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Altered network properties in C9ORF72 repeat expansion cortical neurons are due to synaptic dysfunction

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Abstract

**Background:** Physiological disturbances in cortical network excitability and plasticity are established and widespread in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) patients, including those harbouring the C9ORF72 repeat expansion (C9ORF72RE) mutation – the most common genetic impairment causal to ALS and FTD. Noting that perturbations in cortical function are evidenced pre-symptomatically, and that the cortex is associated with widespread pathology, cortical dysfunction is thought to be an early driver of neurodegenerative disease progression. However, our understanding of how altered network function manifests at the cellular and molecular level is not clear.

**Methods:** To address this we have generated cortical neurons from patient-derived iPSCs harbouring C9ORF72RE mutations, as well as from their isogenic expansion-corrected controls. We have established a model of network activity in these neurons using multi-electrode array electrophysiology. We have then mechanistically examined the physiological processes underpinning network dysfunction using a combination of patch-clamp electrophysiology, immunocytochemistry, pharmacology and transcriptomic profiling.

**Results:** We find that C9ORF72RE causes elevated network burst activity, associated with enhanced synaptic input, yet lower burst duration, attributable to impaired pre-synaptic vesicle dynamics. We also show that the C9ORF72RE is associated with impaired synaptic plasticity. Moreover, RNA-seq analysis revealed dysregulated molecular pathways impacting on synaptic function. All molecular, cellular and network deficits are rescued by CRISPR/Cas9 correction of C9ORF72RE. Our study provides a mechanistic view of the early dysregulated processes that underpin cortical network dysfunction in ALS-FTD.

(Continued on next page)
Background

C9ORF72 hexanucleotide repeat expansion (C9ORF72\(^{RE}\)) is the most common mutation found within the ALS-FTD spectrum, giving rise to incurable, rapidly progressive and fatal disease pathologically characterised by degeneration of cortical neurons and upper and spinal motor neurons. Cortical circuit dysfunction is a consistent and prominent finding in C9ORF72\(^{RE}\) patients [5, 42, 50, 62]. Altered cortical network excitability is considered to be an early pathogenic driver of ALS and FTD contributing directly to excitotoxicity-mediated neurodegeneration of upper motor neurons and cortical neurons [21, 22, 36, 37, 58–60, 62]. Furthermore, clinical neurophysiological studies of C9ORF72\(^{RE}\) patients have demonstrated notable impairments in cortical network plasticity at the pre-symptomatic stage [5]. For many progressive neurodegenerative diseases, including ALS-FTD, functional impairments in plasticity are thought to manifest early in disease progression, being representative of altered synaptic homeostasis that precede and potentially cause neuronal dysfunction and/or loss, and lead to cognitive impairments [38, 55, 56].

Our current mechanistic understanding of potential sources of altered cortical network excitability in ALS-FTD is derived largely from mutant murine models (SOD1 and TDP-43 mutations) of ALS and ALS-FTD [17, 30, 45, 48, 65], but does not extend to provide a physiological basis for altered network excitability. Similarly, our understanding of the potential synaptic plasticity dysregulation that may occur in ALS-FTD has come from studies that use ex vivo brain slice preparations from rodent models of rare genetic mutations [24, 54]. Functional impairments in synaptic plasticity in ALS-FTD have yet to be examined in a human-based model system. Identified physiological disturbances in ALS motor neurons [55] also may provide insights into cortical neuron pathophysiology though this must remain highly tentative given that diverging potential pathophysiological mechanisms in C9ORF72\(^{RE}\) cortical and spinal neurons are established [52]. Despite its proposed pathogenicity and early prominence, cortical dysfunction in ALS-FTD remains poorly defined at both the synaptic and network level.

To address this, we have used human induced pluripotent stem cell (iPSC) derived cortical neurons from patients harbouring C9ORF72\(^{RE}\) mutations, combined with gene-edited isogenic paired lines [52], to interrogate the consequence of C9ORF72\(^{RE}\) on cortical neuronal physiology. In view of dysregulation of glutamate homeostasis being a major hypothesis underlying ALS-FTD [9, 55], we have examined physiological perturbations in iPSC-derived glutamatergic cortical neurons. We determine that C9ORF72\(^{RE}\) neurons display altered network properties that are underpinned by synaptic dysfunction, but not altered intrinsic cellular excitability, and display impairments in synaptic plasticity. Our transcriptomic analysis highlights dysregulated molecular pathways in accordance with physiological observations. Our observations are notably different from those previously reported for C9ORF72\(^{RE}\) motor neurons and provide evidence of cortical-specific pathophysiology that may contribute to cortical dysfunction in ALS-FTD.

Methods

iPSCs

Dermal fibroblasts from patient and control individuals were obtained under full Ethical/Institutional Review Board approval at the University of Edinburgh. Fibroblasts were reprogrammed to iPSCs by either Sendai virus or retrovirus expressing OCT4, SOX2, C-MYC, and KLF4. iPSCs were maintained in Matrigel (BD Biosciences)-coated plastic dishes in E8 medium (Life Technologies) at 37 °C and 5% CO\(_2\). Lines were derived from three patients harbouring repeat expansions in the C9ORF72 gene [52] and a healthy individual with no known association with neurodegenerative disease.

Anterior precursor (aNPC) derivation

Human iPSCs were maintained on Matrigel (Corning), with Advanced DMEM/F12, 20% Knockout Serum Replacement, FGF-2 (10 ng/mL), L-glutamine (1 mM), 2-mercaptoethanol (100 mM) and 1% penicillin/streptomycin (P/S). All media were obtained from Life Technologies. Human iPSCs were neurally converted in suspension in chemically defined medium as described [6]. The media was changed to base media (Advanced-DMEM/F12, 1% P/S, 1% Glutamax, 1% N-2), 0.5% B-27, FGF-2 (2.5 ng/mL) upon observation of radially organised structures in neurospheres (10–21 days) and plated on Laminin (Sigma) coated tissue culture plates (Nunc) a week later. Neural rosettes were mechanically isolated, dissociated with Accutase
They were permeabilised with 0.1% tritonX-100, blocked and fixed in 4% PFA at room temperature (RT) for 20 min.

Differentiation of aNPCs into cortical neuronal cultures
aNPCs were plated in default media on poly-ornithine (Sigma), laminin (Sigma), fibronectin (Sigma) and Matrigel-coated coverslips in which primary mouse astrocytes have been propagated. Primary mouse astrocytes were prepared as previously described [26]. Density of astrocytes was 100,000 per 13 mm coverslip at least 48 h prior to plating aNPCs. Cultures were fed twice a week. Default medium was supplemented with forskolin (10 μM, Tocris) from days 7–21 after aNPC plating down (200,000 per coverslip) and with BDNF and GDNF (both 5 ng/mL) from day 28 onwards. Coverslips were then processed fixed and stained as previously described [6]. Multielectrode arrays were first coated with poly-D-lysine then the laminin, fibronectin and Matrigel-coating was applied to the region containing the electrode arrays (60MEA200/30 irr-Ti, Multi Channel Systems). aNPCs were plated to the coating spot and left for 2 h to adhere. Array wells were then flooded with default media containing suspended DIV14 mouse astrocytes.

Immunohistochemistry
Five–six weeks old cultures on glass coverslips were fixed in 4% PFA at room temperature (RT) for 20 min. They were permeabilised with 0.1% tritonX-100, blocked with 6% goat serum and stained with primary antibodies against βIII-tubulin (dilution 1:500, Sigma), human nuclei (dilution 1:200, Millipore), nestin (dilution 1:200, Millipore), GFAP (1:400, Sigma), synapsin-1 (dilution 1:500, Sigma) and PSD-95 (dilution 1:250; Neuromab) sequentially for 2 h at RT. These were then probed with appropriate secondary antibodies and mounted with FluorSave and imaged in Zeiss LSM Z10 confocal microscope using 20X objective. For synaptic density analysis, 5 fields of 20 μm region across 3 coverslips were analysed for the co-localised puncta of synapsin-1 and PSD-95 using colocalization plugin in ImageJ.

RNA extraction, RNA sequencing and transcriptomic analysis
Total RNA was extracted from cortical neurons from 2 independent isogenic corrected paired cell lines at day 35 post platedown using RNeasy Mini kit (Qiagen), according to the manufacturer’s instructions. RNA samples were assessed for concentration (NanoDrop ND-100 Spectrometer, NanoDrop Technologies) and quality (Agilent 2200 Tapestation, Agilent Technologies) before library preparation. Library preparation and sequencing were carried out by Edinburgh Genomics (Edinburgh, UK). For each sample, cDNA was converted to a sequencing library using the TruSeq stranded mRNA-seq library. Barcoded libraries were pooled and sequenced on an Illumina HiSeq 4000 using 75 base paired-end reads to generate at least 111 million raw reads per sample. The reads were mapped to the primary assembly of the human (hg38) reference genome contained in Ensembl release 90 [12]. Alignment was performed with STAR, version 2.5.3a [16]. Tables of per-gene read counts were generated from the mapped reads with featureCounts version 1.5.2 [33]. Differential gene expression analysis, using DESeq2 version 1.18.1, specifically examined the intersection in commonly and concordantly differentially expressed genes between the two mutant-isogenic pairs, using a false discovery rate of 20%, achieved by setting a Benjamini-Hochberg corrected p-value threshold of 0.2 (genes with an average FPKM < 1 were disregarded). Gene ontology (GO) analysis was performed on all the differentially expressed genes to identify putatively altered pathways or processes using topGO version 2.30.1 [1]. RNA-seq data are available upon request to the corresponding authors.

Morphology
Cortical NPCs were sparsely transduced with lentivirus expressing GFP in order to label individual cells for analysis (ca. 1 viral particle to 10 cells). Following labelling with GFP, NPCs were differentiated as mentioned above and immunohistochemistry was performed against GFP and β3-tubulin. These were then probed with appropriate secondary antibodies and mounted with FluorSave and imaged in Zeiss LSM Z10 confocal microscope using 20X objective. Total neurite length (sum of all the processes) in the GFP channel was manually traced using ImageJ.

Multi-electrode array (MEA) electrophysiology
Extracellular recordings from 59 channels per array were acquired at 37 °C in the culture media using a Multichannel Systems MEA system at a sampling rate of 20 kHz. Data was analysed using the Multichannel Systems software and in-house custom Matlab scripts.

Patch-clamp electrophysiology
For other electrophysiological experiments, whole-cell patch-clamp recordings were performed as described [6, 34] using electrodes filled with (in mM): 155 K-gluconate, 2 MgCl₂, 10 Na-HEPES, 10 Na-PiCreatine, 2 Mg₂-ATP, and 0.3 Na₃-GTP, pH 7.3, 300 mOsm. For spontaneous action potential activity, cells were typically bathed in an extracellular recording comprising (in mM): 152 NaCl, 2.8 KCl, 10
HEPES, 2 CaCl$_2$, 10 glucose, pH 7.3, 320–330 mOsm. For mEPSC recordings, the extracellular solution was supplemented with TTX (1 nM), picrotoxin (50 μM) and MgCl$_2$ (1.5 mM). For intrinsic membrane and excitability properties, the extracellular solution was supplemented with CNQX (5 μM) and D-APV (50 μM). Recordings were performed at room temperature (20-23 °C). Current and voltage measurements were typically low-pass filtered online at 2 kHz, digitized at 10 kHz and recorded to computer using the WinEDR V2.76 Electrophysiology Data Recorder (J. Dempster, Department of Physiology and Pharmacology, University of Strathclyde, UK; www.strath.ac.uk/Departments/PhysPharm/). Series resistance compensation was applied up to 75%. Recordings were omitted from analysis if the series resistance changed by more than 20% during the experiment, or if they exceeded 20 MΩ.

### Burst analysis

Burst detection for both single cells and MEAs were performed using custom-written Matlab scripts. For patch-clamp recordings action potentials were identified using threshold detection (routinely set at −10 mV) and bursts were defined as groups of action potentials with a minimum inter burst period set as log10 of the intra spike interval. For each MEA, 4–10 active channels were selected for further analysis. Bursts were identified as activity 2–5 times the standard deviation of the baseline (as determined by the signal-to-noise ratio) with a minimum quiet period set to define separate bursts. This was routinely set to 5 s given the lowest observed inter burst period was 9.6 s. The spike threshold was variable, but consistent between each of our isogenic and C9 pairs for each experiment and optimal to detect as many, but variable, number of channels with robust activity. On all MEAs there were both active and inactive channels (likely due to some electrodes not having active cells close enough) but all the active channels showed the same pattern of activity with low standard deviation in the burst start times across channels ranging from 0.10 to 0.78 s, indicating a synchronous network across the area of the MEA electrodes.

### mEPSC analysis

mEPSC recordings were analysed offline using the WinEDR software stated above. A dead time window of 10 ms was set and individual mEPSCs were detected using an algorithm that selected for mEPSCs below a – 4 to – 6 pA amplitude threshold and greater than 1 ms in duration. mEPSCs that had a monotonic rising phase with a 10–90 rise time of lower than 6 ms and a T-decay with a decay time constant of lower than 25 ms were selected for analysis. Recordings were then visually inspected for validity. For mEPSC analysis (Fig. 2c), data were obtained from at least 2-min recordings and neurons that displayed mEPSC frequencies under 0.05 Hz were omitted from the analysis. For sucrose experiments, baseline mEPSC properties were determined from a 2-min stretch of mEPSC activity of at least 0.05 Hz. The transient phase was determined from the onset of sucrose application to the transition of mEPSC activity to steady-state activity. Steady-state data were determined from at least a 30 s stretch of mEPSC activity in continued presence of sucrose.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Data are represented as mean ± s.e.m. *p < 0.05, **p < 0.01, ***p < 0.001. The number of experimental replicates (for MEA recordings, this indicates number of plates; for patch-clamp recordings this indicates number of cells) is denoted as n and N represents the number of independent de novo preparations of batches from which n is obtained. Data were initially determined to be parametric or non-parametric before applying either one-way ANOVA with Bonferroni’s multiple comparisons test or unpaired t-tests or Welch’s t-test, as appropriate.

### Results

**C9ORF72$^{RE}$-derived cortical neurons display network dysfunction**

iPSCs from three patients harbouring C9ORF72$^{RE}$ mutations (C9–1,2,3), three paired isogenic control lines (C9–Δ1,2,3) in which the C9ORF72$^{RE}$ mutation had been selectively excised by CRISPR/Cas9-mediated gene-editing [52], and an unrelated healthy individual (Con) were used to generate cultures of excitatory cortical neurons using an established protocol [6, 52]. Cortical neurons were maintained in co-culture with primary mouse astrocytes in order to promote neuronal maturation [35] and experiments were performed at 4-to-6 weeks post differentiation. Cultures efficiently differentiated into enriched populations of neurons by this time point (Supplementary Figure 1) and presented a marker profile consistent with a glutamatergic cortical neuron identity (Supplementary Figure 2).

To examine network dysfunction in C9ORF72$^{RE}$ excitatory cortical neurons we initially used a multi-electrode array (MEA) recording platform. C9ORF72$^{RE}$ excitatory cortical neurons displayed an increase in the rate of burst firing (reduced network burst duration and inter-burst lengths versus healthy and isogenic controls) (Fig. 1a). Although shorter inter-burst lengths are typically associated with increased glutamate-mediated excitatory network activity, the reduced network burst duration in C9ORF72$^{RE}$ cortical neurons (Fig. 1a) is inconsistent with this [3, 43]. Intra-burst spike frequency was comparable across all lines (Fig. 1a) suggesting that intrinsic excitability is unaffected. We then examined whether spontaneous...
network activity was maintained at the single-cell level using whole-cell patch-clamp recording. Burst activity from C9ORF72RE neurons also showed a similarly reduced inter-burst length and burst duration and no change in intra-burst spike frequency versus controls (Fig. 1b). Spontaneous burst firing was blocked by CNQX and was not affected by bicuculline consistent with an enriched population of excitatory glutamatergic neurons and the absence of GABA-ergic interneurons (Supplementary Figure 3 [6, 34, 52]). Our data show that network activity is altered in C9ORF72RE cortical neurons.

A possible determinant of altered network excitability may be due to differences in the intrinsic membrane properties and intrinsic capacity of C9ORF72RE cortical neurons to fire and maintain action potentials. Previously, our work and others has shown such properties to be altered in C9ORF72RE motor neurons [14, 49, 61, 66], but remains to be described in the context of C9ORF72RE cortical neurons. Our network analysis suggests no difference in intrinsic excitability, so we therefore directly examined these intrinsic membrane potentials and recording temperatures at which experiments are conducted and/or the alternate plating order of neurons and astrocytes for each respective approach. Significance determined by one-way ANOVA with Bonferroni’s post hoc test.
C9ORF72<sup>RE</sup>-derived cortical neurons have increased synaptic input

To determine whether the altered network activity in C9ORF72<sup>RE</sup>-derived cortical neurons was of synaptic origin we undertook quantification of the co-localisation of pre- (synapsin-1) and post-synaptic (PSD-95) markers and observed an increase in synaptic densities on C9ORF72<sup>RE</sup> versus control neurons (Fig. 2a, Supplementary Figure 6A). Increased synaptic density may reflect an increase in neuronal morphology and therefore we examined the neurite length in our cultures (Supplementary Figure 6B, C). However, consistent with our whole-cell capacitance measurements, our data did not reveal any morphological changes that could account for the large change in synaptic density in C9ORF72<sup>RE</sup> neurons. Consistent with these data we also found an increase in the frequency of mini excitatory postsynaptic currents (mEPSCs) in C9ORF72<sup>RE</sup> neurons compared to isogenic controls and healthy volunteer-derived neurons (Fig. 2c, d, Supplementary Figure 7). AMPAR-mediated mEPSC amplitudes and kinetics (rise-times and decay-times), and further the expression of AMPA receptor subunits, were not changed indicating that the properties and expression of synaptic AMPA receptors were not altered (Fig. 2c, d, Supplementary Figure 7). These findings indicate that C9ORF72<sup>RE</sup> cortical neurons display increased synaptic density resulting in elevated synaptic input.

C9ORF72<sup>RE</sup>-derived cortical neurons exhibit pre-synaptic dysfunction

Increased pre-synaptic glutamate release could also underlie an increased mEPSC frequency and a central determinant of release properties and network burst properties is the size of the vesicle readily releasable pool (RRP) [10, 32]. The RRP size was functionally estimated using hypertonic sucrose, an established method to generate Ca<sup>2+</sup>-independent exocytosis of the vesicular RRP [40, 47]. We observed sucrose-application-evoked mEPSC activity that can be classified into an initial transient phase (initial depletion of the RRP) and a steady-state phase (on-going replenishment of the RRP; Fig. 2e and Supplementary Figure 8A [40]). Given that conventional measurements of RRP are determined by the integral of the total evoked current and are directly proportional to synaptic density we have examined the fold change in mEPSC frequencies because C9ORF72<sup>RE</sup> cortical neurons exhibit increased synaptogenesis over control lines. A fold reduction in mEPSC frequency (Fig. 2f, Supplementary Figure 8B), but not their amplitude (Supplementary Figure 8C), was observed in C9ORF72<sup>RE</sup> neurons compared to control lines for both the transient and steady-state phases. Thus, despite an observed increase in mEPSC frequency during baseline recordings, C9ORF72<sup>RE</sup> cortical neurons display a functionally reduced RRP size that is replenished at a slower rate. These data potentially explain the shorter network burst durations in C9ORF72<sup>RE</sup> cortical neurons.

Physiological vesicular release is Ca<sup>2+</sup>-dependent and thus we examined this by measuring fold changes in mEPSC frequencies before and after addition of KCl. No differences in the fold change of mEPSC frequencies were found between C9ORF72<sup>RE</sup> versus paired isogenic control neurons (Supplementary Figure 9). These data suggest that overall depolarisation-mediated Ca<sup>2+</sup>-evoked release is equivalent in the pre-synaptic terminals of each line. Nonetheless our data indicate that the RRP size is impacted in C9ORF72<sup>RE</sup> cortical neurons.

Synaptic potentiation in C9ORF72<sup>RE</sup> cortical neurons is impaired

Cortical network plasticity in C9ORF72<sup>RE</sup> patients is impaired and suggests that this is due to perturbed activity-dependent synaptic plasticity [5]. The activity-dependent potentiation of AMPA receptor-mediated mEPSCs is a central feature of classical models of synaptic plasticity and therefore we applied a series of depolarization pulse protocols (DPP) to our neurons, as previously described, that leads to a potentiation of the amplitude of mEPSC in rodent hippocampal neurons [4, 29, 31, 63, 64]. Following this depolarisation burst protocol (DPP), we observed that mEPSCs in control neurons were transiently potentiated by around 20% in amplitude from the initial control period before returning close to control levels (Supplementary Figure 10). No significant shift in mEPSC amplitude was observed when no stimulation was applied. Consistent with previous studies on primary hippocampal neurons [4], we found this mEPSC potentiation to be dependent upon the activation of voltage-gated Ca<sup>2+</sup> channels and intracellular elevations in Ca<sup>2+</sup> as was blocked by voltage-gated Ca<sup>2+</sup> channel antagonist, nifedipine, and supplementation of Ca<sup>2+</sup> chelator BAPTA to the patch pipette, respectively (Supplementary Figure 10E, Supplementary Figure 11A-F). In contrast, we observed that DPP did not induce any potentiation of mEPSC amplitudes in C9–1 and C9–2 neurons (Fig. 3a, c, e-g). The respective isogenic C9–1Δ and C9–2Δ neurons displayed a significant mEPSC potentiation post-DPP (Fig. 3b, d-g). The mEPSC decay time constant did not change post-DPP with respect to baseline activity in either control, C9 or C9–Δ neurons suggesting that the composition of AMPA receptors mediating mEPSCs is unchanged post-DPP (Fig. 3f; Supplementary Figure 11G). These data are therefore consistent with a C9ORF72<sup>RE</sup>-mediated physiological disruption of mEPSC potentiation in C9ORF72<sup>RE</sup>-derived cortical neurons in our model of synaptic potentiation.
RNA sequencing highlights molecular disruption at the synapse in C9ORF72RE cortical neurons

To begin to understand the molecular changes that underpin the observed physiological dysfunction in C9ORF72RE cortical neurons, we next performed transcriptomic analysis of cortical neurons derived from two independent C9ORF72RE iPSCs (C9−1 & C9−2) and their corresponding isogenic controls (C9−Δ1 & C9−Δ2). Principal component analysis (PCA) of gene expression showed segregation of differential gene expression between the two mutant-isogenic pairs, though a high degree of similarity within a mutant-isogenic pair, as expected (Fig. 4a). We therefore assessed our data set in order to determine common dysregulated gene expression between the different lines employed (Fig. 4b, Supplementary Table 1). Our biological process gene ontology analyses (Fig. 4c) revealed dysregulated expression of genes involved in vesicle regulation (Gopc, Vamps5), cell-cell adhesion (Chln1, Poddhc4), negative regulation of ion transport (Htr2a) fatty acid metabolism and regulation of DNA-binding transcription factor activity (Irk1, Sigirr). These novel transcriptomic data reveal dysregulated multiple pathways in C9ORF72RE cortical neurons that may contribute to the observed synaptic dysfunction.

Discussion

Increased synaptic glutamate transmission within the cortex presents an attractive hypothesis to potentially explain cortical network hyperexcitability present in early symptomatic C9ORF72RE patients [42, 50, 62] and ALS in general [22]. Our data provide a human in vitro mechanistic exploration of physiological impairments in C9ORF72RE patient-derived excitatory cortical neurons that reveal that perturbed network activity is underpinned by functional synaptic alterations that impact upon excitability. Furthermore, noting that ALS-FTD patients exhibit impairments in network plasticity, we have determined that C9ORF72RE cortical neurons exhibit impairments in synaptic plasticity. Importantly, the physiological alterations observed in iPSC-derived C9ORF72RE cortical neurons are disparate from that previously observed in iPSC-derived C9ORF72RE motor neurons where intrinsic excitability appears to be primarily affected [55]. Our data reveal that intrinsic excitability is not affected in C9ORF72RE cortical neurons.

An increase in network burst frequency in C9ORF72RE patient-derived excitatory cortical neurons is highly consistent with a mechanism requiring increased excitatory input. Our data demonstrate that C9ORF72RE cortical neurons display an increased synaptic input as a result of an increased synaptic density. Such findings are broadly consistent with murine models of ALS, where increased synaptic input of excitatory cortical neurons are observed in pre-symptomatic mutant TDP-43 mice ([17]; but see [25]) and SODG93A mice [17, 48, 57]. Cortical neurophysiological impairments were not found in a C9ORF72RE murine model though this model does not display classical ALS-FTD pathology or neurodegeneration [44]. Our transcriptomic approach has revealed potential causes to this increase in synaptic density. PCDHG4, a γ-protocadherin, negatively regulates the function of neuroligin-1, a post-synaptic molecule that interacts with pre-synaptic neurexin to maintain and promote synapse structures in forebrain neurons [39]. Reduced expression of PCDHG4 in our C9ORF72RE cortical neurons is therefore compatible with increased neuroligin-1 function and increased synaptic density. Overexpression of neuroligin-1 has previously been shown to increase excitatory synaptic activity in in vitro cortical neurons [8]. Equivalently, CBLN1, is a pre-synaptically expressed molecule that interacts with neurexins and promotes synaptogenesis [51] and is upregulated in C9ORF72RE cortical neurons. Contrastingly, CBLN1 has been reported to be downregulated in C9ORF72RE iPSC-derived motor neurons [49]. Together,
Fig. 3 (See legend on next page.)
these studies indicate that increased cortical glutamate-mediated synaptic input is an early feature of ALS. Future work will require to determine when increased synaptic density alongside altered network excitability presents in ALS progression in patients.

Many ALS-focused studies describing altered glutamatergic input have examined synaptic function without assessing presynaptic function in detail, nor have they examined the consequences for network activity. An increase in excitatory synaptic input might be expected to increase network burst duration in addition to burst frequency [32]. However, our assessment of network activity revealed a decrease in network burst duration and appears to be consistent with a decrease in glutamatergic synaptic transmission. Consistent with this, our evaluation of pre-synaptic function revealed an estimated reduced size and replenishment of vesicular RRP. Importantly, a reduced RRP size and replenishment rate has been previously shown to generate early burst termination to shorten burst duration [10, 32]. This provides the most parsimonious explanation of the observed shorter network burst duration in C9orf72RE cortical neurons. A disruption in the vesicular RRP suggests mechanisms in which synaptic vesicular trafficking are impaired. Noting that C9orf72 is detected in pre-synaptic terminals, our data resonate with previous studies highlighting the role of C9orf72 protein in vesicular trafficking within the trans-Golgi network and endosomal signalling and suggest that C9orf72 haploinsufficiency may result in a reduced RRP [2, 19, 53]. Our transcriptomic data provide further evidence of dysregulated genes associated with impaired vesicular trans-Golgi network and endosomal signalling in ALS, consistent with a growing body of evidence of impaired vesicular trafficking in ALS that may impact on the RRP size [11, 15, 46]. For example, our data set highlights an upregulation of the GOPC gene, a chaperone protein that is expressed across the trans-Golgi network and endosomes. Amongst many interactions, GOPC is associated with syntaxin-6 that regulates endosomal vesicular transport [7]. Furthermore, TDP-43 protein appears to bind GOPC RNA [41]. Collectively, our data show a reduction in the RRP that is consistent with impairments in vesicular trafficking.

Importantly, vesicular release is typically stimulated via \( Ca^{2+} \)-dependent mechanisms. Despite a reduced RRP, our evaluation of depolarisation/\( Ca^{2+} \)-dependent vesicular release appears to be equivalent in C9orf72RE excitatory cortical neurons versus isogenic controls. Indeed, our afterhyperpolarisation (AHP) data suggest that calcium mediated influx is not impacted to influence intrinsic excitability properties. However, we must remain cautious that AHP and exocytosis could be independently calcium regulated processes in our cells, subject to localised intracellular calcium regulation. One potential explanation could be that localised \( Ca^{2+} \)-dependent mechanisms controlling vesicular release in C9orf72RE excitatory cortical neurons are enhanced over control lines to generate the higher release probability required to elevate the fold increase in mEPSC frequency to comparable levels to the control lines. Dysregulated cytoplasmic \( Ca^{2+} \) levels in C9orf72RE-derived motor neurons have been previously reported [13, 28] and this elevation in \( Ca^{2+} \) levels may contribute to an increased release probability. However, our findings contrast with those of Jensen et al. [28] who suggest that KCl-stimulated release is impaired, due to a GA-driven loss of the protein SV2, in C9orf72RE patient-derived cortical neurons.

Our data show that synaptic potentiation in C9orf72RE excitatory cortical neurons is impaired. Notably, transcranial magnetic stimulation-based studies show both presymptomatic and post-symptomatic C9orf72RE patients exhibit an abolishment of activity-dependent cortical network plasticity [5]. Together, these data suggest impairments in functional synaptic plasticity may emerge as an early pathophysiological event in C9orf72RE-mediated disease progression to impair network plasticity. The pathological determinants of the impairments in synaptic plasticity remain unknown in C9orf72RE cortical neurons, though a very recent study has shown synaptic plasticity impairments in murine C9orf72 knock out animals [27], suggesting a role for the
Fig. 4 Transcripottic analysis of C9ORF72RE cortical neurons. a Principal component analysis of gene expression derived from RNA sequencing from C9ORF72RE cortical neurons (C9-1, C9-2; black) and isogenic gene-corrected cortical neurons (C9-1Δ, C9-2Δ; red). Each data point represents a de novo differentiation of cortical neurons. As highlighted, the isogenic controls cluster accordingly with their respective parental C9ORF72RE lines. b Scatter plot showing comparison of gene expression (as average Fragments Per Kilobase of transcript per Million mapped reads) from cortical neurons derived from two independent C9ORF72RE iPSC lines and their corresponding isogenic controls. Green and orange data points denote the overlap of significantly up- and down-regulated genes in both mutant-correction pairs, respectively (false discovery rate, p < 0.2). c Selected gene ontology terms enriched in dysregulated genes.
C9ORF72 protein in plasticity mechanisms. In addition to our own data set indicating impact upon synaptic physiology, data sets highlight that altered gene expression in C9ORF72 RE patient tissue [46] and disrupted cell signalling pathways in iPSC-derived C9ORF72 RE neurons [13] are implicated in synaptic plasticity. Furthermore, recent transcriptomic work has associated the expression of di-peptide repeat proteins with a reduction in expression of a mediator of synaptic plasticity [15]. Our data therefore firmly determines synaptic plasticity impairments are present in human C9ORF72 RE cortical neurons.

Crucially, C9ORF72 RE mutations are causal to both ALS and FTD. In this regard, we note that the vast majority of clinical pathophysiological measurements describing hyperexcitability are made from the motor cortex, which is primarily affected in ALS [22]. Nonetheless cortical hyperexcitability is evidenced in rodent models of FTD [20]. We acknowledge that our data may have preferential relevance for FTD over ALS, or vice versa. This is likely to become more apparent with increased pathophysiological characterisation of C9ORF72 RE ALS-FTD patients. Further, early identification of pre-symptomatic individuals and longitudinal stratification of these observations will allow us to place further confidence upon the pathophysiological staging that our observations are most likely to mirror. Our data represent one in vitro time point, but as discussed, closely align with aspects of presymptomatic cortical neuron impairments evidenced in rodent models of ALS and ALS-FTD. Importantly, the network excitability is not investigated in these models. Our data suggest that increases in synaptic transmission may lead to increased homeostatic network activity adaptation, including im paired synaptic plasticity, is an early feature and precedes that of pathophysiological network failure likely resulting in the manifestation of the clinically observed network hyperexcitability [18]. In this regard, there are intriguing similarities in our data set to cortical neurons derived from Alzheimer's patient iPSCs that display increased synaptic activity at the same time point [23].

Conclusions

In summary, our study provides physiological evidence supporting the involvement of widespread glutamatergic synaptic dysfunction as a potential pathogenic mechanism of C9ORF72 RE-mediated disease, a disease that has considerable impact in cortical neurons in addition to motor neurons. We reveal that pre and post-synaptic defects are highly prominent in cortical neurons in ALS-FTD and are suggested to combine to generate early network excitability alterations and impaired synaptic plasticity. We note these are very different physiological perturbations previously reported for motor neurons derived from C9ORF72 RE patients and therefore this study shows that the pathophysiological processes in different brain regions are likely to show divergence. These early synaptic defects are likely linked to core mechanisms of neurodegenerative disease progression and symptoms in ALS-FTD.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13024-021-00433-8.

Additional file 1: Supplementary Figure 1. Neuronal specification. Example images of immunostaining against neuronal precursor marker nestin (A) and human nuclei (B) with neuronal marker βIII-tubulin and astrocyte marker GFAP. Our cultures at week 5 post-differentiation generate dense human nuclei-positive neuronal populations (mean ± sem % human nuclei+ cells with βIII-tubulin; C9–1, 96.8 ± 4.1; C9–Δ, 97.4 ± 3.0; C9–2, 90.4 ± 3.2; C9–3, 90.2 ± 4.4; C9–3, 98.5 ± 4.9; C9–Δ, 96.4 ± 7.1; data from 3 de novo plate downs) with only negligible detectable levels of nestin (mean ± sem % nestin; C9–1, 1.1 ± 0.01; C9–Δ, 2.5 ± 0.1; C9–2, 2.3 ± 0.1; C9–2Δ, 3.5 ± 0.1; C9–3, 1.0 ± 0.1; C9–Δ, 2.0 ± 0.1; data from 3 de novo plate downs). Data are consistent with previous cortical neuron differentiations with cell lines used in this study and a cortical neuron protocol that gives rise to a highly efficient neuronal differentiation [6, 34, 52]. Scale bars, 100 μm.

Additional file 2: Supplementary Figure 2. A, B and C. Neuronal specification. RNA-seq analysis of established neuronal and glia markers (MAPT, NEFL, ALDH1L1, A04, MYRF), neuronal markers for anterior/cortical development (OTX2, PAX6, FOXP4, BCL6), hindbrain development (HOXB2), cortical layers (CUX1, POUSF2, PCP4, FOXP2), plus glutamatergic (CAMK2A, SLC17A6 and SLC17A7) and GABA-ergic neurons (GAD2, SLC32A1, PVALB) in C9 and C9Δ lines. We note that out analysis obtained extensive detection of cortical transcripts from our cultures and are consistent with predominantly glutamatergic neurons. Note that the y axis is presented using a logarithmic scale. Data in C also show synaptic markers DLG4 and SYN1. Data are representative of mean ± sem from two pooled C9 lines (black bars) and their respective isogenic lines (red bars), as further detailed in Fig. 4. Data were derived from 3 plate downs from each line.

Additional file 3: Supplementary Figure 3. Pharmacological block of network activity. A. Example whole cell current-clamp recordings of effect of AMPA receptor blocker, CNQX (30 μM) upon network activity. Scale bar, 50 mV, 20 s. CNQX generated full block of network burst activity. B. As in A though for GABA A receptor blocker, bicuculline (30 μM). Scale bar, 50 mV, 5 s. C. Mean (± s.e.m) percentage shift in burst frequency in presence of either CNQX or bicuculline for each line type (CNQX – Con, n = 5, N = 3; C9, n = 5; N = 2; C9Δ, n = 5; N = 3 / bicuculline – Con, n = 5, N = 3; C9, n = 5; N = 3; C9Δ, n = 5; N = 3). Bicuculline did not significantly impact upon network burst activity. Expectedly, data are consistent with an enriched population of excitatory glutamatergic cortical neurons [6, 34, 52].

Additional file 4: Supplementary Figure 4. Network burst data. A. Mean (± s.e.m) MEA-determined burst duration, interburst length and spike frequency within the burst for each respective C9ORF72 RE and C9ORF72 RE Δ isogenic pair (C9–1, N = 4; C9–Δ, N = 6; C9–2, N = 6; C9–2Δ, N = 3). Significance determined by unpaired t-test. B. Mean (± s.e.m) patch-clamp-determined burst duration, interburst length and spike frequency.
Additional file 5: Supplementary Figure 5. Intrinsic excitability of C9ORF72Δ-deleted cortical neurons. A, Mean (± s.e.m.) data for each Control (Con), C9ORF72Δ, (C9) and respective C9ORF72Δ gene-edited (C9-Δ) deprived neurons for passive membrane properties (Con, n = 6; N = 2; C9, n = 10; N = 3; C9-Δ, n = 8; N = 2; C9-2Δ, n = 5; N = 2). Significance determined by unpaired t-test.

Additional file 9: Supplementary Figure 9. KCl-evoked release properties. A, Sample tracés from recordings of mEPSC events before and in the presence of KCl (30 mM) from C9ORF72Δ- and C9ORF72Δ-Δ-cortical neurons (C9-2 and C9-2Δ). Scale bars; 50 pA, 5 s. B, Mean ± s.e.m. fold change in mEPSC frequency for each line in the presence of KCl (C9-1, n = 10; C9-2, n = 8; N = 3; C9-2Δ, n = 6; N = 2; C9-3, n = 8; N = 3; C9-3Δ, n = 6; N = 3). Significance determined by unpaired t-test. E, Cumulative probability plots showing the shift (p < 0.001, Kolmogorov-Smirnov test) in mEPSC amplitude data in B from the initial control period (1 light blue) to the 10 min post-DPP period (2 blue) in which there is consistent, transient potentiation of mEPSC amplitude. D, Left, mean mEPSCs for data shown in B for initial control period (1. light blue) and 10 min post-DPP (2 blue). Scale bar; 5 pA, 2.5 s. Right, mean mEPSCs scaled to amplitude and time base. E, To test whether potentiation was Ca2+-dependent we performed DPP in the presence of nifedipine, a blocker of voltage-gated Ca2+ channels, or BAPTA, a Ca2+ chelator, supplemented to the patch pipette. Data shows mean ± s.e.m. fold increase of mEPSC amplitude 10 min post-DPP from initial control period for the control line (n = 17), + nifedipine (n = 14) and + BAPTA (n = 6). Mean traces and data presented in Supplementary Figure 11. Significance determined by one-way ANOVA with Bonferroni’s post hoc test.

Additional file 11: Supplementary Figure 11. Ca2+-dependent mEPSC potentiation. A, Example recordings of mEPSCs prior (1. light blue) and after (2. blue) the depolarisation pulse protocol (DPP, 10 depolarising pulses of 3 s in duration, every 9 s, from −8 to +16 mV). Example post-DPP mEPSCs are sampled from the 8–10 min stretch of data. Scale bar; 10 pA, 2.5 s. B, Individual mEPSC amplitude plots for DPP experiments in the presence of nifedipine. mEPSCs before (1) and after (2) are represented in light blue and blue, respectively. The grey bar indicates the stimulation period. Note the transient increase in mEPSC amplitude post-DPP. C, Cumulative probability plot showing a shift (p < 0.001, Kolmogorov-Smirnov test) in mEPSC amplitude data in B from the initial control period (1 light blue) to the 10 min post-DPP period (2 blue) in which there is consistent, transient potentiation of mEPSC amplitude. D, Left, mean mEPSCs for data shown in B for initial control period (1. light blue) and 10 min post-DPP (2 blue). Scale bar; 5 pA, 5 s. Right, mean mEPSCs scaled to amplitude and time base. E, To test whether potentiation was Ca2+-dependent we performed DPP in the presence of nifedipine, a blocker of voltage-gated Ca2+ channels, or BAPTA, a Ca2+ chelator, supplemented to the patch pipette. Data shows mean ± s.e.m. fold increase of mEPSC amplitude 10 min post-DPP from initial control period for the control line (n = 17), + nifedipine (n = 14) and + BAPTA (n = 6). Mean traces and data presented in Supplementary Figure 11. Significance determined by one-way ANOVA with Bonferroni’s post hoc test.
Abbreviations

ALS: Amyotrophic lateral sclerosis; AMPA: α-(α-amino-3-hydroxy-5-methyl-4-isoxazolopropanionic acid); ANOVA: Analysis of variance; APNC: anterior neural precursors; C5ORF72; C5ORF72 repeat expansion; CNQX: Cyanouaoline (6-cyano-7-nitroquinoxaline-2,3-dione); DPP: Depolarisation pulse protocol; FTD: Frontotemporal dementia; GABA: Gamma aminobutyric acid; GO: Gene ontology; iPS: induced pluripotent stem cell; MEA: Multi-electrode array; mEPSC: mini excitatory post-synaptic current; RNA: Ribonucleic acid; RRP: Readily releasable pool; RT: Room temperature; s.e.m.: standard error of the mean

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Authors’ contributions

Conceptualization, EMP, KB, SC and MRL; Methodology, EMP, KB, PB, ARM, OD, CMH, BTS, DS, JN, GEH, TGH, DJAW, SC and MRL; Investigation, EMP, KB, PB, ARM, OD, BTS, CMH, TGH, and MRL; Writing – Original Draft, EMP, KB, PB, ARM, BTS, CMH, GEH, TGH, DJAW, SC and MRL; Writing – Review & Editing, EMP, KB, ARM, OD, BTS, CMH, TGH, GEH, DJAW, SC and MRL; Funding Acquisition, DJAW, GEH, TGH, SC and MRL; Resources, GEH, TGH, DJAW, SC; Supervision, SC and MRL. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Dermal fibroblasts from patient and control individuals were obtained under full Ethical/Institutional Review Board approval at the University of Edinburgh.

Consent for publication

Not applicable.

Competing interests

None declared.

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