Integrated Molecular and Functional Profiling of ALS in Human iPSC-Derived Motor Neurons for Target Validation and Therapeutic Assessment

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CORTEXA THERAPEUTICS

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease defined by the selective loss of motor neurons. TDP-43 pathology, with cytoplasmic mislocalization and phosphorylation, is found in ~97% of cases, and TDP-43 mutations, including Q331K and M337V, are linked to ALS. Excitotoxic calcium overload is a leading candidate mechanism for motor neuron death, because these cells combine low calcium buffering with strong calcium entry through calcium-permeable AMPA receptors (CP-AMPAR). Two routes to CP-AMPAR expression have been proposed **(Fig. 1)**. Whether the above TDP-43 mutations engage either route in human motor neurons is not known. Here, we asked whether these mutations alter AMPA and NMDA receptor expression, TDP-43 pathology, CP-AMPAR-dependent calcium entry, and whether that entry can be blocked pharmacologically.

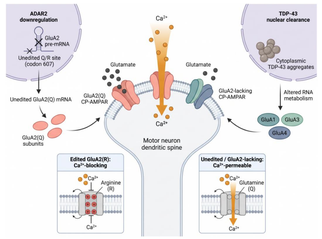


Figure 1. Proposed routes to CP-AMPAR expression in motor neurons. (Left) ADAR2 loss impairs Q/R-site editing of GRIA2 (codon 607), yielding unedited GluA2(Q) subunits. (Right) TDP-43 nuclear clearance, aggregation and/or hypofunction alter RNA metabolism, increasing GluA1, GluA3 and GluA4 and producing GluA2-lacking CP-AMPARs. (Center) Both receptor types let Ca^{2+} enter the dendritic spine on glutamate binding. (Insets) Arginine (R) at site 607 blocks Ca^{2+}; glutamine (Q) or absent GluA2 permits it.

2. Methods

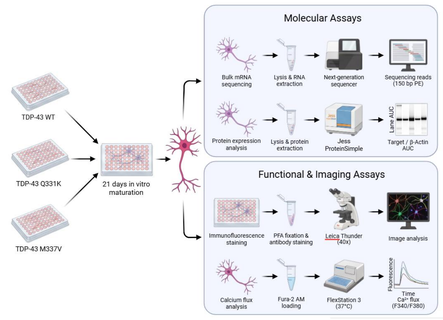
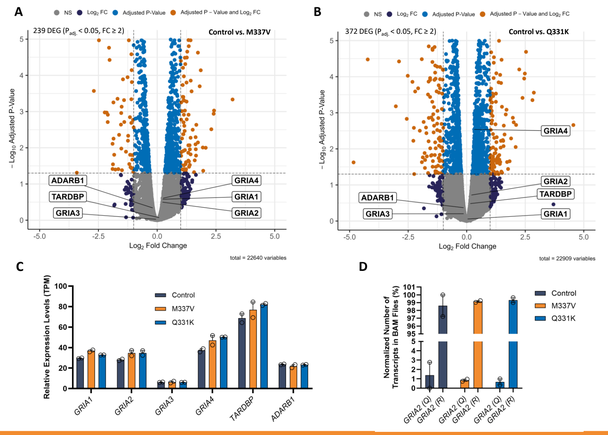


Figure 2. CRISPR-edited human iPSC-derived motor neurons carrying TDP-43 Q331K or M337V, and isogenic TDP-43 wild-type controls, were matured for 21 days in vitro before analysis. For mRNAseq, RNA was extracted in duplicate and sequenced at ~100 million 150 bp paired-end reads per sample, with differential expression analyzed in R. Protein expression was measured by Jess™ Western, with AUC values normalized to β-Actin. For immunostaining, PFA-fixed neurons were labelled with fluorescent primary antibodies and counterstained with CellMask™ and DAPI, then imaged at 40x on a Leica Thunder microscope and analyzed in Arivis Vision 4D. For calcium flux, neurons were loaded with Fura-2-AM and fluorescence at 510 nm was recorded continuously at 37 °C on a FlexStation 3® following dual excitation at 340 and 380 nm.

3. Transcriptional Profiling



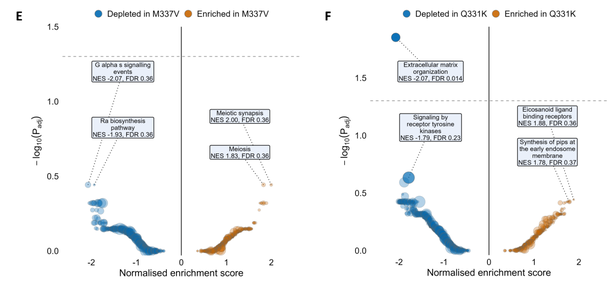
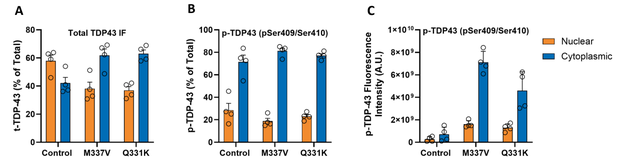


Figure 3: Volcano plots showing upregulated and downregulated differentially expressed genes (DEG) in Control vs. TDP-43 M337V **(A)** or Control vs. TDP-43 Q331K **(B)** motor neurons. **(C)** Transcript per million (TPM) expression levels of selected genes in Control, M337V and Q331K motor neuron. **(D)** Normalized proportion of transcripts at the *GRIA2* Q/R site, expressed as a percentage of total reads, indicating the relative frequency of edited (R) versus unedited (Q) forms. Data is presented as mean ± SEM. Gene set enrichment analysis (GSEA) of Reactome pathway analysis in Q331K **(E)** and M337V **(F)** (dashed line: FDR = 0.05; faint bubbles: FDR > 0.25).

4. TDP43 Protein Expression and Pathology



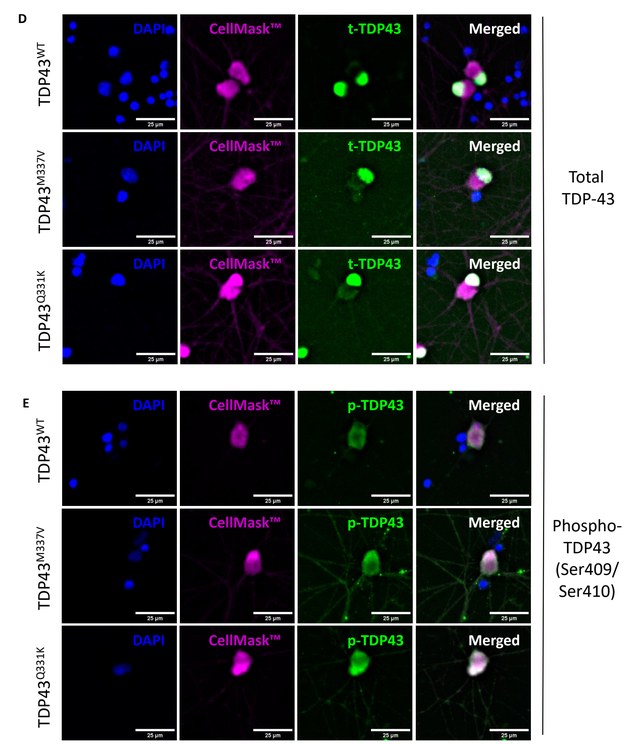


Figure 4: Relative fluorescence intensity levels of total **(A)** and phosphorylated **(B)** TDP-43 in the nucleus and cytoplasm of immunostained Control, TDP-43 Q331K and TDP-43 M337V motor neurons, expressed as a percentage of the total (nuclear + cytoplasmic) fluorescence intensity signal in each genotype. **(C)** Nuclear and cytoplasmic fluorescence intensity signal for phosphorylated TDP-43 in immunostained Control, TDP-43 Q331K and TDP-43 M337V motor neurons. **(D, E)** Representative images of Control, TDP-43 Q331K and TDP-43 M337V motor neurons immunostained with a fluorescently labelled antibody against total TDP-43 **(D)** or TDP-43 phosphorylated at Ser409 and Ser410 **(E)**. TDP-43 (total or phosphorylated) signal is shown in green, CellMask™ signal is shown in purple and DAPI-labelled nuclei are shown in blue. Data are presented as mean ± SEM of 4 technical replicates (culture wells) per condition. Scale bars are 25 µm.

5. Expression of Glutamate receptor subunits

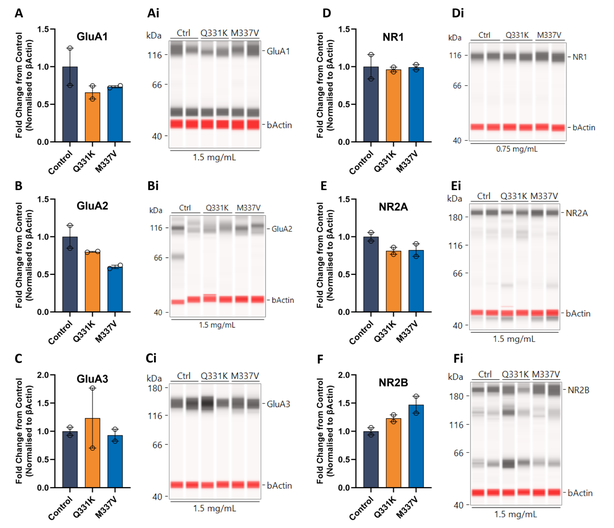


Figure 5: Subunits of AMPA **(A-C, Ai-Ci)** and NMDA **(D-F, Di-Fi)** receptors were quantified by Jess™ ProteinSimple® automated western blotting in Control, TDP-43 Q331K and TDP-43 M337V motor neurons. For all targets, expression levels relative to the loading control β-actin and normalized to expression levels in control neurons are indicated in **(A-F)**. Pseudoblots for each target protein are shown in **(Ai-Fi)**. Data are presented as the mean ± SEM of two culture technical replicates.

6. Measurement of CP-AMPAR-mediated responses

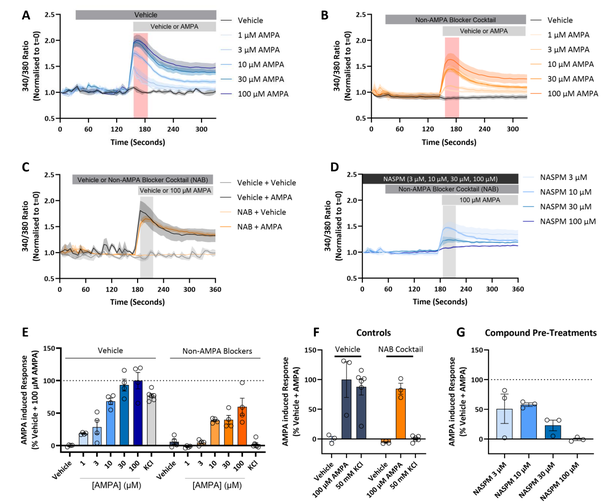


Figure 6: Calcium flux assay optimization. Motor neurons were loaded with Fura2-AM, followed by measurement of the 340/380 fluorescence intensity ratio. **(A, B)** XY plots of the 340/380 fluorescence intensity ratio obtained from cells treated with either vehicle **(A)** or a cocktail of blockers of non-AMPA ion channels **(B)**, prior to AMPA stimulation. **(C)** XY plots of 340/380 ratios obtained from neurons pre-treated with Vehicle, followed by the addition of either Vehicle or NAB cocktail, and either vehicle or 100 µM AMPA. **(D)** XY plots of 340/380 ratios obtained from neurons pre-treated with NASPM, followed by the addition of the NAB cocktail, and 100 µM AMPA. Data in **(A-D)** are normalized to 340/380 fluorescence intensity ratio at t=0. Red shaded area in **(A-D)** indicates the time period used for the AUC analyses shown in **(E-G)**, respectively. Data are presented as the mean ± SEM of up to 6 technical replicates per condition per timepoint.

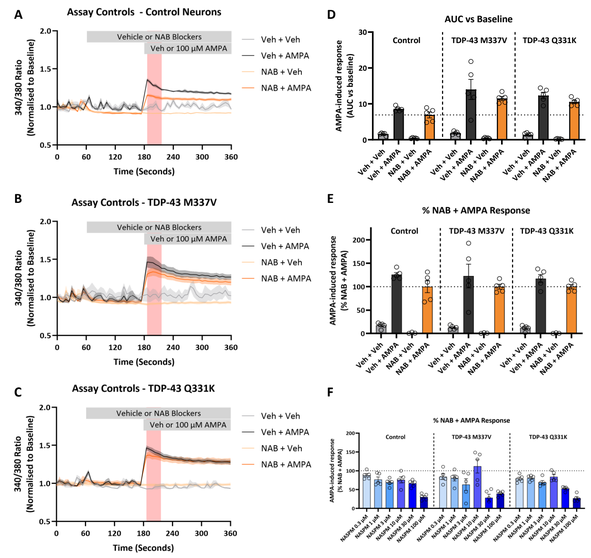


Figure 7: Calcium flux measurements in TDP-43 motor neurons. Control **(A)**, TDP-43 M377V **(B)** and TDP-43 Q331K **(C)** motor neurons were loaded with Fura2-AM, followed by measurement of the 340/380 fluorescence intensity ratios. **(A-C)** XY plots of the 340/380 ratios obtained from neurons pre-treated with vehicle, prior to NAB or AMPA stimulation. Data are normalized to the average 340/380 ratio at baseline. The red shaded areas indicate the time period used for the AUC analyses shown in **(D, E)**. **(F)** AUC analysis of XY plots of the 340/380 ratio obtained from neurons pre-treated with NASPM prior to NAB or AMPA stimulation. Data are presented as mean ± SEM of up to five technical replicates per condition (n=5).

7. Conclusions

Here we present preliminary results suggesting that:

- **TDP-43 Q331K and M337V motor neurons show enhanced AMPA-evoked Ca^{2+} entry** relative to isogenic controls (~1.5–1.7-fold, Fig. 7D).
- **The AMPAR change appears to be driven by a shift in subunit composition, rather than defective GluA2 editing.** *GRIA2* was almost fully edited (R) and *ADARB1* was unchanged in all lines, while GluA2 protein fell (~20% Q331K, ~40% M337V) and *GRIA4* transcript rose.
- **A NASPM-sensitive, CP-AMPAR-driven component contributes to the response in all three lines.** NASPM at 30–100 µM reduced the AMPA response to ~30–50% of the NAB + AMPA response, confirming that the assay can be used to test CP-AMPAR-targeting compounds.
- **NMDAR composition is also altered.** NR2B increased and NR2A decreased, with NR1 unchanged, in both mutants.
- **Both mutations show TDP-43 pathology.** Cytoplasmic and phosphorylated (Ser409/410) TDP-43 were elevated, most strongly in the M337V mutant.

Confirmation of these disease-relevant phenotypes in independent experimental replicates is underway.

8. Acknowledgements

Portions of this research were supported by a charitable grant from Mission Edge San Diego, a 501(c)(3) public charity, through its fiscal sponsorship of Precision ALS. Results from grant-funded work are made openly available to the research community.

All graphics were prepared on BioRender.com