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Dysregulation of AMPA receptor subunit expression in sporadic ALS post-motorneuron brain

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Abstract

Amyotrophic lateral sclerosis (ALS) is characterised by progressive motor neuron degeneration. Although there are over 40 genes associated with causal monogenic mutations, the majority of ALS patients are not genetically determined. Causal ALS mutations are being increasingly mechanistically studied, though how these mechanisms converge and diverge between the multiple known familial causes of ALS (fALS) and sporadic forms of ALS (sALS) and furthermore between different neuron types, is poorly understood. One common pathway that is implicated in selective motor neuron death is enhanced α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPAR)-mediated excitotoxicity. Specifically, human in vitro and pathological evidence has linked the C9orf72 repeat expansion mutation to a relative increase in the Ca2+-permeable AMPAR population due to AMPAR subunit dysregulation. Here, we provide the first comparative quantitative assessment of the expression profile of AMPAR subunit transcripts, using BaseScope, in post–motorneuron lower motor neurons (spinal cord, anterior horn), upper motor neurons (motor cortex) and neurons of the pre-frontal cortex in sALS and fALS due to mutations in SOD1 and C9orf72. Our data indicated that AMPAR dysregulation is prominent in lower motor neurons in all ALS cases. However, sALS and mutant C9orf72 cases exhibited GluA1 upregulation whereas mutant SOD1 cases displayed GluA2 down regulation. We also showed that sALS cases exhibited widespread AMPAR dysregulation in the motor and pre-frontal cortex, though the exact identity of the AMPAR subunit being dysregulated was dependent on brain region. In contrast, AMPAR dysregulation in mutant SOD1 and C9orf72 cases was restricted to lower motor neurons only. Our data highlight the complex dysregulation of AMPAR subunit expression that reflects both converging and diverging mechanisms at play between different brain regions and between ALS cohorts.

Keywords: sporadic; ALS; SOD1; C9orf72; AMPAR; neuron; post-mortem; RNA; BaseScope

Introduction

Amyotrophic lateral sclerosis (ALS) is a rapidly progressive and invariably fatal neurodegenerative disease characterised by degeneration of motor neurons of the brain and spinal cord. The last 10 years has seen considerable progress in the genetic understanding of ALS with over 40 associated genes known to harbour genetic mutations associated with the disease. However, only a small proportion of total ALS cases (5–10%) are linked to hereditary mutations and the proportion of apparently sporadic ALS (sALS) cases having a genetic basis remains ~10% [1,2]. In this regard, modelling sALS has been challenging and the majority of ALS research has been conducted on models based on genetic mutations including the C9orf72 repeat expansion (C9orf72RE) mutation and mutations to SOD1, which represent the two most frequent known familial and sporadic ALS mutations [1,2]. Whilst many mutations share general pathological features with sALS, including TDP-43 pathology [3], there is an appreciation that the diversity of mutated genes are also likely to reflect diverse pathways and mechanisms that underlie the degeneration of motor neurons in ALS [4,5]. The extrapolation and relevance of how these pathways converge and diverge between the multiple known familial causes of ALS and sporadic forms of ALS is largely unknown.

Glutamate-mediated excitotoxicity has been a major hypothesis in ALS motor neuron degeneration for ~25 years, with α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptors (AMPARs) emerging...
as a likely candidate for glutamate receptor-mediated excitotoxicity [6]. Crucially, glutamate-gated AMPAR ion channels are composed of four potential subunits, GluA1–4, each of which genetically encode Ca\(^{2+}\)-permeable subunits [7]. The GluA2 subunit is however almost uniformly subject to post-transcriptional RNA editing such that insertion of edited GluA2 subunits into the AMPAR complex confers Ca\(^{2+}\)-impermeability to the ion channel. Dysregulation of AMPAR subunits to generate increased Ca\(^{2+}\)-permeable AMPAR populations is thought to contribute to excitotoxicity in ALS motor neurons [8–15]. Indeed, recently we showed that C9orf72RE patient motor neurons displayed a vulnerability to AMPAR-mediated excitotoxicity due to a C9orf72RE-dependent increase in Ca\(^{2+}\)-permeable AMPAR expression through an abnormal increase in GluA1 subunit expression [15]. However, altered AMPAR properties were not observed in human cortical neurons suggesting that the C9orf72RE mutation imparts a selective AMPAR-associated mechanism of excitotoxicity onto motor neurons [15]. The degree to which mechanisms are conserved across specific brain areas and furthermore other ALS patients with different familial and sporadic aetiologies remains to be clarified.

Noting sporadic and C9orf72RE ALS patients, but not SOD1 mutation patients, typically exhibit shared TDP-43 pathology [16] we have therefore, for the first time, compared the regional expression of AMPARs in sALS patients together with patients with SOD1 (I114T) and C9orf72RE mutations. To accomplish this we used a high-resolution in situ hybridisation technique, BaseScope, to systematically characterise the expression of AMPAR subunit transcripts at the single-cell level in post-mortem spinal cord (anterior horn), prefrontal cortex and motor cortex from the different ALS cohorts with respect to age- and sex-matched controls with no clinical or pathological evidence of neurological disease. Furthermore, we have used human pluripotent stem cell technology to examine the degree of GluA2 RNA editing within sALS patient-derived neurons. Our data implicate notable regional AMPAR subunit dysregulation across all brain regions examined in sALS patients and a restriction of AMPAR subunit dysregulation to the spinal cord in SOD1 (I114T) and C9orf72RE patients.

Materials and methods

Case identification and ethics

ALS post-mortem samples were obtained from the Medical Research Council (MRC) Edinburgh Brain Bank and had separately undergone whole genome sequencing for genetic identification [2]. Our study used three separate cases for each of C9orf72 repeat expansion, sporadic and SOD1 ALS cases. Age and sex-matched control cases with respect to the ALS cases, exhibited no evidence of neurodegenerative disease pathology and were obtained from the Edinburgh Sudden Death Brain Bank. All clinical data were collected as part of the Scottish Motor Neurone Disease Register and Care Audit Research and Evaluation for Motor Neurone Disease platform (Ethics approval from Scotland A Research Ethics Committee 10/MRE00/78 and 15/SS/0216) and all patients consented to the use of their data during life. All post-mortem tissue was collected via the Edinburgh Brain Bank (Ethics approval from East of Scotland Research Ethics Service, 16/ES/0084) in line with the Human Tissue (Scotland) Act (2006). Use of human tissue for post-mortem studies was reviewed and approved by the Edinburgh Brain Bank ethics committee and the Academic and Clinical Central Office for Research and Development medical research Ethics Committee.

Histology and neuropathological assessment

Brain tissue was taken post-mortem from standardised Brodmann areas (BA), BA4 and BA9 and spinal cord and fixed in 10% formalin for a minimum of 72h. Tissue was dehydrated in an ascending alcohol series (70–100%) followed by three successive 4 h washes in xylene. Three successive 5 h paraffin wax embedding stages were performed followed by cooling and sectioning of the FFPE (formalin-fixed paraffin-embedded) tissue on a Leica microtome into 4 μm thick sections that were collected on Superfrost microscope slides. Sections were dried overnight at 40 °C and immunostaining was performed, following epitope retrieval in citric acid buffer (pH 6) in a pressure cooker for 30 min, using the Novolink Polymer detection system with the Proteintech (Manchester, UK) anti-phospho(409–410)-TDP-43 antibody at a 1 in 1000 dilution and Abcam (Cambridge, UK) anti-glutamate receptor 1 antibody (ab32436) at a 1 in 50 dilution (both incubated for 30 min at room temperature). Counterstaining was performed using DAB chromogen counterstained with haematoxylin, according to standard operating procedures. TDP-43 pathology was graded semi-quantitatively by two independent pathologists, using the following descriptive scoring system: (1) no TDP-43 pathology; (2) mild TDP-43 pathology (up to 5 affected cells in at least one ×40 high power field (HPF) out of three HPFs examined); (3) moderate TDP-43 pathology (5–15 affected cells in at least one ×40 HPF out of three HPFs examined); severe TDP-43 pathology (>15 cells affected in at least one ×40 HPF out of three HPFs examined). Assessors were blinded to all demographic and clinical information. Motor neurons were identified based on anatomical location within the spinal cord and according to established neuropathological criteria including size and morphology.

BaseScope analysis

FFPE tissue was sectioned at 4 μm thickness on to Superfrost slides. BaseScope reagents (Advanced Cell Diagnostics Inc., Newark, CA, USA) were used following the manufacturer’s guidelines according to the
original protocol. In brief, following deparaffinisation, tissue sections were 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 permeabilised using BaseScope protease III at 40 °C for 30 min. Probe hybridisation was then performed by incubating the slides with 4 drops of custom designed BaseScope probe, negative control probe (dihydropicolinate reductase, DapB*) or positive control (peptidyl-prolyl cis-trans isomerase B, PPIB*) 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 haematoxylin and lithium carbonate. 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 ×20 objective magnification on a NanoZoomer slide scanner. An unsharp mask in Photoshop was equally applied to all BaseScope images to improve transcript visualisation. Due to the sensitivity and specificity of BaseScope, one red dot corresponds to one transcript, therefore transcript copy number values were taken to be absolute. Counts were plotted as individual transcripts per cell. GluATOTAL* for each patient was calculated by summing the subunits GluA1–4. The amount of Ca²⁺-permeable AMPAR was estimated by taking a ratio of (GluA1 + GluA3 + GluA4)/GluATOTAL*.

Induced pluripotent stem cell derived motor neurons and GluA2 editing

Dermal fibroblasts from sALS patient and control individuals were obtained under full Ethical/Institutional Review Board approval at the University of Edinburgh. Fibroblasts were reprogrammed to iPSCs using episomal vectors expressing OCT4, SOX2, C-MYC and KLF4. iPSCs were maintained in Matrigel (BD Biosciences, San Jose, CA, USA)-coated plastic dishes in E8 medium (Life Technologies, Renfrew, UK) at 37 °C and 5% CO₂. Motor neurons were differentiated according to a protocol detailed previously [15]. RNA editing of the GluA2 subunit was performed exactly as detailed previously [15].

Statistical analysis

The relative abundance of transcripts was quantified by assessing the mean (±SD) number of red dots per cell across 10 randomly identified cells in three randomly generated fields of view (1 mm²) for each case. Cells within each field of view were randomly identified and to prevent bias the assessor was blinded to all clinical and identifiable data and to the GluA1–4 subunit being assessed. Cases were compared using one-way ANOVA with Bonferroni’s post hoc test to account for multiple comparisons. Significance values; *p < 0.05; **p < 0.01; ***p < 0.001. Fold-change compared to GluA2 expression was performed by correcting each case to its age and sex-matched control.

Results

This study has employed a highly sensitive in situ hybridisation method (BaseScope) to examine the expression of AMPAR subunit mRNA transcripts (GluA1–4) at a single-cell level in post-mortem spinal cord (anterior horn cells), motor cortex (BA4) and prefrontal cortex (BA9) tissue from sporadic, SOD1 11I14T and C9orf72RE ALS patients (three patients for each ALS cohort). AMPAR expression in ALS post-mortem tissue was compared to post-mortem tissue from three age- and sex-matched controls with no clinical history or pathological detection of neurodegenerative disease (neuropathological data summarised in Table 1 and cohort demographics in Table 2).

Control data

Our positive control slides localising PPIB transcripts showed no observable difference in PPIB expression between controls and cases across all sampled regions. No staining was seen in our negative control slides. By using both positive and negative controls, we have confidence that our cases are directly comparable in a robust and reproducible manner. Our PPIB data is described and presented for each of the brain regions in which AMPARs subunits were investigated.

AMPA subunit expression is altered in sporadic, C9orf72 repeat expansion and mutant SOD1 ALS motor neurons

Previously we have shown transcriptional upregulation of the Ca²⁺-permeable GluA1 AMPAR subunit in C9orf72RE patient post-mortem anterior horn cells (spinal motor neurons) compared to controls [15]. We and others have shown that GluA1 mRNA expression in adult spinal cord motor neurons is absent or negligible [8,9,15,17,18]. To determine if this transcriptional dysregulation of AMPAR subunit is C9orf72RE mutation specific, we extended our analysis to sALS and SOD1 11I14T spinal motor neurons. Equivalent to C9orf72RE patients, our data from sALS patients demonstrate an
increased GluA1 mRNA abundance in the anterior horn cells of the spinal cord with respect to controls which displayed no measurable level of GluA1 transcripts, whilst displaying equivalent expression of the positive control transcript, PP1B (Figures 1A and 2A,B). For ease of comparison, we have presented the raw quantified AMPAR subunit transcript data previously published for C9orf72RE patient post-mortem anterior horn cells (and respective controls; [15], Figure 2B). However, SOD1 I114T patient samples did not express GluA1 transcripts in anterior horn motor neurons (Figures 1 and 2B. These data were further confirmed by immunohistochemical (IHC) staining for the GluA1 subunit (protein) in post-mortem spinal cord tissue and finding increased staining for GluA1 in the anterior horn cells (spinal motor neurons) of C9orf72RE and sALS cases, with no staining for GluA1 in control individuals and SOD1 cases (Figure 1B). Given that TDP-43 pathology is a common pathological signature observed in C9orf72RE and sALS and absent in mutant cases (Figure 1B). Given that TDP-43 pathology is a common pathological signature observed in C9orf72RE and sALS and absent in mutant

Table 1. Summary of neuropathological assessment of cases

| Case | TDP-43 immunostaining | p62 immunostaining |
|------|-----------------------|--------------------|
|      | Spinal cord | Prefrontal cortex | Motor cortex | Spinal cord | Prefrontal cortex | Motor cortex |
| 1 – sALS | Anterior horn cells lost. Neuronal inclusions highlighted by TDP-43, p62 and Ubiquitin were seen. GFAP highlighted reactive gliosis | Mild abundance of neuronal and glial TDP-43 inclusions | Mild abundance of neuronal and glial TDP-43 inclusions | No evidence of non-TDP-43 related p62 staining |
| 2 – sALS | Anterior horn cells lost. The few remaining anterior horn cells contained inclusions which immunoreacted for p62, TDP-43 and Ubiquitin. In addition, there was extensive glial pathology highlighted by TDP-43 and p62 | Mild abundance of neuronal and glial TDP-43 inclusions | Mild abundance of neuronal and moderate abundance of glial TDP-43 inclusions | No evidence of non-TDP-43 related p62 staining |
| 3 – sALS | Anterior horn cells lost. Cytoplasmic inclusions were immunoreactive for TDP-43 and were seen in residual anterior horn cells throughout the spinal cord | Mild abundance of neuronal and glial TDP-43 inclusions | Mild abundance of neuronal and glial TDP-43 inclusions | No evidence of non-TDP-43 related p62 staining |
| 1 – SOD1 | Anterior horn cells lost. No TDP-43 inclusions were seen within the neocortical ribbon, brain stem nuclei or within residual anterior horn cells | No evidence of neuronal or glial TDP-43 inclusions | No evidence of neuronal or glial TDP-43 inclusions | No evidence of non-TDP-43 related p62 staining |
| 2 – SOD1 | Anterior horn cells lost. No TDP-43 inclusions were seen within the neocortical ribbon, brain stem nuclei or within residual anterior horn cells | No evidence of neuronal or glial TDP-43 inclusions | No evidence of neuronal or glial TDP-43 inclusions | No evidence of non-TDP-43 related p62 staining |
| 3 – SOD1 | Anterior horn cells lost. No TDP-43 inclusions were seen within the neocortical ribbon, brain stem nuclei or within residual anterior horn cells | No evidence of neuronal or glial TDP-43 inclusions | No evidence of neuronal or glial TDP-43 inclusions | No evidence of non-TDP-43 related p62 staining |
| 1 – Control | No pathology | No pathology | No pathology | No pathology |
| 2 – Control | No pathology | No pathology | No pathology | No pathology |
| 3 – Control | No pathology | No pathology | No pathology | No pathology |
| 1 – C9orf72 | Anterior horn cells lost. TDP-43 inclusions were noted within residual anterior horn cells | Mild abundance of neuronal and glial TDP-43 inclusions | Mild abundance of neuronal and glial TDP-43 inclusions | No evidence of non-TDP-43 related p62 staining from pathology within the amygdala, hippocampus and cerebellum |
| 2 – C9orf72 | Anterior horn cells lost. TDP-43 inclusions were noted within residual anterior horn cells | Mild abundance of neuronal and moderate abundance of glial TDP-43 inclusions | Mild abundance of neuronal and moderate abundance of glial TDP-43 inclusions | No evidence of non-TDP-43 related p62 staining from immunityreactivity throughout the neocortex and within the hippocampus, particularly the dentate gyrus. In addition, p62 expression was noted within the cerebellar cortex particularly within the granule cell layer |
| 3 – C9orf72 | Anterior horn cells lost. TDP-43 inclusions were noted within residual anterior horn cells | Mild abundance of neuronal and no evidence of glial TDP-43 inclusions | Moderate abundance of neuronal and glial TDP-43 inclusions | No evidence of non-TDP-43 related p62 staining from pathology within the amygdala, hippocampus and cerebellum |

Abundance of pathological cortical TDP-43 inclusions (determined using an antibody against the pathologically phosphorylated form of TDP-43) are scored as follows: *Mild (1–5 affected cells in at least one >20 field of view per section); *moderate (5–15 affected cells in at least one >20 field of view per section); *severe (>15 cells affected in at least one >20 field of view per section). Spinal cord TDP-43 inclusion assessment is descriptive due to differences in abundance of anterior horn cells (markedly reduced in number in ALS spinal cord).
SOD1 patient post-mortem brain tissues [16,19], these data support an association between TDP-43 pathology and the transcriptional dysregulation of GluA1 in spinal cord motor neurons. Indeed, prominent pathological TDP-43 aggregation was observed in the anterior horn cells of sALS and C9orf72RE patients but was not present in SOD1 I114T patient samples (Table 1).

The GluA2 subunit typically controls and confers Ca\(^{2+}\)-impermeability onto the AMPAR ion channel [7] and its reduced expression (which would result in calcium permeability) has been previously highlighted as a factor that may underpin increased vulnerability of ALS motor neurons to glutamate excitotoxicity [9,13, 20]. However, the data in our study demonstrate that, in direct contrast to C9orf72RE, sALS patients and controls, GluA2 mRNA expression was not detectable in SOD1 I114T anterior horn cells indicating exclusive expression of Ca\(^{2+}\)-permeable AMPARs. We did not examine protein expression of the GluA2 (and GluA3 and GluA4) subunits given the inability of antibodies to discriminate between these AMPAR subunits. To assess the impact of alterations in AMPAR subunit expression upon the potential functional Ca\(^{2+}\)-permeable versus Ca\(^{2+}\)-impermeable AMPAR identity in each of the ALS patients and control individuals, we initially calculated the ratio of GluA2 subunit mRNA expression with respect to total AMPAR subunit expression (GluA2/GluATOTAL) for each ALS group, where 0 and 1 reflect purely Ca\(^{2+}\)-permeable and Ca\(^{2+}\)-impermeable AMPARs populations respectively (Figure 2C). Surprisingly, despite an abnormal increase in GluA1 transcript level, we found that sALS motor neurons exhibited a GluA2/GluATOTAL ratio that was comparable to that of control individuals. However, C9orf72RE patients had significantly lower GluA2/GluATOTAL, which was therefore consistent with an increased Ca\(^{2+}\)-permeable AMPAR population.

To understand how expression of GluA1, GluA3 and GluA4 subunits individually contribute to the predicted total Ca\(^{2+}\)-permeable versus Ca\(^{2+}\)-impermeable AMPAR identity we normalised each subunit data in human post-mortem anterior horn cells. Figure 2D. This analysis confirmed that GluA1 expression was substantially elevated in anterior horn cells of both sALS and C9orf72RE with respect to control individuals. However, considered together with the GluA2/GluATOTAL data displayed in Figure 2C, the impact of increased GluA1 expression in sALS patients appears to be compensated by a decrease in the relative expression of GluA4 transcripts, which was significantly reduced with respect to control cases. For C9orf72RE patients, the relative expression data for GluA3 and GluA4 subunits were not significantly changed with respect to control patient data. The large standard deviation for the relative expression data of GluA3 and GluA4 subunits is due to a high degree of reciprocal variability between patients (Table 3). The

| Patient | Sex | Age | Disease duration |
|---------|-----|-----|------------------|
| 1 - sALS | M   | 54  | 66               |
| 2 - sALS | M   | 70  | 16               |
| 3 - sALS | F   | 68  | 24               |
| 1 - C9orf72 | F | 62  | 97               |
| 2 - C9orf72 | F | 63  | 100              |
| 3 - C9orf72 | F | 63  | 33               |
| 1 - SOD1  | M   | 64  | 67               |
| 2 - SOD1  | F   | 68  | 127              |
| 3 - SOD1  | F   | 56  | 98               |
| 1 - Control | M | 58  | N/A             |
| 2 - Control | M | 65  | N/A             |
| 3 - Control | F | 68  | N/A             |

Age indicates age at death; disease duration indicates months from symptom-onset to death.
abnormal increase in GluA1 in C9orf72RE patient motor neurons therefore appears to be uncompensated leading to an increase in Ca\(^{2+}\)-permeable AMPARs.

Sporadic ALS post-mortem spinal cord motor neurons also exhibit inefficient RNA editing of the GluA2 subunit [10]. This appears to be an sALS-specific feature since mutant SOD1 post-mortem motor neurons [20] and C9orf72RE patient-derived motor neurons [15] do not exhibit inefficient GluA2 editing. Because BaseScope probe design is not yet conducive for...
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Table 3. Reciprocal expression of GluA3 and GluA4 in C9orf72RE spinal cord motor neurons

| Case     | GluA3 Mean (SD) | GluA4 Mean (SD) |
|----------|-----------------|-----------------|
| 1 – C9orf72 | 4.6 (0.84)      | 19.9 (5.09)     |
| 2 – C9orf72 | 2.8 (0.42)      | 21 (4.24)       |
| 3 – C9orf72 | 12.7 (2.21)     | 5 (0.82)        |
| 1 – Control | 2.6 (1.78)      | 3.7 (2.00)      |
| 2 – Control | 2.6 (1.78)      | 3.1 (1.20)      |
| 3 – Control | 2.6 (1.78)      | 4 (1.83)        |
| 1 – SOD1   | 2.5 (1.58)      | 3.5 (1.08)      |
| 2 – SOD1   | 2.4 (1.43)      | 3.6 (1.35)      |
| 3 – SOD1   | 2.8 (2.25)      | 3.8 (1.03)      |
| 1 – sALS   | 4.7 (0.48)      | 22.6 (4.20)     |
| 2 – sALS   | 5.1 (0.88)      | 21.7 (4.16)     |
| 3 – sALS   | 2.3 (0.67)      | 21.8 (6.65)     |

Mean and SD of the number of GluA2 and GluA4 mRNA transcripts in each case, demonstrating reciprocal expression profiles. Light shading – higher expression; Dark shading – lower expression; No shading – no difference in expression.

A comparison of unedited versus edited GluA2 levels (essentially a single base pair substitution) we examined the efficiency of GluA2 editing in enriched cultures of motor neurons differentiated in vitro from induced pluripotent stem cells obtained from a sALS patient, as described previously [15]. We employed restriction fragment length polymorphism (RFLP) analysis using the restriction enzyme Bbv1 and this indicated that GluA2 editing is highly efficient in sALS patient-derived motor neurons (Figure 2E).

Ca2+-permeable AMPAR subunit expression is elevated in the motor cortex of sporadic ALS patients

The principle feature of ALS is degeneration of both lower and upper motor neurons. We therefore examined whether expression of AMPAR subunit transcripts in cortical motor neurons (upper motor neurons, BA4) are altered in sALS, C9orf72RE and SOD1 I14T patients (Figure 3A). Motor cortex samples displayed equivalent staining for PPIB (Figure 4A) and subsequent quantification of AMPAR subunit mRNA transcripts revealed the variable expression of all transcripts in all ALS cases examined (Figure 4B). For the first time, we showed that C9orf72RE patients expressed comparable levels of AMPAR subunit expression with respect to control individuals in cortical motor neurons with the exception of a modest increase in levels of the GluA4 subunit transcript. Interestingly, we observed a profound reduction of GluA2 subunit expression in sALS cases and modest reductions in GluA3 and GluA4 subunit expression. In contrast, SOD1 I114T patients displayed reduced expression of each of the AMPAR subunits when compared to control individuals (Figure 4B).

To estimate the levels of Ca2+-permeable AMPARs in cortical motor neurons, we assessed GluA2/GluA4 TOTAL, as discussed for anterior horn cells. Importantly, GluA2/GluA4 TOTAL was found to be substantially reduced in sALS, but not C9orf72RE or SOD1 I114T, patients compared to control motor cortex (Figure 4C).

Therefore, despite sALS and C9orf72RE patients both exhibiting TDP-43 pathology (Table 1), sALS neurons of the motor cortex only exhibit a transcript profile consistent with an increased Ca2+-permeable AMPAR population.

To understand this change in AMPAR dysregulation in sALS patients with respect to control individuals, we then normalised each AMPAR subunit data relative to GluA2 (Figure 4D). Notably, we observed a 2- to 3-fold significant increase in the levels for all Ca2+-permeable subunits (GluA1, GluA3 and GluA4) with respect to controls. This is a reflection of normalisation to GluA2, which is substantially reduced with respect to control individuals. Inspection of the raw transcript data in Figure 4B indicates a substantial reduction of GluA2, but not GluA1, GluA3 or GluA4 transcripts with respect to control data. These data indicate that the motor cortex
of sALS patients exhibit a transcriptional downregulation of the GluA2 subunit, which is consistent with a Ca$$^{2+}$$-permeable AMPAR phenotype.

Relative GluA1 AMPAR subunit expression is elevated in the prefrontal cortex of sporadic ALS patients.

To determine whether AMPAR subunit dysregulation is associated with upper and lower motor neurons or, non-motor neurons in other brain regions also affected by TDP-43 pathology in ALS patients we examined AMPAR subunit expression in the post-mortem prefrontal cortex (BA9) of sALS, C9orf72$$^{RE}$$ and SOD1 I114T patients (Figure 5A). Cellular quantification of positive control PPIB (Figure 6A) and AMPAR subunit transcripts was performed as for sALS and SOD1 I114T patients (Figure 6B). Previously published quantified C9orf72$$^{RE}$$ AMPAR subunit data for the prefrontal cortex [15] is presented for comparison (Figure 6B). All AMPAR subunit transcripts were detected in each of the cases and control individuals. AMPAR subunits were not extensively altered in prefrontal cortex neurons in C9orf72$$^{RE}$$ and SOD1 I114T cases. However, in sALS cases, GluA2 and GluA3 transcripts were reduced substantially and there was a significant upregulation of GluA1 mRNA (Figure 6B). These data were further confirmed by IHC staining for the GluA1 subunit (protein) in post-mortem prefrontal cortex, demonstrating a possible increase in staining for GluA1 in the neurons of sALS cases compared to controls (Figure 5B).

Strikingly, the GluA2/GluA$$^{TOTAL}$$ ratio in the prefrontal cortex of sALS patients was lower than in controls, therefore suggesting a substantially larger Ca$$^{2+}$$-permeable AMPAR population (Figure 6C). GluA2/GluA$$^{TOTAL}$$ prefrontal cortex data generated for C9orf72$$^{RE}$$ and SOD1 I114T patients revealed no significant difference in the GluA2/GluA$$^{TOTAL}$$ ratio (Figure 6C). Of interest, we additionally note that for the prefrontal cortex of control individuals that GluA2/GluA$$^{TOTAL}$$ ratios are significantly higher than that of the motor cortex ($p < 0.01$, unpaired t-test) and anterior horn cells ($p < 0.001$, unpaired t-test), indicating the different transcriptional regulation of AMPAR subunits between different brain regions.
There is therefore demonstration that the observed dysregulation of AMPAR subunit expression in the prefrontal cortex. The data for each patient (Figure 6D).

The dysregulation of GluA1 subunit expression in sALS prefrontal cortex is therefore likely specific to sALS patients. Furthermore, the fact that the prefrontal and motor cortex of sALS and C9orf72RE patients shows overlapping TDP-43 pathology (Table 1) suggests that TDP-43 cannot be the sole pathogenic substrate that generates altered Ca\(^{2+}\)-permeable AMPAR subunit expression in the cortical regions of sALS patients.

**Discussion**

This study has provided the first comparative study of AMPAR subunit expression across post-mortem brain regions (spinal cord – anterior horn, motor cortex – BA4 and prefrontal cortex – BA9) of the most prevalent genetic ALS patient cohorts; sporadic ALS, SOD1 mutation and C9orf72RE. To do this we employed a high resolution *in situ* hybridisation technique, BaseScope, which permitted a quantitative assessment of AMPAR subunit expression at the single-transcript and single-cell level previously not detailed in control individuals or ALS patients. Whilst it is not possible in post-mortem tissue, using laser capture dissection or *in situ* hybridisation, to capture a neuron in its entirety, we have sampled multiple cells in multiple regions to try to limit sampling bias, as other studies have done [9]. Using these methods, our data indicate extensive AMPAR subunit dysregulation in all brain regions examined from sALS patients. However, AMPAR dysregulation was observed to be restricted to spinal motor neurons for SOD1 1114T and C9orf72RE patients.

AMPAR subunit dysregulation in spinal motor neurons has been described in several ALS models [20] and in human ALS patients [9], where mechanistic disease hypotheses have centred on an increased vulnerability to excitotoxicity due to an increased Ca\(^{2+}\)-permeable AMPAR population in these cells. Previously [15], we showed that C9orf72RE spinal motor neurons exhibited an increased functional Ca\(^{2+}\)-permeable AMPAR population underpinned by an increase in the abnormal expression of the GluA1 subunit and, in this study, is reflected as a reduction in the GluA2/GluA\(^{\text{TOTAL}}\) expression ratio. Noting the expression of GluA1 in adult spinal motor neurons is consistently reported to be absent or negligible [15], we found that sALS patient spinal motor neurons exhibit equivalent substantial dysregulation of GluA1 transcripts and protein. This indicates that sALS and C9orf72RE spinal motor neurons, which both exhibit TDP-43 pathology, may share pathological mechanisms. Indeed, the GluA2/GluA\(^{\text{TOTAL}}\) expression ratio for sALS spinal motor neurons was, surprisingly, not different to that of control individuals and the observed reduced expression of Ca\(^{2+}\)-permeable GluA4 subunit may instead reflect compensatory mechanisms for the increased GluA1 expression in sALS patients. These specific data suggest to us that sALS patients do not exhibit differences in the relative level of the Ca\(^{2+}\)-permeable AMPAR population in spinal motor neurons. However, a functional Ca\(^{2+}\)-permeable
AMPA population still cannot be ruled out given that AMPAR assembly, trafficking and localisation is highly subunit dependent [21] and that the underlying profile of AMPAR subunit expression is different in sALS patients to that of control individuals.

GluA1 was not expressed by SOD1 I114T patients, which do not exhibit TDP-43 pathology, confirming GluA1 dysregulation is associated with common TDP-43 pathology. However, we observed that only Ca²⁺-permeable AMPARs could be expressed by SOD1 I114T spinal motor neurons since GluA2 was found not to also be expressed. These data are the first to be presented in the context of human ALS patients with SOD1 mutations, though are in broad agreement with previous studies of mutant Sod1 rodent models of ALS that implicate a reduction in GluA2 expression in spinal motor neurons [11,13,23–27]. Despite mechanistic divergence at the AMPAR subunit transcriptional level, it appears that SOD1 mutant and C9orf72RE patient spinal motor neurons show a functional convergence through an increase in the relative Ca²⁺-permeable AMPAR population.

Inefficient (glutamine to arginine) RNA editing of the GluA2 subunit also generates an increase in Ca²⁺-permeable AMPARs and has been evidenced in sALS post-mortem spinal motor neurons [10], but not SOD1 mutation post-mortem motor neurons [21], C9orf72RE motor neurons [15] and whole spinal cord lysates from C9orf72RE patients [27]. We examined GluA2 editing in sALS patient-derived motor neurons and found the editing of GluA2 to be highly efficient. ALS is also typified by the degeneration of upper motor neurons and several other brain regions are also affected by TDP-43 deposition. We therefore performed a comparative quantitative assessment of AMPAR subunit expression in the ALS motor cortex (BA4) and pre-frontal cortex (BA9). Our data indicated that SOD1 I114T and C9orf72RE patients did not exhibit any change in GluA2/GluA TOTAL expression or subunit profiles with respect to control individuals in the cortical areas investigated and indicate that AMPAR dysregulation is restricted to spinal motor neurons. Despite sharing equivalent TDP-43 pathology with C9orf72RE patients, dysregulation of AMPAR
subunit expression in the motor and prefrontal cortex of sALS patients is, however, prominent. The GluA2/GluATOTAL expression ratio in the pre-frontal cortex and motor cortex in sALS is lower due to an increase in GluA1 expression and a reduction in GluA2 expression, respectively. TDP-43 pathology alone therefore cannot account for the observed differential spatial AMPAR dysregulation. These scenarios lead to an increase in Ca2+-permeable AMPARs in sALS motor and pre-frontal cortex and suggest that they may contribute to excitotoxically mediated cell death. In this regard, we highlight that GluA2/GluATOTAL expression ratios are estimations and not direct measurements of the functional Ca2+-permeable AMPAR composition.

Our data indicate that neuronal AMPAR subunit expression is heterogeneously regulated between ALS cases with different genetic backgrounds and between different brain regions within each case type. Furthermore, given that sALS patients exhibited altered AMPAR subunit regulation in all regions examined and C9orf72RE patients only in anterior horn motor neurons, it appears that classical TDP-43 pathology can only be associated with aspects of anterior horn motor neurons, but not cortical, AMPAR subunit dysregulation. Nonetheless, with the potential exception of sALS anterior horn motor neurons, all observed perturbed AMPAR subunit expression is consistent with a convergence on an increased relative Ca2+-permeable AMPAR population, which is thought to underpin an increased vulnerability to glutamate-mediated neurotoxicity.

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Author contributions statement

JMG, MRL, SC and CS designed the study. JMG, MRL, KM, BTS, SB, SC and CS prepared the samples and carried out experiments. JMG, MRL, SC and CS conceived experiments and analysed data. JMG, MRL, BTS, SC and CS interpreted the data. JMG, MRL, SC and CS wrote the manuscript. All authors had final approval of the submitted and published versions. JMG and MRL contributed to the study equally.

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