Monitoring peripheral nerve degeneration in ALS by label-free stimulated Raman scattering imaging

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The study of amyotrophic lateral sclerosis (ALS) and potential interventions would be facilitated if motor axon degeneration could be more readily visualized. Here we demonstrate that stimulated Raman scattering (SRS) microscopy could be used to sensitively monitor peripheral nerve degeneration in ALS mouse models and ALS autopsy materials. Three-dimensional imaging of pre-symptomatic SOD1 mouse models and data processing by a correlation-based algorithm revealed that significant degeneration of peripheral nerves could be detected coincidentally with the earliest detectable signs of muscle denervation and preceded physiologically measurable motor function decline. We also found that peripheral degeneration was an early event in FUS as well as C9ORF72 repeat models of ALS, and that serial imaging allowed long-term observation of disease progression and drug effects in living animals. Our study demonstrates that SRS imaging is a sensitive and quantitative means of measuring disease progression, greatly facilitating future studies of disease mechanisms and candidate therapeutics.

DOI: 10.1038/ncomms13283
Amyotrophic lateral sclerosis (ALS) patients suffer from spreading paralysis and terminal decline often within only 3 years of diagnosis. Mouse strains carrying human disease-related mutations have emerged as important models for understanding the molecular and cellular events that underlie ALS and phenocopy many processes observed in ALS patients including protein aggregation and motor neuron degeneration.

Improved methods for monitoring motor neuron degeneration are much needed, in part due to the high-degree of variability of disease progression in rodent models and ALS patients. End point analyses currently used to monitor disease progression in mouse models are also either laborious histological measurements or behavioural assays that can be impacted by operator-specific variance. Unbiased imaging methods could provide more precise measures of disease onset and progression, as well as reduce the length and ambiguity currently inherent to trials of candidate interventions in these models.

Stimulated Raman scattering (SRS) microscopy is an emerging chemical imaging technique that can map the distribution of molecules including lipids, proteins and nucleic acids in living cells and tissues based on their intrinsic molecular vibration. SRS and its precursor coherent anti-Stokes Raman scattering have been demonstrated as a powerful tool for myelin imaging. Although there is growing interest in the importance of demyelination in ALS, systematic studies of peripheral nerve degeneration have not yet been carried out with SRS.

Here we describe the use of SRS imaging to visualize peripheral degeneration in several mouse models of ALS and human postmortem tissue. We found that peripheral nerve degeneration as monitored by SRS imaging was one of the earliest detectable pathological events in ALS mouse models and that disease progression could be followed reliably over time in living animals through serial imaging. We also demonstrate that SRS imaging could be employed to evaluate candidate therapeutics, confirming that the compound minocycline significantly slows peripheral nerve degeneration in the SOD1 mouse. To demonstrate the potential clinical utility of our approach, we showed that motor nerve degeneration can similarly be monitored in postmortem tissue from ALS patients.

Results

SRS imaging of the sciatic nerve. In SRS imaging, two focused beams of synchronized picosecond pulsed lasers (pump and Stokes) are overlapped in space and time on the sample to produce SRS signal. The energy difference of the photons of the two beams can be tuned to match the vibrational frequencies of molecules to be imaged. As peripheral nerves are characterized by a myelin sheath with a high lipid content, we optimized parameters to allow imaging of lipids in fixed, isolated sciatic nerves (Methods section, Fig. 1, Supplementary Fig. 1). Collection of pump light, after the lasers' SRS interaction with the sample in focus, was achieved by the use of a high numerical aperture condenser and band-pass filter before detection with a photodiode (Methods section and Supplementary Fig. 1). After signal processing, the myelin sheath and nodes of Ranvier were clearly visualized in the nerve of wild-type (WT) animals (Fig. 1d,e) at an improved resolution and ease of execution relative to standard histological methods (Supplementary Fig. 2).

To explore whether SRS imaging might be useful for studying peripheral nerve degeneration in ALS mouse models, we imaged the sciatic nerve from the ventral root to the tibial nerve branch in 16-week-old SOD1 mice exhibiting end-stage paralysis (Fig. 1b). We found minocycline sheath discontinuity and the appearance of oval structures in these animals that seemed to be derived from degenerating nerve fibers (Fig. 1e). These 'ovoid' structures were ubiquitous throughout the regions imaged and were similar to those observed in models of nerve injury and Wallerian degeneration by histology and electron microscopy (Fig. 1b,e and Supplementary Figs 3,4a). The three-dimensional (3D) reconstruction of z-stack SRS images demonstrated that these ovoids were primarily irregular ellipsoids (Fig. 1f–j).

To determine how early degeneration in the SOD1 mouse model could be detected, we performed SRS imaging on dissected sciatic nerves at 4, 8, 12 and 16 weeks in transgenic males and their WT littermates (Fig. 1a). This timecourse suggested early changes in the peripheral myelin of SOD1 mice, as early as 4 weeks, numerous structural changes by 8 weeks and continued degeneration as animals aged (Fig. 1d–g, Supplementary Figs 3 and 5). Similar changes were observed in a smaller cohort of 12-week-old transgenic female mice (Supplementary Fig. 3b).

Ovoid structures are primarily lipids. To further understand the ovoid structures appearing in SOD1 mouse nerves, we performed whole-mount immunohistochemistry with antibodies specific to neurofilament (an axonal protein), myelin basic protein (MBP), an antigen produced by Schwann cells, and CD45, which marks infiltrating macrophages on nerve injury. We then attempted to visualize ovoid structures by SRS followed by imaging of immunofluorescence by confocal microscopy (Methods section). We found that a number of ovoids in the SRS channel were labelled by anti-MBP antibodies, while there was negligible overlap between SRS signal and neurofilament or CD45 staining (Fig. 2a, Supplementary Fig. 6).

To further characterize the chemical composition of ovoids, we conducted a detailed SRS spectral scan in both WT and SOD1 sciatic nerve. Myelin is mainly composed of cholesterol, lipid, protein and water. The lipid components of myelin include galactocerebroside, sphingomyelin, phosphatidylcholine and phosphatidyethanolamine. We used the least square method to dissociate the spectra of myelin and degenerated myelin to the seven chemicals mentioned above (Methods section). We found that ovoids had a chemical composition similar to myelin, but with a trend towards an increased abundance of lipids and a significant decrease in water and cholesterol (Fig. 2b). We, therefore, concluded that the potential clinical utility of our approach, we showed that motor nerve degeneration can similarly be monitored in postmortem tissue from ALS patients.

Quantification of myelin degeneration. We next established a computational method for quantifying lipid ovoids as a means of monitoring peripheral nerve degeneration in longitudinal optical sections. We selected three distinct lipid ovoids with typical sizes and shapes to serve as templates (Supplementary Fig. 7a). These templates were then used to generate spatial correlation maps for each of the SRS images to be quantified. Areas showing strong correlation to the templates were identified as regions of interest (ROI; Supplementary Fig. 7b and Methods section). ROIs that were found to have an area within a pre-established range (size larger than 7.4, 14.8 and 13.0 µm², respectively) were scored as lipid ovoids (Supplementary Fig. 7b).

Quantification was repeated for each of the three templates in the 20 optical slices in a given z-stack (Supplementary Fig. 7b). By summing all counts in a given z-stack, we could calculate the total number of ovoids present in a given location. Using this approach, we quantified myelin degeneration in SOD1 mice and WT littermates between 4 and 16 weeks of age. Consistent with the qualitative changes we observed by manual inspection, there was a trend for an increase in the number of lipid ovoids in...
4-week-old SOD1G93A mice (9 ± 2 ovoids per 50,000 μm² per optical section for SOD1G93A versus 5 ± 0 ovoids per 50,000 μm² per optical section for WT, N = 5 for each strain, P = 0.13; Fig. 1c) that became significant at 8 weeks of age (64 ± 22 ovoids per 50,000 μm² per optical section for SOD1G93A versus 5 ± 1 ovoids per 50,000 μm² per optical section, N = 5 for each strain, P = 0.008; Fig. 1c). As suggested by manual inspection, the extent of myelin degeneration was greater and more significant in SOD1G93A mice at 12 weeks (103 ± 16 ovoids per 50,000 μm² per optical section for SOD1G93A versus 12 ± 5 ovoids per optical section for WT, N = 5 for each strain, P < 0.001; NS is not significant).
50,000 µm² per optical section for WT, N = 5 for each strain, P = 0.00015; Fig. 1c) and 16 weeks (189 ± 52 ovoids per 50,000 µm² per optical section for SOD1G93A versus 20 ± 6 ovoids per 50,000 µm² per optical section for WT, N = 5 for each strain, P = 0.012; Fig. 1c).

As a second means of quantification, we attempted to mimic a classical approach for monitoring peripheral myelin degeneration, the counting of intact nerve fibers in cross section36. To begin, we reconstructed cross sectional images of sciatic nerves from z-stack images (Fig. 1f, Supplementary Fig. 7c). Using SRS imaging, a band-pass Fourier filter and contrast enhancement to improve the reconstructed cross sectional images, we found that we could reconstruct nerve fibers present near the surface of the sciatic nerve (Fig. 1f, Supplementary Fig. 7c). A number of representative normal and blocked nerve fibers were selected as templates and we applied a method similar to that used for lipid ovoid quantification to count the number of blocked and intact nerve fibers. This quantification method could detect a difference between WT and SOD1G93A animals. However, significance was not reached until 16 weeks of age (39.4 ± 13.0% for SOD1G93A versus 5.7 ± 3.0% for WT, N = 5 for each strain, P = 0.0030; Supplementary Fig. 8). Although these cross-sectional measurements were less sensitive than the longitudinal measurement of ovoids, they were consistent with historical counts, which detected alterations in myelinated fibers at a similar time point36.

We next asked whether degeneration occurred with any spatial preference along SOD1G93A sciatic nerves. SOD1G93A mice at 8 postnatal weeks and their WT littermates were studied to capture early changes in myelin (Fig. 1c). All microscopic fields of the freshly perfused sciatic nerves were acquired in the lipid channel and integrated by an image-processing algorithm (Methods section). The resulting images provided a high-resolution global view of myelin sheaths where any detailed regions along the nerve could be individually magnified and examined. (Supplementary Fig. 7). Automated quantification was then employed to count the planar lipid ovoid numbers of eight assigned sub-sections from the proximal to the distal end of the nerve.

We found that sites of degeneration were distributed throughout the 8-week SOD1G93A sciatic nerve (Supplementary Fig. 9a–vi). In contrast, the WT sciatic nerve showed minimal signs of damage (Supplementary Fig. 9ai–aiii). Both the proximal and the distal regions of SOD1G93A sciatic nerve manifested a significant increase of lipid ovoid deposition over the WT sciatic nerve (N = 4, P = 0.0084 for proximal region, P = 0.0018 for distal region; Supplementary Fig. 9b). We did observe a trend for increase in lipid ovoids in distal regions of the nerve, although it was not significant (N = 4, P = 0.20; Supplementary Fig. 9b) and was mirrored by a similar trend for an increased number of distal ovoids in controls.

**SRS imaging in vivo.** We next explored the feasibility of serial imaging in ALS mouse models by attempting to image the sciatic nerve in vivo using a modified optical path (Supplementary Fig. 10a). As an initial end-point imaging study, mice at 8 weeks, 12 weeks and 16 weeks, respectively were anaesthetized and immobilized on the microscope stage (Fig. 3b and Methods...
A small incision was made on the hind limb to expose the sciatic nerve and imaging was carried out avoiding muscle damage (Fig. 3c). To remove motion artifacts from respiring animal, we replaced any movement-distorted portion of images with the average of the previous and the subsequent optical slices (Supplementary Fig. 11). We again found a significant difference between SOD1G93A and WT mice at least as early as 8 weeks (74 ± 23 ovoids per 50,000 µm² per optical section, N = 5 for SOD1G93A versus 4 ± 1 ovoids per 50,000 µm² per optical section, N = 4 for WT, P = 0.031; Supplementary Fig. 12).

Following our initial success with in vivo imaging, we attempted to monitor the rate of myelin degeneration in SOD1G93A mouse.
individual animals by performing serial SRS imaging. We selected several time points for long-term survival imaging in attempt to capture the earliest onset of lipid ovoid deposition (Fig. 3a). We found that repeated imaging was well tolerated (Supplementary Fig. 1b, Supplementary Movie 1) and that imaging did not damage the nerve of control animals over the time investigated (Fig. 3d–g).

In contrast, the progression of nerve degeneration could be readily monitored in SOD1G93A mice (Fig. 3d–f). Lipid ovoid number was significantly increased in SOD1G93A mice as a group at 5 weeks postnatal (12 ± 4 ovoids per 50,000 μm² per optical section for SOD1G93A versus 3 ± 1 ovoids per 50,000 μm² per optical section for WT, N = 5 for each strain, P = 0.043; Fig. 3d,g). By 8 weeks postnatal, each of the individual SOD1G93A animals exhibited a significantly increased number of lipid ovoids relative to the average of WT animals (67 ± 9 ovoids per 50,000 μm² per optical section for SOD1G93A versus 5 ± 1 ovoids per 50,000 μm² per optical section for WT, N = 5 for each strain, P = 0.00012; Fig. 3e,g). This significant increase in myelin degeneration was most apparent at 11 weeks of age (175 ± 28 ovoids per 50,000 μm² per optical section for SOD1G93A versus 4 ± 1 ovoids per 50,000 μm² per optical section, N = 5 for each strain, P = 0.00031; Fig. 3g).

Interestingly, one SOD1G93A animal exhibited a much slower rate of progression (Fig. 3g). This outlier was later confirmed by quantitative PCR to carry a lower copy number of the human SOD1G93A transgene (Supplementary Fig. 13), which was consistent with previous observations that low copy-number SOD1G93A mice show a delayed and less severe phenotype.

Ordering events in nerve degeneration. To further understand the timing of peripheral myelin degeneration relative to other events occurring during disease progression in ALS mouse models, we performed both SRS imaging and electromyography (EMG) in 5-week-old animals. Consistent with previous studies, quantitative measures of motor neuron physiology including compound motor action potential (CMAP), motor unit size and motor unit number estimation (MUNE) did not show significant differences between 5-week-old SOD1G93A and WT mice (Fig. 4a–c and Supplementary Table 1). In contrast, five of six SOD1G93A mice imaged by SRS exhibited an increased number of lipid ovoids relative to the average of their WT littermates. Furthermore, the overall comparison between the SOD1G93A and WT cohorts showed a significant increase in lipid ovoid number within ALS animals (P = 0.034; Fig. 4d).

By needle EMG, three out of six SOD1G93A mice within the same cohort displayed active denervation with fibrillation potentials and positive sharp waves, while all WT littermates were negative (Fig. 4e). These results confirmed that fibrillation potentials and positive sharp waves in needle EMG are one of the earliest detected minimally invasive biomarkers in the SOD1G93A mouse model. Using SRS imaging, degenerative myelin morphology was observed in 4 out of 6 SOD1G93A mice, which again was not observed in any WT controls (Fig. 4f). Thus our results indicated that peripheral nerve degeneration as measured by SRS imaging reached significance at a time comparable to the initial observation of muscle denervation by EMG and weeks before the first detectable decline in motor nerve function. This early onset of nerve degeneration was confirmed in larger group of control and SOD1G93A animals at 6 weeks of age (Supplementary Fig. 14).

Evaluation of minocycline in SOD1G93A mice. The antibiotic minocycline has previously been reported to significantly extend the lifespan of SOD1G93A mice, though it later failed to show efficacy in clinical trial. We wondered whether SRS imaging was sufficiently quantitative and sensitive to be useful in revisiting the efficacy of this compound in a mouse model. As we had reproducibly observed peripheral nerve degeneration at 5 weeks of age by SRS, we initiated minocycline treatment at this age. SOD1G93A animals were randomly assigned to either minocycline (N = 15) or vehicle treatment (N = 13) groups, with daily administration via intraperitoneal injection (Methods section). In addition to control SRS imaging at 5 weeks, just before initial drug administration, we performed two later survival SRS imaging sessions on each animal in the study at 8 and 11 weeks of age (Fig. 5a).

Before the onset of drug administration (5 weeks), we found no difference between the overall number of lipid ovoids in transgenic animals randomized to the minocycline and vehicle treatment groups (11 ± 2 ovoids per 50,000 μm² per optical section for minocycline treated versus 10 ± 2 ovoids per 50,000 μm² per optical section for vehicle treated, P = 0.82; Fig. 5c and Supplementary Fig. 15a), while both surpassed that of WT animals of the same age (3 ± 1 ovoids per 50,000 μm² per optical section). In contrast, following 3 weeks of minocycline treatment animals receiving minocycline showed a significantly lower burden of lipid ovoids compared with the vehicle controls (30 ± 9 ovoids per 50,000 μm² per optical section for minocycline versus 62 ± 12 ovoids per 50,000 μm² per optical section for vehicle, P = 0.036; Fig. 5b,c), though both groups were elevated over WT control animals (5 ± 1 ovoids per 50,000 μm² per optical section). This effect of minocycline was sustained and became more significant at the 11-week imaging time point (64 ± 10 ovoids per 50,000 μm² per optical section for minocycline versus 96 ± 7 ovoids per 50,000 μm² per optical section for vehicle, P = 0.012; Fig. 5c and Supplementary Fig. 15b), although the lipid ovoid deposition in these animals was not completely rescued as demonstrated by comparison with WT animals (4 ± 1 ovoids per 50,000 μm² per optical section for WT at 11 weeks of age; Fig. 3g).

To rule out the possibility that mice with a lower copy number of the SOD1G93A transgene might have been inadvertently randomized to the minocycline group, we determined the relative copy number of the SOD1G93A transgene in each of the study animals. Although we did observed variation in SOD1G93A copy number within the overall cohort, we found that this variation was equitably distributed between the minocycline and vehicle controls, with no significant difference in copy number between the groups (Supplementary Fig. 15c). We next attempted to relate transgene copy number, drug effects and the extent of peripheral nerve degeneration as measured by lipid ovoid accumulation (Fig. 5d). Before initial drug treatment at 5 weeks, we found there was a positive correlation between transgene copy number and lipid ovoid accumulation that was similar in the two separate groups of animals.

When SOD1 copy number was incorporated into analysis of animals at 8 weeks, the positive correlation between SOD1 copy number and ovoid number remained, however, the overall frequency of lipid ovoids was significantly reduced across animals of all copy numbers in the minocycline group (Fig. 5e). A similar pattern was observed at 11 weeks (Fig. 5f). Thus SRS imaging facilitated the finding that minocycline was able to modestly but significantly reduce the burden of peripheral nerve injury regardless of transgene copy number.

Imaging SOD1G37R mice. To determine whether the early degenerative phenotype we observed were conserved in additional ALS models, we imaged dissected nerves from SOD1G37R transgenic mice. These mice exhibit a later disease onset and slower rate of phenotypic progression than observed in
SOD1G93A mice, making it a preferred model for some studies. Intriguingly, at 10 weeks of age, the earliest time point examined, we observed a significant increase in degeneration relative to controls (31 ± 12 ovoids per 50,000 \( \mu \)m\(^2\) per optical section for SOD1G37R versus 4 ± 1 ovoids per 50,000 \( \mu \)m\(^2\) per optical section for WT, \( N = 4 \) for each strain, \( P = 0.043; \) Supplementary Fig. 16d). This greatly preceded the earliest changes thus far reported by histological analysis (after 25 weeks)\(^4,27\). Further imaging at symptom onset (44 weeks) and end-stage (52 weeks) showed a further significant increase in ovoid numbers (Supplementary Fig. 16). We observed a number of lipid ovoids in end-stage SOD1G37R animals that was comparable to that we found in SOD1G93A mice (239 ± 17 ovoids per 50,000 \( \mu \)m\(^2\) per optical section for SOD1G37R 189 ± 52 ovoids per 50,000 \( \mu \)m\(^2\) per optical section for SOD1G93A; Fig. 1c and Supplementary Fig. 16d).

Non-SOD1 mouse models of ALS. We sought to employ SRS imaging in additional ALS models to expand its utility. The next model we examined was created by adeno-associated virus (AAV)-mediated expression of the C9ORF72 hexanucleotide repeat expansion throughout the central nervous system (Fig. 6a). This C9ORF72 model has been reported to exhibit neuronal RNA foci, dipeptide repeat protein inclusions, TDP-43 pathology, cortical and Purkinje cell loss as well as locomotive defects by 24 weeks\(^5\). By imaging ex vivo sciatic nerve samples of AAV-C9ORF72 mice carrying pathogenic (G4C2)\(^66\) repeats (66R) (\( N = 12 \)) and their littermate controls carrying (G4C2)\(^2\) repeats (2R) (\( N = 13 \)) between 26 weeks and 28 weeks, we observed lipid ovoid deposition in the majority of 66R mice (Fig. 6b). Quantification demonstrated a significantly increased number of lipid ovoids in 66R animals (29 ± 6 ovoids per 50,000 \( \mu \)m\(^2\) per optical section for 66R versus 13 ± 3 ovoids per 50,000 \( \mu \)m\(^2\) per optical section for 2R, \( P = 0.024; \) Fig. 6c).

We next examined a mutant FUS transgenic model in which either human WT or P525L mutant FUS is expressed from the mouse MAPT (tau) promoter (Fig. 6d). In this mouse model, it was shown ~30% of motor neurons were lost and ~40% of tibialis anterior neuromuscular junctions were denervated at 52 weeks postnatal\(^41\). By examining sciatic nerves from hFUSP525L mice, we found significant lipid ovoid deposition was already
present in these mice at 12 weeks of age (43 ± 6 ovoids per 50,000 \( \mu m^2 \) per optical section for hFUS P525L versus 15 ± 2 ovoids per 50,000 \( \mu m^2 \) per optical section for hFUS WT, \( P = 0.0008 \); Fig. 6e,f). Overall, these results validate SRS imaging as a powerful resource to study peripheral nerve degeneration in emerging ALS mouse models.

**Imaging Sciatic nerve crush and EAE models.** We reasoned that if the lipid ovoids we observed in ALS models were resulting from injuring of the underlying neuron, then similar changes in myelin morphology might be observed distally to the site of nerve crush, as a result of Wallerian degeneration. We therefore next tested the utility of SRS imaging in the context of a mouse model of axonal injury by sciatic nerve crush. We reasoned that if the lipid ovoids we observed in ALS models were resulting from injuring of the underlying neuron, then similar changes in myelin morphology might be observed distally to the site of nerve crush, as a result of Wallerian degeneration. We therefore next tested the utility of SRS imaging in the context of a mouse model of axonal injury by sciatic nerve crush. The sciatic nerve of one hind limb was crushed in WT and SOD1G93A animals then imaged distally of the injury at both 1 week and 3 weeks after initial injury (Fig. 7a).

We found WT animals exhibited significant peripheral nerve degeneration at 1 week (662 ± 287 ovoids per 50,000 \( \mu m^2 \) per optical section in crush versus 4 ± 3 ovoids per 50,000 \( \mu m^2 \) per optical section in the uncrushed, \( N = 4 \) for each group, \( P = 0.038 \); Fig. 7b-d). At 3 weeks after injury, significant recovery was observed (222 ± 90 ovoids per 50,000 \( \mu m^2 \) per optical section for

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**Figure 5 | SRS imaging of SOD1G93A under minocycline treatment.** (a) Experimental design of long-term serial in vivo SRS imaging for SOD1G93A under minocycline treatment. (b) SOD1G93A versus WT long-term serial in vivo SRS images at 8 weeks postnatal. Scale bar, 50 \( \mu m \). (c) Quantification of lipid ovoids for long-term serial in vivo SRS imaging throughout disease progression. Data are presented as mean ± s.e.m., and error bars show s.e.m. (d-f) Correlation and regression analysis between SOD1G93A copy number and lipid ovoid deposition at 5 weeks (d), 8 weeks (e) and 11 weeks (f) of postnatal age.
3 weeks after crush, \( N = 4 \) for each group, \( P = 0.038 \) versus 1 week of recovery; Fig. 7b, d). We found that the morphology of degenerating fibers after nerve crush was very similar to that observed in SOD1 mouse models, and when whole mount immunofluorescence was compared with SRS signal it again suggested that they were not likely to be infiltrating immune cells (Supplementary Fig. 17a).

We also examined the response to nerve crush in SOD1G93A mice and found that, with the exception of an elevated baseline of lipid ovoids relative to controls, these mice initially responded similarly to injury (28 ± 10 ovoids per 50,000 \( \mu \)m² per optical section for uncrushed versus 590 ± 126 ovoids per 50,000 \( \mu \)m² per optical section for 1 week after crush, \( N = 4 \) for each group, \( P = 0.038 \); Supplementary Fig. 18). However, peripheral recovery in SOD1G93A mice was muted and did not reach significance (590 ± 126 ovoids per 50,000 \( \mu \)m² per optical section for 1 week after crush versus 295 ± 135 ovoids per 50,000 \( \mu \)m² per optical section for 3 weeks after crush, \( N = 4 \) for each group, \( P = 0.62 \); Supplementary Fig. 18c).

We then compared the nature of peripheral nerve degeneration we observed in ALS and nerve crush models with that resulting from the damage of primary myelinating glial cells in the Autoimmune encephalomyelitis (EAE) model of multiple sclerosis (Fig. 7e)\(^{43,44} \). In late stage EAE animals, we clearly...
**Figure 7 | Lipid ovoid deposition could be resulted from initial axon degeneration.**

(a) Experimental design of SRS imaging for sciatic nerve crush model. (b) Lipid ovoid quantification result of sciatic nerve crush experiment. *P*<0.05. N = 4 for each strain or each age point. Data are presented as mean ± s.e.m., and error bars show s.e.m. (c,d) Sciatic nerve SRS images of WT mice after crush and recovery. Scale bar, 50 µm. (e) MS like phenotypes and disease progression of EAE model. (f) Sciatic nerve SRS images of EAE model. The left arrow shows demyelination morphology, and the right arrow shows remyelination morphology. Scale bar, 50 µm. (g) Lipid ovoid quantification result of EAE model SRS imaging.
identified several sites where the morphology of myelin was altered (Fig. 7f). Following cross sectional reconstruction of these regions from optical slices, an apparent ‘onion bulb’ morphology could be visualized (Supplementary Fig. 19a). Our analysis indicated that relative to controls EAE nerves displayed an increased proportion of under-myelinated as well as over-myelinated nerve fibers (Supplementary Fig. 19b,c).

In contrast, we observed only a modest increase in lipid ovoid number even in moribund EAE animals (14 ± 4 ovoids per 50,000 μm² per optical section for untreated versus 25 ± 5 ovoids per 50,000 μm² per optical section for EAE late stage, N = 4 for each group, P = 0.22; Fig. 7g and Supplementary Fig. 17). This change was not comparable with the dramatic increase of lipid ovoids between SOD1 end-stage and non-carrier animals. Our study demonstrated that ALS and MS models showed distinct peripheral myelin phenotypes.

**Figure 8 | SRS imaging identified lipid ovoid deposition in human ALS patient samples.** (a) Experimental design of SRS imaging for human ALS patient samples. (b) Lipid ovoid quantification of ventral (motor fiber enriched) and dorsal (sensory fiber enriched) root samples from either human ALS patients or controls. *P < 0.05, N = 4 for each group. Data are presented as mean ± s.e.m., and error bars show s.e.m. (c) SRS imaging of ventral root nerve fibers from human ALS patients versus controls. Scale bar, 50 μm. (d) SRS imaging of dorsal root nerve fibers from human ALS patients versus controls. Scale bar, 50 μm. (e) Timeline of ALS associated pathological changes and diagnostic methods. EMG, electromyography; IHC, immunohistochemistry; MN, motor neuron; NMJ, neural muscular junction; SRS, stimulated Raman scattering; TEM, transmission electron microscope.
from ALS patients (51 ± 19 ovoids per 50,000 μm² per optical section for ALS ventral versus 2 ± 2 ovoids per 50,000 μm² optical section for control ventral, N = 4 for each group, P = 0.046; Fig. 8b,c). In contrast, no significant difference was observed between dorsal roots from ALS patients and those from controls (7 ± 6 ovoids per 50,000 μm² optical section for ALS dorsal versus 8 ± 5 ovoids per 50,000 μm² optical section for control dorsal, N = 4 for each group, P = 0.88; Fig. 8b,d).

Discussion

Here we show that SRS imaging can be used to visualize peripheral nerve degeneration in ALS mouse models and post mortem materials. The sensitivity of SRS imaging was comparable to EMG for detecting early signs of neuronal degeneration, but also provided a richness of structural information about the peripheral nerve that EMG does not require. Strikingly, SRS imaging identified lipid ovoids in some SOD1G93A mice that showed no active denervation by EMG, suggesting that SRS alone or in combination with EMG may allow for the earliest in vivo detection of peripheral nervous degeneration in mouse models yet reported (Fig. 8c).

In a study of minocycline, we found that SRS imaging enabled detection of the slowing of peripheral nerve degeneration induced by this compound. In the future, SRS could be used to potentially shorten the time frame for the evaluation of candidate ALS therapeutics as well as provide a more quantitative view of drug effects.

Our SRS imaging studies suggest that lipid ovoids accumulating in the peripheral nerves of pre-symptomatic ALS animals were most likely lipids derived from degenerating myelinating cells. We also visualized and quantified the peripheral demyelinating/remyelinating processes in the EAE model of MS, which were substantially distinct from those observed in ALS models. The EAE model did not display any significant lipid ovoid deposition in peripheral nerves. Instead, we found that the pattern of degeneration in ALS mouse models and patient samples was more similar to that which occurs during Wallerian degeneration following axon injury. Notably, it has been shown that introducing the Wallerian degeneration slow (Wld(s)) mutation into the SOD1G93A background slowed disease progression. Similarly, forced expression of ATF3 (activating transcription factor 3), which is normally induced after sciatic nerve injury, also delayed muscle atrophy and extend the lifespan of SOD1G93A mice. More intriguingly but less well substantiated, SAR41, whose activation has been observed after Wallerian degeneration, falls within a region of linkage disequilibrium associated with ALS.

The imaging studies reported here would seem to support the notion that signalling pathways involved in Wallerian degeneration warrant further attention in ALS.

With the development of fiber laser SRS and SRS based histological methods for studying brain tumours, the possibility of moving SRS microscope into a surgery room or clinic for rapid label-free histology is being explored. Our studies have shown that SRS imaging might also eventually have utility for pathologists in rapid analysis of peripheral postmortem samples from ALS patients. However, given the current state of SRS technologies the greatest immediate utility of SRS imaging will likely be in the study of pre-clinical animal models.

Our natural history study of peripheral nerve injury in a variety of ALS models by SRS imaging further indicates that substantial nerve degeneration has occurred before the disease-related changes in the outward behavioural measures that are often used to determine disease onset and the commencement of drug administration studies in SOD1 models. As a result, the therapeutic window remaining at the later time when many studies normally begin may be small, which could explain why detection of drug effects in these models has often been difficult. Incorporating SRS imaging as a means of determining the earliest onset of neural degeneration and then proceeding with initiation of treatment may allow for improved sensitivity in detecting the effects of experimental interventions.

Methods

Mouse models of ALS. The mouse ALS models used in this study were the strain carrying human ALS-associated transgene SOD1G93A (B6.129S7-Tg(SOD1*G93A) 1Gur/J, JAX Laboratory, No. 002726) or SOD1G37R (B6. Cg-Tg(SOD1*G37R) 1Dwe/J, JAX Laboratory, No. 016149). To acquire animals with and without SOD1G93A transgenic in the same litter as the optical comparison, we crossed male SOD1G93A mice to non-transgenic genetic background strain C57BL/6J (JAX Laboratory, No. 000664) and SOD1G37R mice to non-transgenic C57BL/6J mice. The transgenic SOD1G93A using primers specified by JAX Laboratory (olmR0113, olmR0114, olmR3738 and olmR3739) before weaning on day 21 postnatal. After genotyping, mice carrying SOD1G93A and their non-transgenic littermates were fed with special care approved for ALS disease study. Only males (unless specified) were selected and raised for the SRS imaging experiments unless particularly mentioned. Mice with SOD1G93A transgene were weighed every 3 days after 8 weeks postnatal to monitor the onset of muscle atrophy. Normally, SOD1G93A mice start to lose weight varying from 12 to 14 weeks. ALS Behavioural staging monitor was performed starting from 12 weeks postnatal, with regular diet and hydrogel provided ad libitum. Male SOD1G93A mice with ALS level 2 were monitored for 6 weeks, while mice over ALS level 3 were monitored daily until sacrifice for imaging or histology. Unless mentioned specifically, male SOD1G93A animals were subjected to ex vivo and in vivo SRS imaging for this study to reduce the variability resulted from a population of mixed genders. Copy number quantitative PCR tests were performed based on JAX Laboratory protocols. The F6P25FL and AAV-9/C0R/F27 mouse model of ALS has been described previously.41 All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee of Harvard University. These procedures were consulted and supervised by the veterinarian of the Office of Animal Resources.

Ex vivo SRS imaging. Mice carrying SOD1G93A and age-matched non-transgenic WT littermates were imaged at the age of 4, 8, 12 and 16 weeks postnatal. Mice were anaesthetized with 4% paraformaldehyde, and their sciatic nerves were dissected and embedded at the branching point of tibial nerve. The sciatic nerves were rinsed in PBS and mounted on glass slides covered by a coverslip for SRS imaging. As described in the main text, we mainly imaged the midpoint of the dissected sciatic nerve (from the ventral root and ending at the branching point of tibial nerve).

The imaging was performed on an Olympus BX61WI upright microscope with FV300 scanning unit. The laser source employed was pico EMERALD from A.P.E., which provided a combined beam of two 7-ps lasers, whose wavelengths were 1,063 nm for Stokes beam and a tunable output for pump beam respectively. In this study, we focus on the CH2 symmetric stretching vibration in lipids (Supplementary Fig. 4b). The 1,063 nm laser was modulated by an electro-optical modulator at 10 MHz. The lasers were transmitted to sample using a 1060 nm excitation fiber and scanned by x-y galvo scanners and focused on the sample by a high NA objective. The objective we applied in the experiment was the IR version Olympus UPLANAPO 60X water immersion objective (NA 1.2). The transmitted light through the sample was collected by a Nikon oil immersion condenser (NA 1.4) and filtered by a band pass filter (Chroma, coherent anti-Stokes Raman scattering 890/220) to remove the 1,064 nm laser component. The pump laser was subsequently illuminated on a large area silicon photodiode detector (OSI Optoelectronics, S-100CL). The detected photocurrent was amplified by a filtered trans-impedance amplifier (Supplementary Fig. 20) and sent to the lock-in amplifier for demodulation. The demodulated signal was then sent to the data acquisition unit of the microscope and was eventually sent to a personal computer to show the images of the sample tissue. The optical power used on the sample is about 90 mW of pump and 80 mW of IR. The signal we get from myelin is about 110 μV with photodiode connected to a 50-ohm resistor. Our preamplifier will acquire this signal by about 23 times, and the signal level depends on the thickness of sample, so it is varies depending on the samples preparation process. The imaging speed was 1 s/frame or 1.5 s/frame in a few occasions, and the image size was 512 by 512 pixels. The 3D reconstruction was generated by imaged plug-in called volume viewer with the three dimension scales corresponds to real life sizes.

Whole-mount staining. The whole mount staining was performed based on a previously published procedure.32 After being fixed in 4% paraformaldehyde at 4 °C overnight, sciatic nerves were washed in 1% TritonX-100/PBS for 15 min. The nerve samples were then incubated in 10% donkey serum/1% TritonX-100/PBS at 4 °C overnight.
4 °C overnight. On the next day, the nerves were incubated in anti-MBP (catalog number: MAB3865M, dilution factor: 1:500)/anti-Neurolphilament (catalog number: SM-32500/1:500/100% donkey serum/1% TritonX-100/PBS. At 48 h at 4 °C, the nerves were washed with 1% TritonX-100/PBS for two-photon excitation of pump laser at around 816 nm only under SRS microscope. The neurofilament, which was tagged at 488 nm, was imaged by fluorescence imaging of MBP and neurofilament staining are performed at the SRS imaging separately. The SRS lipid imaging and two-photon excited epi-confocal system at Harvard Center for Biological Imaging and our multiple channel SRS imaging separately. The SRS lipid imaging and two-photon excited epi-fluorescence imaging of MBP and neurofilament staining are performed at the same location by blocking different lasers. The MBP, which was excited at 543 nm, was imaged by two-photon excitation of 1.063 nm laser only under SRS microscope. The neurofilament, which was tagged at 488 nm, was imaged by two-photon excitation of pump laser at around 816 nm only under SRS microscope. SRS lipid imaging was obtained when both light beams were on the same channel. The optical power used on the sample was about 90 mW of pump and 80 mW of IR. The imaging speed was 1 s/frame, and the image size was 512 by 512 pixels.

Chemical composition analysis of lipid ovoids. The nerve sample was imaged at 35 wavenumbers from 2,830 to 3,020 cm⁻¹. The first and last spectral point were not used for later spectral analysis. Seven chemicals were used for spectral retrieval. They are cholesterol(Ch), BSA, water and galactocerebroside, sphingomyelin, phosphatidylcholine, phosphatidylethanolamine. The small square method was used to retrieve the component of each chemical. We added the concentration of phosphatidylcholine and phosphatidylethanolamine to get a singlet spectrum. The lipid ovoids were manually selected and myelin sheath was selected from histogram from peak to 50% in the histogram. After point of interest was identified, the mean was then calculated for each chemical component. We imaged five locations on SOD1G93A mice and WT mice. The error bar was the standard deviation of mean in processed chemical content of five imaging locations. We used the one-tailed unpaired student t-test to calculate the P value. The imaging parameter were the same as those used for ex vivo imaging experiment. The optical power used on the sample was about 90 mW of pump in the middle of scanning range, and the power was lower at both ends of spectral scanning range. The IR power was around 80 mW. The imaging speed was 1 s per frame, which means the image for each colour takes 1 s to take, and the image size was 512 by 512 pixels.

Quantification of SRS images. The commercialized image processing program ImageJ was used as a computational tool for SRS images quantification. The plug-in "threshold" was employed to calculate the correspondence between lipid image and templates with the method called 'correlation coefficient'. The correlation pictures were then transformed to a threshold picture with an empirically chosen threshold value for each template. The areas with correlation coefficients higher than the threshold value would be designated as ROIs, which were then selected as the red color. Then the function of 'Analyze Particle' in ImageJ was used to select the ROIs satisfying defined roundness and size requirements. The circularity was assigned to be 0.1 smaller than the circularity obtained by template on the image from where the template had been selected. The size of the ROI was empirically selected, which was normally around 20% smaller than the size of original templates. The results of the 'Analyze Particle' Function for each template were saved to a spreadsheet, and the total number was quantified. If the positions of the red areas counted by 'Analyze Particle' function from two templates overlapped, it would be counted for only once. 20 slices of z-stack images were analysed with this method, and the total number of lipid ovoids was calculated as the number of lipid ovoids of this z-stack image set. All sciatic nerve SRS images of ALS mouse models were quantified based on the same algorithm and template. Different lipid ovoid templates are used in human patients and mouse model quantifications.

Quantification of intact and degenerated axon numbers were performed from the 3D reconstruction of the z-stack images of sciatic nerves. The principle is the same as the one used for lipid ovoid counting. The difference was that we had 14 templates for intact myelinated nerve fibers and 4 templates for blocked nerve fibers. The ratio of blocked nerve fibers versus total myelinated nerve fibers (intact + blocked) was used to evaluate the progression of the disease.

EMG. To minimize electrical artifacts by the wound clips applied to the mice during SRS imaging; we conducted EMG first and subjected the mice for a recovery of 10 min before in vivo SRS imaging. Genotype 5-week old mice were anaesthetized by 1.5% (v/v) avertin (Sigma T8404). Animals were placed immediately on a heating pad to maintain their core temperature at 37 °C. Measurements were performed using a Cardinal Synergy EMG machine. A ground self-adhesive gelged surface electrode was placed over the tail. Potentials were recorded from several sites of the hind-limb muscles with a concentric needle electrode (30G) using a gain of 50 mV per division and a band pass filter with low and high cut-off frequencies of 20 and 10,000 Hz, respectively. The entire recording process took 15–20 min per mouse.

Minocycline and treatment and serial in vivo SRS imaging of SOD1G93A mouse model of ALS. To minimize the variation of SOD1G93A transgene copy number, we purchased 30 SOD1G93A mice at the age of 4 weeks from JAX Laboratory (Charles River Laboratories). Each mouse was in an isolated cage, and high- and low-cutout frequency settings for EMG were removed from the mice. These animals were supposed to carry 21–30 copies SOD1G93A transgene. An equal number of mice (N = 15) were randomly subjected to...
References


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