

Supplementary Materials

Neuronal Activity-Dependent Gene Dysregulation in C9orf72ⁱ³Neuronal Models of ALS/FTD Pathogenesis

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Genotype	GroupID	Age of Onset	Sex	Onset	C9 Repeat Size	Insert / site	Lab Source
C9orf72	ALS/FTD	49	M	Lumbar	>44	Ngn1/2 + mCherry in CLYBL	Barmada lab
C9orf72	ALS/FTD	56	M	Cervical	>25	Ngn1/2 + mCherry in CLYBL	Barmada lab
Control	CTRL	NA	M	NA	normal	Ngn1/2 + iRFP in CLYBL	Barmada lab
Control	CTRL	NA	F	NA	normal	Ngn1/2 + mCherry in CLYBL	Barmada lab
C9orf72	ALS/FTD	NS	F	NA	NS	Ngn2 + mCherry in AAVS1	Ward lab
Control	CTRL	NA	M	NA	normal	Ngn2 in AAVS1	Ward lab

Supplemental Table 1. Source and demographic information for i³Neuron lines used in this study. NA = not applicable, NS = not specified.

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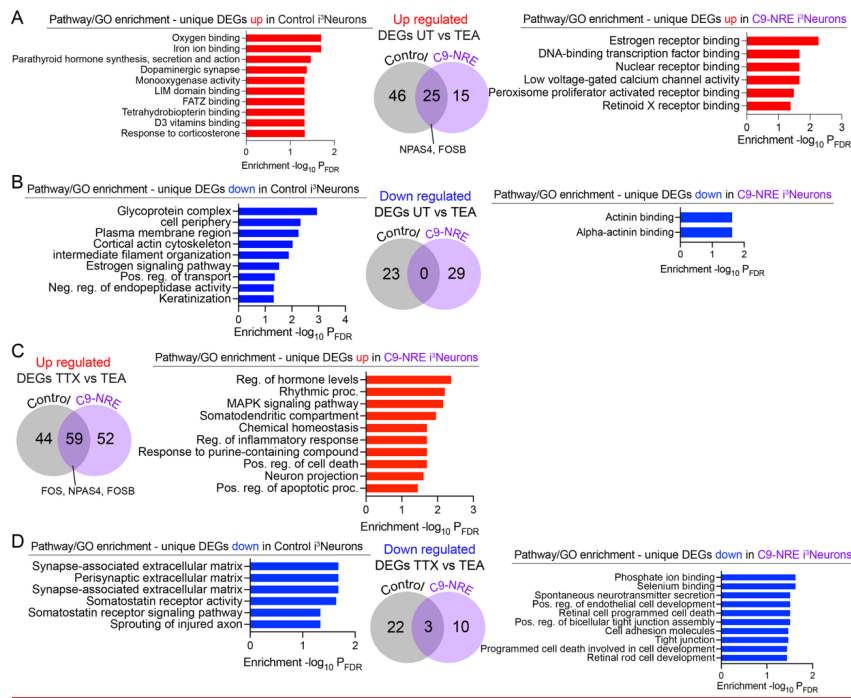
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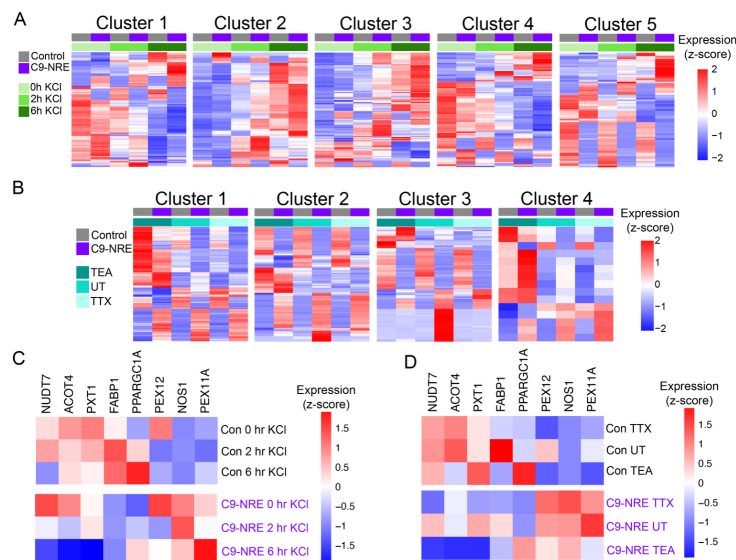
(A) Schema of origin of DEGs from DESeq2 used in Venn diagram comparisons. At each time point (0, 2, 6), Control i^3 Neurons are compared to C9-NRE i^3 Neurons. **(B)** Hierarchical clustering heatmap showing the gene expression (scaled by z-score) of pooled DEGs between listed comparisons in (A). **(C)** Venn diagram of DEGs shows the inherent transcriptomic differences between Control and C9-NRE i^3 Neurons and how depolarization drives differential gene expression. Pathway Enrichment/GO are for transcripts upregulated in C9-NRE i^3 Neurons. **(D)** same as in (B) but for down-regulated transcripts in C9-NRE i^3 Neurons. **(E)** Hierarchical clustering heatmap showing widespread dysregulation of the crystallin family of genes in C9-NRE i^3 Neurons.



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Supplemental Figure 2. Heatmaps of Clusters identified by maSigPro and peroxisomal gene dysregulation.

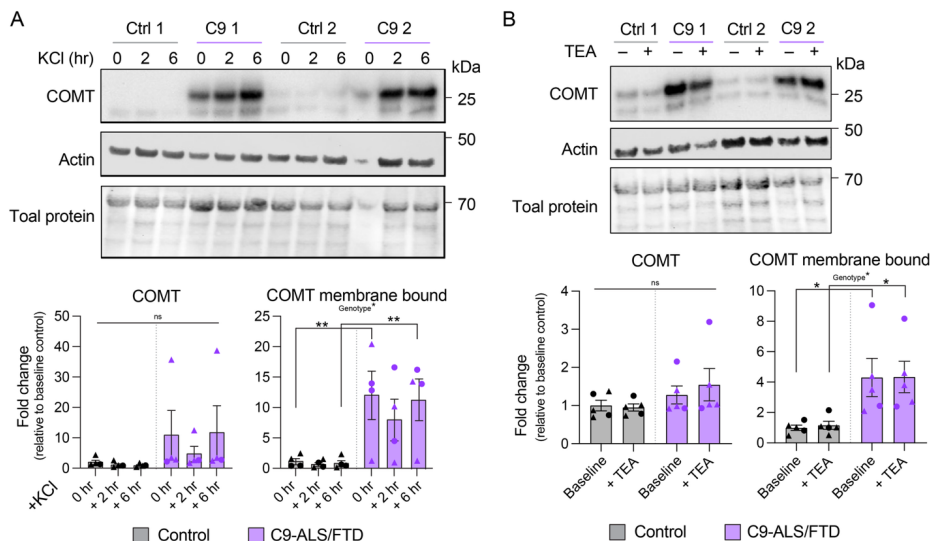
(A) DEG clusters were identified using maSigPro, using levels of neuronal activity as pseudo-time. (B) DEG clusters were identified using maSigPro, using levels of neuronal activity as pseudo-time with TEA stimulation, UT (untreated), and TTX silenced i³Neurons. (C) A heatmap showing the dysregulation of peroxisome-associated genes in Control and C9-NRE i³Neuron samples used in the KCl paradigm. (D) A heatmap showing the dysregulation of peroxisome-associated genes in Control and C9-NRE i³Neuron samples used in the TEA paradigm.



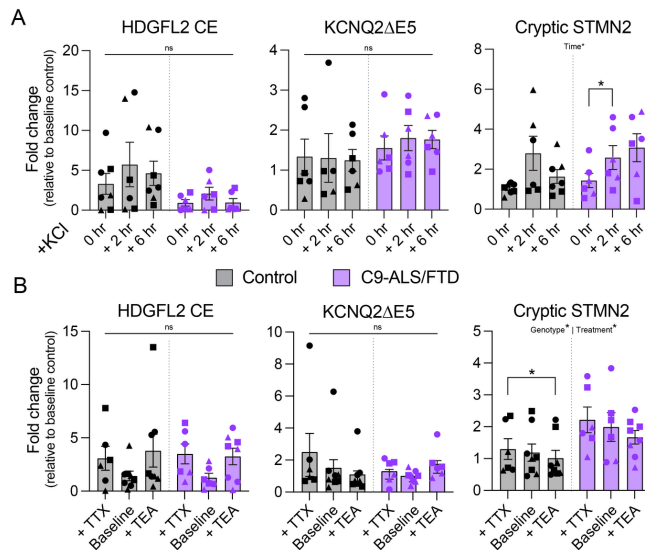
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Supplemental Figure 3. Pairwise differential gene expression of TEA-stimulated and TTX-silenced i³Neurons.

RNA Sequencing was performed on TTX-silenced and TEA-stimulated i³Neurons, and differentially expressed genes (DEGs) were identified using DESeq2. Pairwise comparisons were performed between untreated (UT) and TEA stimulated, or TTX-silenced and TEA stimulated for both Control and C9-NRE i³Neurons. The number of genes uniquely up-regulated or down-regulated in control or C9-NRE i³Neurons are shown in the Venn diagrams. **(A)** Pathway and gene ontology (GO) analysis were performed on the uniquely up-regulated DEGs in either Control or C9-NRE i³Neurons in UT vs. TEA **(B)** Same as in (A) but for down-regulated genes. **(C)** Pathway and gene ontology (GO) analysis were performed on the uniquely up-regulated DEGs in either Control or C9-NRE i³Neurons in TTX vs. TEA. **(D)** Same as in (C) but for down-regulated genes.



Supplemental Figure 4. Membrane-bound COMT is increased in C9 i³Neurons. (A) KCl paradigm. Representative immunoblots of whole-cell lysates from two independent lines per genotype (Ctrl 1–2; C9 1–2) collected at 0, 2, and 6 h after KCl. Blots were probed for COMT, actin, and total protein. Two COMT isoforms are visible: membrane-bound (~28 kDa, upper band) and soluble (~24 kDa, lower band). Molecular weight markers are indicated (25/50/70 kDa). No lanes were rearranged. Quantification (bottom) from $n = 2$ biological replicates and 2 independent differentiations. Intensities were expressed as fold change relative to the average control baseline (0 h) within each replicate. Symbols denote individual lines; color denotes genotype (gray = Control; purple = C9). Bars = mean \pm SEM. Two-way mixed effects ANOVA (genotype \times time) showed a main effect of genotype for membrane-bound COMT, with no effect for total COMT (ns). Sidak–Holm post hoc tests indicated C9 > Control at 0 h and 6 h ($p < 0.01$) for membrane-bound COMT. (B) TEA paradigm. Representative immunoblots from the same two lines per genotype under basal conditions (–) and after TEA (+). Membrane-bound and soluble COMT bands are indicated as in (A). Quantification (bottom) from $n = 2$ biological replicates with 2–3 independent differentiations. Data are plotted as fold change relative to the average basal control within each replicate; symbols and bars as in (A). Two-way mixed effects ANOVA (genotype \times treatment) revealed a main effect of genotype for membrane-bound COMT, with total COMT not significant (ns). Asterisks indicate statistical significance of * $p < 0.05$; ** $p < 0.01$; ns = not significant.



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Supplemental Figure 5. Cryptic splicing is altered in C9 iPSC-derived neurons. (A) KCl stimulation paradigm. qPCR quantification of cryptic exon-containing transcripts (HDGFL2 CE, KCNQ2ΔE5, and cryptic STMN2) from control and C9-ALS/FTD iPSC-derived neurons following 0, 2, and 6 h of KCl stimulation. Data point color denotes cell line; shape denotes independent differentiation replicate. Each point represents the average of two technical qPCR replicates. $\Delta\Delta\text{CT}$ values were calculated by first normalizing raw CT values to RPLP0, then normalizing to the average ΔCT for control lines at 0 h. Bars show mean \pm SEM. Two-way mixed effects ANOVA indicated a main effect of replicate for all three targets. Cryptic STMN2 additionally showed a main effect of stimulation time, with Tukey's post hoc test revealing a significant increase from 0 h to 2 h in C9 lines. **(B)** TEA and TTX paradigm. qPCR quantification of the same cryptic exon-containing transcripts under baseline (+TTX) and TEA treatment conditions. Data plotting and normalization as in (A), using baseline control values for $\Delta\Delta\text{CT}$ calculation. Two-way mixed effects ANOVA showed a main effect of genotype and treatment for STMN2 but not KCNQ2ΔE5 or HDGFL2. Tukey's multiple comparisons revealed a significant difference between TEA and TTX conditions in control lines. Asterisks indicate statistical significance of $*p < 0.05$; ns = not significant.