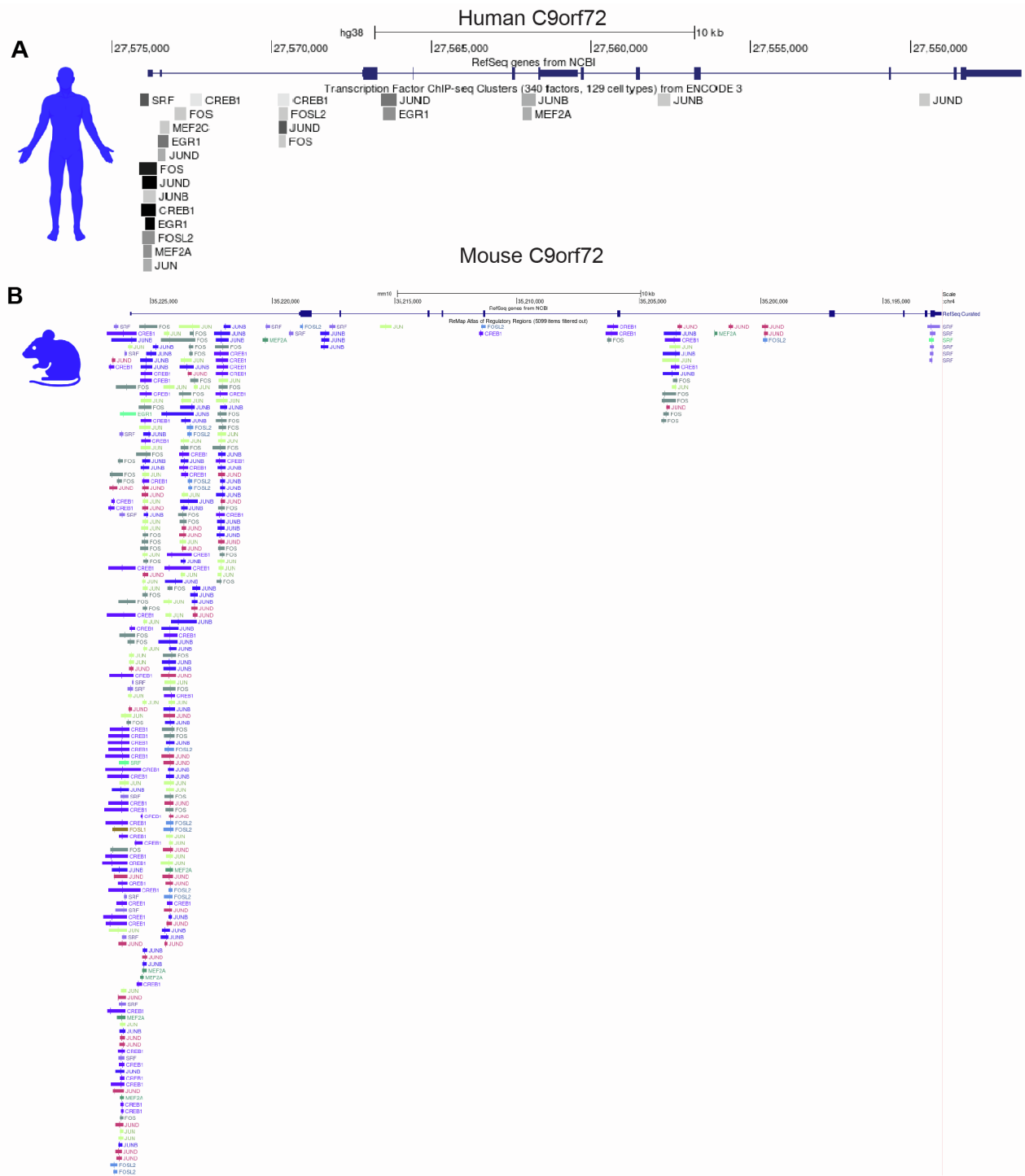# Supplemental information

Differential response of C9orf72 transcripts

following neuronal depolarization

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Supplemental Material

Figure S1. Original screenshots showing ChIP-seq data on the UCSC Genome Browser, related to Figure 1. (**A**) A visualization of ChIP-sequencing results via the ENCODE project shows the human C9orf72 gene is enriched for activity-dependent transcription factors, including SRF, CREB1, MEF2A, MEF2C, EGR1, FOS, FOSL2, JUN, JUNB, and JUND. (**B**) A visualization of ChIP-sequencing results via ReMap Atlas of Regulatory Regions shows the mouse homolog of C9orf72 (3110043O21 Rik) is enriched for activity-dependent transcription factors, including SRF, CREB1, MEF2A, FOS, FOSL1, FOSL2, JUN, JUNB, and JUND.
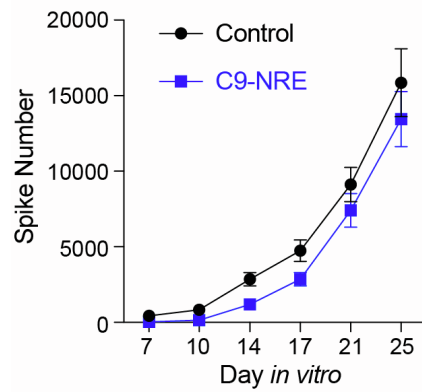
Figure S2. i³Neurons from C9-NRE carriers or controls exhibit similar spontaneous neuronal activity, related to Figure 4. Spontaneous spikes of i³Neurons were recorded via a multi-electrode array longitudinally through differentiation. Spike number increases over time as i³Neurons synaptically mature. There are no statistically significant differences in the number of spikes between control i³Neurons and C9-NRE i³Neurons. For each experiment $n = 3$ biological replicates, $m = 2 – 5$ technical replicates, $o = 4$ wells per experiment. Error bars represent the SEM.
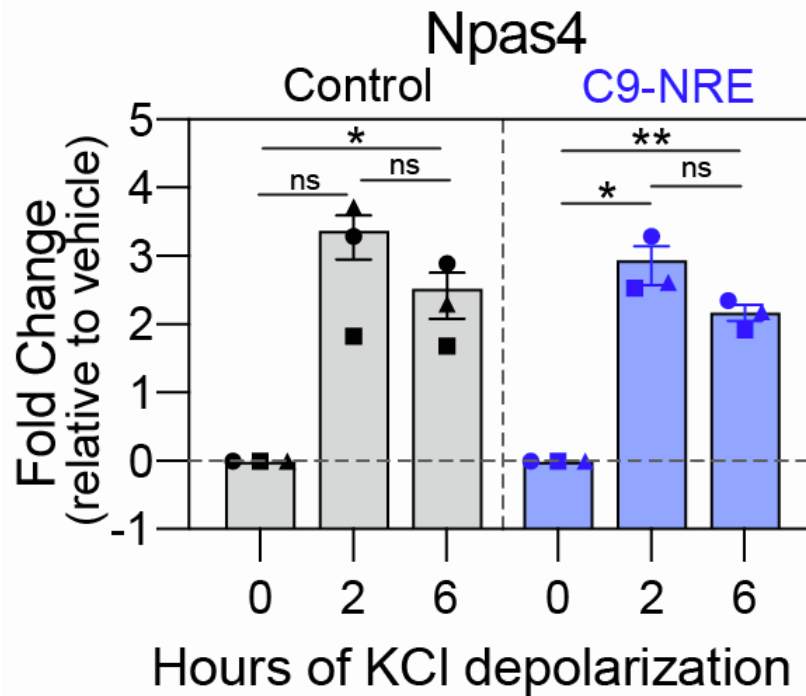
Figure S3. The immediate early gene, Npas4, is induced following depolarization in Control and C9-NRE i³Neurons, related to Figures 4 and 6. The induction of Npas4 following depolarization serves as a positive control for activation of the activity-dependent transcriptional pathway. For this experiment, n = 3 biological replicates, *m* = 1 technical replicate. Data is plotted in log scale. Each biological replicate is indicated by a unique shape. Statistical analyses in (were performed by repeated measures one-way ANOVA with Tukey's multiple comparison test on dCt values. *P < 0.05. Error bars represent the SEM.
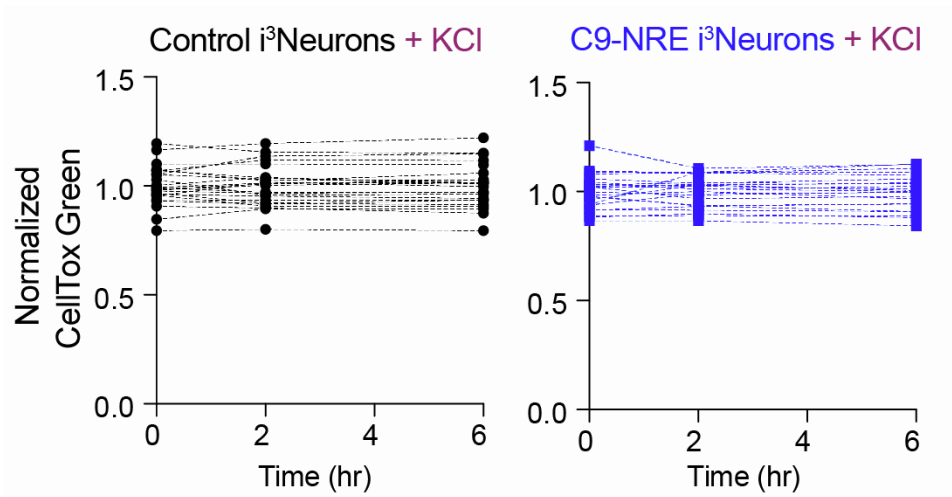
Figure S4. I³Neurons from either C9-NRE carriers or controls do not undergo membrane depolarization-induced cell death over the course of experimentation, related to Figure 6. CellTox Green, a dye that measures membrane integrity and thus serves as a proxy for cell death, was used to determine if i³Neurons were vulnerable to cell death throughout the course of membrane depolarization. There are no observed increases in fluorescence over the time course following depolarization, indicating that i³Neurons are not dying in response to membrane depolarization. For each experiment $n = 3$ biological replicates, $m = 2$ technical replicates, $o = 4$ wells per experiment.
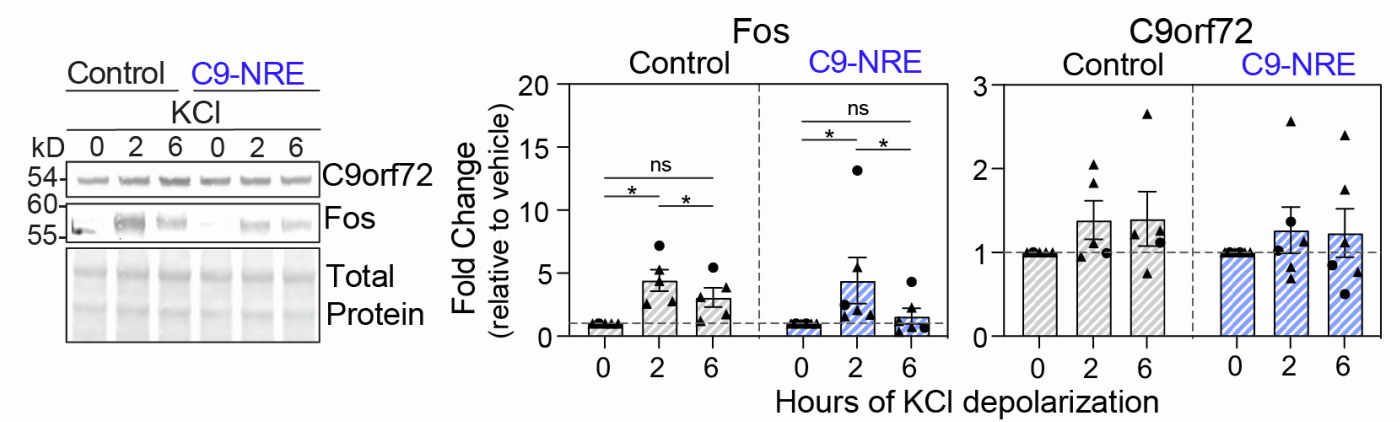
Figure S5. Levels of C9orf72 protein levels are unaltered by membrane depolarization in both Control and C9-NRE i³Neurons, related to Figure 6. Fos protein is increased in tandem with Fos transcripts; however, there is no rapid reduction of C9orf72 protein levels evaluated via western blotting. Band intensity was normalized to Total Protein stain. For each experiment $n = 2$ biological replicates, $m = 1 - 4$ technical replicates. Each biological replicate is indicated by a unique shape. Statistical analyses in (B) and (C) were performed by repeated measures one-way ANOVA with Tukey's multiple comparison test on values normalized to total protein. *P < 0.05. Error bars represent the SEM.
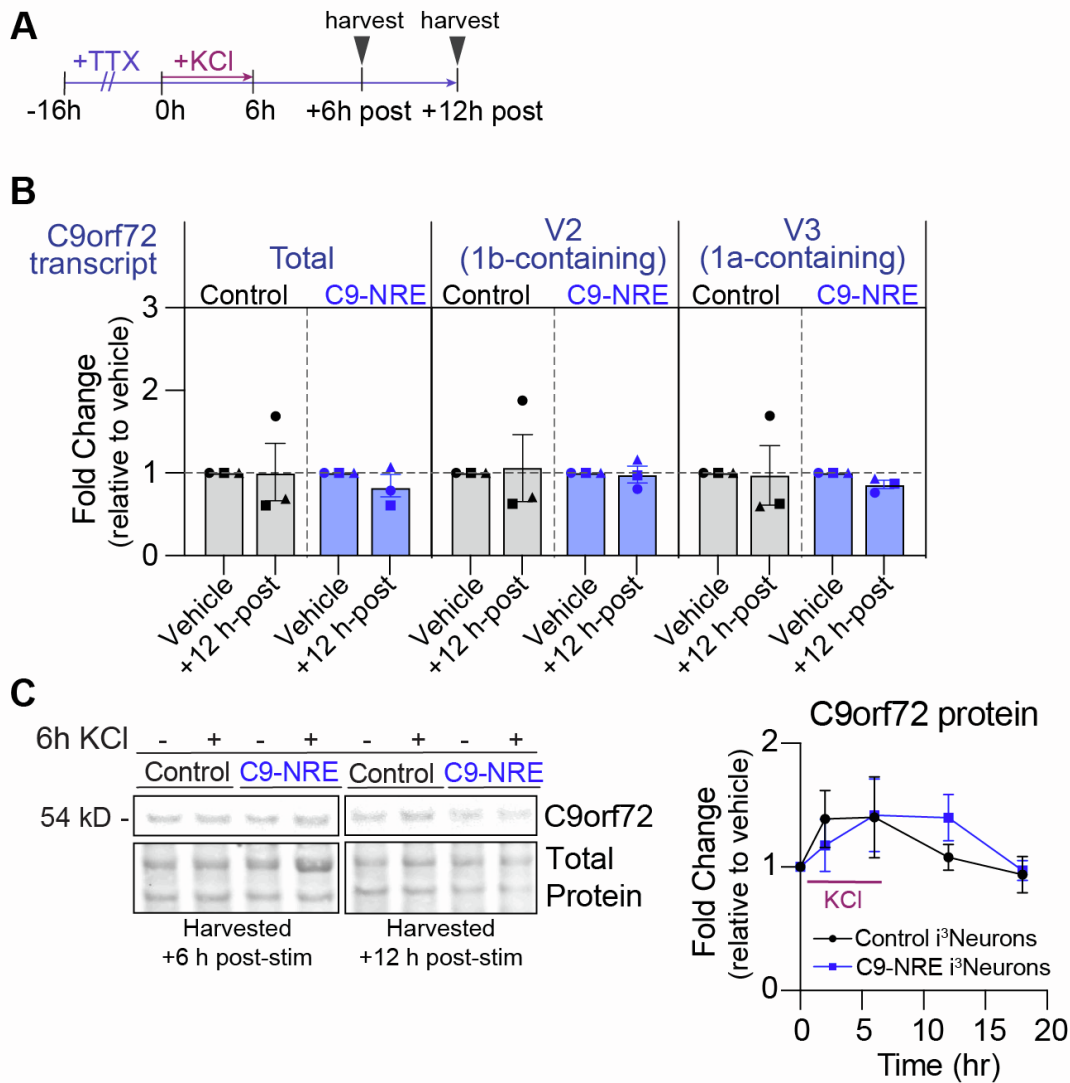
Figure S6. Levels of V3 transcripts return to baseline while C9orf72 protein levels remain unaltered after 12 hours of recovery post-depolarization in both Control and C9-NRE i³Neurons, related to Figure 6. (A) Neurons are silenced with TTX overnight (16 hours) then depolarized by KCl for 6 hours. i³Neurons are then allowed to recover for 6- or 12- hours before being harvested. **(B)** Levels of V3 transcripts return to baseline in both control and C9-NRE i³Neurons after being allowed to recover for 12 hours following depolarization. For each experiment $n = 3$ biological replicates, $m = 1$ technical replicate **(C)** Levels of C9orf72 were examined 6 hours and 12 hours post-6 hours of depolarization. There are no significant differences in levels of C9orf72 at these time points, compared to the time-matched vehicle control via unpaired t-test. For each experiment $n = 2$-3 biological replicates, $m = 1 - 4$ technical replicates. Error bars represent the SEM.

Genotype	GroupID	Age of Onset	Sex	Onset	C9RepeatSize	insert / site	source
C9orf72	ALS	49	Male	Lumbar	>44	Ngn1/2 + mCherry in CLYBL locus	Barmada lab
C9orf72	ALS	56	Male	Cervical	>25	Ngn1/2 + mCherry in CLYBL locus	Barmada lab
Control	CTRL	NA	Male	NA	normal	Ngn1/2 + iRFP in CLYBL locus	Barmada lab
Control	CTRL	NA	Female	NA	normal	Ngn1/2 + mCherry in CLYBL locus	Barmada lab
C9orf72	ALS	NS	Female	NA	NS	Ngn2 + mCherry in AAVS1 locus	Ward lab
Control	CTRL	NA	Male	NA	normal	Ngn2 in AAVS1 locus	Ward lab

Table S1. Source and demographic information for i³Neuron lines used in this study, related to Figures 4-6. NA = not applicable, NS = not specified

Name	Sequence (5' to 3')
Rat C9orf72 forward	GGTCATCCCTGTGATGGAG
Rat C9orf72 reverse	AGTACTGTATCAGCTATATCGAGG
Rat Fos forward	AGCATGGGCTCCCCTGTCA
Rat Fos reverse	GAGACCAGAGTGGGCTGCA
Rat Beta-actin forward	CTAAGGCCAACCGTGAAAAG
Rat Beta-actin reverse	TACATGGCTGGGGTGTTGA

Table S2. Primers used in rat cortical neuron qPCR experiments, related to Figure 3.

Name	Taqman Assay ID	Sequence (5' to 3')
Human C9orf72 total	Hs00376619_m1	
Human C9orf72 variant 2	AII1NCR	
Human C9orf72 variant 3	Hs00948764_m1	
Human Fos	Hs04194186_s1	
Human BDNF	Hs02718934_s1	
Human Beta-actin	Hs01060665_g1	
Human Npas4 forward		AAGGTCCGGCTGTCCTACC
Human Npas4 reverse		GCCGCTACGATGTCCTCAAG
Human beta-actin forward		CACCATTGGCAATGAGCGGTTC
Human beta-actin reverse		AGGTCTTTGCGGATGTCCACGT

Table S3. Primers used in i³Neuron qPCR experiments, related to Figures 4-6.