Transcriptomic evidence of a para-inflammatory state in the middle aged lumbar spinal cord

William Galbavy, Yong Lu, Martin Kaczocha, Michelino Puopolo, Lixin Liu and Mario J. Rebecchi

Abstract

Background: We have previously reported elevated expression of multiple pro-inflammatory markers in the lumbar spinal cord (LSC) of middle-aged male rats compared to young adults suggesting a para-inflammatory state develops in the LSC by middle age, a time that in humans is associated with the greatest pain prevalence and persistence. The goal of the current study was to examine the transcriptome-wide gene expression differences between young and middle aged LSC.

Methods: Young (3 month) and middle-aged (17 month) naïve Fisher 344 rats (n = 5 per group) were euthanized, perfused with heparinized saline, and the LSC were removed.

Results: ~70% of 31,000 coding sequences were detected. After normalization, ~ 1100 showed statistically significant differential expression. Of these genes, 353 middle-aged annotated genes differed by > 1.5 fold compared to the young group. Nearly 10% of these genes belonged to the microglial sensome. Analysis of this subset revealed that the principal age-related differential pathways populated are complement, pattern recognition receptors, OX40, and various T cell regulatory pathways consistent with microglial priming and T cell invasion and modulation. Many of these pathways substantially overlap those previously identified in studies of LSC of young animals with chronic inflammatory or neuropathic pain.

Conclusions: Up-modulation of complement pathway, microglial priming and activation, and T cell/antigen-presenting cell communication in healthy middle-aged LSC was found. Taken together with our previous work, the results support our conclusion that an incipient or para-inflammatory state develops in the LSC in healthy middle-aged adults.

Keywords: Transcriptome, Aging, Spinal cord, Microglia, Neuropathic, Complement, T-cell, Inflammation
lumbar spinal cords (LSC) arises by middle age in healthy rats [14]. This is accompanied by remarkable changes in dorsal horn microglial morphology rarely seen in young adult LSC, that indicated activated M1 and M2 morphologies (shortened, thickened processes, decreased arborization, and hypertrophic cell bodies). In the present study we compared the transcriptomic expression of whole lumbar spinal cords of healthy middle-aged male rats to those of young adults. The results provide further support for a para-inflammatory status in the LSC by middle age and point to development of microglial states previously associated with establishment of neuropathic or inflammatory pain.

Methods
Perfusion, tissue harvesting, RNA extraction and purification
All work conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Stony Brook University Institutional Animal Care and Use Committee and conducted under protocol #203692-23. Three-month and 17-month old Fisher 344 rats were were euthanized and conducted under protocol #203692-23. Three-month and I n s t i t u t i o n a lA n i m a lC a r ea n dU s eC o m m i t t e ea n d Animals and were approved by the Stony Brook University Health Guidelines for the Care and Use of Laboratory All work conformed to the National Institutes of

Transcriptomic analysis
The RNAs were reversed transcribed, and then, using an in vitro transcription reaction, biotinylated nucleotides were incorporated, converting the cDNA to labeled cRNA. The cRNA pool was purified, fragmented and then hybridized to Rat Genome 230 2.0 arrays (Affymetrix) displaying over 31,000 probe sets, representing 30,000 transcripts and variants from over 28,000 well-substantiated rat genes. Hybridized chips were washed, incubated with fluorescently labeled streptavidin probe, laser scanned and probe fluorescence intensities were measured. Quality control analysis was performed using the affyQCReport package in Bioconductor. CEL files were quantified and normalized using GenePattern ExpressionFileCreator function at the setting of RMA method and quantile normalization. The relation of samples was displayed in dendrogram that was generated by hclust package in Bioconductor. Comparison of samples was conducted using R statistical project. The FDR values were calculated using the samr package in Bioconductor. The normalized data were then subjected to t-testing with FDR = 10%. Annotation of probe sets was based on Affymetrix Rat230_2.na34 release.

Pathway analysis
The differential expression gene set was subjected to Pathway analysis using IPA software suite. Three hundred fifty three differentially expressed, annotated genes that were increased or decreased significantly were imported and analyzed using Canonical pathways. Adjusted p values were obtained from a modified Fisher test [15] that compared the ratio of differential gene set to number of pathway members obtained to the probability that such a ratio would be found by chance, after correcting for multiple hypothesis testing.

Results
Transcriptomic expression patterns were determined in LSC from young (3 month) and middle-aged (17 month) male Fisher 344 rats. Of the 28,000 genes probed, over 1,100 showed significant differential expression (Fig. 1). Five hundred twenty eight genes increased or decreased at least 1.5 fold in middle age (Additional file 2). Of these, 353 unique well-annotated genes were subjected to further analyses (Additional file 3). Table 1 lists the C t of a reference gene (GAPDH) of the corresponding samples. The ΔC t values were calculated using the samr package in Bioconductor. The normalized data were then subjected to t-testing with FDR = 10%. Annotation of probe sets was based on Affymetrix Rat230_2.na34 release.

qPCR Analysis
Primers were designed with Primer3 and synthesized by Eurofins MWG Operon LLC (Louisville, KY, USA). The primer pairs used in the qPCR reactions are listed in Additional file 1. cDNA was synthesized with Quantitect Reverse Transcription Kit (Qiagen, Germantown, MD, USA) using the same mRNA that were used in the microarray measurements. qPCR analysis was performed on an Step-One Plus qPCR equipment (Applied BioSystems), using the Quantitect SYBR Green Kit (Qiagen). PCR reactions were followed by melt curve analysis. For data analysis, ΔC t were acquired by subtracting the C t values of the genes of interest from the C t of a reference gene (GAPDH) of the corresponding samples. The ΔC t's for the young and middle-aged groups were subjected to t-testing (one-tailed). Adjusted p value for multiple comparisons was carried out with R using the method of Benjamini and Hochberg [15].
annotated genes with the greatest differential expression. The largest change associated with middle age was expression of Dnajb12 (6.70 fold increase). Dnajb12 and related genes regulate proteasomal degradation of polytopic membrane proteins, particularly ion channels [16]. Gipr transcript, which encodes the receptor for gastric inhibitory polypeptide (GIP), also substantially increased. Agonist analogs of GIP reduce central oxidative stress, and are neuroprotective in Alzheimer’s disease [17, 18], and stroke models [19]. Gpmb transcript levels were elevated over three fold. This mRNA encodes a regulator of immune responses expressed in microglia that appears to have anti-inflammatory properties [20]. Among transcripts reduced in middle age, Herc1 participates in membrane trafficking; its loss increases autophagy and decreases mTOR activity associated with Purkinje cell degeneration [21]. Kif1a expression, which is required for BDNF-induced synaptogenesis in the hippocampus [22], was also significantly diminished in middle age.

To confirm the reproducibility and accuracy of the microarray measurements, we performed qPCR analyses on the same sets of samples measuring the relative change in expression (normalized to GAPDH mRNA). Our results (Table 2) show a close correspondence between qPCR and microarray results for 8 differentially expressed genes: Lgals, Fcr2b, GPNMB, C3, Atf3, Ptprc, Cd163 and Nrg1, as well as induction of the microglial activation marker Cd11b.

The annotated differential gene set consisting of the 353 transcripts, was subjected to pathway analysis (IPA) using its curated pathway database. Of 13 canonical pathways, complement cascade had the highest proportion of differentially expressed genes and lowest adjusted p value (Table 3). In the complement system, 9 differentially expressed aged-related genes were up modulated out of 37 Complement system members (0.243; p = 1.11 x 10^-6). Members of the Classical pathway, initiated by C1q activation and the Alternative pathway, initiated by C3 activation were well populated. The positive Z-score = 1.414, indicated significant complement pathway activation. On the other hand, expression of several inhibitory components, Serping1, a C1 esterase inhibitor, and Cfh, which accelerates C3b inactivation, were increased in middle age (1.55 and 1.79 fold, respectively), and could possibly suppress these complement activation [23].

Among the remaining 12 pathways, four involved cholesterol metabolism, while nearly all remaining pathways were related to the T-helper cell/antigen presenting cell (APC) or T cell signaling (Table 3). These other pathways overlapped with common immune cell markers including Cd3g, Ptprc, Fcer1g, and MHC-I and MHC-II related genes. This is well illustrated in the Ox40-signaling pathway, in which APC, such as microglia, present antigen in the context of MHC and Ox40 receptor ligand to effector T cells, leading to activation of Nfkb, cJun and PI3K/PKB pathways [24].

A number of mechanistic regulatory networks were also populated by the differentially expressed gene set (Table 4), including pro-inflammatory mechanistic networks, IFNG, LPS and TNF. Additionally the inosine network, an immunomodulatory adenosine metabolite, and vancomycin, a neurotoxic antibiotic, were also significantly represented. IFNG, the most well populated
The annotated differential gene set (353) was filtered for largest effect sizes. Mean probe set expression levels (arbitrary units) for Y (young) and M (middle age) genes are shown.

The transcriptional mechanistic regulatory networks were also well represented. Ctnnb1, part of the canonical WNT pathway, is a co-activator of TCF/LEF family transcription factors, which up-modulates expression of WNT-responsive genes [25]. Out of 124 members, this network contained 36 of the differentially expressed, age-related genes ($p = 3.96 \times 10^{-12}$). Major regulatory nodes included Ctnnb1, Fge2, Smad3, Sp1, and Jun. Differentially expressed genes having multiple regulatory inputs included Igfbp5, Bmp2, Socs2, Ccnd2, Gja1, Ctgf, Mmp14 and Cxcl9. Overall this transcriptional network was significantly inhibited in middle age (Z-score = -2.377) which could explain the down-modulation WNT-responsive genes: Ccnd2, Gja1, Igf1, Ppap2b, Socs2, and Tcf7l2. On the other hand, two Ctnnb1 genes, that are also STAT regulated, were up modulated in older LSC, as are Stat6 and a synergistic co-activator Cebpδ.

Table 1: Differential gene expression: largest effect sizes

| Gene     | Y Mean | M Mean | Annotation                                    |
|----------|--------|--------|-----------------------------------------------|
| Abp10    | 0.46   | 677    | Annexin V-binding protein                     |
| Aff4     | 0.45   | 458    | Transcription factor and central SEC component|
| Ago2     | 0.43   | 330    | RISC Catalytic Component 2                    |
| Aqp4     | 0.50   | 2664   | Aquaporin family member                        |
| Arf11    | 2.07   | 94     | ADP-ribosylation factor-like 11               |
| Asap1    | 0.40   | 524    | ADP-ribosylation factor (ARF) GTase-activating protein |
| Bmpr2    | 0.46   | 970    | Bone morphogenetic protein receptor type II    |
| C3       | 2.40   | 830    | Central complement component                   |
| Chrdl1   | 2.48   | 60     | Ventroptin, antagonizes BMP actions            |
| Clec12a  | 2.18   | 99     | CTL/CTLD member, widely expressed in innate immune system |
| Csp1     | 0.49   | 207    | Important for normal neural specific cilia function |
| Dnajb12  | 6.70   | 52     | DNAJ/HSP40 family member, regulates molecular chaperone activity, protein folding and degradation |
| Enc1     | 0.47   | 135    | Kelch-related family of actin-binding proteins, role in oxidative stress response, regulates Nrf2 |
| Falz     | 0.50   | 482    | Transcription/epigenetic regulator, up-modulated in neurodegenerative diseases |
| Fcgr2b   | 2.89   | 303    | Low affinity Fc receptor expressed in microglia |
| Fcrls    | 2.78   | 218    | Fc receptor; part of TGFβ1 microglial signature response |
| Fmo2     | 2.35   | 87     | Flavin-containing monoxygenase                  |
| FstB     | 2.13   | 66     | Secreted form binds/antagonizes members of TGF-β family, e.g., BMP2 |
| Gipr     | 3.49   | 179    | Receptor for neuroprotective polypeptide GIP    |
| Golga4   | 0.42   | 211    | Role in Rab6-regulated membrane-tethering events in the Golgi |
| Gpnmb    | 3.14   | 420    | Expressed in microglia, inflammatory response gene |
| Herc1    | 0.36   | 294    | Regulator of membrane trafficking              |
| Kif1a    | 0.38   | 3058   | Kinesin family protein involved in syaptogenesis |
| Lgals3   | 2.44   | 927    | Member galectin family of carbohydrate binding proteins; microglial marker and alt priming gene |
| Msi2     | 0.49   | 304    | RNA binding protein; role in CNS stem cell proliferation |
| Nrg1     | 0.46   | 553    | Neuregulin-1, signals through erbB2/3 receptors, functions in neural development and plasticity |
| Pitpnm1  | 3.50   | 499    | Transfers PtdIns from ER to plasma membrane    |

Table 1: Differential gene expression: largest effect sizes (Continued)

| Gene     | Y Mean | M Mean | Annotation                                    |
|----------|--------|--------|-----------------------------------------------|
| Postn    | 2.73   | 147    | Aka peristin; enhances TGF-β signaling, facilitates BMP1, expressed in reactive microglia and astrocytes, neuroprotective |
| Pou3f1   | 2.16   | 266    | Transcription factor, promotes stem cell commitment to neural fate |
| RT1-EC2  | 2.50   | 75     | Class I MHC, up-modulated in spinal cord injury and neurodegeneration |
| S100a8   | 2.30   | 85     | Induced in macrophages and dendritic cells by TLR agonists, oxidative stress and corticosterone |
| Slc9a3   | 2.15   | 195    | Sodium/proton exchanger; Cation Proton Antiporter 3 |
| Tnc      | 2.32   | 75     | Extracellular matrix protein, up regulated following CNS trauma |
| Unc5b    | 0.47   | 995    | Netrin receptor family; required for axon guidance; maintains blood–brain barrier |
| Usp7     | 0.46   | 121    | Ubiquitin-specific-processing protease 7, deubiquitinates range Deubiquitinate target proteins |
Bio-functional networks were also explored. The top functional populated networks were systemic autoimmune syndrome and activated immune cell adhesion and migration (Network 01). This network had the highest consistency score (10.104) with a total of 21 nodes and 5 regulators and 12 differentially expressed LSC target genes that formed the core of the immune cell regulatory network, under the control of Tmg2, Ifnαr, Gata4, and/or Hbb-b1. Strong relationships included activation of immune cell adhesion and leukocyte chemotaxis involving S100a8, C3 and Cd74 expression, and migration of antigen presenting cells associated with expression of Tyrobp, Il16, S100a4 and C3. All functional network members are listed in Additional files 4, 5, 6 and 7.

Multiple other functional networks overlapped Bio-functional Network 01. All were related to immune cell function, adhesion and migration. Network 02 had a consistency score of 6.640, with 19 nodes, 5 regulators and 12 targets. Interactions stemming from up-modulation by Nfkb complex of C3, Plau, Tnsf4, Map3k8, and Ctgf expression were associated with activation of macrophage migration; whereas, increased expression of C1qa, S100a8, Cd74, Lgals3, C3 and Plau, under Hrg, Tgm2, Nfκb, or Nfκb complex control, was associated with immune cell adhesion. Functional networks 03 and 04 (consistency scores of 5.078 and 3.618, respectively) overlapped. Network 03, which had 21 nodes, 6 regulators, and 14 target genes, featured Ifng and/or Ifnαr driving expression of Cxcl9, Cd74, C3, S100a8, Stat6 and Aif1 expression associated with leukocyte chemotaxis; whereas Network 04, which had 16 nodes, 3 regulators and 11 targets, featured Tlr9, Tlr3, or transcriptional regulator, Ehf, modulating expression of C3, Map3k8, S100a8, Timp1 and, Abca1 that was associated with activation of myeloid lineage cell migration.

| Table 2 | Confirmation of microarray results by qPCR |
|----------------|------------------------------------------|
| Results        | Lgals | Fcgr2b | Gpnmb | C3   | Atf3 | Ptprc | Cd163 | Nrg1 | Cd11b |
| ΔΔCt, M v Y    | 1.62  | 1.68   | 1.76  | 1.42 | 0.94 | 0.99  | 0.78  | −0.79| 0.87  |
| qPCR Fold, M v Y| 3.08   | 3.20   | 3.40  | 2.68 | 1.92 | 1.99  | 1.72  | 0.58 | 1.83  |
| Microarray, M v Y | 2.44  | 2.89   | 3.14  | 2.40 | 1.73 | 1.84  | 1.59  | 0.46 | N/A   |
| Adjusted p-value| 0.004  | 0.003  | 0.006 | 0.010| 0.004| 0.001 | 0.001 | 0.056| 1.0E-07 |

ΔΔCt values are expressed as described in Methods. cDNA was synthesized from the same RNA samples analyzed in the microarrays.

| Table 3 | Canonical pathways populated by the age-related gene set |
|----------------|------------------------------------------|
| Pathway                  | Target Genes | Pathway Genes | Ratio | z-score | B-H p value | Target Gene ID's |
| Complement               | 9 | 37 | 0.243 | 1.414 | 1.11E-06 | C3, C1QA, C1QB, C1QC, C1S, CFD, CFH, ITGB2, SERPING1 |
| Cholesterol Biosynthesis | 7 | 28 | 0.250 |       | 2.63E-05 | CYP51A1, HMGCR, HMGCS1, HSD17B7, ID1, LSS, SC5D |
| iCOS-iCOSL signaling T helper cell | 10 | 108 | 0.093 |       | 6.64E-04 | CAMK2B, CD3G, FCER1G, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB5, PDPK1, PLEKHA4, PTPRC |
| Cholesterol Biosynthesis I | 4 | 13 | 0.308 |       | 1.84E-03 | CYP51A1, HSD17B7, LSS, SC5D |
| Cholesterol Biosynthesis II (via 24,25-dihydroxlanosterol) | 4 | 13 | 0.308 |       | 1.84E-03 | CYP51A1, HSD17B7, LSS, SC5D |
| Cholesterol Biosynthesis II (via desmosterol) | 4 | 13 | 0.308 |       | 1.84E-03 | CYP51A1, HSD17B7, LSS, SC5D |
| Ca-induced T cell Apoptosis | 7 | 64 | 0.109 |       | 2.40E-03 | CD3G, FCER1G, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB5, PRKCE |
| OX40-signaling           | 8 | 89 | 0.090 |       | 2.40E-03 | CD3G, FCER1G, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB5, RT1-EC2, TNF5F4 |
| NFAT regulation of immune response | 11 | 171 | 0.064 | 1.89 | 2.40E-03 | BLNK, CD3G, FCER1G, FCGR2B, FCGR3A/FCGR3B, GNAS, GSK3B, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB5 |
| B Cell development       | 5 | 33 | 0.152 |       | 4.35E-03 | HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB5, PTPRC |
| Nut77 signaling in T cells | 6 | 57 | 0.105 |       | 6.70E-03 | CD3G, FCER1G, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB5 |
| Cd28 signaling in T Helper Cells | 8 | 118 | 0.068 |       | 1.15E-02 | CD3G, FCER1G, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB5, PDPK1, PTPRC |
| PKC-τ signaling in T cells | 8 | 118 | 0.068 | 1.414 | 1.15E-02 | CAMK2B, CD3G, FCER1G, HLA-DQA1, HLA-DQB1, HLA-DRA, HLA-DRB5, MAP3K8 |

The differentially expressed annotated genes (target gene set) were placed in the contexts of canonical biological pathways using the IPA software and its curated database. Ratio = target genes/pathway genes. P values were calculated using a modified Fisher test that corrects for multiple hypothesis testing. Significant Z-score indicate overall pathway modulation (+) for up (-) for down.
The consistency of each network and the overlaps indicate a strong association of these members of the age-related gene expression set with immune cell adhesion and migration.

We had previously reported that most microglia of healthy middle-aged rat LSC exhibited a activated morphology compared to young adults [14]. Here we find that nearly 10% of the differential gene set was associated entirely or primarily with changing patterns of microglial gene expression (Table 5). Approximately 75% of these transcripts were related to microglial activation [26, 27], and substantially overlapped with neuropathic pain-modulated spinal cord genes that were described in previous transcriptomic studies [28, 29]. In addition to microglial associated genes, other neuropathic pain-related transcripts were differentially expressed, yielding a total of 43 such genes or ~12% of the entire age-related LSC transcriptome (annotated).

**Discussion**

We have previously published cytokine and immune marker gene expression and immunohistochemical evidence that a para-inflammatory state develops in the LSC by middle age [14]. We reported that microglia of healthy middle-aged rat LSC showed a predominately activated phenotype, whereas astrocyte morphology and GFAP protein levels indicated quiescence [14]. Results also showed that Cd2, Cds3e, Cds68, Cd45, Tnf-α, Ile6, Ccl2, Atf3 and Tgfb1 mRNA levels were substantially elevated. Here we extend that study and report that the transcriptomic changes associated with middle age are dominated by up-modulation of the innate immune system, including complement, TLR signaling, T-cell/APC interface, microglial priming, and M1 and M2 activation states. A number of other immune regulatory pathways were significantly up modulated including NFAT transcriptional pathways, important for neuronal excitation-transcription coupling and neurotrophin signaling [30], and the T-cell/PKC-Tau pathway. Components of other pathways involved in the regulatory interface between APC’s and T-cells were similarly affected with increased expression of components in the Ox40, Icos-icosL, Nur77, and Cds28 signaling. Many immune regulatory networks were affected by age. Three of the top six networks control or activate central inflammatory responses involving glia and T-cells, which fit well the view that the innate immune system in the healthy LSC in middle age is in a state of incipient activation.
In a detailed study of the mouse life span, 127 genes were identified as aging-related in brain [31]. Eighteen genes associated with middle age in the spinal cord were similarly modulated in the mouse brain data set, with 8 of these expressed in microglia. A recent combined transcriptomic and proteomic study of aging brain and liver also reported up-modulation of genes associated with antigen processing and presentation and immune response.
system responses in senescent rat brains compared to those of young adults (two of the top three functional classifications) [32]. Thirty of the same genes reported here for middle-aged LSC were identified in the 609 differentially expressed genes in senescent rat brain [32], and 12 of these common genes were related to microglial function and/or establishment of neuropathic pain, including a number of complement components.

While our results are consistent with those previously reported for the senescent brain and other aging tissues, few of the genes we report are represented in the differential gene set previously reported for middle-aged and young adult mouse spinal cord [33]. Only 7, Acd63, Elov6, Jam2, Mapt2, Nedd4, Park3, Packer1, and Ryr2 were found in both differential gene sets. Of these, the directions of modulation in 5 (Elov6, Jam2, Nedd4, Packer1, Ryr2) were not in agreement. The reasons underlying the lack of consistency with our study is unclear, though species differences may have contributed, as well as the many variables explored in the previous work (age, gender, tissue), that could have reduced the power to reliably identify differentially expressed genes in middle-aged mouse spinal cord. In two previous transcriptomic studies of middle-aged brain reported by Loerch and co-workers [34] and by Wood and others [35], only 3 (Csnk2a1, Hmgcr, Sggt) and 4 (RT1-Ba, RT1-Bb, RT1-Da, Sema3b), respectively, were in common with the differential set reported here, suggesting the changes in middle-aged spinal cord we report here may be unique to the spinal cord. It is also possible that dissimilar platforms could have contributed the lack of overlap.

Up-modulation of the complement pathway activation is one of the most common age [36] or neuropathic pain related changes reported for the CNS [37]. A detailed study of complement component expression in aging mouse forebrain also reported significant increases in C1q and C3 transcript levels [38], similar to those reported here. Unlike the LSC, C4 transcript levels were not significantly elevated until 24 months in the mouse forebrain. We also measured significant increases (by qPCR) in transcripts encoding Cd11b or Cr3 (Table 2), a common microglial activation marker and the receptor for C3 complement, which has been reported to increase in a variety of different CNS pathologies [39–41]. While many activating components were up modulated in the middle-aged LSC, so were the transcripts of several counter regulatory components encoding proteins that block formation of C1q protein or increase the degradation of C3a, suggesting that complement activation in middle age could be suppressed.

A substantial fraction of the age-associated LSC transcriptome (Table 5) belongs to the microglial sensome [26, 27]. Most identified here are related to M1 or M2 activation states. Cd74 (MHCII invariant chain) encodes a marker of microglial M1 activation [42–44] and is increased in the brains of aging rodents [27] and non-human primates [45]. MHCII complex, identified in the canonical pathways up modulated in LSC, is key on APC/T-cell interface [24]. Consistent with M1 state activation were the increases in transcripts encoding pro-inflammatory chemokine Cxcl9, Cd16 (Fcg2a, Fcg3b), Cd32 (Fcg2b) and Cd45 (Ptprc) [42–44]. On the other hand, many M2 sub-state activation markers and associated microglial transcripts were also up modulated including Cd163, Trem2, Tyrobp, Lgals3, and Csfr3 [42–44]. Some transcripts identified in the differential gene set are also implicated in activating microglial phagocytosis; these include Trem2, Tyrobp, Fcer1g, Cd32, and Aif1 [44, 46]. Enhanced phagocytosis is associated with M2 activation and with beneficial anti-inflammatory effects and augmented recovery/resolution or lower risk of neurodegenerative changes [42, 46]. Overall the evidence supports the evolution of multiple different microglia activation states during aging in the spinal cord, any one of which could alter the innate immune response to injury or infection, and the trajectory of recovery or resolution.

We also report here a general down modulation of transcripts encoding enzymes involved in cholesterol metabolism including Hmgcr, Hmgcs1, Cyp51, Lss, Sc5d and Scd1; whereas the cholesterol transporter, Abca1, was up-modulated. Implications for spinal cord cholesterol, however, are unclear. Comparable changes have been previously reported, in a study of aging cervical spinal cord, that were associated with perturbation of cholesterol homeostasis (increased white matter cholesterol ester concentrations) and inflammatory activation in the cervical spinal cord [47]. Age-related changes in CNS cholesterol metabolism are believed to play a key role in the development of some forms of Alzheimer’s disease and other degenerative disorders [48].

Overall, these results provide further support for “inflammaging” of the LSC by middle age in rat, which has been amply demonstrated in other senescent tissues of many species, including humans [6]. Such emerging inflammatory changes in the LSC may be most relevant to human spinal cord neuropathies, particularly the increased risk of chronic pain, which is strongly associated with middle age, rather than senescence [8, 10–13, 49, 50]. The spinal cord is the primary site of first order nociceptive signal processing; the first relay in transmission to higher centers; and is the origin of hypteralgesia and spontaneous pain, all driven by activated microglia, astrocytes and the innate immune system [51–53]. Nonetheless, our study did not investigate changes in the aging rat proteome, nor did we explore here any alterations in the responses to injury or to degeneration of the somatosensory system; therefore these potential implications remain speculative. Furthermore, to test
whether our results are relevant to the risks of chronic pain or the failures of current treatments in middle-aged humans will require investigations of post-mortem spinal cords.

Conclusions
Transcriptomic analysis of healthy middle-aged LSC demonstrated up-modulation of complement pathway, microglial priming and activation, and T cell/antigen-presenting cell communication. Taken together with previous work, the results support our conclusion that an incipient or para-inflammatory state develops in the LSC in healthy middle-aged adults.

Additional files

- **Additional file 1:** Primer sequences used in the qPCR measurements. (PDF 34 kb)
- **Additional file 2:** Up and down modulated genes. Description: all genes significantly up or down modulated in LSC comparing young to middle-aged LSC. (XLSX 445 kb)
- **Additional file 3:** Differentially expressed gene set. Description: all annotated genes included in the differentially expressed gene set that were included in the pathway analysis. (XLSX 38 kb)
- **Additional file 4:** Bio-functional Network 01. Description: differentially expressed gene set: pink to red (intensity indicates degree of up-modulation), while green denotes down-modulation. Colors of upstream regulators and downstream biological processes are related to predicted activation states with orange indicating activation and blue denoting inhibition. Lines connecting nodes are orange when leading to activation, blue when inhibition is predicted, and yellow if relationships are not consistent with the downstream node state shown. Gray lines denote lack of evidence to form a prediction. Solid lines show direct relations and dashed indirect. Blunted ends indicate expected inhibition if upstream node is activated. (TIFF 2369 kb)
- **Additional file 5:** Bio-functional Network 02. Description: as for Additional file 4. (TIFF 2216 kb)
- **Additional file 6:** Bio-functional Network 03. Description: as for Additional file 4. (TIFF 2215 kb)
- **Additional file 7:** Bio-functional Network 04. Description: as for Additional file 4. (TIFF 2311 kb)

Abbreviations
- APC: Antigen presenting cell; CNS: Central nervous system; LSC: Lumbar spinal cord; qPCR: Quantitative polymerase chain reaction

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Availability of data and materials
All data generated or analyzed during this study are included in this published article (and its Additional files).

Authors’ contributions
WG extracted RNA, conducted quality controls, performed qPCR assays, assisted in analyzing the results and helped to write the manuscript; YL performed qPCR assays, assisted in analyzing the results and helped to write the manuscript; MK helped design the study, analyzed data and assisted in editing the manuscript; MP assisted in analyzing the data, and editing the manuscript; LL helped design the study and edit the manuscript; and MR designed and supervised the study, analyzed data, and edited the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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