Molecular changes associated with spinal cord aging

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Abstract Age-related muscle weakness and loss of muscle mass (sarcopenia) is a universal problem in the elderly. Our previous studies indicate that alpha motor neurons (α -MNs) play a critical role in this process. The goal of the current study is to uncover changes in the aging spinal cord that contribute to loss of innervation and the downstream degenerative processes that occur in skeletal muscle. The number of α -MNs is decreased in the spinal cord of wildtype mice during aging, beginning in middle age and reaching a 41% loss by 27 months of age. There is evidence for age-related loss of myelin and mild inflammation, including astrocyte and microglia activation and an increase in levels of sICAM-1. We identified changes in metabolites consistent with compromised neuronal viability, such as reduced levels of N-acetyl-aspartate. Cleaved caspase-3 is more abundant in spinal cord from old mice, suggesting that apoptosis contributes to neuronal loss. RNA-seq analysis revealed changes in the expression of a number of genes in spinal cord from old mice, in particular genes

encoding extracellular matrix components (ECM) and a 172-fold increase in MMP-12 expression. Furthermore, blood-spinal cord barrier (BSCB) permeability is increased in old mice, which may contribute to alterations in spinal cord homeostasis and exacerbate neuronal distress. Together, these data show for the first time that the spinal cord undergoes significant changes during aging, including progressive α -MNs loss that is associated with low-grade inflammation, apoptosis, changes in ECM, myelination, and vascular permeability.

Keywords Spinal cord · Aging · Motor neurons · MMPs · Age-related neuronal loss

Introduction

Our previous studies have demonstrated that changes in motor neurons and preservation of intact muscle innervation are important contributing factors in age-related

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muscle atrophy and loss of muscle contractile function. We have reported an age-related loss of neuromuscular junction (NMJ) innervation (Jang and Van Remmen 2011), reduced synaptic transmission (Ivannikov and Van Remmen 2015), and decreased nerve conductance velocity (Walsh et al. 2015). Here, we extend our studies to define age-related changes in spinal cord that could have a direct impact on alterations in the neuromuscular junction and loss of innervation during aging. While there are numerous studies on age-related changes in brain function, aging of the spinal cord remains an understudied topic. Loss of motor neurons (MNs) and an increase in neuronal cell body size with age have previously been reported in aging spinal cord (Zhou et al. 1996; Kawamura et al. 1977a; Kawamura et al. 1977b; Tomlinson and Irving 1977; Machado-Salas et al. 1977); however, the underlying mechanisms and impact of these changes remain unknown.

The goal of this study was to investigate the landscape of changes occurring in aging spinal cord that may contribute to alpha motor neuron loss. We conducted an analysis of gene expression changes using RNA-seq and measured age-related changes in a number of potential contributing factors including inflammation, apoptosis, metabolite changes, demyelination, vascular permeability, and protein synthesis. The RNA-seq results reveal a number of changes in the transcriptome of old mouse spinal cord comparing to young, in particular, induction of MMPs and extracellular matrix (ECM) components. Our comprehensive analysis indicates a number of other changes consistent with alpha motor neuron death including activation of apoptosis, changes in metabolites consistent with neuronal dysfunction, decreased myelination, and an increase in BSCB permeability.

Materials and methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee at the Oklahoma Medical Research Foundation (OMRF). Young (2–8 months), middle-age (16–18 months), and old (24–28 months) wild-type mice (C57BL/6J) mice from the Jackson Laboratory and NIA were used for the experiments (males and females). The mice were maintained in specific pathogen-free barrier environment with unlimited access to food and drinking water.

Immunofluorescence

The lumbar portion of the spinal cord was fixed in 4% PFA overnight, then washed for 15 min in PBS twice, and incubated overnight in 30% sucrose in PBS. The excess of sucrose was removed by blotting the tissue with a moisten kimwipe and the tissue was incubated in OCT (Sakura Finetek, Torrance, CA) for 1 h, then embedded in a mixture of OCT and TFM (EMS, Hatfield, PA) 1:1 and frozen on dry ice. The following day, the blocks were sectioned on cryotome (Leica; 15–20 μ m thick) and mounted on Superfrost Plus Slides (VWR, Radnor, PA) and stored at -80°C . For immunostaining, the frozen sections were rehydrated in ddH₂O for 2 min, washed three times for 5 min in 0.3% Triton X-100 in PBS then blocked for 2 h at RT in blocking buffer (5% normal goat serum or donkey serum, 1% BSA and 0.3% Triton X-100 in PBS), then incubated overnight at 4°C with primary antibodies in the blocking solution. The next day, the slides were washed in 0.3% Triton X-100 in PBS 3×5 min and incubated with secondary antibodies in blocking buffer for 2 h at RT. Then, the slides were washed again in 0.3% Triton X-100 in PBS 3×5 min, air dried, mounted using EMS Glycerol Fluoromount with PPD anti-fading agent mounting medium (EMS, Hatfield, PA), and protected with a coverslip and sealed with nail polish. The cells were then visualized using Nikon C2 confocal microscope.

Motor neuron count and cell body area measurements

Images of every fourth section (4 sections in total per animal) were taken using ($\times 10$ objective) confocal microscope, and NeuN positive cells that were identified as motor neurons were counted for each ventral horn. Motor neurons were distinguished by size, morphology, and location (lamina IX), and their number was averaged per animal. MN area was measured using Nikon NIS-Elements Advanced Research software by tracing around the cell body. Only MNs with a visible nucleus were included in the analysis to avoid underestimating the cell body size. The person that analyzed the images was blinded to sample identity.

Electron microscopy

Electron microscopy was performed by OMRF Imaging Core to visualize white matter adjacent to the ventral horn. All axons visible in the field of view were counted

and the results were averaged. Axon diameter and myelin thickness were measured for each axon using ImageJ software.

Immunoblotting

Tissues were homogenized in RIPA buffer with protease inhibitor and the samples were resolved on a 12.5% polyacrylamide gel, then transferred overnight onto a previously methanol-activated PVDF membrane. The membrane was then stained with Ponceau stain to visualize the lanes, and cut, rinsed in TBS-T, blocked for 1 h in 5% milk in TBS-T at RT, and incubated overnight with primary antibodies in blocking buffer at 4 °C. The following day, the membrane was washed three times for 10 min in TBS-T, incubated for 1 h at RT with secondary antibodies in blocking buffer, washed again three times for 10 min in TBS-T, and developed with ECL. The band intensity was then measured using ImageJ software.

Fluoromyelin staining

Fluoromyelin staining was performed as follows: 20- μ m-thick frozen sections of lumbar spinal cord (the blocks were prepared as described above) were incubated for 20 min in 0.3% Triton in PBS and then with Fluoromyelin Red fluorescent myelin stain (Invitrogen, #F34652) 1:300 in PBS at RT for 10 min. Next, the slides were washed in PBS 3 $\times$ 10 min, mounted and visualized under confocal microscope. The images taken with $\times$ 4 objective were used for the analysis in ImageJ software. Using free-hand selection tool, the white matter area was selected and fluorescence intensity was measured, then the fluorescence intensity in the gray matter was measured and subtracted from the fluorescence intensity in white matter.

Nuclear Fast Red staining

Nuclear Fast Red was performed by Imaging Core Facility at Oklahoma Medical Research Foundation. MNs were distinguished based on their size, shape, and location. The scorer was blinded to sample identity.

Neuromuscular junction (NMJ) staining

Neuromuscular junctions were stained following a previously described method (Falk et al. 2015), with

modifications. Fresh gastrocnemius muscle was dissected, cleaned from connective tissue, and cut in small, flat pieces in cold PBS. The tissue was then fixed in 10% STUmol (Poly Scientific R&D, Bay Shore, NY) in ddH₂O for 1 h on a rocker, washed three times for 5 min, and permeabilized in 2% Triton X-100 in PBS for 30 min. After blocking overnight at 4 °C in 5% NGS, 4% BSA, 1% Triton X-100 in PBS, the samples were incubated with primary antibodies for 24–48 h. Then, the samples were washed six times for 30 min in PBS and incubated overnight with a secondary antibody and bungarotoxin conjugated to a fluorophore. Next, the samples were washed again in PBS (six times for 30 min), blotted with a moistened kimwipe, and mounted onto Superfrost Plus Slides (VWR) with EMS Glycerol Fluoromount with PPD anti-fading agent mounting medium (EMS, Hatfield, PA), and coverslipped.

Neuromuscular junction analysis

Images of NMJs ($\times$ 20) were used for measuring NMJ area, fragmentation, and denervation. NMJ area was measured with Nikon NIS-Elements Advanced Research software by tracing around the acetylcholine receptors (30–50 per animal, n = 3 per group). NMJ fragmentation was assessed by counting AChR fragments. NMJs composed of five or more fragments were considered fragmented. NMJ denervation was determined by assessing the overlap between the nerve terminals (SV2 staining) and AChR and expressed as fully innervated, partially innervated (partial overlapping), or denervated (little or no overlapping).

Cytokine panel

Cytokine abundance in the spinal cord lysates (200 μ g of total protein) was measured using Proteome Profiler Array (Mouse Cytokine Array Panel A #ARY006, R&D Systems, Minneapolis, MN), accordingly to the manufacturer's protocol and visualized by autoradiography. The film exposed for 1 h was scanned and the resulting image was further analyzed. Dot intensity was measured in ImageJ, and after subtracting averaged background, the signal was averaged among the duplicates and compared between the groups. Heatmap was generated in R Studio using pheatmap library.

RNA-seq

Total RNA was extracted from 30 mg of the spinal cord of the young and old mice using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. After the RNA isolation, the samples were processed by OMRF Clinical Genomics Center (<https://omrf.org/research-faculty/core-facilities/next-generation-sequencing/>). To assure proper quality standards, the RNA integrity and concentration were estimated with the use of Agilent TapeStation. Libraries were prepared using the TruSeq Stranded mRNA Library Kit (Illumina) and the samples were subsequently sequenced on Illumina NextSeq 500. The resulting RNA-seq data were then processed by OMRF Core facility and analyzed by OMRF Genomics and Data Science group.

Blood-spinal cord barrier permeability assay

Anesthetized mice were injected ip with 75 mg/kg HRP (type II, #P8250, Sigma, St. Louis, MO) in 0.9% saline and kept on a heating pad for 15 min, then sacrificed and spinal cord was collected and processed the same way as in the immunofluorescence protocol. The blocks were then sectioned (50 μ m thick) and HRP was visualized with the use of DAB. Two hundred fifty microliters of 1% DAB and 250 μ l of 0.3% H_2O_2 were added to 5 ml of 0.01 M PBS of pH 7.2. Slides were incubated for 3 min with 200 μ l of DAB solution at RT, then rinsed twice in PBS-Tween 20 for 2 min, dried, and coverslipped. The images taken with $\times 1$ were used to count HRP extravasation regions. Since the animals were not perfused, the blood vessels remained highlighted in the sections and were not considered during the analysis.

MRI

For MRI assessment, mice were anesthetized with isoflurane (1.0–2.0% with 0.6–0.8 L/min O_2). Mice were placed into the MRI instrument, a 7 Tesla 30-cm-bore Bruker Biospec MRI system (Bruker Biospin Corporation, Billerica, MA) with a standard Bruker volume coil. Twenty microliters of the contrast agent (0.5 mmol gadolinium diethylenetriamine penta-acetic acid/mL (Gado-DTPATM, BioPAL, Worcester, MA)) was diluted to 100 μ L in saline, and was administered intravenously via tail-vein catheter. The following scans

were done: 1H magnetic resonance spectroscopy (1H-MRS), and pre- and post-contrast and enhanced T1-weighted rapid acquisition with relaxation enhancement imaging (CE T1 RARE). A T1-RARE pulse sequence was used with the following parameters: repetition time (TR) of 1500 ms, echo time (TE) of 7 ms, 4 averages, 25 slices with a thickness of 0.8 mm/slice, a matrix of 256×256 , a field-of-view (FOV) of 4.5×4.5 cm², and motion and fat suppression.

For contrast-enhanced blood-spinal cord barrier permeability assessment (CE-MRI) sagittal T1-weighted RARE images were used. MRI signal intensities were measured from circular regions of interests (ROIs) within the spinal cord, and 3–4 ROIs were taken in cervical, thoracic, and lumbar regions of the spinal cord in pre- and post-contrast injection images and recorded as average value for each spinal region of each animal. The percent differences between signal intensities before and after contrast administration were calculated. Percent differences of signal intensity of each spinal region were recorded as mean $\pm$ standard deviation within each age group.

1H -MRS was acquired using a PRESS (Point RESolved Spectroscopy) sequence with a TE of 24.0 ms, a TR of 2500.0 ms, 256 averages, and a spectral width of 4006 Hz. A non-suppressed MR spectrum was acquired beforehand by applying eddy-current correction to maximize signal intensity and decrease the peak linewidths. Water was suppressed with a VAPOR (variable power radio frequency pulses and optimized relaxation delays) suppression scheme. In all cases, the peak width (full width at half maximum) of the water peak was less than 30 Hz following localized shimming, which was conducted by using first- and second-order adjustments with Fastmap. A cubic voxel of $1.5 \times 2.0 \times 1.5$ mm³ was positioned in the mouse spinal cord. To analyze the MRS data, an in-house Mathematica program was used (version 8.0.4.0, Wolfram Research, Champaign, IL, USA). The spectra were scaled in ppm by calibrating against the water peak (4.78 ppm). The major metabolic peaks were identified as Lipids-CH₃ at 0.9 ppm, Lipids/Lactate at 1.3 ppm, N-acetylaspartate (NAA) at 2.02 ppm, glutamate (Glu) at 2.3 ppm, creatine (Cr) at 3.02 ppm, choline (Cho) at 3.22 ppm, taurine (Tau) at 3.43 ppm, and myo-inositol at 3.57 ppm. The peak area measurements of each metabolite were used to calculate ratios against Cr (metabolite/Cr).

Eicosanoids

The spinal cords of young (10 months) and old (24 months) mice were snap frozen and sent to the lipidomics core at UCSD (<http://www.ucsd-lipidmaps.org/>) for analysis. The heatmap of the results was generated using pheatmap library in RStudio. RStudio was also used to perform PCA analysis.

F2-Isoprostanes

The abundance of F2-isoprostanes in spinal cord lysates was established following a method described by Roberts and Morrow (2000) with some modifications. In summary, 45–60 mg of previously frozen spinal cord tissue was homogenized in 10 ml of ice-cold Folch solution (CHCl₃: MeOH, 2:1) with butylated hydroxytoluene (BHT). The samples were then incubated for 30 min at room temperature. Next, 2 ml of 0.9% NaCl was added, mixed vigorously, and the samples were centrifuged at 3000g for 5 min at 4 °C. The aqueous layer was discarded, while the organic layer was dried using N₂ at 37 °C. Solid phase was extracted and F2-isoprostanes were quantified using GC-MS, with [2H₄]8-Iso-PGF₂α as the internal standard.

Protein turnover

Young (6 months) and old (23 months) male C57Bl/6 mice were used for the labeling of newly synthesized proteins with deuterium oxide (D₂O) using our previously published procedures (Drake et al. 2013; Drake et al. 2015; Reid et al. 2019). After an intraperitoneal (ip) bolus injection of 99% D₂O, mice received 8% D₂O enriched drinking water 15, 30, or 45 of days of labeling ($n = 3–5$ per timepoint). The initiation of labeling was staggered so that all mice were sacrificed at the same age. Mice were euthanized by isoflurane, immediately followed by cardiac puncture. The spinal cord was dissected, immediately frozen in liquid nitrogen, and stored at -80 °C. Approximately 30 mg of the spinal cord was pulverized using liquid nitrogen-cooled mortar and pestle. In brief, tissue was fractionated into subcellular compartments by differential centrifugation as previously described (Drake et al. 2013; Drake et al. 2015; Reid et al. 2019) to obtain mitochondrial (MITO) and cytosolic (CYTO) protein fractions. In addition, plasma was prepared using distillation for the determination of precursor pool enrichment. Protein fractions were

derivatized for analysis of deuterium enrichment by gas chromatography-mass spectrometry (Agilent 7890B GC and 5977B MS), while distilled water samples were analyzed by enhanced performance liquid water isotope analyzer (Los Gatos Research LWIA-912). Deuterium enrichment of protein was used to calculate protein fraction new using a MIDA correction for the equilibration of the precursor enrichment. From this data, one-phase associations were generated and were left unconstrained to calculate the plateau value (p , fraction new), or by constraining the plateau to the young value to calculate the rate (k , 1/day). The plateau of the fraction new is representative of the proportion of the protein pool (with 1.0 equal to 100% of the protein pool) that is turning over. For the statistics, synthesis and plateau values used an unpaired two-tailed t test.

Antibodies

The following antibodies were used for the spinal cord immunofluorescence: anti-NeuN (Cell Signaling #D3S3I, rabbit mAB, 1:500), anti-Iba1 (Wako #019-19741, rabbit, 1:500), anti-GFAP (Abcam, ab7260, rabbit, 1:1000), donkey anti-rabbit Alexa 594 (1:200), goat anti-rabbit Alexa-488 (1:200). For neuromuscular junction staining: anti-SV2 (DSHB, #2315387, mouse monoclonal, 1:50), anti-2H3 (DSHB, #2314897, mouse monoclonal, 1:50), α -BTX-Alexa 488 (Invitrogen, #B13422, 1:1000), goat anti-mouse Cy3 (Jackson ImmunoResearch, #115-165-146, 1:250); immunoblotting: anti-caspase-3 (Santa Cruz, #sc7272, mouse, 1:1000), goat anti-mouse IgG-HRP (Santa Cruz, #sc-2005, 1:10000). Anti-CD31 antibody was kindly provided by Lijun Xia from OMRF.

Statistical analysis

After confirming normality, averages (for normally distributed data) or medians (for data that does not follow a normal distribution) were analyzed using Graphpad Prism 8 software. For a comparison between two groups, unpaired Student's t test with or without Welch's correction (depending on unequal or equal variance) was employed for normally distributed data, or Mann-Whitney test for non-normally distributed datasets. Similarly, for comparisons between more than two groups, one-way ANOVA with Tukey's post hoc test or Kruskal-Wallis with Dunn's multiple comparisons post hoc test were performed.

Results

α -motor neuron number and morphology changes with age

We observed an age-dependent loss of α -MNs in the ventral horn of the spinal cord from older mice (Fig. 1a–c). The α -MN number decreases progressively with age, and middle-aged mice (17 months old) exhibit a 17% α -MN loss comparing to 3- to 6-month-old young mice that does not reach statistical significance, while the old mice (24–27 months) have 41% less MNs per ventral horn than young mice. The dynamics of MN loss emerging from this study suggests that MN loss starts early but at first progresses more slowly (between 3 and 6 and 17 months on average there are two MNs lost, while between 17 and 24–27 months, we observe an average loss of 4 MNs). α -MN morphology also changes with age, with the most pronounced change being increased neuronal cell body area (Fig. 1d, e). The timing of the cell body swelling seems to follow a similar pattern as the α -MN loss. As shown in Fig. 1e, there is an age-related right-shift in neuronal cell body area distribution (which indicates an increase in cell body area), as well as an increased variance in α -MN cell body area in old mice. In addition to measuring α -MN count on spinal cord sections immunostained for NeuN, we also performed nuclear fast red staining (Fig. S1) to make sure that the effect we see is not NeuN staining-dependent, and the results of all methods consistently support α -MN loss in aged spinal cord.

Axonal loss and a slight demyelination accompany spinal cord aging.

Axons present in aged spinal cord demonstrate similar changes as those occurring in aging α -MN cell bodies, namely a decrease in number and increase in size (Fig. 2a–c). Analysis by electron microscopy reveals axonal loss (a 40% decrease) and swelling, as well as a decreased myelin content in spinal cord from old mice compared to young mice. The ratio of the axon diameter to myelin thickness increases with age, due to a decrease in myelin thickness and an increase in axonal diameter. The slopes of the regression equation describing the relationship between the myelin thickness and the axon diameter do not differ (Fig. 2b), suggesting that the general property that bigger axons tend to have a thicker myelin sheath remains true in spinal cord from old mice.

However, the intercepts of the regression equation are significantly different ($p < 0.0001$), demonstrating that axons of a similar diameter have a thinner myelin sheath in old mice compared to young mice. Furthermore, we observe an age-related decrease in fluorescence intensity in fluoromyelin staining, which indicates a decrease in myelin abundance in aging spinal cord (Fig. 2d).

Aging spinal cord exhibits increased apoptosis and decreased neuronal health and integrity

Because we observed an age-related MN loss, we measured caspase 3 activation to determine whether an increase in apoptosis plays a role in this process. As shown in Fig. 3a and b, we found a significant increase in cleaved caspase 3 in spinal cord from old mice. In agreement with the loss of motor neurons observed in the old spinal cord, we found changes in metabolites levels revealed by MR spectroscopy, including a decrease in NAA abundance (a 14% decrease in old mice comparing to young) that indicates a decrease in neuronal health (Fig. 3c, d). Moreover, we also found an increase in glutamate levels (a 52% increase) that can also contribute to an increased MN vulnerability with age through an increased excitotoxicity, a potential mechanism of neuronal loss in neurodegenerative diseases. Levels of myo-inositol are elevated (a 37% increase) in spinal cord from old mice, which can influence the levels of secondary messengers, since myo-inositol is a precursor for inositol triphosphate. Both myo-inositol and taurine (which also increased 73% in old spinal cord) are osmotically active molecules, and changes in their concentrations could potentially disrupt ionic balance in the cell and favor cell death, as well as contribute to the swelling of neuronal soma observed in aged MNs. However, we did not observe decrease in blood vessel density with age (Fig. S2), after immunostaining for CD31; therefore, the decreased neuronal health is not due to a decrease in availability of nutrients or oxygen.

Aging spinal cord demonstrates low-grade inflammation which is not accompanied by cytokine release

Aging is associated with increased inflammation (“inflammaging”) and elevated inflammation in brain is a common manifestation of a number of neurodegenerative

diseases (Franceschi et al. 2000; Akiyama et al. 2000). To determine whether there is an increase in inflammation with age in the spinal cord, we measured two common inflammatory markers, Iba1, a marker of microglia, and GFAP, a marker of astrocyte activation, in spinal cord sections (Fig. 4a–d). Surprisingly, the increase in glial proliferation and activation was not associated with an increased cytokine release measured using a cytokine protein array (Fig. 4e, f). Only 3 out of 40 cytokines (sICAM-1, eotaxin, and IL-12p70) changed between young and old spinal cord and reached statistical

significance after adjusting for multiple comparisons. In contrast, our RNA-seq analysis did reveal an enrichment in transcripts connected to the immune system-related pathways, such as dendritic cell maturation, complement system, T helper cell differentiation, granulocyte adhesion, and diapedesis (Fig. 4g; full results of IPA analysis on RNA-seq data can be found in the Supplementary Data Table 1). However, the most enriched pathway in old spinal cord was the fibrotic pathway, which pointed toward changes in extracellular matrix.

Fig. 1 Age-related α -MN loss and morphological changes. **a** Anti-NeuN staining of a spinal cord section ($\times 4$) with marked location of the region of interest, ventral horns, where α -MNs reside; **b** a representative image of a ventral horn ($\times 10$) of a young (3–6 months), middle-aged (17 months), and old (24–27 months) mice, used for α -MN count and area measurement; **c** alpha-MN count. There is a significant α -MN loss (one-way ANOVA, $F = 11.38$, $p = 0.0014$) in old mice ($n = 5$, mean $\pm$ SD = 8.713 ± 0.97) comparing to young ($n = 5$, mean $\pm$ SD = 14.82 ± 3.28 , Tukey's multiple comparisons post hoc test, $p = 0.0010$), and middle-aged

group ($n = 6$, mean $\pm$ SD = 12.25 ± 1.18 , $p = 0.0327$); scorer was blinded; **d** There is an age-related increase in α -MN cell body area (one-way ANOVA, $F = 9.844$, $p = 0.0029$) in old mice ($n = 5$, mean $\pm$ SD = 1318 ± 144.4) comparing to young ($n = 4$, mean $\pm$ SD = 737.9 ± 263.6 , Tukey's multiple comparisons post hoc test, $p = 0.0027$), and the middle-aged mice ($n = 6$, mean $\pm$ SD = 949.2 ± 195 , $p = 0.0260$); scorer was blinded; **e** the distribution of α -MN cell body area is right-shifting with age, indicating age-dependent increase in cell body size

Extracellular matrix components and metalloproteinases levels increase in old spinal cord

The transcripts that showed the largest change with age in the RNA-seq analysis were ECM components such as collagens 28A1 (this collagen was previously associated with dysmyelination in a mouse model of Charcot-Marie-Tooth demyelinating disease, Grimal et al.

2010), 14A1, 3A1, 5A3, or 15A1, as well as matrix metalloproteinases (Fig. 5a). MMP-12 was by far the most upregulated molecule (172-fold increase) and MMP-9 was among other MMPs that change with age. While we were unable to use immunostaining to measure MMP-12 protein levels, we confirmed the age-dependent increase of MMP-9 abundance at the protein level (Fig. 5b, c). The co-immunostaining demonstrated

Fig. 2 Age-related axonal loss and demyelination. **a** A representative electron micrograph ($\times 1000$ upper row, $\times 5000$ lower row) of the white matter adjacent to the ventral horn of young (4 months) and old (24–25 months) mice; **b** Myelin thickness and axon diameter relationship in young and old mice. There is a significant difference between intercepts in linear regression equations ($p < 0.0001$), while the slope remains the same ($p = 0.9935$); **c** Aging is associated with axonal loss, since there is a significant decrease in the number of axons (one-tailed unpaired t test, $t = 4.545$, $p = 0.0052$) between young ($n = 3$, mean $\pm$ SD = $166 \pm$

24.98) and old mice ($n = 3$, mean $\pm$ SD = 99 ± 5.29); **d** A representative image of spinal cord section ($\times 4$, lumbar) stained with fluoromyelin and quantification of fluorescence intensity; we observe a decrease in fluorescence intensity between young (3–7 months, $n = 4$, mean $\pm$ SD = 27.20 ± 0.98) and old mice (27–29 months, $n = 4$, mean $\pm$ SD = 13.57 ± 7.57 ; ANOVA, $F = 9.196$, $p = 0.0067$ with Tukey's post hoc, $p = 0.0054$). The difference between middle-aged (17–18.5 months, $n = 4$, mean $\pm$ SD = 18.90 ± 1.797) and young ($p = 0.0685$), as well as old mice ($p = 0.2697$), was not significant

that most of the MMP-9 signal colocalizes with MNs; however, MMP-9 exhibits also some non-neuronal localization.

Blood-spinal cord barrier permeability increases with age

Elevated MMPs (such as MMP-9 and MMP-12) have been associated with increased blood-brain barrier

permeability in aging, as well as in the basal lamina disruption in cancer metastasis (Merdad et al. 2014; Shay et al. 2015). Thus, we asked whether the blood-spinal cord barrier (BSCB) is also affected in aging mice. Using injection of horseradish peroxidase (HRP) to measure blood vessel permeability, we found an increase in HRP extravasation in spinal cord from old versus young mice (Fig. 6a, b). Since the animals were not perfused, we can visualize blood vessels as well as

Fig. 3 SC aging is associated with increased apoptosis and decreased neuronal health and integrity. **a** Western blot of spinal cord lysates (20 μ g of total protein) of young (4 months) and old (24–25 months) mice probed for caspase-3; **b** Quantification of cleaved caspase-3 band from the blot showed in panel **a** after normalizing to GAPDH. There is a significant increase in cleaved caspase-3 abundance (Mann-Whitney test, $p = 0.0079$) in old spinal cord ($n = 5$, median = 0.1383, IQR = 0.14) comparing with young ($n = 4$, median = 0.02182, IQR = 0.029); **c** A representative MR spectrum of spinal cord of young (3–4 months) and old mice (26–27); **d** Changes in individual metabolites measured by MR spectroscopy. There is a decrease in lipids and NAA in old spinal

cord comparing to young, while the levels of glutamate, taurine, and myo-inositol increase with age (one-tailed t test with Welch's correction when necessary, or Mann-Whitney test, $n = 5$ per group); lipids: $p = 0.0040$ (young, median = 0.51, IQR = 0.10; old, median = 0.21, IQR = 0.13); NAA, $t = 2.325$, $p = 0.0243$ (young, mean $\pm$ SD = 1.766 $\pm$ 0.18; old, mean $\pm$ SD = 1.524 $\pm$ 0.14); glutamate, $t = 2.235$, $p = 0.0398$ (young, mean $\pm$ SD = 0.256 $\pm$ 0.037; old, mean $\pm$ SD = 0.39 $\pm$ 0.13); taurine, $p = 0.0437$ (young, mean $\pm$ SD = 0.164 $\pm$ 0.077; old, mean $\pm$ SD = 0.284 $\pm$ 0.082); myo-inositol, $t = 2.324$, $p = 0.0243$ (young, mean $\pm$ SD = 0.668 $\pm$ 0.016; old, mean $\pm$ SD = 0.918 $\pm$ 0.18)

the regions of HRP extravasation; however, these can be clearly distinguished. Blood vessels are clearly delineated in 2-month-old mice and differ from the large, irregular regions that correspond to HRP extravasation at 18 and 27 months of age. Old mice (27 months) have significantly more regions of HRP extravasation compared to young mice (2 months). Interestingly, the HRP extravasation regions are not uniformly distributed across the spinal cord cross section. The greatest

increase in the number of extravasation regions with age is in the ventral horn, where the α -MNs reside (Fig. 6c). However, BSCB permeability to Gd-DTPA assay using MRI revealed increased permeability only in cervical spinal cord (Fig. S3). This may be due to technical limitations of the method, since the lumbar part of the spinal cord is smaller than the cervical part, which presents a challenge while trying to detect such a change in this region of the spinal cord. Also, HRP

Fig. 4 SC aging is associated with a para-inflammatory state. **a** A representative image of Iba1 immunofluorescence in a ventral horn of young (6–7 months) and old (27–28 months) mice with an inset with a zoom showing a single cell for better morphology visualization; scale bar indicates 100 μ m; **b** Quantification of Iba1-positive microglia cells. Microglia proliferation occurs in spinal cord from old mice (Mann-Whitney test, $p = 0.0143$; $n = 4$, median = 17.58, IQR = 12.15) comparing to young ($n = 4$, median = 4.167, IQR = 2.086); **c** A representative image of GFAP immunofluorescence in ventral horn of young (6–7 months) and old (27–28 months) mice; **d** Quantification of GFAP signal. There is an

increase in GFAP fluorescence intensity in old spinal cord ($n = 3$, mean $\pm$ SD = 3.64 ± 1.114 ; unpaired t test, $t = 2.225$, $p = 0.0450$) comparing to young ($n = 3$, mean $\pm$ SD = 1.235 ± 1.5); **e** A representative image of membranes from cytokine array of young (3–4 months) and old (25 months) mice spinal cord lysates, 1 h exposure; **f** A heatmap of cytokine abundance in young ($n = 5$) and old ($n = 6$) spinal cord lysates measured with the cytokine array; **g** RNA-seq analysis—top ten most enriched pathways that are differentially expressed with age (9 vs 26–27 months) identified with IPA software and ordered by p value (with B-H correction)

visualization using DAB is a method that has much higher sensitivity than Gd-DTPA assay.

Eicosanoids and F₂-isoprostane abundance does not change in aged spinal cord

Eicosanoids are bioactive lipids that play a crucial role in inflammation and homeostasis (Dennis and Norris 2015). An increase in eicosanoid levels was

previously reported in neurodegenerative diseases (Tassoni et al. 2008), MS, and demyelination (Palumbo and Bosetti 2013), as well as in aging (see Das 2018, for review). However, we did not observe a significant change in eicosanoid abundance in aging spinal cord (Fig. 7a–c). Similarly, the level of F₂-isoprostanes, a marker of lipid peroxidation, remained unchanged in the spinal cord between 7 and 27 months of age (Fig. 7d).

Fig. 5 Age-related changes in ECM and the expression of MMPs. **a** Example of ECM components in fibrotic pathway that are differentially expressed in aging (IPA analysis on RNA-seq data); **b** MMP-9 immunofluorescence and co-localization with neuronal marker NeuN in young (6–7 months) and old (27 months) spinal

cord sections; **c** Quantification of MMP-9 fluorescence sum intensity in MNs. There is an increase in MMP-9 in MNs in old spinal cord (one-tailed unpaired *t* test, $t = 7.187$, $p = 0.0010$; $n = 3$, mean $\pm$ SD = $175,678 \pm 16,322$) comparing to young ($n = 3$, mean $\pm$ SD = $87,058 \pm 13,773$)

Aging affects spinal cord protein synthesis rate of mitochondrial but not cytosolic protein fractions

As far as we are aware, there are no previous studies that have examined the effect of aging on protein synthesis in the spinal cord. By using mitochondrial protein synthesis as a direct measure of mitochondrial biogenesis (Miller and Hamilton 2012), we determined that rates of mitochondrial biogenesis were lower in old animals (23 months) compared to young (6 months; Fig. 8a, c). In addition, the fraction of the mitochondrial protein pool that was actively turning over was lower in young mice compared to old mice (Fig. 8e). This finding indicates that for part of the mitochondrial protein pool of older mice became resistant to turnover. However, neither of these parameters were significantly different in the cytosolic fraction (Fig. 8b, d, f), indicating that these age-related differences were only in mitochondria.

Neuromuscular junction structure is affected in aging

Alterations in the interaction between motor neurons and skeletal muscle at the neuromuscular junction is a key candidate culprit behind loss of muscle mass and function during aging. Because our data showed clear loss of MNs during aging, we asked whether the timeline of changes in motor neurons and the NMJs was similar. To determine how early NMJ changes occur, we compared NMJs structure of young (4.5 months), middle-aged (17 months), and old (27 months old) mice by performing an immunostaining on gastrocnemius muscle (Fig. 9). NMJ area increases in both middle-aged and old animals compared to young mice, and this structural change appears to precede NMJ fragmentation that is evident only in old animals (NMJs with 5 or more fragments were considered fragmented). The changes at NMJ (the significant increase in NMJ area in middle-aged mice) precede then the MN loss (we observe some MN loss in the middle-aged mice, but it is not yet statistically significant at this point). We did not observe denervation in the middle-aged mice. The percentage of fully innervated NMJs is decreased compared to young mice, although the difference was not significant. As expected, the percentage of denervated NMJs increases in old mice, while the percentage of fully innervated NMJs decreases significantly.

Discussion

The goal of this study was to define differences between young and old mice spinal cord that may be related to loss of neuromuscular junction structure and function and increased denervation of skeletal muscles in sarcopenia. We measured an age-related loss of α -MNs in the lumbar part of the spinal cord in wildtype mice, characterized by a 41% decrease in α -MN cell body number and axonal loss of 40% by 27 months of age. Our results are in agreement with other studies supporting age-related α -MN loss, e.g., there are reports of α -MN loss (up to 50%) in human lumbar spinal cord from individuals older than 60 years (Zhou et al. 1996; Kawamura et al. 1977a; Kawamura et al. 1977b; Tomlinson and Irving 1977). In contrast, a recent study by Maxwell et al. (2018) reported no age-related α -MN loss based on changes of gene expression and abundance of NeuN protein measured by immunoblotting, and transcript levels rather than using α -MN counts. One possible reason for the contrasting results between this and the other studies may be because NeuN is expressed not only by α -MNs, but by other neuronal populations as well. Thus, changes in NeuN protein or transcript levels in spinal cord lysates would lead to inaccurate conclusions about any specific neuronal population size. Also, the gene expression and protein levels across lifetime are variable and can change in various diseases (e.g., decrease in NeuN protein level and loss of antigenicity was reported in stroke etc.; Duan et al. 2016), and thus may lead to an over- or underestimation of cell number. Overall, the majority of studies support a decrease in α -MN number as a function of age.

The significance of the increase in cell body size, reported by Machado-Salas et al. (1977), and also seen in our study, remains uncertain. It is possible that the increase in cell body size is the first step in neuronal degeneration process. The α -MN with larger cell bodies might be more vulnerable, since it is known that larger MNs are more affected in ALS patients (Conradi and Ronnevi 1993) and are more susceptible to polio virus infection (Hodes 1949; Hodes et al. 1949). Age-related changes in the concentration of osmolytes that we observe in MRS (N-acetyl-aspartate (NAA), taurine and myo-inositol) might be a factor contributing to increased cell body area (Erschbamer et al. 2011). It is also interesting to note that studies reporting no age-related α -MN loss do not report increased cell body size. For example, Liu et al. (1996) found that the number of

“large” neurons (α -MNs) did not change in aged cats, while the cell body area did not increase, suggesting that smaller cell body size could somehow be protective. Similar changes in α -MN morphology and number were recently reported by Gillon et al. (2018).

Our results also suggest that demyelination occurs during aging. This is consistent with our previous report that nerve conduction velocity is decreased in aging mice (Walsh et al. 2015). Demyelination has been reported in aging brain (Lintl and Braak 1983; Sherin and Bartzokis 2011), but studies of demyelination in aging spinal cord are sparse.

In addition to α -MN loss, we also found biochemical evidence of changes in metabolite levels using MR spectroscopy that confirms a decline in neuronal health and integrity. One commonly studied marker of neuronal health is NAA. The decrease in NAA in aged spinal cord we report here corresponds well with the age-

related loss of α -MNs that we observe. NAA was previously shown to decrease in the brain with age (Brooks et al. 1999), as well as in MS (Li et al. 2013) and Alzheimer’s disease (AD) patients, and correlates with cognitive decline (Jessen et al. 2001), it has also been used as a marker of neuronal loss (Singh et al. 2002). NAA is used in myelin lipid synthesis as a source of acetyl groups; thus, the decrease in NAA is also consistent with the slight demyelination that we observe. Furthermore, the decreased levels of lipids (a 56% decrease) observed in MR spectroscopy (Fig. 3c, d) support a chronic demyelination in old spinal cord. Myo-inositol, besides being an osmolyte, is often used as glial cell marker (Best et al. 2014), and the changes in its levels that we report are consistent with glial cell proliferation and activation in aged spinal cord. Moreover, myo-inositol increases in AD patients and in demyelinating diseases (Singh et al. 2002). An increase in myo-

Fig. 6 BSCB permeability increases with age. **a** A representative image of a ventral horn (10x) across different ages after HRP injection; **b** There is an increase in the number of HRP extravasation regions in old spinal cord (27 months, $n = 4$, mean $\pm$ SD = 4.875 ± 1.98 comparing to young (2 months, $n = 4$, mean $\pm$ SD = 1.08 ± 0.88 , one-way ANOVA, $F = 4.086$, $p = 0.0355$, with Tukey’s multiple comparisons post hoc test, $p = 0.0275$). The HRP extravasation at 7 months ($n = 3$, mean $\pm$ SD = 2 ± 0.88) and 18 months ($n = 4$, mean $\pm$ SD = 2.42 ± 2.025) is not statistically different from other ages; **c** Plotting HRP extravasation by location reveals that the SC region with the highest age-related increase in BSCB permeability is the ventral horn

inositol, a precursor for various secondary messengers, and in glutamate levels could contribute to neuronal distress via excitotoxicity. Thus, our MR spectroscopy results are consistent with neuronal loss, increased cell body size, increased apoptosis, demyelination, and para-inflammatory state that we observe in our immunofluorescence and immunoblotting assays.

Previous studies have reported an increase in pro-inflammatory cytokines and glial activation in aging brain, as well as in rat spinal cord (Sparkman and Johnson 2008; Xie et al. 2013; Galbavy et al. 2017; Nacka-Aleksić et al. 2017). We were interested in determining, whether there was an increase in inflammation in spinal cord from aging mice. Astrocyte, as well as microglia activation, has previously been reported in aged spinal cord, as indicated by an increase in GFAP and Iba1 signal in immunostaining in 18-month-old rat dorsal horn and posterior funiculus of the spinal cord (Xie et al. 2013, Duan et al. 2009; Parkinson et al. 2016). In addition to an increase in GFAP and Iba1, there is also an increase in CD68, a marker for microglia

and macrophages, in dorsal and ventral funiculi (Kamiya et al. 2017). GFAP was the most upregulated protein in a proteomic analysis of 2- and 24-month-old rat spinal cord (Lee et al. 2017) and a significant age-related increase in astrocyte number has also been reported (Xie et al. 2013). To investigate the role of cytokine release in our study, we measured a panel of 40 cytokines. Surprisingly, the glial cell activation in aged spinal cord that we measured using immunofluorescence was not associated with a pronounced increase in cytokine levels measured using cytokine panel, as the level of only 3 out of 40 cytokines was increased. These results demonstrate that aging spinal cord differs from the aging brain, as more pro-inflammatory cytokines, such as IL-1 β , IL-6, and TNF α , have been reported to be increased in aging brain (Yin et al. 2016; Czirr and Wyss-Coray 2012). The most increased cytokine in the spinal cord was sICAM, which is a marker of age-related inflammation that decreases in fasting in humans (Stekovic et al. 2019), and its serum level was connected to mild cognitive impairment in diabetic patients (Gorska-Ciebiada et al.

Fig. 7 The abundance of eicosanoids and F2-isoprostanes levels do not change with age in the whole spinal cord lysates. **a** A heatmap of normalized abundance of eicosanoids in young ($n = 6$) and old mice ($n = 6$), measured by lipidomics panel; **b** PCA analysis of eicosanoid abundance in young and old spinal cord; **c** Average levels (geometric mean) of eicosanoids in young and old spinal cord; **d** F2 isoprostanes abundance and young (7 months, $n = 7$), middle-aged (16–17 months, $n = 4$), and old (27 months, $n = 7$) mice spinal cord lysates

2015). It is possible to observe the low cytokine release in presence of glial activation, since there are studies reporting glial activation without increased cytokine production (Bell-Temin et al. 2015), as M2a and M2c-activated microglia were not associated with increased cytokine release. The existing literature, as well as our study, support the presence of both para-inflammatory state and demyelination in the aging spinal cord. Moreover, a recent study by Toedebusch et al. (2019) reports microglia polarization toward a pro-inflammatory phenotype preferentially in the lumbar spinal cord of aging dogs. The reported “primed” state of the microglia corresponds to microglia activation without pronounced cytokine release, similar to what we observed in this study. Toedebusch et al. also report increased microglia number in close proximity to α -MN cell bodies, which is particularly interesting in the light of the recent findings by Cserép et al. (2020) describing a neuroprotective effect of microglia on neurons via purinergic junctions and signaling. The authors demonstrated that neurons and microglia are in close proximity and communicate closely, which would

suggest that any alterations in microglial homeostasis would have a profound influence on neurons by disrupting communication.

One of the most striking changes we observed is the upregulation of ECM components in aged spinal cord revealed by our RNA-seq analysis. This corresponds to changes in spinal cord structure that are visible in electron micrographs. Furthermore, our RNA-seq and IPA analyses demonstrate that the most upregulated pathway in the aging spinal cord is the fibrotic pathway. There is a relevant link between ECM, MMPs, and neuronal viability, as summarized by McCawley and Matrisian (2001). The importance of MMPs in CNS homeostasis and pathology has been noted previously (see Yong 2005 for a review), and recently, MMPs are also getting more recognition in the field of aging (Freitas-Rodríguez et al. 2017). MMPs alter ECM properties, which changes the α -MN environment. Moreover, MMPs and ECM can regulate apoptosis by activating apoptosis-inducing ligands, such as Fas ligand (Musiał and Zwolińska 2011) and by upregulating pro-apoptotic

Fig. 8 Protein synthesis rates of mitochondrial and cytosolic protein fractions of spinal cord tissue. Mitochondrial (a) and cytosolic (b) rise to plateau of protein fraction new, which was

used to calculate synthesis (c and d) and plateau value (e and f); $n = 3-5$ /timepoint, or 11–15 per age in total; * $p < 0.05$ compared to young

genes (Dandachi et al. 2017). It is possible that the dramatic upregulation of MMP-12 (172-fold increase) that we observe in aged spinal cord can contribute to increased apoptosis in α -MN. Also, MMP-12 knock-down mice exhibit decreased apoptosis and increased myelin basic protein (MBP) levels in ischemic brain (Chelluboina et al. 2015b; Chelluboina et al. 2018). Interestingly, MBP is one of MMP-12 substrates, and the main source of MMP-12 is microglia, which undergo proliferation and activation in aged spinal cord, as we demonstrated in our study. MMP-12 was also reported to contribute to increased inflammation in aging brain (Liu et al. 2013). Moreover, MMP-12 was shown to play role in increasing BSCB permeability after spinal cord injury (Chelluboina et al. 2015a). Upregulation of MMP-9 also contributes to barrier disruption (Noble

et al. 2002), which corresponds well with changes in BSCB permeability and MMP-9 abundance that we see in aged mice. Increased BSCB permeability means that toxic metabolites could enter into the spinal cord parenchyma and further favor α -MN death. Therefore, age-related changes in the abundance of MMPs and ECM components may favor α -MN loss, and are an interesting target for future studies.

We did not detect an age-related change in the level of eicosanoids and F_2 -isoprostanes in spinal cord from older mice. An increase in F_2 -isoprostanes levels was previously reported in aging in other tissues, such as skeletal muscle (Jang et al. 2012) and brain (Nishio et al. 2006), which constitutes another difference between aging spinal cord and aging brain. However, due to the technical limitations of the assay, F_2 -isoprostanes

Fig. 9 Age-related changes in NMJ structure. **a** A representative image ($\times 20$) of NMJ of young (4.5 months), middle-aged (17 months), and old (27 months) mice, immunostained for SV2 (nerve terminals) and 2H3 (axons), as well as with bungarotoxin conjugated to a fluorophore (to visualize AChR). Insets show zoom of a single NMJ; **b** There is an increase in NMJ (AChR) area in middle-aged ($n = 3$, mean $\pm$ SD = 1402 ± 70.68 , one-way ANOVA, $F = 11.75$, $p = 0.0084$, with Tukey's multiple comparisons post hoc test, $p = 0.0074$) and old mice ($n = 3$, mean $\pm$ SD = 1295 ± 101.9 , $p = 0.045$) comparing to young mice ($n = 3$, mean $\pm$ SD = 1086 ± 65.74); **c** There is a significant age-related difference in NMJ fragmentation (NMJs with 5 or more fragments were considered fragmented) in middle-aged ($n = 3$, mean $\pm$ SD =

2.623 ± 2.284 ; one-way ANOVA, $F = 20.42$, $p = 0.0021$, with Tukey's multiple comparisons post hoc test, $p = 0.0044$) and young mice ($n = 3$, mean $\pm$ SD = 0 ± 0 , $p = 0.0029$) comparing to old mice ($n = 3$, mean $\pm$ SD = 33.32 ± 12.09); **d** Percentage of fully innervated, partially innervated, and denervated NMJs in aging and comparison of the percentage of fully innervated NMJ across different age groups. There is a decrease in the number of fully innervated NMJs in old mice ($n = 3$, mean $\pm$ SD = 26.33 ± 13.8 ; one-way ANOVA with Tukey's multiple comparisons post hoc test, $F = 48.62$, $p = 0.0002$) comparing to middle-aged ($n = 3$, mean $\pm$ SD = 85.67 ± 7.638 , $p = 0.0006$) and young mice ($n = 3$, mean $\pm$ SD = 96 ± 3.606 , $p = 0.0002$)

measurements were conducted on the whole spinal cord lysate, and thus, we cannot exclude the possibility that F_2 -isoprostanes levels might still increase in a specific cell population, such as α -MNs. Further studies are needed to address this issue.

The current study provides the first direct measure of protein synthesis in spinal cord of young and old mice. We found that mitochondrial protein synthesis, a direct measure of mitochondrial biogenesis (Miller and Hamilton 2012), was lower in older mice than young mice. This outcome is different from our previous studies in brain and muscle where mitochondrial protein synthesis rates are maintained or increased with age (Reid et al. 2019; Miller et al. 2019). Not only was the rate of mitochondrial protein synthesis lower in old than young, but the fraction of the protein pool that was actively turning in the older was also significantly lower in old than young. It is interesting that neither of these changes were present when examining cytosolic proteins. Therefore, it appears that there is some deterioration of proteostatic maintenance in mitochondrial proteins that was not present in non-mitochondrial proteins, which likely negatively impacts mitochondrial function and is consistent with at least one previous study that showed decreased mitochondrial respiration in spinal

cord, but not brain, of older rodents (Yonutas et al. 2015). Future studies should determine if the age-related deterioration of mitochondrial proteostasis is a cause or consequence of the aging process.

Figure 10 summarizes a proposed timeline of the events described in this study. The causality of the events will need to be investigated further. Based on our results, we propose that BSCB permeability may increase early, favoring neuronal loss by allowing the entry to parenchyma of toxic metabolites present in the blood. An upregulation of MMPs and neuroinflammation could possibly contribute to the decreased barrier integrity. α -MN loss corresponds in time with the beginning of demyelination and an increase in NMJ area, which may be related to neuronal loss and decreased stimulation to the muscle. As α -MN loss, demyelination, and changes in NMJ structure continue, the muscle atrophy begins to become apparent (muscle mass data taken from Muller et al. 2007). NMJ fragmentation and denervation follow, disrupting further neuronal input to the muscle which continues to degenerate. Therefore, preventing age-related α -MN loss could potentially contribute to the preservation of muscle mass in aging. One possible target would be prevention of BSCB disruption, which could decrease neuronal loss and help to maintain muscle innervation and function.

Fig. 10 Graphical summary and proposal for a timeline of the interaction between spinal cord and skeletal muscle during aging. Curves represent the measurement for which there are more than two timepoints. BSCB permeability (purple) increases early and becomes significant by 27 months of age. NMJ area (green) increases significantly by 17 months and remains elevated at 27 months. At approximately the same time (by 17 months), we start to observe α -MN loss (light blue), which is not yet significant. However, by 24–29 months, α -MN loss is pronounced and statistically significant. Demyelination (dark blue) follows a

similar pattern (increased but not significant by 17–18.5 months but pronounced by 27–29 months). NMJ fragmentation and denervation (black) occur later, and is measurable by 27 months. The increase in NMJ area and α -MN loss coincide in time with the onset of muscle atrophy (red; muscle mass data taken for reference from Muller et al. 2007—gastrocnemius mass). Concurrently, there is also age-related microglia and astrocyte activation, MMPs and ECM components upregulation, demyelination, and increased apoptosis

Interventions to preserve muscle quality could in turn further support neuronal health, as there is a cross-talk between α -MNs and muscle involving neurotrophic factors, such as NT-4 and BDNF, that are produced by the muscle. The connection between neuronal health, intact innervation, and muscle health is further supported by the recent report by Gillon et al. (2018), where the authors show attenuation in age-related α -MN loss and NMJ preservation with exercise.

In conclusion, in this study, we attempted to give a comprehensive survey of changes occurring in aging spinal cord and α -MNs. The present study represents a starting point to elucidate mechanisms behind age-related α -MN loss. We report for the first time the potential role of MMP-12 in α -MN aging and increased apoptosis. Further studies are needed to determine the meaning of the increased α -MN cell body size, as well as changes in ECM composition.

Author contributions KMP designed research, conducted experiments, analyzed the results, and wrote the paper; SB contributed western blot and EM data; KS contributed to cytokine assay and the RNA-seq experiment; KSt contributed to the RNA-seq experiment; PP performed and analyzed isoprostan assay; DS performed MRI; MZ and RG analyzed MRI data; SK, JL, RP, and BFM performed and analyzed data from the protein turnover assay; RT contributed to MRI experiment design, supervision, and interpretation; HVR supervised experiments and edited the paper.

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Compliance with ethical standards All experiments were approved by the Institutional Animal Care and Use Committee at the Oklahoma Medical Research Foundation (OMRF)

Conflict of interest The authors declare that they have no competing interests.

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