Presentazione di progetto per assegno A2J

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Project title: Investigating the contribution of the DNA damage repair pathway to neurodegeneration in ALS/FTD models

Abstract (max. 150 words)

Along with increased life expectancy, age-related neurodegenerative disorders such as Alzheimer's and Parkinson's disease, and amyotrophic lateral sclerosis (ALS) are more frequent.

Accumulation of DNA damage was observed in the tissues of patients affected by various neurodegenerative disorders including ALS were DNA damage was observed in motor neurons of patients with mutations in TDP-43, FUS, and C9ORF72 genes.

We have recently dissected the roles of FUS in mRNA biogenesis, and more recently in the cellular response to DNA damage (DDR). FUS, EWSR1 and TAF16 are the three members of the FET protein family. Mutations in all three FET proteins have been described in ALS and Frontotemporal dementia (FTD), a related disorder and the second most common form of dementia. Interestingly, literature evidence also suggest a role for these proteins in genome stability.

Our goals are: (i) the elucidation of the roles of FET proteins in DNA damage repair; (ii) the investigation of the neuropathological contribution of DDR activation using advanced cellular models of ALS/FTD.

This study will improve our understanding of the multiple pathological processes leading to neuronal cell death and will contribute to the development of novel therapeutic approaches.

Background, aims and significance of the proposed work (max 300 words)

Accumulation of DNA damage may be particularly prevalent in the central nervous system because of the lower DNA repair capacity of post-mitotic neurons, and may contribute to age-related neurodegeneration. Indeed, activation of the cellular response to DNA damage (DDR) has been observed in the affected tissues of several neurodegenerative disorders such as Alzheimer's diseases (AD), Parkinson's disease, and amyotrophic lateral sclerosis (ALS) (1).

ALS and frontotemporal dementia (FTD) are two genetically and pathologically related neurodegenerative disorders. Notably, FUS (fused in sarcoma) and TDP-43, two diseases proteins involved in both ALS and FTD have been directly linked to the DDR. More recently, DNA damage has been also discovered in C9ORF72 ALS (1). FUS is a member of the FET protein family that consists in addition of EWSR1 (Ewing's sarcoma) and TAF15 (TATA-binding protein associated factor 2N) (2). The FET proteins are DNA/RNA-binding proteins that participate in transcription and RNA processing. Mutations in the genes encoding FUS, EWSR1 and TAF15 have been recently identified not only in ALS and FTD but also in other neurological disorders (3).

Similarly to FUS, loss of EWSR1 causes genomic instability in cell types that undergo physiological DNA breaks such as B cells and meiotic germ cells. Interestingly, Ews–/– mice show premature cellular senescence and aging-like features. Compared to FUS and EWSR1, the biological function of TAF15 is less well-understood but as for FUS and EWSR1 it was originally associated with cancer.

We hypothesise that loss of nuclear function of FET proteins, due to mutations or sequestration, may affect DNA repair leading to the accumulation of DNA damage thus contributing to neuronal dysfunction in ALS/FTD. Therefore, we aim at elucidating the molecular function of FET proteins in DNA damage repair, and the impact of ALS-linked mutations on this pathway.

Experimental plan (max 1000 words)

WP1. Investigate the role of EWSR1 and TAF-15 in the DSB repair

A major open question in ALS is whether neuronal dysfunction caused by FUS mutations is due to a toxic gain of cytoplasmic function of the mutated, mislocalized FUS protein, or by a loss of nuclear function. To address this question, and in particular to elucidated the molecular function of FUS in DNA repair, we generated human HeLa and SH-SY5Y cells in which the FUS gene has been knockout (FUS-KO) by gene editing and we characterized them for their sensitivity to etoposide, a doublestrand breaks (DSBs)-inducing drug. We also assessed DNA repair efficiency using a GFP-based *in vivo* repair assay, and the recruitment kinetics of core DDR factors by time-lapse microscopy at laser-induced DNA damage sites (unpublished results, Lenzken et. al, manuscript in preparation). Based on these results we now want to define the molecular function of the other two members of the FET family in DNA repair.

Task1.1 Generation of gene-edited SH-SY5Y cells for EWSR1 and TAF15 using the CRIPR/CAS9nsystem.
Human neuroblastoma SH-SY5Y cells are a well-established in vitro model because they can be differentiated into neuron-like cells by retinoic acid treatment. We will use the CRISPR/Cas9 gene editing system in this cell line to generate: (i) EWSR1 and TAF15 knock-out cell lines to perform loss-of-function studies (Task 1.3); (ii) cells expressing endogenous mCherry-tagged wild type proteins to monitor the intracellular dynamics of the proteins (Task 1.2). (iii) cells expressing ALSlinked mutations (EWSR1 G511A, P552L; TAF15 D386N, R388H) via CRISPR Homology Direct Repair to be used for the experiments described in task 1.4.

Task1.2. Analysis of the role of EWSR1 and TAF15 in the DDR
To determine their association with DDR components we will perform co-immunoprecipitations of from nuclear extracts of SH-SY5Y cells treated with the DSB-inducing topoisomerase II inhibitor etoposide. To determine the presence of EWSR1 and TAF15 at DSBs we will exploit the AsiSI system to monitor their recruitment by chromatin immunoprecipitation (ChIP). This system is based on the tamoxifen-inducible expression of the DNA endonuclease AsiSI in the nucleus of U2OS cells to induce DSBs at known genomic loci and we have already used it in the lab. As an alternative approach we will use the gene-edited cells generated in task 1.1 to follow recruitment of the endogenous mCherry-tagged proteins at sites of laser microirradiation-induced DSBs. Moreover, we will evaluate the impact of the siRNA-mediated depletion of EWSR1 and TAF15 (i) on the recruitment of DDR effector in the AsiI system; (ii) on DSB repair, using a GFP-based HR and NHEJ reporter system that allows the monitoring of HR and NHEJ repair flow cytometry.

Task 1.3 Characterization of EWSR1 and TAF15 knock-out cell lines
Cells will be initially characterized for the sensitivity to different genotoxic drugs. Cell viability and proliferation will be assessed by MTT and clonogenic assays; cell cycle by FC (Flow Cytometry). Then, we will determine if the absence of EWSR1 and TAF15 affects DDR signalling by Western blotting, and immunofluorescence (by monitoring the formation of γH2AX and 53BP1 foci). Finally, we will characterizes the recruitment kinetics of apical GFP-tagged effectors in the absence of EWSR1 and TAF15.

Task 1.4 Evaluation of the biological effect of the ALS-linked mutations in FET proteins
We will characterize

- the intracellular localization of the mutant mCherry-tagged proteins by confocal microscopy to determine if the ALS-linked mutations, similarly to most FUS mutations lead to aberrant localization and aggregation.

- DDR signalling without and with genotoxic treatment (etoposide) by Western blotting and immunofluorescence as described in Task 1.3.

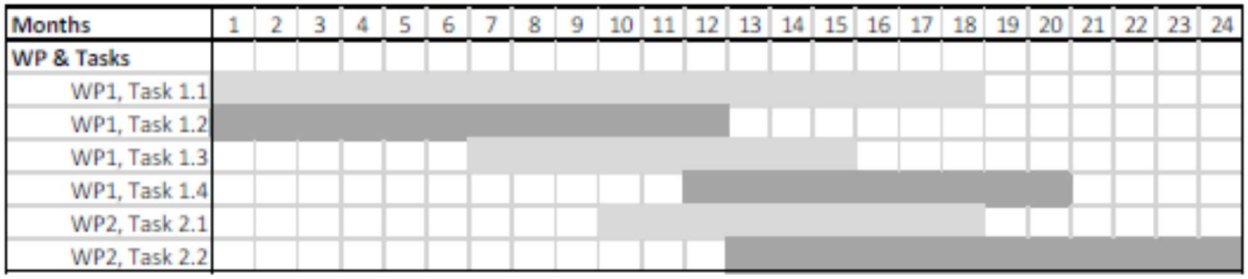
WP2. Characterization of the neuropathological role of DNA damage in advanced cellular models of ALS/FTD.

ALS and FTD are two genetically and pathologically related neurodegenerative disorders. ALS is characterized by the loss of upper and lower motor neurons (MNs). However, besides motor symptoms, ALS is also characterized by cognitive impairment and behavioural disorders, overlapping with the cognitive profile of FTD, the second most common form of dementia after Alzheimer's disease. To characterized the neuropathological effect of DNA damage we will use patients-derived iPSCs and iPSCs differentiated MNs.

Task 2.1 Characterization of the neuropathological role of the DDR in iPSCs and iPSCs differentiated motor neurons (MNs) expressing FUS and C9ORF72 mutations.
We will characterize the level of DSBs, of DDR activation, and of DNA damage-induced cell-death in iPSC-derived MNs carrying FUS (G156E, R244C, P525L) mutations or C9ORF72 expansions. The rational for the use of the C9ORF72 model derives from the fact that expansions of the hexanucleotide (G4C2) in an intron of the C9ORF72 gene are the most common cause of ALS and FTD. Moreover, EWSR1 and FUS are interactors of the GR/PR dipeptide repeat proteins produced from the hexanucleotide repeat expansion of the C9ORF72 gene. MNs will be identified by immunocytochemistry for specific marker (Islet1, Hb9 and ChAT) and by lentiviral transfection with HB9-GFP gene reporter allowing cell selection by FACS. The presence of DNA damage will be determined by immunofluorescence and Western blotting with anti-γH2AX or anti-53B1 antibodies, and as an alternative approach, using the Comet assay. DNA damage-induced toxicity will be evaluated by measuring cell-death (including activation of caspases) and by neurite outgrowth.

Task 2.2 Establishment of a 3D culturing system for investigating neurodegeneration
Gene edited SH-SY5Y cells carrying FUS, EWSR!, and TAF15 mutations will be used to establish a 3D culturing system for in vitro modelling of neurodegeneration. Cells will be pre-differentiated with RA for 5-7 days and then seeded into 3D Matrigel cultures. We will monitor the expression of differentiation markers by RT-qPCR and immunofluorescence compared to undifferentiated or 2Dseeded cells. As initial readout of neurodegeneration, we will monitor intracellular aggregation of the mutant FET proteins and activation of the DDR as described previously. As an alternative approach we will we will perform the experiments using the KO lines stably transfected with plasmids expressing mCherry-tagged wt and mutant proteins. The drawback of this approach is however that the proteins will be expressed from the viral promoter of the plasmid and even if we chose a weak promoter they will still not be expressed at physiological levels

Timetable



Feasibility and financial support (single statement)

The project is supported by University's funding (FAR_QC2020).

The applicant's lab has already performed similar experiments to those outlined in this proposal for the characterization of the role of FUS in the DDR including the generation of KO cell lines (Lenzken et. al, bioRXiv doi: https://doi.org/10.1101/798884).
Knock-in cell lines will be produced in collaboration with Dr. M.D. Ruepp at King's College London. Dr. Ruepp who has developed an efficient protocol for CRISPR/Cas9 gene replacement and will financially support this part of the project. He will also provide iPSCs carrying the FUS mutations and isogenic control. iPSCs lines carrying C9ORF72 expansions and isogenic control lines will be provided by Prof. Stefania Corti (University of Milan), who has an internationally recognized expertise in ALS and in stem cell-based in vitro models. Prof. Corti will provide the expertise for iPSCs differentiation into motor neurons according to their well-established protocol.

Literature references (max 5)

1. Coppede, F. and Migliore, L. (2015) DNA damage in neurodegenerative diseases. Mutation research, 776, 84-97.

2. Schwartz, J.C., Cech, T.R. and Parker, R.R. (2015) Biochemical Properties and Biological Functions of FET Proteins. Annual review of biochemistry, 84, 355-379.

3. Svetoni, F., Frisone, P. and Paronetto, M.P. (2016) Role of FET proteins in neurodegenerative disorders. RNA biology, 13, 1089-1102.

PI recent papers on the topic (max 5)

1. Lenzken SC, Levone BR, Filosa G, Antonaci M, Conte F, Kizilirmak C, Reber S, Loffreda A, Biella F, Ronchi AE, Mühlemann O, Bachi A, Ruepp M-D, Barabino SML (2019) FUS-dependent phase separation initiates double-strand break repair. bioRxiv: 798884
2. Loffreda1 A, Nizzardo M, Arosio A, Ruepp M-D, Calogero RA, Volinia S, Bendotti C, Ferrarese C, Lunetta C, Rizzuti M, Ronchi AE, Mühlemann O, Tremolizzo L, Corti S, Barabino SML "miR-129-5-p: a key factor and therapeutic target in amyotrophic lateral sclerosis" (Prog. Neurobiol., in press)
3. Rizzuti M, Filosa G, Melzi V, Calandriello L, Dioni L, Bollati V, Bresolin N, Comi GP, Barabino S, Nizzardo M, Corti S. "MicroRNA expression analysis identifies a subset of downregulated miRNAs in ALS motor neuron progenitors." Sci Rep. 2018 Jul 4;8(1):10105. doi: 10.1038/s41598-018-28366-1.
4. Reber R, Stettler J, Filosa, G., Colombo M, Jutzi D, Lenzken, SC, Schweingruber C, Bruggmann R, Bachi A, Barabino SML, Mühlemann O, Ruepp M-D "Minor intron splicing is regulated by FUS and affected by ALS-associated FUS mutants." EMBO J. 2016 Jul 15;35(14):1504-21. Doi: 10.15252/embj.201593791.
5. S.C. Lenzken, A. Loffreda, S.M. Barabino "RNA Splicing: A New Player in the DNA Damage Response. " Int J Cell Biol. (2013), 153634. Epub 2013 Sep 12.