Age-related gene expression changes in lumbar spinal cord: Implications for neuropathic pain

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Abstract
Clinically, pain has an uneven incidence throughout lifespan and impacts more on the elderly. In contrast, preclinical models of pathological pain have typically used juvenile or young adult animals to highlight the involvement of glial populations, proinflammatory cytokines, and chemokines in the onset and maintenance of pathological signalling in the spinal dorsal horn. The potential impact of this mismatch is also complicated by the growing appreciation that the aged central nervous system exists in a state of chronic inflammation because of enhanced proinflammatory cytokine/chemokine signalling and glial activation. To address this issue, we investigated the impact of aging on the expression of genes that have been associated with neuropathic pain, glial signalling, neurotransmission and neuroinflammation. We used qRT-PCR to quantify gene expression and focussed on the dorsal horn of the spinal cord as this is an important perturbation site in neuropathic pain. To control for global vs region-specific age-related changes in gene expression, the ventral half of the spinal cord was examined. Our results show that expression of proinflammatory chemokines, pattern recognition receptors, and neurotransmitter system components was significantly altered in aged (24–32 months) versus young mice (2–4 months). Notably, the magnitude and direction of these changes were spinal-cord region dependent. For example, expression of the chemokine, Cxcl13, increased 119-fold in dorsal spinal cord, but only 2-fold in the ventral spinal cord of old versus young mice. Therefore, we propose the dorsal spinal cord of old animals is subject to region-specific alterations that prime circuits for the development of pathological pain, potentially in the absence of the peripheral triggers normally associated with these conditions.

Keywords
Dorsal horn, ageing, aging, inflammation, chemokines, Cxcl13, microglia, astrocytes, glia

Introduction
Chronic pain affects 20–30% of the population \(^1\) and considerably impacts quality of life. The prevalence of chronic pain increases with advancing age reaching 50% in later life.\(^2\) Furthermore, pain more severely interferes with daily activities in the elderly.\(^3\) The dorsal horn (DH) of the spinal cord has been implicated as a source of dysfunction in chronic pain states. For example, after injury to peripheral neurosensory elements the release of proinflammatory molecules from damaged primary afferents into the DH activates spinal glia, particularly astrocytes and microglia.\(^4\)–\(^6\) These activated glia in turn modulate excitatory and inhibitory neurotransmission in the DH\(^7\)–\(^8\) to promote altered sensory processing.\(^7\) Thus, the DH’s ability to discriminate sensory modalities changes following central sensitization and the development of chronic pain.\(^9\)

Glial cells are strongly implicated in the generation of aberrant sensory experience in neuropathic pain. For
example, microglia rapidly adopt a reactive phenotype following nerve injury. This coincides with increased release of proinflammatory cytokines, including interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)-α, and increased expression of a number of proteins involved in local microenvironment sampling. Inhibition of microglia with minocycline prior to nerve injury decreases mechanical hyperalgesia and allodynia. In contrast, astrocytes have been suggested to participate in the maintenance of neuropathic disturbance. They undergo a delayed but prolonged activation following peripheral nerve injury, and evidence shows activated astrocytes are necessary and sufficient to induce hyperalgesia. Together the above work shows that glial-derived neuroinflammatory signalling is a key process in the establishment and maintenance of chronic pain, particularly of neuropathic origin.

Despite the burden of chronic pain falling overwhelmingly on the elderly, the above mechanisms have been largely uncovered in preclinical studies on juvenile and young-adult animals. Therefore, it remains unclear how reliably our current understanding of neuropathic pain mechanisms applies to the elderly. Despite this, work has provided clear evidence of altered excitability in central nociceptive pathways in naïve aged animals. For example, extracellular in vivo recordings show that DH neurons in aged rats exhibit significantly higher background activity and higher discharge rates during noxious stimulation. After-discharge is also enhanced following noxious stimulation and the receptive field of putative nociceptors is larger in aged versus young adult animals. Our group has also shown DH processing circuits are altered in aged mice. Specifically, DH neurons exhibit a more excitable phenotype along with decreased spontaneous excitatory synaptic input, and an increased contribution of GABAergic signalling to inhibition. While the ultimate outcome of these combined changes for sensory processing under normal and pathological conditions remains to be determined, collectively they confirm that the excitability and signalling in the aged dorsal horn differs from its younger counterpart.

What exactly initiates and maintains the differences in dorsal horn signalling and function with advanced age is yet to be determined. Many studies have demonstrated aging in the central nervous system is associated with chronic low-grade inflammation, or inflammaging. How inflammaging impacts spinal dorsal horn circuits normally and during chronic pain signalling is not known. In fact, the effects of aging on the spinal cord in general is relatively understudied, and work that focuses on the dorsal horn, where sensory information is processed, is scant. Nevertheless, the literature on inflammaging in the brain highlights many of the same neuroinflammatory pathways and cell signalling events that are implicated in neuropathic dysfunction of the dorsal horn. This raises the possibility that inflammaging in the spinal dorsal horn influences nociceptive signalling at baseline as well as the propensity of this region for dysfunctional signalling under neuropathic conditions. In line with this view, metabolite levels such as N-Acetyl-aspartate, glutamate and glutamine are disrupted in the aged spinal cord, suggesting neuronal dysfunction and neurodegeneration. Furthermore, age-related functional changes in the spinal cord have been described, along with greater morbidity and mortality, following spinal cord injury in older humans and rats. In summary, the available evidence suggests the mechanisms regulating neuronal excitability and neuroinflammatory signalling are altered in the aged dorsal horn. To determine the extent of any such disruption we quantified expression of a range of genes with known roles in glial function, neurotransmission and inflammation in young, middle aged, and old mouse spinal cords.

Methods
Healthy C57Bl/6 male mice were used for all experiments and were maintained under standard housing conditions, on a 12-hour light-dark cycle, with food and water available ad libitum. Eight mice were used for each of the following age groups: young (2–4 months), middle-aged (12–14 months) and old (>24 months). All animal work was undertaken in strict accordance with the University of Newcastle Animal Ethics Committee guidelines and New South Wales and Australian animal research guidelines.

Tissue processing and RNA isolation
Mice were euthanized with 1mL intraperitoneal injection of Lethabarb, and transcardially perfused with 50mL ice-cold, diethyl pyrocarbonate-treated, phosphate buffered saline (DEPC-PBS). Spinal cords were removed, and snap frozen in dry ice-cooled isopentane and stored at −80°C. Lumbar regions were separated from whole spinal cords using a clean razor blade on a cutting block cooled on dry ice. Tissues were embedded in Tissue-Tek (ProSciTech, Thuringowa, QLD, Aust), frozen on dry ice and cryosectioned at 100μm thickness (Leica CM 1950). Cryosections were thaw mounted on RNase-free glass microscope slides and then stored at −80°C. Between 15 and 18 transverse sections were taken from the spinal cord lumbar enlargement of each animal and mounted on slides for subsequent macrodissection and RNA extraction. Slides were washed briefly in DEPC-PBS on ice, fixed and dehydrated for 3–5 minutes in 100% ethanol. The ethanol fixation/
dehydration step was used to maintain RNA stability during the macro-dissection step. With the aid of a dissecting microscope a single horizontal cut was made on each spinal cord section at the level of the central canal. This produced a dorsal and ventral half of each spinal cord section that contained the dorsal (DH) and ventral horns (VH), respectively. Isolated dorsal and ventral halves of the spinal cord were pooled for each animal, placed into RNA later (Sigma-Aldrich) and stored at -80°C for subsequent RNA extraction. Samples were homogenised using a motorised pestle (Sigma-Aldrich) in 350 μL Buffer RL from the Norgen Single Cell RNA Purification Kit, to which β-mercaptoethanol was added (1:100). RNA was extracted as per the manufacturer’s instructions, eluted in RNAse free water, quantified using a Nanodrop Spectrophotometer (Thermo Scientific), and taken for DNase treatment. To remove any residual contaminating genomic DNA, RNA was DNase treated for 15 mins with DNase I (Invitrogen) according to the manufacturer’s protocol. The DNase was subsequently inactivated by the addition of EDTA (2.5mM) and heated to 65°C for 10 minutes. To generate the first strand of cDNA, reverse transcription (RT) was carried out using the Sensifast cDNA synthesis kit (Bioline) according to the manufacturers’ instructions. Two RT reactions were run, one including the reverse transcriptase enzyme (RT+) and the other without the enzyme (RT-). RNA integrity was determined using the relative transcript abundances of each gene’s 3- and 5-prime ends. This step not only detects RNA degradation, but also serves as measure of cDNA completeness. Samples were diluted in preparation for qPCR analysis.

**qPCR and statistics**

qPCR gene targets were selected to assess a number of functional groups including: pattern recognition receptors; proinflammatory signalling molecules; astrocyte-specific proteins/activation markers; microglia/macrophage-specific proteins/activation markers; growth factors; glycnergic, GABAergic, glutamatergic, and purinergic neurotransmitter signalling systems, and transporters. qPCR primers were designed using the NCBI Nucleotide Primer BLAST program. Primers were designed across exon-exon junctions, and/or towards the 3’ end of the transcript where possible. Primer sequences for all genes investigated in the study are listed in Tables 1 to 3. All qPCR reactions used the SensiFast Low Rox Sybr Green kit (Bioline). Primer pair specificity was confirmed by melt curve analysis. qPCR annealing temperatures were adjusted to ensure a single product for each primer pair. All primer pairs were found to have single peak melt curve. qPCR reaction volumes were 12 μL, which contained 5 μL of sample cDNA and 7 μL of master mix. Master mix contained 6 μL of Sybr Green, 0.25 μL primer stock (forward and reverse at 10 μM each; final reaction concentration was 210 nM for each primer), and 0.75 μL nuclease free water. RT+ samples were run in triplicate for each gene. All RT- samples were run in triplicate for primer work up, and then as singles for experimental samples, on a subset of the genes assessed. The subset included nominal “housekeeping” genes, and genes where the primer did not span over an exon junction. This allowed for determination of gDNA contamination. qPCR reactions were run either on an Applied Biosystems 7500, or Viia7 Real-Time PCR System. Ct cut off in these reactions was set at 35, where Ct averages for all age groups had to be above 35 for a particular gene to be excluded. In some cases, expression of the gene was undetectable or poorly detectable (Ct > 35) in young but expressed in old (Ct < 30), and therefore that gene remained in the analysis. The reference/housekeeping genes were detected in all samples with the highest Ct (lowest transcript abundance) being 26 for reference genes.

RNA integrity was determined using the 3’; 5’ ratio for the succinate dehydrogenase (SDH). Raw qPCR CT values for each gene were normalised to the geomean of beta actin, SDH, and Glyceraldehyde 3-phosphate dehydrogenase (gapdh), to generate a ΔCT. The ΔCT was transformed to its linear form using the formula $\Delta CT = 2^{-\Delta CT}$ for statistical analyses and fold change calculations. SEMs were calculated and adjusted relative to fold change. Heat maps summarise gene expression comparisons. For each transcript, the group average $\Delta CT$ (young, middle age, and old age) was calculated and then the difference of each sample’s $\Delta CT$ from this group average was determined, expressed in standard deviations. Values were transformed to single colour gradients (dorsal spinal cord = white to red, ventral spinal cord = white to blue) to produce finalised heatmaps. IBM SPSS was used for statistical analyses.

| Gene name/group                  | Abbreviation | Forward primer | Reverse primer |
|----------------------------------|--------------|----------------|----------------|
| Actin beta                       | Actb         | GCAAGAGTACGATGAGTCCG | ACGCAGCTCAGTAACAGTCC |
| Glyceraldehyde-3-phosphate dehydrogenase | Gapdh | TAGGGCCTCTTGTGCTAGT | GTGGTCTTCTGGCAGCTTCA |
| Succinate dehydrogenase 3'       | Sdh3'        | CTTGTTGAAGTAGGCCAGGTC | GCACGCTGGCTCAGTACTTACA |
| Succinate dehydrogenase 5'       | Sdh5'        | TAACTGGGGCGTGGCCAGGA | ATGCAGCTCAGGAAGCCTGCC |
was set at a mean between-group differences. All values reported are Howell post-hoc tests performed to determine specific group differences, with Games-Howell ANOVA comparing ΔACT values. Browne-Forsythe ANOVA was used to determine group differences, with Games-Howell post-hoc tests performed to determine specific between-group differences. All values reported are mean ± SEM, unless otherwise stated, and significance was set at α = 0.05.

## Results

Our results report on 46 genes expressed in the dorsal and ventral spinal cord of young, middle-aged, and old aged mice (n = 7, 7, 7, respectively). The outcome of these comparisons identified that age related upregulation (12/46 genes) was more common than downregulation (6/46 genes) in the transcripts assessed, while the remaining did not show statistically significant changes across ages (28/46 genes). Regarding region specificity, although some gene changes were conserved across the dorsal and ventral halves of the spinal cord, some genes showed selective gene expression changes in one region only.

Microglia and astrocytes have been heavily implicated in the mechanisms underlying pathological dorsal horn signalling and age-related neuroinflammation, therefore a number of genes known to be enriched in these cell types were assessed in young, mid- and old-aged tissue (Figure 1). First, related to microglia, Cx3cr1 expression was affected by age in the dorsal spinal cord (F(2, 18) = 7.47, p = 0.004), undergoing upregulation at old age (1.3-fold, p = 0.006) when compared to young samples. Cx3cr1 expression was also affected by age in the ventral spinal cord (F(2, 18) = 11.42, p = 0.001), upregulated between young and old (3.8-fold, p = 0.017) and middle and old age animals (1.8-fold, p = 0.022, Figure 1). Itgam expression, also enriched in microglia, was affected by age in the dorsal spinal cord (F(2, 19) = 10.67, p = 0.001), with upregulation in old age (1.5-fold, p = 0.004) compared to young, and between middle age and old age (1.2-fold, p = 0.022). These differences were mirrored in the ventral spinal cord (F(2, 21) = 7.58, p = 0.003) with Itgam expression increased at middle age (1.3-fold, p = 0.003) and old age (1.6-fold, p = 0.023) when compared to young samples.

Two other microgliae-related genes (Csf1 and Cd4) showed less consistent results, with Cd4 expression exhibiting a biphasic aging effect in the dorsal spinal cord (F(2, 18) = 5.63, p = 0.013), initially being down-regulated at middle age (0.69-fold, p = 0.013), before rebounding in old age and returning to marginally higher levels than in young samples (1.1-fold, p = 0.019, Figure 1). While this same trend was apparent in the ventral spinal cord sample, Cd4 expression levels did not differ statistically across age groups (F(2, 18) = 2.118, p = 0.149). Moreover, age-related expression of Csf1 was static in the ventral spinal cord but changed in the dorsal spinal cord (dorsal: F(2, 18) = 4.008, p = 0.036; ventral: F(2, 18) = 0.238, p = 0.79).

Our analysis also assessed expression of two astrocyte-related genes. Gfap expression in the dorsal spinal cord was significantly affected by age (F(2, 18) = 9.179, p = 0.002), being upregulated at old age (1.9-
| Gene name/group                                      | Abbreviation | Forward primer       | Reverse primer       |
|-----------------------------------------------------|--------------|----------------------|----------------------|
| Glycine receptor alpha 1 subunit transcript variant 1 | Glra1 tv1    | TTGCCTGCTCTTCTGTGTCTT | GCATGCGCGCTCTGTGTAGT |
| Glycine receptor alpha 1 subunit transcript variant 2 | Glra1 tv2    | TGGTTGCTCTTGCTGTGTTG | CCACCCTCATCATCTTCTTGA |
| Glycine receptor alpha 2 subunit                     | Glrn2        | GCTGGAGAGTCTGGGGTACA | GCGCTCCAGGTGAACCTTG  |
| Glycine receptor alpha 3 subunit                     | Glrn3        | GGCTCCTTACAAAGGTTGT | CCGTGCAAAGCTTCCGCT   |
| GABA(B) receptor 1                                   | Gabbr1       | TGGCTCCGTCTTCTTATGG  | TGGTGACAGAGCGGGAATGG |
| GABA(A) receptor alpha 1 subunit                     | Gabra1       | AATAGGGCAAGTGGGGCTTA | ACACAGTAGCAGCCATTGA  |
| GABA(A) receptor rho 1 subunit                       | Gabrr1       | AGTCCCTTAGCCATCACCA | AGTTGACAGCTGTCCTCC   |
| Gephyrin                                             | Gphn         | GCACACCAGGATACCTGT   | GCACAGGGTCCAGTTTACA  |
| Glutamate ionotropic receptor AMPA subunit 1          | Gria1        | AAAAGGGAATGCTGGGAAGCA | AAGCAGAAACCTTCCCTCCGCT |
| Glutamate ionotropic receptor AMPA subunit 2          | Gria2        | ACTCATCGGGATCTGGAGGT | TGGAAAGTAGGCACAGAAG  |
| Glutamate ionotropic receptor NMDA subunit 1          | Gria1        | GCTTACTACAGGACACAGG | GTCTTCTTTAGGGTCGCGGC |
| Glutamate ionotropic receptor NMDA subunit 2a         | Gria2a       | TCTGCTCTCAGTTGTGGTTA | GCCGTCTCATCACCTCTCC  |
| Glutamate ionotropic receptor NMDA subunit 2b         | Gria2b       | AAATCGTCCCTCATATGCTG | TGGAGCCCGTTCTACCTCT  |
| Solute carrier family 1 member 3/Excitatory amino acid transporter 1 | Scla3/Eaat1 | CGAACCACCAAACGACTAC | AAGGTTCTCTCGGTCAATT |
| Solute carrier family 1 member 2/Excitatory amino acid transporter 2 | Scla2/Eaat2 | TATGTCGTTGGCTGGTCGTT | CCGCTCCCTACGCGGTATT  |
| Solute carrier family 2 member 5/Potassium chloride cotransporter 2 | Scla2a/Kcc2 | GAAGGCGAGATGGAAACT | GGGGCTTTGGAGCCAAACT |
| Solute carrier family 32 member 1/Vesicular gaba transporter | Scla32a/Vgat | GAATATACAGGTCCAGGCA | CAGGCGCCAAGTTGAAAGT |
| Solute carrier family 17 member 7/Vesicular glutamate transporter 1 | Scla17a/Vglut1 | TCCACTACCAACGTCGAAA | GAGTGGGAAGTACGACCAC |
| Adenosine receptor a1                                 | Adora1       | GCATACAGAAGGACCTGCT | GGAAGCAACACTCAGGCTCTCA |
| Adenosine receptor a2a                                | Adora2a      | ACAGGGCTATCTCCGGGTA | GTGGTGCTCATACGGTAGTT |
| 5'-nucleotidase eecto                                 | N5e          | GCTGTGACTGAAAGTGACGA | AGGTGGGTGGTCAAGTCCC |
| Purinergic Receptor P2X1                              | P2rx1        | CTACCAGCTGGCGCTGGATTG | GTCAGCTTACCTCCAGCAG |
| Purinergic Receptor P2X4                              | P2rx4        | CTTTGTGGATCGAGGCTCA | ACAAAGGGGTAGGTCTTCTG |
| Purinergic Receptor P2X7                              | P2rx7        | ACAGACGACTAAAGGCAACTG | GAACATCGAGAGACCCAGGCA |
| Purinergic Receptor P2Y1                              | P2ry1        | CAGAGCAACTAACCACCTCGT | AAGGCCACAAACCTCTCTCA |
| Purinergic Receptor P2Y12                             | P2ry12       | TACAGAAACACTAAGGCTGC | TGGTGACAGAGCAGACATCC |
Figure 1. Aging alters expression of glial activation and signalling genes in the dorsal and ventral spinal cord. Heatmaps show gene expression data presented as standard deviations (SD) from mean expression for each gene across all ages (genes listed at bottom). Samples are grouped by age: young (top), middle age (middle), and old age (lower), with mean gene expression for each age group presented at the bottom. Dark values denote elevated expression (max = 4.5 SD above mean) and light values indicate reduced gene expression (min = −2.5 SD below mean). Grey cells denote sample failed QC. Genes with significantly altered expression across age are shaded. (a) Glial activation and signalling related genes are stable in the dorsal spinal cord of the spinal cord between young and middle age, but several of these genes are increased at old age as highlighted by the generally darked expression values at the bottom of the heatmaps. (b) Expression of many glial activation and signalling related genes are also elevated in the ventral spinal cord of the spinal cord with age, with gene expression in this heatmap also appearing generally darked for aged samples, versus young and middle age. (c) Schematics summarise genes that exhibited altered expression in middle (left) and old-age (right) versus young samples in the dorsal (red) and ventral spinal cord (blue).
fold, p = 0.027) when compared to young and middle age (p = 0.013). Gfap was similarly altered by age in the ventral spinal cord (F (2, 18) = 16.989, p < 0.001) at middle age (3.1-fold, p < 0.001) and old age (1.9-fold, p = 0.009), though these differences were far less dramatic than in the dorsal spinal cord. Cc3 expression was affected in the dorsal spinal cord by age (F (2, 18) = 27.457, p < 0.001), increasing with old age (9.3-fold, p = 0.001) and was also affected in the ventral spinal cord (F (2, 18) = 16.989, p < 0.001), increasing at middle (3.0-fold, p < 0.001) and old age (23.3-fold, p = 0.009). Contrasting the conserved changes in cytokine expression across dorsal and ventral spinal cord regions described above, Panx-1 expression did not change with age in the dorsal spinal cord (F (2, 18) = 3.488, p = 0.052), but was altered with age in the ventral spinal cord (F (2, 18) = 5.926, p = 0.011) increasing at old age (1.1-fold, p = 0.037). Similarly, the growth factors assessed were stable in the dorsal spinal cord, with Tgfb1 (F (2, 18) = 3.365, p = 0.057), Tgfb3 (F (2, 18) = 2.364, p = 0.123), Bdnf (F (2, 18) = 0.850, p = 0.443), and Ntk2 (F (2, 20) = 0.532, p = 0.595) all stable across age groups. In contrast, ventral spinal cord expression of Tgfb1 was altered (F (2, 18) = 9.99, p = 0.00241), with upregulation at middle age (2.8-fold, p = 0.026) and old age (2.0-fold, p = 0.001), whereas Bdnf expression changed in the opposite direction (F (2, 21) = 3.82, p = 0.038), downregulated at middle age (0.8-fold) and remaining decreased at old age, though post-hoc tests did not resolve a significant difference in the old age sample.

The remaining category of genes assessed in these experiments included neurotransmitter receptor and transporter proteins (Figure 2). This was motivated by our previous work showing age related changes in both excitatory and inhibitory synaptic drive in dorsal spinal cord neurons. Somewhat surprisingly, the majority of genes assessed remained stable over the age, with only a few exceptions. For example, among the AMPA and NMDA receptor subunits assessed, only Grial1 expression was affected by age in the dorsal spinal cord (F (2, 20) = 4.172, p = 0.031), with middle and old age values ~1.2 fold higher than in the young dorsal spinal cord. No other differences were detected in the dorsal (Gria2 F (2, 20) = 1.084, p = 0.357; Grin1 F (2, 20) = 0.454, p = 0.642; Grin2a F (2, 20) = 0.548, p = 0.586; Grin2b F (2, 18) = 0.359, p = 0.703) or ventral spinal cord (Gria1 F (2, 21) = 1.055, p = 0.366; Gria2 F (2, 21) = 1.903, p = 0.174; Grin1 F (2, 21) = 1.493, p = 0.248; Grin2a F (2, 21) = 1.453, p = 0.257; Grin2b F (2, 18) = 0.269, p = 0.767) expression values.

As fast synaptic inhibition in the spinal cord can be mediated by glycine and GABA, both receptor types were assessed as well as the receptor stabilising protein gephyrin, and a range of transporter proteins. For glycine receptors, Gria2 differed in the dorsal spinal cord with age (F (2, 20) = 14.33, p < 0.001), downregulated at
old age (0.7-fold, \( p = 0.005 \)). Glra2 expression was also affected in the ventral spinal cord (\( F (2, 21) = 7.05, p = 0.005 \)), with a downregulation at middle (0.6-fold, \( p = 0.034 \)) and old age (0.6-fold, \( p = 0.018 \)). Expression of the remaining glycine receptor subunit genes assessed were stable across age in the dorsal (Glra1 tv1 \( F (2, 20) = 0.193, p = 0.826 \); Glra1 tv2 \( F (2, 20) = 2.495, p = 0.108 \); Glyra3 \( F (2, 20) = 1.406, p = 0.268 \)) and ventral spinal cord (Glra1 tv1 \( F (2, 20) = 1.805, p = 0.190 \); Glra1 tv2 \( F (2, 21) = 1.915, p = 0.172 \); Glyra3 \( F (2, 21) = \)
In the ventral spinal cord, Gabral expression did change (F (2, 18) = 8.313, p = 0.003) undergoing downregulation in old age (0.4-fold, p = 0.011), which also differed significantly from middle aged (0.7-fold, p = 0.019). The remaining GABA receptor genes were stable in the ventral spinal cord across age (Gabrr1 F (2, 18) = 0.415, p = 0.667; and Gabbr1 F (2, 18) = 0.265, p = 0.770). Gphn expression was stable in the dorsal spinal cord (F (2, 18) = 0.717, p = 0.501), but differed in the ventral spinal cord (F (2, 17) = 6.90, p = 0.006), showing a modest downregulation with old age (0.8-fold, p = 0.004). The expression of vesicular GABA transporter, Vgt, was not affected in the dorsal spinal cord by age (F (2, 18) = 3.541, p = 0.050), but did differ in the ventral spinal cord (F (2, 18) = 11.21, p = 0.001), with a significant downregulation in old age (0.5-fold, p = 0.004), as well as downregulation between middle age (0.7-fold) and old age (p = 0.019). Also critical to effective inhibition in the dorsal spinal cord, KCC2 (a potassium-chloride co-transporter) expression has been shown to be downregulated under neuropathic conditions, in a microglial dependent manner. Despite these associations, our data suggest KCC2 expression was unchanged by age in the dorsal spinal cord (F (2, 20) = 0.696, p = 0.510) though expression in the ventral spinal cord changed with age (F (2, 21) = 3.808, p = 0.039).

The final neurotransmitter system assessed in our data was purinergic signalling proteins including ATP receptors, adenosine receptors, and associated enzymes. Among ATP receptors, expression of both P2rx1 and P2rx4 ATP-gated cation channels were altered by age (F (2, 16) = 15.42, p < 0.001; and F (2, 16) = 7.20, p = 0.006, respectively), each showing upregulation in the dorsal spinal cord with old age (2.1-fold, p = 0.007, and 1.3-fold, p = 0.007, respectively), and P2rx1 expression also increased in middle age (2.5-fold, p = 0.003). In contrast P2rx7 was unchanged in the dorsal spinal cord across all age groups (F (2, 18) = 0.813, p = 0.459). None of the remaining purinergic related proteins showed age dependent differences in the dorsal spinal cord including the ATP-sensitive G protein-coupled receptors P2ry1 (F (2, 18) = 0.986, p = 0.392) and P2ry12 (F (2, 18) = 2.516, p = 0.109), adenosine receptors Adora1 (F (2, 18) = 1.642, p = 0.221) and Adora2a (F (2, 18) = 1.315 p = 0.293), or the adenosine producing enzyme Nt5e (F (2, 18) = 0.359, p = 0.703). In the ventral spinal cord purinergic signalling gene expression did not change with age for ATP-gated cation channels, ATP-sensitive G protein-coupled receptors, or adenosine receptor and enzyme proteins. Thus, our data shows some age-related changes in glycine, GABA, and purinergic receptor gene expression, though far less pronounced than for cytokine and microglial activation genes.

Discussion

This study was motivated by the need to establish how aging affects signalling pathways in the spinal cord, with a particular emphasis on the phenotype of glial populations and the expression of neuroinflammatory signalling molecules. Comparison across three ages highlighted changes in gene expression that suggest the functional phenotypes of microglia and astrocytes are altered with age and expression of a number of neuroinflammatory molecules and receptors are elevated in naïve, uninjured animals. Altered expression of many of the molecules assessed here has also been reported in the spinal cord of young animals under neuropathic conditions This raises the prospect that these gene expression differences may contribute to the increased incidence of chronic pain in the elderly. Together, these findings provide valuable baseline data for future work assessing pathological pain mechanisms in advanced age.

There are some obvious caveats associated with our study. First, within-group variability was high for many of the genes we assessed, as shown in the intensity ranges within the columns of Figures 1 and 2. With such variability it is possible that this analysis may yield some false negatives and therefore our conclusions provide a conservative overview of these aging effects. This said, previous aging studies have documented that large variability is feature of aging and essentially explains the phenomena of healthy and unhealthy aging. The second caveat is our assessment of only male mice, necessitated by the aging colony used to source animals for the study. This limits translation of our findings as there are now well-established gender differences in chronic pain mechanisms, and chronic pain states are more common in women. Nevertheless, our data provides a necessary first step, with future work needed in aged female mice to extend these insights. Finally, our analysis examined a limited gene panel spanning glial activation and signalling molecules, as well as a number of neurotransmitter systems. (eg. the GABA receptor comes in >20 types - we only examined three). This data still provides insight into the diverse range signalling pathways that are likely altered in the dorsal and ventral spinal cord by age, as well as providing a basis for future work that interrogates these signalling pathways in greater detail.

The most dramatic age-related upregulation of gene expression observed in our work were in cytokine/chemokine and innate immune signalling pathways. For example, C-X-C motif chemokine 13 (Cxcl13) rose in...
the dorsal spinal cord of middle-aged mice by 23-fold, and increased further to 191-fold at old age. This observation was regionally specific, with far less dramatic expression increases in the ventral spinal cord – 3 fold in middle-age (3-fold) and ~2-fold by 24 months of age. It should be noted that the magnitude of the relative increase in expression of Cxcl13 in the DH partly reflects its near undetectable expression in young animals. As to the cellular origin of spinal Cxcl13 expression, microglia and neurons have been identified as potential sources under pathological conditions in a number of studies.14,32,33 Furthermore, Cxcl13 expression has been implicated in the development of neuropathic pain following peripheral spinal- and cranial nerve injury.14 Strikingly, following spinal nerve ligation (SNL), microarray analysis revealed that Cxcl13 was the most upregulated chemokine gene in the spinal cord, showing a 47-fold increase.14 This work also confirmed a corresponding increase in Cxcl13 protein in the DH. Furthermore, Cxcl13 signalling was essential for activation of astrocytes and pain behaviours. Conversely, intrathecal injection of Cxcl13, and of Cxcl13 -stimulated astrocytes from wild type mice, but not Cxcl13 -stimulated astrocytes from Cxcr5 KO mice, induced mechanical allodynia.14 This nerve injury-induced increase in Cxcl13/ Cxcr5 signalling was also demonstrated in the trigeminal ganglion (TG) following partial infraorbital nerve ligation. Intra-TG injection of Cxcl13 also increased TNF-α and IL-1β mRNA.34 Together these findings strongly implicate Cxcl13/Cxcr5 signalling, likely arising from microglia and/or neurons and activating astrocytes, in neuropathic pain and suggest the dorsal spinal cord specific increased expression in aged animals has implications for spinal pain signalling in the elderly.

Another inflammatory chemokine35 exhibiting substantially increased expression in the dorsal and ventral spinal cord (9-fold and 23-fold, respectively) was C-C motif chemokine ligand 3 (Ccl3). The source of Ccl3 is unclear, but IL-1β and TNF-α have been shown to induce Ccl3 production in astrocytes,36 while P2X7 receptor activation can induce the production of Ccl3 in microglia.37 These findings highlight neuroglia as the likely candidate for the enhanced expression of Ccl3 in aging. With respect to its role in pathological pain, expression of Ccl3 was increased following partial sciatic nerve ligation (PSNL), and neutralization of Ccl3 with an antibody ameliorated PSNL-induced neuropathic pain.38 Moreover, the direct peripheral or spinal administration of Ccl338,39 has been shown to induce pain behaviours. Thus, the dramatic increase to Ccl3 and Cxcl13 expression (above), along with both the role of both in neuropathic pain, establishes an altered baseline environment for neuropathic pain mechanisms to develop in the aged spinal cord. Further, regional differences in the degree of upregulation of Cxcl13 and Ccl3, being more pronounced in aged dorsal and ventral cord, carry implications for not just chronic pain but also motor dysfunction in the elderly.

A number of genes showing increased age-related expression relate to activation and switching in microglial and astrocyte phenotype, which would be expected to produce the altered cytokine signalling detected above. For example, Colony stimulating factor 1 (Csf1), also known as macrophage colony-stimulating factor, is a cytokine that increased in the aged dorsal spinal cord (1.3-fold). This molecule is capable of promoting the proliferation, differentiation, and survival of macrophage-derived cells, including microglia.46 The importance of this signalling pathway has been confirmed as genetic removal of Csf1 or Csf1r produces a severe depletion in microglial numbers. Concerning its involvement in pathological pain states, the expression of the receptor for Csf1 (Csf1r) is upregulated following peripheral nerve injury, though Csf1 itself was not increased in this work.46 Regardless, this study showed a nerve injury-dependent induction of Csf1 expression in sensory neurons, which was transported centrally into the DH to activate microglia via the Csf1r and subsequently drive morphological and phenotypic change.46 Thus, elevated Csf1 in the aged dorsal spinal cord could be expected to enhance stimulation of these processes at baseline.

Also contributing to glial activation, expression of C-X3-C motif receptor 1 (Cx3cr1), also known as the fractalkine receptor, increased in both the dorsal and ventral cord (1.3-fold and 3.8-fold, respectively). Fractalkine is constitutively expressed in peripheral and central neurons, while Cx3cr1 is expressed on microglia and astrocytes.47 Accordingly, fractalkine is thought to mediate neuron-glial crosstalk in both the normal and pathological CNS.48 In a rodent prion disease model of chronic CNS inflammation both the fractalkine ligand and receptor upregulated.49 Likewise, soluble fractalkine in the neuropathic dorsal spinal cord is capable of activating microglia, and pharmacological or genetic interventions directed against fractalkine abolish hypersensitivity and the accompanying microglial activation.50–53 Moreover, direct intrathecal administration of fractalkine can induce mechanical allodynia and thermal hyperalgesia, whereas single injection intrathecal Cx3cr1 antagonist can suppress neuropathic pain induced by chronic constriction injury (CCI) and sciatric inflammatory neuropathy (SIN).54–56

The above findings support the existence of an age-related change in the activation state of microglia. However, an expanding view of microglial activation has now begun to consider these cells as a continuum of distinct, overlapping phenotypes.57–59 Importantly, a group of microglia referred to as disease-associated microglia (DAM) are now described among these
phenotypes. These DAM have been associated with neurodegenerative diseases, such as Alzheimer’s and ALS, as well as in ‘aging induced’ brain damage.\textsuperscript{57,60} DAM can be identified by specific gene expression patterns with fully competent DAM requiring Triggering receptor expressed by myeloid cells (Trem2) expression. Expression of Trem2 in our data increased 3.8-fold in DH and 5-fold in the ventral cord of old mice, suggesting the presence of the DAM phenotype in both regions. Furthermore, the DAM gene signature also includes elevated expression of C-type lectin domain family 7 member A (Clec7a) and Toll Like Receptor 2 (Tlr2), which were both expressed at elevated levels in the dorsal and ventral spinal cord in aged samples. Together, these findings suggest that DAM feature in both the dorsal and ventral spinal cord of aged mice, raising the likelihood that these cells modify the vulnerability of both regions to insult and injury with advanced age. Reinforcing this point, Trem2 has been implicated raising the likelihood that these cells modify the vulnerability of both regions to insult and injury with advanced age. Reinforcing this point, Trem2 has been implicated with fully competent DAM requiring Triggering receptor expressed by myeloid cells (Trem2) expression.

In addition, evidence supports a role of TLR-mediated immune responses with neuropathic pain, as nerve transection upregulates TLR2 expression in the spinal cord.\textsuperscript{61} Furthermore, nerve injury-induced increase in Trem2 expression paralleled the increase in microglial numbers in the ipsilateral DH, and intrathecal administration of a Trem2 agonist antibody in the microglial numbers in the ipsilateral DH, and intrathecal administration of a Trem2 agonist antibody in the absence of injury, reduced paw-withdrawal thresholds. In addition, evidence supports a role of TLR-mediated immune responses with neuropathic pain, as nerve transection upregulates TLR2 expression in the spinal cord.\textsuperscript{61} Furthermore, nerve injury induced pain behaviours, microglial activation, and the induction of proinflammatory cytokines are all diminished in TLR2-KO mice.\textsuperscript{62} Finally, the ability of Clec7a to induce the production of proinflammatory cytokines,\textsuperscript{63} and its responsiveness to damage-associated molecular patterns (DAMPs), may further contribute to a heightened responsiveness of the aged spinal cord and nerve-injury responses.

Consistent with the above findings a number of other genes were also increased in the spinal cord including: Glial fibrillary acidic protein (Gfap), an astrocyte-specific cytoskeletal protein increased by activation and a range pathological states\textsuperscript{64}; cluster of differentiation 86 (Cd86), a transmembrane glycoprotein expressed by activated microglia and a recently identified populations of resident brain dendritic cells (bDC)\textsuperscript{65}; and the P2X purinoceptor (P2rx4), an ionotropic receptor expressed in microglia and neurons and increased in spinal microglia following nerve-injury.\textsuperscript{66} Collectively these findings add to the above interpretation that the spinal cord, just like the brain, exhibits age-related priming of glial populations and neuroinflammatory pathways.\textsuperscript{66,67} Accordingly, these alterations may be due to adaptations in response to, or involvement in chronic inflammatory processes. In either case they will dramatically alter the responses of the aged dorsal and ventral spinal cord to peripheral and central insults.

As noted above, interpretation of our data also comes with a number of caveats. As is the case with any gene expression study, it cannot be assumed that these differences are expressed as changes, at a protein level, required to influence function.\textsuperscript{58} Thus, future work in young, middle and old age spinal cords prepared to preserve protein for analysis will be required to verify the age-related dorsal and ventral spinal cord phenotypes we describe. When such cross-checks have been performed in the brain, assessing age-related gene expression that overlaps with our study, there has been good correspondence between mRNA and protein levels.\textsuperscript{57,58} In addition, although we have used the literature describing the expression of genes in different cells to attribute gene expression changes to cell types such as microglia and astrocytes, the cellular origin of these signals was not directly assessed in our experiments. Immunolabelling of the related proteins in spinal cord sections, RNAscope analysis, qPCR in identified cells, or single cell RNASeq will be required to confirm these associations. Future work using the single cell RNASeq approach would also dramatically increase the coverage of gene expression from the relatively small and targeted panel of genes we assessed. This may unmask expression changes with the capacity to compensate for the largely proinflammatory network of disrupted genes we detected. In support of this premise, transforming growth factor-beta 1 (Tgf-\textbeta 1) expression was increased \textasciitilde 2-fold in the aged ventral spinal cord. Given Tgf-\textbeta 1 is a potent anti-inflammatory cytokine,\textsuperscript{69} it is tempting to suggest this indicates motor circuits may be somewhat protected from proinflammatory signalling. Finally, the expression of some of the genes we assessed did not show any age-related change in our analysis, including neuroinflammatory and glial related proteins, and the majority of synaptic proteins. This was surprising for the dorsal spinal cord, as our previous work has identified alterations in synaptic function in these circuits with age\textsuperscript{17} that predict altered gene expression. This discrepancy could be explained by incomplete translation of gene expression through to protein as suggested above. Alternatively, age-dependent posttranslational modification of synaptic proteins, altered trafficking or distribution of these proteins at synaptic versus extrasynaptic sites may help to explain these differences.

**Conclusions and future directions**

The present findings clearly indicate aging causes changes in the expression of a range of transcripts involved in inflammatory responses, and glial function with some regional specificity in the spinal cord (dorsal versus ventral spinal cord). The expression changes in
the dorsal spinal cord relating to glial activation and proinflammatory cytokine signalling bear striking similarities with those seen in neuropathic pain. Of particular interest, the most upregulated chemokine following spinal nerve ligation, Cxcl13, underwent a more pronounced increase in gene expression with age alone. It remains to be determined whether the neuroinflammatory remodelling underlies a heightened susceptibility or response to neuropathology. Moreover, given the identified roles in the pathophysiology of neuropathic pain, it will be important to establish the magnitude of neuroinflammatory responses to nerve injury in aged animals. For example, the altered neuroinflammatory status of the aged spinal cord may limit its capacity to increase inflammatory and glial gene expression, or further potentiate expression when a neuropathic perturbation is superimposed on an elevated baseline. Furthermore, the altered neuroinflammatory baseline may precipitate the recruitment of alternative or additional pathways that could exacerbate or prolong pathology. Future work in aged animals will be required to resolve these issues, but the ability of age to drastically alter key neuroinflammatory cytokines and glial activation, replicating aspects of the pathogenesis of neuropathic pain is undoubtedly relevant. Together, these new insights provide a potential mechanism to alter sensory signalling in dorsal horn circuits and appear of relevance to the heightened incidence of chronic pain states in the elderly.

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