Mutations in C9ORF72 are the most common cause of familial amyotrophic lateral sclerosis (ALS). Here, through a combination of RNA-Seq and electrophysiological studies on induced pluripotent stem cell (iPSC)-derived motor neurons (MNs), we show that increased expression of GluA1 AMPA receptor (AMPAJ subunit occurs in MNs with C9ORF72 mutations that leads to increased Ca\(^{2+}\)-permeable AMPAR expression and results in enhanced selective MN vulnerability to excitotoxicity. These deficits are not found in iPSC-derived cortical neurons and are abolished by CRISPR/Cas9-mediated correction of the C9ORF72 repeat expansion in MNs. We also demonstrate that MN-specific dysregulation of AMPAR expression is also present in C9ORF72 patient post-mortem material. We therefore present multiple lines of evidence for the specific upregulation of GluA1 subunits in human mutant C9ORF72 MNs that could lead to a potential pathogenic excitotoxic mechanism in ALS.
ALS is a relentlessly progressive and invariably fatal neurodegenerative disorder characterized by the selective death of MNs. Recognition that familial and sporadic ALS are largely phenotypically indistinguishable and share pivotal pathological features highlights the value of the study of familial ALS to shed mechanistic insight into ALS. Expansion of a GGGGCC (G4C2) intronic hexanucleotide in the C9ORF72 gene is the most common cause of familial ALS and accounts for ~10% of sporadic ALS cases.1,2 

Although the precise underlying mechanisms of cell-type specificity and death is unknown in ALS, enhanced MN vulnerability to glutamate-mediated excitotoxicity leading to intracellular Ca2+ overload has been implicated.3–7 Rodent studies have suggested that stoichiometry of AMPAR subunits is central to excitotoxicity noting that GluA1, 3, and 4 AMPAR subunits are Ca2+-permeable and that GluA2 subunit typically is Ca2+-impermeable.8–14 Inefficient RNA editing of the GluA2 subunit, resulting in Ca2+-permeability, in sporadic ALS patient samples

**Fig. 1** Excision of the G4C2 repeat expansion. a Strategy for generating C9-Δ from iPSC harboring G4C2 repeat expansion mutation. Two gRNAs were designed flanking the G4C2 repeats in intron-1 of the C90RF72 gene to generate a double-strand break (depicted by //). b PCR of the G4C2 locus in HEK-293 cells transfected with Cas9:gRNA-1 and 2 plasmids generated truncated PCR product (blue arrow) depicting both gRNAs are functional. c Repeat primed PCR and PCR flanking G4C2 locus showed absence of hexanucleotide repeat expansion in C9-Δ iPSC. d Southern blot analysis of C9ORF72 alleles in control, mutant C9ORF72, and C9-Δ iPSC lines. e Sanger sequencing of G4C2 locus in C9ORF72 gene-edited lines confirmed excision of hexanucleotide repeat expansion in mutant allele. G4C2 repeats in wild-type allele were also excised by CRISPR/Cas9 editing strategy (C9-1: 6 repeats, C9-2: 2 repeats, C9-3: 1 repeat)
Fig. 2 | Excision of the G4C2 repeat expansion reverses RNA foci pathology in mutant MNs.

**a** Schematic representation of MN generation and timings of various experiments performed.

**b** Representative images of Isl1/2 and Tau immunostainings in 1-week-old MNs (scale bar: 50 µm).

**c** Percentage of Isl1/2+ MNs from various iPSC lines (data represented as mean ± s.e.m., Con-1, N = 3; Con-2, N = 3; C9-1, N = 3; C9-1Δ, N = 3; C9-2, N = 3; C9-2Δ, N = 2; C9-3, N = 3; C9-3Δ, N = 3).

**d** RNA fluorescent in situ hybridization showed mutant C9ORF72 MNs exhibit intra-nuclear RNA foci (scale bar: 5 µm).

**e** Representative dot-blots for poly-GA, poly-GP, and poly-GR DPRs in mutant C9ORF72 MNs. RA retinoic acid, SAG smoothened agonist, DAPT N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, BDNF Brain-derived neurotrophic factor.
further highlights the importance of improved understanding of the role of AMPARs in ALS15,16.

A powerful approach to interrogate the consequences of G4C2 expansion in human MNs is the study of iPSC-derived MNs from ALS patient carrying the C9ORF72 mutation17,18. Generation of isogenic lines using gene editing technologies, where the only variable is the mutation of interest, thus allowing direct causality to be assigned to any phenotype, further extends the biological relevance of iPSC studies19-22. In this study, we have used CRISPR/Cas9 technology to excise G4C2 repeats from ALS patient-derived MNs to enable direct study of the pathogenic role of G4C2 repeats with particular reference to AMPAR-mediated excitotoxicity. We show that mutant C9ORF72 MNs exhibit increased GluA1 AMPAR expression leading to enhanced MN-specific vulnerability to excitotoxicity. This vulnerability is abolished by CRISPR/Cas9-mediated correction of the C9ORF72 repeat expansion in MNs. Finally, we demonstrate that AMPAR expression is also selectively dysregulated in spinal cord, but not cortical, post-mortem tissue from patients harboring C9ORF72 repeat expansions.

Results
Gene targeting and correction of the C9ORF72 mutation. A CRISPR/Cas9-mediated targeting strategy was used to correct the mutation in three independent C9ORF72 iPSC lines. Two CRISPR guide (g) RNAs (gRNA-1 and gRNA-2) were engineered to target either side of the G4C2 repeats in the intron1 of the C9ORF72 gene. We selected gRNAs that have at least three mismatches to potential off-target sites, which are highly homologous to on-target sites. In the absence of a repair template the double-strand breaks were ligated by non-homologous end joining resulting in deletion of G4C2 repeats (Fig. 1a). First, gRNAs (1 and 2) along with Cas9 plasmid were transfected in HEK-293 cells, which have 3 × G4C2 repeats, to test if the gRNAs were functional. PCR amplification of G4C2 locus in control HEK-293 cells generated a 420 bp amplicon, whereas HEK-293 cells transfected with gRNAs and Cas9 generated both an undated 420 bp amplicon (red arrowhead) and a truncated PCR amplicon (blue arrowhead) suggesting both gRNAs were able to target and delete G4C2 repeats (Fig. 1b). Given that the gRNAs were functional, we next nucleofected Cas9 and gRNAs (1 and 2) into the mutant C9ORF72 iPSC lines that harbor G4C2 repeats (C9-1, up to ~638 repeats; C9-2, up to ~960 repeats; C9-3, up to ~760 repeats; Supplementary Fig. 1A). Repeat primed PCR and G4C2 locus PCR were used to screen individual single-cell-derived clones. PCR of G4C2 locus in mutant C9ORF72 iPSCs lines yielded a single amplicon of ~420–450 bp from the wild-type allele (C9-1, 8 repeats; C9-2, 2 repeats; C9-3, 2 repeats). The mutant allele is not amplified due to larger amplicon size. Deletion of the repeat expansion by CRISPR/Cas9 yields additional PCR amplicons (Fig. 1c). Efficiency of deletion of G4C2 repeats varied between the different iPSC lines ranging from 0.6-4.5% (C9-1, 2.27%; C9-2, 4.5%; C9-3, 0.6%). For each mutant C9ORF72 iPSC line, we selected 1 clone that was confirmed by repeat primed PCR to have a lack of G4C2 repeats. All such lines are hereafter termed C9-1Δ, C9-2Δ, and C9-3Δ (Fig. 1c). In all three C9ORF72 repeat expansion edited lines the wild-type allele was also modified since we observed truncated PCR amplicons (Fig. 1c). Southern blot analysis using a probe outside the G4C2 repeats confirmed absence of repeat expansion in the positive clone (Fig. 1d). We further sequenced the G4C2 locus of the alleles in the positive clones to determine the deleted sequence. C9-1Δ contains 2 × G4C2 repeats in both alleles; C9-2Δ contains 1 × G4C2 repeat in one allele and absence of G4C2 repeat in other allele; and C9-3Δ contains 2 × G4C2 repeats in one allele and 1 G4C2 repeat in the other allele (Fig. 1e). This confirmed that the gene-corrected iPSC clone was karyotypically normal (Supplementary Fig. 1B) and retained pluripotency markers such as OCT3/4, TRA-1-60 (Supplementary Fig. 1C). RNA-guided genome editing, although efficient, has higher off-target activity when compared to other genome editing tools23. Assessment of highest candidate off-target sites, two for gRNA-1 and two for gRNA-2, by Sanger sequencing confirmed no off-target activity for the chosen gRNAs in all three mutant C9ORF72 iPSC lines (Supplementary Fig. 1D).

C9ORF72 mutant MNs display RNA foci. Having corrected the mutation, we next generated, using a recently reported protocol24 MNs from two unrelated healthy controls (Con-1, Con-2) and from three patients harboring the C9ORF72 mutation (C9-1, C9-2, C9-3) and their isogenic pairs (C9-1Δ, C9-2Δ, C9-3Δ) (Fig. 2a). All lines efficiently differentiated into highly enriched neuronal cultures (>95% Tau+, devoid of astrocytes) that expressed the MN markers Isl1/2 (~60%) and ChAT at 1 and 3 weeks post-plating, respectively (Fig. 2b, c; Supplementary Fig. 2A, B). Three principal hypotheses have been proposed to explain how mutant C9ORF72 is pathogenic: haploinsufficiency leading to reduced levels of C9ORF72 protein, RNA foci binding essential RNA-binding proteins, and direct toxicity of dipeptide repeat (DPR) generated by repeat-associated non-ATG (RAN) translation of G4C2 repeats25. To evaluate whether expansion results in loss of protein, western blot analysis was undertaken. This revealed comparable amounts of C9ORF72 protein expression in MNs derived from all control, mutant, and gene-corrected iPSC lines (Supplementary Figs. 2C, 8B). Intranuclear RNA foci in MNs are a characteristic pathological finding in post-mortem tissue of C9ORF72 patients1. Using fluorescent in situ hybridization we confirmed abundant intranuclear RNA foci in mutant MNs that were absent in controls and gene-corrected lines (Fig. 2d) and absent in RNAse-treated cultures. Recent reports have observed DPRs in mutant C9ORF72 material and indeed protein lysates from mutant C9ORF72 MNs expressed higher levels of GA, GP, and GR RAN peptides when compared to control and gene-corrected MNs (Fig. 2e). Collectively, these findings demonstrate a direct causal link between C9ORF72 mutation and the presence of intranuclear foci and RAN peptides. Next, we undertook survival analysis to determine if the C9ORF72 mutation confers selective cellular vulnerability on MNs under basal conditions. Survival, measured using either single-cell longitudinal analysis or absolute cell counts, showed no difference between control and mutant MNs (Supplementary Fig. 2D). Thus, although mutant MNs display pathological RNA foci and DPRs these do not impact upon cell viability or survival under basal conditions.

Mutant C9ORF72 MNs display no difference in excitability. We and others have previously shown that mutant C9ORF72 iPSC-derived MNs exhibit differences in excitability when using differentiation protocols which result in mixed cultures of neurons and glia17,26,27. In the present study using distinct protocols that produce neuronal only, MN-enriched cultures, whole-cell patch-clamp recordings of MNs were undertaken to assess their firing properties (Fig. 3a). No significant differences were observed in the proportions of firing types (Fig. 3b) or intrinsic properties (Supplementary Table 1) of MNs derived from control or mutant iPSCs. Frequency–current (F–I) relationships were also comparable between control and mutant C9ORF72 MNs (Fig. 3c, d; Supplementary Table 1). Collectively, these findings reveal no difference in excitability between control and mutant C9ORF72 MNs in MN-enriched cultures lacking glial cells and therefore implicate aberrant neuron–glia interactions in the functional deficits reported previously17,26,27.
Altered GluA1 subunit expression in mutant C9ORF72 MNs.
To begin to understand the molecular consequences of C9ORF72 mutation on MNs, we next undertook RNA-Seq analysis. Hierarchical clustering and heat map analysis showed segregation between control (including MN from an isogenic line, C9-3Δ) and mutants (C9-1, C9-3) (Fig. 4a). The high degree of similarity between C9-3 and C9-3Δ further indicates comparison between isogenic control overcomes the transcriptional heterogeneity observed in different iPSC lines and observed differential expression is driven by the mutation (Fig. 4a). We filtered differentially expressed genes using the following stringent criteria: (1) genes must have significant differential expression in both independent mutant C9ORF72 lines (p < 0.05) when compared to independent control lines, (2) there must be significant difference between C9-3 and C9-3Δ (p < 0.05), and (3) no significant difference observed between C9-3Δ and controls (p > 0.05). Using this approach 85 upregulated and 80 downregulated genes, collectively 165, were identified (Fig. 4b, c, d; Supplementary Data 1, 2). Gene ontology analysis revealed that genes involved in RNA processing, protein targeting, nuclear transport, and ion transport were significantly upregulated in mutant C9ORF72 MNs. Among downregulated genes in mutant C9ORF72 MNs there was enrichment for genes involved in synaptic transmission, cell–cell signaling, and regulation of phosphate metabolism (Fig. 4f, g).

The finding that the GluA1 subunit was significantly upregulated (>2-fold) in C9ORF72 MNs (red star, Fig. 4d) is consistent with multiple lines of evidence implicating dysregulation of glutamate receptor homeostasis in the pathogenesis of ALS. RNA-Seq analysis of the other AMPAR subunits and their auxiliary subunits showed that only GluA1 mRNA was differentially expressed in mutant C9ORF72 MNs (Supplementary Fig. 3). Subsequent RT-qPCR confirmed increased expression of GluA1 in all week 3 mutant C9ORF72 MNs with gene-corrected lines showing reversal to control levels. Moreover, we also observed increases in GluA3 mRNA expression in mutant C9ORF72 MNs for two out of the three lines examined (Fig. 5a). Western blot analysis also confirmed increased levels of GluA1 in all week 3 mutant C9ORF72 MNs with gene-corrected lines displaying levels comparable to controls (Fig. 5b, c; Supplementary Fig. 8A). Importantly, relative expression of GluA2 mRNA was not altered in any of the lines (Fig. 5a). As would be expected, given the neuronal maturation profile of AMPAR subunit composition, we did not observe altered expression of Ca2+-permeable AMPAR subunits in week 1 MNs (Supplementary Fig. 4A).

The increased proportion of Ca2+-permeable AMPARs in mutant MNs at week 3 could also be due to inefficient RNA editing of GluA2 subunit in addition to increased expression of non-GluA2 subunits. We found, however, that GluA2 subunits were efficiently RNA-edited in all mutant cultures (Fig. 5d; Supplementary Fig. 4B). Together, these findings demonstrate that mutant C9ORF72 MNs exhibit an increased expression of Ca2+-permeable GluA1 AMPAR subunits, but not inefficient editing of the GluA2 subunit.
Mutant C9ORF72 MNs are more vulnerable to excitotoxicity. To determine whether the observed changes in the AMPAR subunit expression profiles had physiological consequences we next examined whether week 3 mutant MNs exhibited enhanced relative functional expression of Ca\(^{2+}\)-permeable AMPARs. MNs from all lines expressed robust AMPA-evoked currents that were potentiated by the allosteric modulator, cyclothiazide, and blocked by AMPAR antagonist CNQX (Supplementary Fig. 5A). The presence of one or more edited GluA2(R) subunits in the AMPAR complex confers Ca\(^{2+}\)-impermeability, reduced single-
channel conductance, and reduced sensitivity to channel-blocking polyamines. We assessed the proportion of Ca2+-permeable AMPARs in MNs by measuring the block of AMPAR-mediated currents by NASPM, a selective blocker of Ca2+-permeable AMPARs (Fig. 6a, b), and estimated their mean single-channel conductance using non-stationary fluctuation analysis (Fig. 6c, d). Comparison of weeks 1 and 3 control MNs showed a reduction in both mean NASPM block and single-channel conductance that is consistent with a transition to a predominantly Ca2+-impermeable AMPAR population (Fig. 6b, d). Strikingly, the maturation of AMPAR composition in C9-1Δ, C9-2Δ, and C9-3Δ MNs reflected that of control MNs. Importantly the retention of Ca2+-permeable AMPARs in mutant C9ORF72 MNs was not due to a slower maturation of AMPAR composition as prolonging culture times for a further 2 weeks did not reveal any changes in the biophysical properties of AMPARs (Fig. 6b, d). Furthermore, we did not observe any impairment in the maturation of intrinsic properties or excitability of MNs across all lines, indicating that AMPAR-mediated-induced firing is unlikely to be a differential source of excitotoxicity in our cultures. These data demonstrate that the C9ORF72 mutation increases persistence in the relative expression of Ca2+-permeable AMPARs. Given the alteration in AMPAR composition we next examined whether C9ORF72 patient-derived MNs showed increased vulnerability to AMPA-induced excitotoxicity. Exposure to AMPA resulted in greater levels of cell death in week 3, but not week 1, mutant C9ORF72 MN cultures vs. control and C9-Δ cultures (Fig. 6c, e; Supplementary Fig. 5B, C). Importantly, enhanced susceptibility of mutant C9ORF72 MNs to excitotoxicity was not due to elevated AMPAR current flux as evidenced by no differences in AMPA-mediated current densities in each of the cell lines at week 3 (Supplementary Fig. 5D). Excitotoxicity experiments performed in the presence of joro toxin (JTX), a selective blocker of Ca2+-permeable AMPARs significantly reduced cell death of week 3 mutant MNs (Fig. 6f). These data therefore indicate the C9ORF72 mutation is associated with a higher susceptibility to excitotoxicity due to an increase in relative expression of Ca2+-permeable AMPARs through altered AMPAR subunit expression.

To determine whether persistent expression of Ca2+-permeable AMPAR was selective to MNs we next generated cortical neurons from Con-1, C9-3, and C9-Δ iPSC lines (Supplementary Fig. 6A, B, C). The extent of NASPM block of AMPAR-mediated currents and the estimated single-channel conductance of AMPARs in C9-3 cortical neurons, unlike MNs, did not demonstrate an elevated expression of Ca2+-permeable AMPARs (Supplementary Fig. 6D, E). Taken together these findings are consistent with a MN-specific cellular pathophysiology caused by the presence of mutant C9ORF72.

**Elevated GluA1 expression in spinal cord of ALS patients.** To evaluate whether findings in iPSC-derived neurons are recapitulated in pathological samples from ALS patients, AMPAR subunit expression was next examined by RNAscope. Post-mortem cervical spinal cord and prefrontal cortex (BA9) sections from three C9ORF72 mutation-carrying ALS patients and three age-matched controls with no known neurodegenerative disease were treated with fluorescence in situ hybridization (FISH) probes against AMPAR subunits and the positive control enzyme PPIB (Supplementary Fig. 7). In agreement with previous reports of control samples displayed negligible GluA1 expression in adult MNs (Fig. 7a). However, high expression of GluA1 RNA was observed in the anterior horn neurons of all ALS patients (Fig. 7b). No difference was found in GluA2, 3, and 4 RNA expression between ALS and control spinal cord samples (Fig. 7c). Conversely, in prefrontal cortex sections, expression of all AMPAR RNA transcripts was similar between ALS and control samples (Fig. 7c, d). These data confirm in human post-mortem tissue that the C9ORF72 expansion is associated with an increase in GluA1 RNA expression in spinal cord MNs but not in prefrontal cortical neurons.

**Discussion**

The ability to study human disease-causing mutations under genetically defined conditions allows causal rather than correlative associations to be ascribed. Using CRISPR technology to correct the C9ORF72 mutation from three patient-derived iPSC lines we show that the C9ORF72 mutation is associated with an increase in GluA1 AMPAR subunit expression, functional expression of Ca2+-permeable AMPARs, and MN vulnerability to excitotoxicity. C9ORF72 mutant post-mortem tissue also demonstrates a marked selective upregulation in GluA1 RNA expression in spinal cord MNs. Both iPSC and pathological data further reveal that dysregulation of AMPAR expression is not found in cortical neurons and thus is MN specific.

There are three major hypotheses as to how C9ORF72 repeat expansion might be pathogenic: loss of, as yet still unknown, normal C9ORF72 function, sequestration of RNA binding proteins by RNA foci, and direct toxicity mediated by DPRs. These processes are not mutually exclusive. We observed RNA foci and DPRs in mutant MNs whose expression was reduced or abolished by gene correction. These findings, consistent with the idea that RNA-binding proteins sequestered by RNA foci in mutant C9ORF72 MNs dysregulate RNA metabolism, are supported by the differential transcriptional findings between mutant, control, and corrected MNs revealing an enrichment for genes involved in RNA metabolism, stress response, and nuclear transport. Collectively our data are consistent with recent studies including those from human pathological findings. Moreover, our RNA-Seq data reveal new candidate genes that will require future validation and functional assessment. These include genes involved in synaptic transmission, protein targeting, and cell–cell signaling. In view of recent findings, it would be of interest to determine whether the increased expression of GluA1 is related to pathological RNA foci and/or DPRs. Furthermore, it will be of considerable interest to study whether other genetic cause of ALS results in AMPAR dysregulation.
In mixed cultures of neurons and glia derived from \textit{C9ORF72} repeat expansion patient iPSCs, MNs first exhibit intrinsic hyperexcitability\cite{27}, then progress to a hypoexcitable state\cite{17}. In contrast, here we have used a modified protocol for the generation of highly enriched neuronal cultures that contains negligible numbers of glial cells\cite{34,41}. Under these conditions we found no difference in MN intrinsic excitability. A likely explanation for the lack of changes in excitability in the present study is that glial-mediated non-cell autonomous mechanisms underlie changes in MN function. This hypothesis is supported by recent findings in murine cell culture models where exposure of wild-type MNs to conditioned medium derived from astrocytes expressing mutant SOD1 alters ion channel function and MN excitability\cite{42}.

Multiple lines of evidence suggest that dysregulated glutamate homeostasis plays a role in ALS. These include patient studies showing reduced expression of glial glutamate transporter, enhanced CSF glutamate levels, and in vitro and in vivo studies that reveal selective MN vulnerability to glutamate or AMPAR-mediated, but not NMDAR, excitotoxicity\cite{34,43}. AMPAR subunit composition is dynamic and changes in development and is dysregulated in disease. In early post-natal life, there is a switch from predominantly Ca\(^{2+}\)-permeable to Ca\(^{2+}\)-impermeable AMPARs which reflects increased GluA2 containing AMPARs; the dominant functional determinant of AMPAR-mediated Ca\(^{2+}\)-permeability\cite{29}. Indeed, this maturation is recapitulated in this study with control and gene-corrected iPSC MNs showing a shift from predominant Ca\(^{2+}\)-permeable to Ca\(^{2+}\)-impermeable AMPAR from 1 to 3 weeks in culture. In contrast, mutant \textit{C9ORF72} MNs display persistent high levels of Ca\(^{2+}\)-permeability at 3 and 5 weeks in culture. The blockade of AMPA-mediated MN cell death by JTX treatment, a selective blocker of Ca\(^{2+}\)-permeable AMPARs, confirms that \textit{C9ORF72} mutation susceptibility to excitotoxicity is due to an increase in relative expression of Ca\(^{2+}\)-permeable AMPARs. Our findings are consistent with an earlier study that showed an enhanced vulnerability of iPSC-derived mutant \textit{C9ORF72} MNs to glutamate-mediated excitotoxicity\cite{18}.

One of the proposed mechanisms of MN death in ALS is through inefficient post-transcriptional editing of the Q/R site within the GluA2 subunit with the resulting AMPAR becoming Ca\(^{2+}\)-permeable\cite{16,44}. Our finding of efficient RNA editing does not support reduced editing as an explanation for increased Ca\(^{2+}\)-permeability in mutant \textit{C9ORF72} MNs and is consistent with findings in native neurons and SOD1 familial cases\cite{45,46}. Alternative explanations for increased Ca\(^{2+}\)-permeability include disease-related alterations in AMPAR subunit composition. Experimental evidence in support of this idea is provided by findings in the SOD1 model of ALS, although these reports do not agree on the precise identity of dysregulated AMPAR subunit\cite{6,10,11,47,48}. Against this background, we show multiple lines
of evidence that demonstrate upregulation in the GluA1 AMPAR subunit in C9ORF72 mutant MNs. The maintenance of functional expression of the GluA1 subunit strongly correlates with an increased Ca\(^{2+}\)-permeable AMPAR identity in our cultures. Although GluA1 upregulation has previously been reported in the spinal cord and hippocampus of hSOD1G93A mice\(^{11,49}\), our study now demonstrates potential functional consequences of increased GluA1 in an experimental ALS model. Furthermore, our data demonstrate that mutant C9ORF72 cortical neurons possess predominantly Ca\(^{2+}\)-impermeable AMPARs, indicating that mutant C9ORF72-dependent increases in Ca\(^{2+}\)-permeable AMPARs are selective to MNs. Moreover, our findings are consistent with recent reports from C9ORF72 FTD patient iPSC neurons and post-mortem cortices that also do not show altered AMPAR subunit transcript regulation\(^{37,50}\). Furthermore, our earlier report that oligodendrocytes generated from the same
Our RNA-Seq study uncovered a mutant C9ORF72-associated increase in GRIA1 (GluA1) expression, which led to the hypothesis of increased Ca\(^{2+}\)-permeable AMPA receptor currents and elevated vulnerability to AMPA-mediated excitotoxicity. However, other genes whose expression are perturbed may also contribute to this phenotype. Our data also revealed a C9ORF72 mutation-associated reduction in PICK1 expression. PICK1 regulates the surface trafficking of GluA2-containing AMPARs,

so a reduction in its expression may modify the proportion of GluA2-lacking AMPARs at the surface. Further genes within this data set may contribute to, or reflect other aspects of mutant C9ORF72-associated cellular phenotypes. For example, we observe a C9ORF72 mutation-associated increase in HSPB8, SERP1, and HSP90B1. The small heat shock protein HSPB8/HSP22 plays a role in protein quality control and is capable of promoting the clearance of aggregates of C9ORF72 dipeptides, as well as mutant SOD1 and TDP-43. HSP22 plays a role in protein quality control and is capable of clearing misfolded proteins. Changes in glial glutamate transporter expression remain unknown.

Our in vitro and post-mortem data suggest that GluA1 subunit upregulation may be contributory to selective MN death in ALS. Notwithstanding our observation of selective upregulation of GluA1 in post-mortem mutant C9ORF72 spinal cord MNs, we are unable to determine whether this is causal or a consequence of a separate disease pathology. It is also important to note that our study cannot exclude other AMPA-related mechanisms that could potentially play a role in mutant C9ORF72-related MN pathology. Furthermore, the degree to which other mechanisms may concurrently contribute to excitotoxicity via elevated glutamate levels at synapses, such as changes in glial glutamate transporter expression remains unknown.

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AMPAR subunit expression in post-mortem spinal cord of healthy patients and ALS patients with C9ORF72 repeat expansion. a Representative RNAscope® images showing the expression of AMPAR GluA1–four transcripts in the anterior horn of cervical spinal cord sections from an ALS patient (C9ORF72) and an age-matched healthy patient and reveals increased expression of GluA1 in ALS patient sample. Scale bar, 25 µm. b Quantification of the mean ± s.e.m. expression levels of AMPAR transcripts in spinal cord samples from three independent patients and controls. Note the lack of GluA1 transcript expression in control spinal cord samples and strong expression in patient samples. c Representative RNAscope images showing the expression of AMPAR GluA1–four transcripts in the prefrontal cortex from an ALS patient (C9ORF72) and an age-matched healthy patient. Scale bar, 25 µm. d Quantification of the mean ± s.e.m. expression levels of AMPAR transcripts in prefrontal cortex samples from three independent patients and controls showing equivalent expression of all transcripts were separated on an ABI 3130xl analyzer (Life Technologies) and data were analyzed using GeneMarker software (Soft Genetics).

A G4C2 flanking PCR for screening targeted clones was also performed using Qiagen multiplex PCR kit. The PCR reaction was set up as per manufacturer’s guide lines using the following primers G4C2 Fw: 5′-CAAGAGGGAGAAGAC CGG-3′ G4C2 Rev: 6-FAM-5′-GGAAAGCAAGGAAGGC-3′. PCR products were separated on an ABI 3130xl analyzer (Life Technologies) and data were analyzed using GeneMarker software (Soft Genetics).

Southern blot. Southern blot analysis was used to analyze the C9ORF72 repeat expansion mutation as described69. DNA was isolated using Wizard® genomic DNA purification kit (Promega). 20 µg of DNA was digested overnight with XbaI endonuclease, separated on a 0.8% agarose gel electrophoresis, gel was subsequently depurinated, denatured, and transferred overnight on to a positively charged nylon membrane (Roche). The membrane was UV cross-linked and hybridized overnight at 47 °C with a digoxigenin (DIG)-labeled PCR probe. PCR probe was synthesized from genomic DNA using PCR DIG probe synthesis kit (Roche) using following primers South Fw: 5′-CTTGCAGATGACCAAGGCCACA-3′ and South Rev: 5′-TTCAGGCACCCTCTTCCTTC-3′. Following hybridization, membrane was washed and labeled with anti-DIG antibody and developed using CDP-Star reagent as per manufacturer’s recommendation (Roche).

MN differentiation. MN differentiation from iPSCs was performed using established protocol24 with minor modifications. iPSCs were dissociated into single cells using 1× Accutase, 2 × 10⁵ iPSCs were neutralized as a suspension culture using SB-431542 (20 µM), LDN-193189 (0.1 µM), and CHIR-99021 (3 µM) in N2/B27 media (0.5× Neurobasal, 0.5× Advanced DMEM/F12, 1× Antibiotic-Antimycotic, 1× Glutamax, 100 µM beta-mercaptoethanol, 1× B27, 1× N2, and 10 µM ascorbic acid). On day 2, neural spheres were simultaneously patterned to spinal cord fate by treating with retinoic acid (RA, 0.1 µM) and smoothened agonist (SAG, 1 µM) as per manufacturer’s recommendation (Roche).

RNA sequencing. Total RNA from iPSC-derived MNs was assessed for quality (Agilent Bioanalyzer) and quantity (Invitrogen Qubit) before library preparation. Illumina libraries were prepared from 1 µg of total RNA using TruSeq RNA Sample Prep Kit v2 with a 10 cycle enrichment step as per the manufacturer’s recommendations. Final libraries were pooled in equimolar proportions before Illumina sequencing on a HiSeq 2500 platform using 75 base paired-end reads. Reads were mapped to the primary assembly of the human (hg38) reference genome contained in Ensembl release 83. Alignment was performed with STAR, version 2.4.0a. Tables of per-gene read counts were generated from the mapped reads with featureCounts version 1.4.6-p2. Differential expression analysis was then performed using DESeq2 (R package version 1.10.0).

Immunocytochemistry. Cells were cultured depending on the experiment, washed once with PBS to remove media components, and fixed with 4% PFA for 20 min at room temperature. Cells were washed three times with PBS and blocking buffer.
containing 5% goat serum and 0.25% Triton X-100 in TBST (TBS-Tween) sub-
 sequently added for 30 min. Primary antibodies diluted in blocking buffer were then added and incubated overnight at 4 °C. Cells were then washed three times with TBS-Tween, and corresponding secondary antibodies coupled with fluor-
ophores were added and incubated at room temperature for 1 h. Coverslips were washed and mounted on glass slides with FluorosaveTM reagent and imaged using Zeiss observer Z1. Images for cell count and neurite outgrowth were analyzed using ImageJ. Immunofluorescence was performed using electrodes either AMPA (100 µM) or AMPA/CNQX (30 µM) or AMPA/JTX (1 µM) for 5–12 week/s. Before the assay was performed, cells were further cultured overnight

**Excitotoxicity assay**

MNs (25,000 per well of a 96-well plate) were cultured for 1 or 3 week/s. Before the assay was performed, cells were further cultured overnight with minimal essential media containing neurobasal, B27, and N2. Live cells were labeled with 4μM Calcein-AM (Invitrogen) for 5 min and imaged using Zeiss Observer Z1. Cells were then treated in the presence of cytochalasin (100 µM) with either AMPA (100 µM) or AMPA/CNQX (30 µM) or AMPA/JTX (1 µM) for 24 h. Followed by washing, cells were incubated again with Calcein AM and reimaged. Cell counts before and after treatment were analyzed using ImageJ.

**Human post-mortem tissue analysis**

Formalin-fixed paraffin-embedded cervical spinal cord and prefrontal cortex (BA9) from three ALS patients carrying the C9ORF72 repeat expansion and three age and sex-matched controls were sectioned at 5-μm thickness on to superfrost slides. BaseScope reagents (Advanced Cell Diagnostics) were used as per manufacturer’s guidelines according to the original protocol. In brief, following deparaffinization, tissue was incubated with hydrogen peroxide for 10 min at room temperature and target antigen retrieval was performed by submerging slides in BaseScope 1 × target retrieval reagent at 99 °C in a Braun Multiquick FS 20 steamer for 15 min. The tissue was then permeabilized using BaseScope Pepsin protease in F1 at 40 °C for 30 min. Probe hybridization was then performed by incubating the slides with four drops of custom-designed BaseScope probe for AMPAR subunits, negative control probe (DapB) or positive control (PP1B) probe for 2 h at 40 °C. Following successive probe amplification steps, transcripts were detected using the BaseScope RED detection kit and slides were counterstained using 50% Gills haematoxylin and 0.02% ammonium water. The slides were then cleared in xylene and mounted with a 24 × 50 mm coverslip using two drops of VectaMount mounting medium. Sections were then imaged at 80× magnification on a NanoZoomer and relative number of transcripts was quantified by counting the dots observed in the cell manually by two independent neuropathologist. Three random fields were chosen and ten cells from each field were counted (in total 30 cells per post-mortem sample) and averaged for each patient. Data were plotted as mean number of transcripts (dots per cell) per post-mortem sample.

**Data analysis**

Statistical analysis was performed using GraphPad Prism software. Data are represented as mean ± s.e.m., **p < 0.05, ***p < 0.001, ****p < 0.0001, one-way ANOVA with Bonferroni’s multiple comparisons test or uncor-
rected Fisher’s LSD test.

**Data availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Electrophysiology**

Experiments investigating excitability were performed as previously described. In short, whole-cell patch-clamp recordings were made in the current-clamp mode to determine firing properties of MNs. Recordings were performed using electrodes filled with internal pipette solution contained (in mM): 140 potassium methansulfonate, 10 NaCl, 1 CaCl2, 1 MgCl2, 26 NaHCO3, 1.25 NaH2PO4, 40 potassium methane-sulfonate, 10 NaCl, 10 HEPES, 1 EGTA, 3 Mg2+–ATP, and 0.4 Na2–GTP, pH 7.2–7.3 (adjusted with KOH; osmolality adjusted to 300 mOsm with sucrose). Cells were bathed in an extracellular recording comprising (in mM): 127 NaCl, 3 KCl, 2 CaCl2, 10 Na–gluconate, 2 MgCl2, 26 NaHCO3, 1.25 NaH2PO4, and 10 D-glucose (equilibrated with 95% O2 and 5% CO2, pH 7.45; osmolality, 310 mosm). Recordings were low-pass filtered at 4 kHz and digitized at 50 kHz using pClamp software (Axon Instruments). F–I relationships were generated by recording the responses to a series of 1 s duration current steps of 5–65 pA (10 pA increments).

For other electrophysiological experiments, recordings were performed as described using electrodes filled with internal pipette solution contained (in mM): 155 K-gluconate, 2 MgCl2, 10 Na–HEPES, 10 Na–PiCreatine, 2 Mg–ATP, and 0.3 Na2–GTP, pH 7.3, 300 mosm. Cells were bathed in an extracellular recording comprising (in mM): 152 NaCl, 3 KCl, 1 CaCl2, 10 MgCl2, 26 NaHCO3, 1.25 NaH2PO4, and 10 D-glucose (equilibrated with 95% O2 and 5% CO2, pH 7.45; osmolality, 310 mosm). Recordings were low-pass filtered at 2 kHz and digitized at 10 kHz and recorded to computer using the WinEDR V2.7 6 Electrophysiology Data Recorder (J. Dempster, Department of Physiology and Pharmacology, University of Strathclyde, UK; www.strath.ac.uk/Departments/PhysPharm/).

Non-stationary fluctuation analysis of slowly rising whole-cell currents evoked by AMPA were used to estimate the AMPAR unitary single-channel current. The analysis of AC and DC currents was performed as described previously in detail with the exception that the AC-coupled signal was filtered with a 1–1200 Hz band-pass frequency.

All experiments were carried out at room temperature. Patch electrodes were manufactured from borosilicate glass to have resistances of 3–6 MΩ. Recordings were made with a series resistance less than 25 MΩ. Reported potential values are corrected for liquid junction potential.

**Human post-mortem tissue analysis**

Formalin-fixed paraffin-embedded cervical spinal cord and prefrontal cortex (BA9) from three ALS patients carrying the C9ORF72 repeat expansion and three age and sex-matched controls were sectioned at 5-μm thickness on to superfrost slides. BaseScope reagents (Advanced Cell Diagnostics) were used as per manufacturer’s guidelines according to the original protocol. In brief, following deparaffinization, tissue was incubated with hydrogen peroxide for 10 min at room temperature and target antigen retrieval was performed using Zeiss observer Z1. Images for cell count and neurite outgrowth were analyzed using ImageJ.
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