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TURIN 4TH-5TH NOVEMBER 2022 II EDITION

MOTOR NEURON DISEASES

UNDERSTANDING THE PATHOGENETIC MECHANISMS TO DEVELOP THERAPIES

HYBRID MEETING

ORGANIZERS: M. Boido & S. Stanga, NICO, Univ. Turin

Organizers: Marina Boido & Serena Stanga

Contacts: marina.boido@unito.it & serena.stanga@unito.it

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2nd International Congress on

"MOTOR NEURON DISEASES: UNDERSTANDING THE PATHOGENETIC MECHANISMS TO DEVELOP THERAPIES"

Torino, Italy, 4-5 November 2022

Lectures: Palace of the Anatomical Institutes, Corso Massimo D'Azeglio 52, Torino
Practical activities: Fondazione Cavalieri Ottolenghi (NICO), Regione Gonzole 10, Orbassano (TO)

PRESIDENTS OF THE CONGRESS

Marina Boido and Serena Stanga

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SCIENTIFIC PROGRAM DAY 1 (4th November 2022)

13.00-14.00: Registration

14.00-14.15: Opening ceremony
Welcome by the Organizers
Greetings by A. Vercelli (Deputy Rector for Biomedical Research, Univ. Turin), A. Mauro (Director Dept. Neuroscience, Univ. Turin) and A. Calvo (PhD Neuroscience Coordinator, Univ. Turin)

14.15-14.30: Brief introduction by the organizers (Marina Boido and Serena Stanga, Univ. Turin)

14.30-15.30: Session I - "SMA disease mechanisms and therapeutic approaches"

- 14.30-14.50: Stefania Corti, Univ. Milan "Spinal Muscular Atrophy: new molecular therapeutic targets"
- 14.50-15.10: Thomas Gillingwater, Univ. Edinburgh "Developing the second generation of therapies for SMA"
- 15.10-15.30: Cécile Martinat, I-STEM, AFM, Evry "Human pluripotent stem cells for neuromuscular diseases"

15.30-15.45: Q&A Session I

15.45-16.10: Coffee break

16.10-16.50: Session II - "SMA: a look at the clinical research"

- 16.10-16.30: Gabriele Pistillo, Novartis Gene Therapies, Milan "Zolgensma journey for SMA patients access in Italy"
- 16.30-16.50: Lorenzo Maggi, Foundation IRCCS Neurological Institute Carlo Besta, Milan "SMA in adult age - treatments and new challenges"

16.50-17.00: Q&A Session II

17.00-17.20: Lecture Francesco Biancardi, Zeiss: "New approaches for Volume Imaging in Neuroscience: Lab based X-Ray and Serial Block Face Electron Microscopy"

17.20-17.40: Lecture Cinzia Gellera, Foundation IRCCS Neurological Institute Carlo Besta, Milan – "Genetics of Motor Neuron Diseases: what we have learned"

17.40-19.30: Poster session

20.00: Social dinner

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SCIENTIFIC PROGRAM DAY 2 (5th November 2022)

8.30-10.00: Session III - "ALS pathogenetic mechanisms"

8.30-8.50: Manuela Basso, Univ. Trento "It's all a matter of communication! What extracellular vesicles are revealing about Amyotrophic Lateral Sclerosis"

8.50-9.10: Ludo Van Den Bosch, VIB-KU Leuven Center for Brain & Disease Research "Using patient-derived induced pluripotent stem cells to get insights into the pathogenic mechanisms of ALS"

9.10-9.30: Maria Pennuto, Univ. Padua "Motor neuron diseases: a journey from the central nervous system to periphery"

9.30-10.10: Session IV – "ALS therapeutic strategies"

9.30-9.50: Françoise Piguet, NEUROGENCELL, INSERM, ICM, Paris "Cyp46a1 overexpression as a relevant target for ALS independent from its origin"

9.50-10.10: Adriano Chiò, Univ. Turin "Is it time for a personalized medicine approach in ALS?"

10.10-10.25: Q&A Sessions III-IV

10:25-10:45: Lecture Francesco Girardi, Media System Lab: "Video Killed the Imaging Star"

10.45-11.30: Closing Lecture Aaron D. Gitler, Univ. Stanford "Expanding mechanisms and therapeutic targets for motor neuron disease" online

11.30-11.35: Awards ceremony and goodbye to the online attendees

11.35-12.15: Brunch

12.15-12.45: Transfer TORINO-NEUROSCIENCE INSTITUTE CAVALIERI OTTOLENGHI (NICO, ORBASSANO)

13.00-16.00: Practical activities at Neuroscience Institute Cavalieri Ottolenghi (with Marina Boido and Serena Stanga, Univ. Turin)

(I) How to set up a 2D nerve-muscle co-cultures: tips and tricks

(II) Label-free live cell imaging of mitochondria dynamics (sponsored by Media System Lab)

(III) Different approaches for mitochondria analysis: from the staining to in silico tools (sponsored by Zeiss)

16:00-16:30 Transfer NICO-TORINO

Goodbye to the next edition (in 2024)!

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ABSTRACTS – POSTER (in alphabetical order)

NON-CELL AUTONOMOUS REGULATION OF PRE-MOTOR INTERNEURON DEVELOPMENT IN THE EMBRYONIC SPINAL CORD

Angla Navarro A, Toch M, Hidalgo-Figueroa M, Francius C and Clotman F

Laboratory of Neural Differentiation, Institute of Neuroscience, LIBST Institute, UCLouvain, Brussels, Louvain-le-Neuve, Belgium

The Onecut (OC) factors are transcriptional activators that regulate several aspects of neural development. In the spinal cord, they play key roles during differentiation and migration of motor neurons and of ventral or dorsal interneuron populations. Their inactivation induces alterations in the development of spinal interneurons that do not express the Onecut genes in control embryos. Furthermore, conditional ablation of the 3 Onecut factors from the spinal motor neurons results in similar interneuron development defects, leading to the conclusion that Onecut factors control in motor neurons a non-cell autonomous process that regulates the development of ventral interneurons. The goal was to identify molecules that contribute to this non-cell autonomous regulation of Interneurons by the Motor neurons. To characterize this mechanism, a RNAseq comparison of control and of Onecut-deficient motor neurons was performed and a list of candidates was selected and analyzed. The most promising candidate was Ntf3, and to assess its impact on the embryonic spinal cord, first the Ntf3 was overexpressed specifically in motor neurons using chicken embryonic spinal cord electroporation, which resulted in alterations on the ventral interneuron populations that did not receive the vector. Moreover, the conditional inactivation of the Ntf3 in the motor neurons in a mice model was also studied and the cell types development was assessed by immunofluorescence for population-specific markers. The results of these experiments will validate a possible function of this gene in the non-cell autonomous control of spinal interneuron development.

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COMPROMISED RESILIENCE OF ASTROCYTES IN AMYOTROPHIC LATERAL SCLEROSIS

Belo do Nascimento I., Desmet, N., Hermans, E.

Laboratory of Neuropharmacology, Institute of Neuroscience, Université catholique de Louvain, Brussels, Belgium

A profound dysregulation of energy metabolism has been reported in amyotrophic lateral sclerosis (ALS) patients and animal models of the disease. Thus, considerable weight loss due to low food intake associated with a systemic hypermetabolism have been observed in this context. Besides, neurodegeneration in ALS is known to involve “non-cell autonomous” mechanisms, as the compromised activity of glial cells has been shown to dramatically impact on neuronal survival. Considering the key role played by astrocytes in the control of energy metabolism in the nervous system, it is surprising that glial cell metabolism has received little attention in the context of ALS. We herein propose that a deficiency in the metabolic plasticity of astrocytes in ALS contributes to the loss of physiological support of nearby neurons, exacerbating their vulnerability to stress.

The AMP-activated protein kinase (AMPK) is a key modulator of cell metabolism and alterations of its expression and/or activity have been reported in motor neurons from both patients and animal models of ALS. However, little is known about this enzyme in glial cells. We first characterized its expression in primary cultures of astrocytes derived from wild-type or transgenic rats carrying the ALS-associated mutated superoxide dismutase (hSOD1G93A). We found decreased mRNA levels of the AMPK catalytic subunit isoforms in astrocytes from ALS rodents, suggesting that they are poorly equipped to face metabolic stresses compared to their wild-type counterparts. Then, as AMPK responds to variations of the energy status of the cell, we characterized its activity in astrocytes subjected to metabolic stress conditions (i.e., glucose deprivation). In parallel, the ATP levels as well as the metabolic activity of these astrocytes were evaluated. Our findings suggest that astrocytes from ALS rodents are more vulnerable to metabolic stress compared to wild-type cells, and that may compromise their ability to provide support to neurons.

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SOMAscan PROTEOMICS IDENTIFIES PLASMA BIOMARKERS WITH DIFFERENT EXPRESSION PATTERNS IN FAMILIAL AND SPORADIC AMYOTROPHIC LATERAL SCLEROSIS

Berrone E.[1], Benedetti V.[1], Palmitessa C.[1], Gallo M.[1], Calvo A.[2,3], Casale F.[2], Guana F.[2], Manera U.[2,3], Favole A.[1], Crociara P.[4], Testori C.[1], Carta V.[1], Tessarolo C.[1], D'Angelo A.[5], De Marco G.[2,3], Guana F.[6], Caramelli M.[1], Chiorino G.[6], Chiò A.[2,3], Casalone C.[1], Corona C.[1].

[1]S.C. Neuroscienze, Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Turin, Italy; [2]Rita Levi Montalcini Dept. Neuroscience, Univ. Turin, Turin, Italy; [3]Neurology, Hospital Dept. Neuroscience and Mental Health, Città della Salute e della Scienza Hospital of Turin, Turin, Italy; [4]ASL TO4. Chivasso, Turin, Italy; [5]Dept. Veterinary Sciences, Unive.Turin, Grugliasco, Italy; [6]Cancer Genomics Lab, Fondazione Edo ed Elvo Tempia, Biella, Italy

Amyotrophic lateral sclerosis (ALS) is a fatal progressive neurodegenerative disease caused by loss of upper and lower motor neurons that leads to death within 2–3 years, mainly due to a respiratory failure. ALS starts generally between 50 and 70 years old and it is one of the most common motor neuron diseases (MNDs) among adults. Most ALS cases (90-95%) are sporadic (S-ALS) with unknown etiology, while approximately 10% of patients have a familial form of the disease (F-ALS); however, they are clinically and pathologically indistinguishable from one another, suggesting similar disease mechanisms. Therefore, unravelling other potential pathogenetic mechanisms and searching reliable markers are high priorities. In this contest, we employed the SOMAscan assay, an aptamer-based proteomic technology, to determine the proteomic background of ALS patients. The expression levels of ~ 1300 proteins were assessed in plasma and a deep bioinformatic analysis revealed significant differences between ALS patients and controls. The study identified 42 proteins with statistically significant differential expression between ALS patients versus controls. Among these, four proteins were selected as putative biomarker candidates with putative biological relevance. These biomarkers were all upregulated and their overexpression in patients was afterwards validated by ELISA assays in an independent cohort. Following deep statistical analyses, different expression patterns of these proteins were observed in familial and sporadic ALS patients. The proteins identified in this study might provide insight into ALS pathogenesis and represent potential candidates to develop novel therapeutic targets. Acknowledgement: This research was funded by the CRT Foundation and 2014-2020 ROP ERDF.

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THE ROLE OF CHI3L1 PLASMATIC LEVELS IN AMYOTROPHIC LATERAL SCLEROSIS

Bombaci A., De Marco G., Casale F., Salamone P., Fuda G., Marchese G., Moglia C., Calvo A., Chiò A.

ALS Centre, Department of Neuroscience "Rita Levi Montalcini", University of Torino, Turin, Italy and.

Background and aim: motor neuron diseases (MND) are neurodegenerative diseases characterised by complex and heterogeneous pathological mechanisms. Biomarkers could help in defining patients' prognosis and stratification. Recently, besides neurofilaments, chitinases seem to be a promising family of biomarker. They correlate with neuroinflammation status and they include CHIT1, CHI3L1 and CHI3L2. In one study CHI3L1 CSF levels have been correlated with cognitive impairment. Since blood samples are easy and less invasive to obtain compared to CSF, we wanted to evaluate CHI3L1 plasma levels in MND, MND mimics and healthy controls (HCs).
Methods: sandwich ELISA was used to quantify plasma CHI3L1 from 44 MND, 7 HSP, 9 MND mimics and 19 HCs. ALSFRSr, MRC, spirometry, genetic tests, disease progression rate at diagnosis (PR), blood examinations, neuropsychological tests (MMSE, ECAS, TMT-A, TMT-B, RAVLT, ROCF, FAB, Digit Span, FRSBE). We analysed data using Kruskal-Wallis, ANCOVA and Cox regression analysis.
Results: CHI3L1 plasma levels result to be different between groups ($p = 0.029$), particularly MND mimics have higher levels of CHI3L1 compared to MND and HCs. No difference between HSP, MND and HCs ($p > 0.05$). A sub-group analysis of MND patients (divided in PLS, ALS and PMA) do not show any difference in CHI3L1 levels. Moreover, CHI3L1 do not correlate to ALSFRSr, MRC, FVC, FEV1, PR, blood examination and neuropsychological tests.
Discussion: CHI3L1 plasma levels result to be increased in acute myelopathy, radiculopathy and neuropathies, compared to MND, HSP and HCs. This is consistent with the increase of CHI3L1 in neuroinflammatory processes. Contrarily to CHI3L1 CSF levels, CHI3L1 plasma levels are not able to differentiate between ALS and HCs and do not correlate with neuropsychological impairment. Further multicentre studies are needed to better explain the role of CHI3L1 in diagnosis and prognosis of MND and, also, in neuropathies.

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

Cabras Paolo[1*], Spatafora Mauro G.[1], Dimartino Agnese[1], Gazzano Andrea[1], Peviani Marco[1#].

[1]Cellular and Molecular Neuropharmacology lab, Dpt Biology and Biotechnology "L.Spallanzani", University of Pavia, Pavia, Italy
[*]Presenting author [#]Corresponding author

Background. 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.
Aims. 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.
Discussion. 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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MITOCHONDRIAL *SMN1*-ANTICORRELATED ANALYSIS SHEDS LIGHT ON POSSIBLE GLYCINERGIC SYSTEM ALTERATIONS IN SPINAL MUSCULAR ATROPHY

Caretto A.[1,2], Di Cunto F. [1,2], Boido M. [1,2] and Vercelli A.[1,2]

[1]Department of Neuroscience Rita Levi Montalcini, University of Turin, 10126 Turin, Italy.
[2]Neuroscience Institute Cavalieri Ottolenghi, University of Turin, 10043 Orbassano (TO), Italy.

Spinal Muscular Atrophy (SMA) is a neurodegenerative disease caused by *SMN1* gene mutation. It affects CNS [causing lower motor neuron (MN) loss], but also peripheral districts (e.g. skeletal muscles and heart). Unfortunately, despite the effectiveness of the available therapies, they still show some limitations. Thus, the identification of new targets and therapeutic strategies is necessary. Recently it has been observed that SMA shares mitochondria alterations with other neurodegenerative diseases (e.g. Parkinson's Disease, Alzheimer's Disease and Amyotrophic Lateral Sclerosis) and therefore these organelles can be considered new promising therapeutic targets. In this context, by performing bioinformatic analyses, we identified 8 mitochondrial *SMN1*-anticorrelated genes as potential candidates: *COX7A1, GCSH, BAG1, GOLPH3, DNAJC5, SLC25A36, GLRX2* and *UQCRC2*. Then, rt-PCR on SMNdelta7 mice (representative of a severe form of SMA) revealed a significant *GCSH* upregulation in lumbar spinal cord of SMA mice, at early symptomatic pathological stage (postnatal day 5, P5). Western Blot analysis confirmed the trend of upregulation and immunofluorescence staining on spinal cord slices showed a significant higher expression of GCSH in MN cell bodies. Since GCSH is a glycinergic cleavage system subunit, the obtained results persuaded us to search for other possible alterations in the glycinergic system. In fact, we hypothesized that a reduction in MN inhibition, together with the lack of SMN, could determine the loss of MNs due to their hyperexcitability. Therefore, to verify our hypothesis, we morphologically analyzed Renshaw Cells, the glycinergic inhibitory interneurons involved in MN recurrent inhibition in spinal cord: we observed a cellular shrinkage at P5, that even worsened in the late stage of the disease (P12). Based on these preliminary results, we hypothesize that glycinergic system is early altered in SMA and could represent a new drug target for SMA therapy.

This work is supported by Girotondo and SMArathon-ONLUS foundations to AV and MB.

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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

Casarotto E.[1], Garofalo M.[2], Messa L.[3], Sproviero D[2], Carelli S.[3], Cozzi M.[1], Chierichetti M.[1], Cristofani R.[1], Ferrari V.[1], Galbiati M.[1], Piccolella M.[1], Rusmimi P.[1], Tedesco B.[1,4], Pramaggiore P.[1], Cereda C.[5], Gagliardi S.[2], Poletti A.[1], Crippa V.[1]

1 Dipartimento di Scienze Farmacologiche e Biomolecolari (DiSFeB), Department of Excellence 2018-2022, Università degli Studi di Milano, Milano (MI), Italy
2 Genomic and post-Genomic Center, IRCCS - Mondino Foundation, Pavia (PV), Italy
3 Centro di Ricerca Pediatrica "Romeo ed Enrica Invernizzi", Dipartimento di Scienze Biomediche e Cliniche "L. Sacco", Università degli Studi di Milano, Milano (MI), Italy
4 Unit of Medical Genetics and Neurogenetics, Fondazione IRCCS – Istituto Neurologico "Carlo Besta", 20133 Milano (MI), Italy
5 UOC Screening Neonatale e Malattie Metaboliche, Dipartimento della Donna, della Mamma, del Neonato, ASST Fatebenefratelli Sacco - Ospedale dei Bambini "V. Buzzi", Milano (MI), Italy

Extracellular vesicles (EVs), classified in large (LVs) and small vesicles (SVs), are membrane-enclosed particles released from all eukaryotic cells that carry proteins, RNA and DNA. In our previous study, we demonstrated that both LVs and SVs, have a role in the disposal of the TAR DNA-binding protein 43 (TDP-43) and its C-terminal fragments of 35 (TDP-35) and 25KDa (TDP-25): the main components of ALS and FTD toxic cytoplasmic aggregates. We also demonstrated that 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, further contributes to their secretion. Since TDP-43 is an RNA-binding protein involved in miRNA biogenesis, and knowing that EVs also contain miRNAs, we decided to evaluate the miRNA content of both LVs and SVs and their possible alteration after PQC impairment. Through the differential ultracentrifugation method, we isolated LVs and SVs from an immortalized neuronal cell line treated or not with MG132 and NH4Cl (respectively UPS and autophagy inhibitors). We generated miRNA libraries using Small RNA-Seq Library Prep Kit (Lexogen) and sequenced them on a NextSeq 500/550 (Illumina). Interaction prediction was carried out on TarBase v.8 database. 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, the most enriched pathway targeted by commonly DE SVs-miRNAs is the prion disease. In conclusion, our results suggest that, in pathological condition, EVs, containing both toxic TDP-43 species and potentially harmful miRNA, may contribute to the propagation of the disease from affected to healthy cells.

Fundings: Fondazione Cariplo, Italy n. 2017_0747; Italian Ministry of University and Research (MIUR), PRIN - Progetti di ricerca di interesse nazionale n. 2017F2A2C5 and n. 2020PBS5MJ

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A 3D BIOPRINTED MODEL OF SPINAL CORD: POSSIBLE APPLICATIONS IN THE MN DISEASE FIELD

Chiappini V.[1,*], Traldi C.[2,*], Tonda Turo C.[2], Boido M.[1]

[1] Neuroscience Institute "Cavalieri Ottolenghi", University of Turin, 10043 Orbassano (TO), Italy; Department of Neuroscience "Rita Levi Montalcini", University of Turin, 10126 Turin, Italy.
[2] Department of Mechanical and Aerospace Engineering, Politecnico di Torino, 10129 Torino, Italy.
*These authors share first authorship.

By providing a higher degree of structural complexity, *in vitro* three-dimensional (3D) models have been recently introduced as powerful tools for recreating more physiological conditions and for achieving *in vitro* more predictable results, compared to the conventional 2D models. Moreover, they allow to reduce the use of animals and to preliminary test several therapeutic approaches. The aim of this work is to recapitulate *in vitro* the main 3D features of the spinal cord, by reproducing its multicellular network composition.
For the 3D model development, we selected three murine cell lines: a motor neuron (MN) cell line (NSC-34), an astrocyte cell line (NE-4C) and an oligodendroglial cell line (Oli-Neu). At first, we selected the optimal co-culture conditions in 2D (DMEM enriched with growth factors and retinoic acid 20 µM for differentiation). Then, for each line, we formulated and tested specific hydrogels, able to support the growth and differentiation of a specific cell subtype. The MN-hydrogel (specifically developed for these cells) was a blend of thiolated hyaluronic acid (0.5% w/v), a major component of the CNS extracellular matrix, and gelatin methacryloyl (5% w/v), that thanks to its cell-binding sites promotes cell adhesion. Preliminary viability tests have been performed (CellTiter-Blue Assay, Live/Dead viability/cytotoxicity kit) and showed the high biocompatibility of the MN-hydrogel. Once optimized, the three cell-laden hydrogels will be bioprinted (R-GEN 200, REGEN HU), following a precise geometry specifically designed for recapitulating the spinal cord architecture.
This complex 3D model can be also useful in the field of MN diseases: for example, by using NSC-34 cells expressing hSOD1(G93A) gene under the control of a doxycycline-inducible promoter (already available in the lab) we will be able to develop a model of Amyotrophic Lateral Sclerosis spinal cord, for analyzing neurodegenerative pathways and performing drug screening.
The work was supported by Fondazione CRT (grant RF 2020.1801).

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ALTERNATIVE TRANSLATION INITIATION AS A NOVEL STRATEGY TO BLOCK TOXICITY OF THE MUTANT ANDROGEN RECEPTOR IN SBMA

Chierichetti Marta[1], Cristofani Riccardo[1], Rusmini Paola[1], Ferrari Veronica[1], Tedesco Barbara[1 2], Cozzi Marta[1], Casarotto Elena[1], Pramaggiore Paola[1], Crippa Valeria[1], Galbiati Mariarita[1], Piccolella Margherita[1], Poletti Angelo[1]

[1]Dipartimento di Scienze Farmacologiche e Biomolecolari-Centre of Excellence on Neurodegenerative Diseases, Università degli Studi di Milano, Italia
[2]Unit of Medical Genetics and Neurogenetics, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy

Spinal and Bulbar Muscular Atrophy is a neurodegenerative disease linked to a CAG repeat expansion in the Androgen Receptor (AR) gene, which is translated into a polyglutamine tract (polyQ) in the AR N-terminal region. ARpolyQ acquires neurotoxic properties and aggregates after testosterone binding.
Different start codons (AUGs) are involved in AR translation. I-AUG leads to translation of a full-length AR (AR-B) which includes the pathogenic polyQ tract in SBMA. II-AUG is an alternative AUG leading to the translation of an alternative isoform named AR-A, which does not contain the polyQ tract.
This project aims to characterize AR-A behaviour and to develop an effective strategy to selectively drive the AR translation from the II-AUG via antisense oligonucleotide (ASO) and a library of FDA approved drugs blocking ARpolyQ toxicity (GOF) without causing AR loss of function (LOF).
When exogenously overexpressed AR-A and AR-B have similar expression levels using Western Blot. We then compared the transactivation activity of AR-A through luciferase assay, and we found that AR-A had a lover transactivation capability compared to AR-B, but similar to ARpolyQ. We then have demonstrated that AR aggregates are not present in cells expressing AR-A using Filter Retardation Assay and Confocal Microscopy in addition, co-transfection with ARpolyQ and AR-A stabilize AR dimers after testosterone treatment leading to a reduction of aggregate formation in ARpolyQ:AR-A ratio-dependent manner
These data suggest that depletion of the AR N-terminal region in AR-A: *i.* did not affect AR-A translation and stability *ii.* did not completely reduce its testosterone response and *iii.* Lead to the reduction of aggregate formation.
Future prospective is to use a double report screening vector designed to detect different AR isoforms expression in relation to the signal obtained to perform ASO and drugs screening.

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B-RAF/LIN-45 IS A SIGNALING HUB FOR GENES INVOLVED IN NEURODEGENERATION

Cieri F.[1,2], Santonicola P.[1], Zampi G.[1], Hensel N.[3], Tapken I.[4,5], Detering N.T.[4,5], Claus P.[4,5] and Di Schiavi E.[1]

[1]Institute of Biosciences and BioResources, IBBR, CNR, Naples, Italy [2]Department of Biology, University of Naples Federico II, Naples, Italy [3]Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Canada [4]SMATHERIA gGmbH –Non-Profit Biomedical Research Institute, Hannover, Germany [5]Center for Systems Neurosciences (ZSN), Hannover, Germany

Current treatments for SMA consisting in restoring SMN protein levels result in impressive, but limited effects. To improve SMA therapeutic strategies and better elucidate its molecular pathomechanism, complementary SMN-independent approaches are needed. Model organisms and bioinformatics are essential tools. Using a phospho-array approach on SMA mice and networkbiology, we identified 38 dysregulated proteins involved in motoneuron degeneration, and B-Raf as major signaling hub. We characterized the in vivo role played by B-Raf/lin-45 in neurodegeneration using C. elegans and we demonstrated that lin-45 plays a cell-autonomous neuroprotective role when smn-1 is down-regulated. We found that lin-45 protects from motoneuron loss postsymptomatically rather than interfering with neurogenesis. Finally, we demonstrated that he MAPK/ ERK pathway mediated the lin-45 rescue, and these data were confirmed in mammalian settings, strongly supporting a role of B-RAF in neurodegeneration and confirming C. elegans as an important tool to identify new SMA targets. To elucidate the role played by the other 37 proteins identified in the phospho-array experiment, we set up a new model of SMA in C. elegans that allows to rapidly identify modifier genes among those candidates. We successfully obtained a new quadruple mutant strain for RNA-interference mediated genetic screen, allowing to obtain an enhancement or a suppression of the neurodegeneration. Multiple negative and positive controls were established to monitor the consistency of the screening and we improved the capacity of the analysis up to 14 genes screened per week. We identified 9 suppressors and 9 enhancer genes among all the 37 genes tested. Using cell-specific RNAi, we demonstrated that 11 play a role in neurons and in particular, 3 of those specifically in MNs. These data unveil a neuroprotective role of a network of genes in MNs which are candidate targets for future therapies in SMA.
We thank SMA Europe for funding.

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ONE GENE, MANY PHENOTYPES: INVESTIGATING KIF5A-LINKED NEURODEGENERATION MECHANISMS

<u>*Cozzi M.*</u>[1], Tedesco B.[1,2], Cristofani R.[1], Ferrari V.[1], Casarotto E.[1], Chierichetti M.[1], Pramaggiore P.[1], Piccolella M.[1], Galbiati M.[1], Rusmini P.[1], Crippa V.[1], Gellera C.[2], Magri S.[2], Santangelo S.[3,4], Ratti A.[3,4], Taroni F.[2] , Poletti A.[1]

[1]Dipartimento di Scienze Farmacologiche e Biomolecolari, Università degli Studi di Milano, Milan, Italy; [2]UOC Genetica Medica – Neurogenetica, Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy; [3]Dipartimento di Biotecnologie Mediche e Medicina Traslazionale, Università degli Studi di Milano, Milan, Italy; [4]Dipartimento di Neurologia e Stroke Unit – Laboratorio di Neuroscienze, IRCCS Istituto Auxologico Italiano, Milan, Italy

KIF5A is a neuron-specific kinesin involved in anterograde axonal transport. It comprises an Nterminal motor domain for ATP-dependent microtubule binding, a coiled-coil stalk for dimerization, and a tail domain for cargo/adaptor binding and autoinhibition. Mutations targeting the three KIF5A domains are associated with distinct motor neuron diseases (MNDs) – including spastic paraplegia, Charcot-Marie-Tooth disease, and amyotrophic lateral sclerosis (ALS) – but the molecular mechanisms underpinning such phenotypic heterogeneity are not fully understood yet. Our aim is to functionally characterize four KIF5A mutants (R17Q, R280C, R864X, N999Vfs*39) to gain insight into KIF5A-related neurodegeneration. Upon overexpression in NSC-34 cells, the R864X and N999Vfs*39 mutants displayed abnormal distribution by preferentially localizing within neurites instead of being diffused in the whole cytoplasm like wild-type (WT) KIF5A. Such pattern is consistent with impaired KIF5A autoinhibition, respectively depending on loss or alteration of the tail domain for the two mutants. More in detail, the N999Vfs*39 variant formed p62-positive puncta, while R864X KIF5A was diffused within neurites. Notably, both mutants also showed limited colocalization with mitochondria, whose axonal transport relies on KIF5A, and sequestered WT KIF5A within cell protrusions. Cycloheximide chase in SH-SY5Y cells evidenced shorter half-life for the R17Q and N999Vfs*39 mutants compared to WT KIF5A, hinting at altered protein turnover. In line with this observation, proteasomal blockage induced R17Q and N999Vfs*39 KIF5A accumulation into detergent-insoluble inclusions, indicating that the two mutants are preferentially degraded by the ubiquitin-proteasome system and that they may form harmful aggregates upon proteostasis impairment. Interestingly, some features characterizing the ALS-associated N999Vfs*39 mutant are recapitulated by another frameshift variant, C975Vfs*73 KIF5A, linked to a severe neurodevelopmental disorder. Indeed, C975Vfs*73 KIF5A, too, was found to aggregate into large, detergent-insoluble, p62-positive inclusions sequestering WT KIF5A and to display limited colocalization with mitochondria. Together, our results suggest that both unique and shared pathogenetic mechanisms underlie KIF5Alinked MNDs.

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BOOSTING NERVE REGENERATION IN ALS BY TARGETING THE PERIPHERY

D'Este G.[1], Negro S.[1,2], Fabris F.[1], Zanetti G.[1], Megighian A.[1,3], Bertoli A.[1,4], Massimino M.[1,4], Nardo G.[5], Caterina Bendotti[5], Basso M.[6], Bonetto V.[7], Corona C.[8], Montecucco C.[1,4] , Pirazzini M.[1,9] and Rigoni M.[1,9]

1Department of Biomedical Sciences, University of Padova, Padova, Italy; 2 O.U.C. Clinica Neurologica,Azienda Ospedaliera, Padova, Italy; 3 Padua Neuroscience Center, University of Padova, Padova, Italy; 4 Neuroscience Institute, CNR, Padova, Italy; 5 Department of Neuroscience-IRCCSIstituto di Ricerche Farmacologiche "Mario Negri", Milano, Italy; 6 Department of Cellular, Computational and Integrative Biology - CIBIO, University of Trento, Trento, Italy; 7 Department of Molecular Biochemistry and Pharmacology, IRCCS-Istituto di Ricerche Farmacologiche "Mario Negri", Milano, Italy; 8 Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta, Torino, Italy; 9 Myology Center (Cir-Mio), University of Padova, Padova, Italy.

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 CXCL12α 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. To test our hypothesis we used both behavioural tests, functional analysis and immunofluorescence. We found that: i) In SOD1G93A mice, CXCR4 is expressed in nerve terminals of both fast and slow MNs in a presymptomatic 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. These results prompts 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.

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KNOCKDOWN OF TDP-43G376D MUTATION USING A SMALL INTERFERING RNA AS A POTENTIAL THERAPEUTIC TOOL IN THE TREATMENT OF FAMILIAL AMYOTROPHIC LATERAL SCLEROSIS

Victoria S. Del Fiore[1], Roberta Romano[1], Maria De Luca[1], Martina Pecoraro[2], Serena Lattante[3,4], Mario Sabatelli[5,6], Vincenzo La Bella[2], and Cecilia Bucci[1]

1Dept. of Biological and Environmental Sciences and Technologies (DiSTeBA), Via Provinciale Lecce-Monteroni n.165, 73100 Lecce, Italy; 2 ALS Clinical Research Center, P Giaccone University Hospital and Department of Biomedicine, Neuroscience and advanced Diagnostic (BIND), University of Palermo, via Gaetano La Loggia n° 1, 90129, Palermo, Italy; 3 Section of Genomic Medicine, Department of Life Sciences and Public Health, Università Cattolica del Sacro Cuore, Rome, Italy; 4Unit of Medical Genetics, Department of Laboratory and Infectious Disease Sciences, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy; 5Adult NEMO Clinical Center, Unit of Neurology, Department of Aging, Neurological, Orthopedic and Head-Neck Sciences, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome, Italy; 6 Section of Neurology, Department of Neuroscience, Faculty of Medicine and Surgery, Università Cattolica del Sacro Cuore, Rome, Italy.

Mutations in the TARDBP gene, which codes for TDP-43, are related to several cases of amyotrophic lateral sclerosis (ALS). A heterozygous mutation (c.1127G>A; p.G376D) in the TARDBP gene leads to the onset of ALS in an Italian family. TDP-43 is a highly conserved protein involved in RNA metabolism. Accordingly, TDP-43 localizes in the nucleus, but it can shuttle between nucleus and cytoplasm. Under pathological conditions, mislocalization of TDP-43 occurs and toxic aggregates accumulate in the cytosol. In this study we aimed to investigate the use of allele-specific RNA interference (RNAi) as a potential therapeutic tool to specifically silence TDP43G376D. We designed different siRNAs and we evaluated their ability to silence specifically the mutated allele in the HEK293T cellular model, and in dermal fibroblasts from patients. The efficacy of the siRNAs was tested using qPCR and western blotting. Among the siRNAs analyzed, the siRNA called M10 reduced the mutated protein and mRNA levels without affecting wt mRNA and protein. Using confocal microscopy, we observed that M10 is also able to reduce the pathological aggregates both in transfected cells and in patients' cells. Furthermore, we performed a cell viability assay and a dichlorodihydrofluorescein diacetate (DCFH-DA) staining to investigate oxidative stress before and after the treatment with M10. The G376D mutation leads to a reduction of viability in Neuro2A cells and it increases oxidative stress in patients' fibroblasts. M10 was able to restore these two fundamental aspects. Thus, in this study we demonstrated that M10 is able to reduce the formation of toxic aggregates and to ameliorate general conditions of cells carrying the G376D mutation suggesting that RNA interference could be a potential therapeutic tool in the treatment of fALS. The work was partially supported by Association 2HE and by Regione Puglia-Malattie Rare DUP n. 246 of 2019.

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BLOCKING THE β6/β7 LOOP EPITOPE OF MISFOLDED SOD1 STRONGLY DELAYS DISEASE ONSET AND EXTENDS SURVIVAL IN A MOUSE MODEL OF ALS

Shamchal Bakavayev, Alexandra Stavsky, Zeev Barak, Adrian Israelson, Stanislav Engel

Ben-Gurion University of the Negev, Beer-Sheva, Israel

The current strategy to mitigate the toxicity of misfolded SOD1 in familial ALS is by blocking SOD1 expression in the CNS. Being indiscriminative toward misfolded and intact SOD1 proteins, such treatment, however, entails a risk of depriving the CNS cells of their essential antioxidant potential.
Here, we developed scFv-SE21 intrabody to block the β6/β7 loop epitope exposed exclusively in misfolded SOD1, as an alternative approach to neutralize misfolded SOD1 species and spare unaffected SOD1 proteins. ScFv-SE21 expression in the CNS of hSOD1G37R mice rescued spinal motoneurons, reduced the accumulation of misfolded SOD1, decreased gliosis, and thus delayed disease onset and extended survival by 90 days.
Our results provide evidence that the exposure of the β6/β7 loop epitope is part of the pathogenic mechanism of misfolded SOD1, and raise the possibility that its blocking may constitute a novel therapeutic approach for ALS, with a reduced risk of collateral oxidative damage to the CNS.
The research was funded by the Israel Science Foundation.

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

Gazzano A.[1*], Davide C.[1], Spatafora M.G.[1], Lamacchia A.[1], Marsala A.[1], Doria E.[2], Sponchioni M.[3], Auriemma R.[3], Lasciafari A.[4], Filibian M.[4], Moscatelli D.[3], Peviani M.[1#]

[1]Cellular and Molecular Neuropharmacology lab, Dpt Biology and Biotechnology “L.Spallanzani”, University of Pavia, Pavia, Italy [2]Pharmacology lab, Dpt Biology and Biotechnology “L.Spallanzani”, University of Pavia, Pavia, Italy [3]Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano, Milano, Italy [4]MR and micro-CT Imaging Laboratory, University of Pavia, Pavia, Italy

Background. Neuroinflammation is recognized as a pathological hallmark and potential therapeutic target for many neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS). However, neuroinflammatory responses are heterogeneous and reflect not only the extent of neuronal demise but also variable engagement glial cells in the attempt to cope with the neuronal damage. Thus, new pharmacological tools targeting specific cell subpopulations are warranted. We hypothesized that TSPO ligands, already widely used in the clinic to track neuroinflammation through PET, could be exploited to achieve selective cell targeting via a novel theranostic platform based on MRI/PET traceable nanoparticles (NPs). Aims. In this work we aimed at: i) obtaining an in-depth analysis of TSPO distribution and correlation with disease stage in ALS animal models; ii) validating TSPO-targeted nanoparticles as potential novel cell-specific pharmacological tool. 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, 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 TSPOselective 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.

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AGONIST OF GROWTH HORMONE-RELEASING HORMONE IMPROVES THE DISEASE FEATURES OF SPINAL MUSCULAR ATROPHY MICE

Boido M.[1], Gesmundo I. [2], Caretto A.[1], Pedrolli F. [2], Schellino R.[1], Leone S.[3], Cai R.[4,5], Sha W.[4], Ghigo E.[2], Schally A.V.[4,5], Vercelli A.[1], Granata R.[2]

[1]Department of Neuroscience "Rita Levi Montalcini", Neuroscience Institute Cavalieri Ottolenghi, University of Turin, Turin, Italy; [2]Division of Endocrinology, Diabetes and Metabolism, Department of Medical Sciences, University of Turin, Turin, Italy; [3]Department of Pharmacy, G. D'Annunzio University, Chieti, Italy; [4]Endocrine, Polypeptide, and Cancer Institute, Veterans Affairs Medical Center, Miami, FL, United States; [5]South Florida VA Foundation for Research and Education, Veterans Affairs Medical Center, Miami, FL, United States.

Spinal Muscular Atrophy (SMA) is a degenerative neuromuscular disease, affecting children and young adults, which entails progressive muscle deterioration and general weakness. SMA is caused by the deletion or mutation of the telomeric survival motor neuron 1 gene (SMN1), resulting in a progressive degeneration of spinal alpha motor neurons (αMNs) and, consequently, skeletal muscular atrophy and neuromuscular junction (NMJ) loss. Both growth hormone-releasing hormone (GHRH) and its potent agonistic analog, MR-409, exert protective effects on muscle atrophy, cardiomyopathies, ischemic stroke, and inflammation; however, their possible role in SMA remains to be elucidated. Thus, aim of this study was to assess the protective role of MR-409 in SMNΔ7 mice, a widely used model of SMA. Daily subcutaneous treatment with MR-409 (1 or 2 mg/Kg), from postnatal day 2 (P2) to sacrifice (P12), increased body weight and improved motor behavior in SMA mice, particularly at the highest dose tested. Moreover, hematoxylin/eosin staining of quadriceps and gastrocnemius muscles also revealed a significant increase in muscular fibers size induced by MR409. These effects are correlated with an upregulation in gene expression of myogenic regulatory factors (Myod1 and Myog) and myosin heavy chain (Myh) isoforms and a reduced expression, at both mRNA and protein levels, of muscle atrophy markers (atrogin-1 and MuRF1) in quadriceps and gastrocnemius muscles. MR-409 also promoted the maturation of neuromuscular junctions (NMJs), by reducing multi-innervated endplates and increasing those mono-innervated. Lastly, treatment with MR-409 delayed αMNs death and blunted neuroinflammation in the spinal cord of SMA mice. In conclusion, these results demonstrate the protective effects of MR-409 in SMNΔ7 mice, providing insight into the possible role of GHRH agonists as therapeutic agents for the treatment of SMA, possibly in combination with SMN-dependent strategies.

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NON-CELL AUTONOMOUS REGULATION OF NEURONAL CIRCUITS FORMATION AT EARLY STAGES OF DEVELOPMENT IN THE VENTRAL SPINAL CORD

Luca Goffin, Andrea Angla-Navarro, Mathilde Toch and Frederic Clotman

Laboratory of Neural Differentiation; Animal Molecular and Cellular Biology group (AMCB) - UCLouvain, Louvain Institute of Biomolecular Science and Technology (LIBST), Place Croix du Sud 5, 1348 Louvain la Neuve, Belgium

So far, there are very few studies that have explored non-cell autonomous mechanisms involved in the formation of neuronal circuits during the embryonic development of the spinal cord.
Previous work of the lab demonstrated that the conditional inactivation of Onecut transcription factors (OC) in spinal motor neurons causes perturbations in the development of ventral interneuron populations in a non-cell autonomous manner. Moreover, genes downstream of the Onecut factors that could be implicated in this process were identified by RNA-seq.
In this work, we studied the potential role of two of the candidate genes, tac1 (tachykinin precursor 1) and nt3 (neurotrophin-3) in ventral interneuron differentiation and distribution in the developing spinal cord. Possible cell-autonomous modulation of the motor neurons was also analyzed. Their expression was first validated in the spinal cord by in-situ hybridization both in mouse and chick embryos. Then, vectors that allowed us to overexpress the genes of interest in the ventral spinal motor neurons (pHb9-Nt3/Tac1-IRES-GFP) were electroporated in the spinal cord of chicken embryos. The possible effects of the induced perturbations on the different neuronal populations were studied at HH27-28 by immunofluorescence techniques.
Results showed that the overexpression of neurotrophin-3 in spinal motor neurons alters the differentiation of V2 interneurons in a non-cell autonomous manner.
Further experiments in mice are needed to assess that these genes regulated by Onecut factors contribute to non-cell autonomous regulation of spinal interneuron development.

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SYNCRIP/HRPR-1 RESCUES NEURODEGENERATION VIA RTN/RET-1 IN A C. ELEGANS SMA MODEL

La Rocca F.[1,2] , Santonicola P.[1], Cieri F.[1,3], Zampi G.[1], Nizzardo M.[4], Corti S.[4,5], Di Schiavi E.[1]

[1]Institute of Biosciences and Bioresources, IBBR, CNR, Naples, Italy; [2]Department of Environmental, Biological and Pharmaceutical Sciences and Technologies, University of Campania "L. Vanvitelli", Caserta, Italy; [3]Department of Biology, University of Naples "Federico II", Naples, Italy; [4]Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy. [5]Dino Ferrari Centre, University of Milan, Italy.

The molecular mechanisms underlying the relationship of motor neurons (MNs) degeneration and splicing in SMA are still elusive. Small model systems can be essential to identify the molecular pathways regulating splicing and responsible for neurodegeneration, but also to find new potential therapeutic strategies. In our previous work, RNA-sequencing of induced pluripotent cell-derived motor neurons (iPSC-MNs) from SMA patients and healthy people allowed the identification of differentially spliced genes, enriched in RNA motif 7. This motif is specifically bound by hnRNP Q/SYNCRIP, a spliceosomal component, physically interacting with SMN. To investigate in vivo SYNCRIP role in SMN pathway, we used C. elegans. hrpr-1 and smn-1 are the C. elegans homologs of SYNCRIP and SMN. We determined that hrpr-1mutant animals show pleiotropic phenotypes similar to the one observed in smn-1(KO), arresting as larvae, with a severe decrease in lifespan and locomotion defects. hrpr-1 mutants also show a reduction in MNs and axonal defects, suggesting a specific role in neuron survival. We demonstrated that hrpr-1 and smn-1 genetically interact in MNs, where the overexpression of hrpr-1 rescues the neurodegeneration caused by the MNs-specific silencing of smn-1. Since hrpr-1 is involved in regulating alternative splicing, we investigated the role in smn-1 pathway of a well-known hrpr-1 target, ret-1/RTN. We determined that in smn-1(KO) and hrpr-1(RNAi) animals the splicing pattern of ret-1 is similarly altered and that the rescue of MNs degeneration obtained after hrpr-1 overexpression in smn-1(MNs RNAi) animals, is mediated by ret1. Interestingly the role of ret-1/RTN in neurodegeneration and SMA may be conserved between species, since we observed that RTN transcription levels are altered in SMA mice and iPSC-MNs from SMA patients. These data support a neuroprotective role of hrpr-1 and ret-1 in SMA and their possible role as potential new therapeutic targets.
We thank the PON project for funding.

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INTRACEREBROVENTRICULAR TRANSPLANTATION OF NEURAL STEM CELLS IN AN EXPERIMENTAL MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

Lombardi I.[1], de la Morena S.[2], Pastor Campos D.[3], Carletti R M[4], Gelati M[5], Profico D C[5], Grespi V[6], Muzi G[6], Rasà D M[2, 7], Rosati J[8], Perciballi E[1], Mazzini L[9], De Marchi F[9], Vercelli A[2, 7], Martinez Perez S[10], Vescovi A L[1, 4], Boido M[2, 7] and Ferrari D[1]

1 Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan, Italy; 2 Neuroscience Institute Cavalieri Ottolenghi, University of Turin, Turin, Italy; 3 Department of Sport Science, Miguel Hernández University, Avinguda de la Universitat d'Elx, Alicante, Spain; 4 Fondazione IRCCS, Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy; 5 Fondazione IRCCS Casa Sollievo della Sofferenza, Production Unit of Advanced Therapies (UPTA), Institute for Stem-Cell Biology, Regenerative Medicine and Innovative Therapies (ISBReMIT), San Giovanni Rotondo, Italy; 6 Cell Factory and Biobank Santa Maria Hospital, Terni, Italy; 7 Department of Neuroscience “Rita Levi Montalcini”, University of Turin, Turin, Italy; 8 Cellular Reprogramming Unit, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Italy; 9 Centro Regionale Esperto SLA Azienda Ospedaliero-Universitaria “Maggiore della Carità”, Novara, Italy; 10Instituto de Neurociencias de Alicante, Alicante, Spain

Human Neural Stem Cells (hNSCs) treatment for neurodegenerative and neuroinflammatory diseases might exert antagonizing effects on inflammation and neurodegeneration. We showed that hNSCs transplantation in the spinal cord of SOD1G93A rats can delay disease progression, motor functions deterioration and significantly extends animals survival. These clinical improvements were associated with a reduction of ALS histopathological markers and motor neurons preservation in the transplanted areas. Moreover, we demonstrated that the procedure is feasible in ALS patients. The patient cohort was too small to draw final conclusions, however we observed a significant transitory decline of the ALS- FRS-r score progression for up to 4 months, in accordance with another comparable trial (NCT01348451). To improve the hNSCs efficacy, a conceivable hypothesis is to increase cell dosage. This objective has been tackled by increasing the number of spinal cord injections (NCT01730716), however, this approach is limited due to the backbone destabilization consequent to the surgery. Here, we are evaluating the implementation of intracerebroventricular delivery of hNSCs, as an effective strategy to increase cell dosage, favor a broader spread of transplanted cells and of their secreted healing factors throughout the motor neuraxis by exploiting the liquor circulation. We show that hNSCs (300,000 cells/mice) transplanted into the lateral ventricle of immunodeficient mice are well tolerated and not tumorigenic after 6 months, can extensively migrate and adhere to the ventricle wall occasionally migrating into the parenchyma. The same dosage was injected into the brain of SOD1G93A mice using a transient immunosuppression protocol. hNSCs survived for at least 2 months and preliminary data suggest that the treatment improved mice motor performances. However, cell survival was not optimal, thus, the reduced sample size prohibits any conclusions on survival. We are currently evaluating the safety and efficacy of increased dosage of hNSCs (up to 1x106 cells) in nude and SOD1G93A mice.

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ASTROCYTES' EFFECT ON MOTONEURONS MORPHOLOGY AND NEURO-ACTIVITY IN RIBOFLAVIN TRANSPORTER DEFICIENCY PATIENTS

Maglioccca V.[1] , Marioli C.[3,1], Bertini E.[2] , Tartaglia M.[1] , Persichini T.[3] and Compagnucci C.[1]

[1]Genetics and Rare Diseases Research Division, IRCCS Ospedale Pediatrico Bambino Gesù, 00146 Rome, Italy. [2]Dept. of Neuroscience, Unit of Neuromuscular and Neurodegenerative Diseases IRCCS, Children's Research Hospital Bambino Gesù, 00146 Rome, Italy [3]Dept. of Science, University Roma Tre, 00146 Rome, Italy

Riboflavin (Rf) is an important B2 vitamin and precursor of Flavin Adenine Dinucleotide (FAD) and Flavin Mononucleotide (FMN), two biological cofactors involved in different metabolic redox reactions, especially in mitochondrial processes. Mutations in the genes which encode for human Riboflavin Transporters hRFT2 and hRFT3 (called SLC52A3 and SLC52A2 respectively), cause a rare autosomal recessive disease that arises in childhood, known as Riboflavin Transporter Deficiency (RTD). To better understand the patho-mechanisms which characterize this neurodegenerative disease, the induced pluripotent stem cells (known as iPSCs) were used as in vitro cellular model to recapitulate the pathology. RTD is classified as motoneuronal progressive disease, because motoneurons (MNs) represent the most affected cell type: it is known that RTD MNs, obtained from iPSCs, are shorter than healthy ones, have less branches and have impaired neuronal activity. Since it has been highlighted the role of the antioxidant response of astrocytes (ASTROs) in the protection against neurotoxicity, as well as in the improvement of morphology and neuronal activity, we aimed our study to understand if RTD MNs phenotype could ameliorate when RTD MNs are grown in coculture with ASTROs. So, we differentiate RTD iPSCs into ASTROs and examine their effect on RTD MNs. Evaluating of neurites length (using Neurotrack application of Incucyte SX5 system) and intracellular calcium (Ca+2) levels after Ionomycin stimulation (using Fluo4-AM vital probe) in cocultures with ASTROs, we demonstrate that RTD MNs show increased neurites length and improved intracellular Ca+2 influx if compared to RTD MNs alone, obtaining similar results in two different conditions: co-cultures with and without direct contact. Therefore, these results underline ASTROs have a supporting role toward MNs, in particular to establish a morpho-functional rescue when in coculture. In conclusion, we suggest that in RTD pathology there are cell-autonomous defects in MNs, while ASTROs have no evident alterations.

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THE INFLUENCE OF ARABLE CROPS ON ALS RISK AND AGE AT ONSET: A POPULATION-BASED STUDY

Manera U[1,2], Callegaro S[1], Vasta R[1], Grassano M[1], Canosa A[1,2,3], Cabras S[1], Di Pede F[1], Matteoni E[1], De Marchi F[4], Mazzini L[4], Moglia C[1,2], Calvo A[1,2], Chiò A[1,2,3]

[1] ALS Centre, "Rita Levi Montalcini" Department of Neuroscience, University of Torino, Turin, Italy
[2] ALS Centre, SC Neurologia 1U, AOU Città della Salute e della Scienza of Torino, Turin, Italy
[3] Institute of Cognitive Sciences and Technologies, C.N.R., Rome, Italy
[4] Department of Neurology and ALS Centre, University of Piemonte Orientale, Maggiore della Carità Hospital, Novara, Italy

Several environmental risk factors, such as exposures to neurotoxic chemicals (i.e pesticides), and some professional categories, such as agricultural workers have been related to ALS risk, but with inconsistent results. We investigated the effect of living near to specific agricultural crops on the ALS risk and age at onset. We collected the residence at diagnosis of all ALS patients belonging to the Piemonte and Valle d'Aosta Register (PARALS), diagnosed between 2007 and 2014. Using data from the Regional Environmental Protection Agency (ARPA), we gather data on the geographical distribution of agricultural areas in the same period. First, for all the municipalities we calculated the area covered by each culture and patients smoothed incidence and we compared them using linear regression. Second, a proximity score for each environmental factor was calculated using the area of the environmental component enclosed by a circle centered on the residence address, considering variable radii (ranging from 100 to 2000 meters).
The regression model for arable crops confirmed a linear increase in ALS incidence in the municipalities with a larger area covered by arable crops. Median incidence increased from 0.75 (IQR 0.00-1.26) cases/100.000/year in municipalities with no area covered by arable crops to 1.81 (IQR 0.75-4.11) cases/100.000/year where arable crops covered more than 60% of total municipality area. The proximity score analysis confirmed that arable crops proximity (considering a 500, 1000, 1500 meters radii) significantly reduced the age at onset by about 2 years. Linear regression significantly confirm this trend (p=0.0245).
An higher ALS patients incidence was found in the municipality with high percentage of arable land. Arable land proximity scores resulted to be the related to a significantly reduced median onset age, confirming the presence of possible environmental factors that could anticipate disease onset.
The European Union's Horizon 2020 research and innovation programme (Brainteaser Project, grant GA101017598). Our special thanks to the Regional Environmental Protection Agency (ARPA Piemonte).

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INCREASED APOPTOTIC CELL DEATH IN RIBOFLAVIN TRANSPORTER DEFICIENCY

Marioli C.[1,2], Muzzi M.[1], Colasuonno F.[2], Magliocca V.[2], Bertini E.[2], Tartaglia M.[2], Compagnucci C.[2] and Moreno S. [1]

[1]Dept. of Science, University Roma Tre, Rome, Italy. [2]Genetics and Rare Diseases Research Division, IRCCS Bambino Gesù Children's Hospital, Rome, Italy

Riboflavin Transporter Deficiency (RTD) is a rare, neurological disorder characterized by hearing loss and sensory ataxia associated with spinal motor neuron (MN) degeneration. The disease is caused by loss of function mutations in SLC52A2/3 genes, respectively encoding riboflavin transporters hRFT2/3. As RF is the precursor of the coenzymes FMN and FAD, its abnormally low levels result in defective functionality of flavoproteins which are involved in cellular bioenergetics and cell survival processes. We previously demonstrated altered energy metabolism pathways depending on mitochondria and peroxisomes, accompanied by cytoskeletal derangement. As this disorder lacks dependable in vivo models, we took advantage of iPSC technology to recapitulate human neuronal features of RTD. More specifically we studied MNs differentiated from patient-specific iPSCs to perform combined ultrastructural and confocal analyses, aimed at characterizing the pathomechanisms associated to RTD. Patient-specific iPSCs and iPSC-derived MNs have been analysed by Focused Ion Beam/Scanning Electron Microscopy (FIB/SEM) and conventional SEM. RTD cells displayed profound alterations including neurite swellings, typical neurodegeneration hallmarks suggesting impaired intracellular trafficking. Increased apoptosis is observed in RTD cells, confirmed by the presence of vesicles and blebs budding from the cell surface of RTD cells and by activated caspase-3 immunofluorescence and TUNEL assays. Consistent with this results, ultrastructural characterizations revealed aberrant mitochondrial features confirming persistence of mitochondrial damage after differentiation, compatible with the fact that energy metabolism is impaired. Overall, our work contributes to the knowledge on the multiple cellular features associated to RTD neuronal phenotype, supporting a central role played by mitochondrial apoptosis in its pathogenesis, thus indicating potential targets for future therapies.
This work was supported by Cure RTD Foundation and MIUR fellowships to Marioli C.

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ABERRANT AXON MORPHOLOGY IN AN IMPORTIN MUTANT MOUSE

Marvaldi L[1-5], Panayotis N[1], Rishal I[1], Dagan SY[1], Rother F[2], Brumfeld V[3], Addadi Y[4], Bader M[2] and Fainzilber M[1]

[1]Dept.of Biomolecular Sciences, Weizmann Institute of Science, Rehovot, Israel [2]Max-Delbrück-Centre for Molecular Medicine, Berlin, Germany [3]Dept. of Chemical Research Support, Rehovot, Israel [4]Dept. of Biological Services, Weizmann Institute of Science, Rehovot, Israel [5]Dept. Veterinary Resources, Weizmann Institute of Science, Rehovot, Israel[5] Department of Neuroscience "Rita Levi Montalcini", Neuroscience Institute Cavalieri Ottolenghi, University of Turin, 10043 Orbassano (TO), Italy.

Importin alpha3 (Imp α3) is a nucleocytoplasmic protein that is essential for transduction of pain signals. Imp α3 knock out mice revealed specific sensorimotor deficits, including a delayed response to noxious heat stimuli (Marvaldi et al., 2020), and reduced performance in tests of proprioception or sensory-motor coordination (Panayotis et al., 2018).
Morphological analysis showed reduction of numbers of CGRP fiber endings in the skin and increased ChAT nerve terminals in the gastrocnemius muscle. Consistently, we observed a reduction of unmyelinated axons in the Imp α3 ko mice using electron microscopy.
Confocal and light sheet microscopy revealed strikingly abnormal growth patterns of sciatic nerve axons in the mutant mice, with marked reductions in fasciculation and repeated divergence of axons from the normal directionality of growth along the nerve axis.
Together, the lack of Imp α3 causes severe impairment of sensory and motor fibers suggesting that Imp α3 might be a key regulator of sensory and motor development.

DOTTORATO IN NEUROSCIENZE
PhD IN NEUROSCIENCE

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DRUG REPOSITIONING STRATEGY IN SPINAL MUSCULAR ATROPHY: THERAPEUTIC EFFECTS OF THE ANTIBIOTIC MOXIFLOXACIN IN SMNΔ7 MICE AND IN PATIENT DERIVED-CELLS.

Giovanna Menduti[1], Piotr Konieczny[2,3,4], Camille Januel[5,6], Cecile Martinat[5,6], Ruben Artero[2,3,4], Marina Boido[2]

1 Department of Neuroscience "Rita Levi Montalcini", Neuroscience Institute Cavalieri Ottolenghi, University of Turin, Torino, Italy
2 Interdisciplinary Research Structure for Biotechnology and Biomedicine (ERI BIOTECMED), University of Valencia, Valencia, Spain
3 Translational Genomics Group, Incliva Health Research Institute, Valencia, Spain
4 Incliva-CIPF Joint Unit, Valencia, Spain
5 INSERM/UEPS UMR 861, Paris Saclay Université, I-STEM, 91100 Corbeil-Essonnes, France
6 Université Paris-Saclay, Université d'Evry, U861, 91100 Corbeil-Essonnes, France

Spinal Muscular Atrophy (SMA) is a neurodegenerative disease affecting children, characterized by motor neuron (MN) impairment, skeletal muscle atrophy and premature death. It is caused by the mutation of the survival of motor neuron 1 (*SMN1*) gene. Its homologous *SMN2* is unaffected, but due to a preferential exon 7 skipping mostly generates a truncated and rapidly degraded protein delta7-SMN (SMNΔ7) and only about 10% of FL-SMN. Improving therapeutic strategies aimed at the increase of SMN2 function are still a hot topic in the SMA field. A screening of FDA-approved drugs revealed that the antibiotic Moxifloxacin exerts positive effects on *SMN2* exon 7 splicing, increasing SMN protein level in SMA models (Drosophila-base reporter and patients' fibroblasts). Here we demonstrated that daily subcutaneous injections of moxifloxacin in delta7 SMA mice increased the SMN levels in spinal cord (≥34%) and quadriceps (≥91%), compared to untreated mice, also leading to improved motor skills and extended lifespan. In addition, stereological and immunohistochemical analyses performed on lumbar spinal cord sections showed a delay in MN degeneration and a significant reduction in the levels of the apoptotic marker cleaved-caspase 3 (≤54%) in treated mice compared to controls. Moreover, also the astrogliosis (GFAP signal) was significantly reduced (≤48%), as well as a different degree of microglia ramification/activation was morphologically assessed in treated mice. Finally, moxifloxacin SMA skeletal muscles showed significant increase in trophism along with a decrease in neurofilament accumulation and denervation at neuromuscular junction level. We further confirmed the Moxifloxacin therapeutic effects in muscle cells and MNs derived from SMA type I patients-induced pluripotent stem cells (iPSCs) by improving the SMN2 splicing. The overall results support the drug repositioning strategy value for discovering new therapies for rare diseases and show that the antibiotic Moxifloxacin can be potentially repositioned for the SMA treatment.
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ACONITASE INACTIVATION AND IRON PERTURBATION IN THE SPINAL CORD OF SMAΔ7 MOUSE MODEL

Mezzanotte M.*, Pavarino G.*, Stanga S., Vercelli A., Boido M.

Neuroscience Institute Cavalieri Ottolenghi, 10043 Orbassano (TO), Italy; Department of Neuroscience "Rita Levi Montalcini", University of Turin, 10126 Turin, Italy
*These authors share first authorship.

Spinal Muscular Atrophy (SMA) is a pediatric and juvenile-onset neurodegenerative disease due to the autosomal recessive mutation/deletion of the Survival Motor Neuron 1 (SMN1) gene, causing the decrease of functional SMN protein levels, followed by the selective and early death of spinal cord (SC) motor neurons. Despite the genetic cause of SMA is known, many aspects of its pathogenesis are still not fully understood. During the pre-symptomatic stages of the disease, mitochondrial dysfunctions have been found in terms of functional and cellular homeostasis alterations, which are considered risk factors for SMA. In this context, we decided to study such dysfunctions in the SC of postnatal day 7 SMNΔ7 mice, a severe SMA model. First, by western blotting (WB) analysis, we identified alterations in mitochondrial dynamism such as increased fission (Drp1) and decreased fusion (Mfn2 and OPA1), without any change in mitochondrial content (Tom20). Furthermore, from a screening with 2-DE-MALDI-TOF-MS on pure mitochondria, we observed the altered expression of the Aconitase (mAcn) enzyme, which we then found post-translationally ubiquitinated and with a strong reduction (<40%) of functionality. mAcn is an iron-sulfur-containing protein and a responsive redox sensor, which plays a major role in mitochondrial and cellular homeostasis by acting in the Krebs cycle. Since mAcn is inactive when it loses its cofactor (iron), we measured by WB analysis the level of the mitochondrial iron storage protein (mFt), which we found decreased. This suggests that iron is not sufficiently stocked causing alteration and oxidative damage in SMA mitochondria. Interestingly, we identified a decreasing trend in the respiratory chain Complex V and Complex II levels, suggesting an impact on the efficiency of mitochondrial respiration in SMA. Overall, our data show alterations of mAcn activity together with iron perturbations in mitochondria from the SC, suggesting this enzyme as a potential target/marker of the disease.
The work was supported by Girotondo/ONLUS and SMArathon-ONLUS foundations, and by Fondazione CRT (grant RF 2017.2052).

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NERVE INJURY RAPIDLY INDUCES HYDROGEN PEROXIDE PROMOTING AXON REGENERATION VIA CONNECTIVE TISSUE GROWTH FACTOR

Negro S [1,2], , Lauria F[3], , Stazi M[1], , Tebaldi T[4,5] , D'Este G[1] , Pirazzini M[1,6] , Megighian A[1,7] , Lessi F[8] , Mazzanti CM[8] , Sales G[9] , Romualdi C[9] , Fillo S[10] , Lista F[10] , Sleigh J[11,12,13] , Tosolini A[11,12] , Schiavo G[11,12,13] , Viero G[3] and Rigoni M[1,6]

1Department of Biomedical Sciences, University of Padua, 35131 Padua, Italy, 2U.O.C. Clinica Neurologica, Azienda Ospedale, University of Padua, 35128 Padua, Italy, 3 Institute of Biophysics, CNR Unit at Trento, 38123 Povo, Italy, 4Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, 38123 Povo, Italy, 5Section of Hematology, Department of Internal Medicine, Yale Comprehensive Cancer Center, Yale University School of Medicine, 06520 New Haven, CT, USA, 6Myology Center (Cir-Myo), University of Padua, 35129 Padua, Italy, 7Padua Neuroscience Center, University of Padua, 35131 Padua, Italy, 8Laboratory of Genomics, Pisa Science Foundation, 56017 San Giuliano Terme, Italy, 9Department of Biology, University of Padua, 35131 Padua, Italy, 10Center of Medical and Veterinary Research of the Ministry of Defense, 00184 Rome, Italy, 11Department of Neuromuscular Diseases, Queen Square Institute of Neurology, University College London, WC1N 3BG, United Kingdom, 12UCL Queen Square Motor Neuron Disease Centre, University College London, WC1N 3BG, United Kingdom, 13UK Dementia Research Institute, University College London, WC1E 6BT, United Kingdom

Regeneration of the neuromuscular junction (NMJ) is orchestrated by signals released from motor axon terminals(MATs), muscle fibers and perisynaptic Schwann cells (PSCs), among which neuronal hydrogen peroxide (H2O2) is a major amplification signal. To identify critical determinants of NMJ remodeling in response to injury and the H2O2-activated gene signature, we performed temporal transcriptional profiling of the NMJ during MAT degeneration/regeneration, and cross-referenced the differentially expressed genes with those elicited by H2O2 in SCs. By Gene Ontology analysis we identified an enrichment in extracellular matrix (ECM) transcripts, including Connective Tissue Growth Factor (Ctgf). CTGF is a matricellular protein that do not participate in ECM structural integrity, rather it acts as modulators of a variety of cellular responses such as development and injury. Then, to test the role of CTGF in nerve peripheral nerve regeneration we employed electrophysiological, immunohistochemistry and in vivo live-imaging assay. By these approaches, we discovered that CTGF expression is increased in a YAP-dependent fashion in response to rapid, local H2O2 signaling generated by stressed mitochondria caused by injury (crush) to the sciatic nerve. By neutralizing CTGF or inactivating H2O2, we delayed the recovery of neuromuscular junction functionality by impairing SC migration and, in turn, axon-oriented re-growth. Our results indicate that H2O2 and its downstream product CTGF are pro-regenerative factors that enable axonal growth along CTGF-expressing SCs, and reveal striking ECM remodeling during nerve regeneration upon local H2O2 signaling.

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REPURPOSING POMALIDOMIDE AS A NEUROPROTECTIVE DRUG IN AN ALPHA-SYNUCLEIN-BASED MODEL OF PARKINSON'S DISEASE

Palmas M.F.[1] , Burgaletto C.[2], Casu M.A.[3], Cantarella G.[2], Etzi M.[1], De Simone A.[4], Fusco G.[5], Cardia M.C.[6], Lai F.[6], Tweedie D.[7], Scerba M.T.[7], Greig N.H.[7], Pisanu A.[8], Carta A.R.[1]

1Department of Biomedical Sciences, University of Cagliari, Cagliari, Italy 2Department of Biomedical and Biotechnological Sciences, University of Catania, Catania Italy 3CNR Institute of Translational Pharmacology, Cagliari, Italy 4Department of Pharmacy, University of Naples "Federico II", Naples, Italy 5Centre for Misfolding Diseases, Department of Chemistry, University of Cambridge, Cambridge, UK 6Department of Life and Environmental Sciences, University of Cagliari, Cagliari Italy 7Drug Design & Development Section, Translational Gerontology Branch, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, MD, United States 8National Research Council, Institute of Neuroscience, Cagliari, Italy

The development of therapeutic approaches to slow or even stop disease progression remains the greatest unmet therapeutic need in Parkinson's disease (PD). However, given the high cost and low success rate in new drug development, a complementary strategy based on repositioning drugs that are approved for other indications should be taken into consideration. Here, we tested for the first time the disease-modifying properties of the immunomodulatory imide drug (IMiD) pomalidomide in a translational rat model of PD based on the intranigral bilateral infusion of toxic oligomers of human α-synuclein (H-αSynOs). The neuroprotective effect of pomalidomide (20 mg/kg; i.p. three times/week) was tested in the first stage of disease progression by means of a chronic two-months administration. The infusion of H-αSynOs induced an impairment in motor performance that was fully rescued by pomalidomide, as assessed via a battery of motor tests. Moreover, H-αSynOs-infused rats displayed a 40–45% cell loss within the substantia nigra (SN), as measured by stereological counting of TH+ and Nissl-stained neurons, that was largely abolished by pomalidomide. The inflammatory response to H-αSynOs infusion and the pomalidomide treatment was evaluated both in CNS and peripherally. A reactive microgliosis was present in the SN three months after H-αSynOs infusion as well as after H-αSynOs plus pomalidomide treatment. However, microglia differed for their phenotype among experimental groups. After H-αSynOs infusion, microglia displayed a proinflammatory profile, producing a large amount the cytokine TNF-α. In contrast, pomalidomide inhibited the TNF-α overproduction and elevated the anti-inflammatory cytokine IL-10. Moreover, the H-αSynOs infusion induced a systemic inflammation with overproduction of serum proinflammatory cytokines and chemokines, that was largely mitigated by pomalidomide. Finally, we provide evidence of the disease modifying potential of pomalidomide in a progressive model of PD, thus adding a possible rationale for clinical testing of this drug in PD patients. This research was supported in part by (i) the Intramural Research Program at the University of Cagliari, Italy; (ii) the Intramural Research Program of the National Institute on Aging, National Institutes of Health, Baltimore, MD, USA; (iii) European Research Council, Award number: ERC CoG BioDisOrder 819644.

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

Raffaele S.[1], Nguyen N.[2], Boccazzi M.[3], Frumento G.[2], Milanese M.[2,4], Bonanno G[2,4], Abbracchio M P.[3], Bonifacino T[2,4], Fumagalli M[1]

[1]Department of Pharmacological and Biomolecular Sciences, Università degli Studi di Milano, Milano, Italy; [2]Department of Pharmacy, Unit of Pharmacology and Toxicology, Università degli Studi di Genova, Genova, Italy; [3]Department of Pharmaceutical Sciences, Università degli Studi di Milano, Milano, Italy; [4] Inter-University Center for the Promotion of the 3R Principles in Teaching and Research (Centro 3R), Genova, Italy

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the progressive loss of motor neurons (MN) and consequent muscle atrophy, for which no effective therapies are currently available. Recent findings revealed an early role of oligodendrocyte (OL) dysfunction in promoting MN degeneration and 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 for ALS. An important regulator of OPC differentiation is the P2Y-like GPR17 receptor, which drives the initial steps of this process, and it is then downregulated to allow OPC maturation. Our recent results revealed that an abnormal increase of GPR17 expression is associated to OL dysfunction in the spinal cord of the SOD1G93A murine model of ALS. Accordingly, primary OPCs isolated from SOD1G93A mice displayed differentiation defects compared to wild-type cells, which were rescued by in vitro exposure to the non-selective GPR17 antagonist montelukast (MTK). Overall, these results suggest that the GPR17 receptor may represent a promising therapeutic target in ALS. Here, we evaluated in vivo the effects of the oral administration of MTK (30 mg/kg/day), from symptom onset until end stage, in male and female SOD1G93A mice compared to vehicle-treated littermates. 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. Noteworthy, immunohistochemical analysis revealed that MTK administration significantly counteracted the pathological GPR17 upregulation in the spinal cord of female SOD1G93A mice in vivo, improving OL differentiation into CC1+ mature cells. In addition, MTK markedly enhanced the regenerative properties of microglia and astrocytes in the spinal cord of SOD1G93A mice. Globally, these data support the relevance of a GPR17-based pharmacological approach for ALS treatment.
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AN IN VITRO SET-UP STUDY TO ASSESS STRESSOR EFFECT ON AMYOTROPHIC LATERAL SCLEROSIS ONSET AND PROGRESSION

Rasà D.M.[1,2,3], Stoppa I.[2], Boido M.[1,2]

[1]Department of Neuroscience Rita Levi Montalcini, University of Turin, Turin, Italy
[2]Neuroscience Institute Cavalieri Ottolenghi, University of Turin, Orbassano (TO), Italy
[3]University School for Advanced Studies IUSS Pavia

Nowadays, our body continuously undergoes many stressors, due to physical, social and environmental events. These conditions can trigger several cellular alterations, in turn predisposing to neurodegenerative diseases, as Amyotrophic Lateral Sclerosis (ALS). ALS is a motor neuron (MN) disease, determining weakness, muscle atrophy and premature death. It is characterized by excitotoxicity, oxidative stress and neuroinflammation, cellular processes also activated by stressor exposure.
The purpose of the study is to clarify the stressor contribution in causing/anticipating ALS. To this aim, an in vitro experimental model of ALS has been set-up using NSC-34 cells expressing hSOD1(G93A) gene under the control of a doxycycline-inducible promoter. To differentiate the cells in MN-like cells, different retinoic acid (RA) concentrations have been tested: RA (1, 5, 10, 15 or 20µM) was added to the culture medium for 2, 4, 6 and 8 days. Based on the MTT assay results, 20µM RA for 4 days represented the most proper condition to induce cell maturation. Concerning the overexpression of hSOD1(G93A), the cells were grown in complete medium and 5µg/ml of doxycycline for 24h: Western Blot analysis confirmed hSOD1 expression, in both undifferentiated and differentiated cells. To reproduce a stress condition (oxygen deprivation, together with low or high glucose concentration), $CoCl_2$ (100 µM) was used as a hypoxic agent and its toxicity was measured by MTT. Finally, to evaluate the cell damage, we analyzed the mitochondrial activity by MitoTracker Red and total antioxidant capacity assay.
With these preliminary experiments, we have set-up the conditions for the next analyses, to evaluate genetic/epigenetic mutations and cellular/molecular alterations, and to clarify the stressor impact on the neurons and on the predisposition to neurological pathologies.

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BROWN ADIPOSE TISSUE: A NOVEL ACTOR IN THE PATHOGENESIS OF AMYOTROPHIC LATERAL SCLEROSIS

Rosina, M.[1,2], Scaricamazza, S.[3], Nesci, V.[3,4], Valle, C.[3,5], Lettieri Barbato, D.[2,6], Ferri, A.[3,5], Aquilano, K.[2], Mercuri, N.B.[1,4,7]

[1]Neurology Unit, PTV Foundation Tor Vergata University Hospital, Rome, Italy
[2]Department of Biology, University of Rome Tor Vergata, Rome, Italy
[3]Laboratory of Neurochemistry, Santa Lucia Foundation I.R.C.C.S., Rome, Italy
[4]Department of Systems Medicine, University of Rome Tor Vergata, Rome, Italy
[5]Institute of Translational Pharmacology (IFT), National Research Council, Rome, Italy
[6]Laboratory of Molecular Neuroimmunology, Santa Lucia Foundation I.R.C.C.S., Rome, Italy [7]Laboratory of Experimental Neurology, Santa Lucia Foundation I.R.C.C.S., Rome, Italy

Amyotrophic Lateral Sclerosis (ALS) is characterized by an excessive energy expenditure mainly related to a catabolic switch to fatty acid oxidation (FAO) in adipose tissue and skeletal muscle. Brown adipose tissue (BAT) play a pivotal role in controlling systemic energy expenditure and thermogenesis dependent on uncoupled respiration (UCP1) and/or SERCA/acto-myosin proteins. When activated, BAT secretes soluble factors and extracellular vesicles (EVs) that can alter molecular and metabolic pathways in target cells. In our study, we hypothesized that BAT could be involved in the altered metabolic phenotype of ALS mouse model SOD1-G93A through secretion of pro-catabolic factors. To this aim, we performed multi-omics analysis of BAT and BAT-EVs from 120 d.p.p. mice (SOD1- G93A vs wt). Moreover, we performed isolation and culture of BAT-derived primary adipocytes to monitor their metabolic profile through Seahorse Technology. Results show a functional enrichment of genes and protein related to SERCA/acto-myosin proteins and Sarcolipin, while UCP1 remains unaltered. Moreover, mitochondrial proteins resulted to be under-represented. Extracellular flux analysis through Seahorse Technology allowed us to determine a lower mitochondrial efficiency in SOD1-G93A BAT primary adipocytes, in terms of reduced Maximal Respiration and Spare Capacity. In parallel, proteomics profiling of BAT-EVs showed that EVs from SOD1-G93A mice contain increased abundance of mitochondrial proteins, maybe dependent on increased intracellular mitochondrial damage. Our results suggest that ALS-related environment has a detrimental effect on BAT functionality. Such alteration results in secretion of damaged mitochondrial proteins trough EVs. We propose that increased circulation of mitochondrial proteins could be at the basis of increased mitochondrial metabolism in target organs and a valuable biomarker for disease prediction and prognosis.
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AI-PROTEIN STRUCTURE PREDICTION INTEGRATED IN A PERSONALIZED DRUG DISCOVERY PIPELINE: A RARE IAHSP CASE

Matteo Rossi Sebastiano, Giuseppe Ermondi, and Giulia Caron

Molecular Biotechnology and Health Sciences Dept, University of Torino, Quarello 15, 10135 Torino, Italy

Infantile onset ascending hereditary spastic paralysis (IAHSP) is a neurodegenerative autosomal recessive rare disease characterized by a progressive degeneration of the upper motoneuron. Key events responsible for IAHSP are mutations to the gene *ALS2*, encoding for the cell trafficking protein Alsin. Being this protein a pivotal player for the development of the central nervous system, mutations characterize other juvenile ALS-like motor neuron disorders too. The variety of the mutational landscape can induce different effects: NMD mRNA degradation, truncated protein products and missense mutants, all specific for each patient. Especially missense mutations could benefit from the study of the corresponding 3D protein structures, but a major obstacle remains: the structure of Alsin has not been experimentally resolved yet.

Our approach consists in coupling 3D modeling with virtual drug discovery strategies for selected IAHSP cases. In the presenting case, we applied different protein modeling techniques generating the first tetramer model of Alsin, the active form. Furthermore, we used this information to clarify specific pathogenic mechanisms and investigate the druggability of representative mutants. A patient case harboring the missense mutation R1611W in the C-terminal VPS9 domain was studied. Once characterized its typical pathologic aggregation mode, we performed a drug virtual screening, repurposing an already commercialized molecule. This compound can bind the sidechain of the pathologically acquired amino-acid and re-establish the physiologic tetramerization, subcellular localization and downstream pathways of alsin. After *in vitro* proof-of-concept experiments it received compassionate use approval.

In our view, our research highlights the potential of a synergic application of modern structure prediction of difficult protein targets, in silico drug design strategies, and experimental tests providing concrete solutions to case studies.

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DIFFERENTIAL AUTOPHAGIC MARKERS REGULATION BETWEEN SPINAL MUSCULAR ATROPHY MUSCLE AND MOTONEURONS

Sansa A., Soler RM. and Garcera A.

Neuronal Signaling Unit, Departament de Medicina Experimental, Universitat de Lleida-IRBLleida, Av. Rovira Roure 80, 25198, Lleida, Spain.

Spinal Muscular Atrophy (SMA) is a severe degenerative neuromuscular disease and the first genetic cause of infant death. SMA is caused by the loss or mutation of the Survival Motor Neuron 1 gene (SMN1) and the decrease of the Survival Motor Neuron (SMN) protein. SMN levels contribute to determining the disease phenotype and severity. A centromeric duplication of this gene, SMN2, is responsible for SMN protein production in SMA conditions. The systemic decrease of SMN results in the alteration of various cells and tissues. Spinal cord motoneurons (MNs) are the cells with the greatest dysregulations in SMA disease. To develop new therapeutic strategies for preventing MN degeneration and disease progression, studying the cellular and molecular mechanisms underlying the collapse of these cells is needed. Here we propose to evaluate autophagy in SMA tissues and cells, including; muscle biopsies from SMA patients, gastrocnemius, spinal cords, and cultured MNs from a severe SMA mouse model, and human MNs differentiated from SMA iPSCs. We analyzed by western blot and immunofluorescence autophagy proteins involved in different stages of the process: mTOR, LC3, and p62. Muscle biopsies from SMA patients showed a reduction of the autophagy marker LC3-II. SMA mouse gastrocnemius presented lower levels of LC3-II and p62 at pre-symptomatic stage. However, we observed increased LC3-II and p62 levels in human-cultured SMA MNs. When analyzing mTOR pathway, mouse SMA muscle and spinal cordsshowed decreased mTOR phosphorylation at Ser2448, while in mouse and human cultured SMA MNs, mTOR phosphorylation was increased compared to the control condition. Altogether, these results suggest a differential regulation of the autophagy process and mTOR phosphorylation in muscle and MNs in SMA disease. These differences may reflect a specific response to SMN reduction, implying diverse tissue-dependent reactions to therapies that should be considered when treating SMA patients.
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CYTOARCHITECTURE OF THE CEREBRAL CORTEX OF A MURINE MODEL OF SPINAL MUSCULAR ATROPHY: FOCUS ON PROJECTION NEURONS AND INTERNEURONS

Schellino R.[1,2], Menduti G.[1,2], Boido M.[1,2], Vercelli A.[1,2]

[1]Department of Neuroscience "Rita Levi Montalcini", University of Torino, Torino, Italy. [2]Neuroscience Institute Cavalieri Ottolenghi, University of Torino, Orbassano (TO), Italy.

Spinal Muscular Atrophy (SMA) is a severe neurodegenerative disease of the early childhood, caused by the mutation/deletion of the survival motor neuron (SMN1) gene. It has been well established that the loss of functional SMN protein induces selective degeneration of spinal motor neurons (MNs), resulting in progressive skeletal muscle denervation and atrophy. However, little is known about the effects of SMN deficiency in the brain. Some functional alterations in cerebral neural networks have been documented in SMA models, and imaging studies on patients have demonstrated the occurrence of a reorganization of cortical grey matter. Thus, we focused on the sensorymotor cortex of SMAΔ7 mice, as a model of severe SMA, to investigate the effect of SMN deficiency on the cortical cytoarchitecture. We analyzed symptomatic animals (postnatal day 11), comparing SMA mice with their wild-type (WT) littermates. We investigated both projection neuron and interneuron distribution by immunofluorescence analysis, using different markers to identify specific neuronal subtypes. We confirmed a lower cell density in layer V of SMA cortex. Looking at different projection neuron subtypes, we discovered that both corticospinal (Ctip2-positive) and callosal (Satb2-positive) neurons are reduced by about 40% in SMA cortex, suggesting that SMN reduction could affect the upper MNs as well. Furthermore, we observed a differential distribution of interneuron populations (parvalbumin and somatostatin-positive cells), in both supragranular and infragranular layers, in SMA sensorymotor cortex compared to WT. Overall, we found a remodeling in cortical cytoarchitecture in SMA which could contribute to the etiopathology of the disease. Knowing the involvement of cerebral cortex in SMA will contribute to unravelling the dynamics of progressive degeneration occurring in the disease and will be also useful in designing new comprehensive treatments strategies, for better clinical outcomes.
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NOVEL INSIGHTS ON THE ROLE AND THERAPEUTIC POTENTIAL OF GLYCOPROTEIN NONMETASTATIC MELANOMA PROTEIN B (GPNMB) IN AMYOTROPHIC LATERAL SCLEROSIS

Mauro G. Spatafora[1*], Paolo Cabras[1], Gianluca Di Nolfi[1], Andrea Gazzano[1], Loris Bandirali[1], Bruno M Custode[1], Agnese Dimartino[1], Daniela Curti[1], Alessandra Biffi[2], Teuta Domi[3], Nilo Riva[3], Marco Peviani[1#]

[1]Cellular and Molecular Neuropharmacology lab, Dpt Biology and Biotechnology "L.Spallanzani", University of Pavia, Pavia, Italy [2]Gene Therapy Program, Dana-Farber/Boston Children's Cancer and Blood Disorders Center, Boston, MA, USA [3]INSPE, San Raffaele Scientific Institute, Milano, Italy
[*]Presenting author, [#]Corresponding author

Background. 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.
Aims. 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 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.
Discussion. 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.

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NEUROPROTECTIVE EFFECT OF EXTRACELLULAR VESICLES DERIVED FROM ADIPOSE STEM CELLS DIFFUSED THROUGH NASAL EPITHELIUM ON INJURED NEURONAL CELLS

Virla F.[1], Versuraro M.[1], Dabrowska S.[2], Scambi I.[1], Turano E.[1], Royo F.[3], Falcon-Perez J.[3], Mariotti R.[1]

[1]Department of Neuroscience, Biomedicine and Movement Sciences, University of Verona, Verona, Italy
[2]NeuroRepair Department, Mossakowski Medical Research Centre, PAS, Warsaw, Poland
[3]Exosomes Laboratory, Center for Cooperative Research in Biosciences (CIC bioGUNE), Basque Research and Technology Alliance (BRTA), Derio, Spain

A promising therapeutic option for neurodegenerative diseases is represented by stem cells that are able to differentiate and to migrate to damage tissues to stimulate reparative and regenerative processes. However, it is well-accepted that stem cells act through paracrine mechanisms releasing extracellular vesicles (EVs). Indeed, EVs are considered important mediators in intercellular communication as they can transfer their cargo (proteins, miRNAs and mRNAs) to nearby cells promoting neurogenesis, inhibiting apoptosis, enhancing immunomodulation in different pathophysiological contexts, recapitulating the effect of origin cells. An EVs-based therapy needs to identify an advantageous route of administration for therapeutic delivery: in this context we explore the intranasal (i.n.) route as a non-invasive strategy to deliver therapeutic agents directly to the brain. In particular, the aim of this study was to set up an in vitro model of nasal epithelium using RPMI 2650 cells and to evaluate the neuroprotective effect of EVs derived from murine adipose stem cells (ASCs) after their passage through the epithelial barrier on an injured model of both motor neuron (NSC-34) and neuron (SH-SY5Y) cells. Regarding the study of i.n. route, it is also crucial to identify the mechanisms by which EVs are able to cross the epithelium and reach the central nervous system. To do that we set up a fluorescent labelling protocol to isolate and characterize labelled EVs and to evaluate their uptake by injured NSC-34 cells. The results showed that EVs neuroprotective effects observed in previous studies were maintained after their passage through the nasal epithelium as well, with a rescue of the neuronal cells viability after oxidative stress. Moreover, the EVs labelling protocol allowed us to isolate fluorescent EVs that will pave the way for further studies in order to clarify their passage after i.n. administration and their capture by injured cells in the central nervous system.

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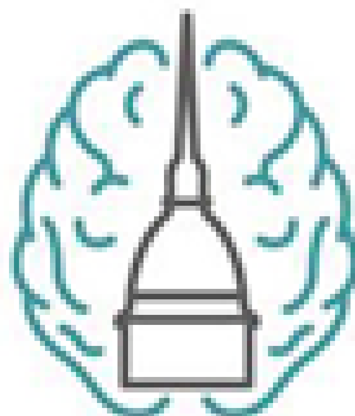

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