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Oligodendrocytes contribute to motor neuron death in ALS via SOD1 dependent mechanism

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Abstract

Oligodendrocytes have recently been implicated in the pathophysiology of ALS. Here we show that, *in vitro*, mutant SOD1 mouse oligodendrocytes induce wild-type motor neuron hyperexcitability and death. Moreover, we efficiently derived human oligodendrocytes from a large number of controls, sporadic and familial ALS patients using two different reprogramming methods. All ALS oligodendrocyte lines induced motor neuron death through conditioned medium and in co-culture. Conditioned medium-mediated motor neuron death was associated with decreased lactate production and release, while toxicity in co-culture was lactate independent, demonstrating that motor neuron survival is not only mediated by soluble factors.

Remarkably, human SOD1 shRNA treatment resulted in motor neuron rescue in both mouse and human cultures when knockdown was achieved in progenitor cells, while it was ineffective in differentiated oligodendrocytes.

Early SOD1 knockdown, in fact, rescued lactate impairment and cell toxicity in all lines tested with exclusion of samples carrying C9orf72 repeat expansions. These did not respond to SOD1 knockdown nor showed lactate release impairment.

Our data indicate that SOD1 is directly or indirectly involved in ALS oligodendrocyte pathology and suggest that in this cell type some damage might be irreversible. In addition, we demonstrate that C9ORF72 patients represent an independent patient group that might not respond to the same treatment.

Significance Statement

Oligodendrocytes have been implicated in disease pathology in amyotrophic lateral sclerosis (ALS) using transgenic mouse models. To date there is no human co-culture system available to investigate oligodendrocyte involvement in motor neuron death in ALS. Our data highlight that oligodendrocytes derived from familial and sporadic patients from iPSCs and iNPCs play an active role in motor neuron death in ALS. Oligodendrocyte toxicity is mediated through soluble factors as well as cell-to-cell contact, thus identifying multiple mechanisms of action and therapeutic opportunities. Their pathogenic phenotype can be reversed by achieving SOD1 knockdown in early oligodendrocyte progenitors in both familial and sporadic cases, but not C9orf72 samples. This study provides important insights for patient sub-grouping and timelines for therapeutic approaches.

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Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult onset motor neuron disorder. Patients are initially affected by muscle weakness and fasciculations, rapidly leading to paralysis and eventually death by respiratory failure within 2-5 years from symptom onset. Approximately 10% of patients have a family history of the disease. Mutations in superoxide dismutase 1 (SOD1)(1), TAR DNA-binding protein 43 (TDP43)(2, 3), Fused in sarcoma (FUS)(4, 5), and hexanucleotide repeat expansions in C9orf72(6, 7) are responsible for about 65% of these cases. On the contrary, the etiology of sporadic ALS, affecting about 90% of patients, is still largely unknown. Interestingly, familial and sporadic ALS are clinically indistinguishable, thus leading to the hypothesis that common mechanisms might be involved in disease etiology and progression(8). Nonetheless, the staggering complexity of this disorder and its fast progression have hampered the efforts to find an effective treatment. As a result, Riluzole is the only FDA approved drug for this disease, leading to a modest increase in survival(9).

Although motor neuron degeneration is the most striking event occurring in ALS, *in vitro* and *in vivo* murine models of ALS have demonstrated that astrocytes(10, 11) and microglia(12, 13) play a crucial role in motor neuron degeneration during disease progression. Recently, the availability of human samples has confirmed the toxic role of human astrocytes *in vitro*(14, 15). Elegant studies have shown that oligodendrocytes are also involved in the non-cell autonomous nature of ALS using mouse models of the disease (16-18). In fact, oligodendrocytes are severely affected during disease and their degeneration has been shown to precede motor neuron death in the mutant SOD1 (mSOD1) mouse model (17, 18). Moreover, it has been reported that oligodendrocyte progenitors rapidly proliferate in the spinal cord of mSOD1^{G93A} mice, but fail to replace degenerating oligodendrocytes, thus leaving motor neuron axons demyelinated(17, 18).

Interestingly, removal of mSOD1^{G37R} from only the oligodendrocyte lineage using the Cre-recombinase system under the PDGFαR promoter resulted in a significant delay in disease onset and increase in survival(17). Although the SOD1 mouse models of ALS have greatly helped identify the contribution of individual cell types to disease onset and progression, the complexity of the *in vivo* system makes it difficult to unravel the role of each cell type leading

to motor neuron degeneration. Co-culture methods to evaluate oligodendrocytes *in vitro* may be beneficial to uncover novel therapeutics and may also help determining the timing of disease intervention for maximal therapeutic effect. Since mouse and cell models can only be used to model a minority of ALS cases, it remains unknown whether the same observations hold true in a broad spectrum of ALS patients including sporadic cases without known genetic cause.

To address these questions, we developed a co-culture *in vitro* model to study both mouse and human ALS oligodendrocytes and their role in motor neuron death. Our data show that oligodendrocytes can be successfully differentiated from mouse neural progenitor cells (NPCs) and human induced pluripotent stem cells (iPSCs), as well as induced NPCs (iNPCs)(19), from both non-ALS and ALS samples.
We find that oligodendrocytes from ALS samples convey toxicity towards motor neurons *in vitro* independent of their origin and that the toxicity can be rescued by reducing SOD1 in the oligodendrocyte precursor cells, but not in differentiated oligodendrocytes. However, the toxicity derived from cells carrying the C9orf72 repeat expansion seems to be SOD1 independent since no response was seen in those cases.

Our work provides the first *in vitro* co-culture model of mouse and human oligodendrocytes for ALS, as well as the first oligodendrocytes-motor neuron electrophysiology recordings. We demonstrate that oligodendrocytes from ALS samples induce motor neuron death via distinct mechanisms of toxicity mediated by soluble factors and cell-to-cell contact when no sign of oligodendrocyte degeneration can be observed. Finally, this study provides insight into the detrimental role of oligodendrocytes on motor neurons in ALS and the involvement of SOD1 in different genetic variants of this disease supporting the finding that C9orf72 mutations define a discrete subgroup of ALS patients.

Results

Oligodendrocyte differentiation from mouse and human samples does not differ between ALS and controls *in vitro*

Oligodendrocyte degeneration and impaired regeneration have been previously reported as contributors to ALS pathology (17). In order to study oligodendrocyte differentiation and maturation in ALS samples and their involvement in motor neuron death, we developed a protocol to obtain myelin basic protein (MBP) positive cells from both mouse and human samples *in vitro*.

Oligodendrocyte progenitors cells (OPCs) were isolated from the cortex of neonate mSOD1 G93A mice and WT littermates and cultured in proliferation medium containing PDGFaa. After 48h, the cultures were stained for the oligodendrocyte progenitor marker NG2, showing 95% $NG2^+$ cells. These cells were then cultured in medium depleted of PDGFaa and supplemented with IGF-1 for 3 additional days to promote differentiation into MBP^+, highly ramified cells (See SI Appendix Fig. S1a). During the differentiation protocol about 5-10% of the OPCs died. Of the surviving cells, approximately 84% were MBP^+ and negative for microglia or astrocyte markers (See SI Appendix Fig. S1b, Table 1 and see SI Appendix Fig. S2a-d). At the end of this 5-day differentiation protocol, cells were harvested and tested for RNA expression of oligodendrocyte, astrocyte and microglia markers compared to the expression of the same markers in whole spinal cord homogenates as well as microglia and astrocytes isolated from the same preparation (See SI Appendix Fig. S1b). QPCR data showed that the cell population obtained was highly enriched for cells expressing oligodendrocyte markers and there were no differences between WT and mSOD1 oligodendrocytes in expression levels (See SI Appendix Fig. S3).

Following successful differentiation of murine cells, an adaptation of the same protocol was tested on human neural progenitor cells (NPCs) derived either from iPS colonies (20) or from human skin fibroblasts that were directly converted to induced neural progenitor cells (iNPCs) as previously described(19). NPCs obtained with either method were cultured with low concentrations of FGF-2 and high concentrations of PDGFaa (15ng/ml) for 7 days. Immunostaining revealed that after only 7 days, the NPCs had considerably changed

morphology from triangular to a definite bipolar shape and 82% were already positive for the late OPC marker O4 (Fig. 1a). For the following 13 days, these cells were cultured with reduced amounts of PDGFaa (10ng/ml) and IGF-1 (20ng/ml). At day 20 of the differentiation protocol, ~90% of the surviving cells expressed GalC, an important component of myelin. For the following 10 days, the cultures were treated with high concentrations of IGF-1 (50ng/ml) without PDGFaa, leading to a definite morphologic change, accompanied by expression of MBP. This differentiation protocol yielded 50-65% survival compared to the initial number of NPCs or iNPCs plated, with 96% of the surviving cell population being positive for MBP (Fig. 1a and See SI Appendix Table S1). MBP^+ cells from ALS patients and controls, derived from iPSC or iNPCs, were analyzed for oligodendrocyte as well as astrocyte and microglia marker expression in comparison to whole spinal cord homogenate (Fig. 1b, see SI Appendix Fig. S2e-h and S4a-b). QPCR results showed that the cellular population obtained was highly enriched for cells expressing oligodendrocyte markers, independent of the genotype. Additionally, no difference in markers expression was detected between iPS-derived and iNPC-derived oligodendrocytes, as well as between ALS and control samples in agreement with data recently published (21). Of note, the iPS lines as well as the fibroblasts used for direct conversion were obtained from various sources, i.e. some were purchased from Coriell (http://www.coriell.org/) and some were obtained from national and international collaborators (See SI Appendix Table S2 for a detailed description of each patient line). Despite the heterogeneous origin of the samples, no significant differences in differentiation patterns, including NPC production, oligodendrocyte yield nor marker expression, were noticed. Of note, the fibroblasts from the two sporadic patients 002 and 009 were reprogrammed with both the classical iPS differentiation protocol and direct differentiation to iNPCs, with no differences observed in the ability to generate oligodendrocytes.

To further confirm that the cells obtained with this protocol express the gene signature of oligodendrocytes, we performed a small gene expression study limited to 4 iOligodendrocyte lines from 2 controls (155 and 170) and 2 patients (12 and 17), 4 iAstrocyte lines from the same samples and 4 fibroblast lines from one of our previously published studies (22). We focused on cell-type specific gene expression rather than pathology-related transcriptional changes and we found that iOlogodendrocytes, iAstrocytes and human fibroblasts identify 3 distinct cell populations that significantly differ in gene expression as shown by the principal component analysis (PCA) carried out using Qlucore (Fig. 1c). Moreover, using a two-way ANOVA multi-group comparison analysis ($p<0.001$)

3361 transcripts were identified as differentially expressed between the 3 groups. These were visualised in a heat map that demonstrated that the three cell types are clearly distinguishable (Fig 1d).
In particular, oligodendrocyte marker genes such as myelin basic protein and several enzymes involved in lipoproteins and sphingolipids synthesis, were highly enriched in the iOligodendrocytes and were not found in the corresponding iAstrocytes differentiated from the same iNPCs or fibroblasts (See SI Appendix Table S3).
Gene enrichment analysis carried out using The Database for Annotation, Visualization and Integrated Discovery (**DAVID**) (https://david.ncifcrf.gov) also identified membrane and lumen maintenance as well as mitochondrial proteins as the most enriched categories (See SI Appendix Fig. S5), thus identifying iOligodendrocytes as highly metabolic demanding cells with significant membrane remodeling characteristics as expected from previous studies (33, 34), that were not found in iAstrocytes or fibroblasts.
In conclusion, the method presented here produces high purity MBP^+ oligodendrocytes from murine and human samples independent of the method used to establish the NPCs.

Mouse $SOD1^{G93A}$ oligodendrocytes induce motor neuron death *in vitro*

In vivo studies have shown that oligodendrocytes are affected by the pathogenic mechanisms involved in ALS and removal of mSOD1 from this cell type improves survival in the $mSOD1^{G37R}$ ALS mouse model(17). However, it is still unclear whether oligodendrocytes are actively inducing MN damage or whether their own degeneration is contributing to an inevitable cascade of events leading to MN death. Having established a reliable protocol where ALS and control oligodendrocytes do not seem to differ in differentiation efficiency and survival, we proceeded to test MN viability in this co-culture system.

Wild type Hb9-GFP^+ mouse MN were plated onto 90-95% confluent MBP^+ mouse oligodendrocytes. Forty-eight hours post-plating, MNs extended long axons, presenting no differences in cell number or branching, regardless of the genotype of the oligodendrocytes (Fig. 2a). However, 11 days post-plating, a significant 40% decrease in MN survival, along with decreased axonal length and branching, was detected in the cultures with mSOD1 oligodendrocytes (Fig. 2a).
Whole cell patch clamp analysis was used to determine whether oligodendrocytes expressing mSOD1 affected the electrophysiological profile of MNs in co-culture conditions

before cell death was detectable. Electrophysiological recordings were performed at day 7 post MN seeding. This time point was chosen to allow MN maturation and at the same time to determine if MN distress could be detected before cell death. Wild type MNs in co-culture with either WT or mSOD1 oligodendrocytes were excitable and produced action potentials in response to current injection (See SI Appendix Fig. S6a). Yet, MNs cultured with mSOD1 oligodendrocytes displayed enhanced excitability compared to those cultured with WT oligodendrocytes (See SI Appendix Fig. S6a-b). To determine if ion channel activity in MNs was also affected by co-culture with mSOD1 oligodendrocytes, the cells were voltage clamped in the presence and absence of TTX. Transient TTX-sensitive currents were present but at a lower density in MNs co-cultured with mSOD1 oligodendrocytes (See SI Appendix Fig. S6c). However, the sustained, inward TTX-sensitive current was larger in MNs co-cultured with mSOD1 oligodendrocytes (See SI Appendix Fig. S6d). This would result in greater excitability in response to a depolarizing stimulus. The reversal potential was also shifted suggesting that the identity or ion selectivity of the channels contributing to these sustained currents was altered. The density of TTX -insensitive currents, evoked by voltage-gated potassium channels or through leak channels, was not significantly different between the two co-culture conditions (See SI Appendix Fig. S6e-g). Together, these results indicate that mSOD1 oligodendrocytes can actively induce motor neuron death.

Human ALS oligodendrocytes derived from multiple genetic and sporadic cases induce motor neuron death *in vitro*

To determine if human oligodendrocytes from patients were also able to induce MN death, we performed co-cultures of human MBP+ oligodendrocytes and Hb9-GFP$^+$ MNs. Wild type Hb9-GFP$^+$ MN were plated onto 60-65% confluent iPS or iNPC-derived human oligodendrocytes from sporadic and familial ALS cases. After 24 hours the MNs displayed neuritic extensions with no significant differences between ALS and control samples. However, 72h post plating, a significant difference in survival (50-60% decrease) with a striking axonal beading phenotype was detected in cultures with iPS-derived oligodendrocytes from three sporadic patients and one familial case carrying a mutation in *FIG4* (Fig. 2b). Of note, one of the two non-ALS lines was derived from fibroblasts from a patient affected by Becker muscular dystrophy and no difference in MN survival compared to the control line was detected.

An identical phenotype was observed in cultures with iNPC-derived oligodendrocytes, where samples with a wider spectrum of genetic variants were available, i.e. four sporadic ALS samples, three carrying *C9orf72* repeat expansion, as well as one carrying *SOD1* and one *TARDBP* mutations (Fig. 2c). Motor neurons showed reduced survival in the presence of these oligodendrocyte lines, with 40-60% of MNs perishing on all lines. Of interest, two sporadic lines 002 and 009 were reprogrammed with both, the classic iPSC procedure followed by production of NPCs, or the new conversion protocol to make iNPCs directly from fibroblasts as previously described[19]. Oligodendrocytes from both iPSCs and iNPCs displayed similar toxicity regardless of the different reprogramming procedure used, thereby further validating the value of direct conversion to generate NPCs.

To determine if oligodendrocyte death was associated with the MN loss observed *in vitro*, mouse OPCs were transduced with Lv-MBP-RFP at the beginning of differentiation and cell number was determined using the InCell Analyzer 2h prior to MN seeding (i.e. 5 days post-RFP infection). Similarly, human oligodendrocytes were transduced 5 days before co-culture and RFP^+ cell number was determined 2h prior MN seeding. RFP^+ cell numbers were also determined at the end of co-culture and no difference between ALS and control oligodendrocyte numbers were detected in mouse or human cultures (See SI Appendix Fig. S7).

Oligodendrocyte contribute to MN death via soluble factors and cell-to-cell contact through separate mechanisms

To test whether MN death required cell-to-cell contact, we tested the effect of mouse and human oligodendrocyte conditioned medium onto GFP^+ MN monocultures.
MN were plated in 96-well plates and conditioned with increasing concentrations of growth medium from mouse WT or mSOD1 oligodendrocytes. After 6 days we detected a significant ($p=0.013$) 20% decrease in cell survival when MNs were treated with 100% conditioned medium from oligodendrocytes expressing mSOD1, while no significant difference was detected when replacing MN medium with 50 or 75% oligodendrocyte conditioned medium (Fig. 3a).

Similarly, conditioned medium from human ALS oligodendrocytes both familial and sporadic cases, induced a significant increase in MN death of WT GFP^+ MNs in monoculture within 4

days (Fig. 3c). The percentage of conditioned medium inducing MN death varied between patients (See SI Appendix Fig S8), but in general correlated with the amount of MN death the conditioned medium could cause, the higher the MN death, the more the conditioned medium had to be diluted to lose toxicity. With complete oligodendrocyte conditioned medium replacement, MN death increase ranged between 15-40%, with the mildest increase in cell death associated with the conditioned medium from the oligodendrocytes carrying a mutation in *FIG4*, and the highest increase associated with the conditioned medium from the oligodendrocytes carrying a SOD1 mutation.

In light of the reported involvement of lactate release impairment in ALS oligodendrocyte pathology(16), we examined the lactate content in the conditioned medium of both mouse and human oligodendrocytes throughout differentiation and one week after MBP expression was achieved.

We found that there is no difference in the amount of lactate released by progenitor cells at the beginning of differentiation regardless of their disease state (Fig 3b, d and e). Lactate secretion increases as progenitor cells differentiate into oligodendrocytes for both, mouse and human, and reaches a plateau upon expression of MBP.

Interestingly, in ALS samples the lactate production is reduced starting from day 3 in mouse and week 3 in human samples. These time points correspond to the expression of early oligodendrocyte progenitor markers Ng2 in mouse and GalC in human cells (See SI Appendix Fig S1a and Figure 1a). This failure in lactate release developed during differentiation results in significantly lower levels of lactate secreted by mouse and human ALS MBP+ cells compared to controls (Fig 3b, d and e). Intracellular lactate measurements from cell lysates revealed that ALS oligodendrocytes overall produce less lactate (See SI Appendix Fig S9a) and, in agreement with previous findings, display lower levels of *MCT1* transcript (See SI Appendix Fig S9b).

Interestingly, although the conditioned medium from C9orf72 mutant oligodendrocytes induced increased MN death, no reduction in lactate intracellular or extracellular content was detected.

To determine whether lower lactate levels in the medium are the only or main responsible for MN death in monoculture and co-culture, we supplemented mouse and human cultures with 1 or 2mM lactate to maintain the same range of lactate concentration observed in control oligodendrocyte monocultures (Fig 3b, d-e).

The addition of lactate to the monocultures led to a slight, but significant, increase in MN survival even when MNs were grown in both mouse wild type and human control

oligodendrocyte conditioned medium. 2mM lactate supplementation resulted in complete MN rescue in monocultures treated with $SOD1^{G93A}$ (Fig 4a-d) and ALS patient-derived oligodendrocyte conditioned medium with exception of C9orf72 samples (Fig 4h-l).
On the contrary, addition of lactate to co-cultures only partially increased MN survival, did not improve the axonal beading phenotype originally observed and did not affect C9orf72 cultures at all (Fig 4e-g and 4m-o).
This indicates that MN survival is likely mediated by both soluble and insoluble factors that require cell-to-cell contact or very close vicinity and that oligodendrocytes from patients carrying C9orf72 repeat expansions affect MNs via different pathways compared to other ALS cases.

Mutant SOD1 irreversibly causes oligodendrocyte-mediated motor neuron death

It has been shown that genetic knock down of mSOD1 specifically in the oligodendrocytic lineage in the $mSOD1^{G37R}$ mouse resulted in delayed onset and increased survival when the knock down was driven by PDGFαR, a marker of oligodendrocyte progenitors(17). To test whether mSOD1 knock down resulted in MN rescue *in vitro* as one would expect from the *in vivo* experiments, and also to determine whether timing is important in this process, mSOD1 knock down was performed either in primary mouse OPCs or in MBP^+ cells (5 days post isolation). Knock down was achieved by transducing OPCs on the day of isolation or differentiated MBP^+ cells 48h before MN co-culture with an adenovirus expressing a human SOD1 shRNA. An adenovirus expressing RFP was used as control. Motor neurons were plated onto fully differentiated oligodendrocytes for all conditions. Wild type oligodendrocyte co-cultures did not affect MN survival regardless of the treatment (Fig 5a-b). Interestingly, mSOD1 knock down in fully differentiated MBP^+ cells did not prevent oligodendrocyte-mediated MN death (Fig 5a-b). However, mSOD1 knock down in OPCs, before differentiation into oligodendrocytes, completely rescued MN survival (Fig 5a-b). To confirm that indeed the transgene expression had been decreased in both conditions, human SOD1 levels were measured by ELISA and the results showed in both cases a 40-50% decrease in mutant protein (Fig 5c).
Of interest, the reported increase in MN survival was accompanied by near baseline excitability and transient TTX-sensitive sodium current density compared to untreated oligodendrocytes (Fig 5d-e).

Interestingly, early mSOD1 knock down also resulted in an increase in lactate content in the oligodendrocyte-conditioned medium (Fig. 5f), while mSOD1 knock down after full differentiation had no effect (See SI Appendix Fig S10a).
These results indicate that mSOD1 damage to oligodendrocytes, at least *in vitro*, is related to their maturation and it is irreversible via transgene knock down in fully differentiated cells.

SOD1 is a common target in oligodendrocytes of sporadic and familial ALS cases with different genetic origins, but not for C9orf72 cases

One of our previous studies indicated that SOD1 could play an important role in various variants of ALS, not only in cases carrying mutations in SOD1(14). The availability of several human cell lines gave us the opportunity to test this finding in our new co-culture model by knocking down human SOD1 in oligodendrocytes from patients affected by sporadic as well as familial ALS. Similar to the murine study, SOD1 was knocked down at the OPC stage (i.e., 7 days into the differentiation protocol) or at the final stage of differentiation (i.e. 30 days post NPC plating) and MNs were seeded in both cases on fully differentiated MBP^+ cells. Oligodendrocytes derived from iNPCs (3 non-ALS samples, 3 sporadic, 3 samples carrying C9orf72 repeat expansion, 1 familial SOD1 and 1 familial TDP43 case) were tested. SOD1 knock down did not affect survival of MNs on the control lines (Fig. 6a-b). Strikingly, oligodendrocytes from sporadic ALS patients, as well as the familial cases, were responsive to SOD1 knock down. Motor neuron survival was approximately doubled to 70-80% on sporadic ALS oligodendrocytes, while complete rescue was achieved in mutant SOD1 and TDP43 cases (Fig. 6a-b). Again, similar to the mouse oligodendrocytes, this rescue was only observed if the knock down was performed early during the differentiation of the oligodendrocytes and not after completion of maturation. In contrast, SOD1 knock down in oligodendrocytes carrying C9orf72 mutations did not ameliorate MN survival in co-culture, regardless of the timing of knock down (Fig 6a-b). This indicates that SOD1 is not involved in the pathway leading to MN death caused by this mutation.
Consistent with these findings, the conditioned medium of oligodendrocytes from sporadic as well as familial cases associated with mutations in SOD1, TARDBP and FIG4 lost its toxicity to MNs when SOD1 knock down was achieved at the progenitor stage (Fig. 7a). This loss of toxicity/lack of support also correlated with restored levels of lactate in the

growth medium (Fig 7b,c), which were not achieved when SOD1 shRNA treatment was performed in MBP^+ cells (See SI Appendix Fig. S10b).
Consistent with the lack of MN rescue in co-culture after SOD1 knock down, the conditioned medium of oligodendrocytes carrying C9orf72 mutations remained toxic/unsupportive of WT MNs.
To strengthen the association reported here between sporadic ALS and SOD1, we tested our oligodendrocytes for the presence of misfolded SOD1 with the antibody B8H10 (MediMabs) raised against misfolded $SOD1^{G93A}$. Several reports have, in fact, brought to light that spinal cord biopsies from sporadic ALS patients display considerable levels of WT SOD1 misfolding (23, 24).
Oligodendrocytes derived from familial patients carrying mutant SOD1, or TDP43 or FIG4, as well as sporadic patients, displayed B8H10 positive signal, indicating presence of misfolded SOD1 (Fig. 8a-c). Oligodendrocytes carrying C9orf72 repeat expansion did not display misfolded SOD1 (Fig. 8d). Interestingly, SOD1 knock down at the early stage of differentiation successfully eliminated these aggregates (Fig 8e-h) as well as restoring MN survival (Fig. 7a). On the contrary, SOD1 knock down after differentiation was ineffective in eliminating SOD1 aggregates (See SI Appendix Fig. S10c), thus suggesting that misfolded SOD1 is implicated in the pathogenic mechanism leading to MN death.

Discussion

Oligodendrocytes have been implicated in the pathogenic mechanisms occurring in ALS only recently(16-18). It has been shown that oligodendrocytes are severely affected during disease and their degeneration occurs before motor neuron death. In an attempt to compensate for oligodendrocyte loss, progenitor cells have been reported to be highly proliferative, but also fail to reach maturation. As a result, the motor fibers in both mouse models and in ALS patient spinal cords show signs of evident demyelination(17). Interestingly, neither the ALS mouse model nor ALS patients show defects in developmental myelination. The data collected in the mSOD1 mouse, in fact, suggest that only the adult-born progenitors are unable to differentiate.

The present study provides the first *in vitro* model of mouse and human oligodendrocyte-motor neuron co-cultures to investigate the role of this cell type in ALS. In this study we

analyzed the effect of oligodendrocytes from mSOD1 mice and WT littermates on WT motor neurons.

Importantly, human oligodendrocytes were differentiated from human fibroblasts reprogrammed using two different methods, i.e. the classical iPSCs reprogramming(25) and the recently published direct conversion from fibroblasts to induced neural progenitor cells(19). This new and fast reprogramming method enabled us to include a high number of human cell lines. Taking advantage of these two methods, we analyzed oligodendrocytes from non-ALS controls, including a Becker muscular dystrophy patient, and several ALS patients, including both sporadic and familial cases, carrying mutations in *SOD1, TARDBP, C9orf72 and FIG4*.

Our *in vitro* data indicate that both mouse and human progenitors from non-ALS and ALS samples can efficiently differentiate into oligodendrocytes that express the main typical cellular markers in agreement with a recent report (21).
Although we did not detect any difference in oligodendrocyte survival between ALS and control samples during the time the cells were differentiated and kept in co-culture, we did observe that MBP^{+} cells from mSOD1 mice and ALS patients, induced motor neuron death. In addition, because the mouse model provided a slower *in vitro* assay, we were able to perform whole cell patch clamp recordings, which showed that the oligodendrocytes expressing mSOD1 can induce substantial electrophysiological changes in WT motor neurons prior to cell death. Similar results have been reported in mSOD1 mice(26), where increased persistent sodium currents were identified as selectively altered and leading to hyperexcitability. The pattern described in this study *in vitro*, and previously reported in the mouse model(26), is in perfect agreement with the findings that cortical hyperexcitability is one of the first alterations detected in ALS patients(27), even before disease onset(28). While the origin of this phenomenon is still unknown, our results suggest that oligodendrocytes are involved in this pathologic mechanism. Moreover, consistent with data from the mouse model(17, 18), both mouse and human ALS oligodendrocytes *in vitro* display impairment in lactate production and release along with downregulation of the lactate transporter MCT1.

Human oligodendrocytes from both sporadic and familial cases carrying different mutations, i.e. SOD1, TDP43, FIG4 and C9orf72, and obtained through different reprogramming

protocols, all induced a significant decrease in motor neuron survival within 72h from motor neuron seeding. While motor neurons plated on non-ALS oligodendrocytes develop long and highly branched neurites over time, most cells plated onto ALS oligodendrocytes die without extending processes or making neuritic connections. This result is particularly interesting in light of the new finding that oligodendrocytic connexin expression is significantly decreased in the spinal cord of the mSOD1 mouse model, whilst the inhibitory molecule Nogo-A is upregulated(29).

In contrast to the mechanisms of human astrocyte toxicity against motor neurons (15, 19), we found that decreased lactate levels account for a large part of the conditioned medium-mediated toxicity associated with oligodendrocytes. In fact, not only motor neuron survival improves with ALS oligodendrocyte conditioned medium dilution, but addition of lactate to the monocultures completely rescues motor neuron survival in all ALS-derived cases, with exception of samples carrying C9orf72 mutations.

Interestingly, we had previously reported that lactate secretion deficiency is also involved in astrocyte-mediated toxicity in mutant SOD1 astrocytes from the $SOD1^{G93A}$ mouse(30), thus indicating that lactate production and secretion might be an impairment common to multiple cell-types. Indeed oligodendrocytes secrete trophic factors and metabolic substrates that promote motor neuron survival. In fact, wild-type oligodendrocyte conditioned medium supports motor neurons in monoculture as well, if not slightly better, than motor neuron medium enriched with several growth factors and supplements.

Similarly to astrocytes (15, 19, 30-32), however, we report that oligodendrocytes affect motor neurons through two distinct mechanisms of action: soluble factors and cell-to-cell contact, with the latter being more aggressive. Addition of lactate to the co-cultures, in fact, does not rescue motor neurons. Another potential explanation for the data here presented is that, in addition to lactate, the toxic factor(s) secreted by oligodendrocytes are short-range diffusible factor(s) or highly labile soluble factor(s), which need the two cell types to be in a close range. In this case, even if all toxic factors were soluble, they would need the two cell types to be in close vicinity.

Particularly interesting is the finding that SOD1 knockdown in finally differentiated mSOD1 carrying murine oligodendrocytes did not improve motor neuron survival. While on the other hand, SOD1 knock down was effective when performed in the progenitor cells of the very same animals, resulting in restored levels of lactate secretion from oligodendrocytes and rescue of motor neuron survival as well as their electrophysiological properties. Although

the aberrant characteristics of mouse ALS oligodendrocytes did not alter classic oligodendrocyte marker expression, the lower levels of lactate in the growth medium revealed that ALS oligodendrocytes are partially dysfunctional. Of note, the difference between secreted lactate levels in control and ALS samples builds up during cell differentiation, indicating that the presence of SOD1 during ALS-derived oligodendrocyte differentiation causes an intrinsic damage interfering with their functionality, thus resulting in a phenotype that is irreversibly deadly to motor neurons.

Of therapeutic interest is that SOD1 knock down has the same effect on human oligodendrocytes from various sporadic and familial ALS patient samples. Our group previously reported that SOD1 knock down in human post-mortem NPC-derived astrocytes has beneficial effects on motor neuron survival in culture(14). In this current study, a different virus and a different, commercially available, shRNA sequence were used to further strengthen that the effect was specific to SOD1 knock down rather than potential off target effects. Again, this study mirrors results that were obtained in the astrocytes differentiated from the post-mortem NPCs. The novelty of the data presented here is the finding that in oligodendrocytes, timing for SOD1 suppression is important. Although the mechanisms behind this process are unknown, our study suggests that ALS oligodendrocyte function is affected directly or indirectly by SOD1.

Our microarray data, which is in line with multiple previously published studies, show that even after maturation, oligodendrocytes are highly energy demanding cells, sensitive to ER stress (33, 34), both mechanisms that have been implicated in the early phases of ALS (35). Consistently with the hypothesis that metabolic failure might be responsible for the reported dysfunction of ALS oligodendrocytes, we show that these cells are unable to produce and provide metabolic substrates to motor neurons.

In agreement with observations from post-mortem tissues (24), which identified misfolded SOD1 and other protein aggregates predominantly in periaxonal oligodendrocytes in the spinal cord of sporadic ALS cases, our human ALS oligodendrocytes display misfolded SOD1 aggregates, mostly with perinuclear localization.

Importantly, samples carrying C9orf72 repeat expansions did not display misfolded SOD1 aggregates, did not respond to SOD1 knock down at any time point and also did not display dysfuction in lactate release. This adds to the evidence indicating that this mutation defines a specific subgroup of ALS patients within a neuropathological spectrum (36, 37). Of importance, conditioned medium from C9orf72 mutant oligodendrocytes still causes motor

neuron death, but likely through different mechanisms compared to sporadic and SOD1-related familial ALS, as they did not react to the same treatment.
The finding that the presence of misfolded SOD1 in sporadic and familial oligodendrocytes, but not C9orf72 samples, correlates with the metabolic ability of oligodendrocytes to produce and secrete lactate suggests that WT SOD1 misfolding might be implicated in sporadic ALS through a dysregulation of metabolic pathways. In support of this hypothesis, using yeast and human cell lines, Reddi and Culotta identified a new role in cellular metabolism for SOD1: to integrate signals from oxygen and glucose to repress respiration within cells (38). The action of SOD1 knockdown, however, is not limited to the restoration of secreted lactate levels, as this approach successfully rescues motor neurons in co-cultures, while simple addition of lactate to the medium only marginally improves neuronal survival.
Although more work needs to be done to determine how WT SOD1 is implicated in sporadic ALS, the present results support a potential role of SOD1 in metabolic pathways, which might lead to a cascade of events altering the signaling pathways between oligodendrocytes and motor neurons.

In conclusion, the present study provides the first *in vitro* model to study the pathogenic features of human ALS oligodendrocytes and their contribution to motor neuron death. The new and fast direct conversion method proved equally efficient in producing differentiated oligodendrocytes compared to classical reprogramming and allowed for inclusion of multiple ALS samples carrying different mutations.
Indeed, our results indicate that there are not only distinct therapeutic windows to target different cell types involved in ALS pathology, but also different patient populations that might need to be considered separately for future clinical trials.

Materials and Methods

Mouse cultures

Primary cultures of cerebral cortical oligodendrocytes were prepared from c57/bl6 SOD1^{G93A} mice and littermate newborn mice (1-3 days old). Pups were screened for human SOD1 transgene at P1 and 3 brains from mSOD1 or control mice were pooled together.
Mixed cortical cultures were grown to confluence in DMEM containing 10% fetal bovine serum in T75 flasks and oligodendrocyte progenitors and microglia were separated from the

astrocyte monolayer through shaking (250 rpm, 37C O/N). The following morning the supernatant was collected and plated in an untreated Petri dish for 40 minutes to allow microglia to attach. Oligodendrocyte progenitor cells (OPCs) were collected in the supernatant, spun at 200g for 4 min, counted and plated in 96well plates for co-culture (30,000 cells/well) or on 1cm^2 coverslips for staining and electrophysiology recordings (150,000 cells/well).

OPCs were cultured in DMEM with 10% serum for 4h. Subsequently, the cells were washed twice with PBS to remove traces of serum and the medium was switched to DMEM/F12 supplemented with 2% B27, 20 ng/mL PDGFaa for 48h. The cells were then cultured without PDGFaa and with IGF-1 (20ng/ml) for 72h.

Detailed descriptions of all methods, reagents, and information about the cell lines, as well as analysis, are provided in SI Methods.

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Titles and legends to figures

Figure 1. Efficient differentiation of human neural progenitors into MBP$^+$ oligodendrocytes. Schematic representation of human NPC differentiation into MBP+ oligodendrocytes (a) and expression of oligodendrocyte markers at the end of differentiation determined by Q-PCR and normalized to b-actin (b). Expression levels are relative to whole spinal cord homogenates. Transcripts were investigated in 4 lines, 2 derived from iNPCs (AG08 and 201) and 2 from iPSCs (iPS4 and 009). Two lines were healthy controls (AG08 and iPS4), and 2 were ALS patients (201 and 009). N=3 per sample; error bar=SD. Scale bar =30 μm.

Principal component analysis reveals that iOligodendrocytes, iAstrocytes and fibroblasts are 3 distinct cell populations (c) based on a two-way ANOVA multi-group comparison analysis ($p<0.001$). Differentially expressed transcripts were visualised in a heat map, identifying iOligodendrocytes and iAstrocytes as more closely related cell types than they are to fibroblasts, even if significantly different (d).

Figure 2. Oligodendrocytes from ALS samples reduce motor neuron survival. Co-culture of mouse oligodendrocytes from mSOD1^{G93A} mice and WT Hb9-GFP motor neurons result in reduced motor neuron survival after 11 days compared to WT oligodendrocyte co-cultures. This is accompanied by reduction in axonal length and branching (a). Scale bar 100 μm, error bar = SD, n=6

Co-culture of human iPSC and iNPC-derived oligodendrocytes from sporadic and familial ALS patients results in 50% increased cell death 72h after plating the motor neurons (b, c). Scale bar 50 μm, error bar = SD, n=3 per line.

Figure 3. Oligodendrocyte conditioned medium from ALS samples induces motor neuron death and is associated with decreased lactate levels. Hb9 GFP$^+$ MN treated with increasing percentages of oligodendrocyte conditioned medium (CM) from the mSOD1^{G93A} mouse model displayed a significant increase in cell death, while increasing percentages of CM from WT oligodendrocytes to MN medium slightly improved, but did not significantly change MN survival (a), thus we are representing the 100% CM condition. Increased mSOD1^{G93A} oligodendrocyte CM-induced MN death was accompanied by significantly lower levels of lactate in the CM (b). As levels of secreted lactate increase in WT cells as they differentiate into oligodendrocytes, mSOD1^{G93A} cells display lower increments resulting in a significant difference in secreted lactate at the end of the differentiation protocol.

Hb9 GFP$^+$ MN treated with CM from human fully differentiated oligodendrocytes from both iNPCs and iPSCs also display increased cell death when treated with increasing percentages of ALS CM (c). Similarly to the mouse data, human ALS cells secrete progressively less lactate than control cells as they differentiate into oligodendrocytes with exception of samples carrying C9orf72 repeat expansion (d, e). Error bar = SD, n=3-4 per line

Figure 4. Lactate is a major component of CM-mediated toxicity, but not in co-culture. Monocultures (mc) (a-d) of Hb9 GFP$^+$ MN were treated with 100% WT or SOD1^{G93A} oligodendrocyte conditioned medium (CM) with addition of 1 or 2 mM lactate, resulting in MN rescue, while co-cultures (cc) treated with 2mM lacate (e-g) only showed a minimal increase in MN survival.

Similarly, monocultures of Hb9 GFP$^+$ MN treated with 100% CM from human control or ALS oligodendrocytes plus 1 or 2 mM lactate showed increase in MN survival with exception of MNs treated with C9orf72 CM (h-l). Lactate supplementation only marginally, but significantly, rescued MN survival in co-culture (m-o). Statistical significance refers to One-way ANOVA with multi-comparison test of each treated sample against it own untreated control.

Figure 5. Knock down of the human SOD1 transgene in mSOD1^{G93A} oligodendrocyte progenitors, but not finally differentiated oligodendrocytes, results in motor neuron rescue. Knock down of SOD1 in oligodendrocyte progenitors (before starting differentiation) results in complete rescue in motor neuron survival (a, b) as well as electrophysiological properties (n= 8) (d, e) and secreted lactate levels (f). No difference between mSOD1^{G93A} oligodendroctes infected with Ad-RFP and Ad-shSOD1 at the end of differentiation was detected in co-culture with motor neurons, i.e. no motor neuron rescue is achieved when mSOD1 in knocked down at the end of differentiation before co-culture (a, b). SOD1 protein levels were quantified at the end of co-culture (c). n=3 per co-culture condition. Error bar=SD, scale bar=100μm.

Figure 6. Knock down of human SOD1 in oligodendrocyte progenitors results in motor neuron rescue in sporadic and familial ALS patients, but not in patients carrying C9orf72 repeat expansions. Knock down of human wt SOD1 in human oligodendrocyte progenitors obtained from iNPCs results in a significant rescue in motor neuron survival in sporadic and familial ALS cases carrying mutations in SOD1 and TDP-43, but not C9orf72 repeat expansion (a, b). SOD1 knock down was ineffective when performed

at the end of differentiation. n=3 per co-culture condition per cell line. Error bar=SD, scale bar=50μm.

Figure 7. Knock down of human SOD1 in oligodendrocyte progenitors results in normal levels of lactate in the growth medium throughout their differentiation. Knock down of human wt SOD1 in human oligodendrocyte progenitors obtained from iNPCs or iPSCs results in rescue of MN monocultures treated with oligodendrocyte conditioned medium from sporadic and familial ALS cases carrying mutations in SOD1 and TDP-43, but not C9orf72 repeat expansion (a). This is accompanied by restoration of normal levels of secreted lactate (b, c). n=3 per culture condition per cell line (a) and lactate measurements at all timepoints (b, c)

Figure 8. Oligodendrocytes from sporadic, SOD1 and TDP43-linked ALS patients display misfolded SOD1. Oligodendrocytes from sporadic, SOD1 and TDP43-linked ALS patients, but not C9orf72-linked and unaffected individuals, display misfolded SOD1 aggregates (a-d). The pattern is mostly perinuclear (b-c). SOD1 knockdown in progenitor cells successfully eliminates such aggregates as shown via immunocytochemistry (e-g) and ImageJ analysis software (h). Scale bar=10μm