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Parkinson, Gemma M.; Dayas, Christopher V.; Smith, Doug W. "Perturbed cholesterol homeostasis in aging spinal cord", Published in *Neurobiology of Aging* Vol. 45, Issue 24 May 2016, p. 123-135. (2016)

Available from: <http://dx.doi.org/10.1016/j.neurobiolaging.2016.05.017>

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Perturbed cholesterol homeostasis in aging spinal cord.

Gemma M. Parkinson ^{a,b,c}, Christopher V. Dayas ^{b,c} and Doug W. Smith ^{a,b,c}

^a Neurobiology of Aging and Dementia Laboratory
School of Biomedical Sciences and Pharmacy,
University of Newcastle,
Callaghan, NSW, 2308, Australia

^b Preclinical Neurobiology Research Program
Centre for Brain and Mental Health Research,
University of Newcastle,
Callaghan, NSW, 2308, Australia

^c Hunter Medical Research Institute
1/Kookaburra Circuit,
New Lambton Heights, NSW 2305, Australia

* Corresponding author
Doug Smith
School of Biomedical Sciences and Pharmacy
Medical Sciences Building, Room 306C
University of Newcastle
Callaghan, NSW 2308, Australia
douglas.smith@newcastle.edu.au
Ph. 61 2 4921 7108

Author email addresses:

GMP: gemma.parkinson@uon.edu.au

CVD: christopher.dayas@newcastle.edu.au

DWS: douglas.smith@newcastle.edu.au

Abstract

The spinal cord is vital for the processing of sensorimotor information and for its propagation to and from both the brain and the periphery. Spinal cord function is affected by aging, however, the mechanisms involved are not well understood. To characterise molecular mechanisms of spinal cord aging, microarray analyses of gene expression were performed on cervical spinal cords of aging rats. Of the metabolic and signalling pathways affected, cholesterol associated pathways were the most comprehensively altered, including significant down-regulation of cholesterol synthesis related genes and up-regulation of cholesterol transport and metabolism genes. Paradoxically, a significant increase in total cholesterol content was observed – likely associated with cholesterol ester accumulation. To investigate potential mechanisms for the perturbed cholesterol homeostasis, we quantified expression of myelin and neuroinflammation associated genes and proteins. While there was minimal change in myelin related expression, there was an increase in phagocytic microglial and astrogliosis markers, particularly in the white matter. Together, these results suggest that perturbed cholesterol homeostasis, possibly as a result of increased inflammatory activation in spinal cord white matter, may contribute to impaired spinal cord function with aging.

Keywords

Aging, spinal cord, cholesterol, myelin, gene expression

1. Introduction

Aging is characterised by progressive decline in physiological function and impaired ability to respond to changes and stressors in the environment. For example, age-related changes to motor functionality result in altered gait patterns, slower movement, and impaired reaction times, all of which contribute to increased incidence of falls and reduced quality of life in the elderly. While these motor impairments are likely attributable in part to brain neurodegeneration or senescence, and to age-related degeneration of peripheral effectors such as skeletal musculature, a role for the spinal cord is also evident. The spinal cord is a vital connector and integrator, carrying and manipulating efferent motor and afferent sensory information between the brain and the periphery to control a multitude of physiological processes. Perturbed spinal cord function impacts many, if not most, of our behaviours, yet the spinal cord has received surprisingly modest interest in neurobiological studies of aging.

Research on the effects of aging on the spinal cord has mostly focused on ventral horn motor neurons and their connections to skeletal musculature, together known as the ‘motor unit’. With aging, there is thought to be a decline in motor unit number, but a compensatory increase in collateral axonal and terminal sprouting, resulting in an increase in motor unit size (Deschenes, et al., 2010; McNeil, et al., 2005; Vandervoort, 2002). Studies of lower motor neurons in aging humans and rodents have identified alterations to the cell soma size (Miyata, et al., 2008; Rodrigues de Amorim, et al., 2010), degeneration of dendritic and axonal integrity or number (Miyata, et al., 2008), alterations in protein expression (Finazzo, 1994; Hideyama, et al., 2012; Portiansky, et al., 2006; Rodrigues de Amorim, et al., 2010; Rygiel, et al., 2014; Vogelsberg, et al., 1997; Xie, et al., 2000), as well as changes in electrochemical properties and motor neuron firing rates (Christie and Kamen, 2006; Kalmar, et al., 2009; Kamen, et al., 1995; Kido, et al., 2004; Tracy, et al., 2005). However, the literature is not consistent regarding the effects of age on motor neuron number with some studies reporting no change (Chai, et al., 2011; Chopek and Gardiner, 2010), and others indicating substantial loss (Hashizume, et al., 1988; Ishihara, et al., 1987; Jacob, 1998; Kanda, 2002; Lexell, 1997; Tomlinson and Irving, 1977; Xie, et al., 2000). The reasons for the discrepancy are not clear but probably reflect species, gender, and quantification method differences. What is not disputed is the age-related decline in motor unit function, which is thought to be due, in part, to neuromuscular junction (NMJ) changes including motor neuron retraction, terminal disfigurement and sprouting, post-synaptic density fragmentation, and decreased junctional acetylcholine receptor expression (G. Valdez, et al., 2010), not to mention a host of muscle myofibre changes (Ryall, et al., 2008). Reduced motor unit recruitment and discharge rate, and premature fatigability of the NMJ are observed in middle aged rats, before changes in muscle mass (sarcopenia), suggesting that central motor system changes, potentially at the spinal cord level, are an important contributor to reduced strength and motor function with aging (Tamaki, et al., 2014). The underlying mechanisms contributing to these changes

in morphological and functional properties of motor neurons in the spinal cord and periphery with aging are not well understood.

Although much less studied than motor neurons, it has been shown that spinal cord sensory, inter and projection neurons, axonal pathways and various glial cell types are also vulnerable to aging. For example, studies of metabolite profiles suggest that neurotransmitter concentrations are perturbed with reduced GABA and glutamate levels found in spinal cords of old rats (Jing, et al., 2013). Magnetic resonance spectroscopy analysis of human spinal cords also found perturbed metabolite levels indicative of neurodegeneration or neuronal dysfunction with aging (Abdel-Aziz, et al., 2014). Axonal fibres make up the ascending and descending white matter tracts, which comprise about half of the spinal cord mass and altered structural integrity of these bundles of myelinated axonal fibres is thought to contribute to age-related decline in central nervous system (CNS) function. For example, using magnetic resonance imaging techniques, a number of studies have reported a decline in white matter integrity in cervical spinal cord of aged humans (Agosta, et al., 2007; Lindberg, et al., 2010; Van Hecke, et al., 2008; Vedantam, et al., 2013). The causes of age-related decline in spinal cord myelin integrity are not fully understood, but a loss of myelin related proteins and increased neuroinflammation have been demonstrated (Chung, et al., 2010; Lee, et al., 2013; Xie, et al., 2013).

The extent to which aging can impact spinal cord function can be readily appreciated from the altered response of spinal cords to spinal cord injury (SCI) in old animals and humans. For example, age at time of SCI is associated with delayed recovery, greater levels of disability post-injury and increased mortality in humans (Furlan, et al., 2014; Rodakowski, et al., 2014; Scivoletto, et al., 2003; Wilson, et al., 2014) and rats (Gwak, et al., 2004; Siegenthaler, et al., 2008a; Siegenthaler, et al., 2008b). Morphologically, animal studies of SCI have identified hyper activation of inflammatory mediators, greater myelin pathologies and delayed wound regeneration and repair in old animals (Genovese, et al., 2006; Hooshmand, et al., 2014; Siegenthaler, et al., 2008a). Unfortunately, the mechanisms are not well understood, but these studies suggest that aging related changes make the spinal cord more susceptible to injury and hinder recovery.

Collectively, the studies discussed above indicate that aging has a marked effect on spinal cord function. In order to further our understanding of the potential mechanisms underlying normal aging-related functional decline in the spinal cord, we carried out microarray analysis of gene expression in the cervical spinal cords from young, middle age and old rats, followed by biological pathway analysis. The cholesterol synthesis pathway was profoundly affected by aging, with the majority of genes in this pathway down-regulated with age. As cholesterol is a key constituent of myelin, and myelinated fiber tracts make up the white matter that is a major structural and functional component of the spinal cord, the cholesterol synthesis and associated pathways were selected for further investigation. Quantitative analyses of gene and protein expression and cholesterol content in the

spinal cord were performed to characterise age-related changes in cholesterol synthesis and metabolism pathways, and to investigate potential contributors to these changes including perturbed myelin integrity and inflammation.

2. Materials and Methods

2.1. Animals and tissue preparation.

Male Fischer 344 (F344) rats were used for all experiments and were group housed in a temperature and humidity controlled environment with a standard 12-hour light/dark cycle. All animals had *ad libitum* access to standard rat chow and water. At appropriate ages, 7 young (3-5 months), 5 middle aged (15 months) and 7 old (26-30 months) rats were lethally anaesthetised with Lethobarb (1 mg/kg; Virbac) and the brain and cervical spinal cord rapidly removed and cooled in ice-cold phosphate buffered saline (PBS). Tissues were blocked into forebrain, brainstem, and spinal cord segments, snap-frozen in dry-ice cooled isopentane, and stored at -80°C. Fresh frozen spinal cords were cryosectioned in the coronal plane at 50 µm thickness, along the full rostro-caudal extent of the cervical region, and sections mounted onto clean, RNase-free glass microscope slides. Sections were stored at -80°C until required for RNA, protein or cholesterol extraction.

In addition, 3 young and 3 old animals were lethally anaesthetised as above and prepared for immunohistochemistry by transcardial perfusion with 50 mL of ice-cold PBS followed by 200 mL of ice-cold 4% paraformaldehyde (PFA) in PBS. Spinal cords and brains were dissected and post-fixed for 3 hours in 4% PFA at room temperature. Cervical spinal cords were dissected at the medulla-cervical junction, and immediately below the 8th cervical spinal nerve, serially sectioned at 20 µm thickness, and stored in PBS at 4°C until required for immunolabeling.

Animal housing and procedures were carried out in strict accordance with the University of Newcastle's Animal Care and Ethics Committee regulations, the NSW Animal Research Act and Regulations, and the Australian Code of Practice for the care and use of animals for scientific purposes.

2.2 Analysis of age related gene expression changes in spinal cord.

Age related mRNA expression changes in cervical spinal cord were analysed by microarray. Briefly, twelve 50 µm thick fresh frozen sections were taken from equidistant locations along the rostro-caudal extent of the cervical spinal cord of 5 young, 5 middle age and 5 old animals. For each animal the sections were pooled and immediately homogenized in Qiazol (Qiagen) in preparation for RNA purification. RNA was extracted from all samples using the miRNeasy Mini Kit (Qiagen) according to manufacturer's instructions, quantified using the NanoDrop 1000, and stored at -80°C. Approximately 600 ng of total RNA was DNase I (Invitrogen) treated. RNA integrity analysis, cRNA amplification

and hybridisation on Affymetrix GeneChip Rat Gene 2.0 ST microarrays, were carried out at the Ramaciotti Centre for Genomics (University of New South Wales, Australia). RIN values are shown in Supplementary File 1. Microarray background correction, robust multi-array average (RMA) normalisation, and gene expression level determination, were performed using Expression Console software (www.affymetrix.com). Statistically significant changes in gene expression between the age groups were calculated using Transcriptome Analysis Console software (www.affymetrix.com).

Age-related changes in expression were confirmed by qPCR for a subset of genes identified by microarray analysis. Total RNA (400 ng) from the 5 animals of each age group used for microarray, and an additional 2 young and 2 old animals (extracted as described above and included to improve statistical power), was DNase treated (as above) and reverse transcribed using Life Technologies' SuperScript III Reverse Transcription kit, according to the manufacturer's instructions. An *18s*-rRNA reverse primer was also included in the reaction to improve transcription of non-adenylated *18s*-rRNA (Zhu and Altmann, 2005). A reverse transcriptase enzyme-negative control reaction was also performed for each sample. cDNA was diluted 1:80 in molecular biology grade water and stored at -20°C.

qPCR primers were designed using Primer Blast (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and are listed in Table 1. For each animal, qPCR reactions were performed in triplicate for each gene, using 1x SensiMix SYBR Lo-Rox qPCR mix (Bioline, Australia), 200 nM of each forward and reverse primer, and 5 µL of cDNA sample, in a total volume of 12.5 µL per reaction. Amplification was performed using an Applied Biosystems 7500 Real Time PCR machine with an initial denaturation and activation step at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60-68°C for 32 s according to preliminary primer optimisation. Melt curves were generated to confirm amplification of a single gene product. The average threshold cycle (Ct) value was calculated using the 7500 SDS software v2.0.6 (Applied Biosystems, Australia) for each gene. The relative expression of each gene's transcript abundance was determined using the delta delta CT method (Schmittgen and Livak, 2008), with the geometric mean of *18s*-rRNA, *Actb* and *Gapdh* used as the internal reference. RNA integrity was confirmed using the 3':5' ratio method for the *Actb*, *Gapdh* and *Thy1* genes, and all ratios are reported in Supplementary File 1. All ratios were < 5, which is considered acceptable for qPCR (Nolan, et al., 2006).

2.3 Spinal cord subregion-specific gene expression analysis.

Age-related gene expression changes identified by microarray analysis and confirmed by qPCR in whole cervical spinal cord sections were also examined in spinal cord subregions - white matter, dorsal grey, and ventral grey matter – to identify subregion specific patterns of expression. Briefly, four slides equidistant along the rostro-caudal axis of the cervical spinal cords (from the same animals used for microarray), each mounted with five, 50 µm thick sections, were selected. Sections were

fixed in ice-cold acetone and incubated in 2M NaCl-PBS for five minutes on ice to protect RNA integrity (Brown and Smith, 2009). Using a dissecting microscope, the white, dorsal grey, and ventral grey matter regions were dissected and collected such that individual subregions were pooled for each animal, and then homogenised in Qiazol. Total RNA was extracted and gene expression analysed (as described in section 2.2 for whole sections) including 3':5' analysis of RNA integrity. 3':5' ratios are listed in Supplementary File 2.

Gene	Forward Primer	Reverse Primer
<i>18s-rRNA</i>	CCCGAAGCGTTTACTTTGAA	CCCTCTTAATCATGGCCTCA
<i>Actb</i> 3'	CCTAGCACCATGAAGATCAAGA	GCCAGGATAGAGCCACCAATC
<i>Actb</i> 5'	ACCCAGATCATGTTTGAGACCTT	CAGAGGCATACAGGGACAAC
<i>Gapdh</i> 3'	GGCTGGCATTGCTCTCAA	GAGGTCCACCACCCTGTTG
<i>Gapdh</i> 5'	GACAGCCGCATCTTCTTG	CACCGACCTTCACCATCTTG
<i>Thy1</i> 3'	AGTTGGACTGTGTCTGTGCC	TTCGGTGATAGAAGGGGGGCT
<i>Thy1</i> 5'	CAGTCTTGCAGATGTCCCGA	GCAGTCCAGTCGAAGGTTCT
<i>Hmgcr</i>	AACCTTCTACCTCAGCAAGCC	TCTTGGTGCATGTTCCCTGC
<i>Hmgcs1</i>	CAAGATGCCACACCAGGATCT	CCGGGCGTAAGTTCTTCTGT
<i>Cyp51</i>	TTGCCTATGTGCCGTTTGGA	AACTGGCTCCTTGTTCTT
<i>Abca1</i>	CAGCAACTACAGTGGCGGTAACA	AATGCTTAGGGCACAATTCCACA
<i>Soat1</i>	CCCTTTAGTGCTCGTGTCTT	GGAACAGCATCAGCACACCT
<i>Apoe</i>	GGCTACCAACTCCATTGCCT	TTAAGCAAGGGCCACCAGAG
<i>Ch25h</i>	CTGCTCTTCGACACCGAGAT	AGGAGGACGAGTTCTGGTGA
<i>Srebf1</i>	CACCGTTTCTTCGTGGATGG	CAGTTCAATGCTCGCTCCAG
<i>Srebf2</i>	TCAATGTCAGCGGCACTACT	AGTGACAGTAGCAGATCACAG
<i>Mbp</i>	TTAAGCTGGGAGGAAGAGACAG	ATTAAGAAGCCGAGGGCAGG
<i>Mobp</i>	AAGACCAGATTGAGAAGGAGGAG	GCGGTGAAGCAGTAAATCCAC
<i>Mog</i>	TGTTCTTGACCCCTGGTTG	CAAAAGGGGTTTCTTAGCTCTTCA
<i>Mag</i>	TTGCTGAGAGCCTGTACCTG	AGAATAGGGTCCGGGTGCT
<i>Iba1</i>	ATGCTGGAGAACTTGGGGT	CCAGTTGGCTTCTGGTGTTT
<i>Cd68</i>	TGTGTCCTTCCCACAAGCAG	CAGGTCCAGGTGAATTGCTG
<i>P2rx7</i>	TGTGTCCTTTGTGGACGAGC	AAGAAGTCCGTCTGGGGTCT
<i>Trem2</i>	TCTGCGTTGAGTGGGGAAGA	CAGTCCTGGGTGTTGTGTGA
<i>Gfap</i>	CAACCTCCAGATCCGAGAAA	GCTCCTGCTTCGACTCCTTA
<i>Vim</i>	GAATTGCAGGAGCTGAATGACC	ACGGGCCTTGTCATTGGTGA

Table 1: Primer sequences for qPCR.

2.4 Western blotting.

Selected gene expression changes, assessed by qPCR, were evaluated at the protein level using western blotting. Four slides of 50 μm thick cryosections, 5 sections per slide, from 4 of the young, middle and old, fresh-frozen spinal cords (randomly selected from cords processed in Section 2.1) were taken equidistant along the rostro-caudal axis of the cervical spinal cord and proteins extracted in RIPA buffer. After brief sonication, samples were centrifuged for 15 min at 4°C and supernatants collected and stored at -80°C. 30-50 μg of protein was heated to 95°C for 5 min in Laemmli loading buffer and electrophoresed in a 4-20% SDS gel (BioRad). Proteins were transferred to nitrocellulose membranes for 60 min at 100V at 4°C. Membranes were blocked in 5% BSA or non-fat milk for 1 hour at room temperature and incubated with primary antibodies (1:500 Rab anti Acat1 (Cayman Chemical); 1:1000 Rab anti Mbp (Sigma); 1:1000 Rab anti Cnp (Sigma); 1:1000 Rab anti Olig2 (Millipore); 1:5000 Mse anti Mog (Millipore); 1:10 000 Mse anti Gfap (Millipore); 1:400 Rab anti Iba1 (Wako)) at 4°C for up to 72 hrs. Secondary antibodies were applied for 1.5 hrs at room temperature and protein bands visualized using enhanced chemiluminescence. Protein was normalized to β -Actin (1:10 000, GE Healthcare Life Sciences) and bands were analyzed using Image J.

2.5 Immunofluorescence labeling of spinal cord sections.

Selected protein changes observed by western blot were further investigated using immunolabeling so that spinal cord subregion effects could be determined. Briefly, 3 - 5 PFA-fixed sections taken equidistant along the rostro-caudal extent of the cervical cord from each of the 3 young and 3 old animals were blocked with 10% donkey serum in PBS for one hour at room temperature. Sections were then incubated in primary antibodies overnight at room temperature (1:200 Rab anti Iba1 (Wako); 1:200 Mse anti Cd68 (Abcam); 1:500 Mse anti Gfap (Millipore)), washed in PBS and incubated with fluorescent secondary antibodies (1:200, Jackson ImmunoResearch). To minimize background autofluorescence, which was particularly problematic in tissue from old animals due to accumulated lipofuscin, sections were washed in PBS after secondary antibody incubation, rinsed in water, and incubated in 1 mM copper sulfate in 50 mM ammonium acetate for 5 minutes at room temperature and briefly washed in water and PBS (Schnell, et al., 1999). Labeled sections were mounted, coverslipped, and imaged on an epifluorescence microscope.

For quantification of immunofluorescent labeling, whole sections from 3 young and 3 old animals were imaged using a 4x objective. Fluorescence intensity was determined using Image J for whole sections, grey matter, and the dorsal, lateral and ventral funiculi of the white matter. An overall average intensity was calculated for each region for each individual animal.

2.6 Cholesterol assay.

To determine whether the age-related changes in expression of cholesterol associated genes affected cholesterol levels, we quantified the relative abundance of total and free cholesterol in the cervical

spinal cord. Ten 50 µm fresh frozen sections per animal (7 young, 5 middle age and 7 old animals; Section 2.1), were homogenised in 100 uL of cholesterol extraction buffer – chloroform:isopropanol:NP-40 (1:11:0.1). Samples were centrifuged at 15,000g for 10 min. The organic liquid phase was removed, air dried at 50°C, and vacuum centrifuged to remove all traces of organic solvents. Dried lipids were resuspended in 200 uL of 1x assay diluent (Total Cholesterol Assay Colorimetric kit, Cell Biolabs) and cholesterol content determined according to manufacturer's instructions. For total cholesterol measures, the cholesterol esterase enzyme is included in the reaction prior to spectrophotometric measurement to ensure all cholesterol is available for reaction. A separate measure of free cholesterol excludes the esterase from the reaction. Post-centrifugation sample pellets were used for protein determinations using the BCA method and cholesterol measures were normalised to sample protein amounts.

2.7 Data analysis.

Microarray data: The expression levels of a total of 30429 unique RNA transcripts were quantified on each gene array. To identify significantly, differentially expressed genes with age, first poorly annotated transcripts were excluded, and then normalised expression values for the remaining 17520 genes were compared between the three age groups using one-way ANOVA. 7208 genes met the ANOVA $p < 0.05$ criterion. Multiple comparison testing effects were minimized using the Benjamini-Hochberg False Discovery Rate (FDR) correction with an $\alpha < 0.1$, filtering the dataset to 6192 genes. Then a fold-change (FC) cut off of ± 1.2 between at least two age groups (i.e., young vs middle age, middle age vs old age, or young vs old age) was also applied, resulting in 3808 genes for further analysis.

Expression patterns: The normalised expression of each significantly differentially expressed gene (DEG) was correlated with 12 theoretical expression patterns (A – L; Figure 1), with each gene being assigned to the pattern for which it had the strongest correlation (Kadish, et al., 2009). For example, pattern A represents DEGs with unchanged expression between young and middle age (represented by an expression value of 1), and an increase between middle and old age (with expression at old age represented by a value of 3). Thus, DEGs that correlated with the pattern 1, 1, 3 (Pearson's r -value ≥ 0.9) were assigned to pattern A.

DEGs assigned to each individual pattern were subject to pathway enrichment analysis using the Database for Annotation, Visualisation and Integrated Discovery (DAVID; (Huang da, et al., 2009a; Huang da, et al., 2009b)) and any significantly over-represented KEGG pathways and Gene Ontology Biological Processes for each expression pattern were identified ($p < 0.05$ after Bonferroni correction for multiple testing).

qPCR analyses: Selected age-related gene expression changes were evaluated by qPCR, using RNA from whole spinal cord sections, and significance determined by one-way ANOVA and Tukey's post

hoc analysis. Relative age-related changes in gene expression between spinal cord sub-regions (dorsal grey, ventral grey and white matter regions) were analysed using two-way ANOVA with age and spinal cord region as main effects, followed by Tukey's post hoc analysis.

Western Blot: Differential expression of proteins between the three age groups was determined using one-way ANOVA and Tukey's post hoc analysis.

Immunofluorescence: For each antibody used, the normalised intensity for whole sections was compared between young and old animals using two-tailed T-tests. Expression in the grey matter and each white matter funiculi were compared between young and old animals using two-way ANOVA and Tukey's post-hoc analysis.

Cholesterol quantification: Total cholesterol levels (normalised to total protein) and the relative abundance of total to free cholesterol in each age group were compared by one-way ANOVA and Tukey's post hoc analysis.

3. Results

3.1. Age-associated changes in mRNA expression in spinal cord.

3.1.1 Microarray results.

A total of 3808 significantly differentially expressed genes (DEGs) were identified with age in the spinal cord (ANOVA $p < 0.05$, FDR < 0.1 and minimum FC ± 1.2 criteria). Similar to a previous study, we constructed theoretical expression patterns to characterise expression changes at middle and old age, relative to young (A to L; Figure 1; (Kadish, et al., 2009)). Normalised expression values of each DEG were correlated with twelve theoretical expression patterns and each DEG was assigned to the pattern to which it most strongly correlated ($r \geq 0.90$). All DEGs were assigned to one pattern. The complete list of DEGs, their mean expression values and p-values are organised by expression pattern in Supplementary File 3.

3.1.2 Functional grouping of differentially expressed genes.

To identify potential age-related biological processes, the DEGs from each of the 12 expression patterns (A – L, Fig. 1) were subjected to biological enrichment analysis using DAVID functional analysis tools. Significant enrichment for KEGG pathways and/or Gene Ontology Biological Processes (GO BP) were identified for 9 expression patterns, with 3 patterns showing no enrichment. Representative KEGG pathways, GO BP terms, and DEGs are listed in Table 2, with a complete list of KEGG pathways, GO BP terms, and associated genes are included in Supplementary File 4. Patterns characterized by down-regulation of gene expression with age (G, H, I), were associated with

processes involved in cholesterol and lipid biosynthesis, for example, *cholesterol biosynthetic process* and *steroid biosynthesis*. Notably, there was a striking down-regulation of almost all genes involved in the cholesterol synthesis pathway (e.g. *Hmgcr* and *Cyp51*, Fig. 2). Gene expression patterns characterized by age-related up-regulation (A, B, C), were predominantly associated with inflammation and immune related functions, for example, *defense response* and *immune response*. Of the remaining 3 expression patterns showing significant enrichment (E, J, K), all 3 showed significant change by middle age that was subsequently reversed by old age (Fig. 1). Pattern E (neurotransmitter-receptor interaction) was significantly up by middle age and then returned to young levels by old age, whereas patterns J and K (protein translation) were significantly down at middle age before returning to normal by old age (K) or increasing further (J). The reasons for these bidirectional patterns are not known and we found a much greater percentage (83%) of our included genes mapped to these patterns than previously reported for mouse spinal cord (Xu, et al., 2007). The bidirectional patterns may reflect a transition where the spinal cord is initially able to compensate for age-related changes but is eventually overwhelmed – the latter potentially resulting in dysfunction.

Cholesterol is a major constituent of cell membranes, particularly of myelin, and perturbed white matter integrity is increasingly thought to be a major contributor to age-related nervous system dysfunction (for example, see review by (Lockhart and DeCarli, 2014)). White matter tracts are abundant in the spinal cord, and altered cholesterol homeostasis may affect myelin integrity and spinal cord function with age. Therefore, the cholesterol synthesis and metabolism pathways were selected for further investigation.

Figure 1: Twelve gene expression patterns, relative to young, were identified in aging spinal cords. The number of DEGs associated with each expression pattern (A to L) is indicated adjacent to its representative pattern.

Pattern	Functional Annotation	P-value	Example genes
A	K: NA GO: Defense response	2.1E-04	P2rx7, Tnfrsf1b, Stat3
B	K: NA GO: Immune response	1.5E-14	Ccl2, Cd74, RT1-Da
C	K: Natural killer cell mediated cytotoxicity GO: Cell activation	4.4E-05 3.1E-07	Cd48, Fcgr2a, Ifngr1 Elf1, Tnfsf4, Tlr4
D	K: NA GO: NA		
E	K: Neuroactive ligand-receptor interaction GO: NA	4.9E-02	Chrm4, Drd2, Gabrb3
F	K: NA GO: NA		
G	K: Phosphatidylinositol signaling system GO: NA	4.8E-02	Dgka, Pik3c2g, Plcd4
H	K: Steroid biosynthesis GO: Lipid biosynthetic process	1.1E-04 3.5E-02	Fdft1, Nsdhl, Sqle Fabp5, Mvd, Scd1
I	K: Steroid biosynthesis GO: Cholesterol biosynthetic process	9.2E-06 1.5E-10	Fdps, Hmgcr, Hmgcs1 Dhcr7, Dhcr24, Idi1
J	K: Ribosome GO: Translational elongation	5.4E-28 7.9E-26	Fau, Rpl10, Rpl36A-Ps2 Rpl10a, Rps14, Rps20
K	K: Ribosome GO: Translational elongation	1.6E-14 3.4E-10	Ehd2, Rpl38-Ps3, Rps16 Rpl13, Rps16, Rps21
L	K: NA GO: NA		

Table 2: Functional classification of the twelve gene expression patterns (A – L). The most significant KEGG pathway (K) and Gene Ontology Biological Process (GO) are listed for each pattern, along with their associated Bonferroni corrected p-value and three example genes. NA: no significant pathway/GO term.

Figure 2: Genes in the cholesterol synthesis pathway are down-regulated with age in rat spinal cord. Expression of the majority of genes in the pathway was correlated with the down-regulated expression patterns G, H, I and L.

3.2 Perturbed cholesterol homeostasis in aging spinal cord.

3.2.1 Altered cholesterol gene and protein expression in spinal cord.

To confirm the age-related changes in expression of genes involved in cholesterol synthesis, metabolism and transport, as identified by microarray analysis, a subset of genes from these processes were analysed by qPCR. In accordance with microarray data, one-way ANOVA identified significant decreases in expression of cholesterol synthesis genes (*Hmgcr*, $F_{2,16} = 20.78$, $p = 3.6e-05$; *Hmgcs1* $F_{2,16} = 100.82$, $p = 8.5E-10$; and *Cyp51* $F_{2,16} = 12.90$, $p = 0.001$) in old relative to young, but only *Hmgcs1* was decreased significantly by middle age (Fig 3A). In contrast, genes involved in cholesterol metabolism (*Soat1*, *Ch25h*) and transport (*ApoE*, *Abca1*), were significantly up-regulated with age, either by middle (*Abca1* $F_{2,16} = 40.33$, $p = 5.6E-07$; *Ch25h* $F_{2,16} = 10.22$, $p = 0.001$) or old age (*ApoE* $F_{2,16} = 7.13$, $p = 0.006$; *Soat1* $F_{2,16} = 7.90$, $p = 0.004$;

Fig 3C). Expression of *Srebf1* and *Srebf2*, transcription factors known to regulate the expression of cholesterol synthesis genes, was also determined. *Srebf1* increased at both middle and old age in accordance with microarray data, and *Srebf2* decreased at old age (*Srebf1* $F_{2,16} = 76.16$, $p = 6.7E-09$; *Srebf2* $F_{2,16} = 8.47$, $p = 0.003$), although change in *Srebf2* was not apparent by microarray (Fig 3B). Expression of the cholesterol esterification enzyme, sterol O-acyltransferase 1 (encoded by the *Soat1* gene and commonly referred to as *Acat1*), was further confirmed by western blot analysis of spinal cord protein homogenates. *Acat1* protein expression was significantly increased at middle and old ages relative to young ($F_{2,8} = 23.17$, $p = 4.7E-04$; Fig 3D).

Figure 3: Perturbed expression of cholesterol homeostasis genes in aging spinal cord. A) Cholesterol synthesis genes *Hmgcr*, *Hmgcs1* and *Cyp51* are down-regulated with aging. B) Altered expression of cholesterol synthesis gene transcription factors *Srebf1* and *Srebf2* in aging spinal cord. C) Genes involved in cholesterol metabolism (*Ch25h*, *Soat1*) and transport (*Apoe*, *Abca1*) are up-regulated with aging. D) Western blot quantification of Acat1 protein (encoded by the *Soat1* gene) shows increased Acat1 expression in middle (grey) and old (white) age spinal cords relative to young (black). Gene expression graphs (A – C) show fold change (FC) at middle age (grey – qPCR; diagonal stripe – microarray) and old age (white – qPCR; horizontal stripe – microarray) relative to young (red dashed line). For mRNA analyses: $n = 7$ young, 5 middle, 7 old age animals. For protein analyses: $n = 4$ young, 3 middle, 4 old. Error bars represent SEM. * $p < 0.05$ compared to young; # $p < 0.05$ compared to middle age as determined by one-way ANOVA and Tukey's post hoc testing.

3.2.2. Age-related changes in expression of cholesterol-associated genes are more pronounced in white matter of spinal cord.

In order to determine whether age-related changes in cholesterol related genes are influenced by spinal cord sub-region, gene expression changes were assessed in the white matter, and dorsal and ventral grey matter regions by qPCR and analysed by two-way ANOVA and Tukey's post-hoc testing. Significant effects of age for cholesterol synthesis genes were observed (*Hmgcr* $F_{2,24} = 54.74$,

$p = 1.22\text{E-}07$; *Hmgcs1* $F_{2,24} = 138.84$, $p = 1.8\text{E-}11$; *Cyp51* $F_{2,24} = 14.1$, $p = 9.7\text{E-}04$), with each gene down-regulated in old animals in all three regions (*Hmgcs1*), white matter and dorsal grey (*Hmgcr*) or white matter only (*Cyp51*; Fig 4A). A significant main effect of region was also found (*Hmgcr* $F_{2,24} = 223.14$, $p = 3.6\text{E-}06$; *Hmgcs1* $F_{2,8} = 30.34$, $p = 2.7\text{E-}07$; *Cyp51* $F_{2,24} = 5.83$, $p = 8.7\text{E-}03$) with a higher level of expression of *Hmgcr* in dorsal grey and white matter compared to ventral grey, and greater expression of both *Hmgcs1* and *Cyp51* in white matter compared to the grey matter regions. Furthermore, there was a significant interaction between age and region for *Hmgcr* ($F_{2,24} = 13.99$, $p = 9.4\text{E-}05$) and *Hmgcs1* ($F_{2,24} = 35.61$, $p = 6.6\text{E-}08$), indicating the age-related reduction in expression of these genes in old animals was greatest in white matter. In contrast to the genes involved in synthesis, the cholesterol metabolism and transport genes were significantly increased with age (*Soat1* $F_{2,24} = 9.95$, $p = 5.5\text{E-}03$; *Abca1* $F_{2,24} = 45.07$, $p = 6.1\text{E-}07$; *Ch25h* $F_{2,24} = 37.32$, $p = 2.6\text{E-}06$), with all three significantly increased in white matter, *Ch25h* and *Abca1* increased in ventral grey and *Ch25h* also increased in dorsal grey (Fig 4B). A significant effect for region was also observed, with white matter exhibiting higher expression than grey matter regions (*Soat1* $F_{2,24} = 3.98$, $p = 0.037$; *Abca1* $F_{2,24} = 29.34$, $p = 3.6\text{E-}07$; *Ch25h* $F_{2,24} = 38.16$, $p = 3.5\text{E-}08$). A significant age by region interaction was also observed for each gene, indicating the increase in expression with age was greatest in white matter (*Soat1* $F_{2,24} = 6.36$, $p = 8.2\text{E-}03$; *Abca1* $F_{2,24} = 22.40$, $p = 3.2\text{E-}06$; *Ch25h* $F_{2,24} = 16.09$, $p = 3.7\text{E-}05$).

3.2.3. Altered cholesterol levels in aging spinal cord.

Gene and protein expression data indicate cholesterol homeostasis is perturbed in aging rat spinal cord. To determine if this is the case, levels of total and free cholesterol were measured and normalised to total protein content. One-way ANOVAs identified a significant increase in total cholesterol ($F_{2,16} = 5.89$, $p = 0.012$) and a trend towards an increase in free cholesterol ($F_{2,16} = 3.43$, $p = 0.057$) in spinal cords from old animals compared to young (Fig 5A).

The majority of cellular cholesterol (>99.5%; (Bjorkhem and Meaney, 2004)) is present in its ‘free’ form, with a small amount present as either cholesterol esters or oxysterols. Alterations in the balance of cholesterol esters and other cholesterol products have been associated with neurodegenerative disease (for example, Alzheimer’s disease (Astarita, et al., 2011)). The ratio of free cholesterol to total cholesterol content (free:total cholesterol ratio) provides an index of this balance. The small, but significant decrease in the free:total cholesterol ratio identified in rat spinal cords with age (FC 0.93; $F_{2,16} = 4.02$, $p = 0.04$; Fig 5B), suggests that, in old animals, there is a decrease in the relative quantity of free cholesterol compared to the total cholesterol pool, possibly due to an increase in the relative quantity of cholesterol esters and/or oxysterols.

Figure 4: Cholesterol-related gene expression is significantly altered in spinal cord grey and white matter regions with age. A) Decreased expression of cholesterol synthesis gene *Hmgcs1* in spinal cord ventral grey, dorsal grey and white matter of old relative to young animals. *Hmgcr* was significantly decreased in dorsal grey and white matter only and *Cyp51* in white matter only. B) Increased expression of cholesterol transport genes *Abca1* and oxysterol enzyme *Ch25h* in ventral grey and white matter, and of cholesterol esterification enzyme *Soat1* in white matter with age. Red dashed line indicative of relative expression in young animals. Columns depict fold change (FC) in each region relative to young. $n = 5$ in all cases. Error bars represent SEM. * $p < 0.05$ for old compared to young by two-way ANOVA. NB: Region and age x region interactions not shown – see text for details.

3.2.4 Altered expression of myelin-associated genes in aging spinal cord.

Cholesterol is a major and critical constituent of the myelin sheath formed by oligodendrocytes, and the spinal cord, due to its white matter content, has the greatest relative level of cholesterol in the CNS (Quan, et al., 2003). To determine if our age-related cholesterol metabolism findings are associated with myelin alterations, we quantified gene and protein expression for myelin and oligodendrocytes in whole cervical spinal cord. Although our microarray analysis of myelin related genes (myelin-associated oligodendrocytes basic protein *Mobp*; myelin associated glycoprotein, *Mag*; myelin oligodendrocytes glycoprotein, *Mog*; and myelin basic protein, *Mbp*), did not satisfy all inclusion criteria, they all showed small decreases in expression with age. We therefore assessed these genes using qPCR. Significant decreases in gene expression, as assessed by one-way ANOVA, of the myelin genes, *Mbp* ($F_{2,16} = 4.05$, $p = 0.037$), *Mog* ($F_{2,16} = 13.06$, $p = 4.0E-04$), *Mobp* ($F_{2,16} = 6.68$, $p = 0.01$), but not *Mag* ($F_{2,16} = 1.61$, $p = 0.23$), were evident by middle age (*Mog* and *Mobp*), or old age (*Mbp*; Fig 6A). However, when *Mog*, *Mbp*, another myelin associated protein, 2',3'-cyclic nucleotide 3' phosphodiesterase (*Cnp*), and the oligodendrocyte marker, oligodendrocyte lineage transcription factor 2 (*Olig2*), were quantified by western blot, there were no significant differences with age (Fig 6B and 6C; *Mog*, $F_{2,8} = 0.33$, $p = 0.72$; *Mbp*, $F_{2,8} = 2.21$, $p = 0.17$; *Cnp*, $F_{2,8} = 2.80$, $p = 0.12$; *Olig2*, $F_{2,8} = 0.33$, $p = 0.72$).

Figure 5: Cholesterol content in aging spinal cord. A) Increased total cholesterol, and a trend towards increased free cholesterol in aging spinal cord. B) Reduced free to total cholesterol ratio in old spinal cord. Columns represent fold change (FC) in middle (grey) and old (white) animals, relative to young (black). A&B) $n = 7$ young, 5 middle, 7 old animals. Error bars represent SEM. * $p < 0.05$ by one-way ANOVA.

Figure 6: Myelin associated gene and protein expression in aging spinal cord. A) Decreased expression of myelin-associated gene mRNA in whole spinal cord with age. Columns represent qPCR and microarray data (as described in previous figure legends). Note, the microarray results did not meet all of our inclusion criteria, but are included here to allow comparison to qPCR. B) Representative western blot and densitometry analysis for myelin (Mog, Mbp, Cnp) and oligodendrocyte (Olig2) proteins from young (Y), middle (M) and old (O) age spinal cord. Columns represent young (black), middle (grey), and old (white), ages as described in previous figure legends. $n = 7$ young, 5 middle and 7 old animals (mRNA expression). $n = 3$ young, 4 middle age and 4 old animals (protein expression). Error bars represent SEM. * $p < 0.05$ compared to young; # $p < 0.05$ compared to middle age by one-way ANOVA and Tukey's post

3.3 Increased inflammatory-related gene and protein expression in aging spinal cord.

There was a significant increase in total cholesterol and a strong trend ($p = 0.057$) for an increase in free cholesterol in old spinal cords (Fig. 5), but no gross changes in myelin protein expression (Fig. 6B). Since there is a tight balance between cholesterol and myelin in the CNS, this excess cholesterol may be cytotoxic (Tabas, 2002). Functional annotation of microarray data identified inflammation and immune associated categories such as the *defense* and *immune response*, which are consistent with the possibility of cholesterol-induced cytotoxicity. In particular, there was an increased expression of microglial and astrocyte-associated genes (eg *Iba1* and *Gfap* respectively) with age. Lipids, including cholesterol, can induce microglial activation and neuroinflammation (Rackova, 2013). Therefore, we investigated neuroinflammatory processes in aged cervical spinal cords by quantifying gene and protein expression for microglia and astrocyte activation (Fig 7A-C). qPCR analysis of gene expression in whole cervical spinal cord confirmed changes identified by microarray, including significant increases in expression of microglial *Iba1* ($F_{2,15} = 7.47$, $p = 5.6E-03$) and astrocytic *Gfap* ($F_{2,15} = 15.32$, $p = 1.9E-04$) and vimentin *Vim* ($F_{2,15} = 26.54$, $p = 8.3E-06$; Fig 7A-B). Other markers of microglial activation (*P2rx7*, *Trem2*, *Cd68*) were also increased in whole spinal cord (*P2rx7* $F_{2,15} = 3.89$, $p = 0.04$; *Trem2* $F_{2,15} = 36.72$, $p = 1.7E-06$; *Cd68* $F_{2,15} = 10.26$, $p = 1.6E-03$; Fig 7A). Analysis of protein content in whole spinal cord also indicated an increase in *Iba1* ($F_{2,8} = 12.89$, $p = 4.5E-03$) and *Gfap* ($F_{2,8} = 6.49$, $p = 0.02$) with age, with *Iba1* significantly higher in old compared to young and middle age, and *Gfap* significantly higher at old age compared to young.

To identify possible spinal cord region-specific effects of age on expression of microglial and astrocytic markers, *Iba1*, *Gfap* and *Cd68* mRNA expression was quantified in white matter and dorsal and ventral grey matter (Fig 7D). Two-way ANOVA and Tukey's post-hoc analysis of each gene identified significant effects of age (*Iba1* $F_{2,24} = 22.37$, $p = 9.1E-05$; *Gfap* $F_{2,24} = 24.72$, $p = 4.4E-05$; *Cd68* $F_{2,23} = 34.01$, $p = 6.1E-06$), with each gene increased in white matter and ventral grey, but only *Gfap* increased in dorsal grey, with age (Fig 7D). A significant effect of region was also apparent (*Iba1* $F_{2,24} = 14.52$, $p = 8.4E-05$; *Gfap* $F_{2,24} = 98.48$, $p = 2.7E-12$; *Cd68* $F_{2,23} = 16.25$, $p = 3.4E-05$), with greater expression in white matter compared to both dorsal and ventral grey matter. A significant age by region interaction was also observed for *Gfap* ($F_{2,24} = 7.64$, $p = 2.7E-03$) and *Cd68* ($F_{2,24} = 9.66$, $p = 9.0E-04$), but not *Iba1* ($F_{2,24} = 2.30$, $p = 0.12$), indicating that the increase in expression for *Gfap* and *Cd68* in white matter was significantly greater than observed for other regions.

Image analysis of immunofluorescence labeling of young and old spinal cords identified a significant increase in *Cd68* labeling in whole cervical spinal cord sections ($p = 0.007$). To characterise potential region-specific changes, expression of *CD68* in grey matter, and three white matter funiculi (dorsal, ventral and lateral) was also analysed. Two-way ANOVA identified a significant effect for age across

regions ($F_{2,16} = 92.77$; $p = 4.6E-08$), with increased expression of Cd68 in old animals (Fig 8A-C). A significant effect of region was also identified ($F_{2,16} = 5.98$ $p = 6.0E-03$), where expression was higher in dorsal and ventral funiculi compared to grey matter. A significant age by region interaction was also observed ($F_{2,16} = 8.66$ $p = 1.2E-03$), indicating that the increase in Cd68 expression was greater in dorsal and ventral funiculi compared to lateral funiculus and grey matter regions. For Iba1, there was a significant increase in Iba1 labeling in whole sections with age ($p = 0.002$). Two-way ANOVA identified significant main effect for age across regions ($F_{2,16} = 5.84$ $p = 0.028$), in accordance with whole section results. There was a significant effect of region ($F_{2,16} = 28.67$ $p = 1.13E-06$), with grey matter having higher Iba1 expression than all white matter funiculi, and dorsal funiculus expression greater than in ventral funiculus. There was no significant age by region interaction ($F_{2,16} = 1.61$, $p = 0.22$). Furthermore, consistent with increased white matter immunolabeling for Cd68, there appeared to be much greater co-localisation of Cd68 with Iba1-positive microglial cells in white matter of old animals (Fig 8B-C). Increased Gfap labeling was also observed with age in whole spinal cord sections ($p = 0.02$; Fig 8D). Two-way ANOVA identified significant main effects for age ($F_{2,14} = 5.08$; $p = 0.04$) with increased Gfap with age. A region effect was also observed ($F_{2,14} = 4.33$; $p = 0.02$), indicating that Gfap expression in lateral funiculus was greater than grey matter. An age by region interaction ($F_{2,14} = 6.59$; $p = 5.2E-03$) indicates that the increase in Gfap was greatest in the dorsal funiculus with age.

Figure 7: Increased microglia and astrocyte markers in whole cervical spinal cord and spinal cord regions with age. Microglial specific markers (A) and astrocyte markers (B) increase with age in whole spinal cord. C) Representative western blots and densitometric analysis of Iba1 and Gfap in old spinal cords. Both Iba1 and Gfap proteins were increased in old relative to young spinal cords. D) Microglial specific *Iba1* mRNA expression increases in ventral grey and white matter, but does not reach significance in dorsal grey. Expression of astrocyte marker *Gfap* increases in each spinal cord region with age, as does a marker of macrophage and microglial phagocytic activity, *Cd68*, except in dorsal grey. Red dashed line indicates relative expression in young animals. Column colours for (A-C) are described in previous figure legends. Columns for spinal cord regional analysis (D) are for old animals relative to young. Region and age x region interaction (for D) not shown – see text for details. $n = 7$ young, 5 middle and 7 old animals (mRNA expression, whole spinal cord). $n = 3$ young, 4 middle age and 4 old animals (protein expression). $n = 5$ young, 5 old (mRNA spinal cord regions). Error bars represent SEM. * $p < 0.05$ relative to young, # $p < 0.05$ relative to middle age.

Figure 8: Immunofluorescence labeling of young and old cervical spinal cords. Spinal cord sections were immunolabeled for Iba1 (red) and Cd68 (green) or Gfap (green, D), and counterstained with Hoescht nuclei stain (blue, A-C). Images were captured at 4x magnification for analysis. A) No change in Cd68 or Iba1 labeling was observed in grey matter (dorsal horn shown) with age. B & C) Cd68 expression was increased in white matter funiculi including lateral funiculus (B) and dorsal funiculus (C) with age. Cd68 also appeared to colocalise with Iba1-labeled putative microglia (arrows). D) Increased Gfap labeling was evident in old spinal cords, particularly in the dorsal funiculus. E) Representative schematic (adapted from (Watson, et al., 2009)) showing locations of high magnification images. Scale bar is 200 μm.

4. Discussion

Aging is associated with gradual decline in almost all biological systems, including the central nervous system. While considerable research has focused on mechanisms contributing to aging in brain regions such as the hippocampus, relatively little has focused on one of the key structures linking the brain and the rest of the body – the spinal cord. To begin to address this, we have investigated potential mechanisms of aging in cervical spinal cords of young, middle and old age rats.

Our microarray and subsequent gene, protein and cholesterol analyses indicate that alterations in cholesterol homeostasis may be a contributing factor to impaired spinal cord function in aging rats, with many changes evident as early as middle age. Cholesterol is a major lipid constituent of both the peripheral and central nervous systems and is an important structural component of cell membranes,

synapses, axons and myelin, as well as the precursor for oxysterols and steroid hormones (Orth and Bellostà, 2012). With its relatively large white matter content, the spinal cord has the highest cholesterol concentration of any tissue in the body (Dietschy and Turley, 2004; Quan, et al., 2003). Cholesterol homeostasis is tightly regulated within the CNS (Waterham, 2006), as excess free cholesterol is cytotoxic (Tabas, 2002). The majority of brain cholesterol accumulates during perinatal development and maturation, but low-level continual synthesis and metabolism occurs throughout life (Martin, et al., 2010; Morell and Jurevics, 1996). Microarray analysis of gene expression in young, middle age and old rat cervical spinal cord revealed a striking pattern of global down-regulation of genes involved in cholesterol synthesis but, in contrast, up-regulation of genes involved in cholesterol metabolism and transport. The age-related expression changes for a subset of cholesterol synthesis (*Hmgcr*, *Hmgcs1*, *Cyp51*), esterification (*Soat1*), hydroxylation (*Ch25h*) and transport (*Abc1*, *ApoE*) genes were also assessed by qPCR. A number of expression changes observed by microarray were confirmed by qPCR, although *Hmgcs1* was the only cholesterol synthesis gene decreased at middle age, and expression of the hydroxylation gene, *Ch25h*, was increased at both middle and old age, by qPCR (Fig. 3). Furthermore, increased Acat1 protein levels (encoded by the *Soat1* gene) were found at middle and old age, relative to young (Fig. 3). As a number of these changes occurred by middle age, perturbed cholesterol homeostasis may be an early event in aging. Other studies have identified age and region specific changes in the expression of cholesterol associated genes and proteins in the CNS of normally aging rats (Kadish, et al., 2009; Perovic, et al., 2009; Segatto, et al., 2013) and mice (Xu, et al., 2007; Zahn, et al., 2007), as well as in the hippocampus of prematurely aging, DNA repair-deficient mice (Smith, et al., 2012). For example, Kadish et al, (2009) showed similar results to the current study, with decreased expression of cholesterol synthesis genes (e.g. *Cyp51*) and increased expression of the cholesterol transporter (*ApoE*) in rat hippocampus. Perovic et al., (2009) reported increased brain *Hmgcr* gene expression in the cortex, hippocampus and cerebellum of aged rats, and Xu et al., (2007) and Zahn et al., (2007) found increased *Cyp51* and *Hmgcr*, respectively in aging mouse spinal cord. Together, these studies suggest that cholesterol homeostasis is altered in the aging CNS and, therefore, further investigation of age-related cholesterol changes may provide insight into the mechanisms of CNS aging.

Expression of cholesterol synthesis genes is regulated by a number of factors including *Srebf1* and *Srebf2* – two transcription factors that regulate both cholesterol and other lipid related gene expression. While both Srebp1 and Srebp2 (the proteins encoded by *Srebf1* and *Srebf2*, respectively) are sterol-responsive transcription factors, Srebp1 preferentially targets fatty acid synthesis genes, whereas Srebp2 preferentially targets cholesterol synthesis genes, including *Hmgcr* (Goldstein, et al., 2006; Horton, et al., 2003). Reduced *Srebf2* expression in our rat spinal cord is consistent with the down-regulation of its target cholesterol synthesis genes. Interestingly, Srebp2 may play a role in regulating the activity of cholesterol synthesis pathways and myelin protein synthesis during

myelination (Camargo, et al., 2009; Salles, et al., 2003; Verheijen, et al., 2003) and may also be down-regulated in response to increased cholesterol ester loading (Iddon, et al., 2001).

To determine whether altered expression of cholesterol-related genes was associated with cholesterol levels, we quantified cholesterol content in aging spinal cord. A significant increase in total cholesterol content between young and old animals was found, with a trend for an increase at middle age (Fig. 5A). The apparent increase in free cholesterol levels did not quite reach statistical significance, but the small reduction in the ratio of free to total cholesterol did (Fig. 5B). In normal adult brain, the vast majority of cholesterol is present in its unesterified, 'free' form, for example, as a structural component of myelin and plasma membranes (Bjorkhem and Meaney, 2004). However, with an overabundance of free cholesterol, for example, as a result of ongoing low-level synthesis that exceeds the accretion rate (Quan, et al., 2003), cholesterol is esterified to promote intracellular storage or exocytosis, or oxidised (hydroxylated) for extracellular transport. Our reduced free:total cholesterol ratio, and the increased expression of *Soat1*, *Ch25h*, *Abca1*, and *ApoE* genes and Acat protein, is consistent with the notion that the spinal cord is dealing with excess free cholesterol through esterification, hydroxylation, and transport. Similar age-related increases in cholesterol content have been reported for rat and mouse brain (Cutler, et al., 2004; Zhang, et al., 1996), however, the spinal cord has not previously been examined. Interestingly, perturbed cholesterol homeostasis, in particular increased abundance of cholesterol esters, has been observed in the CNS of patients with neurodegenerative disease including Alzheimer's disease (AD, (Puglielli, et al., 2001)) and amyotrophic lateral sclerosis (ALS, (Cutler, et al., 2002)). While the consequences of perturbed cholesterol homeostasis are not well understood, these results suggest that it may contribute to neurodegenerative processes, in disease states as well as normal aging.

Cholesterol is required for normal structure and function of all cell types, however individual neuronal or glial cell types differ in their specific requirements (Lund, et al., 1999; C.M. Valdez, et al., 2010). To determine the effects of aging on cholesterol gene expression in functionally distinct regions of the spinal cord that have vastly different cellular make up (e.g. neuron:glia ratios, glial cell type etc), we dissected the dorsal grey, ventral grey, and white matter regions, and quantified cholesterol associated gene expression by qPCR. We found distinct regional patterns in the expression of cholesterol synthesis genes *Hmgcr*, *Hmgcs1*, and *Cyp51* and in cholesterol metabolism and transport genes *Soat1*, *Ch25h*, and *Abca1*. In general the age-related expression changes were more profound in white matter compared to the two grey matter regions. Interestingly, while *Soat1* was unchanged in grey matter with age, it was significantly up-regulated in white matter. Notably, a previous study in aging rat hippocampus found increased Acat1/*Soat1* expression in astrocytes located adjacent to, or within, white matter tracts (Kadish, et al., 2009). While the present study did not investigate which spinal cord cell types show the change in Acat1/*Soat1*, the lack of change in both grey matter regions may indicate that the white matter specific up-regulation of cholesterol esterification may be occurring

preferentially in white matter enriched glial cell types such as oligodendrocytes or astrocytes, with neurons relatively spared.

The preference for cholesterol associated gene expression changes in the white matter is perhaps not surprising, given that 70% of CNS cholesterol resides in myelin (Orth and Bellosta, 2012). Indeed, cholesterol is an essential myelin lipid (Saher, et al., 2005). Age-related ultrastructural changes have been demonstrated in myelin in multiple species in both the central and peripheral nervous systems (Lintl and Braak, 1983; Peters, 2009; Verdu, et al., 2000). Additionally, changes in white matter integrity with aging are consistently reported in diffusion tensor imaging (DTI) studies of humans (Bhadelia, et al., 2009; Madden, et al., 2012). The sensitivity of myelin to aging and its dependence on cholesterol for structure and function suggests that the altered cholesterol homeostasis observed in our aged spinal cords may be associated with perturbed myelin integrity. We therefore determined whether aging impacted the expression of myelin-related genes and proteins in our rat spinal cords. We found small but significant decreases in mRNA expression of *Mbp*, *Mobp* and *Mog*, but no change in *Mag*, with age. However, when *Mbp* and *Mog* proteins, along with oligodendrocyte specific markers, *Cnp* and *Olig2*, were quantified by western blot, no significant differences were found with age. These protein data suggest there is neither a loss of oligodendrocytes, nor a gross breakdown of the myelin sheath, at least in terms of its protein composition, in the aging spinal cord of F344 rats. In contrast, a recent study of aging Sprague-Dawley rats reported a loss of *Mbp* and *Mog* protein expression in the spinal cord dorsal funiculus, but no change in *Mbp*, and an increase in *Mog* in the dorsal horn (Xie, et al., 2013). Additionally, the discrepancy between the spinal cord and the increased myelinogenesis gene expression observed in aging F344 rat hippocampus (Kadish, et al., 2009) may indicate CNS region specific differences in response to aging changes. Overall, our cholesterol perturbations may not be associated with gross changes in myelin protein abundance in old spinal cords, however, this does not preclude potential region, tract specific, or microstructural changes.

In addition to perturbed cholesterol homeostasis, we found up-regulation of neuroinflammatory markers with aging. Both microglia- and astrocyte-specific mRNA and proteins, including *Iba1* and *Gfap*, were up-regulated in old spinal cords. Astrocytes have the highest rate of cholesterol synthesis in the adult rat brain (Nieweg, et al., 2009) and are important for cholesterol transfer to neurons for growth and synapse remodelling (Dietschy, 2009). Microglia – the resident immune cells of the CNS – are activated by many factors, including cholesterol and oxysterol levels (Rackova, 2013). Indeed, we found expression of the microglia-specific, lipid-sensing receptor, *Trem2*, was significantly increased in old spinal cords and these receptors are crucial for sensing myelin breakdown (Poliani, et al., 2015). Furthermore, expression of *Cd68* – a marker of phagocytic microglia (Kettenmann, et al., 2011) – was significantly increased with age, particularly in white matter. Consistent with these changes in glial gene expression, increased *Cd68* and *Gfap* immunolabeling was observed in the white

matter of our aging spinal cords. Reactive astrogliosis increases in the aging CNS (Kohama, et al., 2012), particularly in the white matter of the spinal cord (Kullberg, et al., 2001) and brain (Hart, et al., 2012) and an increase in activated microglia has also been reported in the white matter of the aging rat brain (Kullberg, et al., 2001; Perry, et al., 1993). In support of the present Gfap and Cd68 findings, other studies have also reported that the dorsal funiculus is the most vulnerable to micro- and astrogliosis in aging rodent spinal cord (Kullberg, et al., 2001; Xie, et al., 2013), whereas lateral and ventral funiculi may be less susceptible (Kullberg, et al., 2001; Lasiene, et al., 2009). The colocalisation of micro- and astrogliosis with altered expression of cholesterol associated genes in spinal cord white matter suggests the perturbed cholesterol homeostasis observed in aging spinal cord, may be the cause or result of aberrant glial activation, possibly in a white matter funiculus-specific manner.

In summary, aging in rat spinal cord is associated with altered cholesterol homeostasis, including reduced expression of cholesterol synthesis genes, and increased expression of cholesterol esterification, hydroxylation, and transport genes. Furthermore, the balance of free:total cholesterol is perturbed, likely a result of increased cholesterol ester and oxysterol production. Notably, the effects of aging on the expression of genes associated with cholesterol and neuroinflammation (micro- and astrogliosis) were more pronounced in white matter. Cholesterol is essential for myelination and the life-long maintenance of myelin structure, with breakdown of myelin leading to inflammation and neural dysfunction. Although there was minimal evidence for gross myelin-associated gene and protein changes in whole spinal cord, it is likely specific white matter tracts, such as the dorsal funiculus, are significantly affected, potentially contributing to altered white matter integrity and spinal cord signaling observed with age. Future studies need to determine the roles of specific CNS cell types in cholesterol homeostasis, in particular oligodendrocytes, and how the aging process affects these roles.

Acknowledgments

We gratefully thank the following contributors to the technical aspects of this study: Mitchell Cummins, Ethan Cresswell, Mark Bigland, Millicent Andrews and Dr Matt Dunn.

Funding

Funding for the project was provided by the University of Newcastle and Hunter Medical Research Institute (HMRI). Neither institute had a role in study design, data collection, analysis and interpretation, or in writing the report and submission of the article.

Disclosure Statement

The authors declare that there are no conflicts of interest.

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