reflects the phenotypic difference between the two mouse models.

In this work, we compared the functional, morphological, and molecular profiles of the gastrocnemius muscle (GCM) from these two SOD1G93A mouse strains at the presymptomatic, onset, and symptomatic stages of the disease.

Data collected showed that the slow-progressing (SP) mice, despite the premature denervation and muscle atrophy, activated different compensatory mechanisms, including the increased expression and clustering of the Acetylcholine (ACh) receptors, coupled with the enhanced ACh-evoked currents and the preservation of compound muscle action potential. We suggest that part of this phenomenon depends on prompt and sustained myogenesis coupled with reinnervation, enhanced by an early inflammatory response rapidly switching toward an M2 pro-regenerative phenotype. In contrast, despite a comparable muscle denervation level, the fast-progressing (FP) mice failed to activate a compensatory muscle response, exhibiting a rapidly progressive deterioration of muscle force. Noteworthy, FP mice exhibited a lower macrophage M2/M1 ratio than SP mice, suggesting an unproductive muscle repair. This trend was confirmed in ALS patients' muscle biopsies, where we found an inverse correlation of the M2/M1 ratio with the disease progression rate.

These data indicate new biomarkers and potential therapeutic targets for ameliorating the disease course.

CALL 2019 - MUSALS-ACHR, STUDIARE I RECETTORI DELL'ACETILCOLINA E LA RIGENERAZIONE MUSCOLARE NELLA SLA PER SVILUPPARE MARCATORI PROGNOSTICI E POTENZIALI TERAPIE CHE OSTACOLANO LA PROGRESSIONE DELLA MALATTIA

La Sclerosi Laterale Amiotrofica (SLA) è una malattia eterogenea con elevata variabilità nella velocità di progressione anche nei casi con una causa genetica definita come le mutazioni della superossido dismutasi 1 (SOD1). I topi transgenici SOD1G93A con background genetico distinto (C57 e 129Sv) mostrano differenze consistenti nella velocità di insorgenza e progressione della malattia, simili a quanto osservato nei pazienti con SLA. Noi ipotizziamo che la differenza nel sistema neuromuscolare periferico rifletta la differenza fenotipica tra questi due modelli murini.

In questo lavoro, abbiamo confrontato i profili funzionali, morfologici e molecolari del muscolo gastrocnemio (GCM) dei due ceppi di topi SOD1G93A nella fase prima, all'esordio e durante i sintomi della malattia. I dati ottenuti mostrano che i topi a lenta progressione (SP), nonostante la prematura denervazione e atrofia muscolare, attivano diversi meccanismi compensatori, tra cui l'aumentata espressione e aggregazione dei recettori dell'acetilcolina (ACh), accoppiati con correnti evocate da ACh potenziate e con la conservazione del potenziale d'azione muscolare composto. Parte di questo fenomeno dipende da una sostenuta miogenesi, stimolata dalla risposta infiammatoria (M1) che si trasforma in fenotipo antinfiammatorio (M2). Al contrario, i topi a progressione rapida (FP), nonostante un livello comparabile di denervazione, non sono in grado di attivare tutte queste risposte nei muscoli, esibendo un rapido deterioramento della forza muscolare. Inoltre, il rapporto M2/M1, è molto più basso rispetto ai topi SP indicando una ridotta riparazione muscolare. Questo indice è stato valutato anche nelle biopsie muscolari dei pazienti SLA, dove abbiamo osservato una correlazione inversa del rapporto M2/M1 con il tasso di progressione della malattia.

Questi dati permettono di identificare nuovi biomarcatori e potenziali bersagli terapeutici per migliorare il decorso della malattia.

CALL 2015 - RAP-ALS, RAPAMYCIN (SIROLIMUS) TREATMENT FOR AMYOTROPHIC LATERAL SCLEROSIS

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In preclinical studies rapamycin was found to target neuroinflammation, by expanding regulatory T cells (Tregs), and autophagy, two pillars of ALS pathogenesis. Rapamycin has never been tested in ALS patients.
In this multicenter, randomized, double-blind trial, ALS patients with symptoms onset within 18 months were randomly assigned in a 1:1:1 ratio to receive rapamycin 2 mg/m2/day,1 mg/m2/day or placebo. The primary outcome was the number of patients exhibiting an increase >30% in Tregs from baseline to treatment end. Secondary outcomes included the changes from baseline of T,B,NK cell subpopulations, inflammasome mRNA expression and activation status, S6-RP phosphorylation, neurofilaments, comparing rapamycin and placebo arm. Clinical outcome measures of disease progression, survival, safety and quality of life were also collected.
After screening 70 persons, 63 were randomly assigned to rapamycin or placebo. In intention-to-treat analysis, twice as many patients treated with rapamycin showed an increase of at least 30% of Tregs compared to placebo (p=0.236). Although this change was not significant, several immunological parameters involved in disease pathogenesis and prognosis showed meaningful favorable changes. Treatment with rapamycin 1 mg/m2/day reduced CD8 T-lymphocytes (p=0.064), intermediate monocytes (p=0.076), IL-18 (p=0.006) and increased B cell memory switched (p=0.004) and classical monocytes (p=0.038). Patients treated with rapamycin 1 mg/m2/d showed a mean monthly difference of 0.50 points in the ALSFRS-R total score with respect to placebo during treatment (p=0.110). Rapamycin was safe, and treated patients had a better quality of life. Rapamycin 1 mg/m2/day resulted the best and safer dosage in this study.
A short treatment of 18 weeks with rapamycin showed interesting effects on immune system and quality of life. Further studies are necessary to evaluate the role of this already available drug in ALS.

CALL 2015 - RAP-ALS, IL TRATTAMENTO CON RAPAMICINA PER LA SCLEROSI LATERALE AMIOTROFICA

Studi preclinici hanno mostrato che rapamicina ha come bersaglio la neuroinfiammazione, tramite espansione delle cellule T regolatorie (Tregs), e l'autofagia, due pilastri della patogenesi della SLA. La rapamicina non è mai stata testata in pazienti con SLA.
In questo studio multicentrico, randomizzato, in doppio cieco, i pazienti con SLA con esordio dei sintomi entro 18 mesi sono stati randomizzati con un rapporto 1:1:1 a ricevere rapamicina 2 mg/m2/giorno, 1 mg/m2/giorno o placebo. L'outcome primario era rappresentato dai pazienti che mostravano un aumento >30% dei Treg dal baseline a fine del trattamento nel braccio dei trattati rispetto al placebo. Gli outcomes secondari includevano i cambiamenti delle sottopopolazioni di cellule T, B, NK, dell'inflammasoma, fosforilazione della S6-RP, neurofilamenti, confrontando rapamicina e placebo. Sono state valutate anche progressione di malattia, sopravvivenza, sicurezza e qualità della vita.
Su 70 persone screenate, 63 sono state randomizzate a rapamicina o placebo. Nell'analisi intention-to-treat, il doppio dei pazienti trattati con rapamicina ha mostrato un aumento >30% dei Tregs rispetto al placebo (p=0,236). Inoltre, il trattamento con rapamicina 1 mg/m2/die ha ridotto i linfociti T CD8 (p=0,064), i monociti intermedi (p=0,076), e IL-18 (p=0,006), mentre ha determinato aumento dei linfociti B memory switched (p=0,004) e dei monociti classici (p=0,038). I pazienti trattati con rapamicina 1 mg/m2/die hanno mostrato una differenza media mensile di 0,50 nel punteggio totale ALSFRS-R rispetto al placebo durante il trattamento (p=0,110). La rapamicina è risultata sicura e i pazienti hanno mostrato una migliore qualità della vita. Il dosaggio migliore è risultato rapamicina 1 mg/m2/die.
Un breve trattamento con rapamicina ha mostrato effetti interessanti sul sistema immunitario e sulla qualità della vita. Saranno necessari ulteriori studi per valutare il ruolo di questo farmaco nella SLA.

LECTIO MAGISTRALIS - CLINICAL TRIAL DESIGN ADVANCES IN ALS

Lucie Bruijn
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This is a particularly exciting time with many new pharmaceutical companies entering the field. Significant advances have been made in the understanding of disease mechanism in ALS, leading to an increase in the number of ALS clinical trials to test novel compounds. This emphasizes the need to re-evaluate how we conduct clinical trials. Rapid enrolment into an increasing number of clinical trials necessitates consideration of burden to patients and the potential of digital end points; the need for limited numbers of patients on placebo and expanded access to all ALS patients entered in the clinical trial. In the presentation I will provide an overview of the current clinical trial landscape and highlight approaches that address these considerations with a focus on platform trial designs and biomarkers. Platform trials have been initiated in US, UK and Europe with a goal to expedite the identification of new treatments. Biomarkers form a critical decision tool to rapidly identify treatments in conjunction with the ALS FRS-R and survival data. Increasing evidence has emerged for neurofilaments as an early diagnostic in the case of ALS patients carrying C9orf72 and SOD1 mutations and as a potential tool for stratification. In addition, several novel biomarkers are being explored. Identification of target engagement biomarkers as well as markers of disease progression will enable early decisions as to whether a particular pathway is of relevance and intervention of this pathway will have impact on improved quality of life and survival.

LECTIO MAGISTRALIS - PROGRESSI NEL DISEGNO DEGLI STUDI CLINICI NELLA SLA

Questo è un momento particolarmente emozionante con molte nuove aziende farmaceutiche che entrano nel campo della SLA. Sono stati compiuti progressi significativi nella comprensione dei meccanismi alla base della malattia, portando a un aumento del numero di studi clinici sulla SLA per testare nuovi composti. Ciò sottolinea la necessità di rivalutare il modo in cui conduciamo le sperimentazioni cliniche. La rapida inclusione in un numero crescente di studi clinici richiede di considerare quale sia l'impatto per i pazienti e di valutare potenziali endpoint digitali, oltre che la necessità di limitare il numero di pazienti in trattamento con placebo e di ampliare l'accesso alla terapia a tutti i pazienti con SLA inseriti nella sperimentazione clinica. In questa presentazione fornirò una panoramica sullo stato attuale degli studi clinici ed evidenzierò gli approcci che affrontano queste considerazioni, con particolare attenzione ai progetti in corso nelle piattaforme multitrial e ai biomarcatori. Le piattaforme multitrial sono state avviate negli Stati Uniti, nel Regno Unito e in Europa con l'obiettivo di accelerare l'identificazione di nuovi trattamenti. I biomarcatori costituiscono uno strumento decisionale fondamentale per identificare rapidamente i trattamenti in combinazione con l'ALSFRS-R e i dati di sopravvivenza. Sono emerse prove crescenti per i neurofilamenti come biomarcatori di diagnosi precoce nel caso di pazienti con SLA portatori di mutazioni C9orf72 e SOD1 e come potenziale strumento per la stratificazione. Inoltre, sono allo studio diversi nuovi biomarcatori. L'identificazione di biomarcatori che definiscano il raggiungimento del target prestabilito di una terapia e di marcatori di progressione della malattia consentirà di prendere decisioni tempestive per comprendere se un particolare meccanismo sia rilevante e se intervenire su questo meccanismo possa avere un impatto sul miglioramento della qualità della vita e della sopravvivenza.

THE BIOGEN ROADMAP FOR A DIVERSIFIED AND SYNERGIC PIPELINE

Paola Marcon
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We believe that no other disease area holds as much need for medical breakthroughs as neuroscience. The emergence of promising modalities, including antisense oligonucleotide (ASOs), gene therapy and advancements in biologics and small molecules, are opening new target opportunities with potential applications in critical targets currently in research.
For nearly a decade, Biogen has remained persistent in its commitment to furthering ALS research in an effort to bring therapies to people living with this rapidly progressing neurological condition. Starting in 2012 the company began discovery and development, carrying out a Phase 3 clinical trial called EMPOWER for broad ALS. Unfortunately, the trial failed to meet its primary endpoint and no efficacy was seen in individual components of function or survival. Though the valuable data collected on the disease course and the insights provided on the individual level, shifted Biogen's focus to genetic ALS, with the goal of bringing a potential therapy to patients in need. At present, about 10 percent of ALS cases are caused by a known genetic mutation that can be passed from one generation to another, while 90 percent have no known cause. Nevertheless, scientists believe it is likely that additional genetic mutations have yet to be identified, underscoring the importance of Biogen's continued work in understanding ALS genetics. By applying emerging learnings on appropriate modalities and sensitive endpoints, Biogen is pursuing ASO strategy that selectively targets known genetic drivers of ALS. The most advanced of these programs thus far has been BIIB067 targeting adult ALS with a confirmed superoxide dismutase 1 (SOD1) mutation. Biogen is also investigating ataxin-2 (ATXN2), a gene that may be more broadly involved in ALS, and other undisclosed targets.

LA ROADMAP DI BIOGEN PER UNA PIPELINE DIVERSIFICATA E SINERGICA

Siamo convinti che non ci sia altra area terapeutica che abbia un maggiore bisogno di scoperte mediche quanto le neuroscienze. L'emergere di modalità terapeutiche promettenti, tra cui gli oligonucleotidi antisenso (ASO), la terapia genica, i progressi in biologia e nello studio delle piccole molecole, stanno aprendo nuove opportunità per l'identificazione di target con potenziali applicazioni in target critici attualmente in fase di ricerca.
Da ormai un decennio, Biogen persiste nel suo impegno a promuovere la ricerca sulla SLA nel tentativo di portare terapie alle persone che vivono con questa condizione neurologica in rapida progressione. A partire dal 2012 l'azienda ha iniziato il suo percorso di ricerca e sviluppo clinico, conducendo uno studio clinico di Fase 3 chiamato EMPOWER nella SLA sporadica. Purtroppo, lo studio non ha raggiunto il suo endpoint primario e non è stata osservata alcuna efficacia nelle singole componenti funzionali o di sopravvivenza. Grazie ai preziosi dati raccolti sul decorso della malattia e le informazioni raccolte sui singoli individui, l'attenzione di Biogen si è spostata sulla SLA genetica, con l'obiettivo di portare una potenziale terapia ai pazienti bisognosi. Attualmente, circa il 10% dei casi di SLA è causato da una mutazione genetica nota che può essere trasmessa da una generazione all'altra, mentre il 90% non ha una causa nota. Tuttavia, gli scienziati ritengono che sia probabile che ulteriori mutazioni genetiche debbano ancora essere identificate, sottolineando l'importanza del lavoro continuo di Biogen nella comprensione della genetica della SLA. Applicando le conoscenze emergenti con modalità appropriate ed endpoint sensibili, Biogen sta perseguendo la strategia ASO che prende di mira selettivamente le cause genetiche note della SLA. Il più avanzato di questi programmi finora è stato BIIB067 che ha con obiettivo le forme di SLA adulta con una mutazione confermata della superossido dismutasi 1 (SOD1). Biogen sta anche studiando l'atassina-2 (ATXN2), un gene che potrebbe essere più ampiamente coinvolto nella SLA, e sta perseguendo altri bersagli genetici non divulgati.

Clinical research

P1

DEFINING MOTOR CORTICAL PATTERNS OF UPPER MOTOR NEURON PATHOLOGY IN AMYOTROPHIC LATERAL SCLEROSIS USING A 3T-MRI WITH IRON-SENSITIVE SEQUENCES

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BACKGROUND-AIM

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder involving upper and lower motor neurons. Neurodegeneration may spread among motor neurons with a prion-like behavior. Thus, single or multiple foci of neurodegeneration are followed by disease diffusion to contiguous or connected regions, possibly being related to symptoms occurrence and to body regions' involvement. The evaluation of T2* signal intensity of the primary motor cortex (M1), may be associated with clinical UMN burden and neuroinflammation.

Here we investigated cortical patterns of upper motor neuron pathology in ALS using iron-sensitive MR imaging, correlating MRI data to body sites of disease onset.

METHODS

-78 ALS patients in charge at our Centre for Motor Neuron Disease in Pisa, underwent a brain 3T

-Signal intensity and magnetic susceptibility of M1 were assessed using respectively 3D GRE multi-echo T2*- weighted images and Quantitative Susceptibility Maps.

-The signal intensity of the whole M1 and each of its functional regions was rated as normal or reduced, and the magnetic susceptibility of each M1 region was measured.

RESULTS

The highest frequencies of T2* hypointensity were found in M1 regions associated with the body sites of symptom onset. Homologous M1 regions were both hypointense in 80–93 % of patients with cortical abnormalities, and magnetic susceptibility values measured in homologous M1 regions were significantly correlated with each other. Rarely, T2* hypointensity was detectable in two non-contiguous M1 regions

CONCLUSIONS

Iron sensitive MR imaging of the brain at 3T often show alterations in M1 regions associated with the body site of disease onset. The simultaneous involvement of both homologous M1 regions is frequent, followed by that of adjacent regions; the affection of non-contiguous regions, seems rare. These findings suggest the interhemispheric connections as one of the preferential paths for the UMN pathology diffusion in ALS.

Clinical research

P2

EVTESTINALS - EXTRACELLULAR VESICLES IN ALS: TESTING THEIR USE AS BIOMARKERS FOR PROGNOSIS AND DISEASE PROGRESSION

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BACKGROUND-AIM

Extracellular vesicles (EVs) are naturally released nanoparticles produced by all the cells in our body that circulate into biofluids. Considering that the EV cargo reflects the composition of the donor cell, EVs could be used to deduce the pathophysiological state of the brain, avoiding the problem of impracticable biopsies. The Blood brain barrier (BBB) protects the central nervous system (CNS) from the periphery, the possibility to purify neuronal EVs in the blood is under investigation. EVs can cross biological barriers, moreover the integrity of the BBB decreases with aging and neuroinflammation, observed in Amyotrophic Lateral Sclerosis (ALS). In a previous work, we demonstrated that peripherally circulating EVs features can be exploited to differentiate ALS patients from other conditions, e.g. healthy subjects or patients affected by muscular dystrophies or spinal and bulbar muscular atrophy. In addition, these features can also be used to stratify patients according to the rate of the disease progression. Now we are studying EVs specifically released by the cells of the CNS that can be detected in the peripheral blood and used as biomarker for the diagnosis and prognosis of ALS.

METHODS

We characterized neuronal EVs obtained from primary culture of murine cortical neurons. We isolated EVs exploiting different techniques, e.g. differential ultracentrifugation(UC), density gradient UC and size exclusion chromatography. We performed the EVs proteomic analysis by mass spectrometry.

RESULTS

We have specifically investigated the transmembrane proteins present in our EVs samples focusing on proteins with a reported brain-specific expression.

CONCLUSIONS

We have obtained a list of proteins that characterize neuronal EVs. We are now verifying the possibility to detect these candidates with the final goal to validate them in biofluids. It can be assumed that the identification of target proteins using this approach will give us reliable candidates that can be used as biomarker for clinical purpose.

Clinical research

P3

ALS COMMUNITY SUPPORT ASSESSMENT TO IMPROVE ONLINE ACCESS TO INFORMATION AND RESOURCES IN ITALY

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BACKGROUND-AIM

Access to real time ALS scientific research news and support resources in non-English languages is often a challenging endeavour for people living with ALS (PALS), their families, and caregivers (PALS-related).
To assess the ALS community needs to initiate a new series of virtual seminars promoted by a patient-led, multi-disciplinary Italian ALS association.

METHODS

PALS and family members were asked to fulfill a five-minute anonymous survey online seeking feedback on their interests in several ALS-related topics. The survey was launched in May 2022 and included nine questions about: updates from clinical trials and scientific meetings, patient journey and other social supports, two open questions about scientific publications and other topics of interest. Answer scores ranged from 1 (not interested) to 5 (very interested).

RESULTS

Up to October 2022, 65 participants fulfilled the survey: 31% PALS, and 69% PALS-related people. Overall, 85% of participants expressed high interest in the proposal to launch a new series of virtual seminars on ALS-related topics. Most participants (90%) expressed interest on updates about new clinical trials and on receiving more information concerning local and national support services offered for PALS and PALS-related. Also, updates on the ongoing research were considered favorably.

CONCLUSIONS

Thanks to this survey, we have received significant interest from the Italian community about our proposal to develop a new online seminar series on ALS. Most participants seek information on clinical trials, therapeutic approaches and activities from researchers, suggesting the need for a discussion with experts. Accordingly, we are now building an online seminar series to address the interests expressed in this survey using a design-build approach.

Identification of novel therapeutic targets

P4

A GENE THERAPY APPROACH TARGETING TDP-43 PATHOLOGY FOR ALS

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BACKGROUND-AIM

Up to 97% of ALS cases present TDP-43 inclusions suggesting that TDP-43 is part of a converging pathogenetic pathway with a pivotal role in the disease. Gain of cytoplasm functions and loss of nuclear functions of TDP-43 seem to contribute to pathogenesis and impairment in its trafficking be a key event driving pathology. To restore normal TDP-43 trafficking may be an effective therapeutic approach. We demonstrated that PPIA is an interactor of TDP-43 that regulates its trafficking and function. PPIA knockout (PPIA-/-) mice develop a neurodegenerative disease which resembles ALS-frontotemporal dementia with marked TDP-43 pathology. We found that the PPIA-TDP-43 interaction depends on PPIA Lys-acetylation, which is impaired in experimental models of ALS and in patients, where low level of acetylated PPIA (acetyl-PPIA) is associated with TDP-43 mislocalization.

METHODS

The effect of an acetyl-PPIA mimetic (PPIA K125Q) will be investigated on TDP-43 mislocalization and nucleocytoplasmic transport in cortical neurons expressing different TDP-43 pathogenic mutants. An AAV9 vector carrying PPIA K125Q will be characterized for its biodistribution and effect on TDP-43 pathology in the PPIA-/- mouse and the TAR4/4 TDP-43 mouse model of ALS. The effect of the AAV9-mediated PPIA K125Q transduction will be evaluated on the onset and progression of the disease in the TAR4/4 TDP-43 mouse.

RESULTS

We expect to show that the expression of the acetyl-PPIA mimetic normalizes TDP-43 trafficking in cellular paradigms of TDP-43 pathology. We predict to see a significant amelioration of the TDP-43 pathology in both mouse models and an effect on the onset and progression of the disease in the TAR4/4 TDP-43 mouse. Generation of the AAV9 vector carrying the acetyl-PPIA mimetic is in progress.

CONCLUSIONS

With this project, we will contribute to improve knowledge on the molecular mechanisms at the basis of TDP-43 pathology and possibly lay the foundation for a highly innovative therapeutic approach for ALS.

Identification of novel therapeutic targets

P5

AZYGOS 2.0, AUTOZYGOSITY MAPPING FOLLOWED BY NEXT-GENERATION SEQUENCING IN UNRELATED CONSANGUINEOUS INDIVIDUALS TO IDENTIFY NOVEL ALS-ASSOCIATED GENES

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BACKGROUND-AIM

The majority of ALS cases results from a complex interplay of genetic, environmental, epigenetic and other stochastic factors. Despite this, much of our current understanding of ALS etiopathogenesis comes from the study of Mendelian cases. Thus, the identification of novel disease genes is crucial to advance our knowledge on ALS.
The AZYGOS project aims to perform autozygosity mapping followed by whole genome sequencing (WGS) on a cohort of 115 unrelated consanguineous (inbred) ALS patients of Italian descent in order to identify novel causative recessive genes. This combined approach increases the likelihood of identifying novel recessive ALS genes in loss-of-heterozygosity (LOH) regions.

METHODS

The AZYGOS project includes distinct steps: 1) performing autozygosity mapping using high-density SNP genotyping data on a selected inbred ALS cohort to identify regions in LOH; 2) performing WGS to identify homozygous variants in these regions; 3) prioritization of ALS-associated homozygous variants by in silico analysis; 4) validation of candidate variants in replication cohorts; 5) functional assays to assess the pathogenic role of the new identified genes.

RESULTS

During the first year of the project, the preliminary analysis performed on the whole cohorts showed an increased loss of heterozygosity in cases vs controls, clearly suggesting a contribution of recessive genes in ALS pathogenesis. A total of 115 inbred individuals were extracted for autozygosity mapping and WGS analysis. Standard procedures of quality controls, genomes alignment, and variant calling were adopted to avoid identification mismatches, missing or duplicated information, and false-positive calls. Regions in LOH were a) selected with no stringent parameters, b) crossed with the controls pool, and c) prioritized on ALS disease. Furthermore, WGS and alignment were performed as planned.

CONCLUSIONS

WGS data will be used to confirm and validate the first-year results.

Identification of novel therapeutic targets

P6

BOOSTING NERVE REGENERATION IN ALS BY TARGETING THE PERIPHERY_BREATH.

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BACKGROUND-AIM

Amyotrophic Lateral Sclerosis (ALS) is a spectrum of diseases with different aetiology and phenotypical heterogeneity, lacking a unifying pathogenic mechanism. Early instability and denervation of the neuromuscular junction (NMJ) are common features both in patients and in many animal models. In SOD1G93A mice, loss of functional NMJs begins before the onset of symptoms, and it is preceded by cycles of denervation/reinnervation, until the complete loss of nerve-muscle contacts. Moreover, MNs retain the regeneration competence for some time, and then lose it with disease progression. Our rationale is to support nerve regeneration at the periphery to entail benefits also at central levels, protecting MNs from cell death. We exploit a molecular axis composed by the chemokine CXCL12〈 and its receptor CXCR4 that we recently reported to be crucial for NMJ regeneration. CXCR4 is barely expressed in adult, healthy NMJs, reappearing upon acute nerve injuries representing a marker of neuronal stress and regenerative capability. Moreover, its engagement by a novel agonist, NUCC-390, allows a faster neurotransmission recovery.

METHODS

To test our hypothesis we used both behavioural tests, functional analysis and immunofluorescence.

RESULTS

i) In SOD1G93A mice, CXCR4 is expressed in nerve terminals of both fast and slow MNs in a pre-symptomatic stage, persisting longer in slow MNs and eventually disappearing with disease progression.
ii) CXCR4 is expressed at the NMJs of hFUS+/+ and TDP43Q331K mice in the early stages and in a swine model of ALS, appointing it as a unifying target for ALS therapy.
iii) NUCC-390 treatment shows beneficial effects in motor performance of SOD1G93A mice, preserving innervation and NMJ integrity.

CONCLUSIONS

These results prompt us to propose CXCR4 as a common unifying target for ALS, and NUCC-390 as a powerful compound to support NMJ plasticity and regenerative capability to counteract MN death.

Special thanks to AriSLA for founding this research.

Identification of novel therapeutic targets

P7

EFFECTS OF THERAPEUTIC HYPOTHERMIA IN AN ANIMAL MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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BACKGROUND-AIM

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative syndrome that causes motor neuron degeneration and muscle atrophy. Despite the huge knowledge on the putative molecular mechanisms driving disease, there are no cures or effective treatments for ALS. Therapeutic hypothermia is a neuroprotective intervention employed routinely in a variety of clinical conditions. Induction of hypothermia has also been shown to rescue neurological signs in experimental models of stroke, spinal cord trauma and spinal muscular atrophy. The mechanisms of action of therapeutic hypothermia are diverse, including protection against excitotoxicity, oxidative stress, proteinopathy, cell death, and inflammation.
ALS is a multifunctional syndrome in which neuroinflammation, oxidative stress and excitotoxity are relevant pathogenic mechanisms, consistently identified in patients and animal models. We therefore investigated whether therapeutic hypothermia had a protective effect on the ALS mouse model $SOD1^{G93A}$.

METHODS

We induced hypothermia pharmacologically in $SOD1^{G93A}$ mice combining the effect of 5'AMP on energy metabolism with low temperature. We tested three different schedules of treatment and selected the one with the highest neuroprotective effect to investigate the clinical outcome in the animal model in a preclinical trial.

RESULTS

Although a clear motor neuron protection was achieved, we observed only a small delay in disease onset, a moderate increase both in survival and motor behavior. The hypothermic treatment in $SOD1^{G93A}$ mice reduced inflammation but promotes the impairment of autophagy, with a consequent increase of protein aggregation and muscle denervation.

CONCLUSIONS

In conclusion, hypothermic treatment in $SOD1^{G93A}$ mice showed both protective and harmful effects. Although there is a consistent protection of motor neurons, this does not seem to be sufficient to counteract the effect of the impaired autophagy, which translates only into a mild clinical efficacy.

Identification of novel therapeutic targets

P8

HUMAN IN VITRO MODELS OF TDP-43 PROTEINOPATHY FOR DRUG SCREENING APPROACHES

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BACKGROUND-AIM

Aggregates of phosphorylated TDP-43 protein in the cytoplasm of neurons are an ALS neuropathological hallmark. Response to stress and formation of stress granules (SGs) have been proposed as initiators of TDP-43 pathological aggregation. We previously showed that chronic oxidative stress by arsenite (ARS) induces the formation of SGs and phospho-TDP-43 aggregates in primary fibroblasts and iPSC-motor neurons from ALS patients.
Aim of our study was to generate a robust and reproducible in vitro model of TDP-43 pathology to be used for drug screening.

METHODS

We induced a chronic oxidative insult in human neuroblastoma SK-N-BE cells by exposure to low doses of ARS for 9-24 hours.

RESULTS

Our data showed TDP-43 mislocalization from the nucleus to the cytoplasm in both a dose- and time-dependent manner and increase of P62. We also observed a defective splicing activity of TDP-43 towards its target genes UNC13A and POLDIP3, a readout of TDP-43 nuclear loss-of-function, upon chronic ARS treatment. We tested candidate drugs involved in promoting autophagy, namely rapamycin, lithium carbonate and metformin in our in vitro model of TDP-43 proteinopathy. Only rapamycin was able to rescue ARS-induced loss of TDP-43 splicing activity on its target genes and to reduce P62 accumulation.
We then tested rapamycin in C9ORF72 patient-derived fibroblasts and iPSC-motor neurons, where its efficacy in rescuing ARS-induced loss of TDP-43 splicing activity was confirmed. Rapamycin also significantly reduced ARS-induced phospho-TDP-43 aggregates and SGs formation.

CONCLUSIONS

In conclusion, we have set up human cell models of TDP-43 pathology in which rapamycin was proven to be beneficial in rescuing chronic oxidative stress-induced alterations in TDP-43 splicing activity and cytoplasmic mislocalization by modulating autophagy. Human SK-N-BE and ALS patient-derived cells chronically treated with ARS can therefore be exploited as valuable in vitro platforms for future drug screening approaches.

Identification of novel therapeutic targets

P9

INHIBITION OF CLASS I HISTONE DEACETYLASES AMELIORATES TDP-43 PATHOLOGY IN EXPERIMENTAL MODELS OF ALS

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BACKGROUND-AIM

TDP-43 pathology is a neuropathological hallmark in 97% of ALS and in 50% of FTD patients. Cyclophilin A (PPIA), a foldase and molecular chaperone, once acetylated, is a functional interacting partner of TDP-43. PPIA-/- mice develop an FTD-ALS phenotype with TDP-43 pathology. Moreover, circulating lymphomonocytes of ALS patients, which also display TDP-43 pathology, show a reduction in acetyl-PPIA and an impaired PPIA/TDP-43 interaction, indicating acetylation as a potential pharmacological target to be hit using histone deacetylase inhibition.

METHODS

We investigated the effect of an array of histone deacetylase inhibitors (HDACis) on TDP-43 pathology models, focusing on inhibitors that target single HDAC or some specific classes.
First, we performed an HDACi screening in a cellular model where TDP-43 mislocalization, fragmentation and aggregation were induced by a chronic nutrient starvation protocol.
Next, we treated the homozygous TDP-43$^{Thy1/Thy1}$ mouse, a model with a severe ALS phenotype, with the most effective HDACi in vitro to evaluate its therapeutic efficacy and effect on TDP-43 pathology in vivo.

RESULTS

The class I HDAC inhibitor is effective in reducing TDP-43 pathology in the cellular model in a dose-dependent manner.
In TDP-43Thy1/Thy1 mice, the HDAC inhibition attenuates TDP-43 pathology, neuroinflammation and denervation, with a strong reduction of Nf-L levels at the early stage of disease; this is associated with a mild delay in symptom onset, as detected by body weight monitoring. However, the effect of the treatment on TDP-43 pathology is not maintained during disease progression.

CONCLUSIONS

Class I HDACi reduces TDP-43 pathology both in the cellular model and in mouse model at an early stage of disease, where it induces a neuroprotective effect and a mild delay in symptom onset. As disease progresses, the effect on TDP-43 pathology is lost, even if the reduction in Nf-L levels is still present, indicating class I HDAC inhibition as a potential pharmacological approach for ALS.

Identification of novel therapeutic targets

P10

INVESTIGATING BASAL MITOPHAGY IN ALS: FROM FLIES TO HUMAN NEURONS

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BACKGROUND-AIM

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder that induces loss of motor neurons in the spinal cord and brain, leading to skeletal muscle atrophy. Pathogenic ALS proteins seem to cause impaired transcription and RNA processing, protein aggregation, impaired autophagy and mitophagy, oxidative stress and neuroinflammation. One of these proteins, TDP-43, is taken up by mitochondria, causing cytosolic release of damage-associated molecular patterns (DAMPs) that triggers neuroinflammation and neurodegeneration. Approaches that boost basal mitophagy are therefore expected to clear damaged mitochondria and prevent neuroinflammation and cell death. We can enhance basal mitophagy in cells by inhibiting the proteasome-associated deubiquitinating enzyme USP14. Genetic and pharmacological inhibition of USP14 restores mitochondrial function and ultrastructure of two Drosophila models of mitochondrial dysfunction. In these models, USP14 inhibition extends lifespan and rescues locomotor defects of these flies, presumably by boosting mitophagy.

METHODS

We will use selective inhibitors of USP14 to exploit the mitophagic effect of USP14 inhibition. We will measure mitophagy in vivo in the fly brain of Drosophila ALS models by fluorescent-based approaches (mitoQC and mtKeima). We will address mitochondrial structure and function by TEM analysis and mitochondrial respiration efficiency.

RESULTS

Our preliminary results show that dTDP-43 KO mutant develops locomotor defects in climbing ability. We also observed abnormal mitochondrial morphology in the muscle of the flies' thorax.

CONCLUSIONS

Starting from these observations, we want to investigate whether inhibition of USP14 rescues the pathological phenotype of Drosophila models of ALS, and specifically in TDP-43 gain and loss of function models. If the aims of this proposal will be achieved, we will clarify whether inhibition of USP14 can be considered a druggable target for ALS.

Identification of novel therapeutic targets

P11

KIF5A EXON 27 SPLICING MUTATIONS: MOLECULAR ANALYSIS AND SPLICING CORRECTION WITH MODIFIED U1 SNRNAS

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BACKGROUND-AIM

In the human kinesin family member 5A (KIF5A) gene, exon 27 splicing mutations represent a genetic cause of amyotrophic lateral sclerosis (ALS). The defective ⊗Exon27 motor protein has an altered C-terminal tail and is neurotoxic. Since gain-of-function is the underlying disease mechanism, classical substitutive gene therapies are not beneficial.

METHODS

To eliminate the ⊗Exon27 mRNA, we investigated the exon 27 skipping mechanism in seven mutants and evaluated a splicing correction strategy based on modified U1 snRNAs (named ExSpeU1). ExSpeU1s are novel molecules for splicing correction effective and safe in mouse models when delivered by AAV vectors (SMA and Familial Dysautonomia).

RESULTS

In the normal KIF5A gene, analysis of splicing intermediates showed that intron 26 is preferentially removed before intron 27 and that intronic, but not exonic enhancers define the 28bp-long microexon 27. Six ALS exon skipping mutations interfere with both intron 26 and 27 splicing intermediates whereas one (3020 G>A) blocked intron 27 splicing only. We have identified active ExSpeU1s molecules that restore the first splicing intermediate improving intron 26 splicing. This effect on intron 26 resulted in complete rescue of the exon 27 skipping defect and production normal amount of spliced mRNA in four out of seven mutants.

CONCLUSIONS

We have identified a novel strategy based on ExSpeU1 that improving the intron 26 spliced intermediate eliminate the ⊗Exon27 mRNA in four out of seven KIF5A mutants. ExSpeU1 targeting specific KIF5A mutations might represent a novel therapeutic strategy for splicing precision medicine in ALS.

Identification of novel therapeutic targets

P12

MONTELUKAST COUNTERACTS PATHOLOGICAL GPR17 UPREGULATION, OLIGODENDROCYTE DYSFUNCTION AND DELAYS DISEASE PROGRESSION IN SOD1G93A AMYOTROPHIC LATERAL SCLEROSIS FEMALE MICE

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BACKGROUND-AIM

Recent findings revealed an early role of oligodendrocyte (OL) dysfunction in promoting MN degeneration and ALS disease progression. On this basis, restoring proper myelination and trophic support to MNs by fostering oligodendrocyte precursor cell (OPC) maturation may open new therapeutic perspectives. Results from our previous pilot project revealed that an abnormal increase of GPR17 expression, an important regulator of OPC differentiation, is associated to OL dysfunction in the spinal cord of the SOD1G93A murine model of ALS. Accordingly, primary OPCs isolated from SOD1G93A mice displayed maturation defects, which were rescued by in vitro exposure to the non-selective GPR17 antagonist montelukast (MTK). Here, we evaluated the effects of the in vivo oral administration of MTK in SOD1G93A mice.

METHODS

Male and female SOD1G93A mice and wild-type controls were orally treated with MTK (30 mg/kg/day) or vehicle from early symptomatic stage (P90) until end stage. The effects of MTK on survival probability (Kaplan-Meier analysis), body weight loss, and motor functions (motor coordination, motor skills, and muscle strength) were analyzed in a gender-specific manner. Immunohistochemistry and confocal microscopy were employed to evaluate the impact of MTK on oligodendrocyte maturation, microglia activation, and astrogliosis in the ventral lumbar spinal cord gray matter.

RESULTS

MTK treatment was found to significantly increase survival probability, delay body weight loss, and ameliorate motor functionality of female SOD1G93A mice, while no effects were observed in males. In parallel, MTK significantly counteracted the pathological GPR17 upregulation in the spinal cord of female SOD1G93A mice, improving OL differentiation into CC1+ mature cells, and markedly fostered the shift of microglia and astrocytes toward pro-regenerative traits.

CONCLUSIONS

Globally, these data support the relevance of a GPR17-based pharmacological approach for ALS treatment.

Identification of novel therapeutic targets

P13

NATURAL KILLER CELLS MODULATE MOTOR NEURON-IMMUNE CELL CROSS TALK IN MODELS OF AMYOTROPHIC LATERAL SCLEROSIS

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BACKGROUND-AIM

In amyotrophic lateral sclerosis (ALS), immune cells and glia contribute to motor neuron (MN) degeneration. Here, we investigate the functional role of NK cells in disease onset and progression in the hSOD1G93A mice.

METHODS

Starting at 8 weeks of age, male and female hSOD1G93A, TDP43A315T or not transgenic C57BL/6 J mice were randomly grouped for the treatments. NK cell depletion was performed using a blocking Ab against NK1.1, which recognizes an epitope of the NKR1Pc-activating receptor (PK136). Mice were i.p. injected with 200 ⌈g (in 100 ⌈l) of anti-NK1.1 Ab every 2 days the first week, every 4 days the second week and then repeated once a week until the age described in the text or until sacrifice for the survival analysis experiments. NK cell depletion from the blood sample was monitored by FACS.

RESULTS

We report the presence of NK cells in post-mortem ALS motor cortex and spinal cord tissues, and the expression of NKG2D ligands on MNs. Using a mouse model of familial-ALS, hSOD1G93A, we demonstrate NK cell accumulation in the motor cortex and spinal cord, with an early CCL2-dependent peak. NK cell depletion reduces the pace of MN degeneration, delays motor impairment and increases survival. This is confirmed in another ALS mouse model, TDP43A315T. NK cells are neurotoxic to hSOD1G93A MNs which express NKG2D ligands, while IFN© produced by NK cells instructs microglia toward an inflammatory phenotype, and impairs FOXP3+/Treg cell infiltration in the spinal cord of hSOD1G93A mice.

CONCLUSIONS

Altogether, our data support the critical role of innate immunity in ALS-associated neurodegeneration and demonstrate the neurotoxic properties of NK cells in the hSOD1G93A mice model. Targeting NK cells could revert the vicious cycle among NK cells (producing IFN©) and neurons (producing CCL2), re-boosting the homeostatic functions of microglia and sustaining Treg cell recruitment in the spinal cord. Our results point to innate immunity as a key co-factor in this neurodegenerative disease.

Identification of novel therapeutic targets

P14

NICLOSAMIDE AMELIORATES DISEASE PROGRESSION IN MICE MODELS OF ALS

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BACKGROUND-AIM

The multifactorial nature of ALS could explain the modest results obtained by the treatments proposed so far and highlights the need for multitarget therapies acting synergistically on different aspects of the disease. Niclosamide is on the WHO list of essential medicines, and it has recently been repurposed in clinical trials for its potent anti-inflammatory and anti-fibrotic properties. It is well documented that niclosamide can inhibit different molecular pathways (e.g., STAT3, NF-KB), which are dysregulated in ALS, suggesting its potential use to interfere with these mechanisms in the pathology. We found that niclosamide inhibits microglia reactivity, reduces inflammatory and fibrotic pathways, and promotes autophagy in familial and sporadic ALS fibroblasts. Further, in a proof-of-concept study conducted in a FUS mouse model, niclosamide strongly inhibits inflammation and promotes autophagy in the nervous system and regeneration in skeletal muscles. This work aims to perform a preclinical validation of the drug in two models that recapitulate ALS key pathologies and biological processes, i.e., wild-type FUS and SOD1-G93A mice.

METHODS

We injected niclosamide intraperitoneally at symptom onset and evaluated mice disease progression and pathogenic mechanisms targeted by niclosamide on ALS-affected tissues.

RESULTS

We demonstrated that niclosamide ameliorates neuromuscular deficits and behavioral scores, increasing mice disease duration and survival. In the spinal cord of FUS mice, niclosamide decreases motoneuron loss, axonal damage, and FUS accumulation in motoneurons; moreover, it decreases microgliosis and immune cell infiltrates, increasing blood-spinal cord barrier integrity. Finally, in treated FUS mice, niclosamide decreases the proinflammatory and autophagic markers mTOR, STAT3, and NF-KB.

CONCLUSIONS

These data suggest that niclosamide could be a promising candidate to be deeply investigated as a drug repurposing strategy for ALS.

Identification of novel therapeutic targets

P15

NOVEL FUNCTIONALIZED NANOPARTICLES TARGETED TO 18KDA TRANSLOCATOR PROTEIN (TSPO) TO TRACK AND MODULATE NEUROINFLAMMATION IN ANIMAL MODELS OF FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS

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BACKGROUND-AIM

Neuroinflammation is recognized as a pathological hallmark and potential therapeutic target for many neurodegenerative diseases including ALS. However, neuroinflammatory responses are heterogeneous and reflect not only the extent of neuronal demise but also variable engagement of glial cells in the attempt to cope with the neuronal damage. Thus, new pharmacological tools targeting specific cell subpopulations are warranted.

We exploited TSPO ligands, already widely used in the clinic to track neuroinflammation through PET, as a strategy to achieve selective cell targeting via a novel theranostic platform based on MRI/PET traceable nanoparticles (NPs).

METHODS

We performed in-situ hybridization (ISH) and immunohistochemistry (IHC) experiments to investigate the expression and distribution of TSPO in the CNS of transgenic SOD1(G93A) rat model of ALS, which recapitulates the heterogeneous disease manifestations observed in patients. In parallel, we developed and validated novel polymeric NPs functionalized with TSPO-ligands.

RESULTS

ISH and IHC experiments highlighted a selective increase of TSPO protein expression in reactive microglia after disease onset and correlation with the extent of neuronal demise in different spinal cord districts of SOD1.G93A rats. In parallel, we successfully generated NPs functionalized with PBR-28 TSPO-selective ligand and demonstrated TSPO-dependent cellular internalization in cell cultures.

CONCLUSIONS

We confirmed by ISH/IHC a clearcut upregulation of TSPO in microglia cells in the CNS areas most severely affected by the disease. Functionalization of NPs with two TSPO-selective PET tracers (PBR-28, PK11195) determined a TSPO-dependent NPs internalization in microglia cells both in vitro and in vivo. Based on these results, we launched a proof-of-concept preclinical study (in progress) to test the therapeutic potential of NPs targeting the NF-kB proinflammatory pathway.

Identification of novel therapeutic targets

P16

NOVEL INSIGHTS ON THE ROLE AND THERAPEUTIC POTENTIAL OF GLYCOPROTEIN NONMETASTATIC MELANOMA PROTEIN B (GPNMB) IN AMYOTROPHIC LATERAL SCLEROSIS.

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BACKGROUND-AIM

Increased levels of a peptide derived from Gpnmb in the cerebrospinal fluid (CSF) were recently associated with a poor prognosis in patients affected by Amyotrophic Lateral Sclerosis (ALS). On the other hand, other studies highlighted that upregulation of Gpnmb could play a neuroprotective and immunomodulatory role. In this study we engaged an in-depth characterization of Gpnmb alterations in SOD1.G93A transgenic (TG) rat model of ALS and in patients, to clarify the value of Gpnmb as a prognostic biomarker and to identify a precise time-window, during the disease process, suitable for successful therapeutic intervention.

METHODS

We applied in-situ hybridization (ISH) and immunohistochemistry (IHC) in the central and peripheral nervous system, coupled to the assessment of Gpnmb ectodomain (GpnmbE) in the CSF and blood of TG rats. In parallel, GpnmbE was assessed in a small cohort of ALS patients.

RESULTS

Gpnmb is mainly expressed in MNs in healthy conditions. However, in TG animals there is an early decrease of Gpnmb mRNA and protein levels in MNs and upregulation in reactive microglia after symptom onset. ISH and IHC highlighted a critical role for glial cells in the synthesis and release of GpnmbE. In parallel, we spotted a significant increase of GpnmbE in the CSF and blood of TG rats, as well as in ALS patients, when the pathology is more severe.

CONCLUSIONS

Based on this evidence, we think that Gpnmb could be a part of an insufficient or too delayed response to the neuroinflammatory condition underlying ALS disease. We are currently running a preclinical proof of concept study to verify the therapeutic potential of early administration of recombinant Gpnmb while monitoring GpnmbE as biomarker of target engagement.

Identification of novel therapeutic targets

P17

SWITCHALS - THERAPEUTIC CORRECTION OF ALTERNATIVE SPLICING DEFECTS IN HNRNP A2/B1 AS A WAY TO COUNTERACT AMYOTROPHIC LATERAL SCLEROSIS ASSOCIATED WITH FUS

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BACKGROUND-AIM

Converging evidence indicates that alternative splicing dysregulation has a crucial role in the pathogenesis of Amyotrophic Lateral Sclerosis (ALS) associated with mutations in FUS. Data from our lab demonstrate that a major target of FUS is hnRNPA2/B1, an RNA-binding protein with key roles in RNA metabolism, including splicing regulation. Indeed, mice expressing human wtFUS, which develop an ALS-like disease, are characterized by the progressive skipping of A2/B1 exon 9 during the disease course, leading to the cytoplasmic accumulation of exon 9-lacking isoforms with toxic features that might, in turn, promote motor neuron degeneration.
The general aim of this project is to define a strategy using splicing switching oligonucleotides (SSOs) to correct splicing defects in A2/B1 as a way to counteract FUS-ALS neurodegeneration. SSOs are short synthetic single-stranded nucleic acids that bind splicing regulatory sequences and sterically impede the binding of positive/negative splicing factors, thus modifying the skipping or inclusion of an exon into mature RNA.

METHODS

The appropriate regulatory sites on A2/B1 pre-mRNA to be targeted by SSOs will be identified using both web prediction tools and in vitro deletion analysis. SSOs annealing these sequences will be analysed by semi-quantitative PCR for their ability to modify A2/B1 splicing pattern in vitro. Finally, the most effective SSOs will be tested in FUS-ALS mice to assess their efficacy to modify the disease progression.

RESULTS

Potential splicing regulatory sites have been identified in silico and complementary SSOs have been designed and are under testing in vitro for their ability to modify the splicing pattern of both endogenous A2/B1 pre-mRNA and exogenous minigene containing exon 8-9-10 and inner introns.

CONCLUSIONS

This project will test whether A2/B1 mis-splicing has a direct role in FUS-ALS pathogenesis in vivo, and will provide support to the hypothesis that correction of this defect by SSOs is a therapeutically applicable approach.

Identification of novel therapeutic targets

P18

TARGETING UPPER MOTOR NEURONS: A NOVEL NEURAL STEM CELL THERAPY FOR AMYOTROPHIC LATERAL SCLEROSIS

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BACKGROUND-AIM

Amyotrophic Lateral Sclerosis is a fatal neurodegenerative disease that leads to progressive degeneration of both upper (UMNs) and lower motor neurons (LMNs), resulting in muscle atrophy and paralysis, with no effective therapy nowadays. To date, most studies have focused on LMNs, but recently it has been demonstrated that UMNs hold equal importance in the pathogenesis of the disease and could be targeted to protect the motor cortex but also to extend the possible positive influence on the whole MN circuitry. Here we investigate the mechanism both at phenotypical and molecular level of a novel therapeutic approach based on the transplant of a specific subset of human neural stem cells (NSC) derived from cerebral organoids within central nervous system of a murine ALS model, through intracerebroventricular (ICV) administration or directly into the cortex.

METHODS

Behavioral analyses were performed to assess the beneficial effect of the treatment. To further assess the mechanism of the treatment beneficial effect, bulk-sequencing was performed on total RNA extracted from cortical motor area sections of cortex-treated and untreated animals, and the resulting data were subdivided in human-derived and mouse-derived genes.

RESULTS

The behavioral analyses show improved outcomes in cortex-treated animals compared to ICV-treated and untreated mice. In the human dataset, corresponding to engrafted cells, genes encoding for secreted proteins that could potentially rescue the pathological phenotype have been investigated through DeepLoc, a machine learning model, individuating seven candidates: TAC1, HNRNPA2B1, HMGB1, GNAS, ADCYAP1, FBLN1 and CA10. In the endogenous mouse dataset 74 differentially expressed genes were found between treated and untreated groups.

CONCLUSIONS

Overall, these data point out the potential beneficial effect associated to the transplant of NSCs in targeting UMNs and allow to gain more insight into the molecular mechanisms behind this therapeutic approach.

Identification of novel therapeutic targets

P19

THE P97-NPLOC4 ATPASE COMPLEX PLAYS A ROLE IN MUSCLE ATROPHY DURING CANCER AND AMYOTROPHIC LATERAL SCLEROSIS

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BACKGROUND-AIM

The p97 complex participates in the degradation of muscle proteins during atrophy upon fasting or denervation interacting with different protein adaptors. We investigated whether and how it might also be involved in muscle wasting in cancer or amyotrophic lateral sclerosis (ALS), where motoneuron death causes muscle denervation and fatal paralysis.

METHODS

As cancer cachexia models, we used mice bearing colon adenocarcinoma C26, human renal carcinoma RXF393, or Lewis lung carcinoma, with breast cancer 4T1-injected mice as controls. As ALS models, we employed 129/SvHsd mice carrying the mutation G93A in human SOD1.

RESULTS

The mRNA levels of p97 were induced in tibialis anterior (TA) of all the cachectic models but not in the non-cachectic 4T1-mice. Similarly, p97 was high both in mRNA and protein in TA from 17-week-old SOD1G93A mice. Electroporation of a shRNA for murine p97 in TA muscle reduced the fibre atrophy caused by C26 or ALS. When we interrogated a microarray, we had previously generated for the expression of p97 adaptors, we found Derl1, Herpud1, Nploc4, Rnf31, and Hsp90ab1 induced in cachectic TA from C26-mice. By qPCR, we validated their inductions in TA of cachectic and ALS models and selected Nploc4 as the one also induced at the protein level. Electroporation of a CRISPR/Cas9 vector against Nploc4 into muscle reduced the fibre atrophy caused by C26 or ALS. Because Disulfiram (DSF) uncouples p97 from Nploc4, we treated atrophying myotubes with DSF, and found accumulated mono and polyubiquitinated proteins and reduced degradation of long-lived proteins, including actin. DSF halves Nploc4 in the soluble muscle fraction and given to C26-bearing mice limited the body and muscle weight loss, with no effect on tumour growth.

CONCLUSIONS

Overall, cancer cachexia and ALS seem to display similar mechanisms of muscle wasting at least at the catabolic level. The p97-Nploc4 complex appears to have a crucial role in muscle atrophy and disrupting this complex might serve as a novel drug strategy.

Identification of novel therapeutic targets

P20

THERAPEUTIC EFFECTS OF RETROMER STABILIZATION IN AMYOTROPHIC LATERAL SCLEROSIS

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BACKGROUND-AIM

Cytoplasmic protein aggregation and misfolding are key pathological features of several neurodegenerative diseases, including Amyotrophic Lateral Sclerosis (ALS). We propose the retromer's dysfunction as a key player in the reduced protease-driven degradation of aggregates in the endosomal–lysosomal system. We set up two different strategies to achieve the retromer stabilization thus rescuing motor-neurons (MNs) functionality: (1) investigating new guanyl hydrazone (GH)-based retromer stabilizer promoting aggregates degradation and delivery of missing retromer component;(2) inducing VPS35 gain of function via AAV9-VPS35-gene therapy in SOD1-g93a (SOD1) mice.

METHODS

(1)Cell cytotoxicity of new GHs-compounds were in vitro evaluated by CCK8 and LDH-assay in N2a cell-line. The expression levels of VPS35 were evaluated by Western Blotting and Immunofluorescence assay in GHs-treated N2a cells. (2) The AAV9-CAG2-EGFP [2.8x109 GC/µl] or AAV9-CAG2-VPS35 [1x1010 GC/µl] viruses were injected in intra-cisterna magna (ICM) at postnatal day 65 (P65) in WT and SOD1 mice and tissues harvested at postnatal day 100 (P100). The expression of Choline Acetyltransferase (ChAT) was evaluated by immunohistochemistry (IHC) in anterior brain sections.

RESULTS

From extensive in-vitro profiling of ten GHs-compounds, we selected DN48 as the lead able to significantly increase VPS35 expression, thus stabilizing the retromer complex in N2a cells. In SOD1 mice we observed a significantly increased ChAT expression in MNs belonging to the cranial nerve 7 and to the vagus nerve compared to WT mice upon AAV9-VPS35-mediated gene therapy.

CONCLUSIONS

We selected DN48 lead as the retromer stabilizer that will be further tested in ALS-mouse model. In SOD1-AAV9-VPS35-injected mice the VPS35 levels are significantly enhanced, suggesting an increase in the survival of MNs. These two approaches will lead us to move the GH-compound and AAV9-VPS35 gene therapy into a clinical setting.

Identification of novel therapeutic targets

P21

OMIC CHARACTERIZATION OF PATIENT-DERIVED SPINAL CORD ORGANOIDS TO UNRAVEL NEW THERAPEUTIC TARGETS IN C9ORF72 FORM OF AMYOTROPHIC LATERAL SCLEROSIS

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BACKGROUND-AIM

Amyotrophic lateral sclerosis (ALS) is a rare neurodegenerative disorder involving motor neurons (MNs) in brain and spinal cord, resulting in progressive muscle atrophy and weakness that eventually compromise diaphragm functionality. No efficacious treatment is currently able to halt or reverse disease progression, making it mandatory to develop novel therapeutic approaches that would improve the lives of the patients. The objectives of this work included the characterization of spinal cord organoids (spOrg) to refine the reliability and reproducibility of the differentiation protocol, as well as to delineate the ALS phenotype with omic techniques; over and above, the selection of ALS-related candidates from the outlined transcriptomic and proteomic profiles.

METHODS

In the study of pathophysiology, iPSC-derived 3D models are a promising powerful tool that can recapitulate the complex architecture of tissues in a more accurate manner than 2D cultures.

RESULTS

Our spOrgs displayed neural progenitors that progressively decreased, post-mitotic neurons, MNs, and glia. Organoids were collected at 30, 55, and 80 days in vitro (DIV) and evaluated for their morphology and neurodevelopmental features by IHC and qPCR. Specifically, DIV80-spOrgs expressed SOX2, ISL1, SMI32, TUJ1, MAP2, DCX, OLIG2, PAX6, HOXB4, GFAP, and S100®. Besides astrogliosis, the C9 condition interestingly showed PRPH aggregation, as described in literature. Mass spectrometry and gene ontology depicted an enrichment in pathways related with cytoskeletal coordination, astrocyte reactivity, stress response, and SUMOylation in the C9-ALS condition. Single-cell RNA sequencing and gene annotation disclosed the predominance of neural cell populations in the samples, remarking the potential of this disease model; in addition, C9-spOrgs appeared more immature when compared to controls.

CONCLUSIONS

Overall, this project might allow the assessment of novel candidate genes linked with C9ORF72-ALS pathogenesis and their potential as therapeutic targets.

Pathological mechanism in ALS

P22

MODELLING ALS DISEASE BY 3D ORGANOIDS CULTURE FROM HUMAN- DERIVED IPSCS

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BACKGROUND-AIM

Generation of cerebral organoids from human induced pluripotent stem cells (hiPSCs) has opened new possibilities to investigate brain development in vitro as regards not only neural cell differentiation, but also neuro-glial cell interactions and 3D organization.
Recently, heterozygous loss-of-function (LOF) mutations in the mitotic protein kinase NEK1 gene were identified in ALS patients. However, the pathomechanisms whereby NEK1 LOF leads to neurodegeneration have not been investigated so far.
Aim of our study was to study the biological effect of ALS-related NEK1 LOF mutations in hiPSCs-derived cerebral organoids.

METHODS

We used CRISPR/Cas9 to engineer a heterozygous LOF mutation in a healthy control hiPSCs line and generated organoids following a validated protocol for up to 140 days of differentiation and characterized them.

RESULTS

Immunofluorescence analysis performed on whole organoids at 26 days of differentiation revealed positivity for specific markers of pluripotency and proliferation (SOX2, NESTIN and Ki67), which progressively decreased at later differentiation time points (100-120-140 days).
We observed the expression of markers typical of different neuronal maturation stages such as ßIII-tubulin, double cortin, NeuN and MAP2 as well as the expression of the dopaminergic neuron marker TH. Interestingly, at day 100, organoids expressed also GFAP, marker of astrocytes differentiation. No differences were observed between the mutated NEK1 and the isogenic control organoids in the expression of these neuro-glial markers. Organoids have also been analyzed by electron microscopy to better determine their sub-cellular ultrastructure.

CONCLUSIONS

The cerebral organoids we generated represent a suitable 3D disease model for ALS where the impact of NEK1 haploinsufficiency on different cell pathways associated to DNA damage repair, mitochondria functionality and ciliogenesis can be investigated. Moreover, this human 3D disease model can be exploited also for future pharmacological approaches.

Pathological mechanism in ALS

P23

SPINAL CORD ORGANOIDS FROM SALS PATIENTS SHOW IMPAIRMENT IN MATURATION AND SELF-ORGANIZATION

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BACKGROUND-AIM

ALS is a non-cell autonomous disorder as many cell types contribute to motor neurons death, although the exact pathogenesis is still unkown. The lack of curative treatments is due also to the absence of realistic experimental models. Organoids are pluripotent stem cell-derived self-organizing structures that allow in vitro generation of the tissues. Here we present a new method for the generation of spinal cord organoids (SCOs) with the aim of ALS pathogenesis modeling.

METHODS

iPSCs obtained from healthy controls and sALS patients have been differentiated into neural stem cells (NSCs). NSCs were cultured in floating conditions using an orbital shaker and differentiated in into motor neuron progenitors, immature motor neurons (MNs) and finally mature MNs. SCOs formation and growth was characterized by phase-contrast and confocal microscopy. Finally, we performed bulk RNA-seq analysis.

RESULTS

SCOs derived from sALS were smaller and with irregular morphology compared to healthy controls. By investigating the GFAP marker distribution, sALS SCOs show a thicker glial layer compared to healthy controls. Moreover, sALS SCOs show longer neurites when compared to healthy controls. By RNAseq, a ten-fold increase of deregulated gene in SCOs respect to 2D cell models was found. Moreover, sALS SCOs show an extensive deregulation of genes involved in extracellular matrix organization when compared to controls. Deconvolution analysis highlighted that healthy controls SCOs contain a higher amount of neuronal differentiated cells compared to sALS SCOs.

CONCLUSIONS

Taken together, our data suggest that SCOs show the typical pathological hallmarks of the pathology, such as the presence of neural cells, the smaller length of neurites, decreased level of mature MNs, and deregulation of genes implicated in ALS. In conclusion, SCOs could represent a promising tool for the investigation of pathogenic mechanisms of ALS.

Pathological mechanism in ALS

P24

ONE MOTOR NEURON AT THE TIME: UNCOVERING THE MOLECULAR LOGICS FOR SUBTYPE-SPECIFIC DISEASE VULNERABILITY

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BACKGROUND-AIM

Alpha motor neurons (αMNs) are specialized neurons that reside in the brainstem and spinal cord, and extend axons outside of the central nervous system (CNS) to target muscle and enable contraction. Diseases and insults resulting in αMNs are rapidly debilitating and often fatal. Motor neuron diseases, such as ALS, are characterized by the selective vulnerability of αMNs, and in particular of fast fatigable (FF) MNs whilst other MN types stay functional, even at late stages of the disease. The molecular reasons beyond this selective vulnerability are not known, hampering the discovery of molecular candidates for treatment. This requires a more comprehensive understanding of neuronal subtype-specific molecular profiles than currently available.

METHODS

To contribute in filling this important gap of knowledge and gain a full understanding of the molecular substrate of MN vulnerability, we profile at the single cell level the transcriptome of MNs isolated from the murine spinal cord without employment of genetic labelling, but rather leveraging on the specific expression of nuclear marker.

RESULTS

We were able to successfully identify and isolate molecularly identified neurons, including αMNs, without employment of genetic labelling. Within those, we recognised subclasses of neurons, including FF-MNs.

CONCLUSIONS

This aims at revealing subtype-specific differences that contribute to selective vulnerability of FF-MNs.

Pathological mechanism in ALS

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A KNOCKOUT ZEBRAFISH LINE FOR ALS2 GENE AS A NEW IN-VIVO MODEL FOR JUVENILE AMYOTROPHIC LATERAL SCLEROSIS

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BACKGROUND-AIM

Many increasing lines of evidence support the idea that the maintenance of motoneuron homeostasis as well as the activity of several amyotrophic lateral sclerosis (ALS)-related genes are correlated with the activation of signalling pathways involved in axonal and synaptic development such as Wnt, Bmp, Tgf-ß, Shh and Notch. On this basis, the project was aimed at verifying whether the juvenile form of the disease (JALS) may present alteration of these highly conserved signalling pathways.

METHODS

Mosaic and stable ALS2 KO zebrafish mutants were generated by CRISPR/Cas 9-mediated mutagenesis and analysed through different experimental strategies ranging from phenotypical analysis, in-vivo optical imaging as well as molecular tests.

RESULTS

As first step, survival rate and swimming capacity of the ALS2 crispant embryos were monitored but no significant differences were found compared to matched control animals. Moreover, applying the same CRISPR/Cas 9 protocol against the ALS2 genes, the activity of BMP, Wnt, Shh, Notch and Tgf-ß was assessed using specific fluorescent transgenic lines. Interestingly, ALS2 crispant embryos showed a down-regulation of the Wnt signalling pathway during the embryonic development. In accordance, expression of c-Myc, a Wnt-related gene, showed a similar tendency in stable ALS2 KO animals, which were also characterized by behavioural and morphological alterations together with an increased ER stress.

CONCLUSIONS

In conclusion, here we report the generation and characterization of the first zebrafish JALS model in which the ALS2 silencing resulted to be associated with an activation of the ER-stress pathway and the reduction of Wnt activity. Since the alteration of both these pathways have been independently associated with different ALS variants, the new ALS2 KO zebrafish mutant can become an innovative and useful model to uncover pathogenetic mechanisms that, in future, could represent potential targets of therapeutic intervention for JALS and for other forms of ALS.

Pathological mechanism in ALS

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ASSOCIATION WITH NEURONAL DEVELOPMENT AND ONCOGENESIS IMPLICATED-LNCRNAS AND SOD1-G93A AMYOTROPHIC LATERAL SCLEROSIS PATHOLOGY

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BACKGROUND-AIM

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disorder caused in 10% of cases by inherited mutations considered "familial". The SOD1 gene was the first identified ALS gene, and to this day approximately 18.9% of fALS cases and 1.2% of sporadic ALS (sALS) cases can be attributed to mutations in this gene There is an ever-increasing amount of evidence showing a fundamental role for RNA metabolism in ALS pathogenesis, and long non-coding RNAs (lncRNAs) appear to play a crucial role in ALS development. Here, we aim to investigate the expression of a panel of lncRNAs (linc-Enc1, linc–Brn1a, linc–Brn1b, linc-p21, Hottip, Tug1, Eldrr, and Fendrr) previously implicated in neurodevelopment and oncogenesis.

METHODS

Via Real-Time PCR, we assessed their expression in a murine familial model of ALS (SOD1-G93A mouse) in brain and spinal cord areas of SOD1-G93A mice in comparison with that of B6.SJL control mice, in asymptomatic (week 8) and late-stage disease (week 18).

RESULTS

We highlighted a specific area and pathogenetic-stage deregulation in each lncRNA, with linc-p21 being deregulated in all analyzed tissues. Moreover, we analyzed the expression of their human homologues in SH-SY5Y-SOD1-WT and SH-SY5Y-SOD1-G93A, observing a profound alteration in their expression. Our study is the first characterization of a panel of 8 lncRNAs (linc-Enc1, linc–Brn1a, linc–Brn1b, linc-p21, Hottip, Tug1, Eldrr, and Fendrr) in ALS, indicating a deregulation in all of their expression in specific ALS affected areas or at different stages of the pathogenesis.

CONCLUSIONS

Although a future functional characterization is needed in order to specifically elucidate the lncRNAs role in the pathogenesis, these molecules could be identified as new disease-modifying agents or candidates in ALS pathogenesis.

Pathological mechanism in ALS

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HUD (ELAVL4) GAIN-OF-FUNCTION PHENOCOPIES A SEVERE ALS-FUS MUTATION IN IPSC-DERIVED MUSCLE-NERVE CO-CULTURES

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BACKGROUND-AIM

We previously demonstrated that ALS-FUS mutations alter the activities of two important neural RNA-binding proteins, HuD (ELAVL4) and FMRP. Mechanistically, mutant FUS leads to upregulation of HuD by competing with FMRP for HuD 3'UTR binding. In turn, levels of HuD targets involved in axon development rise. Consequently, mutant FUS motoneurons (MNs) show increased axon growth and branching. Notably, similar phenotypes have been previously described in SOD1 and TDP-43 mutant models. Here we aimed to assess if such axonal alteration might in turn lead to defective neuromuscular junctions (NMJs). We also aimed to clarify the contribution of the altered FMRP and HuD activities to changes in the transcriptome of FUS mutant MNs.

METHODS

Human iPS cells were used to obtain co-cultures of skeletal muscle cells (SkMCs) and MNs. iPS-derived MNs were also cultured in cell culture inserts with a microporous filter to separate soma and neurites for transcripts profiling by digital color-coded molecular barcoding.

RESULTS

We found impaired NMJ formation and increased cell death by apoptosis when wild-type SkMCs were co-cultured with mutant FUS MNs. Notably, these phenotypes were strikingly similar in co-cultures containing MNs overexpressing HuD in the absence of FUS mutations. Gene expression profiling in soma and neurites of MNs showed that many genes were commonly altered upon FUS mutation or HuD overexpression. Moreover, we identified other common targets that, like HuD, were upregulated due to mutant FUS competition with FMRP for 3'UTR binding, including AP2B1 and PTEN.

CONCLUSIONS

These data suggest that in ALS MNs many effects of mutant FUS could be ascribed to HuD gain-of-function. Together with the finding that oxidative stress increased HuD levels in MNs in absence of FUS mutations, these results might be also relevant for sporadic ALS. Moreover, analysis of common FUS and FMRP targets provided new possible biomarkers and therapeutic targets for ALS.

Pathological mechanism in ALS

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INVESTIGATION OF THE LNCRNA ZEB1-AS1 IN SPORADIC ALS: DEREGULATION IN NEURONAL DIFFERENTIATION AND CHARACTERIZATION OF A NOVEL DISEASE PATHWAY

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BACKGROUND-AIM

RNA-seq data highlighting numerous deregulated long non-coding RNAs (lncRNAs) in tissues derived from sALS patients. The oncogenic lncRNA ZEB1-AS1 emerged as one of the most downregulated in peripheral blood mononuclear cells (PBMCs) of sALS patients (Gagliardi et al. 2018). The aim of this work was thus to characterize the role of the lncRNA ZEB1-AS1 in ALS pathogenesis identifying possible disease-modifying targets. The novel ZEB1-AS1 pathway was investigated in multiple patients-derived cellular models (patients-derived peripheral blood mononuclear cells and induced pluripotent stem cells-derived neural stem cells) and in the neuroblastoma cell line SH-SY5Y.

METHODS

Total RNA was extracted using TRIZOL reagent and the genes' expression levels were determined by Real Time PCR. Western Blot analysis and immunofluorescence were also performed to assess protein expression levels. Live&Dead Assay and MTT assay were performed to assess the pathway dysregulation.

RESULTS

In PBMCs, and both undifferentiated and differentiated SH-SY5Y cells silenced for ZEB1-AS1 we validated the downregulation of ZEB1-AS1's expression but we did not observe a concordant downregulation of its sense gene ZEB1. Considering down the-stream regulators of ZEB1-As1 we observed that was upregulated mir200c and BMI1 downregulated, suggesting a specific pathway for sALS. Furthermore, we also report the upregulation of BMI1's downstream mediator GSK3, which is though inactivated. The silencing of ZEB1-AS1 in SH-SY5Y did not impact on cellular viability. Moreover, we found that ZEB1 and ZEB1-AS1's levels change during neuronal differentiation, suggesting also an implication for the lncRNA in this process, where the ZEB1-AS1's silencing results in the reduction of neurite length.

CONCLUSIONS

In conclusion, our results show the implication for ZEB1-AS1's pathway in sALS with a specific role in neuronal differentiation.

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OXYGEN SENSING IN AMYOTROPHIC LATERAL SCLEROSIS: CURRENT MECHANISMS, IMPLICATION OF TRANSCRIPTIONAL RESPONSE AND PHARMACOLOGICAL MODULATION

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BACKGROUND-AIM

Amyotrophic Lateral Sclerosis (ALS) is an adult-onset neuromuscular disease characterized by a progressive degeneration of motor nerve cells in the brain and spinal cord. Oxygen sensing mechanisms in the brain are crucial to maintain tissue homeostasis and their implication in neurodegenerative disorders (NDs) are poorly studied. Thus, this work aims to review and elucidate the presence of pathways related to oxygen sensing involved in ALS.

METHODS

Public transcriptomic datasets related to ALS were explored in GEO repository. Raw FASTQ files were downloaded and reprocessed using a common pipeline to obtain comparable data. Differential expression analysis was performed with DESeq2 R package while functional enrichment analysis with g:Profiler to understand the role of oxygen sensing related pathways in ALS.

RESULTS

The data hereby presented provide a first inspection of the genes and pathways pertaining O2 sensing mechanisms in ALS. Specifically, the pathways analysis of the 187 dysregulated genes in ALS allowed to highlight those related to alterations in oxygen imbalance. It is interesting to observe that alterations in oxygen levels cause not only the dysregulation of metabolic processes but also ALS-disease-related processes such as "Dopamine receptors".

CONCLUSIONS

Our results shed a light on oxygen sensing related pathways in ALS, but also highlight the need of further studies and novel strategies to detect these altered mechanisms and their correlation with pathogenesis.

Pathological mechanism in ALS

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TRANSCRIPTOMIC CHARACTERIZATION OF ALS PHENOTYPES HIGHLIGHTS PATIENT-SPECIFIC GENE EXPRESSION PATTERNS

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BACKGROUND-AIM

A critical need in ALS research is the identification of an adequate strategy to stratify patients, and to discover reliable biomarkers. RNA-mediated toxicity is considered a key event in ALS. Regulation of gene expression represents a novel opportunity to identify specific traits in ALS subgroups. For these considerations, the classification based on specific clinical phenotypes could be associated with different gene expression patterns that are shaped during lifespan, representing a novel opportunity to identify specific ALS subtypes with homogeneous clinical and biological features.
Our objective is to identify the transcriptomic signatures of distinct ALS phenotypes, and to use this information for biomarker assessment and personal therapy development.

METHODS

We characterized 48 unmutated sALS patients by clinical and paraclinical phenotype, and subdivided them in "Classic" (n=12), "Bulbar" (n=10), "Flail Arm" (n=7), "Flail Leg" (n=10) and "Pyramidal" (n=9). Then, RNAs extracted from PBMCs isolated from patients and healthy controls (n=19) were sequenced.

RESULTS

We observed distinctive gene expression pattern of patients and controls, especially for "Flail Leg" group. Moreover, "Bulbar" group was the one showing the higher amount of DE genes, coherently with the peculiar features of this phenotype. Interestingly, we found only one gene commonly deregulated in all groups, while the rest of DE genes were phenotype-specific. Moreover, after gene enrichment analysis, we observed a strong deregulation of genes involved in inflammation-related pathway, probably due to early stage of disease when blood was collected. Thus, in order to monitor disease progression and define the diagnostic and prognostic factors, we aim to include a one year follow-up analysis.

CONCLUSIONS

In conclusion, the identification of phenotype-specific pathogenic mechanisms will be crucial for the prognosis and the identification of new therapeutic targets to delay onset or attenuate disease progression rate.

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U1 SNRNA AS A NOVEL RNA-BASED THERAPEUTIC APPROACH TO MODULATE C9ORF72 PATHOLOGY IN PATIENT-DERIVED IPSC-MOTONEURONS

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BACKGROUND-AIM

A novel class of RNA-based therapeutic molecules is represented by modified spliceosomal U1 small nuclear RNA (snRNA) which, by acting on pre-mRNA splicing, have already proved to be effective in mice models of SMA and Familial dysautonomia.
Aim of this work is to test whether modified U1 snRNAs, designed to bind the C9ORF72 hexanucleotide repeat expansion (HRE), correct C9ORF72-associated pathology as a valuable and alternative RNA-based therapeutic strategy to antisense oligonucleotides.

METHODS

Induced pluripotent stem cell (iPSC) derived from C9ORF72 patients were differentiated into MNs.

RESULTS

Two modified U1 snRNAs (U1C and U1G) were co-transfected in HEK293T cells overexpressing 66 hexanucleotide repeats. By FISH analysis we observed that both U1 constructs significantly decreased the number of cells forming C9ORF72 RNA foci. To assess whether U1C and U1G had an effect also on RAN translation of the HRE-containing transcripts, we used a nonATG-plasmid with the 66 hexanucleotide repeats upstream the GFP tag and a significant reduction also in the formation of polyGP-GFP protein was observed upon co-transfection with both modified U1s. We then tested the efficacy of U1 snRNA in reducing RNA foci formation in C9ORF72 patient-derived iPSC-motoneurons carrying 1200 repeats by lentiviral-mediated delivery of U1s. FISH analysis showed a significant decrease both in the percentage of cells containing pathological RNA foci (40% vs 15%) and in the number of pathological RNA foci per cell.

CONCLUSIONS

We are currently measuring the impact of U1s on polyGP synthesis and confirming our data in other C9ORF72 iPSC-motoneurons carrying different HRE size (150 and 670 units) to assess if U1s efficacy in reducing RNA foci is length-dependent.
Our results suggest that modified U1 snRNAs that target the pathological HRE represent an innovative therapeutic strategy to treat ALS and FTD patients carrying C9ORF72 mutation.

Pathological mechanism in ALS

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IMPROVING MUSCLE REGENERATION BY VIRTUE OF PERIPHERAL MACROPHAGES: A THERAPEUTIC STRATEGY FOR AMYOTROPHIC LATERAL SCLEROSIS

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BACKGROUND-AIM

Macrophages (M√) are the most abundant immune cells recruited in regenerating skeletal muscle, where they directly interplay with muscle satellite cells (SCs), which are responsible for the replacement of damaged fibers.
The overall aim of the study was to define a clear relationship between M√ and muscle regeneration in ALS mice. This goal has been generated based on our preliminary data showing a positive correlation between the extent of M√ infiltration in the skeletal muscle and the rate of disease progression in mSOD1 mice. Here, we focussed on the effect of M√ and their fingerprint on SC myogenic activity.

METHODS

A transwell co-culture system was used to evaluate the in vitro responsiveness of mSOD1-derived SCs to M1 or M2 M√. In parallel, mSOD1 mice were intramuscularly treated with scAAV9-MCP1 or IL-10 to elicit M√ recruitment and M1 to M2 M√ polarisation, respectively. The effect on muscle regeneration and atrophy was evaluated.

RESULTS

Our results showed that M1 M√ were essential to activate SC proliferation whilst M2 M√ elicited their differentiation towards myocytes. Besides, the early scAAV9-mediated MCP1 boosting in the skeletal muscle of mSOD1 mice significantly enhanced M√ infiltration, promoting muscle regeneration and lessening denervation atrophy. Finally, we found that IL-10 enhanced M√ shift to a pro-regenerative M2 profile and directly affected SC differentiation to mature myofibres in the skeletal muscle of ALS mice.

CONCLUSIONS

For the first time in the ALS field, we established the pivotal role of the innate immune response in promoting skeletal muscle regeneration and counteracting muscle atrophy. Our findings highlighted the dynamism of infiltrating M√ in skeletal muscles, which is essential to influence the different stages of development of myogenic precursor cells, providing new possible therapeutical strategies to hinder ALS progression.

Pathological mechanism in ALS

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TRANSLATOME PROFILING REVEALS DEREGULATED NEUROVASCULAR CROSSTALK IN MOTOR NEURON DISEASE

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BACKGROUND-AIM

The pathogenic mechanisms responsible for selective loss of motor neurons in Amyotrophic Lateral Sclerosis (ALS) are largely unknown, but it has been shown that both cell autonomous and non-cell autonomous factors are involved. Of the many cellular components in the microenvironment surrounding motor neurons, we became interested in the possible contribution of neurovascular unit dysfunction to ALS pathogenesis, since vascular abnormalities have been detected in ALS patients and animal models.

METHODS

To unravel the crosstalk between vasculature and motor neurons, we employed Translating Ribosome Affinity Purification (TRAP) technology to profile actively translated mRNAs (collectively termed as translatome) in vascular endothelial cadherin (Cdh5)-expressing cells from the spinal cord of mice carrying the ALS-linked SOD1-G93A mutation during disease progression. A bioinformatic pipeline was developed to isolate vascular transcripts and remove parenchymal genes.

RESULTS

Deconvolution of TRAP-Seq data revealed that the majority of translating mRNAs derived from Cdh5+ endothelial cells and perivascular fibroblasts, in both wild type and mutant mice. Discrete changes in vascular translatome were detected in SOD1-G93A mutants before the onset of motor defects. The number of differentially expressed transcripts increased markedly after symptoms onset, revealing a significant association with pathways related to immune response activation. Among vascular genes induced at disease onset, we focused on the chemokine eotaxin-1/CCL11, an immune cell chemoattractant that might initiate neuroinflammation contributing to motor neuron pathology.

CONCLUSIONS

Altogether, TRAP-mediated translatome profiling unmasks dysfunctional vascular pathways in motor neuron disease, and indicates that aberrant endothelial/perivascular signaling is an early event in ALS pathogenesis.

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"INCREASED ADAM 10/17 ACTIVITY IN AN ANIMAL MODEL OF ALS: RATIONALE FOR TARGETING ADAMS AS POTENTIAL THERAPEUTIC TARGET?"

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BACKGROUND-AIM

ADAMs comprise a large family of transmembrane metalloproteases responsible for ectodomain proteolytic cleavage (shedding) of membrane-tethered proteins. ADAM17 was originally identified as the major enzyme for TNF-alpha release, and ADAM10 could compensate for this function. ADAM 10/17 activity is increased in proinflammatory conditions and has already been implicated in the pathogenesis of Alzheimer's disease and Multiple Sclerosis. However, information on potential involvement of ADAM10/17 in ALS is still scarce. Our goal is to elucidate whether alterations of ADAM10/17 protein expression, distribution and/or enzymatic activity could play a role in ALS. Moreover, ADAM10/17 can be released in circulation, and we explored CSF/blood ADAM10/17 activity as a potential ALS biomarker.

METHODS

We performed immunohistochemistry and western blot analyses in spinal cord districts at different disease stages in the SOD1.G93A transgenic (TG) rat model of ALS. In parallel, we measured ADAM10/17 activity in spinal cord homogenates, in CSF and blood.

RESULTS

We highlighted a selective increase of ADAM10/17 immunoreactivity in motor neurons of TG animals at the onset of the disease; as the disease progresses to the late stages, ADAMs are upregulated in glial cells in the white and gray matter. ADAM10/17 enzymatic activity, measured in tissue homogenats, was increased at the symptomatic stage of the disease. Finally, we measured increased levels of ADAM10/17 activity only in the CSF of TG at the symptomatic stage, whereas in the blood was negligible.

CONCLUSIONS

Although our data are still preliminary, we highlighted alterations of ADAM10/17 distribution at the early symptomatic stage of the disease. We are currently investigating the correlation between ADAMs and some of their substrates, such as TNF-alpha or GPNMB implicated in the disease; in parallel, we are exploring selective pharmacological ADAMs inhibitors as a potential therapeutic approach.

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DNA METHYLATION AND HISTONE POST-TRANSLATIONAL MODIFICATIONS OF TARDBP AND SUBSEQUENT MODULATION OF TDP-43

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BACKGROUND-AIM

TDP-43 levels are tightly regulated; significant variations have deleterious effects in cell viability. The predominant mechanism for the regulation of TDP-43 levels is an autoregulatory negative feedback loop via its binding to a region of its pre-mRNA 3'UTR.
We identify an additional regulatory mechanism that is not involved in maintaining TDP-43 proteostasis but rather changes its expression in defined circumstances.

METHODS

Tissue and age specific variations of TDP-43 levels were examined for the epigenetic make-up of the TARDP promoter via bisuphite conversion analysis and ChIP-qPCR in mice and human tissues. DNA hypo and hyper methylation of the Mouse and Human promoter was investigated in vitro.

RESULTS

We observed tissue specific, age-related reduction of TDP-43 levels concomitant with increased promoter methylation and differential histone modifications.
In mouse and in human cultured cell lines, hypo and hyper methylation of the promoter are associated, respectively, with increased and decreased promoter activity.
In human brain necropsy samples, we observed that expression of TDP-43 is heterogeneous between individuals, this not surprising given the wide genetic and environmental heterogeneity of the human population. Notwithstanding, preliminary data shows a decreased methylation of the promoter associated with increased TDP-43 expression.

CONCLUSIONS

TDP-43 regulation may be relevant in the pathogenesis of ALS. The formation of TDP-43 containing brain inclusions removes functional protein. This phenomenon is continuous but compensated by newly synthesized TDP-43. The balance between sequestration and new synthesis may become critical if accompanied by age-related epigenetic modifications that reduce transcription in brain and hence lowering the newly synthesized TDP-43. Sequestration by aggregates would then overcome the production of TDP-43, reducing the amount of functional TDP-43 to levels below those needed by the cell for normal function and thereby trigger the onset of symptoms.

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SENESCENT ASTROCYTES DRIVE NEURODEGENERATION VIA EXTRACELLULAR VESICLES IN ALS-FTD.

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BACKGROUND-AIM

ALS is a non-cell autonomous neurodegenerative disease characterized by altered intercellular communication. Glial cells play a key role in the progression of ALS pathology, but we still lack a thorough understanding of the underlying molecular mechanisms. Cell cycle dysregulation and senescence are gaining increasing attention in the field. In this context, a recent publication in Cell has identified p53, a transcription factor involved in cell cycle regulation, as a driver of neurodegeneration in several C9orf72 ALS models. In the last years, our lab has been investigating the molecular mechanisms of glia-to-neuron miscommunication in a model of ALS. One possible way in which cells communicate with each other is through extracellular vesicles (EVs), nanoparticles that transport proteins, lipids and nucleotides from one cell to the other.

METHODS

We performed RNA sequencing and toxicity assay of wild-type (WT) primary murine cortical neurons after treatment with EVs derived from primary astrocytes. Astrocytes were obtained from WT or transgenic mice expressing mutant TDP-43 Q331K. We also performed proteomic analysis of astrocytic EVs derived from WT and mutant astrocytes by mass spectrometry. Furthermore, proliferation with EdU staining and senescence-associated beta-galactosidase (SA-beta-gal) assays were performed.

RESULTS

From the RNA sequencing and toxicity assay, we observed that EVs derived from mutant astrocytes are sufficient to induce DNA damage and death in wild-type (WT) neurons. From the EV proteome, we found that the protein cargos differ significantly between control and disease condition. Accordingly, TDP-43 Q331K astrocytes show reduced proliferation in vitro and increased SA-beta-gal compared to WT astrocytes.

CONCLUSIONS

Changes in the protein loading of glia EVs in ALS contributes to neuronal toxicity. Deciphering the mechanisms by which glia EVs affect neuronal viability, could help uncovering novel toxic mechanisms to target therapeutically in ALS.

Pathological mechanism in ALS

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LARGE AND SMALL EXTRACELLULAR VESICLES MAY CONTRIBUTE TO THE PROPAGATION OF ALS AND FTD CARRYING TOXIC TDP SPECIES AND POTENTIALLY HARMFUL MIRNAS

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BACKGROUND-AIM

Extracellular vesicles (EVs) are spherical particles, classified in large (LVs) and small vesicles (SVs), composed by a bilayer proteolipid membrane and carryig proteins, RNA and DNA. In our previous studies we demonstrated that both LVs and SVs play a role in the disposal of the insoluble neurotoxic TAR DNA-binding protein 43 (TDP-43) and its C-terminal fragments of 35 (TDP-35) and 25 KDa (TDP-25), the main components of the Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) associated pathological aggregates. Moreover, we observed an increased in their secretion after the impairment of the protein quality control (PQC) system [i.e. chaperone proteins, the ubiquitin proteasome system (UPS) and the autophagic pathway], a common condition observed both in ALS and FTD. Since TDP-43 is an RNA-binding protein, also involved in miRNA biogenesis, and knowing that EVs also contain miRNAs, we wondered whether PQC impairment could also affect the miRNA content of EVs.

METHODS

We studied the miRNA content of LVs and SVs isolated from the culture medium of immortalized neuronal NSC34 cells treated or not with MG132 and NH4Cl (respectively UPS and autophagy inhibitors). miRNA libraries were generated using Small RNA-Seq Library Prep Kit (Lexogen) and sequenced on a NextSeq 500/550 (Illumina). Interaction prediction was carried out on TarBase v.8 database.

RESULTS

We found a total of 91 Differentially Expressed (DE) (log Fold Change (FC) >1 and <-1) microRNAs in treated-EVs compared to untreated EVs. No DE miRNA were found in NH4Cl-LVs, only 7 miRNA were DE in MG132-LVs and of the 82 miRNAs in MG132-SVs and 66 in NH4Cl-SVs, 43 were in common. Interestingly, one of the most enriched pathway targeted by commonly DE SVs-miRNAs is the prion disease.

CONCLUSIONS

In conclusion, from our observations, we can assume that, in disease condition, EVs, enriched in both toxic TDP-43 species and potentially harmful miRNA, may contribute to the propagation of the disease from affected to healthy cells.

Pathological mechanism in ALS

P38

THE SMALL EXTRACELLULAR VESICLES RELEASED BY MOTONEURON MSOD1-NSC-34 CELLS, IN VITRO MODEL OF AMYOTROPHIC LATERAL SCLEROSIS, INDUCE THE ACTIVATION OF THE PERIPHERAL IMMUNE SYSTEM

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BACKGROUND-AIM

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease, involving abnormalities of metabolism and the immune system, including neuroinflammation. In the last years, in vivo experiments and clinical studies confirm the hypothesis about the presence of abnormalities of the peripheral immune system, an actual area of research in ALS pathogenesis.
Among the various modalities of intercellular communication, extracellular vesicles (EVs) released by cells arouse increasing interest in the scientific community for their role in the pathophysiology of diseases of the central nervous system (CNS), including ALS, whose main feature is the functional damage of alpha motoneurons. EVs have important roles in the paracrine pathway signaling involved in the neuroinflammation burst and exhibit neuroprotective effects, as transporters of misfolded proteins including SOD1, FUS, TDP43.
Here, we explore if NSC-34 cells are suitable model for studying the EVs-mediated neuroinflammation occurrence in ALS as they share several morphological and physiological characteristics associated with mature primary motoneurons upon differentiation.

METHODS

Large and small EVs from NSC-34 motoneuron-like cells transfected with mutant SOD (G93A, A4V, G85R, G37R) cell culture medium through the ultracentrifugation technique were obtained. Several markers were used to distinguish isolated EVs: CD63, calnexin and Alix and Annexin A1. Inflammatory response of Raw 264.7 macrophages was investigated by analysis of mRNA levels of several pro- and anti-inflammatory cytokines.

RESULTS

The EVs from NSC-34 expressing mSOD1, induce in Raw 264.7 macrophages the switch to mixed M1 and M2 subpopulations. Particularly interesting is the observed modulation of IL-1® and TGF-® transcript levels.

CONCLUSIONS

mSOD1 NSC-34 cells show the EVs impact on macrophage processes and suggest the potential relevance of study inflammation driven by peripheral cells in MN degeneration.

Pathological mechanism in ALS

P39

PHASE SEPARATION OF FULL LENGTH TAR DNA-BINDING PROTEIN (TDP-43)

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BACKGROUND-AIM

The TAR DNA-Binding Protein (TDP-43) is a nuclear protein that is hypothesised to form reversible condensates via Liquid-Liquid Phase Separation (LLPS). Disruption of TDP-43 homeostasis, and consequent aggregation, is strictly linked to ageing diseases, mainly Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Lobar Degeneration (FTLD).
Recently, a correlation between condensate formation and misfolded pathological inclusions in the cytoplasm has been suggested. Here, we carried out an array of biophysical experiments in vitro, using techniques such as confocal microscopy, Fluorescence Recovery After Photobleaching (FRAP) and time-course turbidimetry, on self-assembly of full-length TDP-43.

METHODS

We applied a pH jump method previously proposed, in order to shed light on TDP-43 self-assemble, the chemical-physical state of the resulting condensates and the main driving forces that take part in TDP-43 phase separation, through a multiparametric screening.

RESULTS

We found that TDP-43 tended to form rapidly spherical assemblies with low fluorescence recovery, corresponding to slow diffusion, and no coalescence with a tendency to self-assemble in a chain-like arrangement with no defined ordered morphology, during the time course. Multi-parametric screening suggested that TDP-43 self-assemble was strongly dependenced by pH, ionic strength, temperature and protein concentration.

CONCLUSIONS

TDP-43 assemblies have a diameter about 0.5-1 µm, round shape, don't coalesce, have a low fluorescence recovery (~20%) in FRAP experiments. These evidences support the hydrogel-like state of TDP-43 assemblies. Moreover, evidences from the multi-parametric screening suggest that self-assembly of TDP-43 is tuned by electrostatic interactions that can inhibit phase separation because of charge-charge repulsions, therefore favouring it when the repulsions are minimal.

Pathological mechanism in ALS

P40

TOWARDS UNVEILING THE NEXUS BETWEEN AXONAL GRANULES AND POLYSOMES IN ALS

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BACKGROUND-AIM

Amyotrophic Lateral Sclerosis (ALS) and Front Temporal Dementia (FTD) are neurodegenerative disorders characterized by progressive loss of motor neurons and cognitive impairment. RNA¬binding proteins have been identified as major contributors to the development of neurological diseases and are known to modulate RNA synthesis, localization, and translation. However, the cellular mechanisms linking RNA dysregulation to neuropathogenesis remain largely unknown.

METHODS

ALS has been associated to mutations affecting the DNA/RNA-binding protein TDP43. We explored the hypothesis that TDP43 overexpression or mutation causes an imbalance between axonal granule- and polysome-associated RNAs. We developed a tag-free polysomal profiling to identify mRNAs associated to subcellular regions (cell body or axon) and sub-compartments (RNA granules or polysomes) of mouse cortical neurons. Through high-throughput sequencing and dedicated computational pipelines, we investigated translational changes induced by overexpression of TDP43-WT or TDP43-A315T mutant.

RESULTS

We revealed a loss of balance between free and polysome-engaged RNA in the axon. These results, supported by additional data from axonal puromycilation assay and multiple in vivo validation assays, suggest that the imbalance between granule- and polysome-associated mRNAs is caused by robust degradation of specific RNAs. Massive depletion of TDP43 target RNAs leads to a translational burden on non TDP43 targets due to the increased availability of ribosomes and the hyper-translation of the remaining mRNAs. We investigated this hypothesis in cell lines model of ALS by designing ad hoc constructs that revealed the presence of a translational burden effect triggered by reduced levels of TDP43 mRNA targets.

CONCLUSIONS

Our results point to an as yet unexplored translation-centred mechanisms linking TDP43 and ALS pathogenesis and pave the way toward a better understanding of axonal protein synthesis, possibly underlying many neurodegenerative diseases.

Pathological mechanism in ALS

P41

SUMOYLATION REGULATES TDP-43 SPLICING ACTIVITY AND NUCLEOCYTOPLASMIC DISTRIBUTION

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BACKGROUND-AIM

The nuclear RNA-binding protein TDP-43 forms abnormal cytoplasmic aggregates in the brains of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) patients and several molecular mechanisms promoting TDP-43 cytoplasmic mislocalization and aggregation have been proposed, including defects in nucleocytoplasmic transport, stress granules (SG) disassembly and post-translational modifications (PTM). SUMOylation is a PTM which regulates a variety of cellular processes and, similarly to ubiquitination, targets lysine residues.

METHODS

The impact of SUMOylation on TDP-43 structure and functionality was assessed by a variety of complementary cell-free and in vitro experimental approaches.

RESULTS

To investigate the possible regulatory effects of SUMOylation on TDP-43 activity and trafficking, we first assessed that TDP-43 is SUMO-conjugated in the nuclear compartment both covalently and non-covalently in the RRM1 domain at the predicted lysine 136 and SUMO-interacting motif (SIM, 106-110 residues), respectively. By using the SUMO-mutant TDP-43 K136R protein, we demonstrated that SUMOylation modifies TDP-43 splicing activity, specifically exon skipping, and influences its sub-cellular localization and recruitment to SG after oxidative stress. When promoting deSUMOylation by SENP1 enzyme over-expression or by treatment with the cell-permeable SENP1 peptide TS-1, the cytoplasmic localization of TDP-43 increased, depending on its SUMOylation. Moreover, deSUMOylation by TS-1 peptide favoured the formation of small cytoplasmic aggregates of the C-terminal TDP-43 fragment p35, still containing the SUMO lysine target 136, but had no effect on the already formed p25 aggregates.

CONCLUSIONS

Our data suggest that TDP-43 can be post-translationally modified by SUMOylation which may regulate its splicing function and trafficking, indicating a novel and druggable mechanism to explore as its dysregulation may lead to TDP-43 pathological aggregation in ALS and FTD.

Pathological mechanism in ALS

P42

CIRCULATING MUSCLE-DERIVED MIR-206 LINKS SKELETAL MUSCLE DYSFUNCTION TO HEART SYMPATHETIC DENERVATION

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BACKGROUND-AIM

ALS is a fatal disorder with highly variable manifestations and biomarkers of disease subtype/stage are required to improve patient care. Among these, the muscle-specific microRNA, miR-206, is a promising candidate, although unanimous consensus is still lacking. Recently, ALS has been defined as a multicellular disorder, involving several cell types, including skeletal muscle cells. In fact, muscle-restricted expression of the ALS mutation, SOD1G93A, causes NMJ dismantlement, muscle atrophy and paralysis. Alterations in the sympathetic nervous system occur in ALS patients, who frequently display arrhythmias and stress-cardiomyopathy. However, direct involvement of sympathetic neurons (SNs), in ALS, is unexplored. In the heart, SNs control heart function and structure, while, in skeletal muscle, sustain muscle homeostasis and NMJ stability.

METHODS

We used MLC-Atg7-/- male mice and littermate controls. In vivo cardiac functional assays were combined to ex vivo analyses of hearts and stellate ganglia, and in vitro tests in cultured sympathetic neurons (SNs).

RESULTS

We showed that muscle-specific block of autophagy, as occurring in Atg7-/- mice, leads to increased secretion, by muscle fibers, of extracellular-vesicles (ECVs) enriched in miR-206, whose levels were found elevated in the bloodstream. Circulating muscle-derived ECVs are taken up by cardiac SNs, where miR-206 leads to sympathetic denervation and arrhythmias. These effects are due to miR206-mediated downregulation of the NGF receptor p75, causing reduced p75/TrkA dimerization, decreased efficiency of NFG retrograde transport and increased cell death. Notably, SOD1G93A mice, which display muscle autophagy impairment, show increased miR-206 levels in serum and cervical ganglia, and degeneration of muscle- and heart- sympathetic innervation.

CONCLUSIONS

Our data identifies miR-206 as mediator of both paracrine effects on muscle-innervating SNs, likely deteriorating neuromuscular function, and long-range effects on cardiac neurons, favoring arrhythmias.

Pathological mechanism in ALS

P43

SYMPATHETIC NEURONS ARE ADDITIONAL CELL TYPES AFFECTED IN AMYOTROPHIC LATERAL SCLEROSIS

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BACKGROUND-AIM

Amyotrophic Lateral Sclerosis (ALS) is a fatal neuromuscular disorder, whose pathogenesis is incompletely understood. Originally defined as 'a motor neuron disease', current evidence supports the multi-systemic nature of ALS, which involves at least glial cells, astrocytes and skeletal myocytes. Notably, several studies support that sympathetic neuron (SN) activity is altered in ALS patients, and may be responsible of life-worsening and life-threatening manifestations (i.e. arrhythmias and sudden death). SNs innervate almost all tissues, including skeletal muscles, where they sustain muscle trophism and neuromuscular junction structure and function. Whether SNs are directly affected in ALS, and participate to disease pathogenesis, is largely understudied and addressing this point is our goal.

METHODS

Combined confocal IF and morphometric analyses.

RESULTS

We demonstrated that sympathetic innervation is affected in muscles from SOD1G93A mice. In detail, muscle SN processes are thinner, with reduced density (15.0± 2.3%, $p < 0.01$) in ALS mice, compared to controls, already at the early disease stage. Sympathetic muscle denervation worsens in time, reaching a (57.2±5.6%, $p<0.001$) reduction at advanced disease stages. In vitro assays revealed the primary role of SOD1G93A mutation in affecting SN phenotype. Indeed, SOD1G93A expression in normal SNs lead to reduced axonal sprouting and neuronal process fragmentation. Notably, similar alterations were observed in muscle biopsies from ALS patients, indicating the alteration of sympathetic innervation is a common aspect in ALS muscles. Consistently, alterations of Heart Rate Variability and a greater incidence of arrhythmic events were observed in patients, indicating that morphological alterations are accompanied to SN dysfunction.

CONCLUSIONS

Our results indicate that SNs are additional cell types compromised in ALS. Understanding how SNs are implicated in the disease pathogenesis may uncover additional, and yet underappreciated, therapeutic targets.