IVT-01: A Personalized functional NMJ system for ALS Modeling and Drug Testing

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Background:
Over 6000 people in the U.S. are diagnosed with ALS each year and currently there is no cure despite decades of study. Despite of the heterogeneity of this disease, loss of neuromuscular junction (NMJ) function is an early and critical hallmark in all forms of ALS (1).

Objectives:
The study design was to develop a functional NMJ disease model by integrating motoneurons (MNs) differentiated from multiple ALS-patients' induced pluripotent stem cells (iPSCs) and primary human muscle into a chambered system (2).

Methods:
NMJ functionality was tested by recording myotube contractions while stimulating MNs by field electrodes and a set of clinically relevant parameters were defined to characterize the NMJ function. Three ALS lines were analyzed, 2 with SOD1 mutations and 1 with a FUS mutation.

Results:
The ALS-MNs reproduced pathological phenotypes, including increased axonal varicosities, reduced axonal branching and elongation and increased excitability. These MNs formed functional NMJs with wild type muscle, but with significant deficits in NMJ quantity, fidelity and fatigue index. Furthermore, treatment with a holistic combination of compounds called the Deana protocol was found to correct the NMJ deficits in all the ALS mutant lines tested. Quantitative analysis also revealed the variations inherent in each mutant lines.

Discussion:
This functional NMJ system provides a platform for the study of both fALS and sALS and has the capability of being adapted into subtype-specific or patient-specific models for ALS etiological investigation and patient stratification for drug testing.

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IVT-02: ALS-associated GLT8D1 and CAV1/CAV2 variants share a common pathway of pathogenesis in ALS.

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Background:
Amyotrophic lateral sclerosis (ALS) is an invariably fatal and relatively common neurodegenerative disorder without effective therapy. Identified genetic variants cluster in biological pathways including RNA processing, axonal transport, and protein homeostasis. Discovery of genetic variants within new biological pathways highlights new disease biology, and can lead to the identification of novel therapeutic targets. ALS-associated variants were recently discovered within GLT8D1 [1], as well as within enhancers linked to expression of CAV1 and CAV2 [2].

Aims and objectives:
To develop cell models to characterise the pathological pathway(s) linking ALS-associated GLT8D1, CAV1 and CAV2 mutations to motor neuron loss. To identify novel therapeutic targets for the treatment of ALS.

Results:
An ALS-associated R92C mutation in GLT8D1 reduces membrane ganglioside expression, which is indicative of dysregulated neurotrophic signalling. Ganglioside biosynthesis occurs in the Golgi; GLT8D1 localises to the Golgi in neuronal and non-neuronal cells [1], and R92C is linked to Golgi fragmentation. CAV1 and CAV2 encode major components of caveolae, which organise membrane lipid rafts (MLR) that are important for neurotrophic signalling. Gangliosides are a key component of MLR. Discovered enhancer mutations reduce CAV1/CAV2 expression and disrupt ganglioside expression within MLR in patient-derived lymphoblastoid cells. A CRISPR/Cas9 perturbation proximate to a patient-mutation is sufficient to reduce CAV1/CAV2 expression in neurons.

Conclusions:
These results place dysregulated ganglioside metabolism upstream in the pathogenesis of ALS. We propose that GLT8D1 and CAV1/CAV2 share a common pathway of pathogenesis in ALS via disruption of ganglioside recruitment to MLR and impaired neurotrophic signalling.

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IVT-03: Arginine DPRs disrupt the phase separation and transport dynamics of nucleoporins in C9orf72-mediated ALS and FTD

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The G4C2 hexanucleotide repeat expansion in the gene C9ORF72 is the most common cause of Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal dementia (FTD). Major progress has been made since the 2011 discovery of the repeat expansion in C9ORF72 in elucidating the underlying pathogenic mechanisms. The repeat expansion is unconventionally translated in the absence of ATG leading to the production of dipeptide repeat proteins (DPR) that aggregate in the brain. These DPRs, specifically the arginine rich DPRs, poly-GR and poly-PR, have been shown to be highly neurotoxic and disrupt several cellular processes including stress granule dynamics, nucleocytoplasmic transport and nucleolar dysfunction. These cellular processes all have one thing in common, they involve the formation membraneless organelles via liquid-liquid phase separation (LLPS) of low complexity domain containing proteins. The arginine DPRs have been shown to cause aberrant LLPS leading to a disruption in membraneless organelles. The pathological hallmark of C9ALS/FTD is the nuclear depletion and cytoplasmic accumulation of the RNA binding protein TDP-43 in degenerating neurons. Hence impaired nucleocytoplasmic transport has emerged as a key mechanism behind neuronal death. However, how the arginine DPRs impair nucleocytoplasmic trafficking has not been resolved. The FG (phenylalanine–glycine) nucleoporins (FG-Nups) are critical for nuclear pore complex (NPC) function, they are thought to form the permeability barrier of the NPC by LLPS. We show human FG-Nup domains spontaneously phase separate into particles that exclude inert macromolecules but allow the entry of nuclear transport receptors, mimicking the NPC selectivity barrier. We have found the arginine DPRs disrupt both the phase separation behaviour and transport dynamics of these human FG-Nup particles. Hence our results suggest the arginine DPRs are key to the nucleocytoplasmic transport abnormalities seen in C9ALS/FTD via a disruption of the phase separation behaviour of FG-Nups.

IVT-04: Astrocyte-induced DNA damage as a mechanism of motor neuron death in ALS

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Background and Hypothesis:
Astrocytes derived from sporadic and familial ALS patients and models have been consistently shown to be toxic to motor neurons. Interestingly, ALS astrocyte toxicity is also transmitted to motor neurons through conditioned medium, but the mechanisms and molecules involved remain elusive. Several studies have shown increased DNA damage as a consistent feature of sporadic ALS, and most familial ALS variants studied. Our data shows that astrocytes derived from familial ALS patients carrying a repeat expansion in the C9ORF72 gene can induce DNA damage in healthy mouse motor neurons. It was thus hypothesised that astrocyte-induced DNA damage could contribute to motor neuron death in ALS.

Methods:
To test this hypothesis, induced astrocytes (iAstrocytes), which retain epigenetic hallmarks of ageing, were obtained from sporadic ALS (n=3), C9ORF72-ALS (n=3) and SOD1-ALS (n=2) patient fibroblasts and healthy control donor fibroblasts (n=3), and the conditioned media was used to treat healthy human iPSC-derived motor neurons.

Results:
It was found that conditioned media from C9ORF72-ALS and sALS iAstrocytes, but not SOD1-ALS iAstrocytes, could induce a significant increase in DNA damage response activation, measured as γH2AX nuclear activation (sALS vs control p=0.03; C9 vs control p=0.007), in treated motor neurons within 24 hours. DNA damage response activation was found to precede cell death, as a significant increase in activated caspase-3 was only observed in motor neurons 72 hours after treatment (SOD1 p=0.0463, sALS vs control p=0.461; C9 vs control p<0.0001).

We are currently working to confirm whether ALS iAstrocyte conditioned media induces DNA damage as well as the DNA damage response, and determining the molecules involved in astrocyte-induced DNA damage. C9ORF72-ALS dipeptide repeat proteins (DPRs) have previously been shown to induce DNA damage when transfected into cells, and present a compelling candidate for the cause of C9ORF72-ALS iAstrocyte-induced DNA damage. We are also investigating oxidative stress as a potential mechanism of astrocyte-induced DNA damage.

Discussion:
In conclusion, our work identifies DNA damage as a possible mechanism by which ALS astrocytes induce motor neuron death. It is thus possible that preventing astrocyte-induced DNA damage or modulating the motor neuron response to this insult could provide potential therapeutic targets. As such, we will be collaborating with AstraZeneca to test compounds that could potentially rescue astrocyte-induced DNA damage and motor neuron death.

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IVT-05: C9orf72-derived arginine-containing dipeptide repeats associate with axonal transport machinery and impede microtubule-based motility

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A hexanucleotide repeat expansions in the C9orf72 gene is the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). How this mutation leads to these neurodegenerative diseases remains unclear. Here, we use human pluripotent stem cell-derived motor neurons to show that the repeat expansion impairs microtubule-based transport of mitochondria, a process critical for neuronal survival. The expansion decreases the frequency of transport events and increases the incidence of arresting cargos during movement. These cargo transport defects are recapitulated by treating healthy human motor neurons with proline-arginine (PR) and glycine-arginine (GR) dipeptide repeats (DPRs) produced from the repeat expansion. Both arginine-rich DPRs similarly inhibit mitochondrial trafficking in adult Drosophila neurons in situ, suggesting interference with fundamental transport mechanisms. Analysis of physical interactions demonstrate association of the arginine-rich DPRs with motor complexes and the unstructured tubulin tails of microtubules. The evidence includes enrichment of the kinesin-1 motor in DPR inclusions in post-mortem patient tissues. Single-molecule imaging in vitro reveals that microtubule-bound arginine-rich DPRs impede translocation of dynein and kinesin-1 motor complexes. Collectively, our study implicates inhibitory interactions of arginine-rich DPRs with the axonal transport machinery in C9orf72-associated ALS/FTD and thereby points to novel potential therapeutic strategies.

IVT-06: Case study: A new homozygous mutation in the HADHB gene causing a rare form of sensorimotor neuropathy and affecting motor neurons functions in culture

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Mutations in the HADHB gene lead to Mitochondrial Trifunctional Protein (MTP) deficiency. HADHB forms, together with the subunit A (HADHA), the trifunctional mitochondrial protein, which catalyzes crucial steps of beta oxidation.
MTP deficiency is a rare disease affecting long-chain fatty acid oxidation. Patients affected by MTP deficiency are unable to metabolize long-chain fatty-acids and suffer a variety of symptoms exacerbated during fasting. Mutations in HADHB gene and mitochondrial trifunctional protein defects are rare genetics disorders with no treatment and a range of symptoms. the key question is how degradation of long-chain fatty acide may affect in particular motor neuron, which metabolically depend on glucose. Indeed, the three phenotypes associated with complete MTP are an early-onset cardiomyopathy, hypoketotic hypoglycemia, and a sensorimotor neuropathy with episodic rhabdomyolysis.

Methods:
Clinical evaluation of the patients was carried out at the Montreal Neurological Institute clinic. The analysis of the mutation was carried out using next generation sequencing against a gene panel for motor neuron and peripheral neuropathies diseases. Fibroblasts were derived from a skin biopsy of the patient and relatives. Western blot analysis was used to study the expression levels of HADHB and HADHA and formation of the MTP complex in samples from fibroblasts while subcellular localization of HADHB and HADHA were studies by immunofluorescence. Motor neurons models of MTP was generated by intranuclear injection of plasmids encoding CRISPR cas9 and gRNA against HADHB in long-term cultures of murine motor neurons

Case study:
We report a case showing a homozygote mutation in the HADHB gene c.712 C>T. The patient present muscle atrophy and a sensorimotor neuropathy with mildly elevated carnityl- long chain fatty acid. The expression of HADHB variant Arg238Trp was severely diminished in patient's fibroblasts in culture indicating loss-of-function. Patient's fibroblasts show an abnormal mitochondrial and endoplasmic morphology indicative of a general defect in mitochondrial function. Interestingly, HADHA expression is downregulated and HADHA mislocalized in the patient's fibroblasts. We also generated a neuronal model using CRISPR-cas9 to investigate motor neuron viability and mitochondrial transport in culture.

Summary:
Interestingly, we described a novel mutation associated with a mild form of sensorimotor neuropathy. We also provide new insights on the role of lipid beta oxidation in motor neuron survival.
Acknowledgements: we thank the ARSACS foundation for financial support (G249285).

IVT-07: Cerebrospinal fluid cytotoxicity in amyotrophic lateral sclerosis: a systematic review of in vitro studies

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Background:
Various studies have suggested that a neurotoxic cerebrospinal fluid (CSF) profile could be implicated in amyotrophic lateral sclerosis (ALS). Here, we systematically review the evidence for CSF cytotoxicity in ALS and explore its clinical correlates.

Methods:
We searched the following databases with no restrictions on publication date: PubMed, Embase and Web of Science. All studies that investigated cytotoxicity in vitro following exposure to CSF from ALS patients were considered for inclusion. Meta-analysis could not be performed, and findings were instead narratively summarised.

Results:
28 studies were included in our analysis. Both participant characteristics and study conditions including CSF concentration, exposure time and culture model varied considerably across studies. Of 22 studies assessing cell viability relative to controls, 19 studies reported a significant decrease following exposure to ALS-CSF, while three early studies failed to observe any difference. Seven of eight studies evaluating apoptosis observed significant increases in the levels of apoptotic markers following exposure to ALS-CSF, with the remaining study reporting a qualitative difference. Although five studies investigated the possible relationship between CSF cytotoxicity and patient characteristics, such as age, gender and disease duration, none demonstrated an association with any of the factors.

Conclusion:
Our analysis suggests that CSF cytotoxicity is a feature of sporadic and possibly also of familial forms of ALS. Further research is, however, required to better characterise its underlying mechanisms and to establish its possible contribution to ALS pathophysiology.

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IVT-09: Characterisation of stress granule mobility and compactability in stable cell line FlpIn SH-SY5Y-mScarletI-G3BP1 with the use of optical tweezers

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Background:
Stress granules (SG) are dynamic membraneless compartments formed after cell exposure to stressful conditions [1,2]. Their formation is cell's first response to stress and results in stalling mRNA translation and recruitment of more than 140 proteins intertwined with cytoplasmic RNA into their structure [3,4]. Among them are RNA binding proteins involved in ALS/FTD pathology like TDP-43, FUS, TAF15, TIA-1 and ATXN2. It is proposed that SG are involved in the formation of toxic aggregates of those proteins [1].

Objectives:
Characterisation of SG mobility and compactability in FlpIn SHSY5Y cell line stably expressing a SG constituent- mScarletI-GRBP1 protein with the use of optical tweezers.

Methods:
Optical tweezers are highly focused laser used for trapping and manipulation of microsized (0,1-10 µm) objects based on the difference in refractive index between the environment and structure. It is easily incorporated into the microscope and if used for short time does not damage living cells and can therefore be used for characterisation of different cell structures. We exposed FlpIn SHSY5Y-mScarletI-G3BP1 cells to 0,5 mM sodium arsenite for 1,5 h and observed the mobility of lipid droplets- LD (induced by oleic acid) and SG.

Results:
We have successfully determined the parameters for observing the mobility of lipid droplets to see if optical tweezers can be used for membraneless organels. We have shown that higher percentage of LD is mobile when sodium arsenite is added in comparison to control. Simillary we are determining the parameters for the mobility of SG in cells expressing ALS related proteins and controls.

Disscusion:
To check the applicability of the optical tweezers system on our stable cell line we have first performed the experiments on LD, since they are easier to trap. We showed that the system can be used for the characterisation of membraneless organels. Additional expiriments are being performed to observe the difference in the compactability of SG formed in control cells or in cells expressing ALS related proteins. We propose that overexpression of mutated or normal proteins like TDP-43 and FUS increase density of SG and this can be measured with the increased ability to trap SG with optical tweezers. Higher density and compactability can be the reason for toxic aggregation of ALS related proteins in SG.

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IVT-10: Crosstalk of TDP-43 and optineurin

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Background:
Protein aggregation and chronic neuroinflammation are two major hallmarks common to neurodegenerative diseases. Protein aggregates in 95% of amyotrophic lateral sclerosis (ALS) cases contain ubiquitinated and hyperphosphorylated TAR DNA binding protein 43 kDa (TDP-43)1. Mutations in optineurin, an ubiquitin-binding adaptor protein implicated in various cellular functions including inflammatory signaling, protein trafficking, and autophagy, have recently been found in ALS2. Mutated optineurin is thought to act by loss of function, leading to disbalanced inflammatory responses and/or impaired disposal of aggregated proteins by autophagy. However, the putative link between optineurin and TDP-43 proteinopathy is still elusive.

Objectives:
We aim to uncover the molecular mechanism(s) by which the loss of optineurin and/or its function could trigger TDP-43 proteinopathy and predispose to neurodegeneration.

Methods:
To assess the putative link between optineurin and TDP-43, we are using: (1) optineurin knockout (KO) neuronal and microglial cell lines made by CRISPR/Cas9 technology, and (2) primary cells from a mouse optineurin insufficiency model carrying a truncation of the ubiquitin-binding region (Optn470T), which mimics loss-of-function mutations found in ALS patients.

Results:
We found elevated TDP-43 protein levels in optineurin-deficient BV2 microglial cell line, and in primary Optn470T bone marrow-derived macrophages (BMDM). We did not detect differences in TDP-43 mRNA levels, arguing that TDP-43 was post-translationally regulated. Elevated TDP-43 protein levels were specific to myeloid cells and were not observed in neuronal NSC-34 and N2A optineurin-deficient cell lines. Elevated TDP-43 protein levels in optineurin-deficient or insufficient myeloid cells were not caused by autophagy blockade. To test if inflammation can influence TDP-43 accumulation in cells lacking functional optineurin, we stimulated them with lipopolysaccharide (LPS). We observed increased TDP-43 levels in WT BV2 and BMDM upon LPS stimulation, but TDP-43 levels in BV2 KO and OPTN470T BMDM remained at the same elevated state as in basal conditions.

Discussion:
Our results demonstrate that optineurin directly influences basal TDP-43 protein levels in myeloid cells but not in neurons. Further studies are needed to determine if this is a mechanistic link to protein aggregation in ALS.

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IVT-11: Deregulation of TBK1-Mediated Autophagy by ALS-Associated MicroRNA-340

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Background:
Growing evidence implicates microRNA (miRNA) deregulation as a hallmark of amyotrophic lateral sclerosis (ALS) and many studies have evaluated the potential of circulating miRNAs as disease biomarkers. However, whether miRNA expression changes in ALS patients are simply an indication of cellular dysfunction and degeneration, or in fact promote functional changes in target gene expression relevant to disease pathogenesis, is unclear. Our recent review of candidate miRNA biomarkers in ALS patients (1) highlighted that many are predicted to target components of the autophagy pathway, altered function of which is a potential pathomechanism in ALS.

Objectives:
To select a candidate miRNA previously reported to be dysregulated in ALS patients, with implications in autophagy regulation. We aim to investigate whether this miRNA can impact cellular autophagic flux and determine a potential target through which it may exert its effects.

Methods:
To select a candidate miRNA, we employed miRNA-target prediction software to identify miRNAs implicated in the autophagy pathway and cross-referenced these with hundreds of miRNAs previously shown to be dysregulated in ALS patients. Selection of a candidate miRNA was made from those which were additionally predicted to target TBK1, a critical regulator of autophagy and ALS gene product. We subsequently utilised a live cell autophagic flux assay (2) to investigate whether the candidate miRNA impacts autophagy in a live cell model, and utilised the dual luciferase assay to determine its potential to target TBK1.

Results:
Our systematic bioinformatic selection process has identified miR-340-5p as a suitable candidate miRNA. Here, using a live-cell autophagy assay, we show that miR-340 expression is associated with reduced incorporation of a SQSTM1/p62 reporter protein into acidic autophagic vesicles, consistent with an impairment of autophagy. Luciferase reporter assays indicate miR-340 can directly target a predicted TBK1 3'UTR site and western blotting provides further evidence miR-340 targets endogenous TBK1 to reduce its expression.

Discussion and Conclusions:
Investigating the functional relevance of ALS dysregulated miRNAs develops knowledge of the pathomechanisms contributing to ALS. Combined, our observations suggest that patient changes in miR-340 levels may be associated with ALS-relevant deregulation of autophagy, by directly targeting TBK1. This supports the notion that specific miRNAs could be used as clinical biomarkers and therapeutic targets in the future.

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IVT-12: Dysfunctional nucleocytoplasmic transport dynamics in amyotrophic lateral sclerosis and frontotemporal dementia caused by mutation in C9orf72

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A mutation in the C9orf72 gene is the most common genetic cause of ALS/FTD (frontotemporal dementia). This mutation results in the production of neurotoxic dipeptide repeat proteins (DPRs). Modifier and interactome studies suggest that DPRs affect nucleocytoplasmic transport. Altered nucleocytoplasmic transport is likely to contribute to disease pathology as this vital mechanism is the main route of information exchange between the cytoplasmic and nuclear compartments.

Smaller molecules can passively diffuse through the nuclear pore, the protein complex through which transport occurs. Conversely, larger molecules need to be actively transported by receptors. We have optimised an assay to specifically investigate passive nucleocytoplasmic transport monitoring inert fluorescent cargo of different sizes using live confocal microscopy. Studying passive diffusion in a disease context yields information about nuclear pore integrity and function. In addition, passive diffusion is thought to be the main route of nuclear export of key ALS/FTD proteins TDP-43 and FUS.

We have found that arginine containing C9orf72 DPRs significantly alters passive import of cargo molecules in a size-dependent manner. Therefore, while the leakiness of the nuclear pore is altered, its permeability barrier is maintained in the presence of C9orf72 DPRs. We are now investigating more physiological cargo and potential mechanisms of action. Thus, this study will inform on interactions between the nuclear pore and C9orf72 DPRs and identify potential new therapeutic targets.

IVT-13: Scaffolds of Mobile Extracellular Matrix Molecules Enhance Maturation of Human Stem Cells-Derived Motor Neurons

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Human induced pluripotent stem cell (iPSC)-based technologies offer a unique resource for modeling disease and regenerating complex tissues such as the central nervous system (CNS). However, iPSC models are still fraught with significant technical limitations including inefficient maturation, abnormal aggregation and reduced long-term viability of neurons. We reasoned that the lack of physiological extracellular matrix (ECM) conditions contributes to these problems and establishing a stable and bioactive ECM environment would facilitate the functional maturation of iPSC-derived neurons. To test this, we utilized peptide amphiphiles (PAs), a class of biomaterials that have the ability to assemble into supramolecular nanofibers capable of morphologically and chemically mimicking the ECM. We designed a series of PAs scaffolds containing a short bioactive peptide (IKVAV) found in Laminin-1, which plays a major role in neuronal behavior in the CNS. The newly design PAs have an identical chemical bioactivity but the supramolecular fibers that they form drastically differ in terms of mobility. Here, we have specifically explored how the molecular mobility of ECM signals impacts the maturation of different classes of iPSC-derived human neurons. We identified an IKVAV-PA that dramatically enhanced the maturation of iPSC-derived cortical and motor neurons in vitro. The beneficial activity of this IKVAV-PA strongly correlated with its intermolecular hydrogen-bonding domain, which facilitated high molecular mobility, mimicking the dynamic ECM-neuronal interactions. Neurons assembled within the high dynamic IKVAV-PA exhibited reduced aggregation, enhanced arborization, and a mature pattern of electrical activity closely resembling neurons co-cultured with primary glial cells. Using global proteomic analysis and high-resolution microscopy, we traced these effects to specific and relevant molecular pathways that were downstream of laminin-1/Integrin beta-1 signaling pathway. Our PA technology highlights the importance of the ECM in recapitulating in vivo conditions and offers a more physiological and translational platform to study the development, function and dysfunction of the CNS in disease or injury using iPSC-based approaches.

IVT-14: The BAG1 molecular chaperone regulators prevent ALS related neurotoxic misfolded proteins accumulation via proteasome and chaperone mediated autophagy

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Many ALS associated proteins, as SOD1, TDP-43, DPRs from C9orf72, etc. tend to misfold and accumulate into aggregates in neurons. Protein quality control system maintains cellular protein homeostasis preventing protein aggregation and counteracting their toxicity by enhancing degradation via proteasome and/or autophagy. The chaperone assisted selective autophagy (CASA) with its complex formed by HSP70, HSPB8, BAG3 and CHIP is mainly involved in the misfolded protein degradation. Moreover, an efficient dynein mediated transport of misfolded proteins to the site of degradation is required as key point to control their aggregation and degradation. In fact, we found an alteration of SQSTM1/p62 and LC3 expression accompanied by altered localization and a reduction of autophagosome number per cell when the dynein retrograde transport of HSPB8/BAG3 substrates are blocked. Despite this, blockage of dynein function reduced the PBS insoluble fraction of mutated misfolded proteins (SOD1, TDP-43 and DPRs). Dynein inhibition selectively increased the mRNA level of the nucleotide exchange factor BAG1, both in NSC34 and in motoneuron derived from iPS cells. Notably, exogenous BAG1 overexpression reduced misfolded species aggregation increasing UPS dependent degradation of proteins recognized by HSP70. Notably, a dysregulation of HSP70 levels has been recently identified in peripheral cells from ALS patients and its downregulation increases TDP-43 protein levels.

Moreover, dynein inhibition increased mRNA and protein levels of the chaperone mediated autophagy (CMA) receptor Lamp2A, suggesting that CMA may restore misfolded proteins degradation. As we observed in dynein depleted cells, BAG1 mRNA is increased also in Lamp2A depleted cells. We measured the protein levels of alpha-synuclein as CMA substrate. We found that BAG1 overexpression reduced alpha-synuclein level, while BAG1 depletion has an opposite effect. In parallel, Lamp2A depleted cells retained a very efficient proteasome system that rapidly cleared soluble alpha-synuclein, even if we observed alpha-synuclein PBS insoluble species accumulation.
Collectively, these data suggest that BAG1 is an important player to assist the degradation via proteasome and CMA of ALS related misfolded protein.

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Arosio et al., 2020; AMYOTROPH LAT SCL FR
Cristofani et al., 2018; CELL STRESS CHAPERON

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IVT-15: Using a proteomic approach to map the interactome of TDP-43 in the context of ALS

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Amyotrophic lateral sclerosis (ALS) is a progressive and incurable neurodegenerative disease in which there is the pathological accumulation of cytosolic protein aggregates. A major component of these aggregates is TDP-43, an RNA binding protein, which mislocalizes from its normal nuclear locale to the cytoplasm. The nuclear expression of TDP-43 is tightly regulated and its cytosolic accumulation in ALS may be indicative of perturbations in mechanisms needed for normal function. Given that the subcellular localization of a protein is highly dynamic and affected by protein-protein interactions (PPIs), we posit that identifying novel interactors of wild-type and mutant TDP-43 could reveal insight into networks involved in driving neurodegeneration.

Using CRISPR/Cas9, our lab has generated knockin cell lines expressing GFP-tagged wildtype (WT) and an ALS-causing mutant (Q331K), in the endogenous TARDBP locus (coding for TDP-43). On the cellular level, we have shown that the Q331K mutation causes loss-of-function and mislocalization of TDP-43. Using these cells, we have performed immunoprecipitation coupled to mass spectrometry (IP-MS) to elucidate interactors of WT- and Q331K, TDP-43, showing that there is an overall loss of TDP43-protein interactions with the ALS-linked mutation.

From this dataset of identified interactors, we have used a combination of literature searches and bioinformatic analyses to shortlist candidates for validation. We are validating these candidates to confirm interaction using confocal microscopy and IP-western blot and we will characterize (using knockdown and overexpression approaches to test the consequences of disrupting these interactions on TDP-43 function) through cellular and biochemical methods. Using this unbiased approach, we will identify novel interactors that give insight into pathways that can be targeted for therapeutic intervention.

IVT-16: Effects of Microcystis aeruginosa or L-BMAA on ALS cellular models

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Background:
ALS is generally thought to progress as consequence of genetic susceptibility and environmental influences. Until now, a number of environmental triggers have been identified in association with ALS and recent studies suggest that some cyanotoxins, produced by cyanobacteria, are highly neurotoxic although their role in neuropathy are still controversial [1,2].
Objectives. The main goal in this project is to establish a cause-effect relationship among exposure to cyanotoxins, ALS and TDP-43 proteinopathy. These data will contribute to understand if cyanobacteria and the metabolites they produce can be considered an environmental risk factor for ALS. In the light of these premises, we aim to: 1) use purified L-BMAA and cyanobacteria extracts, as environmental toxicity triggers on both neuronal (human neuroblastoma SH-SY5Y cells) and non-neuronal (patient's fibroblast) cell models [3,4]. Moreover, these environmental triggers will be used at subliminal dose to investigate their effect on different biochemical properties of TDP43 WT or carrying TDP43-A382T missense mutation in both SH-SY5Y cells or fibroblast from ALS patients; 2) characterize transcriptome in primary ALS fibroblast upon L-BMAA or cyanobacteria extract treatment by RNA-seq.

Methods:
We took advantages of different and complementary experimental systems: continuous cell lines offer the possibility of performing biochemical and molecular manipulations whereas primary fibroblast from patients can recapitulate many features of human disease. To mimic environmental triggers, we use pure L-BMAA and Microcystis aeruginosa crude extracts (MaCe), a cyanobacterial strain isolated from Baratz' lake (Italy) [5].

Results:
Using ALS cellular models, we demonstrate a dose-dependent toxicity upon L-BMAA or MaCe exposure, exacerbated by ALS causing gene over-expression (SOD1, FUS and TDP-43). By immunofluorescence and biochemical analysis, we highlight TDP-43 delocalization and aggregation and ER stress, but not apoptosis or autophagy. Given these promising experimental evidences, we moved on performing an RNA-seq experiments using RNA extracted from ALS patient's fibroblast exposed to L-BMAA or MaCe. Libraries for RNA-seq were prepared with NEBNext Ultra II Directional RNA Library Prep Kit for Illumina and are currently under analysis.

Discussion:
Our results add new pieces to the molecular mechanisms of ALS pathogenesis, highlighting the relationship among CyanoHAB exposure and ALS and will suggest putative pharmacological targets mainly through the identification of specific RNA deregulated in human ALS fibroblast upon exposure to L-BMAA or MaCe.

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IVT-17: Effects of sacsin domains, a protein mutated in a neurodegenerative disorder, on neurofilament assembly

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Introduction:
SACS gene, encoding the giant protein Sasin, is responsible for the Autosomal Recessive Spastic Ataxia of the Charlevoix-Saguenay (ARSACS), an inherited childhood-onset neurodegenerative disorder presenting ataxia, peripheral neuropathy and spastic paraplegia. Sacsin's functions are poorly understood and the protein is predicted to have a role in protein quality control because of the identification of chaperones domains SSR1 and Sacs J domain (homologous to Hsp90, and DnaJ/Hsp40) and an ubiquitin-like domain (Ubl) at the N-terminus. A key feature of ARSACS pathogenesis is the formation of neurofilament (NF) bundles in neurons, suggesting a deficient NF assembly. NF heteropolymers are the most abundant cytoskeletal element in large axons composed of NFL (light), neurofilament middle chain and neurofilament heavy chain.

Objectives:
To determine the function of SacsJ domain on NF assembly

Methods:
GST-tagged construct containing sacsin domains including Ubl, SRR1, J-domain and HEPN were purified using Glutathione Sepharose Resin. Purification of recombinant NFL in vitro was performed using hydrate Bio-Gel HTP. In order to study NF assembly in vitro, dialysis of NF proteins solubilized in 8M urea was performed using D-Tube Dialyzer Mini against assembly buffer. Negative staining of samples were negatively stained with 1% uranyl acetate, and examined via electron microscopy. Filter trap assay was used to determine interaction between NFs and sacsin domains. Chaperone activity was measured using citrate synthase (CS) (at 0.2 µM) and catalase (1 µm) as protein substrates in a chaperone assay measuring protein aggregation for 90 or 60 min at 45 °C to identify the chaperone efficiency of sacsin domains in vitro.

Results:
SRR1 from electron-dense long filament while the SacsJ-domain prevent the assembly of NF in vitro. In addition, sacsJ domain affected the assembly of recombinant NFL alone by preventing assembly and dismantling assembled NFL filaments. SRR1 and sacsJ-domain had opposite effects in the chaperone assay. SRR1 was an efficient chaperone, preventing aggregation of CS especially at 0.2-0.6 µM and catalase. On the other hand, SacsJ-domain failed to prevent aggregation of CS and catalase, and increased aggregation of these protein substrates at concentrations higher than 0.2 µM, suggesting a different role for sacsJ-domain. We identify that the SacsJ peptide interacts with Rab1a and ARF5 using Mass spectrometry indicating a role in autophagy and ER-Golgi trafficking.

Discussion:
In summary, we characterized the important role of sacsin domains on NFs morphology and assembly and specifically NFL. Although there are a lot of sequence similarities between the chaperone domains, especially between sacsJ-domain and Hsp40 family, the sacsJ-domain behaved differently from this family and shows an anti-chaperone function. Our future strategy is to characterize the assembly process in vitro by identifying the dependence on ATP and kinetic of assembly.

Acknowledgements:
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IVT-18: Effects of Specific miRNAs Shuttled by Exosomes Derived from Mesenchymal Stem Cells on Late Symptomatic SOD1G93A Mouse Astrocyte Primary Cultures

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Background:
Despite the significant progress in genetic studies, managed to explain many cases of ALS through mutations in several genes, the cause of a majority of sporadic cases remains unknown, even if the clinical and biomolecular features of genetic and sporadic ALS are very similar. Currently, epigenetics, involving miRNA studies, shows some promising aspects.We previously reported that intravenous administration of mesenchymal stem cells (MSCs) in the SOD1G93A mouse model of ALS produced positive effects on survival and disease progression, also modulating astrocytes and microglia reactive phenotypes. We proposed that MSC effects were paracrine, possibly involving exosome-mediated cell communication. Indeed, unpublished results substantiate the positive impact of MSC-derived exosomes on SOD1G93A mouse-derived astrocytes. Here, we investigated the activity of nine miRNA, which were found up-regulated in IFNɣ-primed MSCs and shuttled by MSC-derived exosomes, on spinal cord astrocyte primary cell cultures from late symptomatic 120 day-old SOD1G93A mice.

Objective:
To analyze the effect of single synthetic miRNA in modulating the phenotype of cultured astrocytes prepared from the spinal cord of late symptomatic SOD1G93A mice and involved pathways.

Methods:
Adult astrocyte culture preparation: Primary astrocyte cell cultures were prepared from 120 day-olds spinal cord of WT and SOD1G93A mice. Tissue was mechanically dissociated in DMEM. Aliquots were cultured at 37°C and 5% CO2 for 20 days before experiments. Astrocyte transfection: Astrocytes were transfected with synthetic mimics of exosome-contained miRNAs for 48h in serum-free DMEM. Protein determination: Protein content was determined by confocal microscopy and quantitative immunofluorescence.

Results:
Seven out of nine miRNA mimics were able to affect the reactive phenotype of SOD1G93A astrocytes by significantly decreasing the overexpression of GFAP, IL1β, and TNFα, detected by confocal microscopy. Four of these miRNAs (466q, 467f, 466m5p, 466i3p) overexpressed in MSCs were overexpressed also in exosomes. We selected in-silico their relevant pathways (p38, TNFαand NFKB) and validated them by determining the miRNA effects on MAP3K8, MAPK-APK2, MAPK11, and TRAF6 by qPCR. Two of them (466q, 467f) strongly reduced MAPK11 mRNA expression, thus inhibiting TNFαformation.

Conclusion:
Our results suggest that the amelioration of the reactive phenotype of spinal cord SOD1G93A astrocytes, brought about by in-vivo MSC treatment, operates through exosome-shuttled specific miRNAs.

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IVT-19: Elucidating mechanisms of TDP-43 toxicity in embryonic stem cell-derived motor neurons

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Background:
Our group has previously developed a BAC transgenic mouse expressing human TDP-43 (M337V) at low levels. Expression of TDP-43 M337V leads to a progressive motor phenotype, alterations in stress granule dynamics and reduced survival in response to oxidative stress compared to non-transgenic (NTg) and TDP-43 WT controls [1]. Mouse embryonic stem cell-derived motor neurons (mESC-MNs), stably expressing the same BAC constructs, are an excellent model for high throughput screening as they show relevant disease phenotypes and can be generated quickly. We have recently identified several FDA-approved compounds that rescued the oxidative stress-induced survival loss in TDP-43 M337V mESC-MNs. Also, a comparative proteomic study before and after oxidative stress identified distinct interactome profiles between TDP-43 WT and TDP-43 M337V mESC-MNs, showing abolished or reduced interaction of the mutant TDP-43 with proteins involved in stress granule assembly and intracellular transport [2].

Objectives:
This project aims to investigate mechanisms of TDP-43 toxicity in mESC-MNs expressing the M337V mutation compared to NTg and WT controls. We also analysed the effects of a potentially pro-survival drug on relevant disease pathways identified in a recent interactome study of these motor neurons [2].

Methods:
Mouse ESCs (NTg, TDP-43 WT/- and TDP-43 M337V/-) were expanded as embryoid bodies and differentiated to MNs [1]. Immunocytochemistry was performed to investigate TDP-43 mislocalisation and stress granule formation. Mitochondrial respiration and glycolysis were examined using the Seahorse XF Analyser. MTS assays were performed to investigate mESC-MN survival following sodium arsenite treatment. Axonal transport was examined in mESC-MNs using cholera toxin B and live cell imaging. To examine the effects of candidate drugs on cellular phenotypes, mESC-MNs were treated with compounds at optimised concentrations prior to phenotypic analysis.

Results:
Here we perform longitudinal analysis of mESC-MNs from 3-10 days after plating to study the progression of altered phenotypes in “aged” MNs. We first studied the effects of the M337V mutation on TDP-43 mislocalisation, stress granule formation and survival after oxidative stress. The proteomic analysis of TDP-43 WT and M337V interactors revealed alterations in the interaction with components of the mitochondrial electron transport chain as well as proteins involved in vesicle trafficking. To investigate deficiencies in the cellular energy metabolic function we performed mitochondrial respiration and glycolysis assays. Alterations in intracellular transport were analysed by tracking the endocytosis and sorting of cholera toxin B using live imaging. Additionally, the effect of candidate drugs identified through high-throughput drug screening on these pathways has been examined.

Conclusions:
This study investigates the mechanisms of mutant TDP-43 toxicity in ALS and may inform drug discovery by highlighting candidate drugs or targets for therapeutic intervention.

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IVT-20: Enhancing glycolytic flux is benefical in models of familial ALS”

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Mitochondrial abnormalities are seen in patients with Amyotrophic Lateral Sclerosis (ALS) as well as in vitro and in vivo models of disease. This is associated with re-programing of intermediary metabolism to maintain ATP levels and redox status. We deployed a yeast growth fitness assay to screen for modifier metabolism genes. Saccharomyces cerevaise were engineered to inducibly express wild type or Q133K mutant TDP43, and upon transgene induction, mutant TDP43 expressing cells displayed a growth fitness defect. We then studied the effects of loss of different non-essential glycolysis genes in a semi-quantitative manner. We found that loss of 5 genes enhanced growth fitness of upon induction of mutant TDP43 expression: emi2, zwf1, ymr085w, fba1, and pgk1. In 3/5 cases, the deletion also improved the growth fitness upon induction of wild-type TDP43 expression. In contrast, we found that deletion of 2 genes reduced growth fitness of S. cerevaise upon induction of mutant (but not wild-type) TDP43 expression: eno1 and pyk2. We wondered if deletion of any of these genes improve the growth fitness of other yeast-based models of ALS or other neurodegenerative diseases. To address this issue, we studied yeast that inducibly-express two different diamino acid peptides (DPRs) derived from the C9ORF72 mutation, wild-type or R521C or P525L fused-in-sarcoma (FUS), wild type TAF15, wild type EWSR1, wild type or E46K or A53T alpha synuclein, or ATXN3 with 25 glutamines (Q25) or 71 glutamines (Q71). We found that Δymr085w and Δzwf1 had broad growth fitness phenotypes, showing beneficial actions in 8 and 6 different models, respectively. zwf1 is the yeast homolog of the human glucose-6-phosphate dehydrogenase and ymr085w is a yeast homologue of glutamine-fructose-6-phosphate amidotransferase. Ongoing work aims to determine if manipulating these genes in mammalian model systems also confers beneficial effects.

IVT-21: Fus protein with a deletion of the nuclear localization signal at the c-terminus forms stable post-stress cytoplasmic aggregates in SH-SY5Y neuron-like cells

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Aims:
Mutations in the FUS gene have been associated to ALS. FUS is physiologically a nuclear protein. Mutations in its nuclear localization signal (NLS) region at the C-terminus leads to an aggressive disease (1), and are responsible for the protein mislocalization to the cytoplasm, where it may be incorporated into stress granules (SGs) (2,3). Whether SGs are the first step to the formation of the stable FUS-containing aggregates seen in the disease is still unclear.
Here, using a human cell line expressing FUS lacking NLS, we aimed to study the FUS-related SG dynamics and observed that SGs containing mutant FUS can evolve into stable cytoplasmic inclusions.

Methods:
Immunoblot and immunofluorescence assays were performed to study FUS expression after acute stress with 0.5 mM of sodium arsenite (NaAsO2) in two SH-SY5Y-mutated cell lines, an homozygous clone ΔNLS-/- and an heterozygous clone ΔNLS-/+ (4). Control was cell line expressing the wild-type FUS. Nuclear and cytoplasmic fractions were obtained through an established method (3). After exposure to NaAsO2, cells were monitored up to 24 h and immunofluorescence analyses were performed at prefixed time-points. Fluorescence images were acquired through a Zeiss confocal microscope, whereas immunoblot results were processed with ImageJ software.

Results:
In basal conditions, cytoplasmic FUS expression was increased in both mutant ΔNLS-/- and ΔNLS-/+ cells; in the wild-type cells, FUS remained mostly nuclear. The cytoplasmic mislocalization of mutant FUS protein was also verified through immunoblot analysis of the nuclear and cytoplasmic fractions. Moreover, FUS was expressed in the insoluble protein fraction in mutant cells. NaAsO2 treatment induced the appearance of SGs: at time 0 after NaAsO2 exposure, >90% of mutant cells showed abundant FUS-containing SGs, as compared to only ≃10% of the wild-type cells. 3 hours after stress, about 35% of mutated cells still contained SGs as compared to ≤3-4% of wild-type cells. At 6 hours 15-20% of mutant cells still contained FUS-positive SGs. This percent value was maintained even 24 hours after stress exposure and also in the presence of cycloheximide, leading to the suggestion that a number of FUS-containing SGs may evolve to form stable aggregates.

Conclusions:
We have demonstrated here that SH-SY5Y cells expressing FUS protein lacking the NLS still contain stress-related inclusions even after long time after a single stress pulse. These results strongly suggest that the ALS-related FUS-positive inclusions might derive directly from SGs, possible related to their incapacity to dissolve because the abundant incorporation of the mutated protein.

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IVT-22: FUS-ALS mutants alter FMRP phase separation equilibrium and impair protein translation

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Mutations in the RNA binding protein (RBP) FUS cause amyotrophic lateral sclerosis (ALS) and result in its nuclear depletion and cytoplasmic mislocalisation, with cytoplasmic gain of function thought to be crucial in pathogenesis. Here, we show that expression of mutant FUS at physiological levels drives translation inhibition in both mouse and human motor neurons. Rather than acting directly on the translation machinery, we find that mutant FUS forms cytoplasmic condensates that promote the phase separation of FMRP, another RBP associated with neurodegeneration and robustly involved in translation regulation. FUS and FMRP co-partition and repress translation in vitro. In our in vivo model, FMRP RNA targets are depleted from ribosomes. Our results identify a novel paradigm by which FUS mutations favour the condensed state of other RBPs, impacting on crucial biological functions, such as protein translation.

IVT-23: Identification of oxidative stress response modifying drugs from a high throughput screen using an ESC-derived motor neuron model of ALS.

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Background:
The mechanisms underlying the preferential loss of motor neurons (MNs) in ALS may be due, in part, to defective responses to conditions such as oxidative stress. Mutations in RNA binding proteins, such as TDP-43, can impair the normal assembly and function of stress granules, suggesting potential pathogenic mechanisms. We have previously shown that mouse embryonic stem cell (ESC)-derived MNs expressing a human TDP-43 BAC (M337V) display impaired stress granule responses and reduced survival following oxidative insult, compared to non-transgenic and human TDP-43-WT BAC controls[1]. Comparative proteomics further identifies distinct interactome profiles between TDP-43-WT and TDP-43-M337V MNs, identifying impaired interactions between mutant TDP-43 with proteins involved in stress granule assembly and intracellular transport[2].

Objectives:
High throughput screening to identify FDA-approved drugs that modify stress-induced survival loss in TDP-43-M337V motor neurons.

Methods:
Mouse ESCs (TDP-43-WT/- and TDP-43-M337V/-) were expanded as embryoid bodies and differentiated to MNs in 384-well plates. Mature MNs were treated with the PHARMAKON 1600 FDA approved library for 24h, with a final concentration of 2 µM for each drug, followed by the addition of 0.5mM sodium arsenite for 1 h. Resazurin was then added to the culture medium. After a further 24h cell viability was measured by fluorescence on an Envision plate reader (Ex 570nm/Em 584nm). Analysis was carried out using the Target Discovery Institute's plate analysis pipeline, from which z-scores were calculated based on the normalised data.

Results:
16 putative hits were identified from a total of 1600 compounds with a z-score ≥ 1.8, which reflect those drugs that improved motor neuron survival by at least 1.8 standard deviations above the library well mean. Of the 16 putative hits from the original high throughput screen, 6 drugs continued to display improved TDP-43-M337V/- MN survival during secondary hit validation and dose-response testing, compared to controls. Further, 24h treatment of TDP-43-M337V/- MNs with the 6 candidate drugs prior to sodium arsenite-induced stress rescued the number of stress granules/ESC-MN to control levels.

Discussion:
Our data suggest that impaired stress responses may underlie the link between mutant TDP-43 and selective MN loss in our mouse model of M337V-associated ALS. With the Oxford Target Discovery Institute, we performed a screen of FDA approved drugs to identify those which restored survival and the stress granule response in mutant-expressing ESC-MNs. Drugs will continue to be validated through detailed analysis of phenotypic and transcriptional changes in primary mouse MNs, and iPSC-derived motor and cortical neurons from ALS patients carrying TDP-43 mutations.

References:
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Acknowledgements:
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IVT-24: Kinase inhibitors targeting growth factor signalling cascadesrescue axonal transport deficits in the SOD1G93A mouse model of ALS.

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Live Poster Session B, December 10, 2020, 5:10 PM - 5:50 PM

Background:
Axonal transport between the soma and axonal terminal is fundamental for neuronal survival. The long axons of motor neurons make them especially vulnerable to changes in axonal transport. Accordingly, deficits in retrograde axonal transport are one of the earliest pathologies observed in several models of ALS. Presymptomatic and embryonic axonal transport deficits have been reported in the SOD1G93A mouse model of ALS. Agents that accelerate axonal transport are therefore being explored as potential therapeutics in ALS. A screen of protein kinase inhibitors conducted in the Schiavo lab identified several compounds capable of rescuing transport deficits in SODG93A mice, including inhibitors of p38 MAPK (Gibbs et al., 2018) and the insulin-like growth factor receptor 1 (IGF1R) (Fellows et al., 2020). Another target identified in the screen was RET, a kinase receptor for the glial cell-line derived neurotrophic factor (GDNF), a neurotrophin fundamental for motor neuron development and survival.

Objectives:
1) Explore the mechanisms underlying the in vitro SOD1G93A axonal transport deficits.
2) Determine the effect of RET inhibition on axonal transport speeds in motor neurons.
3) Investigate the mechanisms driving axonal transport acceleration following inhibition of IGF1R and RET.

Methods:
Live axonal transport of signalling endosomes was visualised in vitro in primary motor neuron cultures from WT and SOD1G93A mice using a fluorescently-labelled non-toxic fragment of the tetanus neurotoxin (HcT). Cultures were treated with kinase inhibitors and growth factors to determine their impact on axonal transport. Retrogradely transported signalling endosomes were manually tracked using the Fiji plugin, TrackMate, yielding information about endosome speed, directionality and pausing. The effect of inhibitors and growth factors on signalling pathways within motor neuron cultures was assessed by western blot. The effect of inhibitors on neuronal morphology, and target localisation were assessed using immunocytochemistry.

Results:
Inhibition of several different kinases accelerates axonal transport in both WT and SOD1G93A primary motor neurons. Inhibition of the growth factor receptors IGF1R and RET, as well as direct inhibition of their shared downstream signalling effector, AKT, accelerates WT transport speeds. Interestingly, in vitro SOD1G93A axonal transport deficits appear to be dependent upon BDNF stimulation, highlighting the interaction between growth factor signalling and axonal transport, and emphasising a key early difference between WT and SOD1G93A motor neurons.

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Gibbs, K. L. et al. (2018) 'Inhibiting p38 MAPK alpha rescues axonal retrograde transport defects in a mouse model of ALS article', Cell Death and Disease, 9(6). doi: 10.1038/s41419-018-0624-8.

Acknowledgements:
This work is funded by the MRC UCL-Birkbeck DTP studentship programme.

IVT-25: Negative feedback loop between TDP-43 and miR-181c-5p: implications for alterations in TDP-43 metabolism in Amyotrophic Lateral Sclerosis (ALS)

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Live Poster Session B, December 10, 2020, 5:10 PM - 5:50 PM

Background:
The most common pathological hallmark of ALS is the re-localization of TDP-43 from the nucleus to the cytoplasm accompanied by the formation of cytoplasmic inclusions within motor neurons. This occurs in 97% of all ALS cases. A major nuclear function of TDP-43 is its involvement in the production of microRNAs—small RNA molecules that are predominately responsible for post-transcriptional gene silencing [1]. This is interesting as miRNA levels are reduced in ALS motor neurons, suggesting that loss of TDP-43 nuclear function could result in reduced miRNA production [1]. Further, we have previously shown that some ALS-linked miRNAs suppress TDP-43 expression [2]. Therefore, reduced miRNA levels, as seen in ALS, could result in the overexpression of TDP-43, a phenomenon that has been observed in ALS motor neurons [2], and thus contributing to the formation of TDP-43 cytoplasmic inclusions. We hypothesize that that a negative feedback loop between TDP-43 and miRNAs is disrupted in ALS due to the loss of TDP-43 nuclear function, which may contribute to overall TDP-43 pathology.

Objective:
Determine whether a negative feedback loop between TDP-43 and miRNAs exists that is dependent on TDP-43 nuclear localization, which could have major implications for ALS pathogenesis.

Methods & Results:
Microarray analysis was done to determine changes to miRNA expression 48-hours post knockdown of TDP-43 in HEK293T cells. Of interest, miR-181c-5p was significantly reduced after knockdown of TDP-43 and contained a conserved binding site in the TARDBP 3'UTR. Further, miR-181c-5p has been shown to be reduced in ALS spinal cord [3], and here, we show it is expressed in human spinal motor neurons using in situ hybridization. Transfection of miR-181c-5p or its inhibitor into HEK293T cells resulted in a significant decrease or increase in TDP-43 levels, respectively, indicating a negative feedback loop did exist between TDP-43 and miR-181c-5p. Finally, we showed that TDP-43 regulation of miR-181c-5p, and hence the negative feedback loop, is dependent on TDP-43 nuclear localization, potentially through nuclear export of the miR-181c-5p precursor molecule.

Discussion:
We identified a novel negative feedback loop between TDP-43 and miR-181c-5p that is dependent on TDP-43 nuclear localization. This could have major implications for ALS where TDP-43 and miR-181c-5p have been shown to be increased and decreased, respectively [2,3].

References:
1. Hawley ZCE, Campos-Melo D, Droppelmann CA, et al. Front Mol Neurosci 2017; 10:127
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IVT-26: Novel Methodology for Producing Postnatal Spinal Co-Cultures of Astrocytes and Neurons for Use in the Study of ALS

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Live Poster Session B, December 10, 2020, 5:10 PM - 5:50 PM

Spinal circuits support a wide range of functions necessary for survival and are composed of neurons, which are supported by glia. Neuro-glial interactions are critical for the healthy function of the nervous system and emerging evidence implicates roles in the pathogenesis of disease, including but not limited to Motor Neurone Disease; however, there are limited tools available to interrogate their respective contributions to function and disease. Here, we present an in vitro primary culture system for neurons and astrocytes derived from the lumbar region of spinal cords of postnatal mice up to 4 days old. This approach allows the generation of cultures obtained from spinal cords of verified, genetically modified mice, which is challenging using traditional approaches derived from embryonic tissue. Neurons and astrocytes can be isolated to produce 'pure' cultures and then be re-combined as mixed-genotype (wildtype and/or genetically modified) co-cultures at later time points to explore astro-neuronal interactions.

Electrophysiological analysis reveals that primary neuron cultures are functional; cells express sodium and potassium currents and can repetitively fire action potentials. Neurons also form functional networks in culture, as indicated by recordings of inhibitory and excitatory postsynaptic currents. Immunocytochemical analysis indicates neurons in co-culture with astrocytes express postsynaptic markers for glutamatergic (PSD95) and GABAergic (gephyrin) synapses. We also find a subset of neurons express SMI-32 and are likely motor neurons. Glial primary cultures are composed of cells that express glial fibrillary acidic protein (GFAP; 82-96%) and glutamine synthetase (99%) indicating that these cultures are primarily composed of astrocytes. Importantly, GFAP and glutamine synthetase-expressing astrocytes are also colocalized with the excitatory amino acid transporter – 2 and connexin 43; markers of functionally mature astrocytes, as early as 2 weeks-post plating.

These findings confirm that our protocol reliably produces neurons, astrocytes and co-cultures. Obtaining viable cultures of spinal neurons from postnatal mice has been a challenge in the past, making this approach of interest to the broader motor control and MND communities. We expect that this system, in combination with genetic models in mice, will serve as a valuable tool to study neuron-astrocyte interactions in health and disease.

IVT-27: Nucleocytoplasmic Transport Defects in a Mouse Model of Neurodegeneration

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Live Poster Session B, December 10, 2020, 5:10 PM - 5:50 PM

Background:
Nuclear Export Mediator Factor (NEMF) has previously been characterized to play a role in the Ribosome Quality Complex (RQC), as well as an association in nucleocytoplasmic transport (NCT). NCT defects are increasingly being recognized as a critical component underlying multiple forms of neurodegeneration, specifically amyotrophic lateral sclerosis (ALS). A novel ENU-induced mouse mutation in NEMF (R86S) causes motor neuron disease with phenotypic hallmarks of ALS, yet NEMF's implication in the development of this disease has yet to be well understood.(1)

Objective:
To determine the role NEMF plays in nucleocytoplasmic transport and its implication in this model of neurodegeneration.

Methods:
Using a R86S NEMF mouse embryonic fibroblasts (MEFs), we have examined for dysregulation in components in the RQC as well as nuclear transport proteins using western blot analysis and immunofluorescence. Furthermore, we have used transient transfection of MEFs with plasmids containing NLS and NES sequences linked to fluorescent probes to determine how canonical transport systems are affected in R86S NEMF MEFs.

Results:
It was shown that a variety of proteins (60S, LTN1) associated in the RQC, as well as NTF2 in NCT had dysregulated protein expression and were mis-localized to the cell membrane with the presence of puncta co-localized with R86S NEMF. It was also shown that the 40S ribosome, a component of the polysome, was found to remain in the nucleoli, showing disrupted nuclear export and consistent with R86S NEMF mis-localization. Multiple nuclear transport factors (Ran, NTF2, Importin-ß, RanGAP1) were found to be mis-localized to the cytoplasm. Mis-localization of several nuclear pore proteins to the nucleus were also observed (NUP155 and NUP62), potentially compromising the integrity of the nuclear pore complex. Lastly, we found that canonical nuclear import and export are dysregulated in R86S NEMF MEFs.

Conclusions:
These findings suggest that NEMFR86S mutations contribute to the dysregulation of nucleocytoplasmic transport leading to the development of neurodegeneration.

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IVT-28: Potential neuroprotective effects in amyotrophic lateral sclerosis and frontotemporal dementia (ALS/FTD) of retinoic acid receptor ligands (RAR-Ms)

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Live Poster Session B, December 10, 2020, 5:10 PM - 5:50 PM

Introduction:
Amyotrophic lateral sclerosis (ALS) is a heterogenous progressive motor neuron (MN) degenerative disorder that has clinical overlap with the Frontotemporal dementia (FTD) as a neurodegenerative and cognitive disorder of frontal and temporal lobes of brain and the second most common cause of dementia after Alzheimer's Disease (AD). The exact pathogenesis and an effective therapeutic approach for both diseases are lacking.

The retinoic acid (RA) signalling pathway plays crucial roles in the central nervous system to promote cell survival via retinoic acid receptors (RARs) transcription factors and ligands for RARs have been proposed for the treatment of neurodegenerative disorders. It is relatively unexplored however whether it may protect neurons in ALS/FTD. Thus, the neuroprotective effect of novel retinoic acid receptor (RAR) ligands, called RAR-Ms, was investigated.

Methods:
To evaluate the CNS distribution and accumulation of RAR-Ms following IP injection into mice, a novel bioassay using reporter cells was developed. The neuroprotective effect of the RAR-M DC645 was tested in (i) a glutamate-induced excitotoxicity assay using rat primary cortical neurons, (ii) an assay measuring assembly and disassembly of stress granules (SGs) in NSC-34 (MN like) cells transiently transfected with (GFP)-SOD1G93A. In addition, the overall survival rate of the NSC-34 cells transiently transfected with genes associated with ALS/FTD was evaluated.

Results:
Determination of in-vivo distribution of RAR-Ms in the mouse following IP injection showed high levels of accumulation of some of them in different parts of the CNS including the spinal cord and cerebral cortex, indicative of which may be better for ALS or FTD treatment. The RAR-M DC645 showed a neuroprotective effect in the excitotoxicity assay, significantly increasing survival of cortical neurons following glutamate treatment. Furthermore, RAR-Ms affect the dynamic of SGs in the NSC-34 cells expressing (GFP)-SOD1G93A after exposure to arsenate. Further investigation of the RAR-Ms continues as a potential ALS/FTD therapeutic.

IVT-29: Repurposing niclosamide to target inflammatory and fibrotic pathways to affect ALS

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Live Poster Session B, December 10, 2020, 5:10 PM - 5:50 PM

Background:
The process of uncontrolled tissue scarring, called fibrosis, is emerging as a pathological feature in chronic CNS injuries as amyotrophic lateral sclerosis (ALS), where fibrosis and associated inflammation appear to fuel neurodegeneration (1). S100A4, a member of S100 proteins, is recognized as pivotal in the formation of the fibro-glial scar, as it marks reactive glia and it characterizes the conversion of mesenchymal cells into scarring-prone myofibroblasts. Recent studies have associated S100A4 to several diseases besides cancer, including kidney fibrosis, cirrhosis, pulmonary disease, cardiac hypertrophy and fibrosis, arthritis and neuronal injuries. Common to all these diseases is the involvement of fibrotic and inflammatory responses, i.e. processes deeply dependent on tissue remodeling, cell motility and epithelial-mesenchymal transition.

Introduction:
In ALS, both inflammatory and fibrotic processes contribute to the progression of the disease. Our previous results have demonstrated that the S100A4 transcriptional inhibitor niclosamide, a drug undergoing repurposing for chronic inflammatory disorders as an anti-fibrotic agent (2), prevents microglia reactivity and inhibits S100A4, that we found strongly upregulated in mutant-SOD1 models (3).

Methods:
We used primary fibroblasts derived from sporadic and C9orf72 ALS patients to detect protein levels of S100A4, mTOR, SQSTM1/p62, STAT3, α-SMA, NF-κB. The effects of S100A4 inhibition on these pathways was analyzed by S100A4 transient silencing and by the administration of niclosamide to fibroblasts. Transgenic mice overexpressing human wild-type FUS were adopted to test the effects of niclosamide in vivo on ALS inflammatory markers.

Results:
Here we identified S100A4 as a marker of a non-canonical phenotype in ALS fibroblasts, demonstrating that S100A4 mRNA silencing decreases mTOR, the accumulation of p62 and the activation of NF-κB, as well as α-SMA and N-cadherin expression. Moreover, niclosamide interferes with STAT3/mTOR axis and reduces fibrotic markers. Remarkably, niclosamide treatment reduces gliosis and axonal impairment in both central and peripheral nervous tissue of FUS mice.

Discussion:
S100A4 and its downstream factors play important roles in ALS-related mechanisms, and drugs such as niclosamide, capable of targeting inflammatory and fibrotic pathways, could represent a novel strategy to affect ALS.

References:
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IVT-30: Targeting a Novel Gene Therapy: The Role of TDP-43 Recruitment to Stress Granules in ALS/FTD

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Live Poster Session B, December 10, 2020, 5:10 PM - 5:50 PM

Background:
TDP-43 aggregation into insoluble cytoplasmic inclusions is the hallmark pathology in ~95% of ALS cases. TDP-43 is an RNA-binding protein which is recruited to stress granules, a proposed seeding event for the formation of toxic oligomers/aggregates[1]. Various nucleating stress granule RNA-binding proteins have been shown to interact and be regulated by TDP-43 both in vivo and in vitro[2].

Objectives:
To characterise the dynamics of stress and TDP-43 granule formation and dissolution in slice cultures from healthy control mice.

Methods:
Organotypic slice cultures prepared from p9-10 C57Bl6/J mouse pups were exposed to heat shock (42oC; 90 min), and left to recover for 2-48h. Immunohistochemistry was used to detect G3BP, TIA1, and TDP-43 granule formation and dissolution. Proximity ligation assays (PLAs) were conducted to look for evidence of colocalization between TDP-43 and the selected stress granule proteins.

Results:
Heat shock resulted in the formation of TDP-43, G3BP and TIA-1-positive granules, with these granules found to be much longer lasting than previously reported in monolayer cell culture studies. Interestingly, ≥90% of cytoplasmic TDP-43 was also found to be recruited to G3BP+ granules whereas only ≥50% cytoplasmic TDP-43 was recruited to TIA-1+ granules. Similarly, different stress granule markers were identified in different granules suggesting there may be various populations of stress granules formed in response to heat shock stress in these cultures. In addition, PLAs show a significant increase in the interaction between TDP-43 and TIA1/G3BP following heat stress, but no increase in interaction between TIA1 and G3BP despite both being considered key stress granule nucleators, further indicating isolated populations of stress granules may exist following heat shock in this model.

Conclusions:
Stress and TDP-43 granule formation in 3D slice cultures is notably slower, and persists for longer than that reported in monolayer cell cultures, and different core stress granule markers may form different granule populations, demonstrating a clear need to more fully understand the in vivo cellular stress response, and its potential role in TDP-43 linked disease.

References:
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IVT-31: Validation of motor neuron disease patient olfactory neural stem cells as a drug evaluation platform

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Live Poster Session C, December 11, 2020, 12:05 PM - 12:50 PM

Background:
Olfactory neural spheres (ONS) are adult multipotent stem cells that are obtained from nasal biopsies of the olfactory mucosa via a simple in-chair procedure performed by a trained ear nose and throat specialist. The culturing of ONS cells from a nasal biopsy has been established as a method for modelling several neurodegenerative diseases, including Parkinson's disease , schizophrenia and hereditary spastic paraplegia [1]. ONS do not require reprogramming and are expected to more accurately model the aged cell phenotype when compared to iPSCs. Here, we aimed to validate ONS as a model of MND.

Results:
RNAseq evaluation showed MND ONS cells differentially express genes associated with autophagy, response to oxidative stress and mitochondrial function when compared to ONS from controls. A principle components analysis of the RNAseq profiles of the ONS cells demonstrated that MND and control cells separate into two distinct groups. Furthermore, we showed that MND ONS cells are more sensitive to oxidative stress and cell death compared to controls, recapitulating what is known to occur in MND motor neurons in vivo. Importantly, MND ONS cells have a greater propensity for protein misfolding when compared to control cells. Given that patient stem cells do not readily display protein aggregation, including cells from patients with mutations in protein aggregating genes (i.e. SOD1, TARDBP), a cellular or environmental stressor is required to induce and model protein aggregation. We compared sporadic MND and control ONS cells for their propensity to form misfolded SOD1 aggregates using the proteasome inhibitor MG132 (20 µM, 24 hours). Immunostaining for SOD1 revealed that MG132 exposure to ONS cells from multiple MND patients induced the accumulation and misfolding of wildtype SOD1 into defined cytoplasmic aggregations, suggesting that MND cells have a greater propensity for SOD1 misfolding when exposed to cell stress. The observation that MND cells respond to cell stress by inducing protein misfolding is consistent with multiple dysfunctional GO pathways identified by the RNAseq analysis, particularly protein modification, cellular response to stress and regulation of autophagy.

Conclusions:
Currently, there are no approved therapeutics that directly target and modulate MND-associated genes. We have a pipeline of drugs designed to target MND-associated genes for subsets of MND patients. Therefore, establishing a robust cell model for evaluating our drugs is critical to drug efficacy studies. Typically, iPSC-differentiated motor neurons are utilized to evaluate drug candidates, but rarely exhibit misfolded proteins and toxic aggregates and are of limited value in determining drug efficacy. Here, we show the potential of ONS as a model MND, in particular, for evaluating the ability of our drugs to reduce misfolded proteins.

References:
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IVT-32: An Amyotrophic Lateral Sclerosis-Associated Mutant of C21ORF2 Is Stabilized by NEK1-Mediated Hyperphosphorylation and the Inability to Bind FBXO3

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Live Poster Session C, December 11, 2020, 12:05 PM - 12:50 PM

Background:
Amyotrophic lateral sclerosis (ALS) is an adult-onset motor neuron disease characterized by a progressive decline in motor function. Genomic analyses have identified several genes that are mutated in ALS patients. Among them include C21ORF2 [also known as cilia and flagella associated protein 410 (CFAP410)] and NIMA-related kinase 1 (NEK1). Interestingly, both genes are also mutated in certain ciliopathies, suggesting that they might contribute to the same signaling pathways, but the roles of these proteins in ALS pathogenesis remains unknown.

Results and Discussion:
Here we show that F box only protein 3 (FBXO3), the substrate receptor of an CRL1 complex (also known as a SCF complex), a ubiquitin ligase that consists of an adaptor protein (SKP1), a scaffold protein (CUL1), and a RING finger protein (RBX1, also known as ROC1), binds and ubiquitylates C21ORF2, thereby targeting it for proteasomal degradation. Loss of FBXO3 stabilizes not only C21ORF2 but also NEK1, because C21ORF2 binds to and stabilizes NEK1. Conversely, C21ORF2 is stabilized by NEK1-mediated phosphorylation that attenuates the interaction of C21ORF2 with FBXO3, forming a positive feedback loop of stabilization between C21ORF2 and NEK1. Importantly, we found that the ALS-associated C21ORF2 mutant with substitution of valine 58th residue to leucine (V58L) is more susceptible to phosphorylation by NEK1, with the result that it is resistant to ubiquitylation by FBXO3 and therefore accumulates together with NEK1. Expression of C21ORF2(V58L) in induced motor neurons generated from mouse embryonic stem cells impaired neurite outgrowth. Taken together, our data indicate that protein stability of C21ORF2 and NEK1 is regulated by FBXO3, and this regulation is disrupted in ALS patients with C21ORF2 mutation, supporting the idea that inhibition of NEK1 activity is a potential therapeutic approach to ALS associated with C21ORF2 mutation.

IVT-33: Development of autophagy-inducing peptides as a potential therapy for motor neurone disease

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Live Poster Session C, December 11, 2020, 12:05 PM - 12:50 PM

Background:
The pathological hallmark of a range of neurodegenerative diseases, including Motor Neurone Disease (MND) is the presence of protein inclusions and aggregates in affected neurons (1). Aggregates progress continually from the site of onset to disturb cellular processes, ultimately resulting in neuron degeneration. The only intracellular degradative pathway that can purge the cells of these misfolded proteins, aggregates, and dysfunctional organelles is autophagy (2).

Objectives:
Since impaired/reduced autophagy may contribute to MND pathogenesis; upregulating autophagy offers a potential therapeutic option. This study aimed to develop and synthesize novel pharmacological agents that have non-toxic autophagy-inducing properties in motor neuronal NSC-34 cells.

Methods:
A series of peptides analogues of the evolutionary conserved domain of Beclin1 protein, the master regulator of autophagy, were designed and synthesized. In order to enhance the cellular uptake and increase the cytosolic bioavailability, the peptides were cyclized through disulfide, thioether and lactam bond formation, and conjugated to a cell-penetrating peptide (CPP) domain (a fragment of apolipoprotein E (ApoE)), and an endosomal escape (EE) domain (hemagglutinin-2 protein (HA2)). The autophagy-inducing effect of the peptides was determined by measuring the level of autophagosome-associated LC3-II, a marker of macroautophagy, in NSC-34 cells.

Results:
We have shown that the peptides are capable of inducing autophagy at 5 micromolar concentration, which is 10-fold more potent than the previously reported Beclin-1 analogues. We have also shown that cyclization and addition of a CPP and EE domain to the Beclin peptides further increases their autophagy-inducing potency. More importantly, the most potent peptide analogue, HA2-ApoE-Beclin-4, significantly decreased the severe and aggregate-prone C9orf72 dipeptide repeat protein (poly-GR) and mutant SOD1 (A4V) protein level. Thus, HA2-ApoE-Beclin-4 was able to reduce the toxicity associated with the expression of these aggregate-prone proteins by reducing cleaved caspase-3 activation.

Discussion:
This study provides in vitro evidence for a novel peptide-based approach to enhance autophagy as a unique and safe treatment modality with potential therapeutic benefit in MND and other proteinopathies.

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IVT-34: Motor cortex with mSOD1 toxicity and TDP-43 pathology utilize exosomes to communicate a warning signal very early in disease

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Live Poster Session C, December 11, 2020, 12:05 PM - 12:50 PM

Background:
Exosomes are small vesicles, typically 30-150 nm in size, that transport important biomolecular cargo (e.g. protein, RNA, miRNA and lipids) to recipient cells. Thus, they are a major mediator of intercellular communication between different cells, organs and systems in the body. Their ability to cross the blood brain barrier enables flow of information between central nervous system (CNS) and the periphery. Building evidence reveal that cortical dysfunction occurs prior to symptom onset and is one of the contributing factors for the initiation and progression of disease pathology in amyotrophic lateral sclerosis (ALS).

Objective:
The objective of this study was to reveal how ALS cortex communicate their diseased state via exosomes, and to reveal exosomal protein cargo that mediate the warning signal.

Methods:
In this study mouse that develop ALS symptoms due to mSOD1 mediated toxicity and TDP-43 pathology are utilized together with wildtype healthy littermates. Exosomes secreted by cultured dissociated motor cortex obtained from diseased and healthy mice were isolated after 10 days of in vitro culture. We performed proteomic analysis that revealed the content of those exosomes, suggesting the presence of a common message sent out both by hSOD1G93A and prpTDP-43A315T motor cortex, which is modulated by a key transcription factor. We performed gene modulation experiments in both healthy and diseased neurons to investigate the impact of this transcription factor on neuronal survival.

Results:
We find that the motor cortex of both hSOD1G93A and prpTDP-43A315T mouse models of ALS utilize exosomes to communicate their disease state even at postnatal day 3 (P3). In an effort to investigate whether this message is “protective” or “detrimental”, we performed gene modulation. Our results suggest that proteins present in these exosomes appear to signal a message that codes for protection from upcoming neurodegeneration at this early age.

Discussion & Conclusion:
This study not only sheds light on how early cortex is involved in ALS, but also reveals how exosomes can modulate key cellular events that may be targeted to overcome neurodegeneration.

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IVT-35: Neuropeptide Y suppresses hyperexcitability of cortical SOD1G93A mouse neurons in vitro.

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Introduction:
Cortical hyperexcitability has been implicated as an early event which contributes to dysfunction in amyotrophic lateral sclerosis (ALS)(1, 2). Therefore, modulating excitability could potentially slow the progression of ALS in the cortex. Neuropeptide Y (NPY) is an inhibitory neuromodulator which has been shown to protect against hyperexcitability in neurological disorders (ie. Epilepsy) (3-5).

Objectives:
The objective of this study was to investigate whether NPY could modify excitability within the cortex and prevent neuronal hyperexcitability. We hypothesise: exogenous application of NPY suppresses neuronal activity.

Methods:
Cortical neurons were cultured from Thy1YFP x SOD1G93A (E15.5) (n =10) and wild type (WT) littermates (n= 18). At 14 DIV cultures were treated with 100nM, 1uM NPY or vehicle. Network excitability was assessed by multi-electrode array. Patch clamp electrophysiology assessed the effect of NPY on pyramidal (n = 11) and inhibitory neurons (n = 7) from C57BL6 mouse cortical slices. Statistical analysis; Two-Way ANOVA with Bonferroni's comparisons and paired t-tests.

Results:
NPY reduced neuronal firing and spike rate of SOD1G93A ($p < 0.05$) but not WT cultures ($p > 0.05$). Neuronal firing ($p < 0.01$) and spike rate ($p < 0.05$) of cortical neurons was increased in SOD1G93A cultures compared to WT. NPY increased rheobase of pyramidal neurons ($p < 0.05$) and decreased rheobase of inhibitory neurons ($p < 0.01$)

Discussion:
NPY modulates neuronal excitability by altering the action potential threshold of pyramidal and inhibitory neurons. NPY suppressed increased excitability observed in SOD1G93A cortical cultures. Suggesting it as a therapeutic candidate for preclinical excitatory dysfunction in ALS.

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IVT-36: Role of chaperone and acetylation levels and therapeutic potential of HDAC inhibitors for Autosomal Recessive Spastic Ataxia of Charlevoix-Saguenay

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Autosomal Recessive Spastic Ataxia of the Charlevoix Saguenay (ARSACS) is caused by mutations in the gene encoding sacsin (SACS) and affects motor neurons causing amongst other symptoms a peripheral motor and sensory neuropathy. Sacsin is a gigantic protein (4579 aa) whose multiple functions, but evidence points to function associated to protein chaperoning and regulation of protein homeostasis. A cellular characteristic of ARSACS is the formation of neurofilament or bundles in neurons and patients' fibroblasts derived form skin biopsy respectively, which consequently disrupt the cellular cytoarchitecture.

Hypothesis:
Because of the putative chaperone functions of Sacsin, we aimed to determine if chaperone induction or overexpression could resolve neurofilaments (NF) or vimentin (IF) bundling characteristic of ARSACS pathology.

Methods:
Long-term cultures of 6 week-old motor and sensory neurons from Sacs-/- and Sacs+/+ mice embryo were treated with, arimoclomol, the pan HDAC inhibitor, Saha or HDAC6 inhibitor, Tubastatin A, or the HDAC1/3 inhibitor RGFP109. ARSACS fibroblasts derived form skin biopsy of patients show bundling of vimentin IF, a phenotype corresponding to NF bundling in neurons and are used to assess the efficacy of HDAC inhibitor on the formation of IF or NF bundles.

Results:
We identified that expression of HSP70 may compensate for the lack of sacsin expression and resolve NF bundles in motor neurons in culture, while arimoclomol 10micromolar was effective only if cells were prestressed with a heat shock. We next generate a cellular stress using HDAC inhibitors known to activate ER Stress using HDAC inhibitors. Interestingly, treatment with the broad spectrum inhibitor SAHA alone was sufficient to resolve NF bundles in cultured Sacs-/- motor neurons. HDACs are classified as Class I (HDAC 1, 2, 3, 8), Class IIa (HDAC4, 5, 7, 9) and Class IIb (HDAC6, 10), Class III (the sirtuins) and Class IV (HDAC11). The pan HDAC inhibitor SAHA (Vorinostat) resolved IF bundles in cultured Sacs-/- motor neurons and ARSACS patient's fibroblasts. The HDAC6 inhibitor, Tubastatin A, efficiently resolved NF bundles in 6 week-old cultured Sacs-/- motor neurons and ARSACS fibroblasts, but the HDAC1/3 inhibitor RGFP109 did not. Interestingly, HDAC6 inhibitors were the most effective in resolving NF and IF in both neurons and in fibroblasts.

Conclusion:
Our data suggest that HDAC6 inhibitors have an interesting therapeutic potential and that there is an underlying abnormality in the balance of histone acetylation/deacetylation in ARSACS.

Acknowledgements:
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IVT-37: Single-nuclei RNA sequencing of primary human myotubes derived from control individuals and MND patients identifies tissue heterogeneity

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Background:
Skeletal muscle is proposed to play a key role in MND pathology. Primary myotubes are commonly used to model muscle pathology in MND, yet the capacity for multinucleated human primary myotubes to recapitulate tissue heterogeneity remains unknown.

Objectives:
Use single-nuclei RNA-sequencing (snRNA-seq) to determine the extent to which human derived myotubes recapitulate tissue heterogeneity.

Method:
Primary myotubes were grown from myoblast stocks derived from two male MND individuals and two age-matched healthy male control participants. Myotube nuclei were isolated using an optimised nuclear extraction lysis buffer, stained for DAPI and sorted using Fluorescence-Activated Cell Sorting (FACS). Individual myotube nuclei RNA sequencing libraries were established using 10x Genomics snRNA-seq. Data were pre-processed using CellRanger and DIEM [1]. Clusters were identified using the single-cell platform, Seurat [2]. Differentially expressed genes were extracted using MAST [3], followed by trajectory analysis with PAGA [4].

Results:
Our optimised myotube nuclear extraction lysis buffer allows for the isolation of approximately 400,000 nuclei for FACS sorting and establishing of snRNA-seq libraries from between 2000-7000 nuclei per line. Based on our extracted transcriptional profiles, we identified heterogeneous cell populations consisting of myotubes, satellite cells and myoblasts. This was further validated with trajectory analysis, which revealed different stages of in vitro myogenesis.

Discussion and conclusions:
Here, we demonstrate snRNA-seq is a viable tool that provides in-depth reads to profile different cell types in in vitro human myotube cultures. The ability to isolate individual nuclei from a multinucleated, heterogeneous population of myotubes for snRNA-seq provides an avenue to explore MND pathology. Studies that identify potential gene variations between control and MND muscle populations is ongoing.

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IVT-38: Strategy for the in vitro assessment of candidate oligogenic ALS genes

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Background:
Amyotrophic lateral sclerosis (ALS) is a disease with a strong genetic component. Its genetic architecture is complex, ranging from classic Mendelian inheritance, wherein a single gene confers a strong effect, to oligo- and polygenic, wherein a combination of two or more risk variants contribute to disease pathogenicity or increase disease susceptibility. While approximately 60% of familial ALS cases follow classic Mendelian inheritance patterns, the genetic basis of ALS remains to be solved in the remaining 40%. These unsolved ALS families do not exhibit classical Mendelian inheritance patterns and, instead, are likely to have an oligo- or polygenic basis. This genetic complexity has made the design and validation of subsequent pathological studies more challenging due to the introduction of multiple variants within the same cell.
We applied a comprehensive genetic and in silico gene discovery pipeline to an ALS family with reduced disease penetrance and that was negative for known ALS genes and identified two candidate variants. The genomic proximity of both candidate genes suggested that they were likely to be co-inherited, and potentially produce oligogenic disease effects. Our attempts to investigate the compound effect of both candidates in vitro were thwarted by low co-transfection and co-nucleofection efficiency, thus necessitating a change in strategy.

Objectives:
In this study, we aimed to utilise a novel mammalian multicistronic expression system to achieve rapid and reliable expression of our candidate variants for subsequent disease biology studies in vitro.

Method:
To accommodate the large size of one candidate gene and to ensure stoichiometric expression of both genes, we designed a multicistronic vector using two 2A peptide sequences to separate the candidate genes. During translation, ribosomes will skip the formation of a glycyl-prolyl peptide bond at the C-terminus of the 2A motif, resulting in the expression of each candidate protein separately. Furthermore, each candidate gene is also tagged with different fluorescent proteins and flanked by unique restriction sites that match other common destination vectors. This enables downstream subcloning for further studies. Immunocytochemistry and western blot experiments were performed to characterise and validate the vector.

Results:
Preliminary results suggest that transfected cells co-expressed both candidate genes and showed that the spatial-temporal expression pattern of each candidate gene remained consistent with previous findings.

Discussion and conclusion:
We have prioritized novel candidate ALS genes and further developed a strategy to study potential oligogenic effects in vitro. These genes and their associated pathways will be further investigated through the development of animal models, to establish in vivo support for their role in ALS. New ALS genes not only offer fresh diagnostic and therapeutic targets, but also function as tools for the generation of novel animal models to better understand disease biology.

IVT-39: Synaptic and mitochondrial abnormalities due to mutant FUS

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Background:
Increased and aberrantly expressed fused in sarcoma (FUS) are a hallmark of FUS-related amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). It has been shown that normal FUS localises to synapses and interacts with mitochondrial proteins. Recent studies describe aberrant FUS leading to early pathological changes in mitochondria, at the neuromuscular junction and at the synapse indicating it may have a role in regulating synaptic and mitochondrial function. However, the role of FUS in the relationship between synaptic and mitochondrial function is poorly understood.

Methods:
Neurite complexity and synaptic density were studied in vitro in primary rat neurons expressing eGFP-FUSWT or one of two mutants eGFP-FUSR514G and eGFP-FUSΔNLS to determine the downstream effects of FUS mutations. To investigate this in a motor neuron specific in vivo model, zebrafish were co-injected with MNX1:Gal4 (driving expression specifically in motor neurons) and either UAS: eGFP-FUSWT, UAS: eGFP-FUSR514G or UAS: eGFP-FUSΔNLS. Axon growth, branching and neuromuscular junction (NMJ) density were measured. Using live imaging, mitochondrial movement was probed in both neuronal models expressing mutant FUS. Complimentary proximity ligation assays were carried out to investigate the endogenous localisation of FUS and mitochondria. Overexpression of mutant FUS was used to assess if mutant FUS led to a change in these interactions. Lastly, Puromycin assays were performed to investigate how mutant FUS led to a change in global translation.

Results:
We found that mutant FUS leads to alterations in synaptic numbers and neuronal complexity in both primary rat neurons and zebrafish in vivo models. The degree to which FUS is mislocalised leads to differences in the synaptic changes which is mirrored by the accompanying changes in mitochondrial transport. We also show that FUS is localised with a mitochondrial anchor protein and that mutations in FUS decrease this relationship, which may cause the synaptic and mitochondrial phenotypes observed. Finally, we show that mutant FUS led to a change in global translation in primary neurons.

Conclusions:
Overall, these results provide evidence that mislocalised cytoplasmic FUS leads to related mitochondrial and synaptic changes and that FUS plays an essential role in maintaining the health of neurons. Importantly, our results support the 'gain-of-function' hypothesis for disease pathogenesis in FUS-related ALS.

IVT-40: TDP-43 and R-loops: a new interplay in ALS disease

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Background:
During transcription, nascent RNA is prone to hybridize with DNA, displacing and forming a three-stranded nucleic acid structure called R-loops. R-loops are physiologically produced, but excessive R-loops formation can cause genome instability and can be implicated in the pathogenesis of several diseases (1). There is increasing evidence on the pathological role of R-loops in Amyotrophic Lateral Sclerosis (ALS) (2). Loss of function of TDP-43 can lead to accumulation of transcription-associated DNA damage, contributing to motor neuron cell death and progressive neurological symptoms (3).

Objectives:
The aim of the study was to understand the role of TDP-43 mutation and overexpression in R-loops removal and genome instability linked to ALS disease.

Methods:
SH-SY5Y were transfected with mutated TDP-43 (A382T), WT-TDP-43 and mock transfected. Lymphoblastoid cell lines (LCLs) from a sporadic ALS patient (SALS-LCLs), an ALS patient LCL cell carrying mutation on TDP-43(A382T) and an healthy CTRL were used. Immunofluorescence analysis for gH2Ax, FANCD2, S9.6 were carried out on these cell lines. DRIP were run according to the literature (4). Coimmunoprecipitation of S9.6 and TDP-43 were checked by western blot.

Results:
We have demonstrated that mutated TDP-43 (A382T) in transfected neuronal SH-SY5Y and lymphoblastoid cell lines (LCLs) from an ALS patient cause R-loop accumulation, and R loop-dependent increased DSBs. Instead, overexpression of TDP-43 in SH-SY5Y lead to dsRNA increase.

Discussion:
These findings showed a new role for TDP-43 in the control of R-loops removal and in preventing genome instability. Knowing the mechanism of R-loops accumulation in TDP-43 ALS pathology it would be of great advantage, not only for providing new knowledge in the pathogenesis of the disease but also for contributing in giving new targets for drug design and innovative therapeutic approaches.

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IVT-41: The Neuroprotective Effects of Neurotrophin Mimetics in Novel In Vitro and In Vivo Models of Amyotrophic Lateral Sclerosis (ALS)

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Neurotrophins are a class of polypeptides that has been shown to be a potent way to protect neuronal function in neurodegenerative conditions, such as amyotrophic lateral sclerosis (ALS), which is characterised by motor neurone death. Neurotrophin treatment has previously shown efficacy in pre-clinical ALS models. However, clinical trials using neurotrophins produced no major improvements in ALS patients, due in part to limited blood brain barrier penetration and suboptimal target engagement that limited their therapeutic use. A solution to overcome these limitations is the development of neurotrophin mimetics able to bind the neurotrophin receptors

We aim to identify novel neurotrophin mimetics that inhibit motor neuron death in vitro, by screening 100 marine extracts, and 169 small synthetic tropomyosin receptor kinase B (TrkB) agonists in a high-throughput screening assay based on co-cultures of iAstrocytes derived from a panel of patient biosamples carrying different ALS-associated mutations and motor neurones. ALS iAstrocytes create a deleterious microenvironment for motor neurones through the secretion of neurotoxic molecules, exacerbating the pathological condition. Only a selected number of hits showing promising motor neuronal rescue will be further analysed and subjected to biological testing and optimization.

The primary screening has been carried out on co-cultures composed by iAstrocytes differentiated from iNPC lines, obtained from ALS patients carrying mutations in the genes encoding for superoxide dismutase 1 (SOD1) or C9ORF72, and murine GFP+ motor neurons. Precise concentrations of each drug have been delivered using the ECHO 550 Liquid Handler. The number of viable motor neurons that survive after 72 hours in co-culture is calculated as a percentage of the number of viable motor neurons after 24 hours in co-culture. Both measurements have been acquired through the high-content imaging system Incell 2000.

The primary screening on the co-culture system based on human iAstrocytes derived from a C9ORF72 ALS patient showed 8 promising marine extracts and 1 synthetic TrkB agonist able to significantly improve motor neuron survival.

Molecules contained in the marine extract hits will be isolated and screened. Top hits and analogues of the synthetic TrkB agonist hit will be further investigated in a multi-donor screening including 2 more C9ORF72, 3 SOD1 and 3 sporadic ALS iAstrocyte lines. Target engagement and molecular functions will be assessed as well. Lastly, top hits will be also tested on in vivo C9ORF72 or SOD1 ALS zebrafish models to identify the compounds that improve motor behaviour. Histological analysis will be also performed to ascertain the therapeutic efficacy of lead molecules.

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IVT-42: The role of LRRK2 in the phosphorylation of Thr175 tau associated with amyotrophic lateral sclerosis-frontotemporal spectrum disorder.

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Background:
Approximately 50-60% of patients with ALS concurrently develop a range of behavioural or cognitive impairments and are diagnosed along the spectrum of ALS-frontotemporal spectrum disorders (ALS-FTSD) (1). Roughly half of ALS-FTSD patients display evidence of microtubule-associated protein tau metabolic dysfunction, characterized by pathological tau phosphorylation (1). Phosphorylation of Thr175 (Thr175tau) is suggested as an early pathological event in the production of cytosolic tau inclusions. pThr175tau immunoreactive inclusions are present in multiple tauopathies, including ALS and ALS with cognitive impairment (2). pThr175tau triggers a cellular cascade, which subsequently phosphorylates Thr231tau and results in fibril formation (3). Further, this pathway has been experimentally replicated in a rodent model of traumatic brain injury (TBI). It is currently unknown which kinase is involved in the phosphorylation of Thr175tau. Objective:
Determine if LRRK2 directly phosphorylates Thr175tau and if inhibition of LRRK2 prevents phosphorylation of Thr175tau and fibril formation in a rodent model of TBI.

Methods:
HEK293T cells were co-transfected with tau and one of three LRRK2 constructs, WT-LRRK2, KD-LRRK2 (constitutively inactive) or G2019S-LRRK2 (constitutively active). Cells were subject to transfection with or without an LRRK2-directed siRNA. Levels of pThr175tau and fibril formation were measured using Western blot analysis and immunofluorescence. Next, the LRRK2-binding cassette on tau was mutated using site-directed mutagenesis, and then co-transfected with LRRK2. Levels of pThr175 and fibril formation were measured using Western blot analysis and immunofluorescence. Future studies will investigate the neuroprotective effect of LRRK2 inhibition in an LRRK2-knockdown rodent model of TBI.

Results:
Preliminary results show that the co-transfection of tau and G2019S-LRRK2 results in an increase in pThr175tau compared to co-transfection with KD-LRRK2. In the presence of an LRRK2-directed siRNA, levels of pThr175tau return to baseline. We predict that if LRRK2 directly phosphorylates Thr175tau, mutation of the LRRK2 binding site on tau will result in a decrease of pThr175tau.

Discussion:
This study examines the role of LRRK2 in the pathological phosphorylation of Thr175tau. The complete characterization of this pathway, including the kinase involved in the phosphorylation of Thr175tau, is imperative for a greater understanding of the cellular mechanisms of tau pathogenicity related to ALS-FTSD. Further, this study may present LRRK2 as an important therapeutic target to reduce tau phosphorylation and fibril formation.

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IVT-43: The unexplored effects of SUMOylation on TDP-43 aggregation and sub-cellular localization

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Background:
TDP-43 localizes predominantly in the nucleus, rearranging itself in highly dynamic granules due to a mechanism called liquid-liquid phase separation. Moreover, TDP-43 actively shuttles between the nucleus and the cytoplasm [1]. The mechanisms underlying how TDP-43 is compartmentalized in the cell, and how different external stimuli impact this localization remains elusive and crucial for innovative therapeutic strategies. We provide novel evidence that post-translational modifications such as SUMOylation can affect TDP-43 localization [2].

Objectives:
In this study, we aimed to decipher the role of SUMOylation in TDP-43 localization and aggregation in vitro and in vivo. We first developed an ALS in vitro model that presents TDP-43 positive cytoplasmic aggregation by over-expressing GFP-TDP-43WT in cell lines. We used anacardic acid (AA), an inhibitor of the first step of the SUMOylation pathway, as a global approach. TDP-43 contains a putative SUMOylation site at lysine 136, we therefore next over-expressed the mutated form for this site (K136R) as a targeted approach. Finally, to validate these findings in vivo, we expressed human variants of TDP-43 (+/- mutation at the site K136) in spinal motor neurons of zebrafish and assessed their localisation and aggregation properties using confocal microscopy.

Results:
Inhibition of SUMOylation processed by AA (25µM) significantly reduced the presence of TDP-43 aggregates and improved neuritogenesis and cell viability in vitro. Interestingly, cells over-expressing GFP-TDP-43K136R presented nuclear aggregates. The K136R mutant also rescued neurite outgrowth and increased toxicity that was associated with the over-expression of TDP-43WT. Our in vivo results are in line with these findings. We found a significant increase in nuclear localisation of eGFP-TDP43K136R compared to eGFP-TDP43WT (78% versus 62% respectively, $p<0.0001$). We also found that zebrafish motor neurons expressing non-SUMOylated forms of TDP-43 showed less cytoplasmic aggregates, as well as altered phase separation properties.

Discussion:
Posttranslational modifications (PTMs) of TDP-43 are crucial for the proper localization and functioning of proteins inside the cells, and TDP-43 is subject to several PTMs. Our data demonstrates for the first time the importance of the SUMOylation pathway (and the lysine 136 of TDP-43) in the process of cytoplasmic localization of TDP-43 aggregates in vitro and in vivo. Influencing PTMs such as SUMOylation could be a promising medium-term therapeutic target compared to modifying the basic expression levels of TDP-43. Understanding this (and other) protein modification pathways may help to find new ways to either prevent cytoplasmic mislocalisation of TDP-43 or transport it back into the nucleus.

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IVT-44: Using human motor neuron (MN) spheroids to model ALS

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Amyotrophic lateral sclerosis (ALS) is a fatal disease characterized by the selective degeneration of motor neurons (MNs) from the cortex, brainstem and spinal cord. Loss of MNs leads to muscle weakness and paralysis, which typically results in the death of patients due to respiratory failure. Currently, ALS has no cure and the lack of models that effectively recapitulate the mechanisms of the disease observed in humans is a major obstacle in the discovery of new therapeutics. Induced pluripotent stem cells (iPSCs) have emerged as a novel tool to model neurodegenerative diseases because they retain the genetic background of patients and can be differentiated into any cell type, including many cell types found within the central nervous system (CNS). Furthermore, iPSCs can be used to generate 3D models in which cell-to-matrix and cell-to-cell interactions observed within the CNS are recapitulated in a more physiological manner than with 2D models. In our lab, we differentiate iPSCs from healthy control and ALS patients into MN progenitor cells which are seeded into low-attachment plates to promote their aggregation and maturation as MN spheroids. qPCR and immunofluorescence (IF) analysis confirmed that these MN spheroids express specific markers of MNs such as HB9, ISL1, ChAT and SMI-32 both at the mRNA and protein level respectively. Additionally, the MN spheroids have shown to be consistent in terms of size and cell identity across batches. Remarkably, MN spheroids are also capable of protruding axons when they are plated on top of a scaffold, showing their potential to reach and innervate target cells such as skeletal muscle. We are now able to culture human primary myoblasts and differentiate them into skeletal muscle micro-tissue that expresses desmin, MyHC and SAA at mRNA and protein level confirmed as well by qPCR and IF respectively. Our next step will be to co-culture MN spheroids and human primary skeletal muscles to generate a human 3D in vitro model to study the formation and degeneration of the NMJ, an early and primarily affected structure in the development of ALS. Additionally, other cell types like astrocytes and oligodendrocytes can be differentiated from iPSCs and incorporated into the spheroids to create a model that allows for the study of the non-autonomous cell mechanisms of the disease and their impacts on NMJ disruption. With this model established, we will start scaling it for high-throughput assays to test the effects of different ALS-associated mutations on NMJ formation and degeneration, and to screen for small molecules to find compounds with the potential to be taken into clinical trials.